Acta Crystallographica Section F
Structural Biology Communications
ISSN 2053-230X

Membrane proteins, detergents and crystals: what is the state of the art?

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Received 15 September 2014
Accepted 14 November 2014

At the time when the first membrane-protein crystal structure was determined, crystallization of these molecules was widely perceived as extremely arduous. Today, that perception has changed drastically, and the process is regarded as routine (or nearly so). On the occasion of the International Year of Crystallography 2014, this review presents a snapshot of the current state of the art, with an emphasis on the role of detergents in this process. A survey of membrane-protein crystal structures published since 2012 reveals that the direct crystallization of protein–detergent complexes remains the dominant methodology; in addition, lipidic mesophases have proven immensely useful, particularly in specific niches, and bicelles, while perhaps undervalued, have provided important contributions as well. Evolving trends include the addition of lipids to protein–detergent complexes and the gradual incorporation of new detergents into the standard repertoire. Stability has emerged as a critical parameter controlling how a membrane protein behaves in the presence of detergent, and efforts to enhance stability are discussed. Finally, although discovery-based screening approaches continue to dwarf mechanistic efforts to unravel crystallization, recent technical advances offer hope that future experiments might incorporate the rational manipulation of crystallization behaviors.

1. Introduction

During the past 30 years, the views of the crystallographic community regarding membrane proteins have undergone an extraordinary transition. When I began graduate school in the early 1980s, crystallizing membrane proteins was widely thought to be nearly impossible. However, this perception has gradually been eroded by steady progress, fueled by unflagging, sometimes heroic efforts and myriad technical advances. Today, membrane-protein crystallography verges on the routine, and the membrane proteome is finally yielding its secrets. This review aims to provide a brief snapshot of membrane-protein crystallization as it currently exists, slightly over midway through the International Year of Crystallography 2014, focusing particularly on the role of detergents in this process.

2. A survey of recent structures: comparison of crystallization methods

The progress of membrane-protein structural biology has been well chronicled on Stephen White's estimable mpstruc website (Membrane Proteins of Known Structure; http://blanco.biomol.uci.edu/mpstruc/), making this an excellent place to begin taking stock of the current state of membrane-protein crystallization.

To assemble a list of recent membrane-protein crystal structures, the mpstruc website was searched for structures published between the start of 2012 and September 2014 (the time of writing). Non-crystallographic structures were eliminated (*e.g.* those determined by NMR or electron microscopy), as were structures of soluble domains of membrane proteins; this leaves 231 *bona fide* membrane-protein crystal structures. The mpstruc database conveniently breaks these

down on the basis of protein architecture (monotopic, β-barrel or α-helical). For this analysis, it proved useful to further divide the helical proteins into three subcategories, namely G-protein-coupled receptors (GPCRs), microbial rhodopsins and other helical proteins.

After cataloging these structures, I then asked how the crystals were grown. Three general crystallization approaches were identified, which differ in the environments experienced by the proteins. This environment may be micelle-like, in the case of direct crystallization of protein–detergent complexes (sometimes known as the '*in surfo*' approach; Privé, 2007); it may be membrane-like, as in crystallization from lipidic cubic phases or related mesophases (also known as the '*in meso*' approach; Caffrey, 2009); or a hybrid approach may used, in which proteins are embedded in bicelles, providing a membrane-like environment, but in the context of a micelle-like structure (Ujwal & Bowie, 2011). As seen in Table 1, since 2012 the dominant approach has been direct crystallization of protein–detergent complexes, with approximately three quarters of all new structures being produced in this way (a detailed list of the structures contributing to these statistics is shown in Supplementary Table S1[1]). Lipidic cubic phases and other *in meso* approaches account for slightly less than one fifth of new structures, and bicelles were used for the remaining 6% of structure determinations. Importantly, these overall statistics fail to convey certain nuances. For example, even though *in meso* methods are used for only about one in five of all new structures, they have achieved near-hegemony in the GPCR field, accounting for roughly six out of every seven new structures. *In meso* methods also seem to be much more important for microbial rhodopsins than for α-helical proteins in general.

