ABC transporters:

Lessons from a bacterial oligopeptide uptake system

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Contents

Chapter 1

General Introduction

1. Membranes: the barriers of life

The basic building block of life is the cell that with no exception is surrounded by at least one membrane. The cytoplasmic membrane forms a physical barrier between the outside world and the cytoplasm, containing molecules specific to the cell or organism. The membrane is a two-dimensional crystalline matrix composed of a double-layer of many different kinds of lipids and packed with an enormous amount and variety of proteins (Devaux and Seigneuret, 1985). The weight ratio of lipid to protein varies among cellular and organellar membranes but is typically in the range of 1 to 1, implying that each membrane protein (complex) is only surrounded by a few shells of lipid molecules. It is remarkable that despite this huge protein content, the semi-permeable and insulating character of the lipid bilayer remains conserved. The function of the cytoplasmic membrane is three-fold: i) to prevent uncontrolled diffusion of (mostly hydrophilic) molecules into and out of the cytoplasm, ii) to control the energy status of the cell by producing and maintaining electrochemical ion gradients, and iii) to form a matrix for a variety of enzymes.

In prokaryotes, the chemiosmotic ion gradient is often formed by the respiratory chains that, at the expense of redox energy, pump protons or sodium ions out of the cell (Nicholls and Ferguson, 1992). This results in a pH (and/or sodium) gradient and a membrane potential (inside negative), the sum of which is termed the proton- (or sodium) motive force (PMF or SMF). Dedicated membrane proteins use this inwardly-directed ion gradient to transport solutes into or out of the cell, or to generate the universal energy currency of life, ATP, via the proton driven F_oF_1-ATPase/synthase (Fig. 1; Nicholls and Ferguson, 1992). Under anaerobic conditions, or in organisms lacking an electron transport chain, metabolic energy can be supplied in the form of ATP by substrate level phosphorylation (Konings, 2002). ATP is then hydrolysed by the F_oF_1-ATPase/synthase in order to generate a PMF.

In eukaryotic cells ATP is generated by mitochondria in a way similar to aerobic bacteria, and transported into the cytoplasm by dedicated mitochondrial carriers (*i.e*. ATP/ADP exchangers; Nicholls and Ferguson, 1992; Kunji *et al.*, 2004). P-type ATPases then generate electrochemical gradients across the cytoplasmic membrane (Stein, 2002). Plants have, in addition, chloroplasts that harbour thylakoid membranes containing photosystems, composed of antennae responsible for the trapping of photons, and reaction centers, to which the light energy is directed and where the photoinduced charge separation takes place (Nicholls and Ferguson, 1992). The light-dependent electron transfer by the reaction centers together with electron transfer proteins drives the pumping of protons into the thylakoid lumen and results in

The abbreviations used are: ABC, ATP-binding cassette; GUV, Giant Unilamellar Vesicle; LUV, Large Unilamellar Vesicle; MF, Major Facilitator; NBD, nucleotide-binding domain; PEP, phosphoenolpyruvate; PMF, proton motive force; PTS, phosphotransferase system; SBP, substrate-binding protein; SMF, sodium motive force; TMD, transmembrane domain; TMS, transmembrane segment.

a PMF. The PMF is used by the ATP-synthase in the thylakoid membrane to synthesize ATP, which is released in the stroma. ATP/ADP carriers and solute pores have been identified in the inner and outer chloroplast membrane, respectively, allowing shuttling of ATP and ADP between the cytoplasmic and stromal compartments (Neuhaus and Wagner, 2000). In the dark, ATP is imported into chloroplasts and used for anabolic reactions or starch degradation. Algae and photosynthetic bacteria also contain photosystems to generate an electrochemical proton gradient (Nicholls and Ferguson, 1992). Finally, halophilic Archaea use the light-driven proton pump bacteriorhodopsin to generate a PMF across the cytoplasmic membrane (Pebay-Peyroula *et al.*, 2000).
As, in principle, most membrane transport processes are reversible, different energy sources (PMF or other (electro)chemical gradients and ATP (ΔG_{ATP})) can be interconverted depending on the environmental conditions and energy needs of the cell.

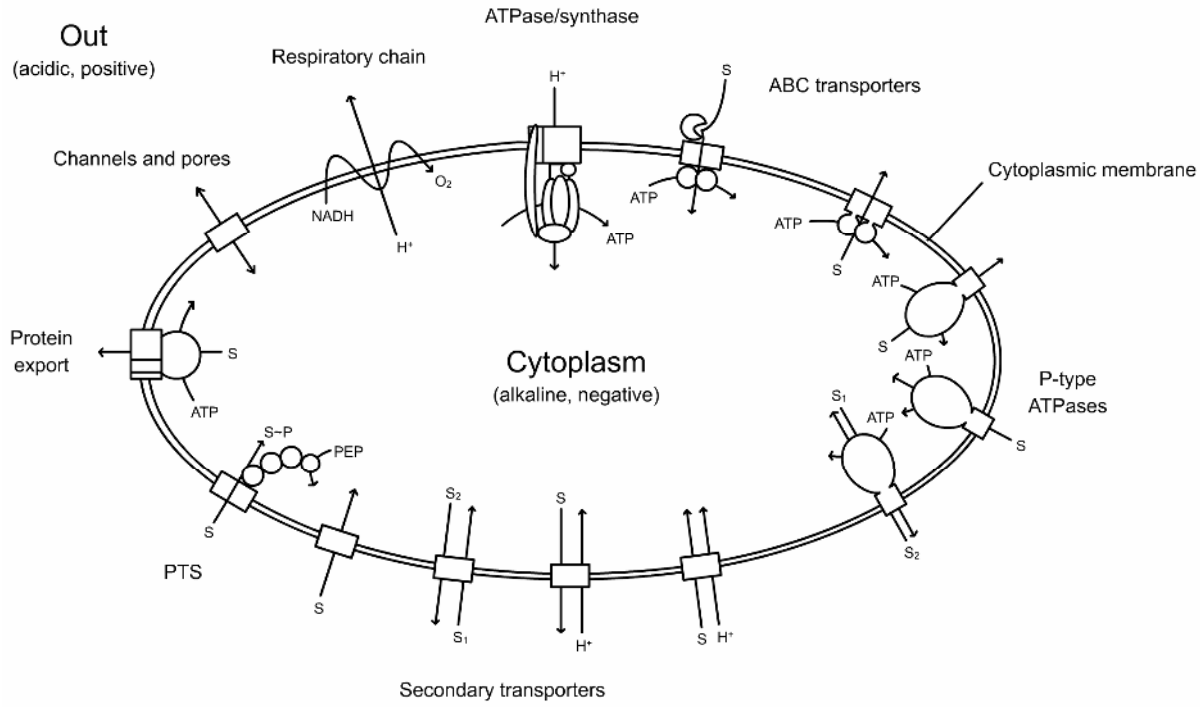

Fig. 1. **Interplay between various energy transducing processes in the cytoplasmic membrane of aerobic bacteria.** See text for details.

2. Membrane transport proteins

Based on which energy source they use, membrane transport proteins can be classified into four groups (Saier, 2000). i) Primary transporters use light (*e.g.,* the proton pump bacteriorhodopsin) or the chemical energy stored in ATP to drive solute translocation. The largest superfamily of primary transporters are the ATP-binding cassette (ABC) transporters (Fig. 1; Higgins, 1992). Members of this superfamily facilitate the unidirectional movement of a

solute into or out of cells or specialized compartments at the expense of, most likely, 2 ATP molecules (Patzlaff *et al.*, 2003). P-type ATPases (*e.g.,* the H^+, Na^+/H^+-, K^+/H^+-, and Ca^{2+}-ATPases; Fig. 1) are primary transporters as well (Stein, 2002). ii) Secondary transporters utilize (electro)chemical gradients to translocate a solute down its concentration gradient (facilitated diffusion), or they couple the transport of one molecule, often a proton or sodium ion, down its gradient to the uptake (symport) or excretion (antiport) of another solute against its gradient (Fig. 1; Poolman and Konings, 1993). The largest group of secondary transporters is formed by the members of the Major Facilitator (MF) superfamily (Pao *et al.*, 1998). The ABC and MF superfamilies constitute the two most abundant and widely spread classes of membrane transport proteins, having been identified in every life form ranging from bacteria to man. iii) Group translocators couple the uptake of substrate to its chemical modification (Fig. 1). This group consists of the bacterial phosphoenolpyruvate phosphotransferase systems (PEP-PTS), that transfer a phosphoryl group from the high energy donor PEP via a cascade of phosphoryl transfer proteins onto the translocated solute (Postma *et al.*, 1993). Finally, iv) channels and pores do not couple energy utilization to the translocation process (Fig. 1), although they can be gated by ion gradients (voltage-gated channels), ATP or other ligands (ligand-gated ion channels), or a mechanic stimulus (mechanosensitive channels; Kung and Blount, 2004). As a consequence, they can not accumulate but only transport solutes down their concentration gradient. They can form selective or non-selective pores in the membrane allowing ions or other low-molecular weight molecules to pass, and are continuously open or gated by external signals.

3. The oligopeptide ABC transporter

ABC transporters are composed of two transmembrane domains (TMDs) plus two nucleotide-binding domains (NBDs) bound to the cytosolic face of the TMDs (for an extensive overview of ABC transporter domain architecture, mechanism and regulation see **Chapter 2**). Bacterial ABC uptake systems use extracytoplasmic substrate-binding proteins (SBPs) that scavenge for substrates and deliver these to the translocator. The oligopeptide translocator is composed of two TMDs, OppB and OppC, each consisting of six transmembrane spanning α-helical segments (TMSs; Pearce *et al.*, 1992), N- and C-termini inside, and two NBDs, OppD and OppF. OppA functions as the SBP that binds oligopeptides and delivers them to OppBCDF where substrate is internalized. The oligopeptide transporter was first identified and characterized in *Salmonella typhimurium* and *Escherichia coli*, Gram-negative organisms in which OppA is present as a soluble protein in the periplasmic space (Higgins and Hardie, 1983; Guyer *et al.*, 1986; Pearce *et al.*, 1992; Tame *et al.*, 1994). Subsequently, oligopeptide transporters of Gram-positive organisms attracted much attention due to their involvement in signalling pathways, affecting for example sporulation in *Bacillus subtilis* (Perego *et al.*, 1991; Koide and Hoch, 1994; Levdikov *et al.*, 2005), and their vital role in nutrition in the industrially important bacteria *Streptococcus thermophilus* (Garault *et al.*, 2002; Juille *et al.*, 2005) and *Lactococcus lactis* (Tynkkynen *et al.*, 1993; Kunji *et al.*, 1995, 1998; Detmers *et al.*, 1998, 2000; Lanfermeijer *et al.*, 1999, 2000; Picon *et al.*, 2000; Charbonnel *et al.*, 2003; Lamarque *et al.*, 2004; Sanz *et al.*, 2004; Doeven *et al.*, 2004, 2005; for a detailed overview see **Chapter 4**). In these organisms, lacking a periplasmic space, OppA is anchored to the cell envelope via coupling of an N-terminal cysteine to a lipid moiety (Sutcliffe and Russel, 1995). This coupling is thought to occur in three subsequent steps: i) diacylglyceryl modification of the cysteine S-H

group, ii) cleavage of the signal peptide, and iii) *N*-acylation (Sankaran and Wu, 1994). Also, many pathogens have been found to encode Opp systems, often employed with multiple OppAs to broaden substrate specificity, and involved in virulence development (Samen *et al.*, 2004; Wang *et al.*, 2004). Examples are *Borrelia burgdorferi*, the causative agent in lyme-disease, and *Bacillus anthracis*, that contain 5 and 16 different OppA variants, respectively (Lin *et al.*, 2001; Read *et al.*, 2003). Although several Archaeal gene clusters have been annotated *opp*, they have not been characterized as such yet. In *Pyrococcus furiosus* and *Sulfolobus solfataricus,* transporters belonging to the Opp family of ABC transporters have been shown to transport a range of oligo β-glucosides rather than oligopeptides (Elferink *et al.*, 2001; Koning *et al.*, 2001).

4. Scope of this thesis

This thesis deals with the molecular mechanism of substrate-binding and translocation by ABC transporters. As a model system the oligopeptide transporter from the lactic acid bacterium *Lactococcus lactis* was studied. **Chapter 2** gives a detailed overview of ABC transporter structure, mechanism and modulation by accessory domains. Whereas the basic architecture of ABC transporters appears conserved throughout all kingdoms of life, several (sub)families of transporters use additional domains that are vital for transport function and/or regulation, or perform additional catalytic activities. **Chapter 3** deals with the overexpression, purification, and functional reconstitution of the oligopeptide ABC transporter in an artificial liposomal system (Large Unilamellar Vesicles (LUVs); average diameter ~ 200 nm). Transport selectivity was found to be largely determined by the specificity of the oligopeptide-binding protein OppA. Taken together with other recent findings, this work clarified discrepancies in older literature on substrate specificity and mechanism of ABC-type peptide transporters. The wider implications for the field of peptide transport in *L. lactis* and other microorganisms are described in **Chapter 4**. The proposed peptide-binding mechanism of OppA remains somewhat controversial in respect to the generally accepted view that ABC transporter SBPs enclose their substrate completely. In lactococcal OppA, the C-terminus of long peptides (> 6 residues) is thought to protrude out of the protein and interact with its surface. **Chapter 5** describes the crystallization of OppA in complex with a high affinity nonapeptide ligand as an initial step towards structure determination. Also, the association state and hydrodyamic radius of OppA in the absence and presence of ligand, as analysed by analytical ultracentrifugation, are reported. **Chapter 6** deals with the functional incorporation of membrane proteins, amongst which the oligopeptide ABC transporter, into Giant Unilamellar Vesicles (GUVs; diameter ranging from ~ 5 – 100 μM). GUVs are formed by subjecting lipids to a drying/rehydration cycle and are widely used to study the physical properties of lipids and lipid mixtures, for example lipid lateral mobility and domain (raft) formation. We took advantage of the stabilizing effects of sugars on proteins and membranes during drying to develop a method that opens up the GUV field to the study of membrane proteins. In **Chapter 7** proteo-GUVs are used to investigate protein:protein interactions in the oligopeptide ABC transporter by fluorescence correlation and cross-correlation spectroscopy, yielding detailed information on the catalytic cycle of this ABC uptake system. **Chapter 8** summarizes the most important findings presented in this thesis, puts them into perspective and ends with an outlook and some suggestions for future research.

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Chapter 2

ABC transporter architecture and regulatory roles of accessory domains

Mark K. Doeven[#], Esther Biemans-Oldehinkel[#], and Bert Poolman

Abstract

We present an overview of the architecture of ABC transporters and dissect the systems in core and accessory domains. The ABC transporter core is formed by the transmembrane domains (TMDs) and the nucleotide binding domains (NBDs) that constitute the actual translocator. The accessory domains include the substrate-binding proteins, that function as high affinity receptors in ABC type uptake systems, and regulatory or catalytic domains that can be fused to either the TMDs or NBDs. The regulatory domains add unique functions to the transporters allowing the systems to act as channel conductance regulators, osmosensors/regulators, and assemble into macromolecular complexes with specific properties.

Introduction

ATP-binding cassette (ABC) transporters are vital to any living system and are involved in the translocation of a wide variety of substrates ranging from ions, sugars, amino acids, vitamins, lipids, antibiotics and drugs to larger molecules such as oligosaccharides, oligopeptides and even high molecular weight proteins (Higgins, 1992). Owing to their function ABC transporters are involved in many crucial processes, including nutrient uptake, lipid trafficking, drug and antibiotic excretion, secretion of macromolecules, antigen presentation to cytotoxic T cells, and cell volume regulation, and they are often major players in complex pathways affecting gene expression (*e.g.,* sporulation, competence and virulence development). In humans a still growing number of diseases is related to (dys)function of ABC transporters, the most common known examples being cystic fibrosis caused by defects in the Cystic Fibrosis Transmembrane conductance Regulator (CFTR/ABCC7), an ABC-type chloride channel, and multidrug-resistance conferred to tumor cells by P-glycoprotein (P-gp/MDR1/ABCB1) activity.

Irrespective of the direction of transport (uptake or excretion), the nature of the transported substrate (low or high molecular weight molecule), or the physiological function, ABC transporters are composed of two hydrophobic transmembrane domains (TMDs) and two

[#]Both authors contributed equally to this chapter. The abbreviations used are: ABC, ATP-binding cassette; CBS, cystathionine-β-synthase; CFTR, Cystic Fibrosis Transmembrane conductance Regulator; ECD, extracytoplasmic domain; ICD, intracellular domain; NBD, nucleotide-binding domain; PTS, phosphotransferase system; SBD, substrate-binding domain; SBP, substrate-binding protein; TAP, transporter associated with antigen processing; TMD, transmembrane domain; TMS, transmembrane segment.

water soluble nucleotide-binding domains (NBDs) bound to the cytosolic face of the TMDs (Fig. 1). In addition to these core domains, hereafter referred to as the translocator or translocation unit, accessory domains or proteins can be part of the ABC transporter. In this chapter we review the architecture of ABC transporters, with emphasis on the types of accessory domains associated with the translocator. These accessory domains provide additional, often regulatory, functionalities to the transporters. The accessory domains can be recognized as additional transmembrane segments or internal or external domains linked to the TMDs, or extensions added to the NBDs. In many cases, it has been shown that the accessory domains can be deleted without affecting the translocation function, confirming that they do not form part of the core of the translocator.

Architecture of ABC uptake systems

The first identified components of ABC transporters were the substrate-binding proteins (SBPs) of ABC uptake systems that reside in the periplasmic space of Gram-negative bacteria, from which they could be released by cold osmotic shock (Neu and Heppel, 1965; Pardee, 1968; Berger, 1973; Berger *et al.*, 1974) . These water soluble proteins bind the substrate in a cleft between two lobes and deliver it to the translocator in the cytoplasmic membrane. In Gram-positive bacteria and Archaea, *i.e.,* microorganisms lacking an outer membrane, SBPs are exposed on the cell surface and attached to the cytoplasmic membrane via a lipid-anchor or a transmembrane-peptide (to date, the latter has been only observed in Archaea), or they can be fused to the TMDs resulting in two substrate-binding domains (SBDs) per functional complex (Fig. 1B; OpuA). In some cases, two SBDs fused in tandem are linked to the TMDs and these systems have a total of four extracytoplasmic substrate-binding sites (*e.g.,* GlnPQ). Systems with SBDs fused to the TMDs can be also found in Gram-negative bacteria but less frequently than in Gram-positives (van der Heide and Poolman, 2002).
In terms of number of different subunits, the most complex ABC type uptake systems known are the nickel- and (oligo)peptide transporters, in which each domain is present as a separate polypeptide, giving a total of (at least) five unique proteins required for transport (Fig. 1A and B; Nik and Opp). In many cases, two copies of the same NBD *plus* two different TMDs (*e.g.,* the maltose, histidine, and glucose transporters) or, less frequently, two copies of one NBD *plus* one TMD are present (the vitamin B12 transporter Btu). Occasionally, the NBDs or TMDs are fused (*e.g.,* siderophore/haem and ribose transporters) such that in total only three unique proteins are used (Fig. 1A). The smallest number of unique subunits of which ABC uptake systems are composed, known to date, is two (Fig. 1B). This is the case for the glycine betaine and glutamine/glutamic acid transporters in which the SBPs are fused to either the N- or C-terminus of their cognate TMDs, and the second unique subunit is formed by the NBD (van der Heide and Poolman, 2002).

Architecture of ABC efflux systems

In contrast to ABC uptake systems, which are present in the cytoplasmic membrane of prokaryotic species (bacteria and Archaea), ABC efflux systems are found in every life-form ranging from microorganisms to man. ABC efflux pumps can be found in the cytoplasmic membrane of prokaryotes and eukaryotes, as well as in the organellar membranes of the higher organisms, that are, the endoplasmic reticulum, inner mitochondrial membrane,

peroxisomal and vacuolar membranes. Although the common four-domain structure of the translocator (two TMDs *plus* two NBDs) is conserved, ABC efflux pumps are somewhat less complex compared to ABC uptake systems in that they do not require a SBP for function (Fig. 1 C and D). The direction of transport can be deduced from the presence or not of this latter component. An exception to this rule has yet to be found. Even more frequently than in ABC uptake systems, the four core-domains of the translocator are fused, yielding only one or two unique polypeptides forming the functional unit (Fig. 1C and D). The most common organization is the "half-size transporter" in which a single TMD is fused to the N- or C-terminus of a NBD. The functional complex is either a homo- or heterodimer of two half-transporters (*e.g.,* the lipid flippases, multi-drug transporters, cysteine exporter, and peptide transporters; Fig. 1C and D). Finally, all four core-domains (two half-transporters) can also be fused as is the case for the multi-drug transporters (*e.g.,* P-gp, MRP1 and 2, and PDR5), and the ABC type chloride channel CFTR (Fig. 1D), and these are termed "full-length transporters".

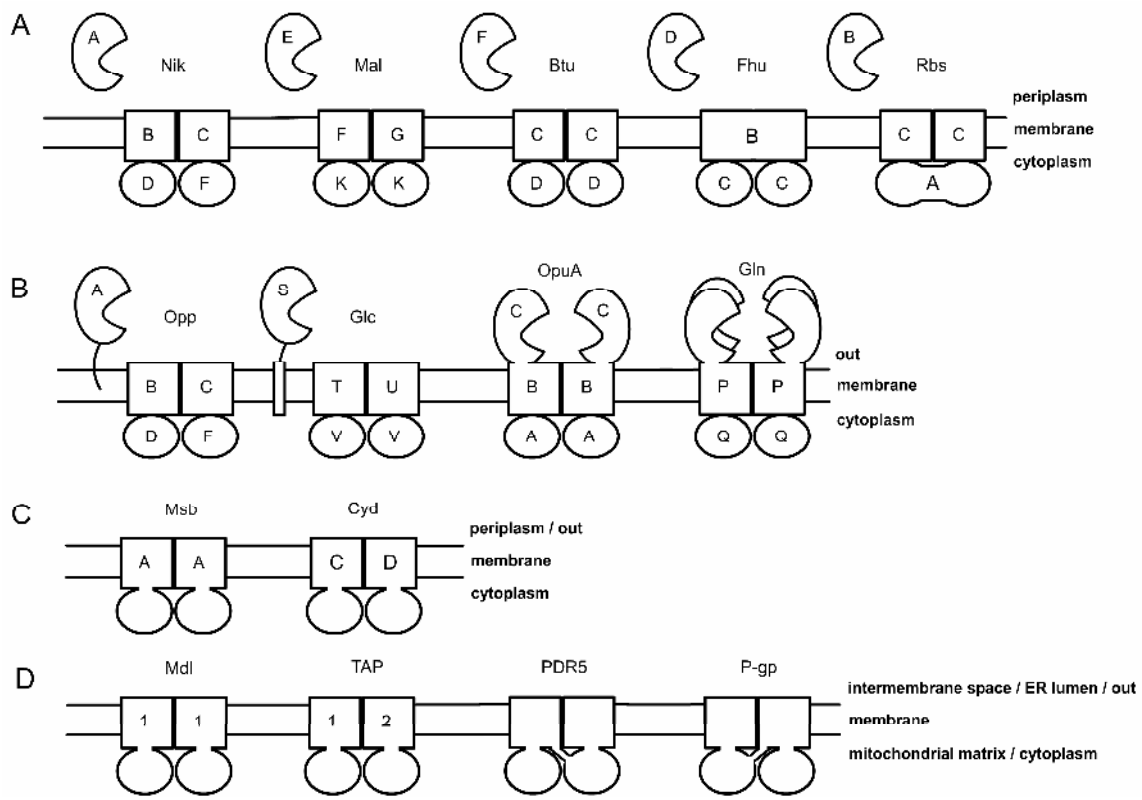

Fig. 1. **Domain architecture of ABC transporters.** Schematically indicated are: SBPs (Pac-man shaped), TMDs (rectangles), and NBDs (ovals). (A) Gram-negative bacteria (all the examples are from *Escherichia coli*): Nik, nickel transporter; Mal, maltose/maltodextrin transporter; Btu, vitamin B12 transporter; Fhu, siderophore/haem/vitamin B12 transporter; Rbs, ribose transporter. (B) Gram-positive bacteria and Archaea: Opp, oligopeptide transporter from *Lactococcus lactis*; Glc, glucose transporter from *Sulfolobus solfataricus*; OpuA, glycine betaine transporter from *Lactococcus lactis*; Gln, glutamine/glutamic acid transporter from *Lactococcus lactis*. (C) and (D) Functional and structural homologues are present in all three kingdoms of life. (C) Msb, lipid flippase from *Escherichia coli*; Cyd, cysteine exporter from *Escherichia coli*. (D) Mdl, mitochondrial peptide transporter from *Saccharomyces cerevisiae*; TAP1/2 (ABCB2/3), human peptide transporter; PDR5, yeast pleiotropic drug resistance transporter; P-gp (MDR1/ABCB1), human multidrug transporter.

Transmembrane domains

The TMDs are two highly hydrophobic proteins or protein domains that create the channel through which the substrate passes during translocation (Fig. 1). In ABC uptake systems they form the docking site for the SBP and transduce a signal to the NBDs to bind and hydrolyze ATP. The TMDs are bundles of α-helices that traverse the membrane multiple times in a zig-zag fashion. Many typical ABC transporters conform to the paradigmatic "two-times-six" α-helices structure, yielding a total of 12 transmembrane segments (TMSs) per functional unit (Table 1). However, the number of TMSs may vary from anywhere between 5 to 11 for each individual TMD.
Whereas the NBDs show considerable sequence identity (see below), the TMDs (as well as the SBPs) have been less well conserved during evolution, reflecting the large diversity of substrates transported. TMDs of ABC uptake systems contain the so-called EAA motif (L-loop), which usually is found in the cytoplasmic loop between TMS 5 and 6 (Saurin and Dassa, 1994). This motif is absent in ABC exporters.
In ABC efflux systems, the TMDs harbor the substrate-binding site(s). Evidence for the existence of a substrate-binding site in the TMDs of ABC uptake systems came from the analysis of SBP-independent mutants (Treptow and Shuman, 1985; Speiser and Ames, 1991; Petronilli and Ames, 1991; Covitz *et al.*, 1994). Mutations in the NBD of the histidine transporter (HisP) or the TMDs of the maltose transporter (MalF and G) resulted in an uncoupled phenotype, in which the SBP no longer interacted with the TMDs and the NBDs hydrolyzed ATP constitutively. In addition, a SBP-independent maltose transporter with a truncated form of MalF had its substrate specificity changed from maltose to lactose, albeit with very high affinity constants (Merino and Shuman, 1998). Finally, the crystal structure of the BtuCD translocator revealed a cleft in the periplasmic half of the TMDs that could accommodate a vitamin B12 molecule and represent a genuine substrate-binding site (Locher *et al.*, 2002).

Nucleotide-binding domains

The engines of ABC transporters are the NBDs as these power substrate translocation by ATP-binding and hydrolysis. Whereas the SBPs and TMDs are poorly conserved, the NBDs are more closely related and group the different transporter families in the ABC superfamily. They contain the ATP-binding cassettes, hence the name ABC transporters. Many crystal structures of isolated NBDs are available, yielding detailed information about the overall fold and the nucleotide-binding site(s) (Davidson and Chen, 2004). An NBD consists of two domains, a RecA-like and a helical domain, the latter being specific for ABC transporters. In the NBD monomer, the ATP-binding site is formed by two conserved sequences, the Walker A (P-loop) and Walker B motifs, present in many ATP-binding proteins and not restricted to ABC proteins (Walker *et al.*, 1982). These motifs bind the phosphates of ATP and ADP, and coordinate a Mg^{2+}-ion via H_2O in the nucleotide-binding site, respectively. A glutamate directly following the Walker B motif might be the catalytic base for hydrolysis, since it binds to the attacking water and Mg^{2+}-ion, and mutation of this residue leads to loss of ATPase activity (Davidson and Chen, 2004). The Q-loop also forms hydrogen bonds with Mg^{2+} and the attacking water. A conserved histidine in the H motif hydrogen bonds with the γ-phosphate of ATP. In the NBD dimer the signature sequence, LSGGQ, (the motif that distinguishes ABC

Table 1. **Overview of TMD topology in well characterized ABC transporters**

		TMD	Transport substrate	Number of TMSs			Total number of TMSs	Reference
				N-terminal	Core-domain	C-terminal		
ABC uptake systems		HisQ	Histidine		**5**		**10** (2 x 5)	Kerppola *et al.*, 1992
		HisM			**5**			
		GlnP	Glutamine/ glutamic acid		**5**[a]		**10** (2 x 5)	Schuurman-Wolters and Poolman, 2005
		ProW	Glycine betaine/Proline betaine	2	**5**		**14** (2 x 7)	Haardt and Bremer, 1996
		OpuABC	Gycine betaine	2	**5**	1[b]	**16** (2 x 8)	van der Heide and Poolman, 2002
		OppB	Oligopeptides		**6**		**12** (2 x 6)	Pearce *et al.*, 1992
		OppC			**6**			
		MalF	Maltose/ maltodextrins	2	**6**		**14** (8 + 6)	Boyd *et al.*, 1987
		MalG			**6**			Dassa and Muir, 1993
		RbsC	Ribose		**10**[c]		**20** (2 x 10)	Stewart and Hermodson, 2003
		BtuC	Vitamin B12		**10**		**20** (2 x 10)	Locher *et al.*, 2002
		FhuB	Siderophores/haem/ vitamin B12		**10** x 2		**20**	Groeger and Köster, 1998
ABC efflux systems	Half-transporters	LmrA	Drugs		**6**[d]		**12** (2 x 6)	van Veen *et al.*, 1996
		BmrA	Drugs		**6**[d]		**12** (2 x 6)	Steinfels *et al.*, 2004
		MsbA	Lipids		**6**		**12** (2 x 6)	Chang and Roth, 2001
		CydC	Cysteine/GSH		**6**		**12** (2 x 6)	Cruz-Ramos *et al.*, 2004
		CydD			**6**			
		HlyB	Haemolysin	(2)[e]	**6**		**12-16**[e] (2 x 6-8)	Wang *et al.*, 1991 Gentshev and Goebel, 1992
	Full-length transporters	TAP1(ABCB2)	Antigenic	4	**6**		**19** (10 + 9)	Koch *et al.*, 2004
		TAP2(ABCB3)	peptides	3	**6**			
		P-gp (MDR1/ABCB1)	Drugs		**6** x 2		**12**	Loo and Clarke, 1999
		BCRP (ABCG2)	Drugs		**6** x 2		**12**	Leslie *et al.*, 2005
		MRP1 (ABCC1)	Drugs	5	**6** x 2		**17** (5 + 12)	Leslie *et al.*, 2005
		MRP2 (ABCC2)	Drugs	5	**6** x 2		**17** (5 + 12)	Leslie *et al.*, 2005
		SUR1 (ABCC8)	None/unknown[f]	5	**6** x 2		**17** (5 + 12)	Conti *et al.*, 2001
		CFTR (ABCC7)[g]	Chloride		**6** x 2		**12**	Sheppard and Welsh, 1999
		Ste6	Mating pheromone		**6** x 2[h]		**12**	Geller *et al.*, 1996
		ABCR (ABCA4)	Retinal/retinal-phosphatidylethanolamine		**6** x 2[i]		**12**	Bungert *et al.*, 2001

[a]Two SBDs are fused in tandem to the first TMS. The N-terminal signal sequence, preceding the SBDs, is cleaved off. [b]The SBD is fused to a C-terminal signal anchor sequence (the 8th TMS). [c]Initially claimed to have 6 TMSs (Park and Park, 1999); later shown to possess 10 TMSs (Stewart and Hermodson, 2003). [d]No experimental evidence; topology based on homology with other multidrug-efflux systems. [e]HlyB is thought to contain 6-8 TMSs; the presence of two N-terminal TMSs has not been unambiguously determined. [f]Sulfonylurea receptors (SURx) associate with K_{IR}6.x to form ATP-sensitive potassium channels. [g]CFTR is a chloride channel rather than transporter although it has the classical two TMDs plus two NBDs structure observed for genuine ABC transporters. [h]Experimental data only for the N-terminal TMD. [i]Two large extracytoplasmic glycosylated domains are inserted between the first and second TMS of both the N- and C-terminal TMDs.

proteins from other nucleotide-binding proteins) of one NBD binds to the γ-phosphate of ATP bound to the other NBD, thereby completing the nucleotide-binding sites (Davidson and Chen, 2004). Thus, two ATPs are sandwiched in head-to-tail orientation between two NBDs in the catalytically active dimer.

ABC transporter mechanism

Given the conserved architecture of two TMDs plus two NBDs across the entire ABC transporter superfamily, one could expect a common translocation mechanism for ABC uptake and efflux systems. Indeed, from the culmination of vast amounts of functional data, in combination with the recent X-ray structures of complete ABC transporters (for both efflux and uptake systems; Chang and Roth, 2001; Locher *et al.*, 2002; Chang, 2003; Davidson and Chen, 2004; Higgins and Linton, 2004; Locher, 2004; van der Does and Tampé, 2004; Reyes and Chang, 2005), a consensus mechanism is emerging. The simplest model of SBP-dependent uptake encompasses four steps (Fig. 2): i) substrate binds to the SBP; ii) the closed, liganded SBP docks onto the TMDs and signals the NBDs to cooperatively bind ATP; iii) upon ATP-binding, a binding site in the TMDs is made available to the outside, the SBP opens and substrate is transferred to the binding site in the TMDs; and iv) ATP hydrolysis dissociates the NBD dimer and results in reorientation of the binding site from an outward- to an inward-facing conformation. The substrate arrives in the cytoplasm and the SBP is released from the TMDs. Alternatively, it cannot be ruled out that upon ATP binding the substrate is transferred directly to the cytoplasm (step iii) and ATP hydrolysis in combination with dissociation of ADP and inorganic phosphate is solely used to reset the system for another translocation cycle. Whereas step i) is unique to SBP-dependent uptake systems, steps ii-iv) could be envisaged for ABC efflux systems as well. In step ii) a substrate bound to the inward-facing binding site would signal the NBDs to bind ATP. Subsequently, step iii), ATP-binding reorients the binding site and substrate is released to the outside. iv) ATP-hydrolysis resets the system by converting the TMDs back to the inward-facing conformation.

Although ABC uptake and efflux systems could share a common translocation mechanism, there are some indications that things might be more complicated. First of all, although in nature TMDs and NBDs appear as separate modules or fused together in almost any possible combination (Fig. 1), to the best of our knowledge, no ABC uptake systems are known that have a TMD fused to a NBD as is the case with half- or full-length efflux systems. For ABC efflux systems, this latter architecture seems to be a universal feature. Second, the conserved EAA motif (see above) is found exclusively in ABC uptake systems. This motif is thought to be important for the interaction of the TMDs with the NBDs. Third, the structures of the ABC efflux system MsbA showed an additional intracellular domain (ICD) between the TMD and NBD that was not present in the structure of the SBP-dependent vitamin B12 uptake system BtuCD (Chang and Roth, 2001; Locher *et al*., 2002; Chang, 2003; Reyes and Chang, 2005). Fourth, NBD2 of CFTR was shown, in addition to hydrolyzing ATP (Mg-ATP + $H_2O \rightarrow$ Mg-ADP + Pi), to display adenylate kinase activity (Mg-ATP + AMP $\leftrightarrow$ Mg-ADP + ADP), which influenced channel activity (Randak and Welsh, 2005). It remains to be established whether the adenylate kinase activity is unique to CFTR or ABC efflux systems, but some important structural differences between ABC uptake and efflux systems seem to exist. The significance of these observations in terms of a general ABC transport mechanism awaits further experimentation.

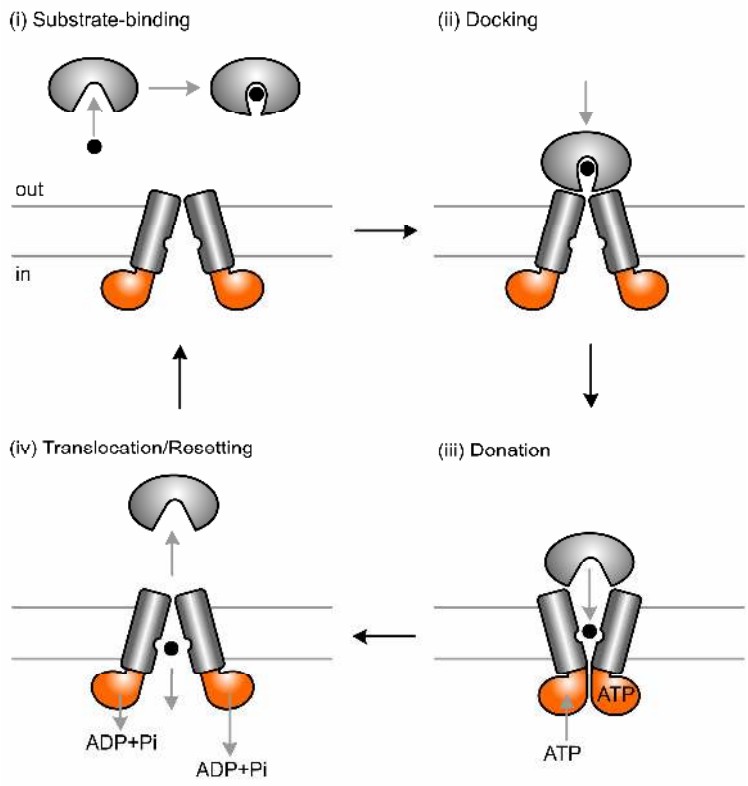

Fig. 2. **Model of the translocation mechanism of a SBP-dependent ABC transporter.** For explanation, see text.

Accessory domains in ABC transporters

In addition to the ubiquitous four core-domains, that are two TMDs *plus* two NBDs, accessory domains can be found in several ABC transporters (Fig. 3). These extra domains often perform different functions and can be found separate from, attached to, or integrated into different parts of the core ABC transporter. On the basis of their location (extrinsic or intrinsic to the membrane) and function (regulatory or catalytic) within the cognate transporters, we divide the well characterized accessory domains of ABC transporters into four different groups: i) extracytoplasmic domains; ii) membrane-embedded domains; iii) cytosolic regulatory domains; and iv) cytosolic catalytic domains.

Extracytoplasmic domains. One of the characteristics of accessory domains is that they are not part of the general ABC transporter core complex. There are three main examples of known extracytoplasmic domains or proteins that can be considered accessory units i) the large extracytoplasmic domains (ECDs) found in members of the ABCA family; ii) the extracellular N-terminal domain of ProW (and closely related homologues), the membrane component of the ProU system from *E. coli*; and iii) the substrate-binding domains or proteins found in prokaryotes.

Members of the ABCA family are full-length transporters consisting of two TMDs of 6 TMSs each and two NBDs, each located C-terminal of one of the TMDs (Table 1; Peelman *et al.*, 2003). The two ECDs are predicted after the first TMS of the N- and C-terminal half of the transporter (Fig. 3C) and are 600 and 275 amino acids long in ABCA4 (ABCR; Bungert *et al.*, 2001). Eight *N*-glycosylation sites were mapped by mutagenesis onto the ECDs of ABCA4, four in the N-terminal half and four in the C-terminal half of the protein (Bungert *et al.*, 2001). Removal of these *N*-glycosylation sites resulted in reduced expression levels, although the mutants retained the capacity to bind ATP. It has been postulated that the two ECDs are linked by at least one disulfide bridge. Intra- and intermolecular crosslinking of cysteine residues in an ECD was shown for ABCG2 (BCRP; Henriksen *et al.*, 2005), a halftransporter with the ECD located between TMS 5 and TMS 6. Two ABCG2 subunits were linked by a disulfide bridge, and two remaining cysteine residues formed intramolecular disulfide bonds within the ECD. Although mutations in the ECDs have been shown to influence the substrate specificity of the transporters, a distinct function is not known for these relatively large extracytoplasmic domains.

The second example of an extracytoplasmic domain is found in the proline and glycine betaine transport system (ProU) of *E. coli*. Here the membrane component, ProW, is composed of an extended N-terminal region of about 100 amino acid residues followed by 7 TMS (Table 1). The function of the N-terminal region is unknown, but a phoA and LacZ fusion study showed that its location is periplasmic (Haardt and Bremer, 1996).

The best-studied extracytoplasmic domains or proteins are the substrate-binding domains or proteins of ABC-type uptake systems. These domains or proteins are considered to be accessory, because these components are not intrinsic to the translocation process as has been demonstrated for ABC transporter mutants that function in the absence of SBPs (see section 'Transmembrane domains'). SBPs are the main determinants of substrate specificity of ABC uptake systems and confer high-affinity on the transport process with dissociation constants (K_d) in the (sub)micromolar range. They have been a gold-mine for structural studies due to their well behavior during overexpression, purification and crystallization trials. Although some early experiments suggested that SBPs might self-associate to form dimers or higher order oligomers (Antonov *et al.*, 1976; Rashed *et al.*, 1976; Richarme, 1982, 1983), crystal structures of numerous SBPs have led to the generally accepted view that they are monomers with one substrate-binding site per molecule (Quiocho and Ledvina, 1996). Even though systems are known that use multiple SBPs to broaden the substrate specificity of the transporter (several peptide transporters; Alloing *et al.*, 1994; Lin *et al.*, 2001; Doeven *et al.*, 2005), only a single SBP is required for transport function. The high resolution structure of the vitamin B12 importer BtuCD confirmed that there is space for only a single SBP to dock onto the TMDs (Borths *et al.*, 2002). The ABC transporters with one or two SBDs fused to the TMD are thought to function similarly, except for that the presence of a second, third or fourth SBD might affect the kinetics of the translocation process (Biemans-Oldehinkel and Poolman, 2003) or perhaps broaden the substrate specificity.

The archetype SBP consists of two domains or lobes connected by a flexible linker. The two lobes close and engulf the ligand upon substrate-binding (Venus's Flytrap model; Mao *et al.*, 1982). Structures are available of SBPs in open-unliganded, closed-unliganded, open-liganded, and closed-liganded forms (Tame *et al.*, 1994; Nickitenko *et al.*, 1995; Heddle *et al.*, 2003; Karpowich *et al.*, 2003; Trakhanov *et al.*, 2005), of which the latter conformation is thought to productively interact with the membrane complex (Prossnitz *et al.*, 1989; Dean *et*

al., 1992; Doeven *et al.*, 2004). In some cases, unliganded SBPs also have access to the TMDs, thereby inhibiting ABC transporter function (*e.g.,* in case of the maltose- and histidine-binding proteins; Merino *et al.*, 1995; Ames *et al.*, 1996). These observations, however, could not be confirmed in similar experiments performed with the oligopeptide ABC transporter (Doeven *et al.*, 2004). Mutational and suppressor analyses suggested that each lobe of the SBP binds to one of the TMDs (Treptow and Shuman, 1985; Park *et al.*, 1999). Site-directed mutagenesis and pre-steady state kinetic analysis indicated that donation of the substrate by the SBP to the TMDs is the rate-limiting step during the ABC uptake catalytic cycle of the oligopeptide transporter Opp (Lanfermeijer *et al.*, 1999; Picon *et al.*, 2000).
Although a typical SBP consist of two lobes (domains) that are connected by a flexible hinge (Quiocho and Ledvina, 1996), exceptions to this rule are the SBPs of the nickel/oligopeptide transporter family (Fig. 3A). OppA contains an extra, third domain (Tame *et al.*, 1994). The function of this additional domain is unknown, although in AppA, an OppA homologue from *Bacillus subtilis,* it has been shown to form part of the binding site for nonameric peptides (Levdikov *et al.*, 2005).
Two ABC transporter families (OTCN and PAO) are known in which one or two SBPs are fused to either the N- or C-terminus of one TMD, yielding a total of two or four SBDs per functional complex (Fig. 1B, OpuA and GlnPQ; van der Heide and Poolman, 2002). Two SBDs are present in OpuA, the osmoregulatory ABC transporter from *Lactococcus lactis*, although for activity only one SBD is needed. The SBDs of OpuA interact in a cooperative manner by stimulating either the docking of one domain onto the TMDs or substrate donation to the TMDs (Fig. 2, steps ii) and iii), respectively; Biemans-Oldehinkel and Poolman, 2003). However, this cooperative interaction has not been established for other SBP dependent ABC transporters, *e.g.,* the glutamate/glutamine transporter GlnPQ from *L. lactis*, containing four SBDs (Schuurman-Wolters and Poolman, 2005). In GlnPQ, the SBD in the primary sequence nearest to the translocator domain (SBD2) is used as high affinity receptor that delivers the substrate to the TMDs. SBD1, on the contrary, is not required for high affinity transport and is not sufficient for transport but does bind glutamine with a low affinity (dissociation constant in the (sub)millimolar instead of micromolar range; Schuurman-Wolters, unpublished results).

