

Review

Protein C-mannosylation: Facts and questions[♣]

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Among the posttranslational modifications of proteins, glycosylation is probably the most abundant one. Two main types of protein glycosylation have been known for several years, namely N-glycosylation and O-glycosylation. Their biochemical properties, structure and biosynthesis, have been described extensively. Their biological functions are also known for a number of proteins, although in many cases the function remains speculative despite continuous efforts.

A few years ago, a new type of protein glycosylation was found, which is different from the above-mentioned ones. It was called C-glycosylation, since the sugar is linked to the protein through a carbon-carbon bond. This article reviews the biochemistry of C-glycosylation, the biosynthetic pathway and structural requirements. Possible biological functions of this modification are also discussed.

Protein glycosylation is probably the most common and widespread posttranslational modification. The best-known types are N- and O-glycosylation, where the sugar moiety is covalently attached, respectively, to the nitrogen atom of asparagine or oxygen atom of a hydroxy amino acid. These types of glycosylation have been studied extensively, their diverse functions are known to some extent

and are described in detail in textbooks (e.g. Gabius & Gabius, 1997).

C-Mannosylation is a novel type of protein glycosylation, which differs fundamentally from previously described types of glycosylation. It involves covalent attachment of an α -mannopyranosyl residue to the indole C2 carbon atom of tryptophan *via* a C-C link (Fig. 1) (Hofsteenge *et al.*, 1994; de Beer *et al.*,

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Abbreviations: Dol-P-Man, dolichylphosphate mannose; ESIMS, electrospray ionization mass spectrometry; ESIMSMS, tandem electrospray ionization mass spectrometry; ER, endoplasmic reticulum; IL, interleukin; RNase, ribonuclease; C²-Man-Trp, C-mannosylated tryptophan; TSR, thrombospondin type I repeat.

1995). This type of linkage was first discovered in RNase 2 from human urine (Hofsteenge *et al.*, 1994). The primary structure of this protein, determined by Edman degradation, and the one deduced from the cDNA sequence (Beintema *et al.* 1988; Hamann *et al.*, 1990) were identical except for residue 7, which was predicted from the cDNA to be a tryptophan. This suggested a modification of this residue. Peptides from different enzymatic digests, which contained Trp-7, were found by ESIMS to have a mass which is 162 Da higher than expected for a non-modified peptide. In addition, the other properties of ESIMS spectra were typical for aromatic C-glycosides:

- ◆ loss of 120 Da, formally corresponding to the loss of four $\text{CH}_2 = \text{O}$ units. This loss is typical for C-glycosidically linked carbohydrate, which was also observed in low molecular mass flavone C-glycopyranosides. (Becchi & Fraisse, 1989; Li *et al.*, 1992). The identification of the monosaccharide was achieved by NMR and it was found to be α -manno-pyranose (Hofsteenge *et al.*, 1994),
- ◆ absence of a loss of 162 Da, characteristic for N- and O-glycosidically linked sugars multiple losses of 18 Da (H_2O) from various ions containing C^2 -Man-Trp.
- ◆ C-Mannosylation can presently be easily detected by protein sequencing, where the (C^2 -Man)-Trp can be identified, without the use of mass spectrometry (Hofsteenge *et al.*, 1996).

A RECIPE FOR C-MANNOSYLATION

The structural features of the protein that determine the specificity of the C-mannosylation reaction were examined next. This was done by analysis of recombinant chimeric RNases (consisting of residues -4 to 13 of human RNase 2 and residues 11 to 119 of porcine RNase 4, which has no N-glycosylation sites; the chimera was called RNase 2.4), iso-

lated and purified from transfected cells in culture. The exact recognition sequence was determined by site-directed mutagenesis of individual amino acids and was found to be WXXW, where the first tryptophan residue becomes C-mannosylated. Replacement of the second tryptophan by other amino acids abol-

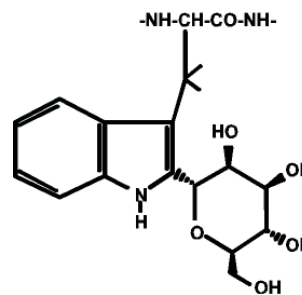


Figure 1. The structure of α - C^2 -mannosyltryptophan (C^2 Man-Trp).

ished (e.g. when the second Trp residue was exchanged for Ala) or substantially reduced (substitution with Phe) the level of C-mannosylation (Krieg *et al.*, 1998). The significance of the amino acids in both X positions is currently studied.