What do these numbers mean? Firstly, the majority of structures currently being determined utilize direct crystallization of protein–detergent complexes, suggesting that this approach should be tried first for most new membrane-protein targets. However, GPCRs and microbial rhodopsins appear to be exceptions to this rule, with *in meso* crystallization being more effective at generating well diffracting crystals for these molecules.[2] Why is this so, when direct crystallization of protein–detergent complexes clearly works well for other types of α-helical proteins? One hint may be found in the unusually dynamic character of GPCRs, which have evolved to twitch back and forth between multiple conformations in response to various stimuli (Manglik & Kobilka, 2014). Their dynamic nature may make them extraordinarily susceptible to inactivation by detergents (see below), whereas a bilayer environment proves more stabilizing. Arguing in favor of this conjecture is the fact that most of the GPCRs that have been crystallized as protein–detergent complexes are engineered variants with enhanced stability (Tate, 2012; Scott *et al.*, 2014). However, the full story is certain to be more complex; for example, many transporters also cycle between multiple conformations and yet have nonetheless been crystallized as protein–detergent complexes. Thus, substantial work remains to be performed in probing the mechanistic differences between lipidic mesophase and protein–detergent complex crystallization. We lack the space in this brief article to explore such questions; happily, many excellent reviews have employed a variety of perspectives to shed light on lipidic mesophase crystallization (Caffrey, 2009; Cherezov, 2011; Johansson *et al.*, 2009; Nollert, 2004).

Table 1
Crystallization methods used for new structure reports from 2012 to mid-2014.

Protein class	Total No. identified	Crystallized as protein–detergent complex	Crystallized using lipidic mesophases	Crystallized using bicelles
Monotopic	12	11 (92%)	0	1 (8%)
β-Barrel	27	22 (82%)	2 (7%)	3 (11%)
α-Helical				
GPCRs	27	4 (15%)	23 (85%)	0
Microbial rhodopsins	6	1 (17%)	3 (50%)	2 (33%)
Other α-helical	159	140 (88%)	13 (8%)	6 (4%)
α-Helical (total)	192	145 (76%)	39 (20%)	8 (4%)

Before completely leaving the topic of proteolipid complex crystallization, however, I will note a recent paper reporting that a variety of different membrane proteins can be induced to assemble into symmetric proteolipid nanoparticles (Basta *et al.*, 2014). These nanoparticles are generated through a reconstitution process in which lipids are added and detergent removed. Their creation appears to involve burial of the transmembrane portions of the proteins in lipid bilayer structures, with the interactions that drive assembly being some mixture of lipid-mediated and/or direct protein–protein contacts. The highly symmetric nature of these particles makes them appropriate for electron tomography; however, they can also be used to produce three-dimensional crystals suitable for X-ray studies. The first example of such crystals was provided a decade ago, with the spinach major light-harvesting complex (Liu *et al.*, 2004). Now that Basta and coworkers have shown that this property is not unique to the light-harvesting complex, and that other proteins can exhibit similar behaviors, we may be seeing more crystal and electron-microscopy structures derived from proteolipid nanoparticles. This process should be facilitated by novel microfluidic devices that employ free-interface diffusion to rapidly remove detergent (Wu *et al.*, 2013).

3. Crystallization of protein–detergent complexes

Given that protein–detergent complexes are the most common species found in new membrane-protein crystal structures, I will focus the balance of this article on the crystallization of membrane proteins in complex with detergent structures (I will also briefly consider the use of bicelles). This topic has also been thoroughly explored in many review articles over the past several decades (see, for example, Bolla *et al.*, 2012; Garavito & Ferguson-Miller, 2001; Loll, 2003; Privé, 2007; Wiener, 2004; Moraes *et al.*, 2014; Kang *et al.*, 2013); thus, the general issues are likely to be familiar to a wide audience, and a brief description of the problem should suffice to set the stage.