Membrane-embedded domains. The majority of ABC transporters are predicted to have six TMSs per TMD core (Table 1). Thus, in a typical transporter a total of 12 TMSs are present. Exceptions are known for ABC uptake systems that contain 5 or 10 TMSs as a single TMD core that is both essential and sufficient for transport. In addition to this somewhat variable number of TMSs in the TMDs, several ABC transporters contain extra TMSs that are not part of the core TMD and can be considered as accessory domains. Although this phenomenon frequently occurs, the function of these extra domains is in many cases unknown. For example, within the OTCN family of ABC transporters (Dassa and Bouige, 2001), sequence alignments reveal a core TMD of 5 TMSs but homologues can have in total either 5, 6 or 8 TMSs. The core TMSs are generally much better conserved than the accessory TMSs (van der Heide and Poolman, 2002; unpublished results). Within the OTCN family, some polypeptides (*f.i.,* the OpuABC subunit of OpuA, Fig. 3B, van der Heide and Poolman, 2002; and the ProW subunit of ProU, Haardt and Bremer, 1996) have two extra TMSs N-terminal of the TMD core but their function is unknown. In addition, some members have an 8th TMS (or 6th TMS in case the first two are missing), C-terminal of the TMD core, that serves as signal anchor sequence for the translocation of the extracytoplasmic SBD. Also, members of the OSP

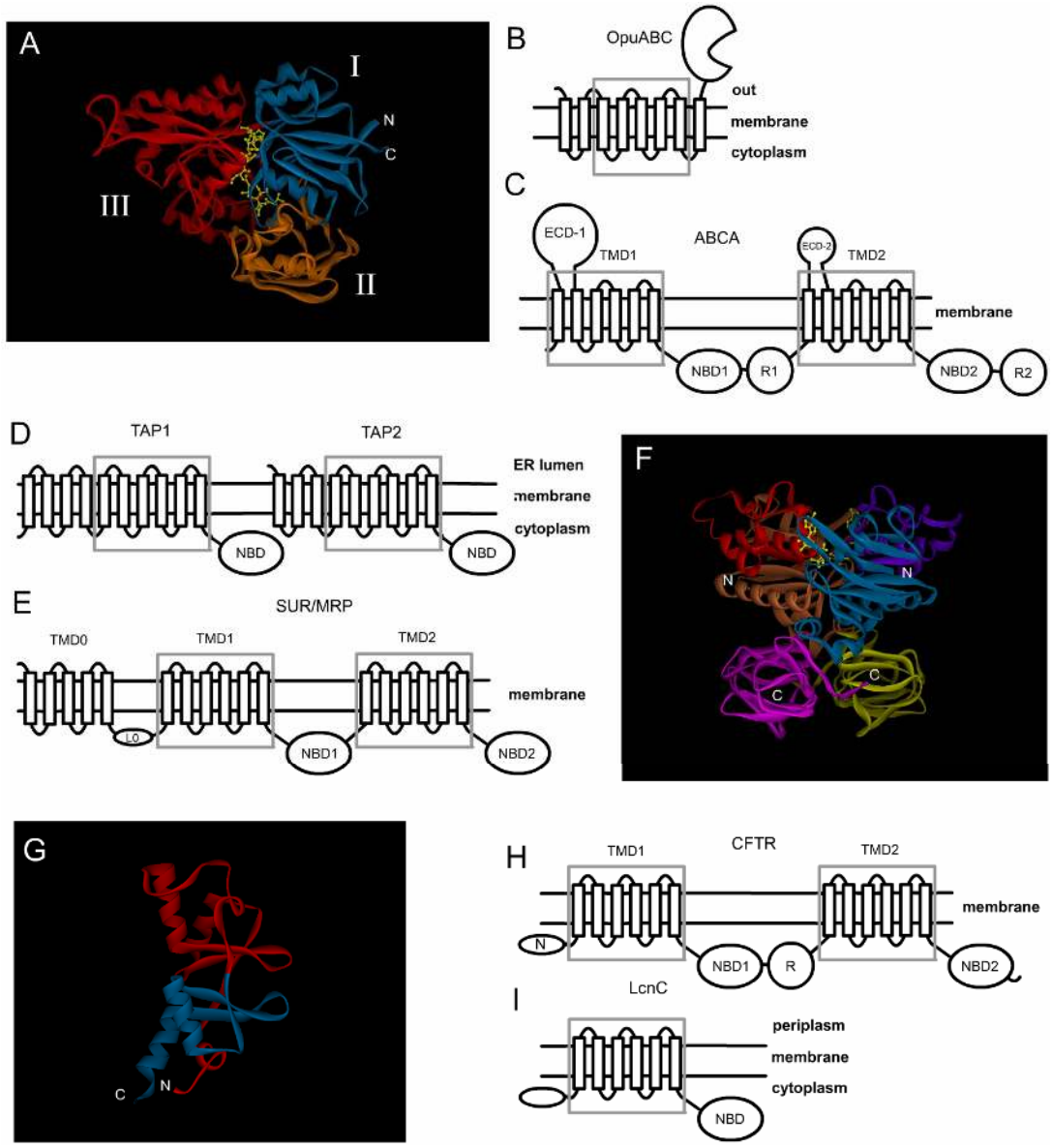

Fig. 3. **Well-characterized accessory domains in ABC transporters.** TMD core domains are boxed (grey lines). (A) Structure of the oligopeptide-binding protein AppA from *Bacillus subtilis* (PDB accession number 1XOC). Domain I and III (blue and red, respectively) are common to SBPs. The extra domain (II, orange) contributes with a few residues to the binding site by interacting with the N-terminal part of the bound nonameric peptide (yellow). (B) Topology of OpuABC, TMD with SBP fused to C-terminus, of the glycine betaine transporter OpuA from *Lactococcus lactis*. The NBD is a separate protein (Fig. 1B). (C) Topology of members of the ABCA family. Extracellular loops (ECD-1 and ECD-2) and cytosolic regulatory domains (R1 and R2) are shown. (D) TMD topology of TAP1 and 2 half-transporters. (E) Topology of the SUR/MRP (ABCC) family. The extra TMD (TMD0) and L0-loop are indicated. (F) Structure of dimeric nucleotide-bound MalK from *Escherichia coli* (PDB accession number 1Q12), the NBD of the maltose/maltodextrin transporter. The common helical and RecA-like domains are shown in red and orange for the first monomer and dark purple and blue for the second, respectively. Two ATPs (yellow) are sandwiched between the NBDs. The additional C-terminal domain is shown in yellow for the first and bright purple for the second dimer subunit. (G) Structure of a tandem CBS domain from *Thermotoga maritima* (PDB accession number 1O50) as present in the C-terminal part of the NBD of OpuA (OpuAA). The N- and C-terminal halves are colored red and blue, respectively. (H) Topology of CFTR. The N-tail and regulatory (R) domain are indicated. (I) Putative TMD topology of LcnC, a bacteriocin exporter with an N-terminal peptidase-like domain.

family to which the maltose transporter belongs can have two extra N-terminal TMSs (MalF; Boyd *et al.*, 1987). For the maltose transporter, the extra TMSs of MalF have been proposed to be an export signal, but most of the two TMSs could be removed without any effect on maltose transport activity (Boyd *et al.*, 1987; Ehrmann *et al.*, 1990; Ehrmann and Beckwith, 1991).
A system in which a clear function could be assigned to the accessory TMSs is the transporter associated with antigen processing (TAP), present in the endoplasmic reticulum membrane. Based on hydrophobicity analysis and sequence alignments 10 (4 accessory plus 6 core) and 9 (3 accessory plus 6 core) TMSs were predicted for human TAP1 and TAP2, respectively (Table 1 and Fig. 3D; Abele and Tampé, 1999; Koch *et al.*, 2004). Although the core-TAP complex (the C-terminal 6 TMSs from TAP1 and TAP2 combined) represented the minimal functional unit for transport, the N-terminal domains of TAP1 and TAP2 were essential for recruitment of tapasin (Koch *et al.*, 2004; Procko *et al.*, 2005), a glycoprotein known to mediate association between TAP and MHC class I molecules (Bangia *et al.*, 1999). Consequently, the extra domains were proposed to mediate the assembly of the macromolecular peptide-loading complex. Subsequently, it was shown that in TAP2 the tapasin-binding site is exclusively located in the N-terminal domain. Upon deletion of the N-terminal domain of TAP1, tapasin could still interact with the TAP1 TMD core (Leonhardt *et al.*, 2005). TAP variants lacking the N-terminal domain of TAP2, but not of TAP1, were disturbed in the quality control of MHC I loading, and formed peptide loading complexes that were disturbed in their physical interaction with calreticulin, calnexin, and ER60.
In stead of a few extra TMSs, some ABC systems, including the sulfonylurea receptors SUR1 and SUR2, and the multidrug-resistance proteins MRP1, 2, 3, 6 and 7, have 5 accessory TMSs that may form a domain by itself (Table 1 and Fig. 3E; Bakos *et al.*, 1996; Stride *et al.*, 1996; Tusnády *et al.*, 1997). These transporters belong to the ABCC family of the ABC transporter superfamily. The core complex in this subfamily is a full-length transporter composed of two TMDs (TMD1 and TMD2, each containing 6 TMSs) and two NBDs (NBD1 and NBD2) that follow TMD1 and TMD2, respectively. The extra TMSs (indicated as TMD0) are linked via a cytoplasmic loop (L0) to the N-terminus of TMD1 (Fig. 3E). In MRP1, the entire TMD0 could be deleted without having any effect on transport activity or on the trafficking and insertion of the protein into the basolateral membrane (Bakos *et al.*, 1998; Westlake *et al.*, 2003). This contrasted results obtained with SUR1 and MRP2, where deletion of TMD0 affected channel activity, processing and trafficking (Sharma *et al.*, 1999; Nies *et al.*, 2002; Fernandez *et al.*, 2002; Babenko *et al.*, 2003; Chan *et al.*, 2003). Recent studies revealed that TMD0 is needed for trafficking if the C-terminal region of MRP1 is mutated or deleted (Weslake *et al.*, 2005). It seems that in MRP1 TMD0 and the C-terminal region contain redundant trafficking signals that become essential when one or the other region is missing or mutated.
In SUR, TMD0 is required for activation of the inwardly rectifying potassium channel (K_{IR}) subunits and anchoring of the K_{IR} subunits to the ABC transporter core of SUR, thereby generating functional K_{ATP} channels (Babenko *et al.*, 2003; Chan *et al.*, 2003). Thus, similar to the TAP system, the accessory membrane-embedded domains of SUR provide additional protein interaction sites and serve a regulatory purpose.

Cytosolic regulatory domains. Regulation of many ABC transporters takes place at the level of gene expression. Most ABC transporter operons encode a transcriptional regulator that connects the level of protein to environmental conditions. Expression of the osmoregulatory ABC transporter OpuA from *Lactococcus lactis* is, for example, regulated in response to

osmotic stress via the transcription factor OpuR (Romeo *et al.*, 2003). However, the activity of ABC transporters, once expressed, may also be regulated. For this purpose, additional (autoregulatory) domains in the transporter itself are required. Examples are the L0 linker in MRP1 and SUR, the C-terminal domain of MalK of the maltose transporter from *E. coli* and *S. typhimurium*, the tandem cystathionine-β-synthase (CBS) domains of OpuA from *L. lactis* and other members of the OTCN family, and the R-domain of CFTR (Fig. 3E, F, G, and H, respectively). Although these domains all regulate ABC transporter activity, they use different mechanisms to do so.

The maltose/maltodextrin transporter from *E.coli* and *S. typhimurium* is directly involved in transcriptional regulation of, amongst others, itself. The regulation takes place at the C-terminal domain of MalK, the NBD of the transporter (Fig. 1A (Mal) and 3F; Chen *et al.*, 2003). Crystal structures are available of MalK (Böhm *et al.*, 2002), and GlcV, the NBD of the glucose transporter from *Sulfolobus solfataricus* (Verdon *et al.*, 2003). These NBDs contain an accessory C-terminal domain with a common tertiary fold. The C-terminal domain of MalK is involved in two regulatory processes, at least in enteric bacteria (Böhm and Boos, 2004). First, via its C-terminal domain, MalK is subject to regulation by IIA^{glc}, a component of the bacterial phosphoenolpyruvate-dependent sugar:phosphotransferase system (PTS) (Nelson and Postma, 1984; Dean *et al.*, 1991; Böhm *et al.*, 2002). IIA^{glc} can be in the unphosphorylated and phosphorylated state, depending on the availability of PTS sugars. In the unphosphorylated form, *i.e.,* when a PTS sugar such as glucose is present, IIA^{glc} binds to MalK and thereby prevents maltose uptake. The inhibition is relieved when glucose is exhausted and IIA^{glc} becomes phosphorylated. This regulatory process determines the hierarchy of sugar utilization and is known as inducer exclusion mechanism. GlcV is unlikely to be involved in this type of regulation, since Archaea lack a PTS. Second, MalK inhibits the activity of MalT, the positive regulator of the maltose regulon, by competing with MalT for maltotriose binding (Reyes and Shuman, 1988; Panagiotidis *et al.*, 1998; Boos and Böhm, 2000; Böhm *et al.*, 2002). Binding of maltotriose to MalT is required for oligomerization of the transcription factor, a prerequisite for the interaction of MalT with its DNA binding sites (Schreiber and Richet, 1999; Danot, 2001). In addition to MalK, two other proteins, MalY and Aes, can also bind MalT (Schreiber *et al.*, 2000; Joly *et al.*, 2002), creating an intricate regulatory network that will not be detailed further in this chapter. MalK is thought to possess two distinct protein binding sites, one for MalT and one for IIA^{glc}, because mutations in MalK affecting inducer exclusion do not interfere with MalT inactivation (Nelson and Postma, 1984; Kühnau *et al.*, 1991). By mapping known mutations onto the structure of MalK (the one from *Thermococcus litoralis*), the MalT interaction site has been proposed to be located on an exposed surface of the C-terminal regulatory domain. Point mutations that confer an inducer exclusion insensitive phenotype form a patch adjacent to and oriented perpendicularly to the MalT interaction site (Böhm *et al.*, 2002).

The osmoregulatory ABC transporter OpuA protects the cell against hyperosmotic stress, ultimately plasmolysis, through uptake of the compatible solute glycine betaine, and thereby reverses the osmotic shrinkage of the cell (van der Heide and Poolman, 2000). When reconstituted in proteoliposomes, OpuA is activated by increasing internal ionic strength. The threshold for activation by ions is dependent on the ionic lipid content (charge density) of the membrane, indicating that the ionic signal is transduced to the transporter via critical interactions of protein domains with membrane lipids (van der Heide *et al.*, 2001). The ABC transporter OpuA, as do other members of the OTCN family, has two CBS domains (Fig. 3G) in tandem at the C-terminal end of the ATPase subunit OpuAA. With two ATPase subunits per

translocation unit, a total of four CBS domains are present in OpuA. This so-called CBS module of OpuA constitutes the sensor that switches the transporter between an inactive, electrostatically locked and an active, thermally relaxed state, by interacting with the negatively-charged membrane surface in response to the ionic strength (unpublished results). The switching mechanism is an effective means for cells to respond to osmotic shifts, as an increase in medium osmolality will result in a decrease in cell volume, and the accompanying increase in cytoplasmic ionic strength will activate the transporter. Glycine betaine accumulation, followed by water influx, will increase the cell volume and decrease the ionic strength in due time, and the transporter will ultimately be switched off. This inherent feedback mechanism prevents over-accumulation of glycine betaine and couples transporter activity with ionic strength in the cell.

CFTR is an ABC-type chloride channel that is regulated by protein kinase A-mediated phosphorylation of its cytoplasmic regulatory (R) domain (Rich *et al.*, 1991). The R-domain is located between NBD1 and TMD2 of the full-length transporter (Fig. 3G), and contains a number of charged residues and multiple consensus phosphorylation sites (Gadsby and Nairn, 1999; Dahan *et al.*, 2001). The unphosphorylated R-domain is thought to inhibit channel activity by interaction with the CFTR core transporter. Release of the R-domain by phosphorylation would activate the channel (Rich *et al.*, 1993; Csanády *et al.*, 2000; Baldursson *et al.*, 2001). Thus far, phosphorylation dependent release of the R-domain has not been demonstrated unequivocally. Correspondingly, a recent study strongly suggested that phosphorylation results in enhanced association of the R-domain with the transporter rather than in its release (Chappe *et al.*, 2005). The mechanism of regulation suggested by Chappe *et al.* was that binding of the R-domain to the transporter induces intramolecular rearrangements that allow nucleotide-induced conformational changes to be transmitted from the NBDs to the TMDs. A second system that was shown to be regulated by phosphorylation of a cytoplasmic domain is ABCA1 (Roosbeek *et al.*, 2004). This half transporter is a member of the ABCA family in which the R-domains (R1 and R2) are located C-terminal of the NBDs of the C- and N-terminal half of the transporter, respectively (Fig. 3C). Phosphorylation of the R1 domain of ABCA1 diminished the phospholipid flip-flop, apolipoprotein binding, and cholesterol/lipid extrusion activities.

The N-terminal tail of CFTR (N-tail; Fig. 3H), next to its R-domain, also plays a role in regulating CFTR channel activity. It modulates the rate of channel openings, probably by interacting with components of CFTR (R-domain or NBD), or with the pore itself (Fu and Kirk, 2001). Next to a suggested physical interaction with a portion of the R-domain (Naren *et al.*, 1999), the N-tail and the C-terminal tails of CFTR have been shown to bind several inhibitors and stimulators (physical modulators) of CFTR activity (Li and Naren, 2005). These interaction partners either bind directly to CFTR or mediated through various PDZ domain-containing proteins. One of the PDZ containing proteins is the Na^+/H^+ exchanger regulatory factor (NHERF) that can form homo- and hetero-oligomers between CFTR and other membrane proteins, *e.g.,* the β_2-adrenergic receptor (Wang *et al.*, 2000; Naren *et al.*, 2003). It has been recently shown that the assembly of the C-terminal domains of CFTR is regulated by ezrin, an actin binding protein (Li *et al.*, 2005). The dimeric CFTR-NHERF-ezrin interaction would provide a pathway to regulate CFTR activity via the cytoskeleton actin network. Moreover, there is evidence that when two subunits of CFTR are brought in close proximity by NHERF, the open probability of the channels is increased (Raghuram *et al.*, 2003). Finally, the increasing number of proteins interacting with CFTR suggests that the chloride conductor is

not only regulated itself, but that CFTR might also regulate the activities of other ion channels, receptors, or transporters.
The N-tail of CFTR contains a cluster of acidic residues which are located onto one face of a predicted α-helix (Naren *et al.*, 1999; Fu *et al.*, 2001). A similar amphipatic α-helix has been predicted for the L0 linker in the sulfonylurea receptor SUR (Bakos *et al.*, 2000). This linker is located between TMD0 and TMD1 (Fig. 3E), and consequently links TMD1 and TMD2 of SUR to the TMD0/K_{IR} complex. Interaction of the N-terminus of K_{IR} with L0 has a bidirectional effect on the open probability of the K_{ATP} channel, *i.e*. structurally distinct segments of the linker can saturate or attenuate the open probability of the channel (Babenko and Bryan, 2002, 2003; Bryan *et al.*, 2004). Additionally, the L0 linker has been reported to be involved in the binding of the sulfonylurea glibenclamide which inhibits K_{ATP} channels (Mikhailov *et al.*, 2001). Similar to CFTR, there is an increasing number of proteins known to interact with K_{ATP} channels (Dhar-Chowdhury *et al.*, 2005). Although these proteins do not bind directly to the ABC component, the sulfonylurea receptor, they form part of the same supramolecular complex. A variety of glycolytic enzymes, adenylate kinase and creatine kinase have been shown to be part of the K_{ATP} channel complex and regulate its function.
The requirement of the L0 linker for transport function has also been demonstrated for MRP1 (Bakos *et al.*, 2000). In MRP1, two α-helices have been predicted in L0, although the first one with low probability (Westlake *et al.*, 2003). L0, and especially the second helix in the loop, are absolutely essential for MRP1 function (Bakos *et al.*, 1998; Gao *et al.*, 1998; Ren *et al.*, 2001; Qian *et al.*, 2001), and might be required for correct folding and trafficking of the protein (Westlake *et al.*, 2003). L0 has been proposed to interact both with the core domains of MRP1 and with the hydrophobic membrane regions of other proteins, or with the membrane itself (Westlake *et al.*, 2003; Bakos *et al.*, 2000). In addition, it has been suggested that at least part of the glutathione binding site is located in L0, although there is no direct evidence for this notion (Haimeur *et al.*, 2004).

Accessory catalytic domains. The type I secretion pathway is used to export competence-stimulating peptides, bacteriocins, haemolysins, and non-proteinaceous material, independent of the Sec system (Holo *et al.*, 1991; Hui and Morrison, 1991; Juranka *et al.*, 1992; Fath *et al.*, 1993). In this pathway, ABC transporters have dual functions, that are, export of polypeptides and removal of leader peptides. Similar to the Sec pathway, the (poly)peptides are synthesized ribosomally and targeted for export by an N-terminal sequence of between 15-30 amino-acids (Dirix *et al.*, 2004), that is removed upon secretion across the membrane. Removal of this signal sequence is taken care of by a peptidase domain associated with dedicated ABC transporters. The signal sequences with the consensus LSXXELXXIXGG are usually cleaved after the two conserved glycine residues (Havarstein *et al.*, 1994) and are therefore called double-glycine-type leader peptides. The polypeptides that are released from the cell, after processing of the leader peptide, range in length between 17 and 80 amino acids (Dirix *et al.*, 2004) and often require post-translational modification prior to secretion to become biologically active. The secreted peptides rarely share significant homology, which is reflective of their functions as molecules that act in a strain- or species-specific manner, typically as competence-stimulating peptides (ComA in *Streptococcus pneumoniae*) or as class I (lantibiotics) or class-II (non-lantibiotics) bacteriocins.
The secretion of ComA and bacteriocins is mediated by dedicated ABC half transporters that possess an accessory N-terminal domain of approximately 160 amino acids to remove the

leader peptide (Havarstein *et al.*, 1995). The peptidase domain has characteristics of a cysteine protease, including conserved cysteine and histidine residues in the active site that are essential for proteolytic activity (Wu *et al.*, 2004). How the peptidase domain interacts with the other components of the transporter to promote secretion is unknown. For LcnC, the ABC transporter responsible for export of the bacteriocin LcnA, the peptidase domain was located at the cytoplasmic face of the membrane on the basis of PhoA and LacZ fusion studies (Fig. 3I; Franke *et al.*, 1999).

In Gram-negative bacteria, the type I secretion systems are composed of a protein complex that spans the cytoplasmic membrane (ABC transporter), periplasm (membrane fusion protein) and outer membrane (porin-like structure). The best characterized system is the one for secretion of HlyA, a 110 kDa haemolytic toxin, in *E. coli*. This system is composed of the ABC transporter HlyB, the membrane fusion protein HlyD, and the outer membrane factor TolC. Contrary to the type I secretions systems for peptides and bacteriocins, HlyB does not contain a peptidase like domain and HlyA does not contain a leader peptide. In stead, type I proteins like haemolysin contain a poorly conserved C-terminal secretion signal (Chervaux *et al.*, 1996). The secretion signal domain of HlyA has been shown to interact with the NBD *in vitro* (Benabdelhak *et al.*, 2003), but the possibility cannot be excluded that the signal region of HlyA may interact with the TMD of HlyB. Also for the interaction between HlyB and HlyD there is not a clearly defined region or domain in the ABC transporter.

ABC transporter domains in other systems

Whereas accessory domains in ABC transporters carry out important regulatory and catalytic functions, (core) components of ABC transporters can have dual functions or be integrated into completely different systems where they are vital for function. The ABC Walker A and B motifs are found in many proteins (Walker *et al.*, 1982), the best known examples probably being myosin, and the DNA repair enzymes Rad50 and MutS. Moreover, Rad50 and MutS not only contain the Walker A and B motifs but also the ABC signature sequence LSGGQ and are genuine ABC proteins, albeit without transport function. In fact, a subset of ABC proteins is involved in DNA maintenance and protein synthesis, *e.g.*, recombination, DNA repair, chromosome condensation and segregation, and translation elongation. These latter proteins exert their functions in the cytoplasm and/or nucleus rather than in the membrane (Holland *et al.*, 2003).

SBPs of bacterial uptake systems have been shown to act as chaperones (Richarme and Caldas, 1997), and can be used as chemotaxis receptors (Kokoeva *et al.*, 2002). In addition, SBP-dependent secondary transporters have been discovered (Driessen *et al.*, 2000), but an evolutionary relatedness of these SBPs to those of ABC transporters is not evident. Finally, domains homologous to prokaryotic SBPs function as receptors in ionotropic glutamate receptors present in the central nervous system of higher eukaryotes (Madden, 2002; Gouaux, 2003).

Conclusions

The core complex of an ABC transporter is composed of two nucleotide binding and two transmembrane domains, each in most cases with five or six transmembrane α-helical segments. A notable exception to this rule is the vitamin B12 transporter BtuCD with two times

ten TMSs in the TMDs, that, on the basis of the crystal structure, all seem critical for the formation of the translocation path. The RbsC and FhuB transporters may also conform to this exception. Additional TMSs associated with the core ABC complex seem to play regulatory roles as is best documented for the transporter involved in antigen presentation and the TMD0 domain of the sulfonylurea receptors.
The SBPs are accessory components of the ABC uptake systems that have evolved to signal substrate availability to (the activation of) the ATPase, following the binding of liganded SBPs to the TMDs. Moreover, the SBPs increase the affinity of the transporters for their substrate(s) by several orders of magnitude. For a number of SBPs, functions are known that are not related to transport, including chaperoning and signal transduction. Several ABC transporters have evolved to play crucial roles in processes such as cell volume regulation, control of channel activity and antigen presentation and therefore acquired accessory regulatory functions. The CBS domains, protein modules linked to a wide variety of proteins (channels, transporters, enzymes and transcription factors), in OpuA and ProU add osmosensing and osmoregulatory functions to the transporters. Finally, ABC transporters (type I secretion systems) can have a peptidase domain that cleaves off the signal sequence prior to translocating the mature peptide (bacteriocin) or protein.

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Chapter 3

The binding specificity of OppA determines the selectivity of the oligopeptide ABC transporter

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Abstract

The purification and functional reconstitution of a five-component oligopeptide ATP-binding cassette (ABC) transporter with a remarkably wide substrate specificity is described. High affinity peptide uptake was dependent of liganded substrate-binding protein OppA, which interacts with the translocator OppBCDF with higher affinity than unliganded OppA. Transport screening with combinatorial peptide libraries revealed that i) the Opp transporter is not selective with respect to amino acid side chains of the transported peptides; ii) any peptide that can bind to OppA is transported via Opp, including very long peptides of up to at least 35 residues; and iii) the binding specificity of OppA largely determines the overall transport selectivity.

Introduction

ATP-binding cassette (ABC) transporters belong to a large superfamily of membrane proteins that are abundantly present amongst all kingdoms of life. Members of this family share a common four-domain architecture composed of two transmembrane domains which span the membrane several times in α-helical conformation and two membrane-associated nucleotide-binding domains (NBDs) which fuel the translocation process at the expense of ATP. The existence and arrangement of this four-domain structure has been confirmed by the high-resolution crystal structure of the vitamin B_{12} ABC transporter BtuCD from *Escherichia coli* (Locher *et al.*, 2002). In addition bacterial ABC importers use substrate-binding proteins (SBPs), located at the extracellular surface or in the periplasmic space, to scavenge for substrate and subsequent delivery to the translocator. The individual subunits of ABC transporters can be present as separate proteins or fused together in almost any possible combination (Higgins, 2001; van der Heide and Poolman, 2002).
The oligopeptide transport system (Opp) from *Lactococcus lactis* is a five-component ABC transporter composed of a membrane-anchored SBP, OppA, two transmembrane proteins, OppB and OppC, and two NBDs, OppD and OppF (Tynkkynen *et al.,* 1993). OppA binds a peptide and delivers it to the translocator complex where upon ATP hydrolysis the peptide is

The abbreviations used are: ABC, ATP-binding cassette; ARS: ATP-regenerating system; NBD, nucleotide-binding domain; SBP, substrate-binding protein; DDM, dodecyl-β-D-maltoside; MALDI-TOF, matrix-assisted laser desorption/ionization-time-of-flight; MS, mass spectrometry.

transported into the cell. Next to nutrition of the cell, in microorganisms the Opp system is also involved in various signalling processes including regulation of gene expression, chemotaxis, competence development, sporulation, DNA transfer by conjugation, and virulence development (for a review see Detmers *et al.*, 2001). In *Lactococcus lactis* (and other lactic acid bacteria), the Opp system is an essential part of the proteolytic system as it transports into the cell β-casein-derived peptides formed by the cell wall-associated protease PrtP, enabling the organism to grow in milk. On the basis of intracellular amino acid accumulation in peptide-containing media, the lactococcal Opp system has been proposed to transport peptides in the range of 4 up to at least 18 residues (Detmers *et al.*, 1998).
The mechanism of peptide binding to OppA has been examined in detail using combinatorial peptide libraries and fluorescence techniques (Lanfermeijer *et al.*, 1999, 2000; Detmers *et al.*, 2000). OppA from *L. lactis* binds oligopeptides as long as 35 residues and has the highest affinity for nonameric peptides. OppA is not able to completely enclose peptides longer than 6 amino acid residues. The remaining residues stick out and interact with the protein surface actively, thereby contributing to the peptide binding affinity. This mechanism of substrate binding by OppA is different from that proposed for other SBPs which, on the basis of crystal structures, are thought to enclose the substrate completely (Tame *et al.*, 1994). Recently, Charbonnel *et al.* (2003) concluded, on the basis of OppA sequence comparisons and peptide utilization patterns of six *Lactococcus lactis* strains, that OppA is not the main determinant of peptide transport specificity of Opp. However, *in vivo* peptide uptake experiments are often complicated by rapid breakdown of internalized peptides by peptidases, subsequent efflux of labeled amino acids and binding of peptides to the cell wall (Kunji *et al.*, 1996; Detmers *et al.*, 1998). These complications prohibit any detailed analysis of transport kinetics, translocation mechanism and substrate specificity of Opp.
Here, we describe an *in vitro* system for the study of peptide uptake via Opp. The purification and functional reconstitution in artificial membranes of this five-component ABC transport system enabled the analysis of the interaction of liganded and unliganded OppA with OppBCDF and determination of the overall transport specificity of Opp. Using radiolabeled combinatorial peptide libraries, we now demonstrate that the transport selectivity of Opp matches the binding specificity of OppA.

Materials and methods

Bacterial strain and growth conditions. Plasmid pAMP42 is a pNZ8048-derivative with a 3804 bp *NcoI-BamHI* insert containing the *oppDFBC* genes under control of the nisin A promoter, yielding OppD, OppF, OppB and C-terminal His_6-tagged OppC (A. Picon, unpublished result). *L. lactis* NZ9000 was used as a host for pAMP42. Cells were grown in 5 % (w/v) whey-permeate, 0.5 % (w/v) yeast extract, 0.5 % (w/v) glucose and 65 mM KPi pH 7.0, supplemented with 5 μg/mL chloramphenicol in a 10 L fermentor with pH control (ADI 1065 fermentor; Applikon Dependable Instruments B.V., Schiedam, The Netherlands). The pH was kept constant at pH 6.5 by titrating with 2 M KOH. Transcription was induced at an $O.D._{660}$ of 2 by adding 1:1000 (v/v) of the culture supernatant of *L. lactis* NZ9700. Cells were harvested by centrifugation, washed with 100 mM KPi, pH 7.0, resuspended in 100 mM KPi, pH 7.0 plus 20 % (v/v) glycerol and stored at -80°C. OppA was produced by *L. lactis* AMP2/pAMP31 as described (Detmers *et al.*, 2000).

Purification of Opp. Membrane vesicles were prepared by rupturing the cells with a high pressure homogenizer (Kindler Maschinen AG, Zürich, Switzerland) in the presence of 20 % (v/v) glycerol. OppBCDF-containing membranes were resuspended to 5 mg/mL of total membrane protein in 50 mM KPi, pH 8.0, 200 mM KCl and 20 % (v/v) glycerol (buffer A) and solubilized with 0.5 % (w/v) DDM. Following 12 minutes of centrifugation at 280,000 x *g*, the supernatant was diluted 5 times with buffer A and incubated with Ni^{2+}-NTA

resin (0.05 mL/mg total membrane protein) for 1.5 hours at 4°C while shaking. Subsequently, the resin was poured into a column and washed with 20 column volumes buffer A, supplemented with 0.05 % (w/v) DDM and 10 mM imidazole. The proteins were eluted with 100 mM histidine, 0.05 % (w/v) DDM in buffer A, pH 7.0. OppA was purified as described (Detmers *et al.*, 2000), except that DDM was not exchanged for Triton X-100 during the washing and elution steps.

Immunoblotting and MALDI-TOF MS. Proteins were separated by SDS-PAGE (12.5 % polyacrylamide) and transferred to polyvinylidene difluoride membranes by semidry electrophoretic blotting. Immunodetection was done using monoclonal antibodies raised against the His_6-tag (Sigma) or polyclonal anti-OppD antibodies at titers of 1:3,333 and 1:10,000, respectively. Detection was performed by using the Western-Light™ chemiluminescence detection kit with CSPD™ as a substrate according to the manufacturers instructions (Tropix Inc., Bedford, MA). Protein identification by peptide mass fingerprinting using MALDI-TOF MS was done by performing in-gel tryptic digestion in combination with CNBr cleavage as described previously (van Montfort *et al.*, 2002).

Membrane reconstitution of the Opp system. Reconsitution of Opp was done in three steps. i) Liposomes (20 mg/mL in 50 mM KPi, pH 7.0) of acetone/ether washed *E. coli* lipid and L-α-phosphatidyl choline from egg yolk in a ratio of 3:1 (w/w) were extruded through 400 nm polycarbonate filters, diluted to 4 mg/mL and titrated using Triton X-100 (Knol *et al.*, 1996). Purified OppBCDF in elution buffer was added at a 1:100 (w/w) protein:lipid ratio, and the mixture was incubated for 30 minutes at RT while shaking gently. To remove the detergent, 40 mg/mL wet weight polystyrene beads (BioBeads SM2) was added, followed by a 15 minutes incubation at RT. Biobeads SM2 were added four more times and the incubation times were 15 minutes, 30 minutes, overnight and 1 hour at 4°C. After 5 times dilution with 50 mM KPi, pH 7.0, proteoliposomes containing OppBCDF were collected by centrifugation for 1.5 hours at 150,000 x *g* and 4°C and stored in liquid nitrogen. ii) OppA was anchored to the liposomes via its lipid moiety, and the final concentration of OppA after membrane reconstitution was estimated from the binding of peptide, as described by Detmers *et al.* (2000). Unless stated otherwise (see legend to Fig. 2), an OppA concentration of ~ 0.35 nmol/m^2 of membrane surface was used. iii) An ATP-regenerating system, composed of 9 mM ATP, 9 mM MgSO4, 24 mM creatine-phospate and 2.4 mg/mL creatine kinase, was included into Opp-containing proteoliposomes by two freeze-thaw cycles. Following extrusion through 400 nm polycarbonate filters, the proteoliposomes were washed twice and resuspended in 50 mM KPi, pH 7.0, to a concentration of 20 mg/mL lipid.

Uptake of bradykinin. Transport assays were started by diluting proteoliposomes into 200 μL 50 mM KPi, pH 7.0 (4 mg/mL final lipid concentration) containing [^{3}H]-bradykinin at various concentrations. Uptake experiments were performed at 30°C. At given time-points, 40 μL samples were taken and mixed 1:1 with 200 μM unlabeled bradykinin in 50 mM KPi, pH 7.0. The sample was then diluted with 2 mL ice-cold 8 % (w/v) PEG-6000, filtered over 0.15 μm pore-size cellulose acetate (OE65) filters (Schleicher & Schuell, Dassel, Germany) and washed twice with 2 mL ice-cold 8 % (w/v) PEG-6000. The amount of radioactivity retained on the filter was determined by liquid scintillation counting. The concentrations of bound and free bradykinin and of liganded and unliganded OppA were calculated from the total concentration of OppA, the total concentration of bradykinin and the K_D for bradykinin binding of 0.1 μM (Lanfermeijer *et al.*, 1999; Detmers *et al.*, 2000). For the calculation of peptide accumulation levels an internal volume of 0.5 μL/mg lipid was used, which corresponds to 50 μL/mg OppBCDF.

Screening of combinatorial peptide libraries. Uptakes were done in a custom made micro-well filtration unit (8 x 6 wells, 300 μL volume each) which allowed a 4-fold scaling down of the assay volumes as compared to the setup described above. After filtration over 0.15 μm pore-size cellulose acetate (OE65) filters (Schleicher & Schuell, Dassel, Germany) samples were washed twice with 200 μL 50 mM KPi, pH 7.0. The amount of radioactivity retained on the filter was determined with γ-counting. Randomized peptide libraries used in this study have been described previously (Uebel *et al.*, 1997; Detmers *et al.*, 2000). Peptides were iodinated as described (Chen *et al.*, 2003) using Chloramin T, and free iodine was removed by DOWEX 1X8 (Serva).

Concentration of OppA at the membrane surface. The two-dimensional concentration of lipid-anchored OppA (in nmol/m^2) at the membrane surface was calculated from the lipid and OppA concentration, and a lipid surface area of 0.6 nm^2. For comparison of affinities with other SBP-dependent ABC transport systems, this

two-dimensional concentration was converted into a local concentration of OppA (in μM). For this, a shell of ~ 3.4 nm around the liposomal surface was used (estimated from the crystal structure of OppA from *Salmonella typhimurium*; Tame *et al.*, 1994).

Miscellaneous. Protein concentrations were determined according to the method of Lowry *et al.* (1951), using bovine serum albumin as a standard. Peptide concentrations were determined using the bicinchoninic acid assay (Pierce). The concentrations of purified OppBCDF or OppA were determined spectrophotometrically by measuring the absorption at 280 nm and using extinction coefficients of 0.990 and 1.605 $(mg/mL)^{-1}cm^{-1}$, respectively.

Results

Purification of OppBCDF. The components of the oligopeptide translocator complex were amplified in *Lactococcus lactis*, using the nisin expression system (Kuipers *et al.*, 1993; Kunji *et al.*, 2003). OppBCDF was overproduced to an estimated level of ~ 5 % (w/w) of total membrane protein content after two hours of induction (Fig. 1A). Membrane solubilization and purification was done with DDM as detergent. Initial conditions for maintaining an intact OppBCDF complex during purification were screened for ionic strength (0-500 mM KCl) and pH (6.0-8.0) using 20 mM imidazole, 0.05 % DDM and 20 % (v/v) glycerol as basal medium. This did not lead to purification of the complete translocator but resulted in purification of the His_6-tagged component OppC only (data not shown). However, when imidazole was omitted from the buffer during binding of the complex to the Ni^{2+}-NTA resin, the OppBCDF proteins could be obtained in an approximate 1:1:1:1 ratio (Fig. 1B). Varying the glycerol concentration from 0 to 40 % (v/v) did not have any effect on the domain stoichiometry obtained after purification (data not shown). The individual translocator subunits were identified by Western blotting, using monoclonal anti-His-tag or polyclonal anti-OppD antibodies for detection of OppC or OppD, respectively (data not shown). OppF was identified by peptide mapping with MALDI-TOF MS using an in-gel digestion procedure developed for integral membrane proteins (van Montfort *et al.*, 2002). The mass spectrum of the putative OppB band did not yield enough peaks to unambiguously confirm the identity of this protein.

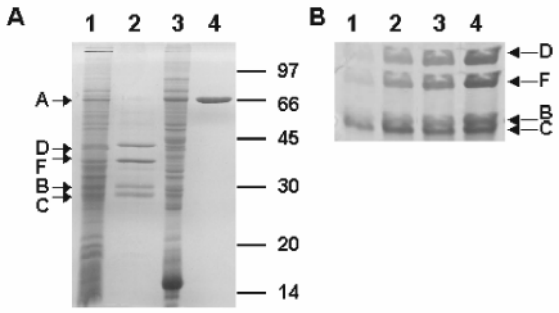

Fig. 1. **Amplification and purification of the Opp system.** (A) Overexpressed (membrane vesicles, lane 1 and 3) and purified (lane 2 and 4) OppBCDF and OppA are shown, respectively. The positions of molecular weight marker proteins are indicated (kDa). (B) Purification of OppBCDF with 10, 5, 2.5 and 0 mM imidazole present (lanes 1, 2, 3 and 4, respectively) during binding to the Ni^{2+}-NTA column.

Functional reconstitution of the Opp system. Membrane reconstitution of the oligopeptide transporter was done via a three step procedure. First, the purified translocator complex OppBCDF was incorporated into Triton X-100 destabilized liposomes. Thereafter, purified OppA was anchored to the outside of OppBCDF containing liposomes via its N-terminal lipid modification by absorbing the purified protein in 0.05 % (w/v) DDM to the (proteo)liposomes and removal of residual detergent with BioBeads. This resulted in proteoliposomes containing all five proteins of which the Opp system is composed. Next, after inclusion of ATP and the ATP-regenerating system (ARS) into the vesicle lumen by two freezing-thawing cycles, bradykinin transport could be measured (Fig. 2A). Control experiments indicated that bradykinin does not interact aspecifically with the liposomes and uptake was not observed when AMP-PNP, a non-hydrolyzable ATP-analogue, was used instead of ATP, ruling out aspecific effects on the membrane of the components of the ARS (Detmers *et al.*, 2000; Patzlaff *et al.*, 2003). To increase the signal-to-noise ratio, background binding of radiolabeled substrate to OppA was diminished by chasing with a large excess (~ 100 fold) of unlabeled bradykinin. Chasing background binding did not lead to an increase of the amount of label inside or efflux of accumulated [^{3}H]-bradykinin from the proteoliposomes (Fig. 2A). The apparent K_M of transport for bradykinin was 0.9 $\pm$ 0.2 μM (Fig. 2B). To verify that bradykinin uptake had taken place, the accumulation level ($[bradykinin]_{in}/[bradykinin]_{out}$) was calculated (see 'materials and methods' section for details). At a bradykinin concentration of 1.5 μM the accumulation level was ~ 50 after 4 minutes of uptake, indicating that the peptide was transported against the concentration gradient.

Peptide uptake as a function of OppA concentration. Since liganded OppA rather than the free peptide is the actual substrate of OppBCDF, the effect of increasing the amount of OppA on the transport rate was determined. For easy comparison with other ABC transporters that employ a soluble SBP, the local concentrations of OppA at the membrane surface (in μM) were calculated as described in the 'materials and methods' section. A concentration of OppA in the assay of 0.36 μM equals 0.35 nmol/m^2 of membrane surface, which corresponds a local concentration at the membrane of about 100 μM. At a bradykinin concentration of 1.5 μM (a concentration more than 10-fold above the K_D for bradykinin binding; K_D = 0.1 μM), the K_M of the translocator OppBCDF for liganded OppA was ~ 50 μM (Fig. 2C). At low substrate concentration, that is, with 0.1 μM bradykinin, the uptake rate increased linearly with the concentration of OppA and no inhibition due to the large excess (up to 4-fold) of unliganded OppA was observed (Fig. 2C). Fig. 2D shows how the ratio of liganded vs. unliganded OppA varied in the titration and confirms that at the highest level of OppA a 4-fold excess of unliganded over liganded OppA was present. These results indicate that the translocator has a much higher affinity for liganded than for unliganded OppA.

Uptake of randomized peptides via Opp. Because unliganded OppA does not inhibit the uptake of peptides, at least under our experimental conditions, the specificity of Opp for peptides can be estimated directly from the rate of uptake. Since the peptide libraries were available in relatively small quantities, a micro-well plate setup was used for these transport assays. The peptide libraries were used at concentrations ranging from 3.2 to 5.0 μM. Randomized peptides were labeled at tyrosine residues with tracer amounts of ^{125}I to monitor the transport reaction. Control experiments showed that uptake and (a)specific binding of randomized peptides could be specifically blocked by the addition of 100 μM bradykinin (Fig.

3). The chase with a large excess of bradykinin allowed us to separate transport from possible non-specific interaction of the peptides with the liposomes.

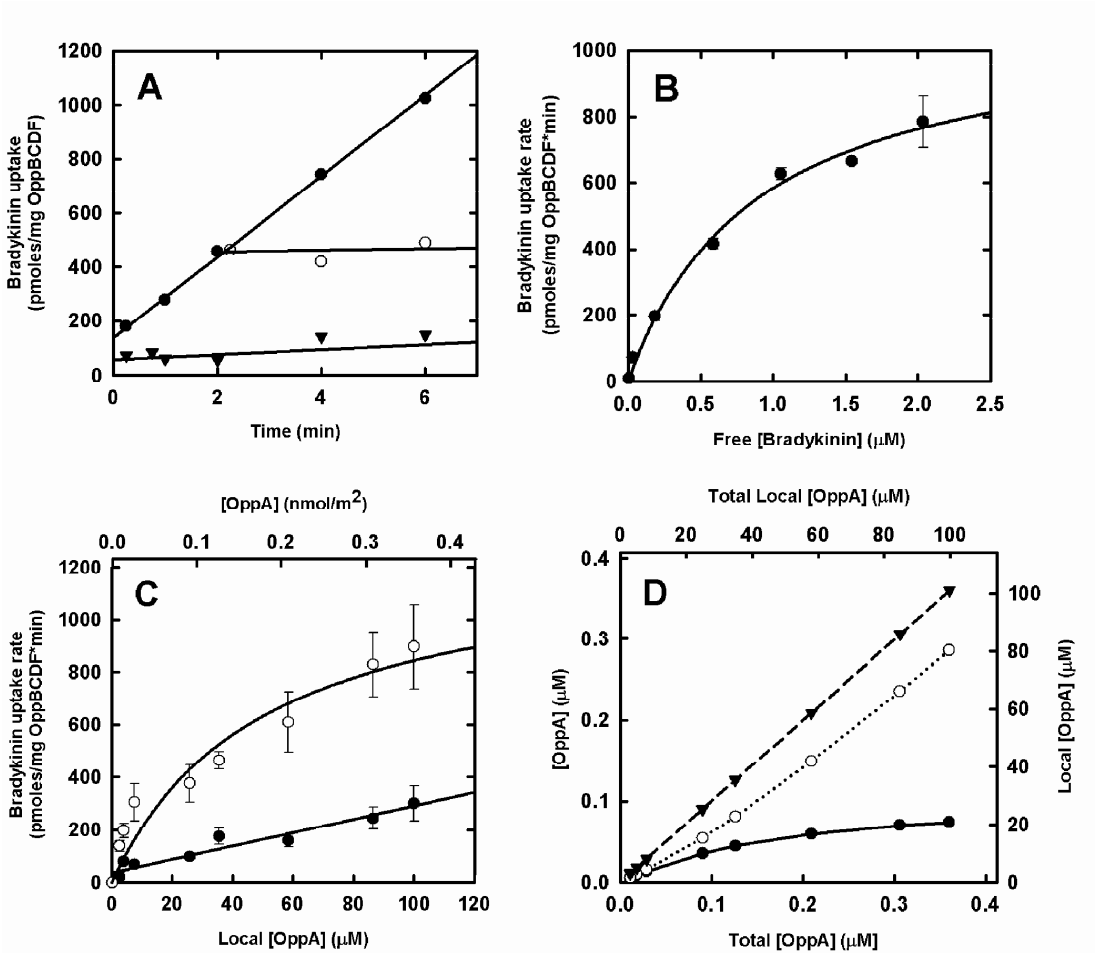

Fig. 2. **Functional reconstitution and characterization of the Opp system.** (A) [^{3}H]-bradykinin (0.7 μM) uptake was assayed in the presence (●) and absence (▼) of ATP plus ATP-regenerating system enclosed in the proteoliposomes. The open circles denote samples to which 100 μM of unlabeled bradykinin was added after 2 minutes of [^{3}H]-bradykinin uptake. (B) Kinetics of [^{3}H]-bradykinin uptake. Initial rates of uptake were calculated from the linear part of the curves as depicted in panel A. Data were fitted with the Michaelis Menten equation. (C) Uptake rates at increasing OppA levels were determined in the presence of saturating (1.5 μM, ○) and low (0.1 μM, ●) [^{3}H]-bradykinin concentrations. The concentration of membrane-anchored OppA was varied from 0 - 0.36 μM, which corresponds to two-dimensional concentrations of 0 - 0.35 nmol/m^2 of membrane surface (top-axis) and local concentrations at the membrane of 0 - 100 μM OppA (bottom-axis; see 'materials and methods' section for details of the calculations). The error bars indicate the standard deviation of experiments performed in duplicate. (D) Calculation of the concentrations of liganded (●) and unliganded (○) OppA at a bradykinin concentration of 0.1 μM. The total OppA concentration (▼) was varied from 0 - 0.36 μM (as in panel C). For easy comparison, the local concentration of OppA at the membrane is shown on the top-axis.

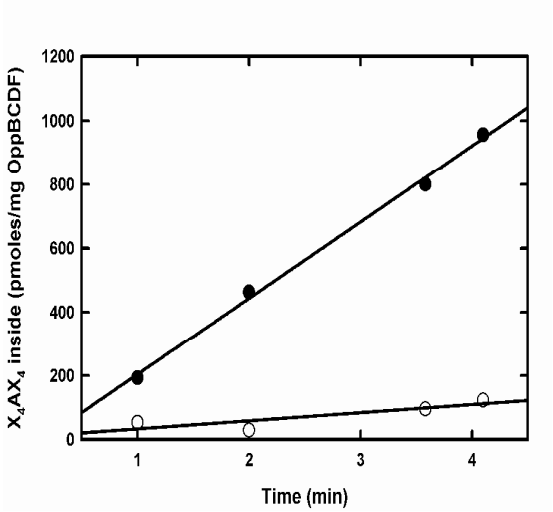

Fig. 3. **Uptake of the randomized nonapeptide X_4AX_4 into Opp containing proteoliposomes.** Transport of 3.2 μM peptide in the absence (●) or presence (○) of 100 μM bradykinin is shown.

Length specificity. To determine the length dependence of transport, randomized peptide libraries ranging from 5 up to 35 amino acid residues were used. All peptides, even the extremely long ones of up to 35 residues, were transported by the Opp system (Fig. 4A). The inhibition of bradykinin binding to OppA by the combinatorial peptide libraries, determined previously by Detmers *et al.* (2000), is shown for comparison. Peptides with nine amino acid residues showed the highest uptake rates and the best binding to OppA. Longer and shorter peptides were transported with slower rates compared to the X_9 library, but, overall, the longest and shortest peptides were transported better than expected on the basis of the binding data. Since partly translocated peptides might be trapped in the transporter (and not be chased by the excess of bradykinin), we calculated the uptake of the largest peptides in terms of accumulation levels and number of peptides translocated per transporter. The accumulation level of X_{35} ($[X_{35}]_{in}/([X_{35}]_{out}$) was 2.5 and at least 30 peptides were transported per translocator after 5 minutes of uptake (see 'materials and methods' section for details). These numbers, and the fact that X_{35} uptake still increased linearly with time after 5 minutes (not shown), demonstrate that even the largest peptides could be fully translocated.

Influence of D-amino acids. The effect of D-amino acids on transport was determined with randomized nonameric peptide libraries containing D-amino acids at a single position. The D-amino acid containing peptides showed lower uptake rates compared to the all L-amino acid containing X_9 library (Fig. 4B). Whereas in previous binding assays the negative effect of the D-amino acids were significant for the positions 4, 5 and 6, the differences in transport were less pronounced.

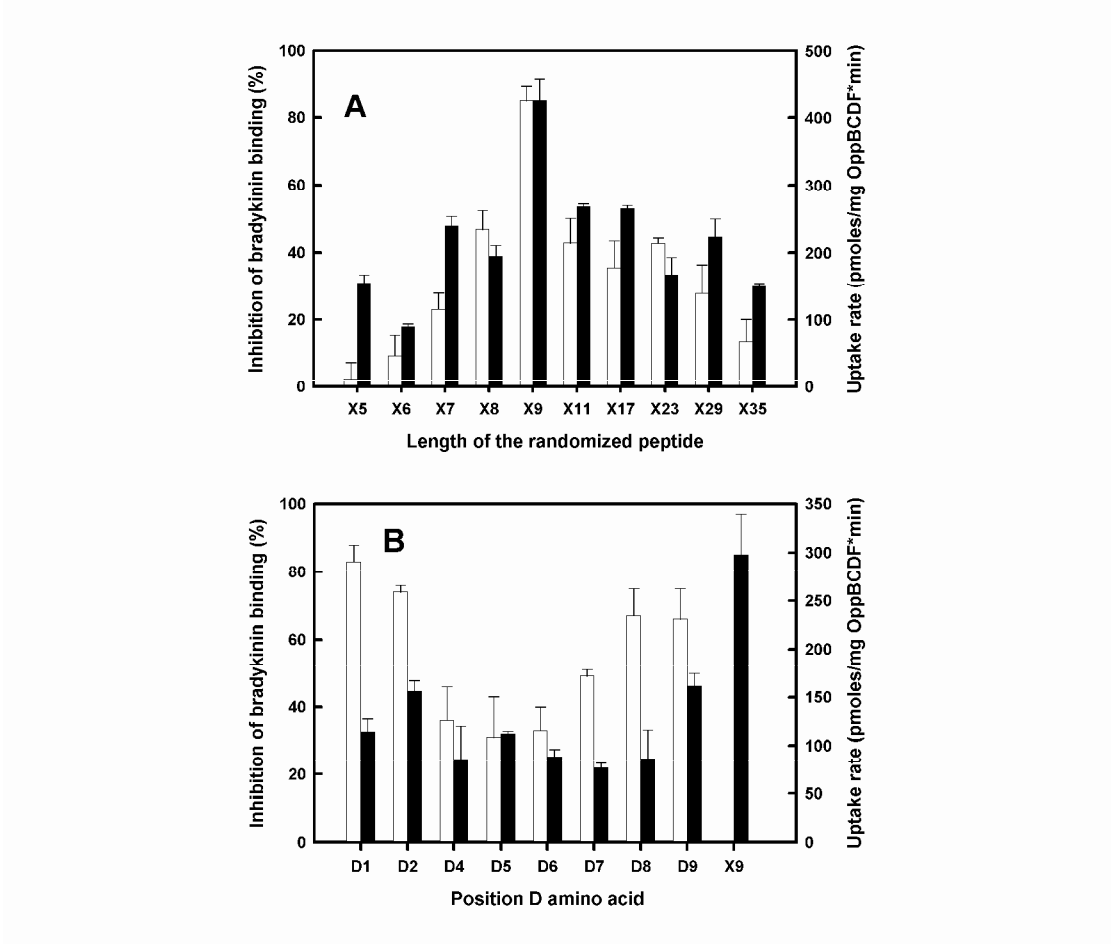

Fig. 4. **Transport of randomized peptide libraries of different length and nonamers with D-amino acids at a defined position.** The peptide concentration was 3.2 (A) or 5 μM (B). Each rate determination was based on four data points, samples taken after 1, 2, 3.5 and 5 minutes, and carried out at least twice; error bars indicate the standard deviation. The transport data are shown as black bars; the binding data are shown for comparison (white bars; taken from Detmers *et al.*, 2000).

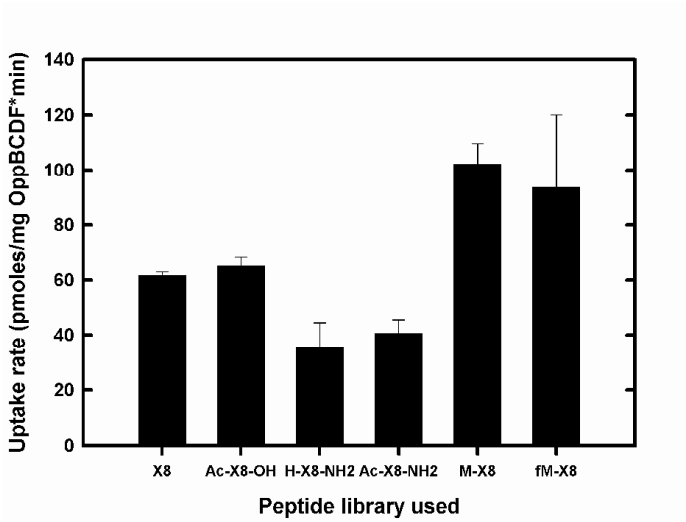

Fig. 5. **Transport of randomized peptide libraries with modified termini.** The peptide concentration was 3.2 μM. For further details, see legend to Fig. 4.

Transport of peptides with modified termini. Randomized peptides with modified termini were used to test whether a free N- or C-terminus was required for transport via Opp. Uptake rates of octameric peptides (X_8) with an acetylated N- or amidated C-terminus or both were compared with the uptake rate of the unmodified randomized octameric peptide. The results indicate that free N- or C-termini are not essential for transport (Fig. 5). Acetylation of the N-terminus had no effect, whereas amidation of the C-terminus reduced the uptake rate by a factor of two. In addition, a nonameric randomized peptide library with a formylated N-terminal methionine, fMX_8, was transported equally well as MX_8.

Nonameric randomized peptides. Nonameric randomized peptide libraries were used in which amino acids at all but one position were random. This position contains one of 19 different amino acids (cysteine was excluded from the libraries). In this way, the effect of a single amino acid at a given position can be determined independent of the sequence context of the rest of the peptide. For comparison the transport via Opp and binding to OppA were screened for two positions. Generally, the transport data differed little from the binding results (Fig. 6A and B). All peptides tested were transported and peptides that bound best to OppA showed the highest transport rates. At position one, bulky and hydrophobic amino acids were somewhat disfavored (His, Leu, Val, Trp, Tyr). At position five, no clear differences between the binding specificity of OppA and the transport selectivity of the Opp system as a whole could be discerned.

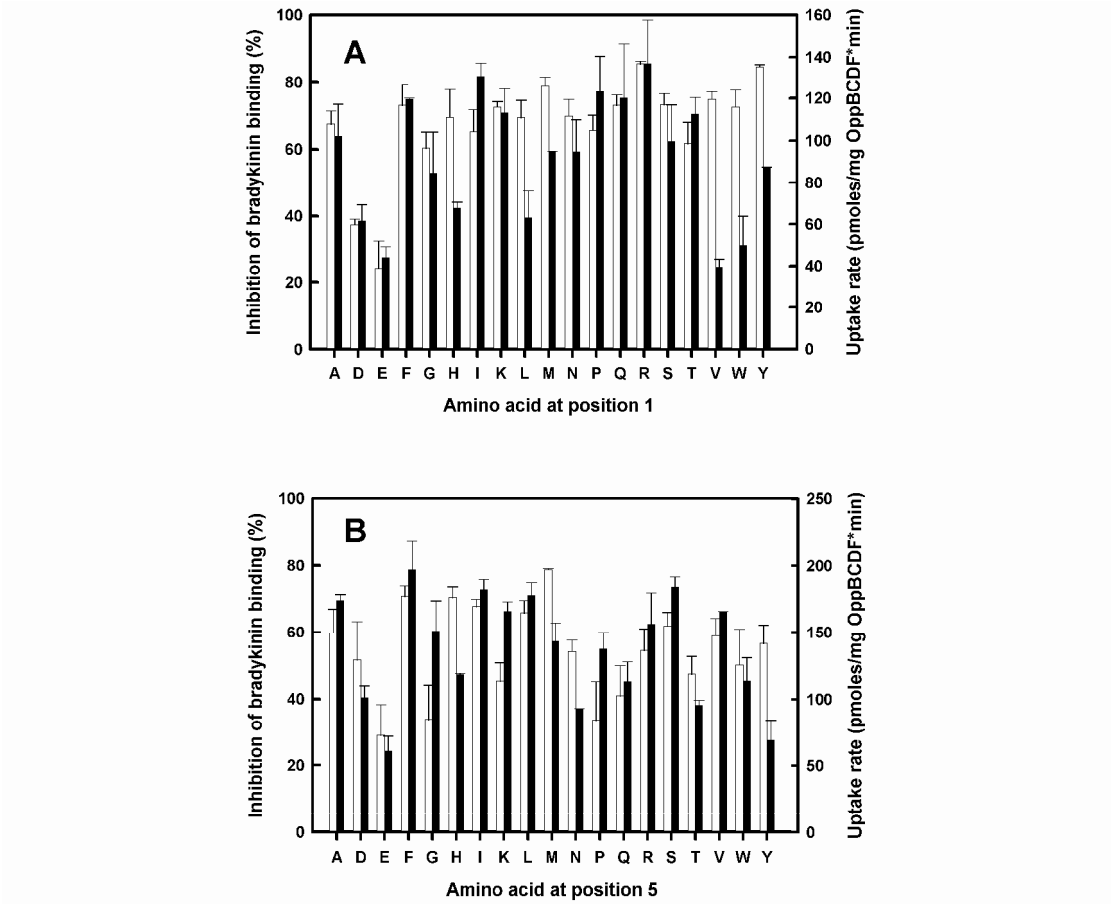

Fig. 6. **Transport of randomized nonameric peptide libraries with a defined amino acid at position 1 or 5.** Peptides were tested at 3.2 μM. For further details, see legend to Fig. 4.

To investigate further if the overall transport specificity differs from the binding selectivity of OppA, two amino acids, methionine and valine, were chosen for screening using randomized nonameric peptide libraries. These two amino acids showed almost no differences in binding specificity, irrespective of the position in the peptide (Detmers *et al.*, 2000). Transport specificity of the methionine containing libraries matched perfectly with the binding selectivity (Fig. 7A). In the case of valine, some differences were observed (Fig. 7B), of which the negative effects of valine at position 1, 4 and 9 on the transport rates were most pronounced. However, again all peptides could bind to OppA and were well transported by the Opp system.

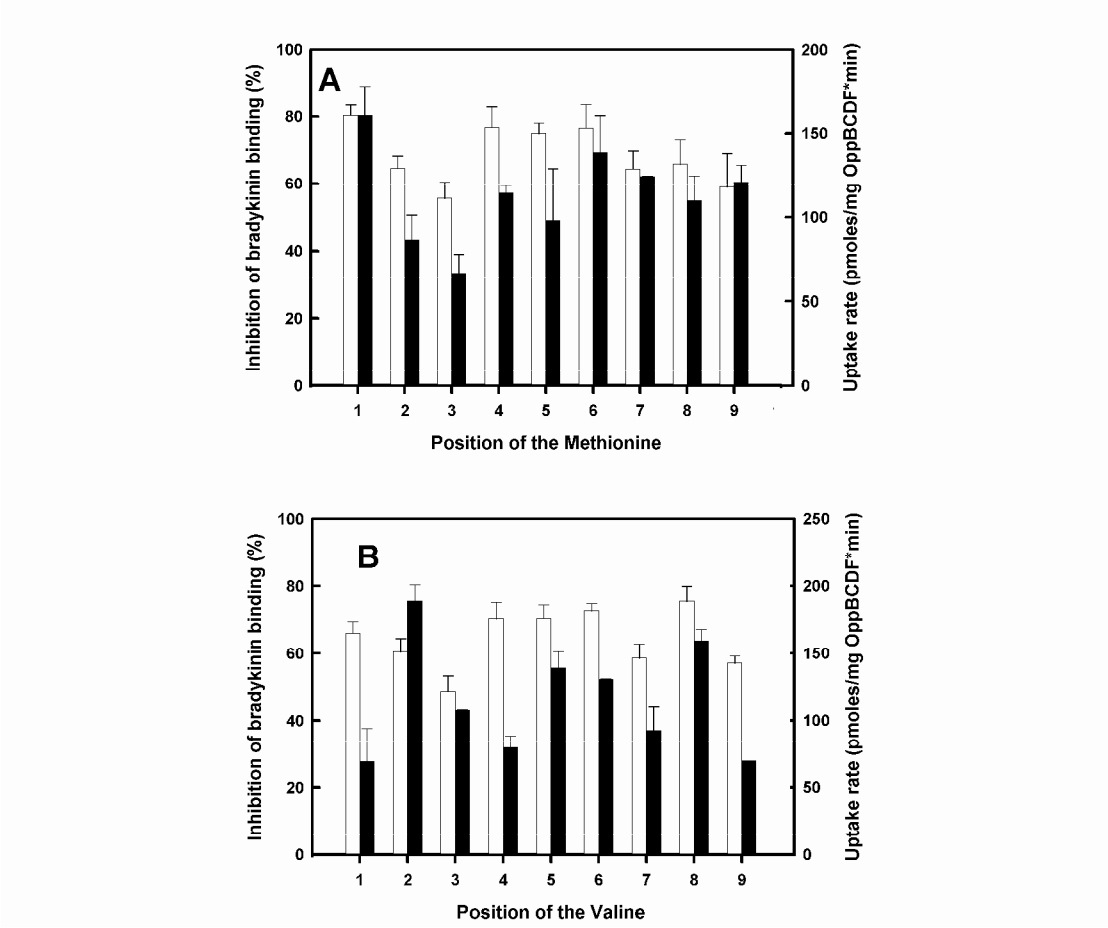

Fig. 7. **Transport of randomized nonameric peptide libraries with Methionine or Valine at a defined position.** Peptides were tested at 3.2 μM. For further details, see legend to Fig. 4.

Discussion and conclusions

We developed an *in vitro* assay system for the analysis of the kinetics, mechanism and specificity of peptide transport, and we present the unique selectivity properties of the oligopeptide (Opp) ABC transporter from *Lactococcus lactis*.

Opp transporters in microorganisms are more complex than most other ABC systems because they are composed of five separate polypeptides, and this has hampered their purification and membrane reconstitution for many years. The translocator complex OppBCDF is embedded in

the membrane, whereas the oligopeptide-binding protein is tethered to the external surface of the membrane. The Opp translocator was produced in *Lactococcus lactis* and purified as a complex using a single His_6-tag on OppC. Although Ni^{2+}-NTA based purification protocols generally employ low concentrations (5-30 mM) of imidazole in the protein binding to the resin and washing steps, imidazole severely compromised the translocator stability. All four proteins could only be co-purified when imidazole was absent in the binding step and histidine was used for the elution of the complex.

Membrane reconstitution of Opp was done by inserting the OppBCDF complex into detergent-destabilized liposomes, after which OppA was anchored to the outside of the membranes via its N-terminal lipid modification. Because of the high affinity of OppA for the reporter substrate bradykinin (K_D = 0.1 μM) and the use of relatively high concentrations of OppA, a ~ 100 fold excess of unlabeled bradykinin was used to stop the transport reaction and chase all OppA-bound [^{3}H]-bradykinin. The bradykinin accumulated inside the proteoliposomes was not affected by the chase with unlabeled bradykinin, because the transporter functions unidirectionally.

The affinity of the translocator for its substrate, that is liganded OppA, was determined by measuring uptake rates at increasing OppA concentrations at the membrane surface and saturating amounts of bradykinin. The K_M of the translocator for liganded OppA was ~ 50 μM, which is in the same range as the values of ~ 65 μM determined for the histidine system $HisJQMP_2$ (Prossnitz *et al.*, 1989) and 25-50 μM for the maltose system $MalEFGK_2$ (Dean *et al.*, 1992). The translocator could already be saturated at relatively low absolute amounts of OppA because OppA is attached to the membrane. Even at an OppA concentration in the assay of only 0.36 μM, which corresponds to ~ 0.35 nmol/m^2 of membrane surface, the local concentration of OppA at the membrane could be as high as 100 μM. Inhibition of transport activity by unliganded SBP has been observed for the periplasmic binding protein-dependent histidine and maltose ABC transport systems (Prossnitz *et al.*, 1989; Merino *et al.*, 1995), and this has been ascribed to competition between unliganded and liganded SBP for the translocator. In the case of the histidine permease, it even was suggested that liganded and unliganded HisJ interact with equal affinity with the translocator complex (Ames *et al.*, 1996). We observed no inhibition at low peptide concentrations, corresponding to local concentrations of 20 μM liganded and 80 μM unliganded OppA, which indicates that the affinity of the translocator for liganded OppA is much higher than for unliganded OppA.

It is often assumed that the SBP of bacterial ABC transporters determines the overall transport selectivity of the system, but, unlike Opp, the specificity of most systems is narrow and binding and transport of only a few substrates have been compared. Recently, Charbonnel *et al.* (2003) proposed, by comparing the peptide utilization patterns of six different *Lactococcus lactis* strains, that OppA is not the main determinant of peptide transport specificity. It was claimed that the translocator OppBCDF plays an important role in determining the overall transport selectivity. It must be noted, however, that several of the used strains were not well characterized and other factors (for example other peptide transporters or lack of certain peptidases) might have interfered with the observed peptide utilization patterns. The sequences of the *opp* genes of two of the used strains, *L. lactis* MG1363 and IL1403, differ for the transmembrane proteins OppB and OppC at only two positions (Tynkkynen *et al*., 1993; Bolotin *et al.*, 2001; the system studied by us is from the MG1363 strain). This makes it unlikely that the observed differences in peptide transport can be ascribed solely to different properties of the transmembrane moieties of the Opp systems. In a follow up paper (Helinck *et*

al., 2003), Juillard and colleagues proposed that some peptides bind to OppA without subsequently being transported, but, with the exception of one tetrapeptide, there is no real evidence for this notion. By using combinatorial peptide libraries, we now clearly show that the binding specificity of OppA compares well with the overall transport selectivity of the Opp system. For the specificity studies, we used saturating OppA concentrations, implying that substrate binding or delivery to the translocator is not rate-determining. This mimicks the *in vivo* situation where overexpression of OppA in wild-type *L. lactis* cells increased the amount of peptide binding but did not affect the rate of uptake (Picon *et al.*, 2000). Furthermore, the K_M for bradykinin transport *in vivo* (0.27 ± 0.03 μM; Picon *et al.*, 2000) and *in vitro* (0.9 ± 0.2 μM) are in the same range. Thus, both *in vivo* and *in vitro* there is a large excess of OppA over OppBCDF and the rate-determining step for bradykinin uptake is in translocation and not in peptide binding.

A striking feature of the *Lactococcus lactis* Opp system is that it binds and transports exceptionally long peptides. Here, we show that the Opp ABC transporter is capable of transporting peptides as long as at least 35 residues. Peptides shorter or longer than nine residues are transported relatively better than expected solely on the basis of the binding data. This fits well with the previously made observation that small peptides are bound relatively poorly but transported rather well (Detmers *et al.*, 1998). On the basis of these experiments and pre-steady state peptide binding kinetics, it was suggested that the dissociation rate constant (k_{-1}) is an important determinant of the rate of transport. Tight binding to OppA was associated with low k_{-1} values which might limit the transfer of peptide from OppA to the translocator and yield a low rate of transport. On the other hand, poorly binding peptides had a high k_{-1} and were transported relatively well. A similar explanation may hold for the difference in binding and transport of the penta- and hexameric peptides.

In a previous report, we showed that there is no strict requirement for a free amino- and carboxyl-group for a peptide to bind to OppA (Lanfermeijer *et al.*, 2000). Acetylation of the N-terminal amino group or amidation of the C-terminal carboxyl group of the peptide SLSQSLSQS decreased the binding affinity only 4 and 7 fold, respectively. Here, we show that a free amino or carboxyl group is also not essential for peptide transport by Opp.

To further analyze the transport specificity of the Opp system, randomized nonameric peptides containing D-amino acids or a particular residue at one single position were used. The uptake rate of D-amino acid containing libraries was 2-3 fold lower than the X_9 library. This is in agreement with the binding studies in which a 5-fold higher peptide concentration had to be used to obtain similar levels of inhibition of bradykinin binding as with all L-amino acid containing libraries. Overall, there are only small differences between the binding and transport profiles. Position 7 and 8 seem a little more selective than expected on the basis of the binding to OppA. The screening of position 1 and 5 of the randomized nonameric libraries revealed little differences between binding and transport specificity. In general, it appears that any peptide that can bind to OppA can also be transported by Opp. There are no indications for any amino acid causing a peptide to be completely excluded from transport.

The fact that some peptides with bulky and hydrophobic amino acids (His, Leu, Trp, and Tyr at position 1 and Val at position 1, 4 and 9) show somewhat reduced uptake rates can be explained in two ways. Either these peptides bind relatively strong to OppA, resulting in low k_{-1} values and consequently lower transport rates, or a peptide-binding site within OppBCDF disfavors these amino acids at the positions 1, 4 and 9.

In conclusion: the affinity of OppBCDF for liganded OppA is in the same range as the corresponding interaction in other SBP-dependent ABC transporters, but because OppA is membrane-anchored much lower amounts are needed to saturate the translocator than with soluble SBPs. The affinity of the transmembrane complex for liganded OppA is much higher than for unliganded OppA. The Opp system has the remarkable capacity to transport peptides ranging from 4 up to at least 35 residues. Overall, the transport selectivity seems to be determined by the binding specificity of OppA.

Acknowledgements

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Chapter 4

Specificity and selectivity determinants of peptide transport in *Lactococcus lactis* and other microorganisms

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Abstract

Peptide transport in microorganisms is important for nutrition of the cell and various signaling processes including regulation of gene expression, sporulation, chemotaxis, competence, and virulence development. Peptide transport is mediated via different combinations of ion-linked and ATP-binding cassette (ABC) transporters, the latter utilizing single or multiple peptide-binding proteins with overlapping specificities. The paradigm for research on peptide transport is *Lactococcus lactis*, in which the uptake of peptides containing essential amino acids is vital for growth on milk proteins. Differential expression and characteristics of peptide-binding proteins in several *Lactococcus lactis* strains resulted in apparent conflicts with older literature. Recent developments and new data now make the pieces of the puzzle fall back into place again and confirm the view that the oligopeptide-binding proteins determine the uptake selectivity of their cognate ABC transporters. Besides reviewing the current data on binding specificity and transport selectivity of peptide transporters in *L. lactis*, the possible implications for peptide utilization by other bacterial species are discussed.

Introduction

Lactic acid bacteria (LAB) contain a proteolytic system that enables them to grow in milk. The milk proteins, α-, κ-, and β-casein, are degraded by the cell wall-associated proteinase PrtP and the resulting peptides, of which the transport of oligopeptides is most important for nutrition (Kunji *et al.*, 1998), are taken up by the cell. Oligopeptides are defined here as peptides of 4 to 35 residues. Internalized peptides are further hydrolyzed by a large array of peptidases (Kunji *et al.*, 1996). Regulation of peptide transport is accomplished via the pleiotropic transcriptional repressor CodY, which senses the intracellular pool of branched-chain amino acids (Guédon *et al.*, 2001b).
L. lactis MG1363 is a plasmid-free derivative of strain NCDO712 and the best characterized LAB in terms of proteolysis, peptide transport, and regulation thereof (Tynkkynen *et al.*, 1993;

The abbreviations used are: ABC, ATP-binding cassette; CDM, chemically defined medium; LAB, lactic acid bacteria.

Hagting *et al.*, 1994; Foucaud *et al.*, 1995; Kunji *et al.*, 1995; Kunji *et al.*, 1996; Detmers *et al.*, 1998; Kunji *et al.*, 1998; Guédon *et al.*, 2001a; Guédon *et al.*, 2001b; Kok and Buist, 2003; Sanz *et al.*, 2003; Sanz *et al.*, 2004). The genome sequence of *L. lactis* MG1363 will be published in the coming year. The chromosome of *L. lactis* IL1403 was the first lactococcal genome to be sequenced (Bolotin *et al.*, 2001), and the genomic data from this strain have been combined with experimental data of MG1363. However, the IL1403 and MG1363 genome sequences are on average only ~ 85 % identical, and the percentages identities vary strongly when individual genes are compared (unpublished result). In recent years, several *L. lactis* strains other than MG1363 have been used in studies of (oligo)peptide transport (Charbonnel *et al.*, 2003; Lamarque *et al.*, 2004). Apparent discrepancies have arisen in the literature due to i) the assumption that there is only one oligopeptide transporter (Opp) that is solely responsible for uptake of oligopeptides in all lactococcal strains, ii) erroneous annotation of the Dpp system of IL1403 as Opt (the *dppAPBCDF* and *optSABCDF* gene products are on average 97 % identical at the amino acid level), and iii) differential expression of (oligo)peptide transporters in the studied *L. lactis* strains.
Although at the moment not recognized as such, recent work (Sanz *et al.*, 2003; Lamarque *et al.*, 2004; this work) provides an explanation for apparent discrepancies in published papers (Charbonnel *et al.*, 2003; Helinck *et al.*, 2003; Doeven *et al.*, 2004). In this chapter, we present an overview of peptide transport and regulation in *L. lactis* species, and provide alternative explanations for published works. In addition, data is presented that supports our current view that the oligopeptide-binding proteins determine the uptake selectivity of the oligopeptide ATP-binding cassette (ABC) transporters.

Peptide transport in *Lactococcus lactis* MG1363

Biochemical analysis indicated that *L. lactis* MG1363 has at least three functional peptide transport systems (Fig. 1). The *opp* and *dtpT* genes were cloned and characterized first and the corresponding transport proteins (Opp and DtpT) were shown to catalyze the uptake of oligo- and di/tripeptides, respectively (Tynkkynen *et al.*, 1993; Hagting *et al.*, 1994). The Opp system belongs to the ABC transporter superfamily and is composed of five proteins: an oligopeptide-binding protein, OppA, two integral membrane proteins, OppB and OppC, and two nucleotide-binding proteins, OppD and OppF. DtpT is a secondary transporter belonging to the PTR family of peptide transporters. An *opp* knockout strain was completely blocked in the uptake of β-casein-derived oligopeptides, indicating that the Opp system is essential for the organism to grow on milk (Tynkkynen *et al.*, 1993).
Analysis of *opp* and/or *dtpT* single or double knockout mutants revealed the presence of a third peptide transport system with preference for hydrophobic (branched-chain amino acid-containing) di- and tripeptides (Foucaud *et al.*, 1995). Peptide transport via this third system was dependent on ATP, or a related energy-rich phosphorylated intermediate, and the system was initially designated DtpP. The genes belonging to the DtpP system were cloned, characterized, and named Dpp (Sanz *et al.*, 2001; Fig. 1). The Dpp system, like Opp, is an ABC transporter. In MG1363, six *dpp* genes are present coding for two peptide-binding proteins, DppA and DppP, two integral membrane proteins DppB and DppC, and two nucleotide-binding proteins DppD and DppF. In *L. lactis* MG1363, the *dppP* gene is not functional due to a nonsense mutation and a frame-shift (Sanz *et al.*, 2001).

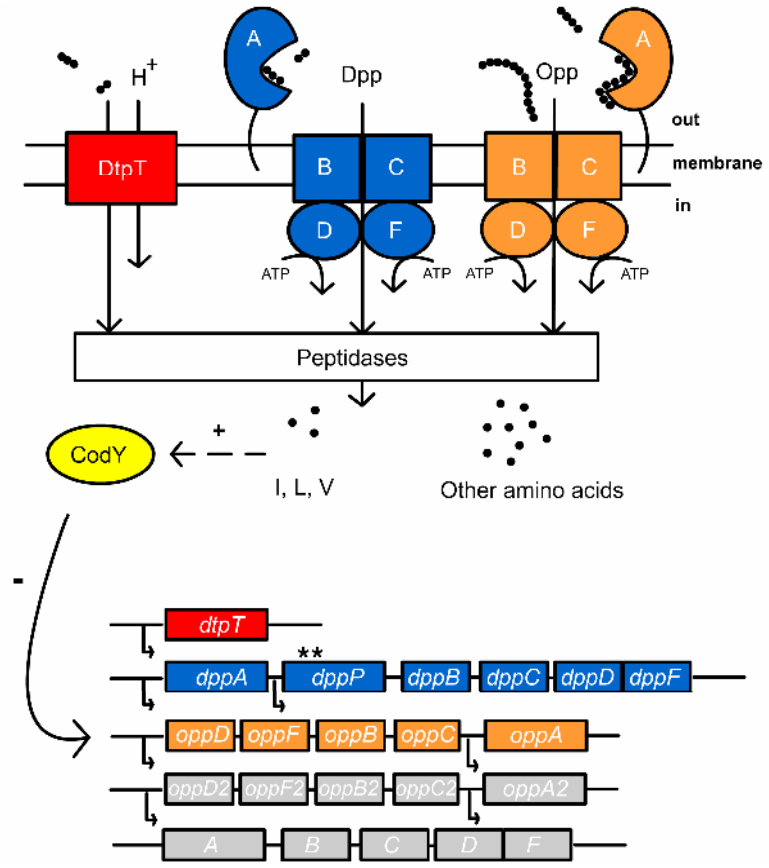

Fig. 1. **Schematic overview of function, regulation and genetic organization of peptide transporters in *Lactococcus lactis* MG1363.** Three functional peptide transport systems have been identified in *L. lactis* MG1363. The ion-linked transporter DtpT and the ABC transporter Dpp facilitate the uptake of di- and tripeptides. A second ABC transporter, Opp, catalyzes the uptake of oligopeptides containing 4 up to and including 35 amino acid residues. The (oligo)peptide-binding proteins (DppA and OppA) are anchored to the membrane via lipid modification of the N-terminal cysteine residue and deliver peptides to their cognate membrane complexes. Internalized peptides are hydrolyzed by intracellular peptidases. The transcriptional repressor CodY senses the internal pool of branched-chain amino acids (I, L and V), and inhibits the transcription of, among others, the *opp* genes. In MG1363, the gene coding for the second peptide-binding protein of the Dpp system, *dppP*, contains nonsense and frame-shift mutations, which are indicated by asterisks. Recently, a second set of oligpeptide transporter genes, designated *oppD2F2B2C2*, and a novel dipeptide/oligopeptide/nickel transporter homologue (genetic organization *ABCDF*) have been found. The Opp2 system and the novel dipeptide/oligopeptide/nickel transporter homologue are either not functional in peptide transport or not expressed.

Recently, a second set of oligopeptide transporter genes was found in *L. lactis* MG1363, presumably originating from a duplication of the *opp* operon, and the genes were designated *oppD2F2B2C2A2* (Sanz *et al.*, 2004; Fig. 1). An *oppA* deletion mutant was fully impaired in oligopeptide utilization, indicating that in MG1363 this second Opp system is either not expressed or not functional. Interestingly, complementation of an *oppA* knockout with the putative peptide-binding protein OppA2, expressed from a plasmid, restored the ability of the strain to utilize oligopeptides. This indicates that the OppA2 protein can interact functionally with the OppBCDF translocator. Genome analysis of *L. lactis* MG1363 revealed that, next to *opp* and *opp2*, MG1363 contains another dipeptide/oligopeptide/nickel transporter homologue

(Fig. 1). At present, it is not clear if this system is expressed and whether or not it is functional as a peptide transporter. On the basis of the oligopeptide null phenotype of the Δ*opp* strains, it is clear that this system does not play a significant, if any, role in oligopeptide uptake.

Regulation of peptide transport in MG1363

Transcription of the genes coding for the proteinase (*prtP*), the oligopeptide transporter (*oppDFBCA*) and several peptidases (*pepC, pepN,* and *pepO*) is repressed 5- to 150-fold upon the addition of peptides to the growth medium, which indicates a negative feedback mechanism of regulation (Detmers *et al.*, 1998; Guédon *et al.*, 2001a). A systematic study, using 67 dipeptides, showed that regulation of peptide transport was accomplished via a pleiotropic transcriptional repressor, CodY, which senses the internal branched-chain amino acid pool (Guédon *et al.*, 2001b). In accordance, deletion of the *dtpT* gene resulted in an upregulation of Opp activity (Kunji *et al.*, 1995), suggesting that di/tripeptide transport indeed plays an important role in the regulation of the proteolytic system. Moreover, expression of Opp was optimal in chemically-defined medium (CDM) containing free amino acids, presumably because these are less efficiently internalized than peptides. *In vitro* experiments confirmed that CodY binds upstream of its target genes, i.e. *pepN*, *pepC*, *opp-pepO1*, *prtPM*, and probably *pepX*, and *pepDA2* (den Hengst *et al.*, 2005). Binding of CodY to the promoter of the *opp* system is strongly affected by the addition of branched-chain amino acids. DNA binding is not influenced by GTP, contrary to what was shown for CodY from *Bacillus subtilis* (Shivers and Sonenshein, 2004). A schematic overview of the different peptide transporter genes, their corresponding transport systems, and the regulation of peptide tranport in *L. lactis* MG1363 is presented in Figure 1.

Peptide transport in *Lactococcus lactis* strains other than MG1363

Homologues of all the peptide transporters that are functional in *L. lactis* MG1363 (DtpT, Dpp and Opp) have been found in the sequenced strain IL1403 (Bolotin *et al.*, 2001; Fig. 2A). Interestingly, the gene coding for the second peptide-binding protein of the Dpp system of IL1403, *dppP*, does not contain the nonsense and frame-shift mutations present in *dppP* from MG1363 (Bolotin *et al.*, 2001; Sanz *et al.*, 2001). Peptide binding experiments showed that DppP bound di-, tri- and tetrapeptides, with highest affinity for tripeptides (Sanz *et al.*, 2003). Peptides longer than four residues were not tested in this study. Importantly, *in vivo* analysis of Dpp in *L. lactis* IL1403 suggests that DppP allows the Dpp system to transport peptides containing up to and including nine amino acid residues (Lamarque *et al.*, 2004). Moreover, Western blotting confirmed that DppP is not produced in MG1363, whereas IL1403 does not appear to synthesize the oligopeptide-binding protein (OppA) of the Opp system. This suggests that the critical activity of oligopeptide uptake in IL1403 is not specified by Opp but rather by Dpp (Fig. 2B). Three other *L. lactis* strains (SK11, SKM6, and Wg2) were also tested for their capacity to express (oligo)peptide transport systems. SK11 and Wg2 produced both DppP and OppA whereas SKM6, like IL1403, only synthesized DppP (Fig. 2B, C). Thus, *L. lactis* SK11 and Wg2 are equipped with at least two functional oligopeptide transport systems. It is not clear whether IL1403 fails to produce only OppA or also its cognate membrane complex OppBCDF. If OppBCDF is expressed, DppP might function not only with the DppBCDF complex but also with OppBCDF, thereby providing the cell with a higher capacity to

transport oligopeptides. An overview of the genetic organization and the types of (oligo)peptide transporters in *L. lactis* IL1403 and other lactococcal strains is presented in Figure 2.