Membrane proteins possess both large hydrophobic surfaces, through which they interact with the lipid bilayer, and substantial hydrophilic surfaces, which are exposed to various aqueous compartments in or around the cell. This amphipathic duality makes them insoluble in any single solvent. Thus, in order to carry out the biochemical procedures required to purify these molecules, they must be solubilized by detergents. Detergents facilitate the extraction of proteins from membranes by disrupting the bilayer structure (Lichtenberg *et al.*, 2013). After extraction, they help to maintain these proteins in a soluble form by adsorption of their hydrophobic portions onto the hydrophobic faces of the protein, thereby assembling a ring-like structure around the membrane protein (le Maire *et al.*, 2000). The detergent is a major component of this protein–

[1] Supporting information has been deposited in the IUCr electronic archive (Reference: EN5558).
[2] It is difficult to definitively prove that *in meso* crystallization is superior to *in surfo* crystallization for GPCRs, since negative results are usually not reported for crystallization experiments. However, the intense and widespread interest in GPCR structures would seem to ensure that many different groups will have tested many different tools in tackling this problem. In this case, the emergence of *in meso* methods as the pre-eminent approach may be seen as the legitimate result of a rigorous selection process.

detergent complex, and can in many cases represent over half of the mass of the complex (Ilgü *et al.*, 2014). The protein–detergent complex is a dynamic structure, like any detergent micelle (Lang *et al.*, 1975), with detergent monomers exchanging in rapid equilibrium between protein–detergent complexes and free detergent micelles. Thus, in order to provide coverage of detergent over the hydrophobic parts of the protein, a reservoir of free detergent must always be maintained in the solution. To accomplish this, detergent levels are typically kept above the critical micellar concentration (CMC).[3] Excessively high concentrations of detergent, however, can lead to protein inactivation, highlighting the importance of tools to accurately monitor detergent concentration (Prince & Jia, 2013). Maintaining detergent in all protein solutions contributes significantly to the cost of these experiments, and the necessity for controlling detergent concentration leads to many of the technical complications that attend working with membrane proteins.

Regardless of whether the membrane protein of interest is inserted into cellular membranes as the protein is expressed, or isolated from inclusion bodies and subsequently refolded *in vitro*, essentially all proteins destined for crystallographic analysis pass through the protein–detergent complex state. For some this is merely an interlude, after which they will be transferred from detergent micelles into a lipidic mesophase for crystallization experiments. For most, however, the protein–detergent complex is the species that will be packed directly into a crystal lattice. We know from radiolabeling and neutron diffraction studies that the detergent accompanies the protein into the lattice (Popot, 2010; Timmins, 2006); however, because the majority of the detergent molecules are disordered, we have relatively little direct information about the details of detergent structure within crystals of protein–detergent complexes. Over 30 years ago, arguing from first principles, Michel identified several possible packing arrangements for membrane proteins, namely 'type I' and 'type II' crystals (Michel, 1983). In type I crystals the proteins assemble *via* their hydrophobic faces into two-dimensional membrane-like arrays; these two-dimensional sheets then pack atop one another to form a three-dimensional crystal. In contrast, in type II crystals protein–detergent complexes assemble *via* crystal contacts between the polar portions of the proteins that protrude from the detergent structures. In type II crystals, the detergents do not form ordered crystal contacts, as the micelle structures are dynamic and 'squishy'. However, it would be a mistake to think that the detergent components are inert passengers that exert no influence on the process; as Michel argued in 1983,

> the size and chemistry of the detergent play an essential role in the crystal packing

(Michel, 1983). When protein–detergent complexes assemble into a crystalline lattice, their micellar portions will necessarily be brought into close apposition, and whether these structures attract or repel each other will have a profound effect on if and how the lattice is formed (Berger *et al.*, 2005; Hitscherich *et al.*, 2000).

The classification of packing within membrane-protein crystals has recently been revisited and expanded. Schulz performed a statistical analysis of all available membrane-protein structures and proposed a new classification scheme based on the detergent structure within the crystal (Schulz, 2011*a*). The approximate detergent structure, while not directly visualized in the diffraction experiment, can be inferred from the arrangement of the membrane-immersed portions of the proteins. This analysis revealed a variety of different possible packing arrangements, wherein the detergent micelles are either isolated or fused into one-dimensional or two-dimensional structures; the isolated and two-dimensionally fused structures correspond to Michel's type II and type I packings, respectively. Interestingly, different types of membrane-protein structures (monotopic *versus* β-barrel *versus* α-helical) show distinct preferences for the different possible packing arrangements. Further, crystal packing density is seen to be specific to the different micelle-packing geometries (Schulz, 2011*b*), driving home the point that interactions between detergent groups must play key roles in the crystallization process.