In vivo or *in vitro* studies of peptide transport?

The specificity of peptide transport via the different uptake systems is often evaluated in growth experiments using either wild-type or transport-null strains (Tynkkynen *et al.*, 1993; Foucaud *et al.*, 1995; Sanz *et al.*, 2001; Charbonnel *et al.*, 2003). Since *L. lactis* is auxotrophic for several amino acids such as Glu (Gln), Met, His, Leu, Ile and Val, the ability to grow in CDM with one of these amino acids in the form of a peptide is an indication that the peptide is transported. Complications might arise when peptides are (partly) degraded outside the cell or contain the branched-chain amino acids Leu, Ile or Val. In the latter case, a reduced growth rate can be explained in two ways: either the peptide is not efficiently transported, or the expression of the transport system is reduced via the action of CodY (see above). Colonies of an *L. lactis* MG1363 strain containing a transcriptional fusion of the *oppA* promoter with *E. coli lacZ* are, for example, blue on CDM whereas they are white on M17, which reflects a 150-fold repression of transcription from P*oppA* when the cells grow in peptide-rich media such as M17 (Guédon *et al.*, 2001a, Guédon *et al.*, 2001b).
Peptide transport assays in whole *L. lactis* cells are done either by measuring the increase in the internal amino acid pool by reversed-phase high-performance liquid chromatography (Kunji *et al.*, 1993) or by using radiolabeled peptides (Detmers *et al.*, 1998; Picon *et al.*, 2000). *In vivo* uptake experiments, however, are complicated by rapid breakdown of internalized peptides by intracellular peptidases, subsequent efflux of (labeled) amino acids, and binding of peptides to the cell wall (Detmers *et al.*, 1998; Picon *et al.*, 2000). Accumulation of leu-enkephaline (YGGFL) via Opp, for example, was followed by a rapid efflux of radiolabeled amino acids from the cells (Detmers *et al.*, 1998). These complications prevent any detailed kinetic analysis of *in vivo* peptide transport in *L. lactis*. For these reasons, *in vitro* approaches using purified proteins are critical for a comprehensive understanding of all aspects of peptide transport and utilization (Lanfermeijer *et al.*, 1999; Detmers *et al.*, 2000; Fang *et al.*, 2000; Sanz *et al.*, 2000; Sanz *et al.*, 2003; Doeven *et al.*, 2004).

The oligopeptide-binding protein determines the selectivity of the Opp system

To determine the factor(s) that impose peptide uptake selectivity, Charbonnel *et al.* (2003) expressed OppA proteins from four distinct *L. lactis* strains in a *L. lactis* MG1363Δ*oppA* strain. Surprisingly, irrespective of the strain from which the OppA protein originated, the peptide utilization patterns were similar to that of the host strain. This suggested that the oligopeptide-binding protein does not play a significant role in determining the transport selectivity of its corresponding ABC uptake system. It was therefore proposed that the membrane complex, OppBCDF, imposes specificity on the transport process. On the contrary, an *in vitro* study, using purified and membrane-reconstituted Opp, clearly showed that OppA determines the uptake selectivity of the Opp ABC transporter (Doeven *et al.*, 2004). Binding and transport profiles with combinatorial peptide libraries matched perfectly.
The finding that several of the strains used by Charbonnel *et al*. (2003) employed DppP in stead of OppA for the uptake of oligopeptides (Lamarque *et al.*, 2004; strains IL1403 and

SKM6), or use both Opp and Dpp (strains SK11 and Wg2), clarifies why, in these *in vivo* experiments, OppA was not identified as the main determinant of oligopeptide transport selectivity. The additional transporter (Dpp) transported the oligopeptides. In fact, the observation that an additional peptide-binding protein (DppP) in *L. lactis* IL1403 enables the Dpp system to transport oligopeptides, in addition to di- and tripeptides (as is the case in MG1363), is a further argument that (oligo)peptide-binding proteins determine the uptake selectivity of their cognate ABC transporters.

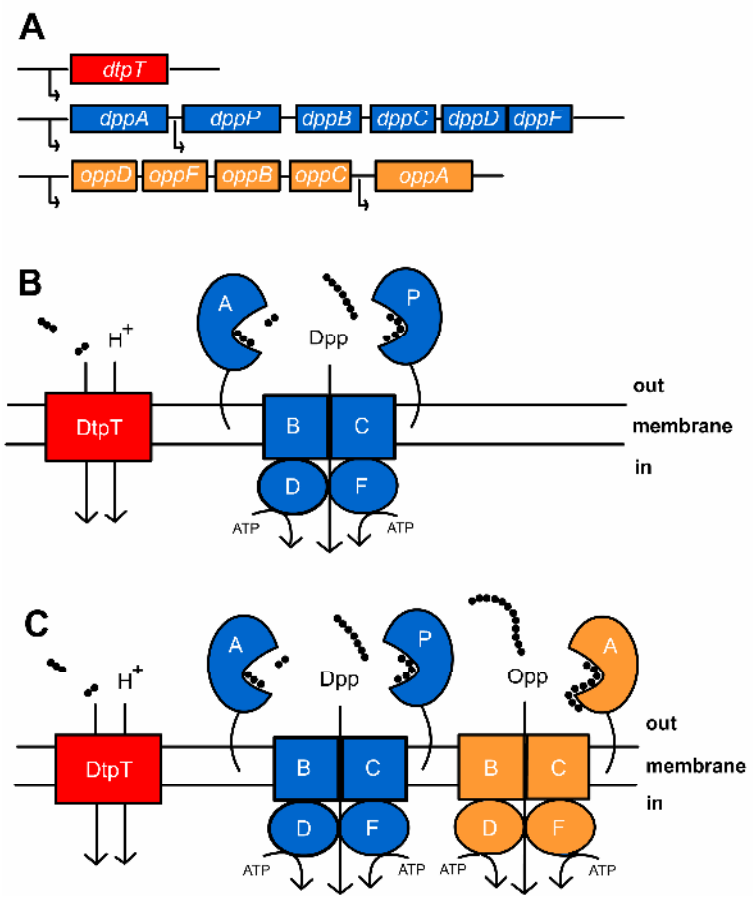

Fig. 2. **Schematic overview of genetic organization of peptide transporter genes and function in *Lactococcus lactis* IL1403 and other lactococcal strains.** Genetic organization of peptide transporter genes in *L. lactis* IL1403 (A). The *dppP* gene does not contain the nonsense and frameshift mutations observed in MG1363. In IL403 and SKM6 the Dpp system is used for the uptake of di/tripeptides (DppA) *and* oligopeptides (using DppP)(B). The Opp system is not used due to lack of OppA production. *L. lactis* strains SK11 and Wg2 are equipped with at least two functional oligopeptide transporters, that are Opp and Dpp; the latter employing DppP as an oligopeptide-binding protein (C). DtpT is presumed to be functional in all lactococcal strains.

Competitive inhibition of peptide binding or transport

An indirect way of evaluating binding and/or transport of peptides is to measure competitive inhibition of a reporter peptide, as has been done for the Opp system (Detmers *et al.*, 1998; Detmers *et al.*, 2000; Helinck *et al.*, 2003). Impairment of peptide binding to OppA, leading to

reduced transport rates, is the result of competition between the peptide of interest and the reporter peptide for the peptide-binding site of OppA. In one remarkable case however, a tetrapeptide (VGDE) was found that binds to the oligopeptide-binding protein OppA, inhibits transport of the reporter peptide Leu-enkephaline (YGGFL) but, as indicated by growth experiments, is not transported (Charbonnel *et al.*, 2003; Helinck *et al.*, 2003). *In vitro* binding studies showed that OppA has a low affinity for short peptides and that negatively charged residues reduce the binding affinity even further (Detmers *et al.*, 2000). At pH 6.5, which is the common pH of the medium used in growth experiments, the side chains of Asp (pK_a = 3.86) and Glu (pK_a = 4.25) are anionic. Under these conditions, *L. lactis* MG1363 does not grow when VGDE is supplied at submillimolar concentrations (Charbonnel *et al.*, 2003). However, upon increasing the VGDE concentration to 10 mM or when lowering the pH of the medium to pH 5.0 (and thereby increasing the fraction of protonated species) *L. lactis* was competent in growing on this peptide (unpublished result). Growth on VGDE requires the *opp* genes and is specifically inhibited by the addition of 100 μM bradykinin (RPPGFSPFR), a cationic peptide that binds to OppA with high affinity (K_D = 0.1 μM; Lanfermeijer *et al.*, 1999; Detmers *et al.*, 2000) and completely blocks *in vitro* peptide uptake via Opp (Doeven *et al.*, 2004). These observations further strengthen the notion that any peptide that can bind to OppA is transported via the Opp system.

Length specificity and structure of peptide-binding proteins

A list of peptide-binding proteins characterized to date is given in Table 1. Due to low sequence similarity (usually 20-30 % identity) between (oligo)peptide-binding proteins with known peptide-length specificity, it is difficult to predict the specificity of uncharacterized peptide-binding proteins solely on the basis of multiple sequence alignments. Currently, three high-resolution crystal structures of peptide-binding proteins are available: OppA from *Salmonella typhimurium*, DppA from *Escherichia coli*, and AppA from *Bacillus subtilis* (Tame *et al.*, 1994; Dunten and Mowbray, 1995; Nickitenko *et al.*, 1995; Levdikov *et al.*, 2005). These proteins bind peptides with 2-5, 2-3, and 9 amino acid residues, respectively. The structure of AppA with a bound nonapeptide is shown in figure 3A. Although the three-dimensional folds of $AppA_{Bs}$, $OppA_{St}$, and $DppA_{Ec}$ are similar, and the peptide-binding sites appear to be located at essentially the same position in each protein, structure-based predictions of peptide-length specificity remain difficult (Levdikov *et al.*, 2005). Interestingly, in $AppA_{Bs}$, the location of positions 4-7 of a bound nonameric peptide corresponds to the positions 1-4 in an $OppA_{St}$ substrate (Fig. 3B, C). Similarly, positions 1 and 2 of the dipeptide bound in $DppA_{Ec}$ superimpose on positions 4 and 5 in the $AppA_{Bs}$-bound peptide (Fig. 3B, D). Residues 1-3 of the ligand bound to $AppA_{Bs}$ are accommodated in an enlarged binding pocket due to a 3-residue shorter loop (residues 421-427) and displacement of an α-helix (151-156).
Alignment of the three (oligo)peptide-binding proteins of known structure with the well-characterized lactococcal (oligo)peptide-binding proteins OppA, DppA and DppP, and other peptide-binding proteins listed in Table 1 indicates that, in general, residues contributing to the peptide-binding site are poorly conserved (not shown; for alignments of peptide-binding proteins mentioned here or elsewhere, see Detmers *et al.*, (2001) and Levdikov *et al.*, (2005)). A notable exception is Asp^{419} of $OppA_{St}$ (the equivalent of Asp^{408} of $DppA_{Ec}$), which forms a salt-bridge with the N-terminal amino-group of the peptide in the crystal structures (Fig. 3C, D).

Table 1. **(Oligo)peptide-binding proteins with known length specificity**

Organism	Peptide-binding protein	Length of bound peptides	Reference(s)
Escherichia coli[1]	OppA	2-5	Guyer *et al.* (1986)
	DppA	2-3	Dunten *et al.* (1995)
			Nickitenko *et al.* (1995)
	MppA	3	Park *et al.* (1998)
Salmonella typhimurium[1]	OppA	2-5	Tame *et al.* (1994)
Xenorhabdus nematophila[1]	OppA1	n.d.	Orchard and Goodrich-Blair
	OppA2	3	(2004)
Enterococcus faecalis	TraC	8	Nakayama *et al.* (1998)
	PrgZ	7	Leonard *et al.* (1996)
Lactococcus lactis MG1363	OppA	4-35	Detmers *et al.* (2000)
	DppA	2-3	Sanz *et al.* (2000)
	DppP	n.d.[2]	Sanz *et al.* (2001)
	OppA2	5[3]	Sanz *et al.* (2004)
Lactococcus lactis IL1403	OppA	n.d.[4]	-
	DppA (OptS)	n.d.[4]	-
	DppP (OptA)	2-9[5]	Sanz *et al.* (2004)
			Lamarque *et al.* (2004)
Lactobacillus delbrueckii	OppA1	5	Peltoniemi *et al.* (2002)
	OppA2	n.d.	
Streptococcus pneumoniae	AmiA	2-3	Alloing *et al.* (1994)
	AliA	2-7	
	AliB	2-7	
Streptococcus gordonii	HppA	6-7	Jenkinson *et al.* (1996)
	HppG	?	
	HppH	6-7	
Streptococcus thermophilus	AmiA1	6, 11, 22[6]	Garault *et al.* (2002)
	AmiA2	?[6]	
	AmiA3	8, 22, 23[6]	
Streptococcus uberis	OppA1	3-8	Taylor *et al.* (2003)
	OppA2	n.d.	
Streptococcus agalactiae	DppA	2	Samen *et al.* (2004)
	OppA1	2-6	
	OppA2	2-6	
Bacillus subtilis	OppA	3-5	Picon and Van Wely (2001)
	AppA	9[7]	Picon and Van Wely (2001)
			Levdikov *et al.* (2005)
Borrelia burgdorferi	OppA1	2-7[8]	Lin *et al.* (2001)
	OppA2	2-7[8]	Wang *et al.* (2004)
	OppA3	2-7[8]	
	OppA4	3	
	OppA5	3	

[1]In Gram-negative organisms, mostly short peptides (2-5 residues) have been tested due to the inability of longer peptides to pass the outer membrane and reach the transport system. [2]Truncated and therefore not functional. [3]Only one peptide was tested. Probably similar to OppA from *L. lactis* MG1363. [4]Most likely similar to their homologues in *L. lactis* MG1363. [5]Only a few peptides longer than 4 amino acid residues were tested. [6]The Ami system of *S. thermophilus* transports peptides from 3-23 residues. The length specificities of individual AmiA proteins have not been fully established. [7]A limited number of peptides were tested. Genetic analysis indicated that AppA might bind a range of peptide lengths. [8]Longer peptides might also be bound since binding experiments were done using phage libraries

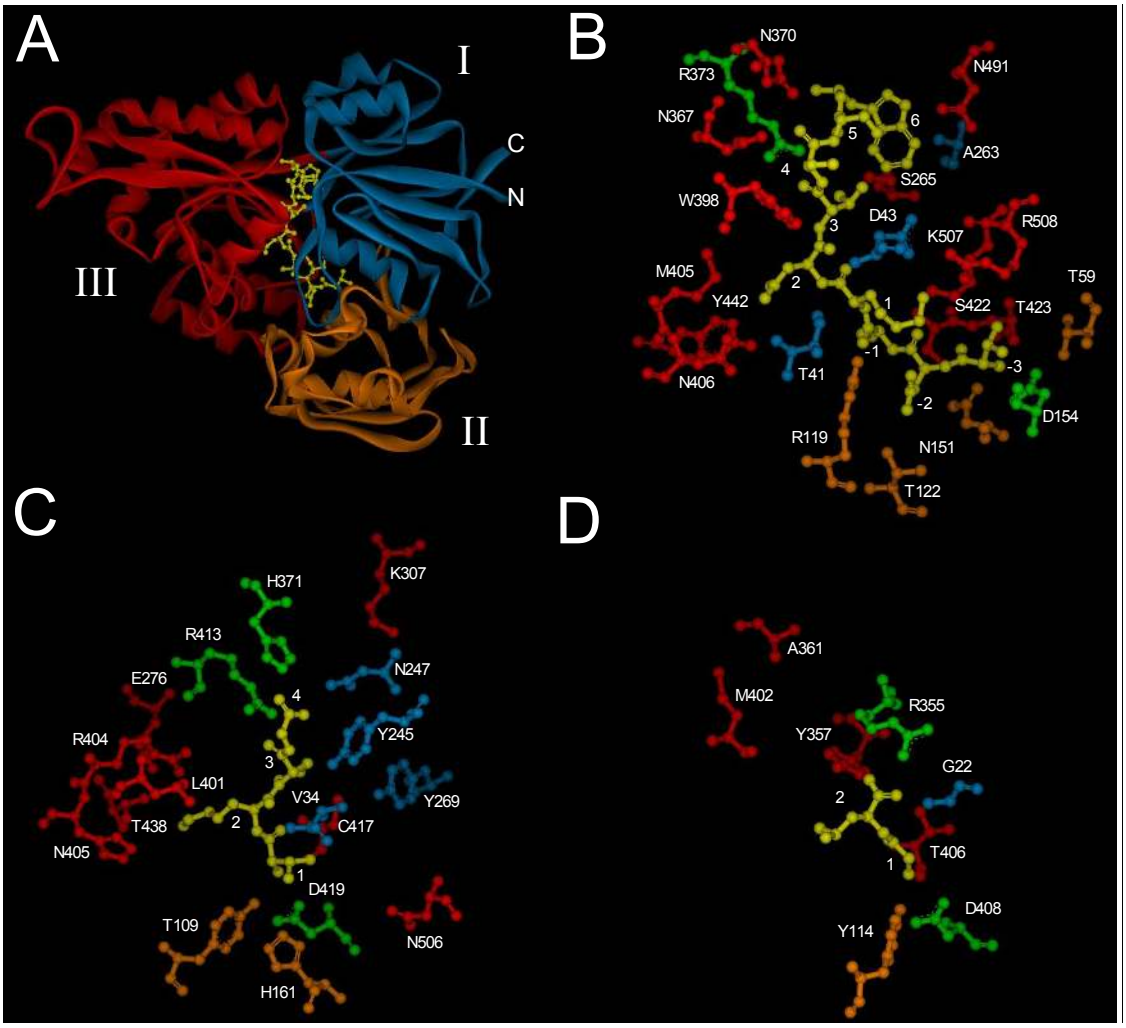

Fig. 3. **Structure and binding site architecture of (oligo)peptide-binding proteins.** Example of the fold of an (oligo)peptide-binding protein (A). The structure of $AppA_{Bs}$ with a bound nonapeptide (yellow) is shown (Levdikov *et al.*, 2005). Domain I and III engulf the substrate and are common for ABC-type substrate-binding proteins. Domain II is a specific feature of the dipeptide/oligopeptide/nickel transporter family. Architecture of the peptide-binding sites of $AppA_{Bs}$ (B), $OppA_{St}$ (C), and $DppA_{Ec}$ (D). Colour coding is the same as in (A) except for charged protein residues that are involved in salt-bridging either the N- or C-terminus of the bound peptides; these are indicated in green. Residues 4-7 of the nonameric peptide bound to $AppA_{Bs}$ are accommodated in pockets 1-4 (based on the structure of $OppA_{St}$), and the additional pockets are numbered -3, -2, -1, and 5, 6 for easy comparison. Even though the role of domain II in substrate binding and/or transport is not clear, in $AppA_{Bs}$ a few residues contribute to the extended peptide-binding site (B).

Although not present in $OppA_{Ll}$ and $AppA_{Bs}$, this residue is conserved in many other characterized peptide-binding proteins (Table 1) as well as in other (putative) (oligo)peptide-binding proteins (Detmers *et al.*, 2001). This suggests that the 'anchoring' of peptides via their N-terminal α-amino groups is similar in most (oligo)peptide-binding proteins. Furthermore, substrate-binding proteins that do not bind peptides but are homologous to (oligo)peptide-binding proteins, for example the heme-binding protein HbpA from *Haemophilus influenzae*, the agrocinopines-binding protein AccA from *Agrobacterium tumefaciens*, and the nickel-binding protein NikA from *E. coli*, do not contain the conserved aspartate, which thus seems to be specific for peptide-binding proteins (Detmers *et al.*, 2001). The residue in $AppA_{Bs}$ that is at

the equivalent position of Asp^{419} in $OppA_{St}$ is Ser^{422}, which is involved in hydrogen bonding with the N-terminus of the peptide (Fig. 3B). This serine is also present in $OppA_{Ll}$ which might indicate that binding of peptide N-termini in $AppA_{Bs}$ and $OppA_{Ll}$ is somewhat different from that in other (oligo)peptide-binding proteins. Asp^{154}, which forms a salt-bridge with the N-terminal residue of the nonameric peptide bound to $AppA_{Bs}$ (Fig. 3B), is not present in $OppA_{Ll}$.
Charged residues that were found to form salt-bridges with C-terminal residues of the bound peptide (like Arg^{373} in $AppA_{Bs}$, Arg^{413} and His^{371} in $OppA_{St}$, and Arg^{355} in $DppA_{Ec}$; Fig. 3B-D), are often absent in other (oligo)peptide-binding proteins (Detmers *et al.*, 2001; Levdikov *et al.*, 2005). Despite these possible differences in the interactions of the proteins with the N- and C-termini of the peptides, the majority of the protein-peptide contacts are formed via the backbone C=O and N-H groups, which facilitates sequence-independent binding of peptides. This phenomenon occurs similarly in each of the peptide-binding proteins for which a structure is available. Interactions with the side-chains of the peptide would impose a high level of specificity toward peptides containing specific amino acid residues. The fact that the peptide-protein interactions are preferably of a non-specific nature might explain why the peptide-binding sites are so poorly conserved and predictions of length specificity of (putative) (oligo)peptide-binding proteins are so difficult. It would be important to know the binding mechanisms of proteins that are thought to be highly specific for certain peptides, such as the pheromone-binding proteins TraC and PrgZ of *Enterococcus faecalis* (Leonard *et al.*, 1996; Nakayama *et al.*, 1998; Clewell *et al.*, 2000). However, detailed peptide binding studies are not available for any of these proteins.
Phylogenetic analyses of characterized (oligo)peptide-binding proteins indicate that binding proteins belonging to different transport systems can be clearly distinguished from each other (Fig. 4). The lactococcal Dpp binding proteins cluster together, and appear distantly related to the OppA proteins from *Streptococcus agalactiae*, *Streptococcus uberis*, and the pheromone-binding proteins TraC and PrgZ from *E. faecalis*. The lactococcal OppAs (including OppA2 and the novel dipeptide/oligopeptide/nickel-binding protein from MG1363) together with those from *Lactobacillus delbrueckii*, form a loose cluster with $AppA_{Bs}$, $DppA_{Sa}$, and $DppA_{Ec}$ but, overall, the identity between the proteins is low. The remaining streptococcal proteins (Ami and Ali from *S. thermophilus* and *S. pneumoniae* and Hpp from *S. gordonii*) all fall in one large group. The borrelial OppAs seem closely related to OppA and MppA from *E. coli,* and OppA from *S. typhymurium*, as do OppA1 and OppA2 from *Xenorhabdus nematophila*. Clearly, proteins with different peptide-length specificities cluster together in the phylogenetic tree, again exemplifying the non-conservative nature of the peptide-binding sites. The most striking example is probably $AmiA_{Sp}$, which, although it binds only di- and tripeptides, clusters with other Ami/Ali proteins, that bind peptides of up to and including 23 amino acids. Generally, (oligo)peptide-binding proteins using the same membrane complex cluster closely together. This is emphasized by the observation that OppAs from *Borrelia burgdorferi* could complement an *E. coli oppA* deletion strain by functionally interacting with the *E. coli* OppBCDF complex (Lin *et al.*, 2001) and by the fact that $OppA2_{Ll}$ could restore function to the Opp permease in a MG1363Δ*oppA* strain (Sanz *et al.*, 2004).
Multiple sequence alignments of all the (oligo)peptide-binding proteins listed in Table 1 reveal that the lactococcal OppAs and streptococcal Ami and Ali have several extensions compared to the other peptide-binding proteins (not shown). These particular sites in the proteins could be involved in the specific recognition by their cognate membrane complexes or be involved in the binding of extremely long peptides (up to and including 23 or 35 amino acid residues for

the Ami or Opp binding proteins, respectively), which has been reported only for these two systems.

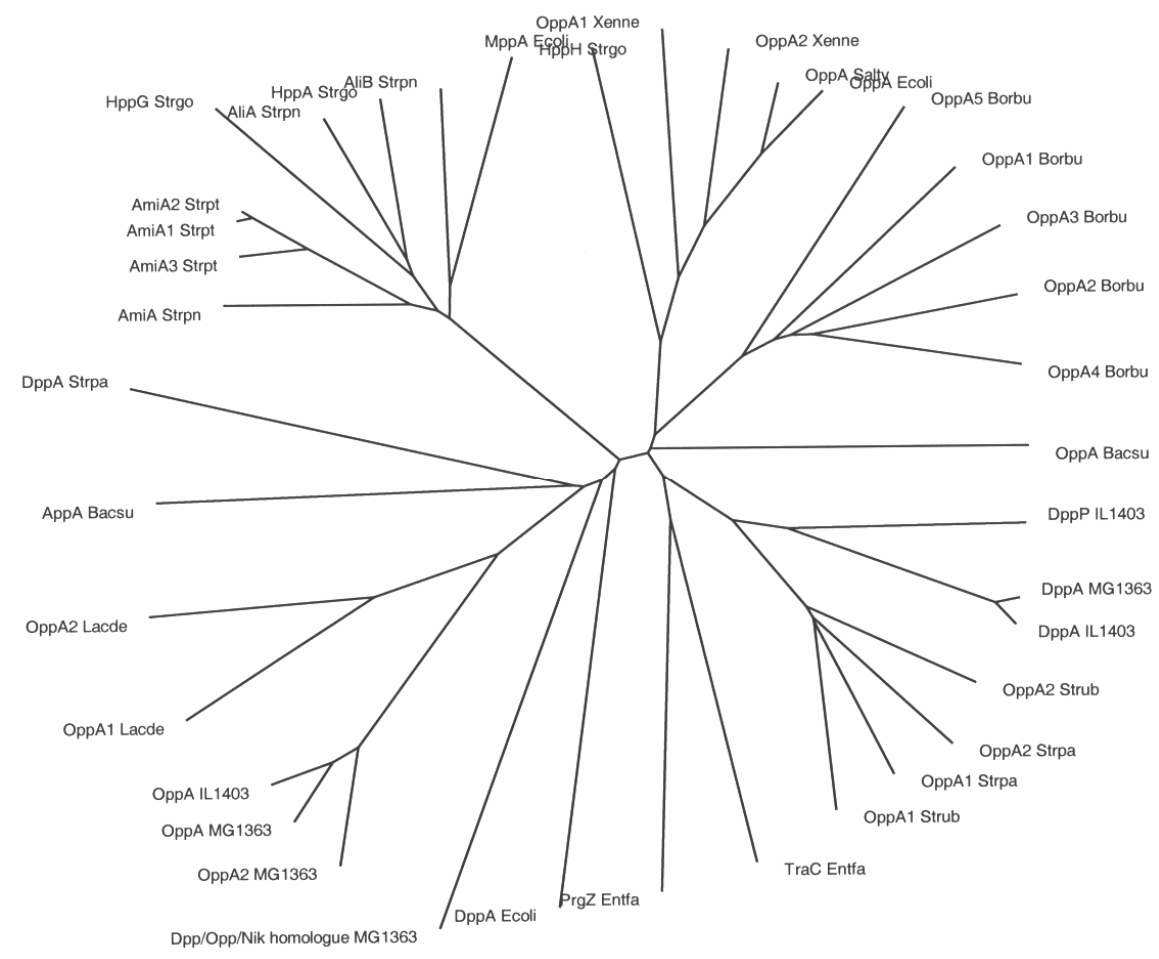

Fig. 4. **Phylogenetic tree of (oligo)peptide-binding proteins with known length specificity.** Bacsu: *B. subtilis*; Borbu: *B. burgdorferi*; Ecoli: *E. coli*; Entfa: *E. faecalis*; IL1403: *L. lactis* IL1403; Lacde: *L. delbrueckii*; MG1363: *L. lactis* MG1363; Salty: *S. typhymurium*; Strgo: *S.* gordonii; Strpa; *S. agalactiae*; Strpn: *S. pneumoniae*; Strpt: *S. thermophilus*; Strub: *S. uberis*; Xenne: *X. nematophila*.

Conclusions

An overview of peptide transport in *L. lactis* species has been presented, and the implications of recent findings for previously proposed hypotheses were discussed. *L. lactis* MG1363 uses DtpT and Dpp for the uptake of short peptides (2-3 residues) and Opp for the uptake of oligopeptides. Some other lactococcal strains also use the Dpp system for oligopeptide uptake. Dpp appears to be a versatile peptide transporter that employs multiple peptide-binding proteins (DppA and DppP), at least in *L. lactis* IL1403, SK11, SKM6, and Wg2. This situation is similar to that of streptococcal and borrelial peptide uptake systems (Ami/Ali/Opp), which use multiple peptide-binding proteins with overlapping specificities (Alloing *et al.*, 1994; Garault *et al.*, 2002; Samen *et al.*, 2004; Wang *et al.*, 2004). Contrary to some recent reports (Charbonnel *et al.*, 2003; Helinck *et al.*, 2003), we provide further evidence for the notion that the specificity

of the peptide ABC transporters is imposed by the selectivity of the (oligo)peptide-binding proteins and that the membrane-embedded translocator complexes are non-selective.

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Chapter 5

Hydrodynamic properties and crystallization of the oligopeptide-binding protein OppA from *Lactococcus lactis*

Mark K. Doeven, Dirk-Jan Slotboom, Andy-Mark W. H. Thunnissen, and Bert Poolman

Abstract

OppA is the receptor that delivers peptides ranging in length from 4-35 amino acid residues to the ATP-binding cassette transporter involved in oligopeptide uptake in *Lactococcus lactis*. The binding mechanism of lactococcal OppA seems distinct from that of other peptide-binding proteins because it lacks conserved binding site residues, and is thought to not completely enclose peptides longer than six amino acids. Analytical ultracentrifugation experiments revealed that purified OppA is a monomer that adopts a more compact shape upon peptide-binding. To obtain direct information on the binding site and to establish whether or not peptides protrude out of OppA in its "closed" conformation, we have crystallized the protein in complex with a nonapeptide. Crystals grew at pH 4.0 in the presence of polyethylene glycol (PEG) 1500, and at pH 7.0 with PEG6000 plus 0.2 M NaCl. Both crystal forms diffracted to a resolution better than 2.9 Å.

Introduction

The ability of *Lactococcus lactis* to grow in milk critically depends on its capacity to take up oligopeptides from the medium (Kunji *et al.*, 1998). To serve this purpose, lactococcal strains utilize transporters belonging to the ATP-binding cassette (ABC) superfamily (Doeven *et al.*, 2005). The translocation units of these systems are formed by complexes of two transmembrane domains plus two cytoplasmic nucleotide-binding domains (Higgins, 1992; Biemans-Oldehinkel *et al.*, 2005). Specificity determinants are the extracytoplasmic substrate-binding proteins (SBPs), that capture peptides and deliver them to the translocator (Doeven *et al.*, 2004). Whereas in Gram-negative bacteria peptide-binding proteins float freely in the periplasmic space, in *L. lactis* and other Gram-positive organisms they are anchored to the membrane via lipid-modification of an N-terminal cysteine.
Depending on the *L. lactis* strain, either the Dpp system, the Opp system, or a combination of

The abbreviations used are: ABC, ATP-binding cassette; BN-PAGE, blue native polyacrylamide gel electrophoresis; Gdm, guanidinium; MWCO, molecular weight cut-off; NTA, nitrilotriacetic acid; PEG, polyethylene glycol; SBP, substrate-binding protein.

both, is used for oligopeptide uptake (Lamarque *et al.*, 2004; Doeven *et al.*, 2005). The lactococcal Dpp system is equipped with a SBP for di- and tripeptides, DppA, and a second SBP for di-, tri-, and oligopeptides, DppP (Sanz *et al.*, 2001, 2003; Lamarque *et al.*, 2004). The Opp system is solely dedicated to oligopeptide uptake by its SBP OppA (Doeven *et al.*, 2004).

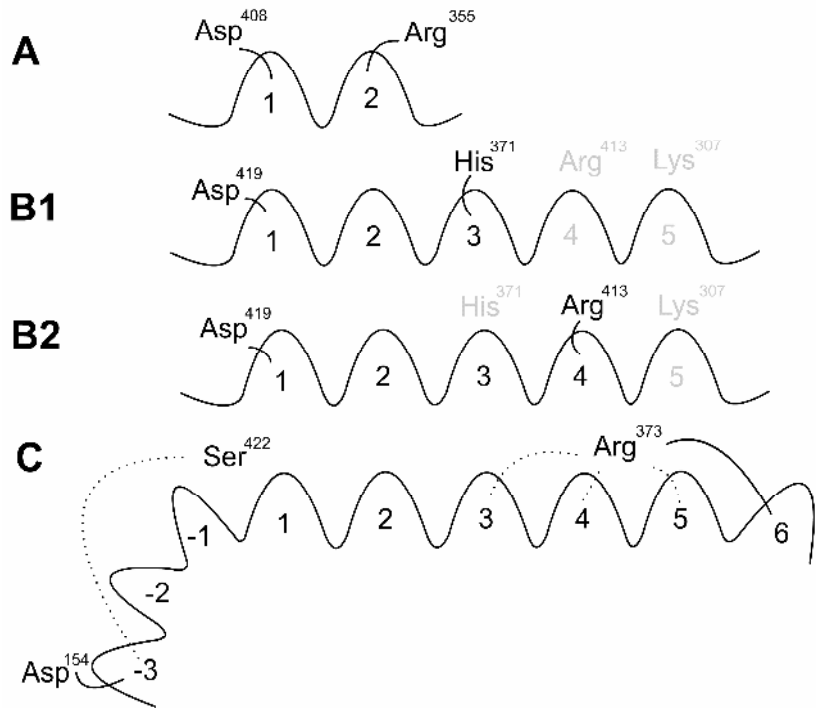

Fig. 1. **Cartoon of the peptide-binding sites in (oligo)peptide-binding proteins for which a crystal structure is available.** Binding pockets for the amino acid side-chains are numbered starting at the peptide N-terminus. Charged peptide-termini are stabilized by opposite charges in the proteins (solid lines), and the positioning of these protein residues determine peptide length preference. The aspartate salt-bridging the peptide N-terminus (A and B1/B2) is conserved in almost all (oligo)peptide-binding proteins. In addition, many residues hydrogen bond to the peptide backbone, but, for clarity, only those involved in binding the peptide termini are indicated (dotted lines). (A) Dipeptide bound in DppA from *E. coli*. (B1) and (B2) tri- and tetrapeptides bound in OppA from *S. typhimurium*. The C-terminus of a pentapeptide might be bound by Lys^{307}. (C) Nonapeptide accommodated in AppA from *B. subtilis*. The peptide is shifted towards the interior of the protein. The conserved aspartate is replaced by a serine, which is present in OppA from *L. lactis* as well. The N-terminus is bound by an aspartate at a different position in the protein, which is not conserved and also not present in lactococcal OppA.

Crystal structures of OppA homologues from *Salmonella typhimurium*, *Escherichia coli*, and *Bacillus subtilis* revealed that the peptide-bound protein is in a closed conformation, with the substrate buried in a cleft between two lobes (Tame *et al.*, 1994; Dunten and Mowbray, 1995; Nickitenko *et al.*, 1995; Levdikov *et al.*, 2005). In each case, an extensive network of H-bonds is formed with the peptide backbone, and charged residues in the protein stabilize the peptide termini (Fig. 1). These latter amino acids, however, appear missing in OppA from *L. lactis.* Moreover, the residues interacting with the peptide backbone are not conserved as well, and studies with modified and fluorescently-labelled peptides imply a binding mechanism for OppA from *L. lactis* where the peptide C-terminus protrudes out of the protein and interacts with its surface (Fig. 2; Detmers *et al.*, 2000; Lanfermeijer *et al.*, 2000). Thus, the binding mechanism

for lactococcal OppA appears fundamentally different from that observed in crystal structures obtained with homologous proteins. To shed more light on this issue, we have crystallized OppA from *L. lactis* in complex with a high affinity nonapeptide ligand as an initial step to structure determination.

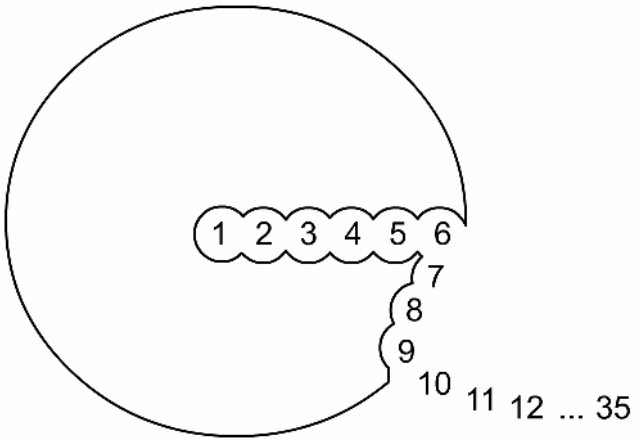

Fig. 2. **Proposed peptide-binding mechanism of OppA from *L. lactis*.** Peptide residue numbering as in figure 1. The 6 N-terminal residues of the peptide are enclosed by the protein. Additional C-terminal amino acids stick out and interact with the protein surface. Peptides as long as 35 residues can be bound. Picture adapted from Detmers *et al.* (2000).

Materials and methods

Bacterial strain and growth conditions. OppA was produced by *Lactococcus lactis* AMP2/pAMP21 as described (Lanfermeijer *et al.*, 1999), yielding OppA*, the protein lacking the N-terminal signal sequence required for export and lipid-modification. The N-terminal cysteine that in mature OppA is lipid-modified was replaced by a methionine. OppA* was constitutively produced in the cytoplasm under control of the lactococcal p32 promoter. The protein contained a C-terminal factor Xa cleavage site followed by a His_6-tag to facilitate purification. Cells were grown to a final $O.D._{660}$ of ~ 5 in 5 % (w/v) whey-permeate, 0.5 % (w/v) yeast extract, 0.5 % (w/v) glucose, and 65 mM KPi, pH 7.0, supplemented with 5 μg/mL erythromycin in a volume of 10 litres in a fermenter with pH control (ADI 1065 fermenter; Applikon Dependable Instruments B. V., Schiedam, The Netherlands). The pH was kept constant at pH 6.5 by titrating with 2 M KOH. Cells were harvested by centrifugation, washed with 100 mM KPi, pH 7.0, and resuspended to an $O.D._{660}$ of ~ 100 in 50 mM KPi, pH 8.0, 100 mM KCl, 10 % (v/v) glycerol (buffer A), flash frozen in liquid nitrogen and stored at -80°C.

Isolation of cytoplasmic fraction. Cells were thawed and treated with 2 mg/mL lysozyme for 30 minutes at 30°C. After adding 1 mM phenylmethanesulfonylfluoride, 0.1 mg/mL DNase, and 10 mM $MgSO_4$, the cells were ruptured using a high-pressure homogenizer (Kindler Maschinen AG, Zürich, Switzerland). Following an incubation of 15 minutes at room temperature, 15 mM Na-EDTA was added and the cytosolic fraction containing OppA* was separated from the membranes and cell debris by 45 minutes centrifugation at 280,000 x *g* and 4°C. The supernatant was frozen and stored at -80°C or, when used for setting up crystallization trials, directly purified further.

Purification of OppA*. The supernatant (~ 400 mL at 11.5 mg of protein/mL) was incubated for 1.5 h at 4°C in buffer A, plus 15 mM imidazole with 1 mL nickel-NTA resin (Qiagen) per 10 mL supernatant. Subsequently, the column material was collected by centrifugation for 10 min. at 1,000 x *g* and 4°C and poured into 10 mL Biorad columns such that each contained ~ 1 mL nickel-NTA after which excess supernatant was drained. The column was washed three times with 10 column volumes of the same buffer, pH 7.0, containing 15, 20 and 40 mM

imidazole, respectively. OppA* was eluted by lowering the pH to 6.0 and increasing the imidazole concentration to 200 mM. Peak fractions (~ 30 mL in total) were pooled and dialyzed overnight against 25 mM KPi, pH 6.0, 10 % (v/v) glycerol (buffer B). Subsequently, the protein was loaded onto 8 mL S-sepharose (Amersham Biosciences) and eluted with a 10 column volume gradient from 0 to 1 M KCl in buffer B. OppA*-containing fractions were pooled and diluted to lower the [KCl] to ~ 200 mM, and raise the pH to ~ 7.5. Endogenously bound peptides were removed by partially unfolding the protein with guanidinium-HCl (Gdm-HCl), according to Lanfermeijer *et al.* (1999) with some modifications. Briefly, the purified OppA* (~ 2.5 mg of protein) was incubated for 1.5 h with 1 mL Ni-NTA/mg protein in a 10 mL Biorad column. The column was drained and washed with 4 column volumes buffer B, pH 7.5, plus 100 mM KCl (buffer C), followed by 40, 4, 4, 4, and 8 column volumes of the same buffer plus 2.0, 1.5, 1.0, 0.5, and 0 M Gdm-HCl, respectively. After this, the protein was eluted from the column using buffer C, pH 6.0, plus 200 mM imidazole. The protein was concentrated on 30,000 MWCO centricon devices (Millipore) to ~ 1.3 mg/mL and dialyzed against buffer C, pH 6.0. Removal of endogenously bound ligands was confirmed by intrinsic protein fluorescence studies and native cationic electrophoresis according to Lanfermeijer *et al.* (1999).

Purification for crystallization. The cytoplasmic extract (~ 400 mL; see above) was incubated for 1 h at 4°C in buffer A plus 10 mM imidazole with 5 mL nickel-sepharose resin (Amersham Biosciences). The column material was collected by centrifugation for 10 min. at 1,000 x *g* and 4°C, suspended in a few mL of the same buffer and packed into a XK 16/20 column (Amersham Biosciences) after which the column was washed with the same buffer, using the Äkta Purifier system (Amersham Biosciences), until the absorbance at 280 nm reached a baseline level. OppA* was eluted by lowering the pH to 6.0 and increasing the imidazole concentration to 200 mM. OppA*-containing fractions were pooled, concentrated on 30,000 MWCO centricon devices (Millipore), and further purified on a Superdex 200/10 size exclusion column (Amersham Biosciences) pre-equilibrated in 20 mM MES, pH 6.0, 150 mM NaCl. OppA*-containing fractions were pooled, concentrated ~ 10-fold, and diluted such that the final buffer composition was 10 mM MES, pH 6.0, 10 mM NaCl, and further concentrated to ~ 14 mg/mL of protein. 1/10 volume of 10 mM bradykinin (nonapeptide with the amino acid sequence RPPGFSPFR; Bachem) in milli Q water was added such that the final concentrations were ~ 12.5 mg/mL OppA*, 1 mM bradykinin, 9 mM MES, pH 6.0, and 9 mM NaCl.

Native protein electrophoresis. Native cationic electrophoresis (Lanfermeijer *et al.*, 1999), and blue native polyacryl amide gel electrophoresis (BN-PAGE; Schägger *et al.*, 1991, 1994; Heuberger *et al.*, 2002) were performed as described.