4. The detergent repertoire

Given the importance of the detergent component to the overall process of membrane-protein crystallization, it behooves us to ask what information about detergents can be gleaned from our survey of new membrane-protein structures in 2012–2014. One obvious result is that the palette of detergents in common use has expanded, but not hugely (Supplementary Table S1). In the early days of membrane-protein crystallization, the detergents for which successful structure determinations were reported were quite limited: a handful of alkyl glycosides, LDAO and some polyoxyethylene detergents accounted for the majority of structures. In recent years, however, more variety has been introduced. For example, the list of commonly encountered alkyl glycosides now includes detergents that were rarely seen a few decades ago, such as nonyl glucoside, undecyl maltoside, various alkyl sucrosides and the cyclohexyl maltoside CYMAL detergents (Parker & Newstead, 2012).

Apart from this modest expansion in the number of alkyl glycoside detergents, the other detergent trend seen in the survey of recent structures is the presence of a group of detergent newcomers, namely the neopentyl glycol detergents. These molecules consist of dual sugar head groups and dual alkyl tails arrayed around a neopentyl glycol core and tend to have markedly lower CMC values than comparable alkyl glycosides (Chae *et al.*, 2010, 2013). For a diverse group of different membrane proteins, neopentyl glycol maltosides proved superior to conventional detergents at maintaining the proteins in a native active form, providing a straightforward explanation for their success in crystallization experiments (Chung *et al.*, 2012; Jiang *et al.*, 2012). Neopentyl glucosides are not as stabilizing as their maltoside counterparts (although they are by no means destabilizing); however, the glucosides give rise to smaller micelles and protein–detergent complexes than the maltosides, which should prove easier to incorporate into crystal lattices (Chae *et al.*, 2013).

Substantial effort has been devoted to developing new detergents for membrane-protein biochemistry and crystallization (Zhang *et al.*, 2011), so it is interesting that so few truly novel detergents are represented in the pool of recent structures. One obvious explanation is selection bias. A related possibility is that detergent availability presents a chicken-and-egg barrier: a new detergent might not be commercially distributed until it is known to be useful, but its utility cannot be demonstrated unless the broader community has access to it. However, the gradual expansion of the detergent toolkit seen in the survey of recent structures argues that the field will benefit from the continued introduction of novel detergents; hence, paths for distributing and testing new surfactants would be welcome. It is worth noting, however, that having more detergent options increases the

[3] Note that this implicitly assumes that the CMC value for the micelle-like structure on the protein–detergent complex is the same as that for pure detergent micelles. This assumption has proven difficult to test but, as a rule of thumb, maintaining detergent concentrations above the CMC works in most cases.

complexity of the experiment,[4] illustrating the importance of high-throughput tools to test multiple detergents early in the structure-determination pipeline (Lewinson *et al.*, 2008).

5. Lipids as additives

Another trend that can be observed in the survey of recent membrane-protein structures is the increasing use of lipids as additives in crystallization cocktails (Supplementary Table S1). It has been known for a long time that endogenous lipids carried along with the protein through the purification process can profoundly affect crystallization. Some early work highlighted the unfavorable effects of such residual lipids (see, for example, the drive to remove bacterial lipopolysaccharide in order to crystallize OmpF porin; Garavito & Rosenbusch, 1986). Such examples gave rise to the notion that proteins should be delipidated prior to crystallization, so as to reduce heterogeneity (Garavito *et al.*, 1996). However, in time this was replaced by a more nuanced view, which recognizes that removal of structural lipids can also have deleterious effects on crystallization (Garavito & Ferguson-Miller, 2001). This was well illustrated in the case of the GlpT transporter, wherein it was found that the removal of some lipid was required for crystallization but the removal of too much lipid caused protein precipitation (Lemieux *et al.*, 2003). Such negative effects probably reflect a loss of stability associated with the removal of structural lipids (Popot, 2010). In many instances, these detrimental effects can be reversed by adding back lipid; an example of this can be seen with cholesterol hemisuccinate, which stabilizes a variety of GPCRs and other membrane proteins (Kulig *et al.*, 2014). Today, various lipid mixtures are being routinely used as additives in the crystallization experiment (Stroud, 2011). Addition of lipid produces mixed detergent–lipid micelles, through which the lipid molecules can readily migrate to favored binding sites on the protein surface. Further details of protein–lipid interactions can be found in a number of review articles (Hunte & Richers, 2008; Lee, 2005; Wiener, 2004; Yeagle, 2014).

6. Bicelles

Another approach to the lipid-depletion problem is to move the protein into a bilayer environment prior to crystallization. This can be accomplished through the use of lipidic mesophases (discussed above). Another path is to use bicelles, which can be viewed as an elegant compromise, balancing the technical ease of working with protein–detergent complexes with the desirability of providing a bilayer environment for the proteins (Agah & Faham, 2012; Ujwal & Bowie, 2011). Bicelles are essentially small disks composed of lipid bilayers, with a ring of detergent or short-chain lipid protecting the edge of each disk. Many proteins are readily transferred from detergent to bicelles, and the resulting solutions can be manipulated in a straightforward manner (in contrast to lipidic cubic or sponge phases, which, for example, cannot be pipeted easily). Protein–bicelle complexes thus formed can be crystallized in much the same way as protein–detergent complexes. Given this ease of manipulation, along with the advantages associated with the native-like bilayer environment, it is therefore surprising that bicelles contribute to such a small fraction of the structure determinations published in the past ∼2.75 years (Table 1). Is this because bicelles are a niche method, only useful for a subset of protein types? Certainly not: bicelles have been used to crystallize all three major membrane-protein architectures (monotopic, β-barrel and α-helical). Further, while no GPCR structures were published using bicelles in the 2012–2014 window, these molecules have been crystallized using bicelles in the past (*e.g.* Rasmussen *et al.*, 2007). In an effort to understand why bicelles appear to be under-represented in the 2012–2014 survey, the papers describing the 12 new protein-bicelle structures were examined. However, few clues were found as to why bicelles were the favored crystallization method in these cases. Three papers indicated that other methods had been tried but failed to yield appropriate crystals (Noinaj *et al.*, 2013; Chen *et al.*, 2013; Spudich *et al.*, 2012), while a fourth described the structure determination of a protein that had previously been crystallized as a protein–detergent complex (Wang *et al.*, 2012). Reading between the lines, one might speculate that bicelles are viewed as a second-tier method to be tried if other methods fail; perhaps, despite reports to the contrary, perceptions linger that bicelle crystallization experiments are more complex or challenging than those involving, say, protein–detergent complexes (certainly, the technical manipulations required for bicelle crystallization are much simpler than those involved in harvesting crystals from lipidic mesophases). In any case, it will be interesting to see whether the percentage of crystal structures determined using bicelles grows in the coming years.

7. Stability

As mentioned above, the removal of lipids can destabilize membrane proteins. However, stability effects on crystallization are not limited to protein–lipid interactions; any factor that affects the stability of a membrane protein will likely affect the crystallization behavior of that molecule. In order for a molecule to be crystallizable, it should be conformationally homogeneous, *i.e.* it should significantly populate a single low-energy state that ideally reflects a native or native-like conformation. The more stable a protein is, the easier it is to accomplish this (of course, factors other than intrinsic stability may also come into play, such as the presence of disordered regions). It can be a challenge to achieve conformational homogeneity with membrane proteins, since many of these molecules are signaling or transport machines that function by toggling between multiple conformers. The problem is further exacerbated when working in detergents, which are at best imperfect mimics of the native bilayer environment of the protein and may therefore allow the protein to access non-native low-energy states. This issue is particularly acute for short-chain detergents; while these detergents form small micelles that are favorable for crystallization, they also have a propensity to denature marginally stable membrane proteins. Thus, major efforts have been devoted to (i) engineering membrane proteins so as to increase their intrinsic stability and (ii) searching for detergents and/or ligands that enhance membrane-protein stability. To support these efforts, it has also been critical to develop assays that allow the facile assessment of stability.

Protein-engineering approaches aimed at increasing stability spring from the observation that it is relatively easy to find stability-enhancing mutations in membrane proteins (Bowie, 2001). This implies that membrane proteins are not evolutionarily optimized for stability, and provides an opportunity to derive stable variants that should prove more amenable to biophysical characterization than their wild-type counterparts. In particular, the expectation is that increased thermostability should translate directly into increased resistance to the denaturing action of 'harsh' detergents. Indeed, this

[4] A character in the British television comedy *Absolutely Fabulous* once said, 'I don't want more choices, I just want nicer things'. An equivalent sentiment for the structural biologist would be 'I don't want more detergents, I just want better crystals'.

expectation appears to have been borne out (Tate, 2012). Importantly, stability in one detergent seems to correlate with stability in many different detergents, so that results obtained with one detergent can be extrapolated to others (Kang *et al.*, 2013).