Analytical ultracentrifugation. Analytical ultracentrifugation experiments were performed in a Beckman Optima XL-I, using an AN-50 Ti rotor with 2-channel charcoal-filled centerpieces. Sedimentation velocity experiments were done at 38,000 rpm and 4°C on sample volumes of 400 μL with loading concentrations ranging from 0.020 to 1.300 mg/mL in buffer C, pH 6.0. Absorbance data were collected at 280 and 230 nm in a continuous mode with a radial step size of 0.005 cm and 10 min. time intervals.
The effective mass of a particle (M_{eff}) is given by:

$$M_{\text{eff}} = \frac{M(1 - \upsilon\rho)}{N_A}, \qquad \text{(equation 1)}$$

where M is the molar weight, u is the partial specific volume of the protein (in dilute solute solutions, u is the volume in mL that each gram of solute occupies), ρ is the solution density, and N_A is Avogadro's number. The definition of the sedimentation coefficient (s) of a macromolecule is given by the Svedberg equation:

$$s = \frac{u}{\omega^2 r} = \frac{M(1 - \upsilon\rho)}{N_A f}, \qquad \text{(equation 2)}$$

where u is the observed radial velocity of the protein, ω the angular velocity of the rotor, r the radial position, and f the frictional coefficient. s-values are reported in Svedberg units (S), that correspond to 10^{-13} sec. The f value can be determined for smooth, compact spherical proteins using the Stokes equation:

$f_0 = 6\pi\eta R_0$, (equation 3)

where f_0 is the frictional coefficient of the spherical particle, η the viscosity of the solution, and R_0 the radius of the sphere. Observed sedimentation coefficients (s_{obs}) can be converted to a standard state in water at 20°C ($s_{20,w}$) using the following equation:

$$s_{20,w} = s_{obs}\left(\frac{\eta_{exp}}{\eta_{20,w}}\right)\frac{(1-\upsilon\rho)_{20,w}}{(1-\upsilon\rho)_{exp}}, \quad \text{(equation 4)}$$

where the subscripts '20,*w*' and 'exp' denote standard and experimental conditions, respectively.
Data analysis was done using the XL-I data analysis software (Beckman). s_{obs} values were determined using the second-moment method of analysis, and converted into $s_{20,w}$ values using the SEDNTERP programme (developed by Hayes, Laue, and Philo, and available at http://www.jphilo.mailway.com/). The partial specific volume (*u*) of OppA* was 0.7227 mL/mg at 4°C as calculated from the primary amino acid sequence using SEDNTERP. Values for the solvent density (ρ) and viscosity (η) of buffer C were determined using the same programme and were 1.03878 g/L and 2.2348 x 10^{-2} Poise, respectively, at 4°C.
Sedimentation equilibrium experiments were performed at rotor speeds of 8,000, 10,000, and 12,000 rpm on sample volumes of 100 μL with loading concentrations of 0.020, 0.050, and 0.100 mg/mL OppA* in buffer C, pH 6.0. The absorbance optics was used to collect data every 0.001 cm with 10 replicates at 280 nm. At sedimentation equilibrium, the protein concentration (*c*) as a function of the radial position (*r*) is given by:

$$\left(\frac{2RT}{\omega^2}\right)\frac{\mathrm{d}\ln c}{\mathrm{d}r^2} = M(1-\upsilon\rho), \quad \text{(equation 5)}$$

with *R* being the gas constant, *T* the absolute temperature, and ω, *M*, *u*, and ρ the same as above. The molecular weight of OppA* was determined from the sedimentation equilibrium experiments by global fitting of nine data sets, using the XL-I data analysis software (Beckman).

Crystallization and X-ray diffraction analysis. Screening was done using 96 well plates designed for sitting drop vapour diffusion experiments (Hampton Research). The reservoir contained 100 μL of mother liquor and drops were made by mixing 1 μL protein solution with 1 μL precipitant solution. The commercially available Structure Screen 1 and 2 (both sparse matrix screens; Molecular Dimensions), and PACT *premier*™ (PEG, pH, ion-screen; Molecular Dimensions) were used at 4°C and room temperature. X-ray diffraction analysis was carried out with Cu Kα radiation from a Bruker-Nonius rotating anode generator equipped with a DIP2000 image plate detector.

Miscellaneous. Protein concentrations were determined according to the method of Lowry *et al.* (1951), using bovine serum albumin as a standard. The concentration of purified OppA* was determined spectrophotometrically by measuring the absorption at 280 nm and using an extinction coefficient of 1.605 $(mg/mL)^{-1}cm^{-1}$. The pH of buffers used for crystallization were set by titrating with either NaOH or HCl.

Results

Purification and characterization of OppA*. A soluble version of OppA that lacks its lipid-anchor, termed OppA*, was produced in the cytoplasm of *L. lactis* as a result of the removal of the N-terminal signal sequence that normally targets the protein for export and lipid-modification (Lanfermeijer *et al.*, 1999). The purification protocol used by Lanfermeijer *et al.* (1999) involved 2 steps: i) passing the protein over an anion exchange column thereby

removing the bulk of cytoplasmic proteins, followed by ii) purification of the protein by cation exchange chromatography. The C-terminal His_6-tag was used to immobilize OppA* on a

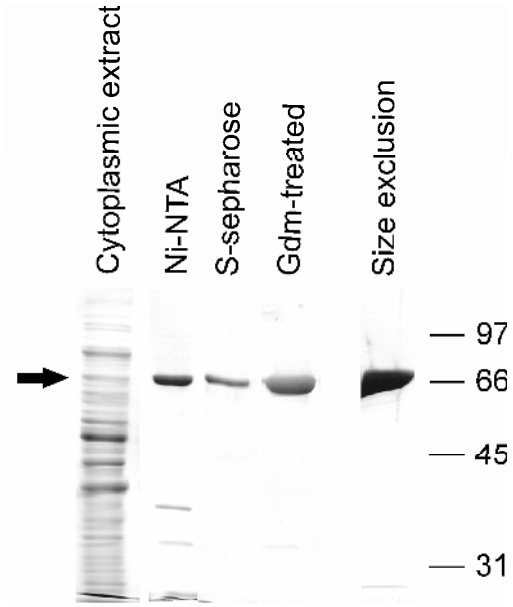

Fig. 3. **Purification of OppA*.** SDS-PAGE analysis of OppA*-containing samples before and after different purification steps. Proteins were visualized by Coomassie brilliant blue staining. Endogenously bound peptides were removed by partial unfolding/refolding of the protein using Gdm-HCl (Gdm-treated) according to Lanfermeijer *et al.* (1999). The positions at which OppA* (arrow) and molecular weight marker proteins (right; masses indicated in kDa) migrate are indicated.

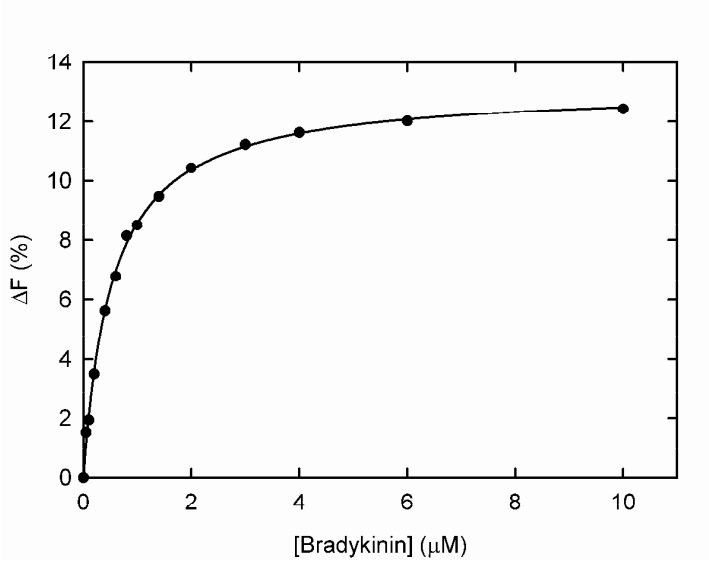

Fig. 4. **Peptide-binding activity of OppA* monitored by intrinsic protein fluorescence measurements.** Titration of Gdm-treated OppA* with bradykinin. The protein concentration was 0.5 μM. The change in protein fluorescence (ΔF) was measured and data fitted according to Lanfermeijer *et al.* (1999).

nickel-NTA column and the protein was treated with Gdm-HCl to remove endogenously bound peptides. Here, we utilized the His_6-tag on OppA* in the first step of our purification by performing metal-affinity chromatography (Fig. 3). The second and third step involved again cation exchange chromatography and removal of co-purified peptides by partial unfolding of OppA* by Gdm-HCl (Fig. 3). Protein functionality and the removal of endogenous bound substrate was verified by monitoring changes in intrinsic protein fluorescence upon titration with peptide (Fig. 4) and native cationic electrophoresis (not shown) both performed according to Lanfermeijer et al. (1999). The dissociation constant (K_D) and maximum change in fluorescence (ΔF_{max}) upon bradykinin (RPPGFSPFR) binding were 0.26 μM and 12.3 % which is close to the values of 0.10 μM and 12.6 % that were obtained previously (Lanfermeijer *et al.*, 1999). For crystallization purposes (see below), the second purification step was chosen to be size-exclusion chromatography (Fig. 3) and the protein was not treated with Gdm-HCl.

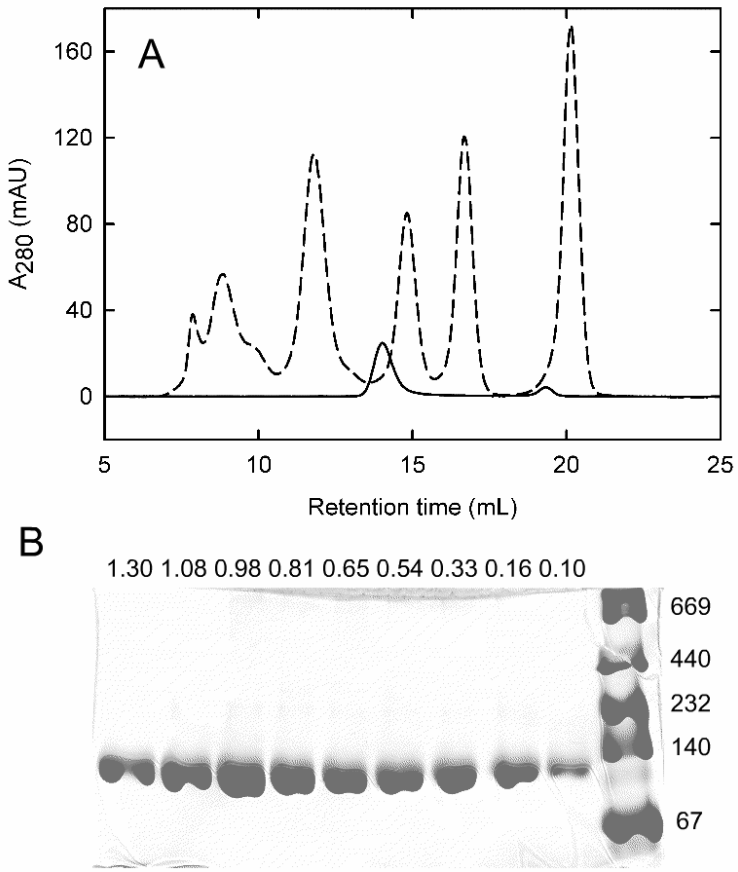

Fig. 5. **Analysis of OppA* by size-exclusion chromatography and BN-PAGE.** (A) Elution profile of ~ 200 μg nickel-NTA purified OppA* (solid line) injected on a Superdex 200 gel-filtration column at room temperature. Molecular weight markers (dashed line) were thyroglobulin (670 kDa), Bovine gamma globulin (158 kDa), Chicken ovalbumin (44 kDa), Equine myoglobin (17 kDa), and vitamin B12 (1.35 kDa). The buffer composition was 50 mM KPi, pH 7.0, 200 mM KCl, 10 % (v/v) glycerol. The flow rate was 0.5 mL/min. (B) BN-PAGE analysis of nickel-NTA and S-sepharose purified, ligand-free OppA*. The protein concentration was varied from 0.10 – 1.30 mg/mL in buffer C as indicated on top of the gel and 3 μg of protein was loaded per lane (except for the 0.10 mg/mL sample that contained 2 μg). The masses of the marker proteins are indicated on the right of the figure (kDa).

Oligomeric state of OppA*. Although crystal structures of numerous SBPs have led to the generally accepted view that they are monomers with one substrate-binding site per molecule (Quiocho and Ledvina, 1996), several early experiments suggested that SBPs self-associate to form dimers or higher order oligomers (Rashed *et al.*, 1976; Richarme *et al.*, 1982, 1983), which might be a way to regulate their activity (Antonov *et al.*, 1976). Moreover, in ABC tranporters containing multiple SBPs fused to the translocator cooperative interactions between these domains have been found to stimulate transport activity (Biemans-Oldehinkel and Poolman, 2003). On a size-exclusion column OppA* eluted at an apparent molecular mass of ~ 54 kDa (Fig. 5A), which is somewhat smaller than the 65,066 Da predicted for the monomeric protein based on its primary structure. BN-PAGE, on the other hand, showed that the OppA*:Coomassie G complex migrates at the position of a possible dimer (~ 130 kDa; Fig. 5B) over the OppA* concentration range from 0.10 – 1.30 mg/mL. Addition of 30 μM bradykinin did not detectably alter the migration of OppA* in the BN-gel (not shown). Since OppA* is cationic with a pI of 8.8, it might be possible that the protein binds a lot of anionic Coomassie G dye, which results in an overestimation of the apparent molecular mass. To resolve this issue, we used sedimentation velocity and equilibrium centrifugation.
Analytical ultracentrifugation sedimentation velocity profiles of OppA at a concentration of 0.65 mg/mL are shown in Fig. 6A. From the sedimentation of the protein in the time period from 100-200 min., s_{obs} values were calculated using the second-moment method of analysis. The sedimentation behaviour of OppA* did not change significantly over the concentration range from 0.02 to 1.30 mg/mL (Fig. 6B), indicating that the protein was present as a single-species not undergoing reversible self-association. The average sedimentation coefficient ($s_{20,w}$) was 4.2 ± 0.1 S, and increased to 4.6 ± 0.2 S upon the addition of a saturating amount of bradykinin (Fig. 6B). The faster migration of OppA* in the centrifugal field suggests a change in hydrodynamic radius: the protein adopts a more compact ("closed") shape upon peptide-binding. The $s_{20,w}$ values of 4.2 and 4.6 are most consistent with OppA* being monomeric (Stewart and Johns, 1976). Sedimentation equilibrium experiments (Fig. 7) confirmed that OppA* is a monomer. The estimated molecular weight was 68 ± 2 ($*10^3$), which is close to the expected molecular weight for the monomer of 65,066, based on amino acid sequence. In the presence of bradykinin, the apparent molecular weight increased from 68 ± 2 ($*10^3$) to 69 ± 3 ($*10^3$). This can be due to the binding of bradykinin (molecular weight is 1,060), although the increase in apparent molecular weight was not significant.

Crystallization. To reveal how peptides are accommodated by lactococcal OppA, the protein was crystallized in complex with the high affinity nonapeptide ligand bradykinin. Crystals were obtained in approximately two weeks of incubation at room temperature with two conditions of the PACT *premier*™ screen: 0.1 M MIB buffer, pH 4.0, plus 25 % (w/v) PEG1500 (B1), and 0.2 M NaCl, 0.1 M Hepes, pH 7.0, plus 20 % (w/v) PEG6000 (C7) (Fig. 8). Raising the pH of condition B1 by one unit resulted in needles, while at higher pH values crystals were not observed. Replacing Na^+ of condition C7 with NH_4^+ yielded needles as well, whereas Li^+, Mg^{2+}, and Ca^{2+} gave no crystals under otherwise identical conditions.

X-ray data collection and optimization of the crystals. Crystals from both conditions (B1 and C7) diffracted to better than 2.9 Å resolution with X-rays from an in-house rotating anode diffractometer (Fig. 9). Their Bravais lattice types are primitive orthorhombic with unit cell dimensions of a = 42.7, b = 64.3, c = 99.3 Å, and a = 42.7, b = 76.0, c = 182.3 Å for condition

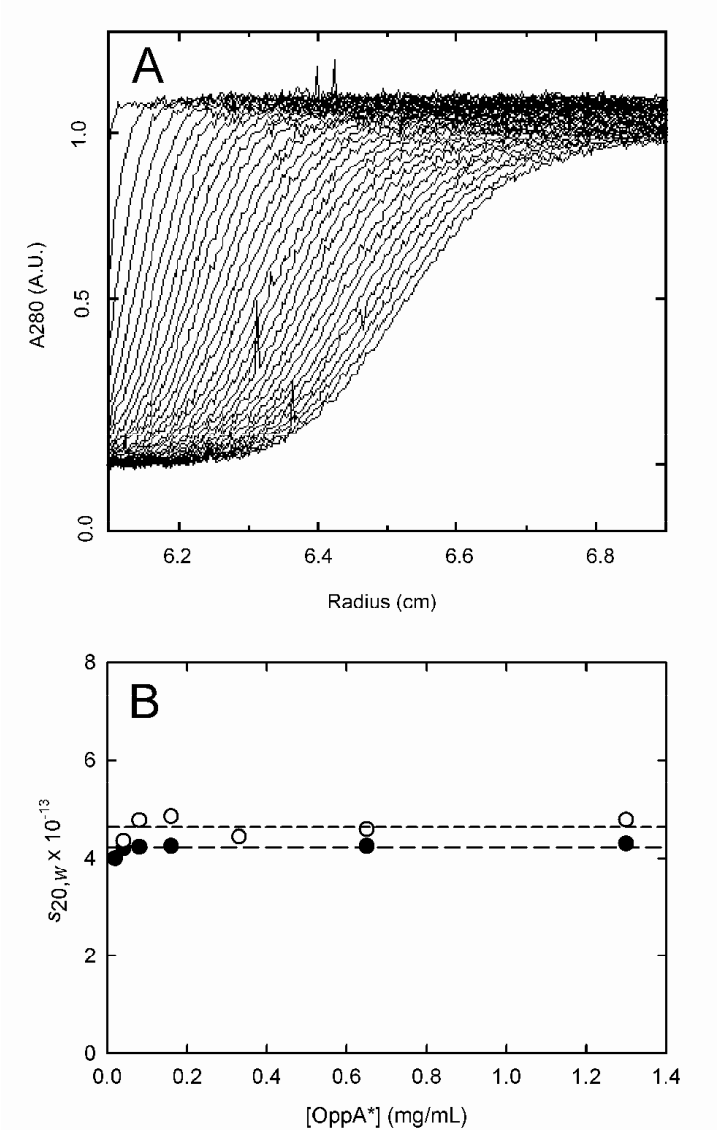

Fig.6. **Velocity sedimentation analysis.** (A) Velocity sedimentation profiles of 0.65 mg/mL OppA* at 38,000 rpm and 4°C in 25 mM KPi, pH 6.0, 100 mM KCl, and 10 % (v/v) glycerol. Time intervals were 10 min. (B) $s_{20,w}$ values plotted vs. OppA* concentration in the absence (filled symbols) or presence (open symbols) of a saturating concentration bradykinin. The horizontal lines indicate the average $s_{20,w}$ values obtained in the absence (long dash) or presence (short dash) of ligand.

B1 and C7, respectively. Unfortunately, both crystal forms displayed high levels of mosaicity (~ 1.2° for B1 and at least 1.5° for C7), and at room temperature in the X-ray beam the crystals were not stable long enough to obtain a complete data set. An attempt to optimize the crystals of C7 was made by varying salt concentration, pH and PEG concentration independently or in various combinations from 0.14-0.26 M NaCl, 0.1 M Hepes, pH 6.4-7.6, and 14-26 % (w/v) PEG6000, respectively, but this yielded no crystals presumably due to the use of a batch of PEG different from that of the manufacturer of the initial screening kit. As an alternative strategy seeding was tried, but this yielded no improvement of the crystals (needles). Further optimization was done for both conditions by using additives [mixing 90 μL of the PACT *premier*™ solutions with 10 μL of stocks from the Additive Screen™ (Hampton Research) in

the reservoir] and setting up crystallization drops with these solutions. In condition B1 plus 4 % (v/v) 1,4 butanediol and C7 plus 0.01 M cobalt-chloride new crystals appeared, but these still have to be tested for X-ray diffraction. For condition C7 cryo-conditions were established by raising the PEG6000 concentration to 40 % (w/v), possibly allowing full data collection as soon as new crystals are available.

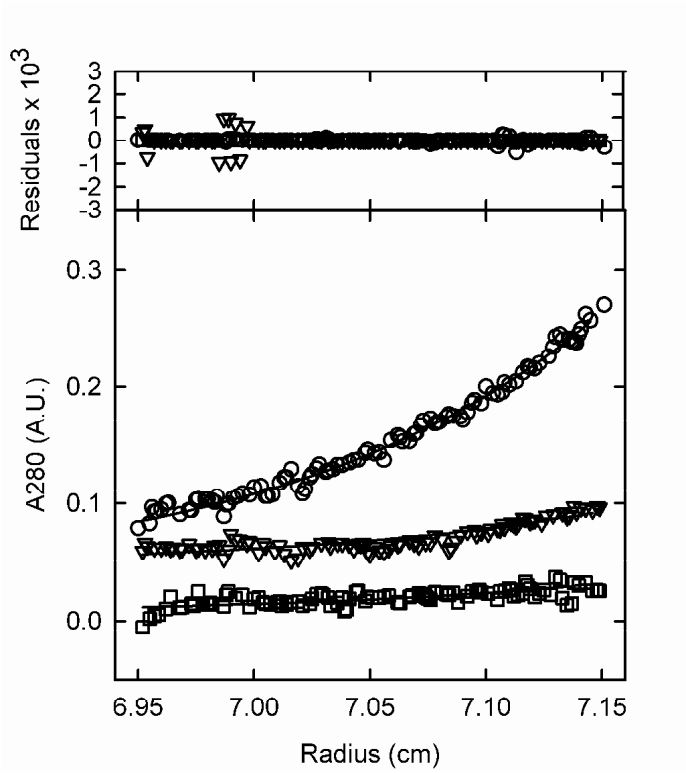

Fig. 7. **Sedimention equilibrium analysis.** Radial distribution of OppA* at 10,000 rpm and 4°C with protein loading concentrations of 0.02 (squares), 0.05 (inverted triangles), and 0.10 mg/mL (circles) in the presence of saturating concentrations of bradykinin. The solid lines represent the best fit described by global analysis of nine datasets collected at rotor speeds of 8, 10, and 12 krpm. Residuals are shown in the top graph.

Discussion and conclusions

OppA from *L. lactis* is the functionally best characterized (oligo)peptide-binding protein to date although a high resolution crystal structure is still lacking. X-ray structures obtained for three homologous proteins, each displaying only ~ 20 % sequence identity with lactococcal OppA and amongst themselves, show a closed, three domain organization with the peptide buried between two lobes (Tame *et al.*, 1994; Dunten and Mowbray, 1995; Nickitenko *et al.*, 1995; Levdikov *et al.*, 2005). Closure of the protein is thought to occur upon peptide-binding, as structures in the absence of ligand show a conformation with the binding site exposed to solvent (Nickitenko *et al.*, 1995; Sleigh *et al.*, 1997). The peptide termini are stabilized by charged residues in the protein, the negatively charged aspartate salt-bridging the N-terminus being the most conserved binding site residue across the entire protein family (Fig. 1; Detmers *et al.*, 2001).

L. lactis OppA has been shown to bind peptides ranging from 4 up to and including at least 35 residues (Detmers *et al.*, 2000), and it seems unlikely that especially these long peptides are completely enclosed by the protein. Furthermore, the charged residues that stabilize the peptide termini in other peptide-binding proteins are not conserved in lactococcal OppA (Detmers *et al.*, 2001; Levdikov *et al.*, 2005). Indeed, for *L. lactis* OppA, a unique binding mechanism was proposed in which only the six N-terminal residues are buried, whereas the C-terminal residues of the peptide stick out and interact with the protein surface (Fig. 2; Detmers *et al.*, 2000; Lanfermeijer *et al.*, 2000).

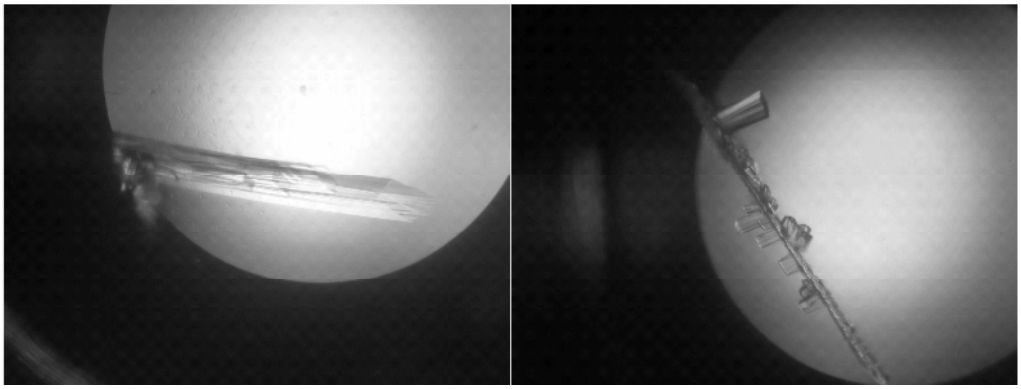

Fig. 8. **Photomicrographs of OppA crystals.** Crystals were grown in 0.1 M MIB buffer, pH 4.0, and 25 % (w/v) PEG1500 (left), and 0.1 M Hepes, pH 7.0, 20 % (w/v) PEG6000, and 0.2 M NaCl (right). Crystals grew at room temperature in approximately two weeks to final dimensions of about 0.1 x 0.1 x 1.0 mm and 0.1 x 0.1 x 0.2 mm, respectively.

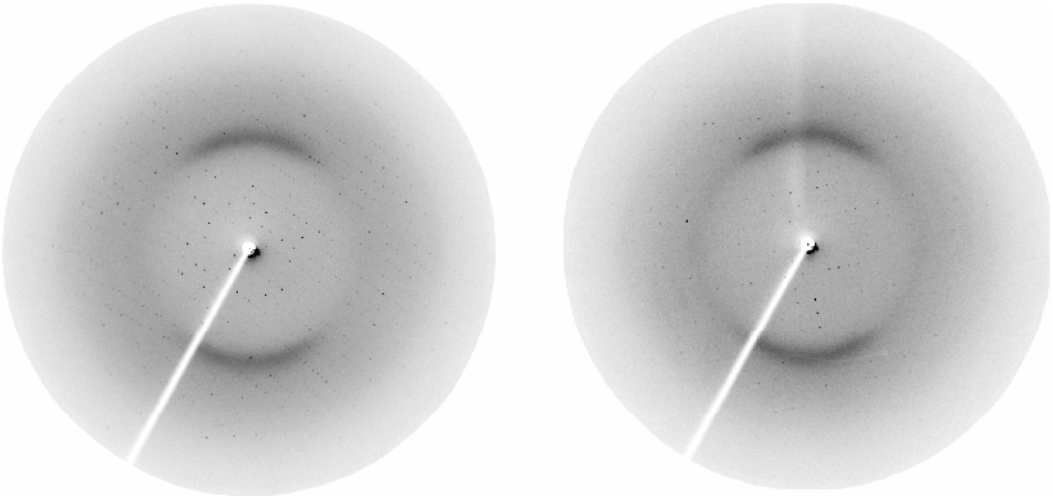

Fig. 9. **Typical X-ray diffraction patterns of OppA*:bradykinin crystals.** The left image was recorded from an OppA* crystal grown under condition C7 using a 0.4˚ oscillation angle and a 10 minute exposure time. The weak spots near the edge of the detector correspond to 2.9 Å resolution. The image on the right was recorded from an OppA* crystal grown under condition B1 using a 1.0˚ oscillation angle and a 10 minute exposure time. The weak spots near the edge of the detector correspond to 2.8 Å resolution.

On a size-exclusion column OppA* eluted at the approximate position of a monomer (Fig. 5A), whereas analysis by BN-PAGE suggested a dimeric quaternary structure (Fig. 5B). It should be noted, however, that it is not clear how the high pI of 8.8 might influence the Coomassie G binding to OppA* and thus the migration of the protein in the BN-gel. Analytical ultracentrifugation experiments (Fig. 6 and 7) confirmed that OppA is a monomer in solution that undergoes a conformational change upon peptide-binding, probably resembling the closed-liganded state observed in crystal structures for many SBPs. This is also consistent with previous experiments with lactococcal OppA in which the protein was shown to migrate faster on native cationic electrophoresis gels in the presence than in the absence of peptides (Lanfermeijer *et al.*, 1999). The resolving power of the BN-gel was not high enough to visualize this protein conformational change (not shown).
The crystallization of lactococcal OppA in the presence of bradykinin, as reported in the present study, is an important step toward determining the structure of a oligopeptide-bound lactococcal OppA complex and understanding its apparent distinct peptide-binding mechanism. Although we have not yet succeeded in collecting a complete data set, either of the two crystal forms might be optimized. Hopefully, we then can solve the structure by molecular replacement using the coordinates of OppA from *S. typhimurium*, DppA from *E. coli*, or AppA from *B. subtilis*, in their closed conformations, or, alternatively, obtain heavy atom-substituted crystals for phasing. If the latter is required, the *L. lactis* nisin-induced expression system should be tested for its capacity to generate seleno-methionine containing proteins. In principle, this should be possible since *L. lactis* strains used for protein production are auxotrophic for several amino acids, including methionine.

Acknowledgements

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Chapter 6

Distribution, lateral mobility and function of membrane proteins incorporated into giant unilamellar vesicles

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Abstract

Giant Unilamellar Vesicles (GUVs) have been widely used for studies on lipid mobility, membrane dynamics and lipid domain (raft) formation, using single molecule techniques like Fluorescence Correlation Spectroscopy (FCS). Reports on membrane protein dynamics in these type of model membranes are by far less advanced due to the difficulty of incorporating proteins into GUVs in a functional state. We have used sucrose to prevent four distinct membrane protein(s) (complexes) from inactivating during the dehydration step of the GUV formation process. The amount of sucrose was optimized such that the proteins retained 100 % biological activity and many proteo-GUVs were obtained. Although GUVs could be formed by hydration of lipid mixtures composed of neutral and anionic lipids, an AC electric field was required for GUV formation from neutral lipids. Distribution, lateral mobility and function of an ATP-binding cassette transport system, an ion-linked transporter and a mechanosensitive channel in GUVs were determined by confocal imaging, FCS, patch-clamp measurements and biochemical techniques. In addition, we show that sucrose slows down the lateral mobility of fluorescent lipid analogues, possibly due to hydrogen-bonding with the lipid headgroups, leading to larger complexes with reduced mobility.

Introduction

Biological membranes are complex matrices mainly composed of lipids and proteins that separate the contents of cells or specialized compartments from the external surroundings.

The abbreviations used are: ABC, ATP-binding cassette; AC, alternating current; DDM, *n*-dodecyl-β-D-maltoside; DiO, 3,3'-dioctadecyloxacarbocyanine perchlorate ($DiOC_{18}(3)$); DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine; DOPE, L-α-dioleoyl phosphatidylethanolamine; DOPG, 1,2-dioleolyl-*sn*-glycerol-3-[phospho-*rac*-(1-glycerol)]; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylserine; FCS, Fluorescence Correlation Spectroscopy; GUV, Giant Unilamellar Vesicle; LSCM, Laser Scanning Confocal Microscope; LUV, Large Unilamellar Vesicle; NBD C_6-HPC, 2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl-1-hexadecanoyl phosphocholine; NTA, nitrilotriacetic acid; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor.

Giant Unilamellar Vesicles (GUVs; diameter ~ 5 – 100 μm) have proven to be useful model systems and have been widely used to study lipid dynamics (Korlach *et al.*, 1999), lipid domain (raft) formation (Bagatolli and Graton, 2000; Kahya *et al*., 2003), elastic properties of membranes (Angelova *et al.*, 1992; Akashi *et al.*, 1996), lipid-DNA interactions (Angelova *et al.*, 1999), vesicle shape changes (Tanaka *et al.*, 2002), membrane tube formation (Roux *et al.*, 2002), and membrane fusion (Tanaka *et al*., 2004). In contrast, reports on membrane protein dynamics in GUVs are rare since for a long time methods suitable for incorporating these proteins in a functional state into GUVs were not available (Kahya *et al*., 2001; Girard *et al.,* 2004).

The mechanism of formation and properties of GUVs have already been studied since the 1980's (Dimitrov and Angelova, 1986; Mueller and Chien, 1983). GUVs can be prepared by drying lipids dissolved in organic solvent (chloroform or choroform/methanol mixtures) followed by addition of distilled water. Water penetrates the dried lamellar structures and GUVs are formed spontaneously due to membrane fusion processes. Formation appears to be optimal if a fraction of anionic lipids is incorporated (phosphatidylglycerol or phosphatidylserine) or can be promoted by the addition of divalent cations (Ca^{2+} or Mg^{2+}) when using only neutral lipids (Akashi *et al.*, 1998). In addition, AC electric fields have been reported to facilitate or impede the formation process (Dimitrov and Angelova, 1986; Dimitrov and Angelova, 1987).

The major bottleneck for direct incorporation of membrane proteins into GUVs is the dehydration step which precedes the formation process. Kahya *et al.* (2001) have circumvented this problem by using peptide-induced fusion of Large Unilamellar Vesicles (LUVs; diameter ~ 200 nm), containing the membrane protein of interest, with preformed GUVs. Although this method has been succesfully applied to study the dynamics and aggregation state of bacteriorhodopsin in GUVs (Kahya *et al.*, 2002), the method is laborious and required the presence of some highly unusual lipids and a fusogenic peptide in the model membranes. Recently, we (Folgering *et al.*, 2004) and others (Girard *et al.*, 2004) developed alternative methods for incorporating polytopic membrane proteins into GUVs by (partial) dehydration of LUVs, containing (purified) membrane proteins, followed by rehydration in the presence of an AC electrical field (Angelova *et al.,* 1992). This method was applied for the Ca^{2+}-ATPase from sarcoplasmic reticulum and two highly stable membrane proteins, the light-driven proton pump bacteriorhodopsin and the mechanosensitive channel of large conductance MscL. A drawback was that electroformation tolerates low (~ < 10 mM) ion concentrations during the GUV formation process. Bacteriorhodopsin and MscL retained their activity during drying of the proteoliposomes, however, activity of the Ca^{2+}-ATPase was reduced by ~ 30 % (Girard *et al.,* 2004). Bacia *et al.* (2004) studied two single-span SNARE proteins incorporated into GUVs by the same method, but the fraction of proteins that survived the GUV formation process was not determined. For single-molecule techniques like Fluorescence Correlation Spectroscopy (FCS), Fluorescence Resonance Energy Transfer (FRET) and Atomic Force Microscopy (AFM), it is essential that heterogeneities due to non-productive protein conformations can be ruled out and that 100 % protein activity can be recovered.

We have made use of the stabilizing properties of disaccharides on membranes and proteins to develop a direct and simple method by which 100 % protein activity was retained during incorporation of four distinct membrane protein(s) (complexes) into GUVs. The proteins studied were the lipid-anchored oligopeptide-binding protein OppA and the translocator complex OppBCDF of the oligopeptide ATP-binding cassette (ABC) transporter from

Lactococcus lactis, the mechanosensitive channel of large conductance MscL from *Escherichia coli*, and the secondary lactose transport protein LacS from *Streptococcus thermophilus*. The distribution, mobility and function of these membrane proteins from distinct protein families were studied in proteo-GUVs by confocal imaging, FCS, patch-clamp and other biochemical techniques. In addition, the effect of sucrose on the lateral mobility of hydrated lipids was analysed by FCS measurements on GUVs prepared in the presence of high concentrations of sugar.

Materials and methods

GUV formation. GUVs were prepared according to standard procedures (Mueller and Chien, 1983; Akashi *et al.*, 1996) with some modifications. In short, 10 μL of 4 mg/mL lipids (w/w), dissolved in chloroform, was deposited on a glass coverslide. For detection of GUVs by fluorescence microscopy, a fluorescent lipid probe, DiO (excitation and emission wavelength maxima of DiO are 484 and 501 nm, respectively), or NBD C_6-HPC (excitation and emission wavelength maxima of NBD C_6-HPC are 460 and 534 nm, respectively), was added (1 μL of 0.5 mM probe dissolved in MeOH/mg lipid). A thin lipid film was formed by evaporating the chloroform under vacuum at 4°C. The lipids were rehydrated at room temperature by the addition of 0.5 mL distilled water or 10 mM KPi, pH 7.0, in a custom-built sample chamber. Optionally 10 mM $MgCl_2$ was added after prehydration with distilled water (Akashi *et al.*, 1998) or electroformation was performed (Angelova *et al.*, 1992). For electroformation, lipids were dried onto ITO-coated coverslips (custom-coated by GeSim, Dresden, Germany). A Pt wire was assembled 1 mm above the ITO-coated slide in the sample chamber, after which an AC electric field was applied (10 Hz, 1.2 V). Formation of GUVs was followed by fluorescence microscopy.

Cysteine mutagenesis. Cysteine mutants of the lipid-anchored oligopeptide-binding protein from *Lactococcus lactis*, OppA I602C, the mechanosensitive channel protein from *Escherichia coli*, MscL K55C, and the secondary lactose transporter from *Streptococcus thermophilus*, LacS C320A / A635C were prepared by standard molecular biology techniques. Each protein contains a C-terminal His_6-tag that was used for purification by Ni^{2+}-affinity chromatography. OppA I602C was made by replacing the 368 baisepair *BamHI-XbaI* fragment of plasmid pAMP42 (Doeven *et al.*, 2004) with a synthetic double-stranded oligonucleotide linker (5'-GATCC**TGT**ATTGAGGGTCGTCATCATCACCACCATCACTGACGCGTCTGCAGT-3' annealed to 5'-CTAGACTGCAGACGCGTCAGTGATGGTGGTGATGATGACGACCCTCAAT**ACA**G-3') containing three extra basepairs coding for cysteine (letters in bold). The linker also contained a *PstI* site (underlined) outside the coding region to facilitate restriction analysis after ligation. Next, the insert of pAMP42 (*oppDFBC*) was replaced with a 1802 basepair *NcoI-BamHI* fragment of pAMP31 (Picon *et al.*, 2000) containing the *oppA* gene. The resulting plasmid was named pNZOppA (I602C). LacS C320A / A635C was made by changing the codon specifying alanine 635 in pSKE8his(C320A) (Veenhoff *et al.*, 2000) into a cysteine codon, using the Quikchange mutagenesis kit (Stratagene). The plasmid coding for MscL K55C was a gift from L. Dijkink from the BioMaDe Technology Foundation.

Protein production and purification. OppA was produced in *Lactococcus lactis* NZ9000, using the nisin expression system (Kuipers *et al.*, 1993; Kunji *et al.*, 2003). Production of MscL K55C was done in *Escherichia coli* PB104 (Blount *et al.*, 1996), using the pB10b expression vector (Sukharev *et al.*, 1994). LacS C320A / A635C was produced by *Escherichia coli* HB101, using the pSKE8his expression vector (Veenhoff *et al.*, 2000). Membrane vesicles were prepared by rupturing the cells with a high-pressure homogenizer (Kindler Maschinen AG, Zűrich, Switzerland) and solubilised by 0.5 % (w/v) DDM (OppA and LacS) or 3 % (w/v) octyl-β-glucoside (MscL), and the proteins were purified by nickel affinity chromatography essentially as described previously (Knol *et al.*, 1996; Detmers *et al.*, 2000; Folgering *et al.*, 2004). Solubilisation buffers were 50 mM KPi, pH 8.0, 200 mM KCl, 10 % (w/v) glycerol, 10 mM imidazole (OppA), 50 mM KPi, pH 8.0, 100 mM NaCl, 10 % (w/v) glycerol, 15 mM imidazole (LacS), and 50 mM KPi, pH 7.0, 300 mM NaCl, 35 mM imidazole (MscL). OppBCDF was produced and purified as described previously (Doeven *et al.*, 2004).

Protein labeling. After 12' centrifugation at 280,000 x *g* the solubilized material was incubated with Ni^{2+}-NTA resin for 1.5 hours at 4°C while rotating. Subsequently, the resin was drained and washed with 10 column

volumes of solubilisation buffer containing 0.05 % (w/v) DDM (OppA and LacS) or 0.2 % (w/v) Triton X-100 (MscL). The columns with OppA and LacS were washed with another 10 column volumes of the same buffer with 25 mM imidazole. For FCS experiments the proteins were labeled with Alexa Fluor 488 C5 maleimide (Molecular Probes) by incubating the proteins after the initial washing steps, while bound to the Ni^{2+}-NTA resin, with a ~ 30 times excess of label for 2 hours up to overnight at 4°C. Labeling was done in solubilisation buffer without imidazole supplemented with 0.05 % (w/v) DDM (OppA and LacS) or (w/v) 0.2 % Triton X-100 (MscL). After labeling the column was washed with 20 column volumes of the same buffer, and the proteins were eluted in the same buffer, pH 7.0, supplemented with 300, 400 or 200 mM imidazole for OppA, MscL and LacS, respectively. The degree of labeling was estimated by measuring the absorbance of Alexa Fluor 488 (extinction coefficient is 71,000 $M^{-1}cm^{-1}$ at 495 nm) and protein concentration and was found to be 80 – 100 % for each protein preparation.

Functional reconstitution of membrane proteins into LUVs. Purified proteins were inserted into Triton X-100 destabilized lipsomes as described (Knol *et al.*, 1996). Activity of the proteins was determined by measuring peptide-binding (OppA; Detmers *et al.,* 2000), peptide transport (OppBCDF plus OppA; Doeven *et al.*, 2004), lactose transport (LacS; Knol *et al.*, 1996), and channel activity (MscL; Folgering *et al*., 2004) as described previously.

Formation of proteo-GUVs. LUVs (10 μL of 20 mg/mL lipids), containing Alexa Fluor 488-labeled membrane protein at given protein-to-lipid ratio in 50 mM NH_4HCO_3, pH 8.0, were dried overnight under vacuum at 4°C on UV-ozone cleaned glass or ITO-coated coverslips. UV-ozone cleaning was not essential for GUV formation but increased the wetting properties of the coverslip surface, making it easier to dry liposomes from aquaeous solution. Sucrose was included in the 50 mM NH_4HCO_3, pH 8.0, buffer at given amounts to stabilize the proteins during dehydration. Rehydration was done by adding 0.5 mL 10 mM KPi, pH 7.0, at room temperature. Optionally, 10 mM $MgCl_2$ was added or electroformation was performed (as described above) when using neutral lipids only. GUV formation was monitored by fluorescence microscopy.

Confocal imaging and FCS measurements. FCS measurements were carried out on a laser scanning confocal microscope (LSCM). The LSCM is based on an inverted microscope Axiovert S 100 TV (Zeiss) in combination with a galvanometer optical scanner (model 6860, Cambridge Technology). The laser beam (488 nm, argon ion laser, Innova 99, Coherent) was focused by a Zeiss C-Apochromat infinity-corrected 1.2 NA 63× water immersion objective for excitation of the Alexa Fluor 488 fluorophore. The fluorescence was collected through the same objective, separated by a dichroic beam splitter (61003bs, Chroma Technology) and directed through an emission filter (HQ 535/50, Chroma Technology) and a pinhole (diameter of 30 μm) onto an avalanche photodiode (SPCM-AQR-14, EG&G). The fluorescence signal was digitized and autocorrelation curves were calculated on a PC using a multiple τ algorithm. The setup was calibrated by measuring the known diffusion coefficient of Alexa Fluor 488 in water (Molecular probes; D = 300 $\mu m^2/s$). Autocorrelation curves were fitted with a one-component two-dimensional diffusion model (Schwille, 2001) using Origin software (OriginLab Corporation, Northampton, MA).

Miscellaneous. Mutations were confirmed by restriction analysis and DNA sequencing. Protein concentrations were determined according to the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. The concentrations of purified OppA, OppBCDF and LacS were determined spectrophotometrically by measuring the absorption at 280 nm and using extinction coefficients of 1.605, 0.990, and 0.926 $(mg/mL)^{-1}cm^{-1}$, respectively.

Results

Investigation of GUV formation from different lipid mixtures. The formation of GUVs from lipid mixtures commonly used for reconstitution of membrane proteins in LUVs was evaluated. GUVs could be formed from all lipid compositions tested, although with different efficiency. When using neutral lipids (DOPC) formation was slow (longer than 1 hour) and GUV yield was

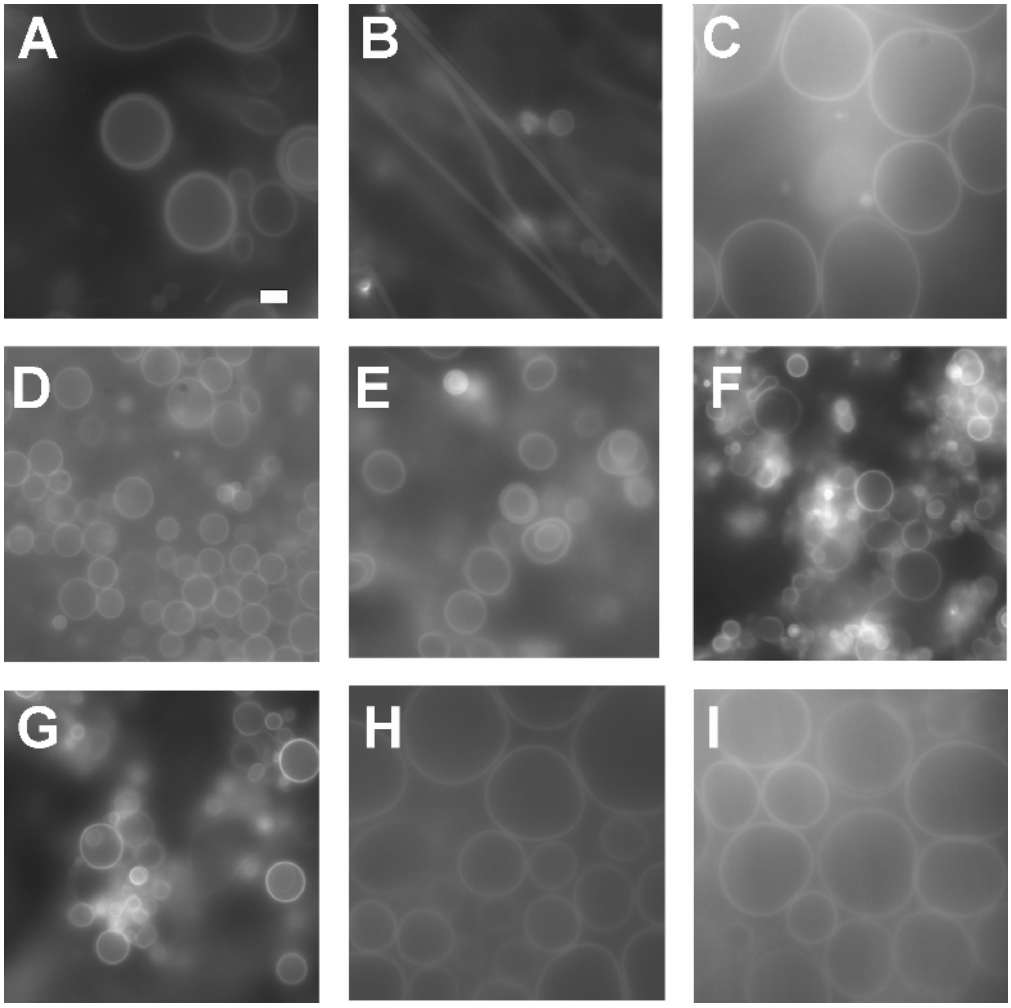

Fig. 1. **Formation of GUVs from lipid mixtures used for proteo-LUV preparation.** Membranes were visualized with a fluorescence microscope equipped with a Zeiss C-Apochromat infinity-corrected 1.2 NA 63× water immersion objective and a CCD-camera. The fluorescent probe DiO was present at a mole ratio of 0.35 μmol DiO / mol total lipid. Scale bar (A) is 20 μm and is the same for all pictures shown. Formation of GUVs from neutral lipids: DOPC (A) and DOPC:DOPE 1:1 (w/w) (B). High yields of GUV formation were obtained when anionic lipids were incorporated: DOPC:DOPG 3:1 (w/w) (C), DOPC:DOPS 3:1 (w/w) (D), and DOPC:DOPE:DOPS 2:1:1 (w/w) (E). Addition of 10 mM $MgCl_2$ after 30 minutes of prehydration in distilled water increased the yield of DOPC (F) and DOPC:DOPE (G) GUVs. Application of an electric field (10 Hz, 1.2 V, distance between electrodes was 1 mm) also dramatically improved the formation of GUVs from neutral lipids: DOPC (H) and DOPC:DOPE 1:1 (w/w) (I).

relatively low (Fig. 1A). With DOPC:DOPE 1:1 (w/w) mixture thread-like structures were observed and GUVs were rarely formed (Fig. 1B). However, when 25 % (w/w) anionic lipids (DOPG or DOPS) was included, formation of GUVs was fast (within 15 minutes) and the yield was high (Fig. 1C, D and E). Formation of GUVs from neutral lipids (DOPC and DOPC:DOPE) could be enhanced by adding 10 mM $MgCl_2$, in accordance with observations made previously (Akashi *et al.*, 1998). Addition of Mg^{2+} collapsed excisting structures, after which many GUVs started to grow (Fig. 1F and G). Electroformation (Angelova *et al.*, 1992) also dramatically increased the yield of GUVs formed from neutral lipids (Fig. 1H and I). For formation of proteo-

GUVs (see below) a lipid mixture of DOPC:DOPS 3:1 (w/w) was used, because most membrane proteins studied to date require anionic lipids for (optimal) activity.

Production, purification and fluorescent labeling of membrane proteins. The four model membrane protein(s) (complexes) used in this study are the oligopeptide-binding protein OppA, and the translocator complex OppBCDF of the oligopeptide ABC transporter from *Lactococcus lactis*, the mechanosensitive channel of large conductance MscL from *Escherichia coli*, and the secondary lactose transport protein LacS from *Streptococcus thermophilus*. In order to selectively label the proteins with fluorescent probes for detection by confocal imaging and FCS measurements, single-cysteine mutants were constructed. The position of the cysteine was located either near the C-terminus of the protein (OppA and LacS), or in an extracellular loop (MscL). Wild-type OppA contains a N-terminal cysteine residue that is lipid-modified and this residue could not be used for labeling; OppA is anchored to the membrane via this lipid-modification. The native cysteine of LacS at position 320 is located in the middle of a transmembrane helix and not accessible or unreactive for labeling. Therefore, this cysteine was replaced by an alanine and a cysteine for labeling was introduced near the C-terminus of the protein. The cysteines in the different proteins were labeled with Alexa Fluor 488 C5 maleimide, after which excess label was removed by nickel affinity chromatography. Figure 2 shows SDS-PAGE analysis of membrane vesicles containing the overexpressed cysteine mutants, the purified proteins, and the proteins labeled with Alexa Fluor 488.

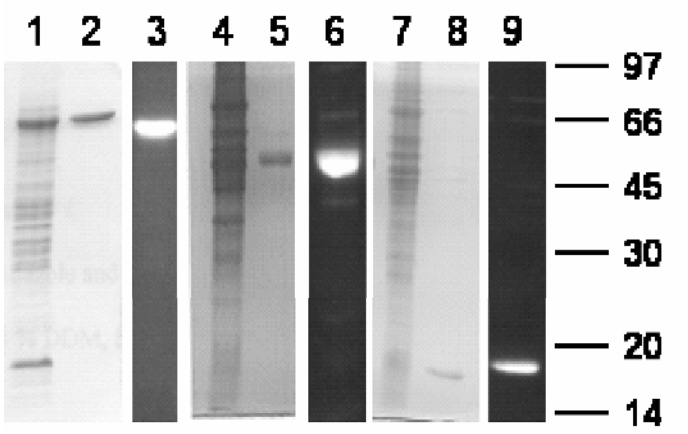

Fig. 2. **SDS-PAGE analysis of overexpressed, purified and labeled cysteine mutants.** Membrane vesicles containing overexpressed cysteine mutants are shown in lanes 1, 4 and 7. Purified proteins labeled with Alexa Fluor 488 C5 maleimide (Molecular Probes) stained with coomassie brilliant blue (lanes 2, 5, and 8) and visualised with a UV lamp (lanes 3, 6 and 9) are also shown. Mutants used were OppA I602C (lanes 1-3), LacS C320A / A635C (lanes 4-6), and MscL K55C (lanes 7-9).

Membrane reconstitution and activity of the labeled mutants. The Alexa Fluor 488 labeled proteins were inserted into Triton X-100 destabilized LUVs (Knol *et al.*, 1996), and protein activity was assayed as described previously (Knol *et al.*, 1996; Detmers *et al.,* 2000; Folgering *et al*., 2004). Peptide binding by OppA, channel activity by MscL, and lactose transport by LacS, were not affected by the mutations and the labeling of the proteins, as is shown in figure 3. In addition, bradykinin uptake via Opp (= OppA plus OppBCDF) was also not influenced by labeling of OppA (not shown).

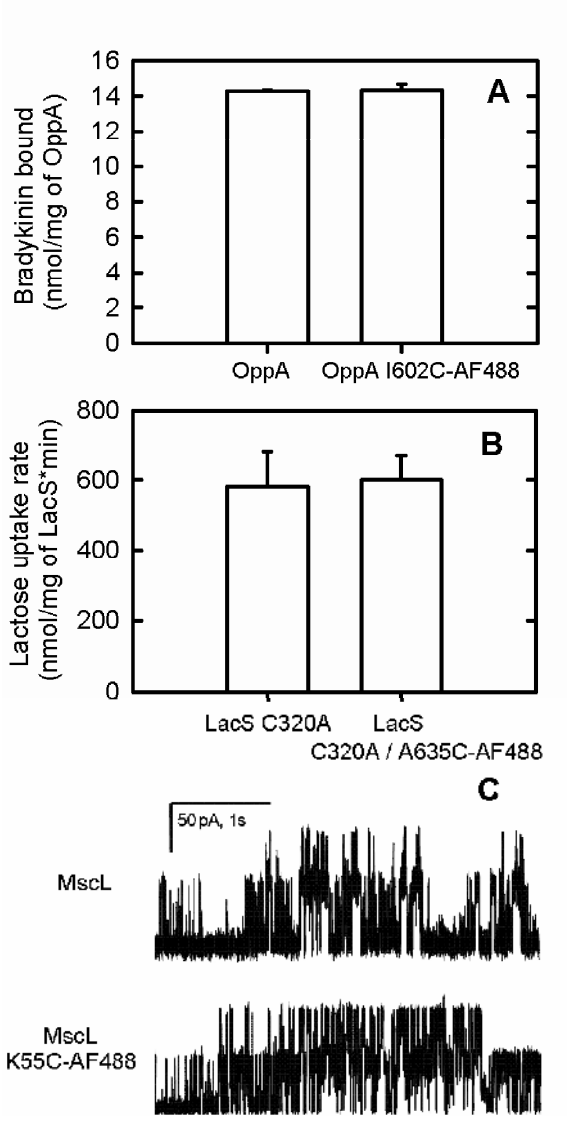

Fig. 3. **Activity of labeled cysteine mutants.** (A) Peptide binding by wild-type OppA and Alexa Fluor 488-labeled cysteine mutant OppA I602C AF488. The concentration of [^{3}H]-bradykinin was 3 μM. (B) Lactose counterflow via LacS C320A and LacS C320A / A635C labeled with Alexa Fluor 488. The internal lactose and external [^{14}C]-lactose concentrations were 10 mM and 100 μM, respectively. (C) Channel activity of wild-type MscL (upper trace) and MscL K55C labeled with Alexa Fluor 488 (lower trace). Patch-clamp measurements were done in 5mM Hepes, pH 7.2, 200 mM KCl, 40 mM $MgCl_2$, and at +20 mV pipette voltage. The unit conductance of a single MscL channel is about 2.5 nS as indicated by the 50 pA scale bar. Protein to lipid ratios were 1:50 (w/w). Proteins were inserted into DOPC:DOPS 3:1 (w/w) lipid mixtures.

Stabilization of membrane proteins by sucrose during dehydration. The major difficulty with incorporating membrane proteins in a functional state into GUVs is the dehydration step of the GUV formation process. During drying labile (membrane) proteins are prone to lose their biological activity (Crowe *et al.*, 1984; Crowe *et al.*, 1988; Crowe *et al.*, 1996b). In our case, only the highly stable channel protein MscL was able to survive completely when water was removed from proteo-LUVs containing this protein (Folgering *et al.*, 2004). OppA and LacS, on

the other hand, were severely affected by the removal of water, resulting in loss of protein activity after de- and rehydration. Protein activity could be preserved by adding sucrose during drying of OppA- and LacS-containing LUVs (Fig. 4A and B), and as little as 20 mg sucrose/g of lipid was sufficient for the recovery of full activity. Notice that during drying the sucrose concentration increases from 1 to an estimated several hundreds of mM. The multi-subunit oligopeptide ABC transporter OppABCDF could be stabilized during dehydration by the addition of 100 mg sucrose/g of lipid (Fig. 4C).

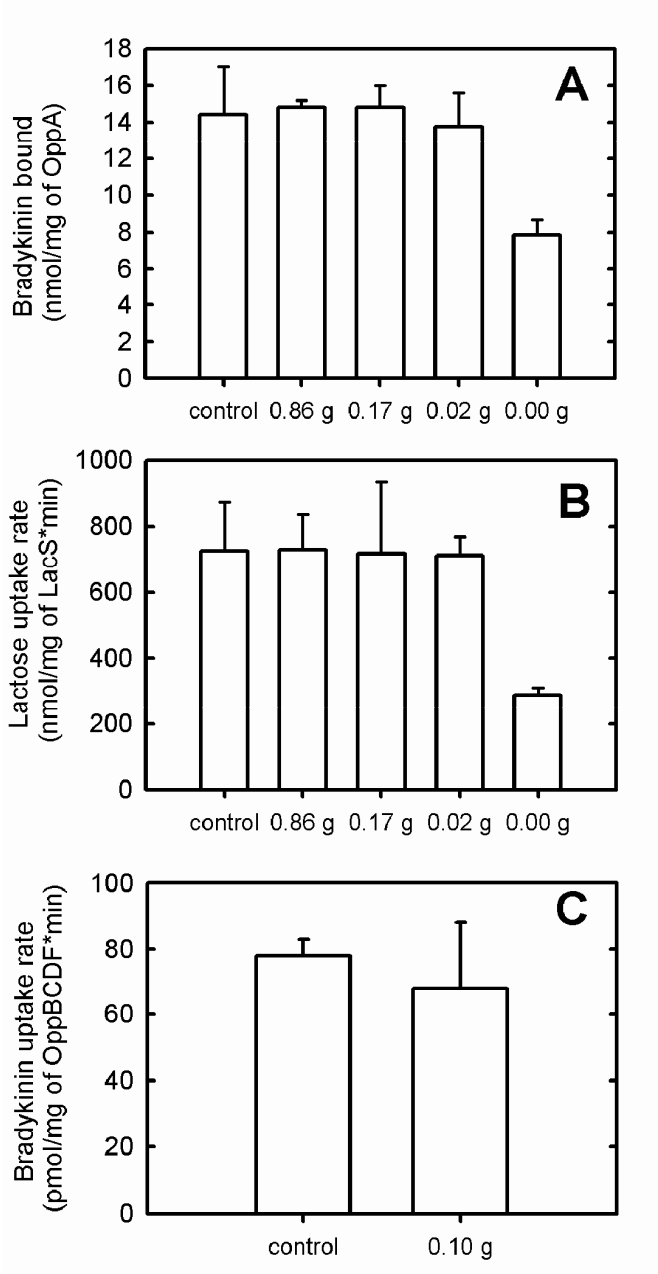

Fig. 4. **Sucrose stabilizes membrane proteins during dehydration.** Peptide binding by OppA (A), lactose counterflow via LacS C320A (B), and peptide uptake by OppABCDF (C; [^{3}H]-bradykinin concentration was 0.7 μM) were measured after dehydration of the membranes in the presence of 0 – 0.86 g sucrose/g lipids and rehydration in 10 mM KPi, pH 7.0. "Control" samples were not dried and rehydrated. For further details see legend to figure 2.

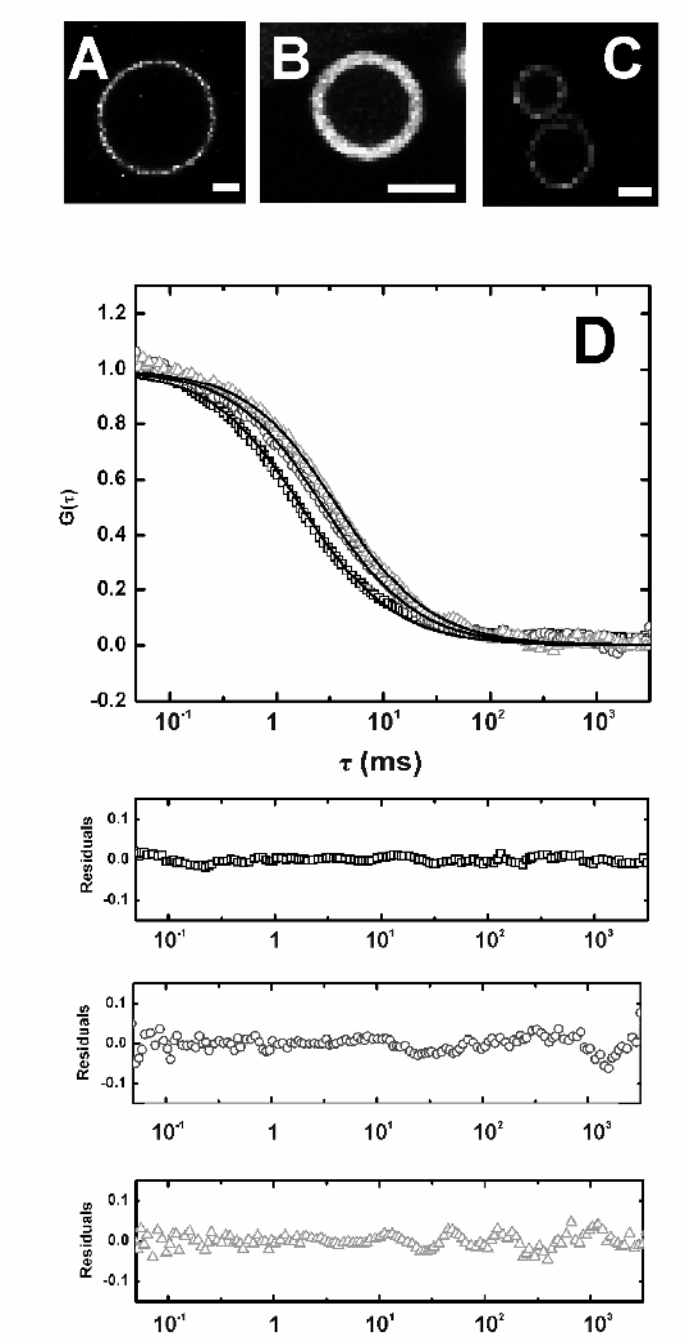

Fig. 5. **Confocal imaging and diffusion measurements of membrane proteins in GUVs.** Confocal images of proteo-GUVs containing functional Alexa Fluor 488-labeled OppA I602C (A), MscL K55C (B), or LacS C320A / A635C (C). Scale bars are 10 μm. GUVs were prepared in the presence of 0.02 (A), 0 (B), or 0.17 (C) g sucrose/g lipids. The protein to lipid ratio was 1:500 (w/w) and the lipid composition was DOPC:DOPS 3:1 (w/w). (D) Autocorrelation curves for OppA I602C (□), MscL K55C (○), and LacS C320A / A635C (Δ) in GUVs. Curves were fit with a one-component two-dimensional diffusion model (solid lines) using Origin software (OriginLab Corporation, Northampton, MA); the residuals of the fits are shown in the panels below the figure.

Confocal imaging of functional membrane proteins in GUVs. After optimization of the amount of sucrose needed to retain 100 % biological activity for each protein (Fig. 4), proteo-GUVs were prepared for confocal imaging. Proteo-LUVs were dried in the presence of stabilizing amounts of sucrose and rehydrated in aqueous buffer. GUV formation was followed by fluorescence microscopy. The addition of Mg^{2+} or application of an electric field was not required for the formation of GUVs, since 25 % (w/w) DOPS was included in the LUVs (see above). Confocal imaging confirmed that the proteins were incorporated homogeneously into the GUVs (Fig. 5A, B and C). Differences in brightness of the GUVs are a result of different amounts of labels attached to the proteins. Pentameric MscL (Fig. 5B), for example, has five labels attached whereas OppA (Fig. 5A) has only one. The presence of sucrose during the drying of the proteo-LUVs inhibited the GUV formation process. When sucrose was used at 0.86 g/g lipid or higher concentration, GUV formation was no longer observed. Addition of Mg^{2+} or application of an electric field also could not induce GUV formation under these conditions. However, the minimal amount of sucrose needed for the protection of the proteins during dehydration was much lower (~ 0.02 g sucrose/g lipid; Fig. 4), making it possible to form GUVs containing fully functional membrane proteins.

Lateral mobility of membrane proteins in GUVs. The mobility of OppA, MscL and LacS in GUVs was determined by FCS measurements. Confocal images were made of proteo-GUVs containing fluorescent labeled protein, and the focal volume was focused on the pole of the GUVs. Representative autocorrelation curves for each of the three proteins studied are shown in figure 5D; the experimental data could be fitted reasonably well with a one-component two-dimensional diffusion model. Occasionally the membranes appeared to move or fluctuate during the autocorrelation measurements which could be observed as a decay or systematic deviation in the count-rate. Autocorrelation curves affected by these membrane undulations were not taken into account when analyzing the data. Undulations seemingly appeared more frequently when measuring autocorrelation curves with very large GUVs (~ 30 – 100 μm) and less when the GUVs were somewhat smaller (< 30 μm). The measured diffusion coefficients (*D*) are summarized in Table 1. Lipid-anchored oligopeptide-binding protein OppA diffused with the same speed as the fluorescent lipid DiO. The mobility of the integral membrane proteins MscL and LacS was lower, with LacS being the slowest. The lateral mobility of the integral membrane proteins was ~ 2-3 fold lower than the mobility of DiO.

Table 1. **Diffusion coefficients for lipid and membrane proteins in GUVs.**

	DiO[1]	OppA I602C AF488	MscL K55C AF488	LacS C320A / A635C AF488
Diffusion coefficient[2] (10^{-8} cm^2/s)	7.7 ± 0.8	7.5 ± 0.9	3.9 ± 0.3	3.0 ± 0.3

[1]Diffusion of DiO was measured in GUVs, that is, in the absence of protein. [2]Diffusion coefficients represent the average of at least five measurements and the SD values are given. FCS setup was calibrated with Alexa Fluor 488.

Interaction of sucrose with hydrated lipids. To investigate the effect of high concentrations of sucrose on the lateral mobility of lipids, we prepared GUVs in the absence and presence of high concentrations of sugar. Although the efficiency of GUV formation was hampered when sugar was present in the rehydration liquid, GUVs could still be formed with up to 1.5 M sucrose (or glucose) in the rehydration medium (Fig. 6A and B).

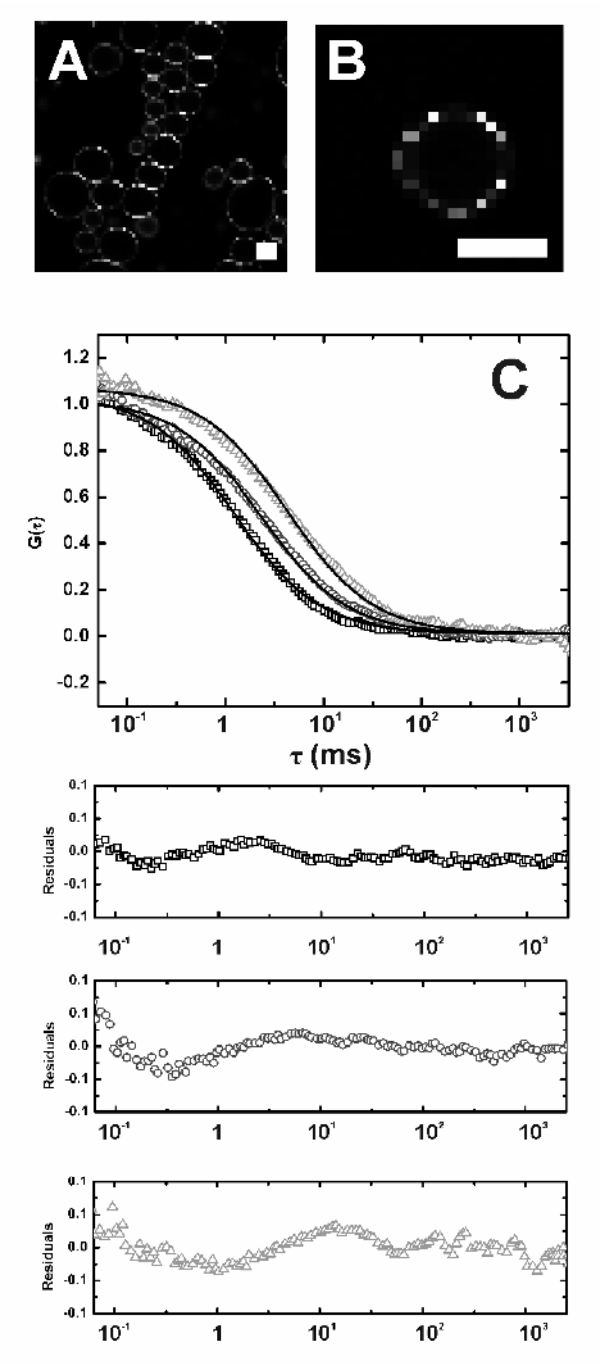

Fig. 6. **Effect of sucrose on lipid lateral mobility in GUVs.** Confocal images of GUVs prepared in the absence (A) or presence (B) of 1.5 M sucrose. Scale bar is 10 μm. The fluorescent lipid probe DiO was used to visualize the membranes. (C) Autocorrelation curves of DiO in GUVs prepared in the absence of sugar (□) or in the presence of 1.5 M glucose (○) or sucrose (Δ). Curves were fit with a one-component two-dimensional diffusion model (solid lines) using Origin software (OriginLab Corporation, Northampton, MA); the residuals of the fits are shown in the panels below the figure. With high concentrations of sucrose (and glucose), the data points deviated significantly from the one-component two-dimensional diffusion model, which may reflect the clustering of the lipids. Excellent fits were obtained when anomalous or two-component diffusion models were used to analyse the data (not shown). The lipid composition was DOPC:DOPS 3:1 (w/w).

Representative autocorrelation curves for the fluorescent lipid probe DiO in GUVs prepared in water or 1.5 M sugar are shown in figure 6C. It can be seen that at high sucrose concentration the lateral diffusion of DiO was slowed by a factor of ~ 3. This suggests that the sugar is binding to the lipids and cluster formation may be taking place. When glucose was used in stead of sucrose the effect on the lateral mobility of DiO was much smaller but still a reduction of ~ 2 fold was observed. In Table 2, a summary of the measured diffusion coefficients is given. Control experiments with another fluorescent lipid analogue (NBD C_6-HPC; Molecular Probes) gave similar results (not shown), indicating that the results were not probe-specific.

Table 2. **Effect of sugars on lipid lateral mobility in GUVs.**

	Water	1.5 M Sucrose	1.5 M Glucose
Diffusion coefficient[1] (10^{-8} cm^2/s)	7.7 ± 0.8	2.5 ± 0.6	4.1 ± 0.7

[1]Diffusion coefficients represent the average of at least five measurements and the SD values are given. FCS setup was calibrated with Alexa Fluor 488. Fluorescent lipid probe was DiO.

Discussion and conclusions

A method for the direct incorporation of membrane proteins into GUVs without losing protein activity is described. Protein activity was retained by drying proteo-LUVs in the presence of sucrose. The distribution, lateral mobility and activity of representative membrane proteins from disinct families in GUVs was analysed by confocal imaging, FCS, patch-clamp and other biochemical techniques. Furthermore, the effect of high concentrations of sucrose on lateral mobility of lipids in GUVs was studied by FCS.

First, we evaluated the formation of GUVs from lipid mixtures commonly used to reconstitute membrane proteins into LUVs (Fig. 1). Formation was optimal when a fraction of anionic lipids (DOPC or DOPS) was incorporated or could be promoted by the addition of Mg^{2+} or by performing electroformation when using only neutral lipids. These results are in accordance with previously made observations (Akashi *et al.*, 1998; Angelova *et al.*, 1992). Importantly, to the best of our knowledge, a systematic comparison of GUV formation in the presence or absence of an AC electric field using a wide range of lipid compositions has not been reported before.

For the formation of proteo-GUVs, we used proteo-LUVs containing purified membrane proteins as starting material. However, after drying and rehydration to form GUVs, three out of the four protein(s) (complexes) had lost biological activity. The only protein which survived completely was the mechanosensitive channel of large conductance MscL, which is not entirely surprising since MscL is a highly stable membrane protein. The other three proteins studied, the lipid-anchored oligopeptide binding protein OppA, the oligopeptide translocator complex OppBCDF, and the secondary lactose transporter LacS, were found to be less stable. Rigaud and colleagues attempted to circumvent the problem of losing protein activity during the drying step by performing partial dehydration of the proteo-LUVs under controlled humidity (Girard *et al.*, 2004). However, of the two proteins studied, the light-driven proton pump bacteriorhodopsin and the Ca^{2+}-ATPase, only the former completely survived the GUV formation process. The Ca^{2+}-ATPase had lost 30 % of its biological activity. We prevented OppA, OppBCDF and LacS from losing activity during dehydration by adding stabilizing amounts of sucrose. Only low amounts were needed to retain 100 % activity (the minimal amount required was 0.02 g sucrose/g lipid; Fig. 4).

Disaccharides (for example sucrose) are known to stabilize the folded state of proteins in solution via a mechanism termed preferential exclusion (Lee and Timasheff, 1981; Arakawa and Timasheff, 1985). At high solute concentration the sugar is excluded from the protein surface and the native state is thermodynamically favored over the unfolded state of the protein. The stabilization of soluble proteins during freezing is thought to occur via a similar mechanism. However, stabilization of soluble proteins by disaccharides during air-drying is thought to occur via direct interaction (hydrogen bonding) of the sugar with polar groups of the protein (Crowe *et al.*, 1988). In addition, disaccharides are known for a long time to stabilize membranes during freezing or drying (Crowe *et al.*, 1984; Crowe *et al.*, 1988; Crowe *et al.*, 1996a; Crowe *et al.*, 1996b). The transitions from liquid-crystalline to gel phase (during dehydration) and gel to liquid crystalline phase (during subsequent rehydration) in the absence of sucrose may cause aggregation of integral membrane proteins and loss of activity. Upon drying the sugar molecules replace water by hydrogen-bonding to the lipid headgroups, thereby maintaining the spacing between the headgroups and preventing the membrane from going from the liquid-crystalline to the gel phase. The interactions of sucrose with the proteins and the maintenance of the liquid-crystalline phase of the membrane are most probably the determining factors for the stabilization of the OppA, OppBCDF and LacS proteins. A side-effect of high concentrations of sugars during drying is that they inhibit membrane fusion (Hincha *et al.*, 2003). This may explain why at high amounts of sucrose during drying (≥ 0.86 g/g lipid) no GUVs were formed. Addition of Mg^{2+} or application of an electric field also could not induce GUV formation under these conditions. However, there appears to be an optimum in sucrose concentration at which membrane protein activity is retained (minimal amount required is 0.02 g sucrose/g lipid) and membrane fusion is still possible (< 0.86 g sucrose/g lipid), which enables proteo-GUV formation.

The distribution of the proteins in the GUVs was assessed by confocal imaging and found to be homogeneous. FCS experiments showed that the diffusion of lipid-anchored OppA was as fast as that of the lipid analogue DiO. This suggests that OppA does not have interactions with the membrane other than through its lipid anchor. The integral membrane proteins MscL and LacS diffused ~ 2-3 times slower compared to DiO. The values found for diffusion of DiO and that of integral membrane proteins in GUVs are in the same range as determined previously (Kahya, *et al.*, 2001; Kahya *et al.*, 2003). The difference in mobility of DiO and the integral membrane proteins is in accordance with the Saffman and Delbrűck model for diffusion in biological membranes (Saffman and Delbrűck, 1975; equation 1):

$$D = \frac{kT}{4\pi\eta h}\left(\ln\frac{\eta h}{\eta_w A} - \gamma\right), \qquad \text{(equation 1)}$$

where k is the Boltzmann constant, η and η_w are the viscosity of the membrane and of the surrounding aqueous medium, respectively, h is the membrane thickness, A the cross sectional surface area (CSSA) of the particle and γ is Euler's constant. The difference in mobility of LacS (dimer with a molecular mass of about 140 kDa and CSSA of ~ 32 nm^2; Friesen *et al.*, 2000; Veenhoff *et al.*, 2001) and that of MscL (pentamer with a molecular mass of about 70 kDa and CSSA of ~ 20 nm^2 for the "closed" state; Chang *et al.*, 1998) was ~ 1.3 times. This difference is expected to be this small due to the logarithmic dependence of the lateral mobility of membrane proteins on the cross-sectional area of the aggregate (Saffman and Delbrűck, 1975; Kucik *et al.*, 1999; Lee and Petersen, 2003).

Surprisingly, GUVs could still be formed when high amounts of sugar (up to 1.5 M) were present during rehydration only. It has been suggested by molecular dynamics simulations that the lateral diffusion of lipids in membranes is reduced in the presence of high concentrations of disaccharides (Sum *et al.*, 2003; Pereira *et al.*, 2004). Clusters of a single sucrose or trehalose molecule bound to 2-3 (Sum *et al.*, 2003) or even more than 5 lipids (Pereira *et al.*, 2004) were observed, with the phosphate group of the lipids as the principal interaction site with the sugars. Consistent with this prediction, we observed a reduction of the lateral mobility of lipids in the presence of 1.5 M sucrose, and the data could no longer be fitted with a one-component two-dimensional diffusion model. However, formation of clusters of 2-3 or even 5 lipids can not explain a ~ 3 fold reduction in diffusion coefficient on the basis of the increased CSSA only (see equation 1). Based on our results, and in accordance with the molecular dynamics simulations, we conclude that high concentrations sucrose (or trehalose) may indeed cluster lipids and hydrogen bonding of sugars to the lipids increases the overall viscosity of the membrane thereby slowing down the lipid mobility even further.
In conclusion, we developed a generic method to insert membrane proteins into GUVs without losing protein activity. Sucrose inhibited GUV formation when present during drying but the amount needed for protein stabilization was lower than the amount that completely blocked GUV formation.

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Chapter 7

Probing the catalytic cycle of the oligopeptide ABC transporter by fluorescence correlation spectroscopy

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Abstract

The oligopeptide transporter Opp is a five-component ATP-binding cassette (ABC) uptake system. Extracytoplasmic lipid-anchored substrate-binding protein OppA delivers peptides to an integral membrane complex OppBCDF, where, upon ATP binding and hydrolysis, translocation across the membrane takes place. OppA and OppBCDF were labeled with fluorescent probes and the catalytic cycle of the transporter was investigated by fluorescence correlation spectroscopy using Giant Unilamellar Vesicles (GUVs) as a model membrane system. Lateral mobility of OppA was reduced upon incorporation of OppBCDF into GUVs and decreased even further upon addition of peptide. Fluorescence cross-correlation measurements revealed that OppBCDF distinguished liganded from unliganded OppA, binding only the former. Addition of ATP, non-hydrolyzable ATP analogue AMP-PNP, or, to a lesser extent, ADP resulted in release of OppA from OppBCDF. In vanadate-trapped 'transition state' conditions, OppA was not bound by OppBCDF. A model is presented in which ATP-binding to OppDF results in donation of the peptide to OppBC and simultaneous release of OppA. ATP-hydrolysis would complete the peptide translocation and reset the transporter for another catalytic cycle. Implications in terms of a general transport mechanism for ABC im- and exporters are discussed.

Introduction

ATP-binding cassette (ABC) transporters comprise one of the largest protein families and their (dys)function is in a number of cases linked to human diseases (Higgins *et al.*, 1992; Biemans-Oldehinkel *et al.*, 2006). They catalyze the unidirectional transport of a wide variety of substrates over cellular and organellar membranes and are widely distributed throughout all forms of life. Two hydrophobic transmembrane domains (TMDs) create the translocation pathway, and two peripherally associated nucleotide-binding domains (NBDs) fuel the

The abbreviations used are: ABC, ATP-binding cassette; DDM. *n*-dodecyl-β-D-maltoside; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylserine; FCS, Fluorescence Correlation Spectroscopy; FCCS, Fluorescence Cross-correlation Spectroscopy; GUV, Giant Unilamellar Vesicle; LSCM, Laser Scanning Confocal Microscope; LUV, Large Unilamellar Vesicle; NBD, nucleotide-binding domain; NTA, nitrilotriacetic acid; SBP, substrate-binding protein; TMD, transmembrane domain.

transport reaction by binding and hydrolyzing ATP. Directionality of the translocation process can be deduced from the presence or not of a fifth component, the extracytoplasmic substrate-binding protein (SBP). ABC efflux systems lack this constituent, whereas in bacterial ABC importers the SBP is essential for function.
A vast amount of experimental data is available for different ABC efflux systems and, although mechanistic details may differ in individual cases, a consensus model on how this type of transporters work is emerging (Higgins and Linton, 2004; van der Does and Tampe, 2004). Substrate is bound at a high-affinity cytoplasmic facing binding site, located within the TMDs. ATP-binding induces NBD dimerization and reorientation of the loaded substrate-binding site from a high affinity inside- to a low affinity outside-facing conformation. After substrate release ATP-hydrolysis resets the transporter by opening the NBDs and reorienting the empty binding site.
Data on SBP-dependent ABC importers are limited to one or two systems as an experimentally well supported transport mechanism is currently available only for the maltose permease $MalEFGK_2$ from *Escherichia coli* (Chen *et al.*, 2001; Chen *et al.*, 2003; Austermuhle *et al.*, 2004) and, to a lesser extent, the histidine permease $HisJQMP_2$ from *Salmonella typhimurium* (Ames *et al.*, 1996; Liu *et al.*, 1999). In a series of elegant experiments, Davidson and colleagues showed that liganded SBP MalE, whilst bound to the TMDs (MalF and G), opens upon ATP-binding to the NDBs (two copies of MalK per functional complex). In the vanadate-trapped transition state for ATP-hydrolysis, MalE, in its open conformation, is bound tightly to $MalFGK_2$, whereas the affinity for maltose is reduced. Under post-hydrolysis conditions MalE dissociates from $MalFGK_2$, thereby resetting the transporter for the next catalytic cycle. These data contrast those of the vitamin B12 transporter Btu, where SBP BtuF binds tightly to BtuCD in the absence and presence of MgATP, and might not dissociate from the transporter during the catalytic cycle (Borths *et al.*, 2005).
The oligopeptide ABC transporter OppABCDF from *Lactococcus lactis* is composed of an extracytoplasmic lipid-anchored SBP, OppA, two TMDs, OppB and C, and two NBDs, OppD and F, bound to the cytosolic face of the TMDs (Tynkkynen *et al.*, 1993). *In vitro* transport assays with purified proteins reconstituted in Large Unilamellar Vesicles (LUVs) suggested that OppBCDF specifically recognizes liganded OppA (Doeven *et al.*, 2004). This contrasted observations for the maltose and histidine transporters, where unliganded SBP seemed to have access to the TMDs as well, thereby inhibiting transport activity (Prossnitz *et al.*, 1989; Dean *et al.*, 1992; Merino *et al.*, 1995).
We now used purified and fluorescent labeled OppA and OppBCDF incorporated into Giant Unilamellar Vesicles (GUVs) to probe the catalytic cycle of the oligopeptide ABC transporter, using fluorescence auto- and cross-correlation spectroscopy. Based on the results, a model for oligopeptide transport is presented which shows similarities but also important differences compared to the mechanism proposed for the maltose permease. The possible origin for the observed differences is discussed, thereby increasing our knowledge on the translocation mechanism of SBP-dependent ABC transporters.

Materials and Methods

Bacterial strain and growth conditions. C-terminal His_6-tagged OppA, OppA I602C and OppBCDF (His_6-tag on C-terminus of OppC) were produced in *L. lactis* AMP2/pAMP31, NZ9000/pNZOppA (I602C) and NZ9000/pAMP42, respectively, as described (Picon *et al.*, 2000; Doeven *et al.*, 2004; Doeven *et al.*, 2005). Plasmid pAMP42 (OppC I602C) was an intermediate in the construction of pNZOppA (I602C), and was used for

production of OppBC(I296C)DF by *L. lactis* NZ9000. Cells were grown in 5 % (w/v) whey-permeate, 0.5 % (w/v) yeast extract, 0.5 % (w/v) glucose, and 65 mM KPi, pH 7.0, supplemented with 5 μg/mL chloramphenicol in volumes of 10 liters in a bioreactor with pH control (ADI 1065 fermentor; Applikon Dependable Instruments B. V., Schiedam, The Netherlands). The pH was kept constant at pH 6.5 by titrating with 2 M KOH. Trancription from the *nisin A* promoter (plasmid constructs in strain NZ9000) was induced at an $O.D._{660}$ of 2 by adding 1:1000 (v/v) of the culture supernatant of *L. lactis* NZ9700. Cells were harvested by centrifugation, washed with 100 mM KPi, pH 7.0, resuspended in 100 mM KPi, pH 7.0, plus 20 % (v/v) glycerol, and stored at -80°C.

Protein purification and fluorescent labeling. OppA and OppBCDF were purified as described (Detmers *et al.*, 2000; Doeven *et al.*, 2004). OppA I605C was purified and labeled with Alexa Fluor 488 C5 maleimide (Invitrogen) as described (Doeven *et al.*, 2005). Labeling of OppA I605C with Alexa Fluor 633 C5 maleimide (Invitrogen) was done in exactly the same way as previously described for Alexa Fluor 488 (Doeven *et al.*, 2005). OppBC(I296C)DF was purified and labeled with either Alexa Fluor 488 or Alexa Fluor 633 as follows. Membrane vesicles were prepared by rupturing the cells with a high-pressure homogenizer (Kindler Maschinen AG, Zürich, Switzerland) in the presence of 20 % (v/v) glycerol. OppBC(I296C)DF containing membranes were resuspended to 5 mg/mL of total membrane protein in 50 mM KPi, pH 8.0, 200 mM KCl, and 20 % (v/v) glycerol (buffer A), and solubilized with 0.5 % (w/v) DDM. After 12 min of centrifugation at 280,000 x *g* and 4°C, the supernatant was diluted five times with buffer A and incubated with nickel-nitrilotriacetic acid (Ni^{2+}-NTA) resin (0.05 mL/mg of total membrane protein) for 1.5 h at 4°C while shaking gently. Subsequently, the resin was poured into a column and washed with 20 column volumes of buffer A, supplemented with 0.05 % (w/v) DDM and 10 mM imidazole. Labeling was done by incubating the purified protein while bound to the column for 2 h up to overnight at 4°C with a ~ 30 times molar excess of probe dissolved in five column volumes buffer A plus 0.05 % (w/v) DDM. The column was washed with 20 column volumes buffer A plus 0.05 % (w/v) DDM to remove free label and the labeled OppBC(I296C)DF complex was eluted in the same buffer, pH 7.0, supplemented with 100 mM histidine. The degree of labeling was estimated by measuring the absorbance of Alexa Fluor 488 and 633 (extinction coefficients are 71,000 $M^{-1}cm^{-1}$ at 495 nm and 100,000 $M^{-1}cm^{-1}$ at 633 nm, respectively) and protein concentration and was found to be 80-100 % for each protein preparation.