A variety of membrane proteins have been subjected to this treatment (Lluis *et al.*, 2013), but during the past few years the greatest structural impact has been observed with GPCRs. Two general approaches have been used: systematic scanning mutagenesis and directed evolution. The former allows mapping of mutational effects onto the entire sequence, allowing rigorous structure–function analyses (Tate, 2012); the latter has the advantage of covering a much larger sequence space than is possible with more systematic mutagenesis approaches (Scott *et al.*, 2013). Each method requires a simple readout of protein function that can be measured in a high-throughput manner, in order to allow efficient screening of a large number of variants. In the case of GPCRs, this need has been filled by the use of ligand-binding assays using either radiolabeled (see, for example, Abdul-Hussein *et al.*, 2013) or fluorescent ligands. In the latter case, automated fluorescence-activated cell sorting (FACS) procedures allow libraries of variant proteins to be screened in intact bacterial cells. This has been exploited to isolate highly expressing variants, for which functional expression was found to correlate with increased stability (Schlinkmann *et al.*, 2012; Schlinkmann & Plückthun, 2013). Direct selection for stability in detergent has also been made possible by encapsulation technologies that seal each bacterial cell within a porous shell; subsequent treatment with detergents and the fluorescent ligand allow isolation of mutants that retain ligand-binding capacity in that detergent (Scott & Plückthun, 2013). Such directed-evolution technologies have recently allowed the crystal structure determination of a form of the neurotensin-1 receptor that, while highly stabilized, nonetheless retains native-like signaling, trafficking and ligand-binding properties (Egloff *et al.*, 2014).

Thermostabilized GPCRs crystallize as protein–detergent complexes far more readily than their unoptimized wild-type versions; indeed, four GPCRs in the 2012–2014 structure survey were crystallized as protein–detergent complexes (Table 1) and, of these, three are thermostabilized versions (Egloff *et al.*, 2014; Huang *et al.*, 2013; Warne *et al.*, 2012). The fourth, notably, was crystallized as a GPCR–antibody complex, for which antibody binding was found to stabilize a specific conformational state of the receptor[5] (Hino *et al.*, 2012).

The linkage between protein stability and crystallizability is of course not limited to GPCRs, nor is mutagenesis the only route to increased stability. Even though stability is largely an intrinsic property of the protein, as opposed to being conferred by a given detergent (Sonoda *et al.*, 2011), the choice of detergent still provides an opportunity to modulate stability, as does the choice of ligand and the choice of the protein target itself. Because many such factors have the potential to modulate stability, there is a strong need for tools to rapidly screen the effects of these variables. Fortunately, a variety of complementary techniques have been developed that approach the question of stability from somewhat different vantages. For example, Vergis and coworkers reported a differential filtration assay that allows rapid, high-throughput assessment of protein aggregation in different detergents; in this case, aggregation serves as a marker for loss of stability (Vergis *et al.*, 2010; note for those readers who are averse to do-it-yourself projects: this assay is now available in kit form). Mancusso and coworkers describe a different technique to measure stability, in which samples of a protein are heated to different temperatures, after which the amount of soluble protein remaining is evaluated using size-exclusion chromatography; this approach allows the calculation of an apparent melting temperature (Mancusso *et al.*, 2011). In this way, the effects of different detergents, ligands *etc.* upon the apparent T_m can be evaluated. In a related approach, the Gouaux laboratory described fusions of their proteins of interest with the fluorescent protein GFP (Kawate & Gouaux, 2006); the fusion protein is subjected to thermal stress and again the remaining soluble protein is quantified using size-exclusion chromatography (Hattori *et al.*, 2012). The GFP tag increases sensitivity and obviates the need for purification of the membrane protein prior to analysis. Another convenient tool for measuring the effects of different detergents and additives upon protein stability has been developed in the Stevens laboratory (Alexandrov *et al.*, 2008) and has subsequently been widely adopted by others (Sonoda *et al.*, 2011; Tomasiak *et al.*, 2014). The tool takes the form of a microscale thermal unfolding assay utilizing the thiol-reactive dye *N*-[4-(7-diethylamino-4-methyl-3-coumarinyl)phenyl]maleimide (CPM). CPM fluorescence is very low in the unreacted state, but when the dye reacts with a thiol its fluorescence is greatly increased. If a protein contains buried cysteines, they will become accessible as the protein is heated and begins to unfold, and thus measuring CPM fluorescence as a function of temperature provides a melting curve for the protein in question.