Reconstitution of (fluorescent labeled) Opp proteins into LUVs and GUVs. Triton X-100 mediated reconstitution of the (fluorescent labeled) Opp proteins into LUVs was done as described (Rigaud *et al.*, 1995; Doeven *et al.*, 2004; Poolman *et al.*, 2005). Next, conversion of Opp-containing LUVs into proteo-GUVs was done essentially as described (Doeven *et al.*, 2005). Briefly, proteo-LUVs in 50 mM NH_4HCO_3, pH 8.0, were freeze-thawed three times in the presence of 0.1 g of sucrose/g lipids such that the sucrose concentration on the in- and outside of the proteo-LUVs was the same. 10 μL proteo-LUVs (5 mg/mL lipids) were dried on an ultraviolet-ozone cleaned cover-slide under a gentle stream of nitrogen gas followed by an overnight incubation under vacuum at 4°C. Rehydration was done by adding 500 μL of 20 mM K-Hepes, pH 8.0. GUV formation was monitored by fluorescence microscopy. Optionally, bradykinin (a nonameric peptide with the amino acid sequence RPPGFSPFR) and/or different nucleotides were included in the rehydration buffer. The distributions of peptide and/or nucleotide on the in- and outside of the GUVs after formation was assessed in control experiments by including tracer amounts (~ 10 nM) of Oregon Green 488 labeled RPPGFSPFC or Alexa Fluor 647-ATP (Invitrogen) in the rehydration buffer, followed by confocal imaging of the formed GUVs (see below). At the concentrations of peptide and nucleotides used, e.g. 20 μM and 0.1 – 3 mM, respectively, no large differences between the amounts of solutes present in- and outside the proteo-GUVs could be detected.

Activity of fluorescent labeled Opp. Activity of the (fluorescent labeled) proteins was determined as described previously by measuring peptide binding (OppA; Detmers *et al.*, 2000) and/or peptide transport (OppABCDF; Doeven *et al.*, 2004).

Confocal imaging, FCS and FCCS measurements. Fluorescence Correlation Spectroscopy (FCS) measurements were carried out on a dual-color laser scanning confocal microscope (LSCM). The LSCM is based on an inverted microscope Axiovert S 100 TV (Zeiss, Jena, Germany) in combination with a galvanometer optical scanner (model 6860, Cambridge Technology, Watertown, MA) and a microscope objective nano-focusing device (P-721, PI). The two laser beams (488 nm, argon ion laser, Spectra Physics and 633 nm, He-Ne laser, JDS Uniphase) were focused by a Zeiss C-Apochromat infinity-corrected 1.2 NA 63×

water immersion objective for excitation of the Alexa Fluor 488 and 633 fluorophores. The fluorescence was collected through the same objective, separated from the excitation beams by a beam-pick off plate (BSP20-A1, ThorLabs) and split into two channels by a dichroic beam splitter (585dcxr, Chroma Technology, Rockingham, VT), and finally directed through emission filters (HQ 535/50 and HQ675/50, Chroma Technology) and pinholes (diameter of 30 μm) onto two avalanche photodiodes (SPCM-AQR-14, EG&G). The fluorescence signals were digitized and auto- and cross-correlation curves were calculated using a multiple τ algorithm.
The setup was calibrated by measuring the known diffusion coefficients of Alexa Fluor 488 and 633 in water (Invitrogen; D = 300 $\mu m^2/s$). The lateral radii ω_{xy}, defined as the point were the fluorescence count rate dropped e^2 times, were 180 nm for Alexa fluor 488 and 240 nm for Alexa fluor 633. Overlap of the two confocal volumes for Fluorescence Cross-correlation Spectroscopy (FCCS) experiments was optimized by maximizing the cross-correlation signal of double-labeled DNA, which was made by annealing equimolar amounts of 5'-ATTATTGAGTGGTCACTTTAAA-3' labeled on the 5'-end with Alexa Fluor 488 and 5' TTTAAAGTGACCACTCAATAAT-3' labeled on the 5'-end with Alexa Fluor 633 (IBA, St. Louis, MO). The overall efficiency of the fluorescence cross-correlation was found to be better than 80% (unpublished result). Crosstalk was lower then 5 %. Auto- and cross-correlation curves were fitted with a one-component three- (Aragón and Pecora, 1975) or two-dimensional diffusion model (Elson and Magde, 1974).

Miscellaneous. Mutations were confirmed by restriction analysis and DNA sequencing. Protein concentrations were determined according to a modified version of the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. The concentrations of purified OppA and OppBCDF were determined spectrophotometrically by measuring the absorption at 280 nm and using extinction coefficients of 1.605 and 0.990 $(mg/mL)^{-1}cm^{-1}$, respectively.

Results

Lateral mobility of OppA is reduced in OppBCDF-containing GUVs. Previous experiments showed that the lateral mobility of lipid-anchored OppA in GUVs was comparable to that of fluorescent lipids (Doeven *et al.*, 2005), which is consistent with the Saffman and Delbrück model for Brownian motion in biological membranes (Saffman and Delbrück, 1975). Since the mobility of integral membrane proteins was 2-3 times lower than that of OppA (Doeven *et al.*, 2005), we monitored the binding of OppA to its cognate membrane complex by autocorrelation analysis. Binding of fluorescent labeled OppA to OppBCDF would result in a reduced mobility of the labeled protein. A single-cysteine mutant of OppA, OppA (I602C), was labeled with Alexa Fluor 488 and reconstituted at a 1:50 (w/w) protein:lipid ratio, which corresponds to ~ 3.5 nmol OppA/m^2 of membrane surface (Doeven *et al.*, 2004). OppBCDF was co-reconstituted at a 1:20 (w/w) protein:lipid ratio, resulting in approximately equal amounts of OppA and OppBCDF complex molecules in the membranes. At these concentrations, according to the estimated K_M of OppBCDF for liganded OppA (~ 1.75 nmol/m^2 (Doeven *et al.*, 2004)), most OppBCDF complexes are interacting with OppA provided the OppAs are in the peptide-bound state (Doeven *et al.*, 2004). In Table 1 the results of the autocorrelation measurements are presented. In OppBCDF-containing GUVs, the mobility of OppA was lowered compared vesicles devoid of OppBCDF, which could reflect binding. The dissociation constant (K_D) for binding of bradykinin (peptide with the sequence RPPGFSPFR) to OppA is 0.1 μM (Lanfermeijer *et al.*, 1999; Detmers *et al.*, 2000). Upon addition of 10 μM bradykinin, that is, when all the OppA molecules are in the ligand-bound state, OppA diffusion slowed down from 3.1 ± 0.4 to 2.3 ± 0.5 (10^{-8} cm^2/s). In vanadate-trapped transition state conditions, that is, in the presence of substrate, MgATP and Vi, OppA mobility was again as fast as without peptide (3.2 ± 0.3 *10^{-8} cm^2/s). In the absence of peptide, however, no distinction could be made between OppA binding and a reduced lipid mobility in general as a consequence of the mere

presence of OppBCDF. A similar reduction in mobility compared to fluorescent labeled OppA was observed for the fluorescent lipid analogue DiO (not shown), suggesting an effect of the increased membrane crowding when reconstituting OppBCDF at protein to lipid ratios of 1:20 (w/w). Therefore, we concluded that autocorrelation measurements alone were not sufficient to perform a detailed analysis of the OppA:OppBCDF interaction.

Table 1. **Lateral mobility of OppA in OppBCDF-containing GUVs.**

Protein composition of GUVs		Additions to rehydration buffer		Diffusion coefficient (10^{-8} cm^2/s)[c]
OppA-AF488[a]	OppBCDF[b]	10 μM bradykinin	3 mM MgATP plus 0.5 mM Vi	
+	-	-	-	7.5 ± 0.9[d]
+	+	-	-	3.1 ± 0.4
+	+	+	-	2.3 ± 0.5
+	+	+	+	3.2 ± 0.3

[a]OppA (I602C) labeled with Alexa Fluor 488 was reconstituted at a 1:50 (w/w) protein:lipid ratio. [b]OppBCDF was reconstituted at a 1:20 (w/w) protein:lipid ratio. [c]Diffusion coefficients represent the average of at least two independent experiments of at least five measurements each, and the SD values are given. The FCS setup was calibrated with free Alexa probes. [d]Taken from Doeven *et al.* (2005) and confirmed in this study (not shown).

Fluorescence cross-correlation measurements. To monitor the binding of OppA to OppBCDF by fluorescence cross-correlation spectroscopy (FCCS), a single-cysteine mutant of the OppC component of OppBCDF was constructed. OppA (I602C) and OppBC(I296C)DF were purified, labeled with Alexa Fluor 488 or Alexa Fluor 633 (Fig. 1), respectively, and functionally incorporated into GUVs. As anticipated from previous results (Doeven *et al.*, 2005), all combinations of (labeled) SBPs and membrane complexes were active in peptide binding as well as transport (Table 2). Again, a relatively high concentration of OppA [1:50 (w/w) protein:lipid ratio] was used, because of the anticipated low affinity interaction of the SBP with its membrane complex (Doeven *et al.*, 2004). The amount of co-reconstituted OppBCDF was lowered to 1:100 (w/w), such that the final concentrations of the different proteins used in the FCCS experiments were equivalent to those used previously in uptake assays with proteo-LUVs and radioactive peptides (Doeven *et al.*, 2004). This also more closely resembles the *in vivo* situation in which an excess of OppA over OppBCDF exists. The maximal fluorescence cross-correlation amplitude G_{cc}^{max} that can be obtained in this situation, assuming that Alexa Fluor 633 labeled OppBCDF binds Alexa Fluor 488 labeled OppA in a 1:1 stoichiometry, is given by:

$$G_{cc}^{max} = 2\frac{\omega_{488}^2}{\omega_{488}^2 + \omega_{633}^2} G_{488}, \qquad \text{(equation 1)}$$

where G_{488} is the amplitude of the green channel, and ω_{488} and ω_{633} the lateral radii of the 488 and 633 confocal volumes, respectively. Note that this equation holds for a two-dimensional system, and is only valid when an excess of Alexa Fluor 488 labeled OppA is present over Alexa Fluor 633 labeled OppBCDF. In the reverse situation, that is with 1:50 (w/w) Alexa Fluor 633 labeled OppA and 1:100 (w/w) Alexa Fluor 488 labeled OppBCDF, the amplitude of the autocorrelation curve from the 633 channel was nearly zero due to the high amount of OppA and cross correlation signal could not be obtained (not shown).

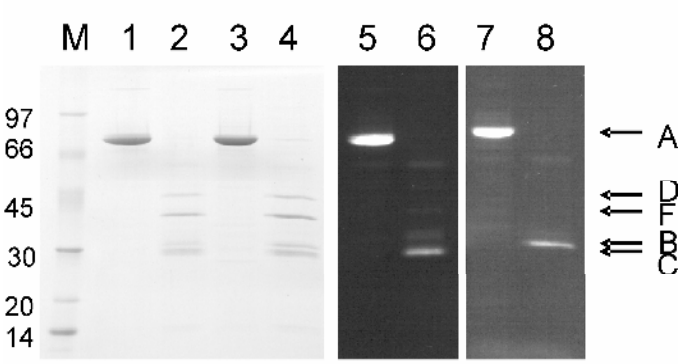

Fig. 1. **Purification and fluorescent labeling of single-cysteine Opp variants.** Ni^{2+}-NTA purified OppA I602C was labeled with Alexa Fluor 488 (lanes 1 and 5) or 633 (lanes 3 and 7). OppBC(I296C)DF labeled with Alexa Fluor 488 (lanes 2 and 6), or 633 (lanes 4 and 8) is also shown. The proteins were visualized with either Coomassie brilliant blue staining (lanes 1-4), or by illumination using UV-light (lanes 5-8). The latter allowed verification of the attachment of the fluorophores to the proteins. The positions of the OppABCDF proteins are indicated as are the masses of the molecular weight marker (M) proteins (kDa).

Table 2. **Peptide binding and transport activity of fluorescent labeled Opp reconstituted into LUVs.**

SBP[a]	Membrane complex[b]	Binding activity (nmol [^{3}H]-bradykinin/mg OppA)[c]	Transport activity (pmol [^{3}H]-bradykinin/mg OppBCDF*min)[d]
OppA	-	14 ± 0[e]	-[f]
OppA-488[g]	-	14 ± 1[e]	n.d.
OppA-633[h]	-	n.d.	n.d.
OppA	OppBCDF	n.d.	70 ± 34
OppA-488	OppBCDF	n.d.	74 ± 26
OppA-633	OppBCDF	n.d.	90 ± 25
OppA-488	OppBCDF-633[i]	n.d.	60 ± 4
OppA-633	OppBCDF-488[j]	n.d.	90 ± 8

[a]OppA and labeled variants were reconstituted at a 1:50 (w/w) protein:lipid ratio. [b]OppBCDF and labeled variants were reconstituted at a 1:100 (w/w) protein:lipid ratio. [c]The concentration of [^{3}H]-bradykinin was 3 μM. [d]The concentration of [^{3}H]-bradykinin was 0.7 μM. Measurements were done in at least two independent experiments; average plus SD values are given. [e]Taken from Doeven *et al.* (2005). [f]Taken from Doeven *et al.* (2004). [g]OppA (I602C) labeled with Alexa Fluor 488. [h]OppA (I602C) labeled with Alexa Fluor 633. [i]OppBC(I296C)DF labeled with Alexa Fluor 633. [j]OppBC(I296C)DF labeled with Alexa Fluor 488. n.d. not determined.

OppBCDF distinguishes liganded from unliganded OppA. Figure 2A shows confocal scans of GUVs containing fluorescent labeled OppA and OppBCDF. The observation volume was positioned on top of a GUV using a *z*-scan (Fig. 2B). Fig. 2C shows representative autocorrelation curves measured on GUVs containing Alexa Fluor 488-labeled OppA and Alexa Fluor 633-labeled OppBCDF. In the absence of peptide no cross-correlation signal was detected indicating that, on the time scale of the experiment, OppA was not associated with OppBCDF. Upon addition of 20 μM bradykinin the cross-correlation signal was 48 ± 15 % with respect to the maximal possible signal (the latter corresponding to ~ 0.72 times the amplitude of the 488 channel as calculated using equation 1), suggesting that liganded OppA was bound to OppBCDF. These results indicate that the membrane complex OppBCDF efficiently discriminates between OppA in its unliganded or liganded form, binding only the latter, under our experimental conditions.

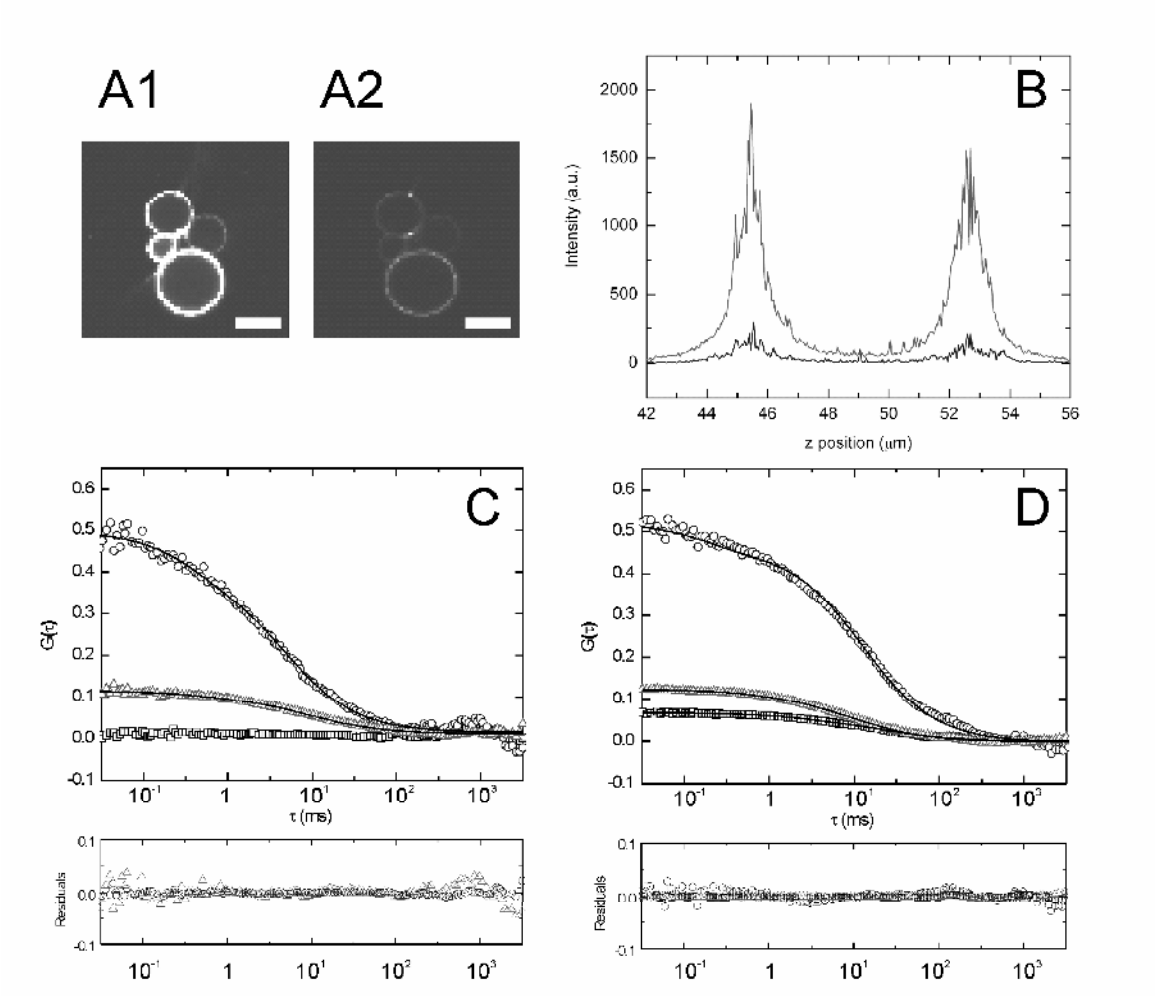

Fig. 2 **Confocal scans and FCCS measurements on fluorescent Opp-containing proteo-GUVs.** (A) Two dimensional cross-sections obtained by (*x*,*y*) confocal imaging of proteo-GUVs containing Alexa Fluor 488 labeled OppA I602C (OppA-AF488; reconstituted at a 1:50 (w/w) protein:lipid ratio) and Alexa Fluor 633 labeled OppBC(I296C)DF (OppBCDF-AF633; reconstituted at a 1:100 (w/w) protein:lipid ratio). Images from the 488 (A1) and 633 (A2) channels are shown. The scale bars are 10 μm. (B) Typical *z*-scan used to localize the proteo-GUV membranes. Signals in the 488 and 633 channels are shown (high and low intensity traces, respectively). An excess of OppA-AF488 over OppBCDF-AF633 was incorporated in the GUVs (see text for details). (C) FCCS experiment in 20 mM Hepes, pH 8.0. The observation volumes were focused on top of a proteo-GUV. Autocorrelation signals from OppA-AF488 (Δ) and OppBCDF-AF633 (○) are shown. No cross-correlation signal (lower curve, □) was detected. (D) FCCS experiment in 20 mM Hepes, pH 8.0, plus 20 μM peptide (bradykinin). The presence of a cross-correlation signal (lower curve, □) indicates that OppA-AF488 and OppBCDF-AF633 were bound. Curves were fit with a one-component two-dimensional diffusion model (solid lines), and the residuals are shown below the graph.

Oligomeric state of lipid-anchored OppA and OppA:OppBCDF binding stoichiometry. To investigate the oligomeric state of membrane-anchored OppA and the OppA:OppBCDF binding stoichiometry, Alexa Fluor 488- and Alexa Fluor 633-labeled OppAs were mixed 1:1 and co-reconstituted into GUVs at a 1:100 (w/w) protein:lipid ratio [total OppA to lipid ratio of 1:50 (w/w)], in the absence or presence of non-labeled OppBCDF. If, for example, OppA were a dimer, 488- and 633-labeled proteins would form 488-labeled homodimers, 488/633 labeled

heterodimers, and 633-labeled homodimers, in a 1:2:1 ratio, respectively. The heterodimers should give rise to a cross-correlation signal. Assuming all OppAs would be present as dimers, G_{cc}^{max} is given by:

$$G_{cc}^{max} = \frac{2}{3}\frac{\omega_{633}^2}{\omega_{488}^2 + \omega_{633}^2} G_{633}\,, \qquad \text{(equation 2)}$$

where G_{633} is the amplitude of the red channel, and ω_{488} and ω_{633} again the respective radii of the 488 and 633 confocal volumes. Note that, as with equation 1, equation 2 describes the situation for a two dimensional system. Thus, if OppA were dimeric, a cross-correlation signal would be observed with a maximal amplitude of ~ 0.24 times that of the 633 curve.
Table 3 shows that no cross-correlation signal was obtained with proteo-GUVs containing 'double'-labeled OppA. Addition of 20 μM peptide had no effect on the fluorescence cross-correlation. This indicates that the oligomeric state of both the unliganded and liganded forms of membrane-bound OppA is monomeric. When OppBCDF was present in the proteo-GUVs as well, also no cross-correlation signal was obtained both in the absence and presence of 20 μM bradykinin. Taken collectively, the data of this and the previous paragraph suggest that a single OppA, in the liganded conformation, binds to OppBCDF.

Table 3. **FCCS results with GUVs containing 'double-labeled' OppA.**

Protein composition of GUVs			Additions to rehydration buffer	Cross-correlation signal (%)[d]
OppA-AF488[a]	OppA-AF633[b]	OppBCDF[c]	20 μM bradykinin	
+	+	-	-	< 5
+	+	-	+	< 5
+	+	+	-	< 5
+	+	+	+	< 5

[a]OppA (I602C) labeled with Alexa Fluor 488 was reconstituted at a 1:100 (w/w) protein:lipid ratio. [b]OppA (I602C) labeled with Alexa Fluor 633 was reconstituted at a 1:100 (w/w) protein:lipid ratio. [c]OppBCDF was reconstituted at a 1:100 (w/w) protein:lipid ratio. [d]Percentage of the maximal possible cross-correlation signal with the combination of fluorescent labels used (equation 2). Average and SD values of at least two independent experiments and five measurements are shown.

ATP-binding induces OppA release from OppBCDF. In order to obtain more detailed information on the catalytic cycle of the Opp system, FCCS experiments were performed under various conditions of energy supply. Whereas in the presence of 20 μM bradykinin-liganded OppA bound to OppBCDF (Fig. 2C), inclusion of 3 mM ATP plus 1 mM EDTA in addition to 20 μM of peptide dissipated the cross-correlation signal completely (Table 4). In the absence of Mg^{2+} ABC transporters bind ATP, but do not hydrolyze it (Austermuhle *et al.*, 2004; Davidson and Chen, 2004; Higgins and Linton, 2004). Addition of 3 mM MgADP, or 3 mM of the non-hydrolyzable ATP-analogue MgAMP-PNP, resulted in the same effect. 100 μM ATP plus 1 mM EDTA appeared more effective in dissipating the cross-correlation signal than 100 μM MgADP. In the latter case a larger fraction of cross-correlation remained. Cross-correlation was also observed in the presence of 20 μM bradykinin and 3 mM of caged-ATP, indicating that the mere presence of a high concentration of adenine nucleotide was not sufficient for dissipation of the FCCS signal. Activation of the caged-ATP by UV light unfortunately destroyed the Alexa Fluor 488 and 633 fluorophores, making it impossible to measure FCS

(not shown). Taken together, these results clearly suggest that binding of ATP to the NBDs results in a conformational change that releases OppA from the translocator (OppBCDF) complex.

Table 4. **FCCS results with GUVs containing Alexa Fluor 488 labeled OppA and Alexa Fluor 633 labeled OppBCDF.**

Additions to rehydration buffer		Cross-correlation signal (%)[a]
20 μM bradykinin	(Mg-)nucleotides +/- EDTA or Vi	
-	-	< 5
+	-	48 ± 15
+	3 mM ATP + 1 mM EDTA	< 5
+	3 mM Mg-AMP-PNP	< 5
+	3 mM Mg-ATP + 0.5 mM Vi	< 5
+	3 mM Mg-ADP	< 5
+	0.1 mM ATP + 1 mM EDTA	32 ± 6
+	0.1 mM Mg-ADP	46 ± 10
+	3 mM caged ATP[b] + 1 mM EDTA	24 ± 15
+	3 mM caged ATP[b] + 1 mM EDTA after UV illumination	n.d.

Protein:lipid (w/w) ratios were the same as in Fig. 2. [a]Percentage of the maximal possible cross-correlation signal with the combination of fluorescent labels used (equation 1). Averages and SD values of at least two independent experiments and five measurements each are given. [b]Adenosine 5'-triphosphate, *P*^{3}-(1-(4,5-dimethoxy-2-nitrophenyl)ethyl) ester (DMNPE-caged ATP; Invitrogen) was used. n.d.: not determined since UV illumination destroyed the Alexa Fluor labels.

Discussion and conclusions

In contrast to the wealth of biochemical data on ABC efflux systems, detailed mechanistic information for ABC importers is more limited. We have analyzed the mechanism of oligopeptide transport by the ABC uptake system Opp by FCS and FCCS measurements under conditions mimicking intermediate stages of the reaction cycle. Importantly, these experiments were carried out with the proteins in the membrane-embedded state and under conditions that the transporters were fully functional. Previous studies on SBP:TMD interactions mostly involved the use of detergent-solubilized proteins or detergent-permeabilized proteoliposomes (Chen *et al.*, 2001; Borths *et al.*, 2002; Austermuhle *et al.*, 2004). The OppBCDF complex was not very stable in detergent solution as concluded from size exclusion and blue-native polyacrylamide gel electrophoresis experiments (unpublished results). Binding of MalE to $MalFGK_2$ was studied in permeabilized proteo-LUVs (Chen *et al.*, 2001). For Opp, this would involve the use of OppA lacking its N-terminal lipid-modification, a more artificial situation compared to when membrane-anchored OppA can be used. Proteo-GUVs proved to be an ideal alternative for studying SBP:TMD interactions of the oligopeptide transporter in a defined, close to native model membrane system.

Using proteo-GUVs, we previously showed that lipid-anchored OppA diffuses with the same speed as lipids whereas integral membrane proteins, such as OppBCDF, have a 2-3 fold lower lateral mobility (Doeven *et al.*, 2005; Poolman *et al.*, 2005). Therefore, we investigated the OppA:OppBCDF interaction by measuring changes in diffusion speed. Binding of fluorescent labeled OppA to non-labeled OppBCDF should result in a shift of the autocorrelation curve towards longer diffusion times. In OppBCDF-containing GUVs the mobility of OppA was indeed slower, and, upon addition of peptide, it reduced even further (Table 1). However, in the

absence of peptide, no distinction could be made between specific binding of OppA to OppBCDF and a reduced lateral mobility due to the mere presence of membrane-embedded protein complex. The latter option is not unlikely since it is known that the presence of integral membrane proteins negatively effects lipid lateral mobility at the lipid-to-protein ratios used in our experiments (Vaz *et al.*, 1984; O'Leary, 1987).
To circumvent this problem we turned to FCCS. Although this remains a technically demanding technique which can easily give rise to artifacts (e.g. crosstalk between the detector channels), experiments with double-labeled fluorescent DNA indicated that in our set-up we had a stable, high degree of overlap between the two laser focal volumes. The overall FCCS efficiency was as high as ~ 80 %. Furthermore, with the Alexa dye couple used in our experiments, the contribution of crosstalk to the cross-correlation signal was low (< 5 %). Single-cysteine mutants of OppA and OppBCDF, the latter bearing the cysteine substitution in one of the subunits of the integral membrane complex (OppC), were used for labeling with Alexa Fluor 488 or 633 maleimide (Fig. 1). In each case the position of the cysteine was chosen in the C-terminal part of the protein just in front of the His_6-tag used for purification and, as expected, labeling had no effect on peptide binding as well as on transport activity (Doeven *et al.*, 2005; Table 2). The fluorophore attached to OppA presumably sticks out from the surface of the protein (based on crystal structures of OppA homologues; Tame *et al.*, 1995; Dunten and Mowbray, 1995; Nickitenko *et al.*, 1995; Levdikov *et al.*, 2005), whereas the one attached to OppC is located in a short stretch of amino acids following the last helix that traverses the membrane back to the cytoplasmic side (Pearce *et al.*, 1992).
SBPs of ABC importers have been crystallized in open-unliganded, open-liganded, closed-liganded and closed unliganded conformations (Nickitenko *et al.*, 1995; Heddle *et al.*, 2003; Karpowich *et al.*, 2003; Trakhanov *et al.*, 2005). In the presence of substrate, the equilibrium is thought to lie strongly towards the closed-liganded state, and only SBPs in this conformation can interact productively with the membrane components. Experiments with the histidine and maltose permeases however, revealed that SBPs in their unliganded form(s) might also have access to the membrane complex, thereby impairing transport function (Prossnitz *et al.*, 1989; Dean *et al.*, 1992; Merino, *et al.*, 1995). BtuF, the SBP of the vitamin B_{12} transporter, was shown to bind to detergent-solubilized BtuCD even in the absence of substrate (Borths *et al.*, 2002), and this SBP is believed not to dissociate from the membrane complex at all during the catalytic cycle (Borths *et al.*, 2005). The FCCS experiments presented in Fig. 2C and D revealed that OppBCDF distinguishes substrate-bound from substrate-free OppA. This in is line with previous results where inhibition of transport activity by high levels of unliganded SBP (as observed for the histidine and maltose transporters) could not be demonstrated for the Opp system (Doeven *et al.*, 2004). Next to the possibility that OppBCDF discriminates liganded from unliganded SBP, a second explanation would be that the equilibrium in the absence of substrate lies more strongly towards the open conformation for OppA compared to other SBPs such as HisJ, MalE, and BtuF, the SBPs of the afore mentioned histidine, maltose and vitamin B_{12} transporters, respectively. This would mean that the inhibition of transport observed with the maltose and histidine transporters was caused by blocking of the membrane complex by SBPs in the closed-unliganded state.
Although it is generally assumed that the oligomeric state of SBPs is monomeric, several early reports on these type of proteins suggest that they are dimeric (Antonov *et al.*, 1976; Rashed *et al.*, 1976; Richarme, 1982, 1983). Moreover, ABC transporters have been found that have either one or two SBPs fused to the translocator, resulting in the presence of two or four SBPs

per functional complex (van der Heide *et al.*, 2002). Interaction between SBPs stimulated transport activity of the osmoregulated ABC transporter OpuA from *L. lactis* (Biemans-Oldehinkel and Poolman, 2003). Table 3 shows that a cross-correlation signal was not detected in proteo-GUVs with Alexa Fluor 488 and 633 labeled OppA co-reconstituted, strongly suggesting that lipid-anchored OppA does not form higher oligomeric aggregates. Addition of 20 μM peptide and/or inclusion of OppBCDF in the OppA-containing GUVs did not change the cross-correlation signals (not shown). Furthermore, sedimentation velocity and equilibrium centrifugation experiments also showed that OppA (without lipid anchor) is monomeric over a wide range of conditions tested (**Chapter 5**). These results indicate that monomeric OppA binds peptide, and that a single (liganded) OppA molecule binds to OppBCDF and is sufficient for transport.

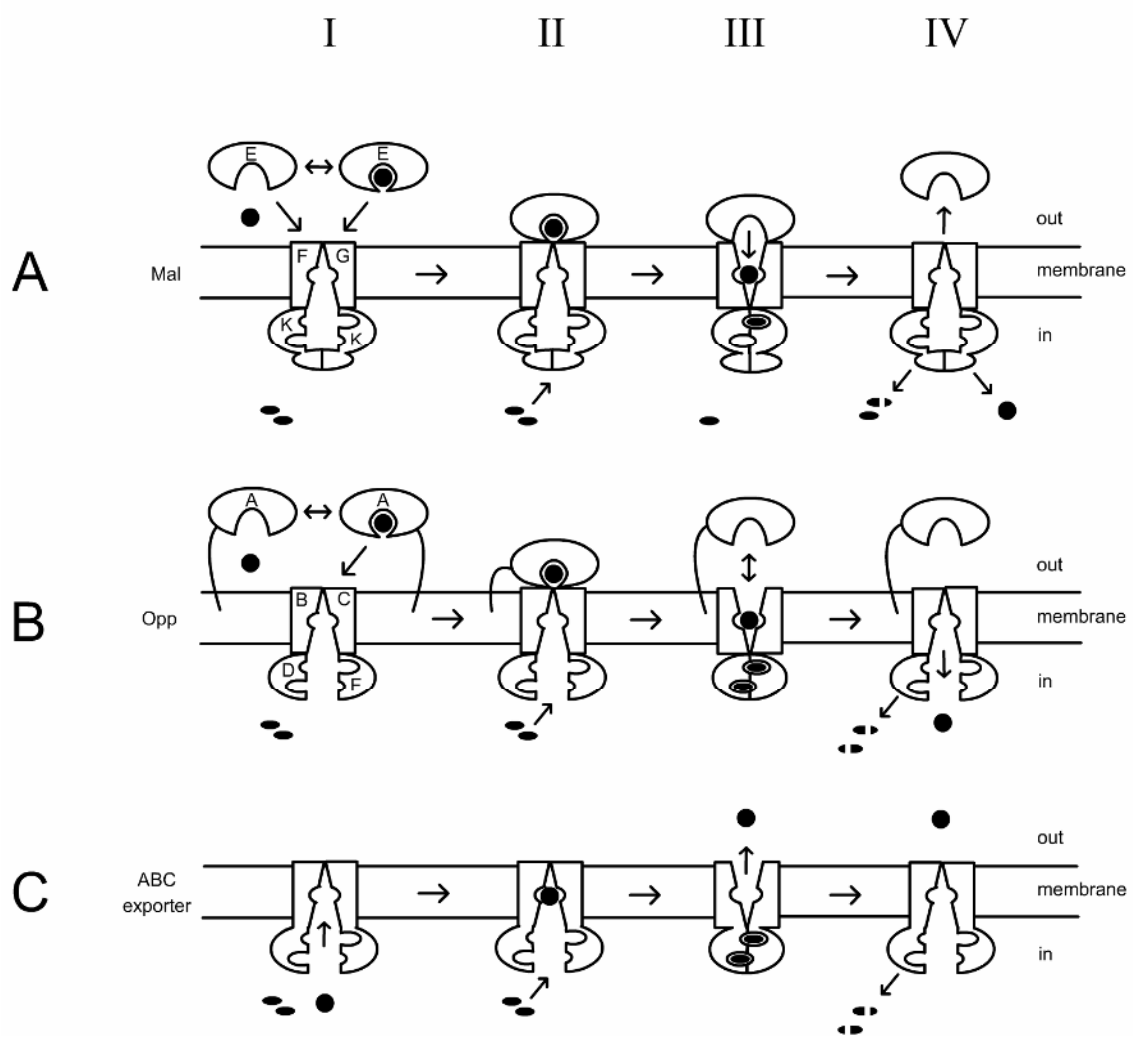

Fig. 3. **Mechanistic models for maltose (Mal) and oligopeptide (Opp) uptake and comparison to an ABC efflux system.** Transport substrate and Mg-ATP are indicated by filled circles and ovals, respectively. Hydrolyzed ATP is depicted as split ovals. For explanation, see text.

In case of the maltose transporter (Fig. 3A), it has been shown that upon ATP binding to the substrate-loaded MalE:MalFGK$_2$ complex MalE opens (step III; Austermuhle *et al.*, 2004). At this stage maltose is thought to be donated to a site in the TMDs, MalF and G, or released into

the cytoplasm directly. ATP hydrolysis is required to dissociate unliganded MalE from $MalFGK_2$ to complete the transport cycle (step IV; Chen *et al.*, 2001). Our results with the oligopeptide transporter (Fig. 3B) revealed that ATP binding is sufficient to dissociate OppA from OppBCDF (step III). This suggests that during this event the peptide must be donated to a binding site in the membrane. ATP-hydrolysis would then reorient this site to complete the catalytic cycle (step IV). The fact that OppA dissociates as soon as ATP is bound whereas MalE merely opens but is not released (yet) might be explained by the previously discussed observation that OppBCDF distinguishes liganded from unliganded SBP (Merino *et al.*, 1995; Doeven *et al.*, 2004). This in turn might be due to the possibility that unliganded OppA exclusively exists in the open conformation whereas MalE could exist in a closed-unliganded state as well. In accordance, the vitamin B_{12} binding protein BtuF does not appear to undergo conformational changes upon substrate-binding (Borths *et al.*, 2002; Karpowich *et al.*, 2003), and is thought not to dissociate from the BtuCD integral membrane complex during the catalytic cycle (Borths *et al.*, 2005).

Although the proposed mechanisms of SBP-dependent uptake systems thus appear somewhat variable with respect to the timing of SBP:TMD interactions during the catalytic cycle, a comparison with the proposed mechanism for ABC efflux systems (Fig. 3C; Higgins and Linton, 2004; van der Does and Tampé, 2004) reveals some conserved features. The largest difference is in the way substrate is bound (Fig. 3, steps I and II). ABC uptake systems require SBPs to signal the availability of substrate on the outside, whereas in ABC exporters the TMD binding site is readily accessible from the cytoplasmic face of the membrane. For both ABC im- and exporters, ATP-binding is thought to result in an altered accessibility of this TMD binding site in respect to the face of the membrane (step III). In ABC uptake systems this means that now substrate can enter the channel, whereas in the case of ABC export substrate is released into the external medium. A second common feature is that upon ATP-hydrolysis the transporter is thought to return to its initial conformation, thereby completing the catalytic cycle (step IV). For ABC uptake systems this results in the actual translocation of the substrate as well.

Acknowledgements

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Chapter 8

Summary and concluding remarks

Introduction

Our knowledge on membrane transport proteins has progressed enormously during the time-span in which this thesis was produced, not in the least due to the determination of high resolution crystal structures for several representatives of important membrane transport protein families.
For primary transporters these were the ATP-binding cassette (ABC) transporters MsbA, a lipid efflux system that was crystallized in three conformations with protein originating from *Escherichia coli* (Chang and Roth, 2001), *Vibrio cholera* (Chang, 2003), and *Salmonella typhimurium* (Reyes and Chang, 2005), and the vitamin B_{12} uptake system BtuCD from *E. coli* (Fig. 1A; Locher *et al.*, 2002). The calcium ATPase (Toyoshima *et al.*, 2000) provided a structural framework for P-type ATPases, whereas many catalytic intermediates of the light-driven proton pump from *Halobacterium salinarum* have been solved over the past four decades (for recent reviews see Kühlbrandt, 2000; Lanyi, 2004). Tremendous progress in the past five years, in terms of structural data, has with no doubt also been made in the secondary transport field. The crystal structure of the paradigm for ion-linked transport, the lactose transporter LacY from *E. coli* was solved (Fig. 1B; Abrahamson *et al.*, 2003), simultaneously with the glycerol-3-phosphate transporter GlpT from the same organism (Huang *et al.*, 2003). The structure of the bovine mitochondrial ADP/ATP carrier was solved at a resolution of 2.2 Å, which is significantly better than most membrane protein structures solved to date (Pebay-Peyroula *et al.*, 2003). Also, the ammonium transporter AmtB was solved at the high resolution of 1.4 Å (Khademi *et al.*, 2004). Two additional proteins from *E. coli*, the multidrug transporter EmrE (Ma and Chang, 2004) and the Na^+/H^+ antiporter (Hunte *et al.*, 2005), followed shortly after, in addition to two prokaryotic homologues of the neurotransmitter transporters Glt_{Ph} (Yernool *et al.*, 2004) and $LeuT_{Aa}$ (Yamashita *et al.*, 2005). All these examples represent major breakthroughs in the membrane transport protein field. The structures of a number of α-helical channel proteins, *e.g.* MscL from *Mycobacterium tuberculosis* (Chang *et al.*, 1998) and the K^+-channel KcsA from *Streptomyces lividans* (Doyle *et al.*, 1998), and several β-barrel porins were already solved before 2000, but also in this field several new structures have appeared in recent years: the mechanosensitive channel of small conductance MscS from *E. coli* (Fig. 1C; Bass *et al.*, 2002) and the mammalian voltage gated Shaker K^+ channel (Long *et al.*, 2005). One class for which no high resolution 3D structure of a complete transporter is available yet,

The abbreviations used are: ABC, ATP-binding cassette; ECD, extracytoplasmic domain; FCS, Fluorescence Correlation Spectroscopy; GUV, Giant Unilamellar Vesicle; LUV, Large Unilamellar Vesicle; NBD, nucleotide-binding domain; TMD, transmembrane domain; TMS, transmembrane segment; SBP, substrate-binding protein.

is formed by the bacterial group translocators (phosphoenolpyruvate:phosphotransferase systems).
Although high-resolution structural models are of utmost importance for progress of research in the membrane transport protein field, it is important to remember that they provide only snapshots of the catalytic cycle. Moreover, several cases are known in which there is serious doubt on whether the crystallographic structure indeed represents an intermediate stage in the reaction pathway, or is an artificial conformation induced by the crystallization conditions. Functional data are still more easily obtained from genetic, biochemical and biophysical experiments, and vast amounts of data were available for all membrane transport protein classes even before any individual member was crystallized. Many times, detailed ideas about structure/function relationships based on functional data appeared to be consistent with and of major importance for the interpretation of 3D structural models.

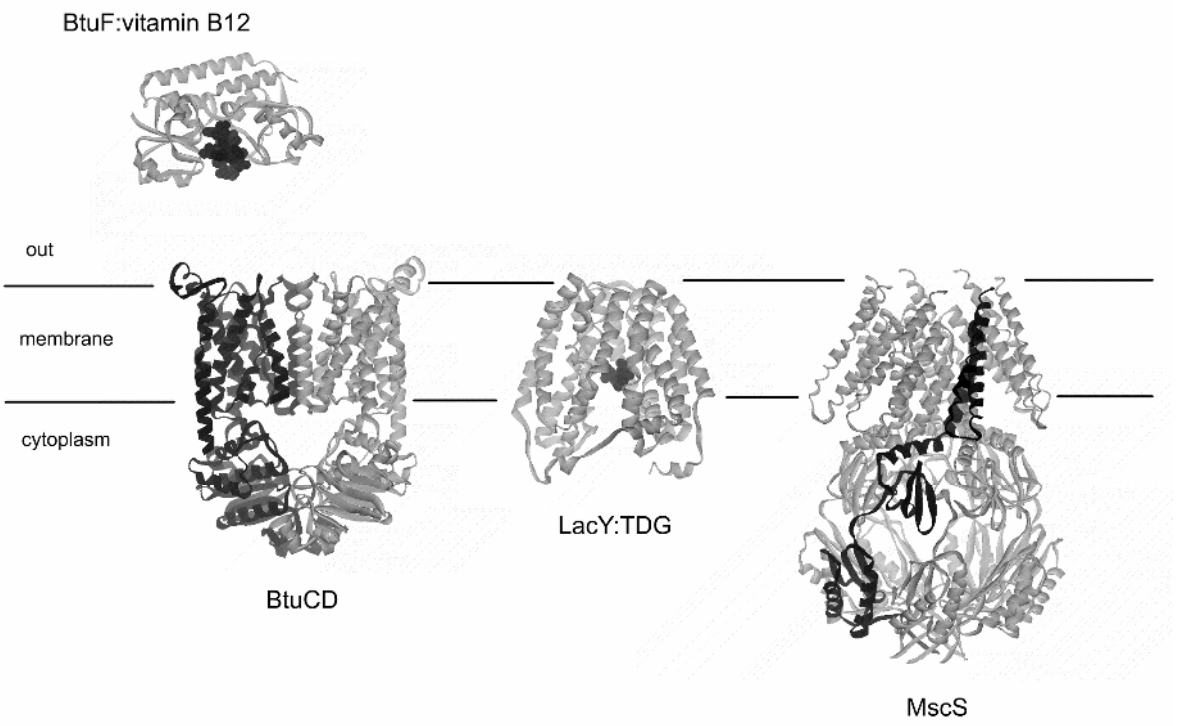

Fig. 1. **3D structural models of membrane transport proteins.** A primary transporter, a secondary transporter, and a channel protein are shown. The approximate position of the membrane is indicated. The ABC transporter BtuCD consists of an NBD, BtuD, bound to a TMD, BtuC. Two copies of BtuCD (shown in black and grey) together form a functional complex. The SBP BtuF is presented in complex with its ligand vitamin B_{12} (black spheres). The secondary transporter mutant LacY C154G is shown in complex with the lactose analogue β-D-galactopyranosyl-1-thio-β-D-galactopyranoside (TDG; black spheres). C. Heptameric channel protein MscS. A single subunit is highlighted in black. The PDB accession numbers for BtuCD, BtuF, LacY, and MscS are 1L7V, 1N4A, 1PV7, and 1MXM, respectively.

ABC transporters

This thesis dealt with the mechanism of substrate-binding, ATP-hydrolysis, and translocation by ABC transporters, in particular that of the oligopeptide transport system from *Lactococcus lactis*. ABC transporters are molecular pumps with a modular architecture (**Chapter 2**). Two hydrophobic transmembrane domains (TMDs) form the translocation pathway and two water-

soluble nucleotide-binding domains (NBDs) are associated to the cytosolic face of the TMDs. These ABC transporter core domains can be present as separate proteins or fused in almost any possible combination. The oligopeptide transporter (Fig. 2A) is composed of a substrate-binding protein (SBP), OppA, that captures and delivers peptides to the translocation unit, which is formed by OppB and OppC (the TMDs) and OppD and OppF (the NBDs). The SBPs of prokaryotic ABC uptake systems can be considered as accessory domains that signal the presence of substrate via the TMDs to the NBDs, which subsequently hydrolyse ATP to fuel the transport reaction. In ABC exporters, substrate can bind directly to a cytoplasmic-facing binding site in the TMDs. Several ABC transporters have extracytoplasmic domains (ECDs), that can be glycosylated or have inter- or intramolecular disulfide bridges, and/or additional membrane embedded and cytosolic regulatory domains that modulate the transport activity or allow the systems to assemble into macromolecular complexes. Examples are the ECDs in the ABCA subfamily of ABC transporters and ABCG2 (BCRP), the extra transmembrane segments (TMSs) in the transporter involved in antigen processing TAP and members of the SUR/MRP family (ABCC), the R-domains that can be phosphorylated in ABCA1 and CFTR, and the C-terminal regulatory extensions of MalK and OpuAA, the NBDs of the maltose and glycine betaine transporters, respectively.

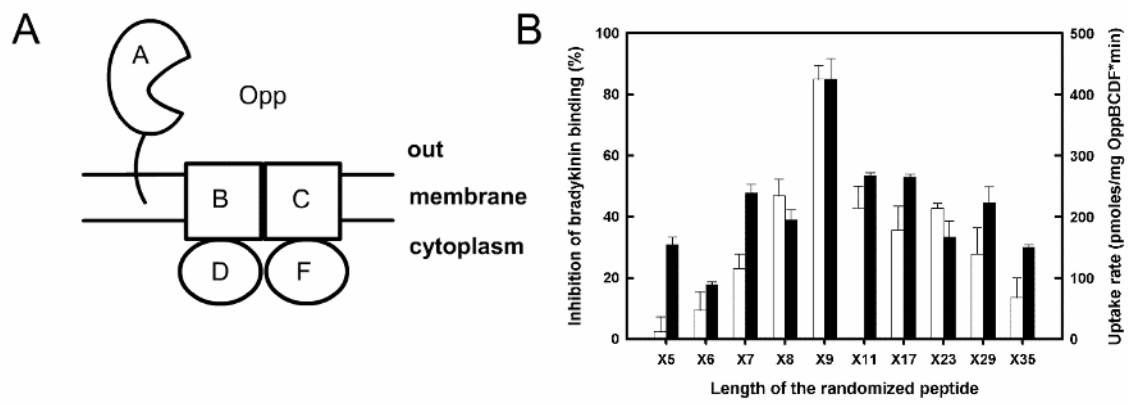

Fig. 2. **Architecture and substrate specificity of the oligopeptide ABC transporter.** A. Cartoon showing lipid-anchored oligopeptide-binding protein OppA and the membrane-embedded translocation unit OppBCDF. B. Comparison of binding (white bars) and transport (black bars) of combinatorial peptide libraries by OppA and OppABCDF, respectively. For experimental details see **Chapter 3**.

Substrate specificity of the oligopeptide ABC tranpsorter

The oligopeptide ABC transporter is an important player in the proteolytic system of lactic acid bacteria that enabled the use of these industrially important microorganisms in milk fermentation already for thousands of years. Milk protein is degraded by cell-wall protease(s) and the resulting peptides are imported, degraded by cytosolic peptidases and used as a source of nitrogen. Although in general it is thought that the SBPs of bacterial ABC uptake systems determine the transport specificity, doubts arose in literature as to whether this indeed was the case with lactococcal OppA (Charbonnel *et al.*, 2003; Helinck *et al.*, 2003). It was postulated that the translocation unit OppBCDF played an important role in determining transport selectivity. Overexpression and purification of the *L. lactis* Opp proteins followed by a thorough comparison of binding and transport of radiolabeled combinatorial peptide libraries by

Opp components, reconstituted into artificial Large Unilamellar Vesicles (LUVs), revealed that the binding specificity of OppA determined the transport selectivity (Fig. 2B; **Chapter 3**). In addition, it appeared that several *L. lactis* strains can also use the di/tripeptide transporter Dpp for oligopeptide uptake by utilizing next to DppA an additional SBP DppP (Lamarque *et al.*, 2004). This latter result clarified apparent contradictions in the scientific literature. Thus, *in vivo* research on peptide transport proved difficult (due to rapid breakdown of internalized peptides, efflux of (labelled) amino acids, and poorly characterized genetic backgrounds), but the combination with *in vitro* experiments, using purified proteins, confirmed that the (oligo)peptide-binding proteins determine the selectivity of their cognate ABC transporters (**Chapter 4**).

Structure and binding mechanism of OppA

OppA was first identified in the Gram-negative organisms *Salmonella typhimurium* and *E. coli* (Higgins and Hardie, 1983), where it is present as a soluble protein in the periplasm. 3D structural models for OppA from *Salmonella* and DppA from *E. coli*, a dipeptide-binding protein, have been obtained from crystallographic analysis more than 10 years ago (Tame *et al.*, 1994; Dunten and Mowbray, 1995; Nickitenko *et al.*, 1995). In contrast to lactococcal OppA, which can bind peptides up to 35 amino acids long with a strong preference for nonapeptides (Detmers *et al.*, 2000), a pentamer is the longest peptide that can be bound by these proteins. Accordingly, in the crystal structures these short peptides appeared buried between two lobes of the protein, whereas in case of lactococcal OppA it is believed longer peptides stick out and interact with the protein surface (Detmers *et al.*, 2000; Lanfermeijer *et al.*, 2000). Surprisingly, a recent publication revealed a nonapeptide bound in the active site of AppA, an OppA homologue from *Bacillus subtilis* (Levdikov *et al.*, 2005). To resolve how peptides are accommodated by OppA from *L. lactis*, crystals were grown in the presence of the high affinity nonapeptide ligand bradykinin (RPPGFSPFR; **Chapter 5**). Although the obtained crystals diffracted to a resolution better than 2.8 Å, a complete data set was not yet recorded. Analytical ultracentrifugation experiments did confirm that monomeric OppA adopts a more compact shape upon peptide-binding, similar to what was observed for the other proteins in their crystal structures.

Catalytic cycle of the oligopeptide ABC transporter

The transport mechanism of ABC uptake systems has been best studied for the maltose permease from *E. coli* (Chen *et al*., 2001, 2003; Austermuhle *et al.*, 2004; Fig. 3A). It is thought that maltose-loaded SBP MalE (step I) binds to the TMDs MalF and G (step II) and opens upon ATP-binding to the NBDs (two copies of MalK; step III). At this point, maltose is transferred to a binding site in the TMDs or released into the cytoplasm directly. ATP-hydrolysis dissociates the MalK dimer and resets the system to its initial conformation by dissociating MalE from MalFG (step IV).
In **Chapter 6**, a method for the functional incorporation of membrane proteins into Giant Unilamellar Vesicles (GUVs; artificial membrane structures ranging in diameter from ~ 5-100 μm) was developed. Upon addition of stabilizing amounts of sucrose to protein-containing LUVs (Large Unilamellar Vesicles; ~ 200 nm in diameter), these could be dried and rehydrated without losing biological activity. Opp-containing proteo-GUVs were used to study the catalytic cycle of the transporter by Fluorescence Correlation Spectroscopy (FCS; **Chapter 7**). The

results suggested that only peptide-bound OppA could interact with its membrane complex OppBCDF (in accordance with the results obtained in **Chapter 3**; Fig. 3B, step I). In contrast to MalE dissociation from the maltose transporter (Fig. 3A, step IV), OppA was released from OppBC already upon ATP-binding by OppDF (Fig. 3B, step III). We argue that the observed variations in timing of SBP:TMD interactions, during the catalytic cycle of various ABC transporters (e.g. Mal, His, Btu, and Opp), could be a consequence of differences in the equilibria between closed and open conformations of their respective SBPs in the absence of ligand.

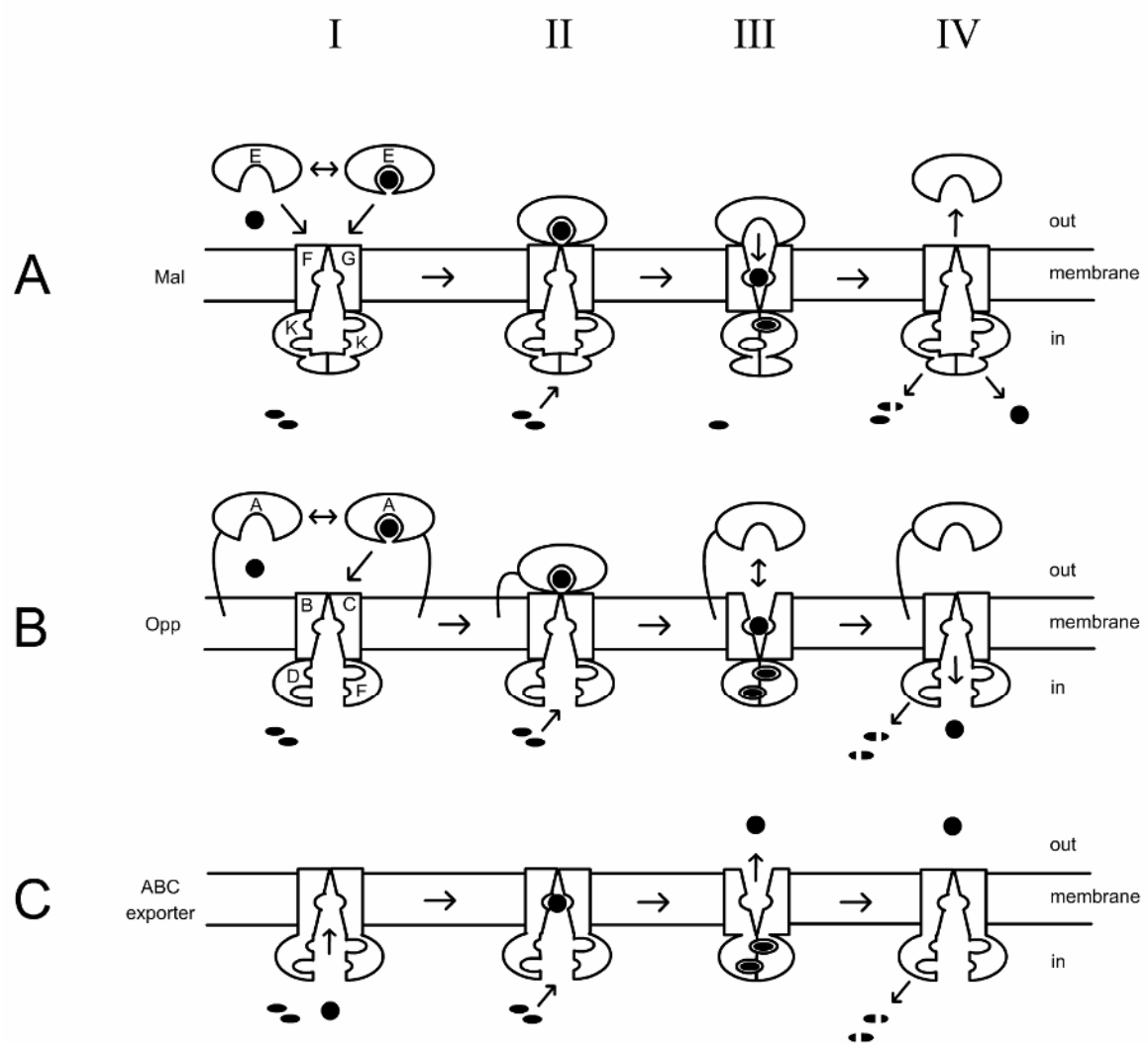

Fig. 3. Models for the translocation mechanism of ABC transporters. A. Maltose transporter Mal from *E. coli*. B. Oligopeptide transporter Opp from *L. lactis*. C. General ABC exporter. Transport substrate and Mg-ATP are indicated by filled circles and ovals, respectively. Hydrolyzed ATP is depicted as split ovals. For explanation see text.

Comparison of the translocation mechanism of SBP-dependent ABC importers (Fig. 3A and B) with the current general view on the catalytic cycle of ABC export systems (Higgins and Linton, 2004; van der Does and Tampé, 2004; Biemans-Oldehinkel *et al.*, 2006; Fig. 3C) suggests that the translocator conformational changes (e.g., NBD dimer association/dissociation coupled to alternate access of the substrate to the channel/TMD binding site) are conserved. The high-

and low affinity binding sites of ABC exporters for substrate on the in- and outside, respectively, appear reversed in ABC uptake systems that, in addition, use the obligatory extracytoplasmic SBPs.

Concluding remarks and future perspectives

The purification of the OppBCDF membrane complex opened up many different possibilities for further research, one of which being the functional reconstitution of the oligopeptide transporter into artificial liposomal systems (**Chapters 3** and **6**). The transport selectivity of Opp was determined and found to match the binding specificity of OppA (**Chapter 3**). Apparent discrepancies with *in vivo* data could be explained by the interference of another peptide transporter, Dpp, in the performed analyses (**Chapter 4**). The catalytic cycle of the Opp system was investigated by fluorescence correlation spectroscopy (**Chapter 7**), and the results indicated similarities but also important differences compared to mechanistic models proposed for other ABC transporters. ABC transporters share a common architecture and conformational changes during the translocation cycle seem to be conserved between ABC im- and exporters, with the exception of the direction of transport and the use of SBPs by uptake systems (**Chapters 2** and **7**).

Future research on lactococcal Opp should be focused to a large extent on obtaining high resolution structural information. The crystals obtained for OppA (**Chapter 5**) can be optimized and used for the elucidation of its seemingly unique peptide-binding mechanism. Although thought to be extremely difficult at the time the research presented in this thesis was started, crystal structures for complete ABC transporters are now being solved. Preliminary results from blue native polyacrylamide gel electrophoresis and size-exclusion chromatography experiments with purified OppBCDF indicated that the complex might be less stably associated in detergent solution compared to for example the maltose and histidine ABC transporters. Analytical ultracentrifugation and gel-filtration experiments could shed more light on the quaternary state of the complex in various detergents. The now more and more common high-throughput strategy of cloning of homologues of a protein (complex) of interest, followed by screening for highly expressed variants, purification, and crystallization seems a good approach to obtain a structure for a representative of the oligopeptide transporter family.

On the level of biochemical characterization of Opp there can also still be made considerable progress, especially when examining data obtained with some of the more intensively studied ABC systems, the maltose, histidine, and multidrug (*e.g.* P-gP) transporters. For SBP-dependent importers it has not yet been demonstrated if, upon release by the SBP, the substrate is donated to a site in the TMDs or directly passed through the channel into the cytoplasm. SBP-independent mutants isolated for the maltose and histidine transporters plead for the first option (Treptow *et al.*, 1985; Petronilli *et al.*, 1991; Speiser *et al.*, 1991; Covitz *et al.*, 1994). Although *L. lactis* mutants that could grow on oligopeptides (GLGL and YGGFL) in the absence of OppA have been obtained (derived from AMP15, a *L. lactis* MG1363Δ*oppA* strain; M. K. Doeven, M. van Waarden, and B. Poolman; unpublished result), the putatively OppA-independent OppBCDF mutants have not been further characterized yet. This was due to inconsistent results obtained in controls performed with the host to be used. *L. lactis* GRS164, an NZ9000Δ*oppA* variant, grew slowly on oligopeptides in the presence of the inducer for gene expression, nisin A, even in the absence of mutagenized *oppBCDF*.

Two other questions specific to Opp might be worth mentioning. First, how does the peptide substrate travel through the membrane? Does the N- or the C-terminus enter the cytoplasm first? Although technically challenging, it might be possible to label for example the N-terminus with a fluorescent probe and the C-terminus with a bulky group that traps the peptide in the OppBCDF complex. Upon addition of quenchers to either side of the membrane it might be possible to resolve where the peptide termini reside. Peptides with a fluorescent label attached have been shown to be able to bind to OppA (Lanfermeijer *et al.*, 2000), although transport assays with C-terminally fluorescent labeled peptides and proteo-LUVs have not been conclusive (M. K. Doeven, J. B. Bultema, and B. Poolman; unpublished results). The second issue concerns the substrate:ATP stoichiometry. Although there has been much debate on this issue in the past, it seems now that 2 ATP molecules are hydrolysed per translocated solute (Patzlaff *et al.*, 2003). Opp substrates, however, vary in size from 4 up to and including at least 35 amino acid residue peptides. Does a 4 amino acid long peptide require the same amount of ATP to be transported as a 35-mer? By performing transport assays with radiolabeled peptides of various lengths and sensitive ATPase assays this question should be possible to answer.

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Chapter 9

Nederlandse samenvatting voor geïnteresseerden buiten dit vakgebied

Samenvatting

ABC transporters zijn eiwitten die betrokken zijn bij de opname van voedingsstoffen en uitscheiding van schadelijke stoffen in de biologische cel. Defecten in ABC transporters kunnen bij de mens ernstige ziektes tot gevolg hebben. Te grote activiteit van ABC transporters kan leiden tot ongevoeligheid van kankercellen voor chemotherapie, en resistentie van bacteriën tegen antibiotica. Van de meest simpele bacterie tot de zeer ingewikkelde mens, alle levensvormen bevatten ABC transporters. In dit proefschrift is een bacteriële ABC transporter bestudeerd om zo meer te weten te komen over de werking van ABC transporters in het algemeen. Het onderzochte eiwit, genaamd de oligopeptide ABC transporter, is gezuiverd uit een bacterie en bestudeerd met biochemische en biofysische technieken. Zo is opgehelderd wat voor stoffen via de oligopeptide ABC transporter worden opgenomen, en wat het werkingsmechanisme van dit eiwit is.

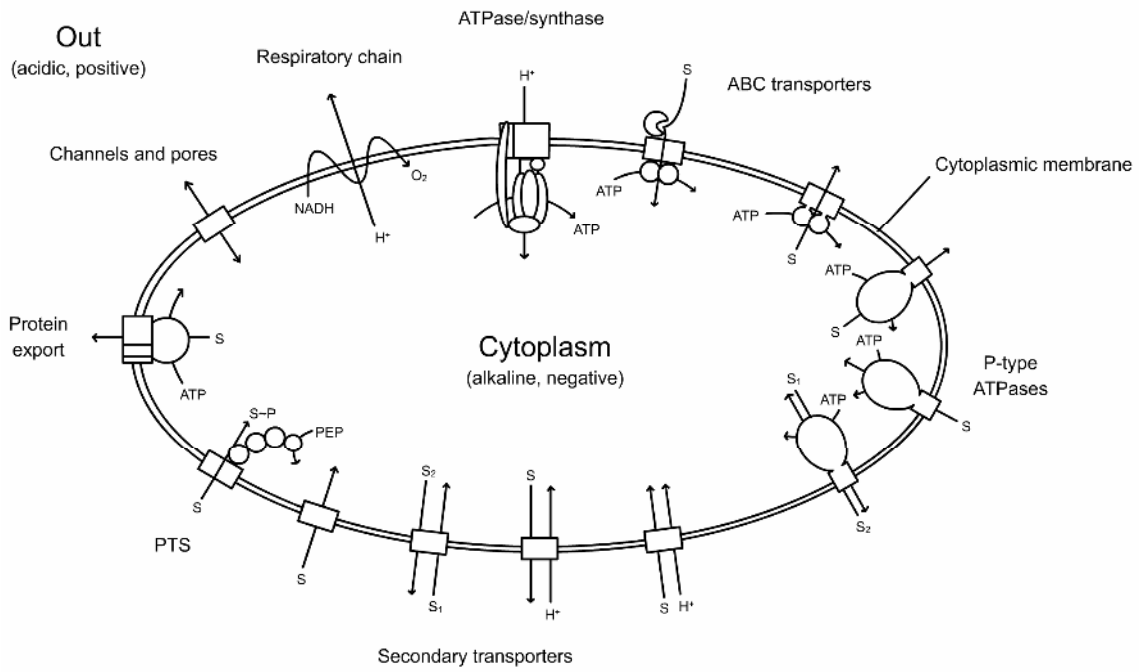
1. De barrière van het leven: het celmembraan

De universele eenheid waaruit alle levensvormen zijn opgebouwd is de cel. Het aantal cellen per individu kan van soort tot soort enorm varieren, van één enkele in het geval van bacteriën tot zo'n slordige 100.000 miljard bij de mens. Iedere cel bestaat uit een stroperige oplossing met hierin een extreem hoge concentratie aan wateroplosbare deeltjes, het cytoplasma, omgeven door ten minste één enkele membraan. In het cytoplasma, en in hogere organismen in de celkern, bevindt zich erfelijke informatie in de vorm van DNA. Coderende gebieden in het DNA, de genen, worden via RNA vertaald in eiwit. De celmembraan is een uit lipiden opgebouwde waterafstotende dubbellaag gevuld met grote hoeveelheden eiwit, en vormt een fysieke barrière voor wateroplosbare stoffen. Zo wordt het binnenste van de cel afgeschermd van de buitenwereld.

2. Transporteiwitten

Om te kunnen groeien moet de cel zich delen. Hiertoe dient de hoeveelheid DNA zich te verdubbelen (1 kopie voor iedere dochtercel) en de biomassa moet voldoende toegenomen zijn. Voedingsstoffen worden daarom opgenomen door de cel via gespecialiseerde eiwitten aanwezig in het membraan (zie figuur 1). Deze 'transporteiwitten' vormen een soort moleculaire machientjes die het verbruik van energie koppelen aan het verplaatsen van een molecuul (het substraat) van de ene naar de andere zijde van het membraan (dus van buiten

de cel naar binnen of andersom). Naast hun belangrijke rol bij de opname van voedingsstoffen, zijn transporteiwitten ook betrokken bij de uitscheiding van afval- of andere schadelijke stoffen, eiwitten met een functie buiten de cel, het invoegen van (transport)eiwitten in het membraan, en het waarnemen en verwerken van signalen uit de buitenwereld. Kleine afwijkingen in genen die coderen voor transporteiwitten kunnen ernstige ziektes tot gevolg hebben bij de mens. Bekende voorbeelden zijn cystische fibrose en resistentie van tumor-cellen tegen cytostatische medicijnen, veroorzaakt door mutaties in een ABC–type kanaaleiwit en een drug-uitscheidings eiwit, respectievelijk. Verder kunnen transporteiwitten er voor zorgen dat bacteriën resistent worden voor veel gebruikte antibiotica. Een van de meest voorkomende types transporteiwitten zijn de ABC transporters.



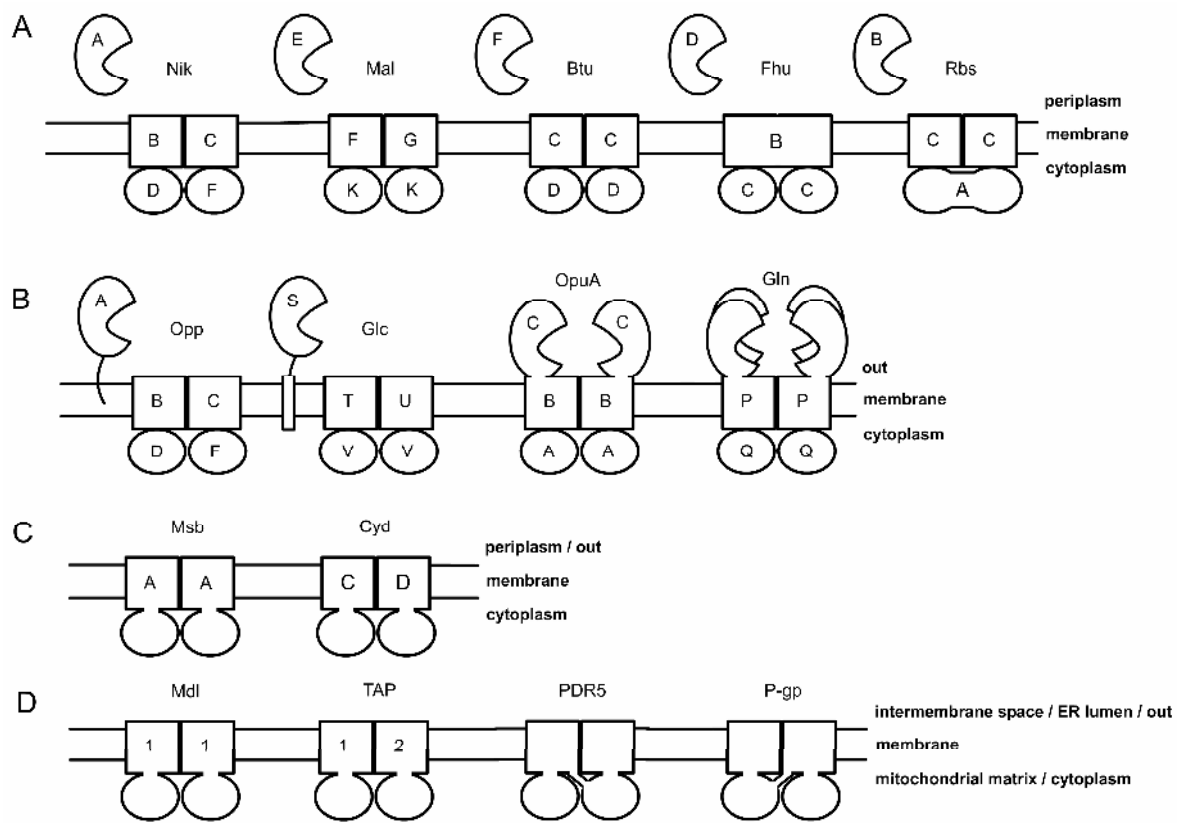
Figuur 1. **Schematische weergave van een bacterie.** Het binnenste van de cel, het cytoplasma, wordt gescheiden van de buitenwereld door een membraan (aangeduid als 'cytoplasmic membrane'). Verschillende soorten transporteiwitten zijn schematisch als blokjes en/of bolletjes weergegeven. Het type energie dat wordt gebruikt voor het opnemen of uitscheiden van substraten (S), ATP of een electrochemische gradient (dit laatste is aangeduid met 'H^+'), en de transportrichting van het substraat zijn aangegeven.

3. ABC transporters en de oligopeptide ABC transporter

3.1 ABC transporters

Alle levende organismen, van de simpelste bacterie tot de meest complexe plant of zoogdier aan toe, bevatten ABC transporters en zijn hier voor hun goed functioneren afhankelijk van. De opbouw van ABC transporters is opvallend genoeg in alle organismen gelijk. Ze worden gebruikt voor zowel opname als uitscheiding van een enorme diversiteit aan substraten (zie **Hoofdstuk 2**). Kleine ionen, suikers, lipiden, drugs, peptiden (stukjes eiwit), en zelfs hele eiwitten kunnen allemaal worden verplaatst over het membraan door ABC transporters. Een ABC transporter bestaat uit een eiwit-complex van vier eiwitten of eiwit-domeinen (figuur 2). In

het membraan vormen twee waterafstotende eiwitten samen het gat waardoor het substraat het membraan kan passeren. Aan de kant van het cytoplasma zitten twee wateroplosbare eiwitten, de zogenaamde ATP-bindende cassettes of ABC-eiwitten. Hieraan danken de ABC transporters hun naam. Twee ABC-eiwitten fungeren samen als de motor die de ABC transporter aandrijft door binding en splitsing van ATP (de brandstof voor transport van het substraat). Elk van de in totaal vier eiwitten kan apart voorkomen of samen als één groot eiwit (figuur 2). Hoewel ABC uitscheidings systemen in alle levende organismen te vinden zijn, zijn ABC opname systemen alleen maar aanwezig in de 'lagere' organismen: de bacteriën en de Archaea; een Archaeon is een soort 'oer-bacterie' die vaak bestand is tegen extreme condities. ABC opname systemen gebruiken een extra eiwit wat zich aan de buitenkant van de cel bevindt. Dit zogenaamde 'substraat-bindingseiwit' (in figuur 2 weergegeven als Pac-man-achtige figuurtjes) pakt het te transporteren substraat en brengt het naar de ABC transporter in het membraan. Veranderingen in de vorm van de ABC transporter als gevolg van ATP binding en splitsing door de ATP-bindende cassettes zorgt er vervolgens voor dat het substraat-bindingseiwit het substraat loslaat zodat het via de ABC transporter in het cytoplasma beland.



Figuur 2. **ABC transporters zoals aanwezig in de membranen van alle levende organismen.** (A) en (B) Opname systemen gevonden in bacteriën en andere 'lage' organismen. (C) en (D) Uitscheidings systemen aanwezig in 'lagere' organismen (C), en in 'hogere' organismen (D) zoals planten en zoogdieren (inclusief de mens). Substraat-bindingseiwitten zijn als Pac-man-achtige figuren weergegeven, eiwit-domeinen die het gat in het membraan vormen waardoor een substraat kan passeren als vierkantjes, en ATP-bindende cassette eiwitten (ABC-eiwitten) als ovale vormen. 3-Lettercodes betreffen de naam van het transport systeem. Letters in de figuren duiden individuele eiwitten aan.

3.2 Het substraat-bindingseiwit OppA bepaalt welke substraten door de oligopeptide ABC tranporter worden opgenomen

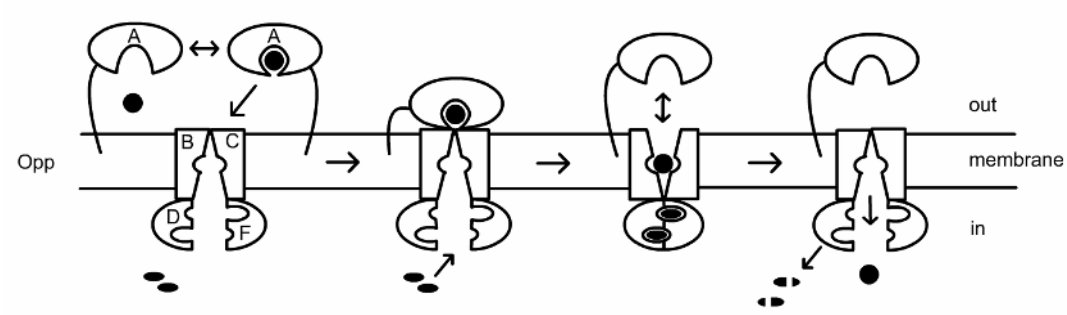
De oligopeptide ABC transporter bestaat uit het substraat-bindingseiwit OppA, en het transportdomein OppBCDF (figuur 2B; Opp). Een algemeen aanvaard beeld is dat de substraat-bindingseiwitten van ABC opname systemen bepalen wat voor substraat er opgenomen wordt door de cel. In het geval van de oligopeptide ABC transporter, een ABC transporter die stukjes eiwit (genaamd oligopeptiden) transporteert, is in de wetenschappelijke literatuur echter twijfel ontstaan omtrent dit punt. Ondanks dat verschillende OppA substraat-bindingseiwitten werden gebruikt, bleek dat toch iedere keer dat dezelfde peptides via de oligopeptide ABC transporter werden opgenomen. Dit leidde tot de conclusie dat het transportdomein OppBCDF (figuur 2B) naast OppA mede bepaalt wat voor peptiden er opgenomen worden. Deze experimenten werden echter uitgevoerd met specifieke bacterie-stammen, welke naast de oligopeptide ABC transporter ook nog vele andere transporteiwitten bevatten. Van deze andere transporteiwitten weten we soms niet eens wat voor substraten ze transporteren. In **Hoofdstuk 3** van dit proefschrift staat de zuivering en herplaatsing in een kunstmatig membraan systeem (bolvormige uit lipiden bestaande blaasjes die 'liposomen' worden genoemd) van de vijf eiwitten behorende bij de oligopeptide ABC transporter beschreven. Dit behaalde resultaat stond toe om de oligopeptide ABC transporter te bestuderen in een gedefinieerde omgeving, zonder andere transporteiwitten die normaal aanwezig zijn in een cel. Wanneer nu binding van verschillende peptiden aan het gezuiverde substraat-bindingseiwit OppA met transport via de gezuiverde oligopeptide ABC transporter, dus OppA plus OppBCDF, werd vergeleken, bleek dat dit volledig met elkaar in overeenstemming was. Het oligopeptide-bindingseiwit OppA bond bij voorkeur peptiden van negen aminozuren lang, en de oligopeptide ABC transporter transporteerde deze vervolgens ook het beste. Deze experimenten spraken dus de eerder door andere groepen gepubliceerde resultaten tegen en bevestigden dat het transportdomein OppBCDF geen extra selectiviteit op het peptide opname proces legt.

3.3 Peptide transport in micro-organismen

Vrijwel gelijktijdig met de hier boven beschreven vinding dat OppA toch de belangrijkste speler is in het bepalen van de transport selectiviteit van de oligopeptide ABC transporter, werden nieuwe resultaten gepubliceerd die lieten zien dat verschillende eerder gebruikte bacteriën naast de oligopeptide ABC transporter ook nog een ander transporteiwit bevatten wat oligopeptide opname kan bewerkstelligen. Deze vinding verklaarde waarom de eerder behaalde resultaten met bacteriën niet overeen kwamen met die beschreven in **Hoofdstuk 3** voor de gezuiverde oligopeptide ABC transporter. Hoe dit precies allemaal in elkaar steekt staat beschreven in **Hoofdstuk 4**. De belangrijkste conclusies van dit hoofdstuk waren dat peptide opname proeven in bacteriën ingewikkeld zijn door de snelle afbraak van opgenomen substraten, uitscheiding van aminzoren afkomstig van eerder opgenomen peptiden, en, zoals net al vermeld, de aanwezigheid van andere transporteiwitten in voor metingen gebruikte bacteriën. Het voorspellen van wat voor peptiden het beste getransporteerd zullen worden door oligopeptide ABC transporters van minder goed bestudeerde micro-organismen, op basis van het vergelijken van de betrokken OppA eiwitten, was vrijwel onmogelijk vanwege een te lage gelijkenis.

3.4 Structuur van het oligopeptide-bindingseiwit OppA

De 3-dimensionale vouwing of structuur van eiwitten kan met behulp van verschillende methoden worden bestudeerd. Eén van de op dit moment meest krachtige methoden om de structuur van een eiwit te bepalen houdt in dat men eiwit-kristallen maakt die vervolgens met röntgen straling worden belicht. Dit resulteert in een stippen-patroon wat gebruikt kan worden om de structuur van het eiwit te berekenen. Er zijn al structuren van OppA eiwitten van verschillende bacteriën beschikbaar. Door deze eiwit structuren te bekijken kan direct duidelijk worden welke aminozuren van het eiwit een belangrijke rol spelen bij bijvoorbeeld het binden van een peptide. Op basis van de verschillende structuren van OppA eiwitten, wordt in het algemeen verondersteld dat OppA eiwitten op zichzelf functioneren en geen grotere structuur vormen door bijvoorbeeld met een tweede kopie van zichzelf een interactie aan te gaan. Er zijn echter indicaties dat substraat-bindingseiwitten, zoals OppA, een interactie aan kunnen gaan met zichzelf wat van belang zou kunnen zijn voor hun functie. In **Hoofdstuk 5** is aan de hand van het gedrag van OppA eiwitten in een centrifugaal veld bepaald dat OppA daadwerkelijk alleen functioneert. Vervolgens zijn eiwit-kristallen van het OppA eiwit uit de bacterie die wij bestuderen in complex met een 9 aminozuur lang peptide gemaakt en belicht met röntgen-straling om de structuur te berekenen. Deze eiwit-kristallen waren helaas nog niet goed genoeg om de structuur van dit OppA eiwit op te helderen, maar vormen een belangrijke stap in de weg hier naar toe.



Figuur 3. **Model voor het transportmechanisme van de oligopeptide ABC transporter.** Eiwitten zijn weergegeven en aangeduid als in figuur 2. Het substraat (zwarte bol) bindt aan de buitenkant van de cel (out) aan het substraat-bindingseiwit OppA, wat vervolgens een interactie aangaat met het transporteiwit OppBCDF in het membraan. OppBCDF bindt en splitst vervolgens ATP (zwarte ovale vormen), met als gevolg dat het substraat van buiten de cel naar binnen (in) wordt verplaatst.

3.5 Analyse van het transportmechanisme van de oligopeptide ABC transporter

In **Hoofdstuk 6** en **7** is de oligopeptide ABC transporter bestudeerd met een techniek genaamd 'fluorescentie correlatie spectroskopie', kortweg FCS. Met behulp van lasers kan men hier mee het diffusiegedrag van eiwitten bepalen. Een probleem met het bestuderen van transporteiwitten met FCS in een kunstmatig systeem zoals liposomen (zie boven), is dat deze kunstmatige structuren vaak veel te klein zijn. Door optische beperkingen moet het te bestuderen liposoom een diameter hebben van minimaal ~ 5 micrometer (1 μm = 10^{-6} m = 0,000001 m), terwijl de liposomen zoals gebruikt in **Hoofdstuk 3** maar ongeveer 200

nanometer groot zijn (1 nm = 10^{-9} m = 0,000000001 m): een verschil van minstens 25 keer! In **Hoofdstuk 6** is een methode ontwikkeld om transporteiwit-bevattende kleine liposomen van 200 nm om te zetten in enorm grote liposomen met een diameter tussen de ~ 5 – 100 μm. Met behulp van FCS is vervolgens aangetoond dat het substraat-bindingseiwit OppA, wat alleen maar via 1 lipide molecuul vast zit aan de buitenkant van het membraan (figuur 2B, OppA), een hogere mobiliteit heeft dan eiwitten die volledig in het membraan zijn ingebouwd (bijvoorbeeld OppBCDF, zie figuur 2B). In **Hoofdstuk 7** is deze techniek verder uitgebreid om de interactie tussen het oligopeptide-bindingseiwit OppA en het bijbehorende transporteiwit OppBCDF te bestuderen. Het bleek dat OppA aan OppBCDF bindt zodra het een peptide gepakt heeft. OppBCDF bindt en splitst vervolgens ATP waardoor het peptide van buiten de cel naar binnen wordt getransporteerd (figuur 3).

4. Slotopmerkingen en toekomstperspectief

Dit proefschrift beschrijft structurele en functionele aspecten van ABC transporters aan de hand van experimenten uitgevoerd met een model systeem: de oligopeptide ABC transporter. Het huidige onderzoek naar membraaneiwitten richt zich zeer sterk op het verkrijgen van structurele informatie, met name met behulp van eiwit-kristallen. Voor de oligopeptide ABC transporter zal ook deze weg ingeslagen moeten worden, zeker nu er hier eiwitkristallen beschreven zijn voor het oligopeptide-bindingseiwit OppA. Gedurende dit promotieonderzoek hebben we veel geleerd over de oligopeptide ABC transporter door het uitvoeren van biochemische en biofysische proeven. Zoals met fundamenteel wetenschappelijk onderzoek echter bijna altijd het geval is, kwamen er ook weer nieuwe belangrijke vragen en uitdagingen naar voren. Men kan zich bijvoorbeeld afvragen hoe extreem lange peptiden van wel 35 aminozuren lang getransporteerd worden. Welk deel van het peptide bereikt het eerste het cytoplasma, het eerste of het laatste aminozuur? Op welk exact moment tijdens transport arriveert het peptide in the cytoplasma? Zijn er voor het opnemen van een kort peptide evenveel brandstof (ATP) moleculen nodig als voor een zeer lang peptide? En, meer algemeen, hoe kan het dat ABC transporters,die allemaal dezelfde universele opbouw hebben in alle levende organismen, zo'n enorme verscheidenheid aan substraten, zowel qua type als grootte, kunnen transporteren? De bevindingen beschreven in dit proefschrift maken de oligopeptide ABC transporter een interessant onderwerp voor verder onderzoek, wat in de toekomst mogelijk zal leiden tot meer inzichten met betrekking tot deze en andere vragen.

List of publications

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Nawoord

Na 4 jaar en een beetje ben ook ik dan nu toe aan het schrijven van het nawoord voor mijn proefschrift. Het was een mooie tijd. Achteraf gezien, en zeker voor degenen die het allemaal wat meer van een afstandje hebben waargenomen, is alles zeer vlot verlopen. Dit neemt niet weg dat het toch allemaal aardig wat moeite heeft gekost, en dat ik nu zeer blij ben dat het af is.

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Verder heb ik 2 à 3 keer per week behoorlijk wat uurtjes doorgebracht ergens op een basketballveld, op borrels en feesten, en bij bestuursvergaderingen van “de Groene Uilen”. Dit was een fantastische tijd waar ik vele goede contacten aan over heb gehouden. Naast “de basketballers” waren er “de biologen”, “de wintersporters”, en, niet te vergeten, “de vrienden uit mijn middelbare school tijd”. Nu we zo goed als allemaal begonnen zijn aan ons werkende leven zijn de ontmoetingen vaak iets minder frequent, maar ik hoop velen van jullie de 21ste april of kort daarna te zien.

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Mark

Curriculum Vitae

Mark Doeven werd op 20 december 1978 geboren te Meppel. De eerste twee jaar van zijn middelbare school heeft hij aldaar doorgebracht op de “C.S.G. Dingstede”, en hij behaalde vervolgens zijn VWO diploma aan “De Waezenburg” te Leek. In 1997 ving hij aan met de studie Biologie aan de Rijksuniversiteit Groningen. Naast zijn studie, en tot enkele jaren hierna, was hij actief als speler, coach en bestuurslid van de Groninger Studenten Basketball Vereniging “de Groene Uilen”. Begin 2002 is hij afgestudeerd na stages in de groep van Prof. Dr. Bert Poolman te Groningen en de groep van Prof. Anthony Watts aan de Universiteit van Oxford. In ditzelfde jaar begon hij met zijn promotieonderzoek naar het moleculaire mechanisme van substraat-binding, ATP-hydrolyse en translocatie door ABC transporters. Vanaf mei 2006 zal hij werkzaam zijn voor het Farber Institute for Neurosciences van de Thomas Jefferson University te Philadelphia in de VS. Mark Doeven woont samen in Groningen met Lilian Eggens.

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