In the case where the effects of different detergents upon stability are being assayed, at least two different approaches can be envisioned. In the first approach, the protein is purified in one detergent and subsequently exchanged for others, so that the stability measurement is conducted completely in the new detergent. In the second approach, the new detergent is added to the existing detergent, and the measurement is conducted using a mixture of the old and new detergents. The latter method is generally faster and easier, but (at least in principle) is liable to confounding effects arising from the use of detergent mixtures (*e.g.* a 'bad' detergent might mask the contribution of a 'good' detergent). The former method avoids this problem, but requires a reliable and generic protocol for detergent exchange (Eshaghi, 2009).

When considering assays that measure detergent effects upon membrane-protein stability, it is natural to ask why certain detergents tend to destabilize proteins while others do not (or do so to a lesser degree). Arguably the most important reason is the ability of detergents to disrupt both protein–protein interactions and protein–lipid interactions (Popot, 2010). The latter effect has been discussed earlier, but the additional point should be made that not only do different detergents delipidate proteins to different degrees, but they also remove different lipids (Ilgü *et al.*, 2014). Other potential sources of destabilization include the inability of a detergent micelle to contribute lateral pressures comparable to those found in membranes (Rosenbusch, 2001), hydrophobic mismatch (Columbus *et al.*, 2009; O'Malley *et al.*, 2011; Stangl *et al.*, 2012) and the possibility that some detergents might exploit transient breathing motions to insert their hydrophobic chains into the protein interior (Polidori *et al.*, 2006). Some responses to these problems have been described above, including the use of bicelles, lipidic mesophases and lipid additives. A different approach that also has the potential to address at least some of these issues is the use of nondetergent fluorinated surfactants (Popot, 2010). Because fluorocarbons are miscible with neither water nor hydrocarbons, they are less likely than hydrocarbon detergents to remove critical lipids, disrupt protein–protein interactions or insert

[5] Crystallization chaperones have proven extremely useful in membrane-protein crystallization; such chaperones include antibodies, related non-covalent binding partners such as nanobodies and darpins, and fusion partners such as T4 lysozyme. Methods employing such chaperones are beyond the scope of this article, but have been ably reviewed elsewhere (see, for example, Bertheleme *et al.*, 2013; Bukowska & Grütter, 2013; Griffin & Lawson, 2011).

into the protein interior. This concept is not new (Chabaud *et al.*, 1998), but the preparation of chemically homogeneous fluorinated surfactants is not trivial (Polidori *et al.*, 2006), so new compounds have been slow to emerge. However, recent results suggest that fluorinated or hemifluorinated surfactants can stabilize membrane proteins more effectively than conventional detergents (Cho *et al.*, 2013; Nehmé *et al.*, 2010; Talbot *et al.*, 2009), while maintaining a compact micelle structure (Abla *et al.*, 2011) compatible with the demands of crystal packing. Hence, this technology merits close attention in coming years.

We cannot abandon this topic without at least a brief mention of amphipols, since the amphipol technology was conceived to address many of the stability issues listed above (for recent reviews, see Popot *et al.*, 2011; Zoonens & Popot, 2014). The amphipol approach has enjoyed substantial success in a variety of solution-based studies of membrane proteins. In addition, proteins can be transferred from an amphipol to a lipidic mesophase and subsequently crystallized (Polovinkin *et al.*, 2014). Direct crystallization of amphipol-trapped membrane proteins has not typically been considered to be feasible, because complexes of proteins with amphipols tend to be polydisperse; however, this view may be too restrictive, based on the recent demonstration that at least one amphipol-solubilized membrane protein can be crystallized (Charvolin *et al.*, 2014). Amphipols may therefore play more substantive roles in future crystallographic analyses of membrane proteins.

In a final comment on how detergents can affect membrane-protein stability and crystallization behavior, it is worth noting that most studies concerned with stability focus on how the detergent interacts with the hydrophobic face of the protein; however, detergents can also interact with the hydrophilic portions of a membrane protein, and in several recent cases these extramembrane interactions have been shown to affect stability and/or aggregation (Yang *et al.*, 2014; Neale *et al.*, 2013).

8. Mechanistic approaches to crystallization of protein–detergent complexes

In the last section of this article, I feel obliged to remark on the relative paucity of mechanistic studies devoted to the crystallization of protein–detergent complexes. Great energy has been expended upon engineering proteins to enhance stability and upon developing clever tools to rapidly screen different proteins in different conditions. By comparison, it appears that relatively little effort has been devoted to deciphering the basic mechanistic principles underlying the crystallization process. Why might this be? To some degree this imbalance reflects biases that favor large-scale screening, even at the expense of mechanism. Clearly, if such biases have prevailed (among scientists, or within the funding agencies that support them) the results have not been disastrous; the continually increasing number of membrane-protein crystal structures is proof of this. However, it is nonetheless true that an improved understanding of mechanism will likely propel future advances. An example of a potentially enabling avenue of research involves the osmotic second virial coefficient, a dilute solution parameter that has been demonstrated to predict crystallization behavior (a recent review in this series was devoted to this topic; Wilson & Delucas, 2014). Because the second virial coefficient reflects pairwise interactions between protein molecules (or between protein–detergent complexes), it provides a window into the molecular interactions driving assembly of the crystal lattice. This parameter could therefore be exploited to rationally manipulate solution conditions in order to favor crystal growth. In the particular case of membrane proteins, several significant successes have been reported in which explicit virial coefficient measurements have driven crystal optimization (Gabrielsen *et al.*, 2010; Barret *et al.*, 2013). However, despite strong evidence of the predictive value of this parameter, the virial coefficient is not yet widely measured during crystallization efforts. This largely reflects technical difficulties associated with the measurement; until fairly recently, most virial coefficient measurements were performed *via* static light scattering or small-angle X-ray scattering and in the batch mode. These experiments require both large quantities of protein and significant expertise on the part of the user. However, this situation might be changed by recent technical advances, which attack the problem from two different directions. One approach uses microfluidics to deliver samples for SAXS (Lafleur *et al.*, 2011), thereby substantially reducing both the sample size and the speed of the measurement, while the other dispenses with diffraction altogether, instead using self-interaction chromatography to measure virial coeficients (Tessier *et al.*, 2002; Payne *et al.*, 2006). Both of these approaches have the potential to simplify and expedite the measurement of virial coefficients. Thus, there remains hope that structural biologists might one day enjoy the ability to systematically manipulate the interaction potentials between protein–detergent complexes so as to produce well ordered crystals of membrane proteins.

9. Conclusions

Just past midway through the 2014 International Year of Crystallography, a snapshot reveals that the number of membrane proteins of known structure continues to rise exponentially. Several key conclusions can be drawn about the current state of the art.

(i) The majority of new structures are still being determined by crystallizing protein–detergent complexes (*i.e.* by the *in surfo* method); however, the use of lipidic mesophases has come on strongly, particularly for GPCRs.

(ii) Crystals of membrane proteins grown using bicelles appear to be under-represented among new structures; perhaps this methodology deserves a closer look?

(iii) The old paradigm of 'less lipid = better crystals' has been overturned and augmentation of crystallization mixtures with exogenous lipids is now a standard tool.

(iv) The stability of the membrane protein to be crystallized is crucially linked to success, and may even be the most important variable that we can manipulate. Stability can be enhanced in many ways, including protein engineering, ortholog screening and systematic testing of different detergents, ligands and lipid additives.

(v) The available detergent repertoire has been significantly expanded since the early days of membrane-protein crystallization, but the number of different detergents actually appearing in new crystal structures has grown only modestly.

(vi) 'Irrational' screening methods still trump 'rational' approaches to crystallogenesis, but new enabling technologies may alter this balance.

In the ∼30 years since the first membrane-protein crystal structure, the field has matured to the point where exciting new structures appear every few weeks, rather than every few years. This trend will only accelerate, and one's imagination boggles at the prospect of the storehouse of structural knowledge that will accrue in the next 30 years. Brace yourselves.

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