The determination of the recognition sequence allowed a database search for potentially C-mannosylated proteins. The search, restricted to the mammalian proteins that cross the endoplasmic reticulum (ER) membrane, yielded 336 proteins. They are present in different organs and body fluids and have many distinct functions. These data suggest that C-mannosylation is a common and widespread form of protein modification (Krieg *et al.*, 1998).

The biosynthetic pathway was also established (Doucey *et al.*, 1998). First, by expressing chimeric RNase 2.4 in NIH-3T3 cells labeled with D-[2- ^3H]mannose, mannose was identified as the earliest precursor in C-mannosylation. Subsequently, the next steps of the pathway were revealed. RNase 2 is a secreted protein, contains a signal sequence for membrane translocation and travels through the endoplasmic reticulum. The sugar precursor would probably be the same as in the other types of protein glycosylation

occurring in ER. Based on this, the hypothesis was ventured that the precursor in the biosynthesis of (C²-Man)-Trp is dolichylphosphate mannose (Dol-P-Man). This was verified by expression of RNase 2.4 in CHO Lec15 cells, in which the Dol-P-Man synthase activity is very low and which contain strongly decreased levels of Dol-P-Man. The analysis of purified protein showed strongly diminished levels of C-mannosylation. It confirmed the hypothesis that Dol-P-Man is the precursor in the biosynthetic pathway of C-mannosyltryptophan (Doucey *et al.*, 1998).

The whole biosynthetic pathway, from GDP-Man, through Dol-P-Man to the C-mannosylated peptide, was reconstructed *in vitro*. The *in vitro* assay was done by incubation of rat liver microsomes with the synthetic peptides containing the recognition sequence [the shortest peptides used consisted of only four amino acids forming a recognition sequence, WAKW (Hartmann, 2000)] and radioactively labeled Dol-P-Man. After extraction with chloroform/methanol, the peptide in the aqueous phase was found to be radioactively labeled. It proved that Dol-P-Man was indeed the precursor and that the recognition sequence WXXW is sufficient for C-mannosylation. The reaction appears to be enzymatically catalysed, since heating at 95°C or treatment with chymotrypsin abolished C-mannosylation (Doucey *et al.*, 1998)

C-MANNOSYLATION OCCURS IN DIFFERENT TYPES OF CELLS

C-Mannosylation of tryptophan residues was not only found in RNase 2 from urine (excluding that it is only present in a metabolized form of an excreted protein), but also in the enzyme isolated from erythrocytes and from HL-60 cells in culture. The modification was also performed by a variety of transfected cultured mammalian cells (HEK293, COS7, CHO, and NIH-3T3), proving that cells derived from different species of mammals,

from various organs and tissues have the machinery necessary for C-mannosylation (Krieg *et al.*, 1997).

After having determined the biochemical pathway, different organisms were examined for C-mannosyltransferase activity on RNase 2.4 derived peptides. The activity was found in *Caenorhabditis elegans*, amphibians, birds, mammals, but not in *Escherichia coli*, insects and yeast (Doucey *et al.*, 1998; Krieg *et al.*, 1997; Hatmann, unpublished results). The *in vitro* assay with membrane fractions from various mouse organs and tissues showed that C-mannosyltransferase activity can be found in most of the parts of the mammalian organism. However, the specific activity of the enzyme differs from organ to organ (Doucey, 1998). These results strongly suggested that the machinery necessary for C-mannosylation exists in many organisms.

The catabolism of (C²-Man)-Trp from C-mannosylated proteins is not known. The recent isolation of (C²-Man)-Trp as a free amino acid from human urine (Gutsche *et al.*, 1999) suggests the minimal pathway leading to the excretion of (C²-Man)-Trp just from the proteolytical degradation of C-mannosylated proteins.

Some of the proteins found in the database search have already been examined for the presence of C-mannosylation. In total, 49 C-mannosylated tryptophan residues were found in 11 proteins (Table 1). The second protein proven to be C-mannosylated was recombinant interleukin-12 (IL-12) from CHO cells (Doucey *et al.*, 1999).

An especially interesting group of proteins are those that contain complex recognition motifs (consensus sequence, TSP-1: WXXWX-XWXXC) in the so-called thrombospondin type 1 repeat (TSR) (Adams & Lawler, 1993; Adams & Tucker, 2000). Among them are: extracellular matrix proteins (thrombospondin-1 and -2), axonal guidance proteins [UNC 5 (Leung-Hagesteijn *et al.*, 1992), F-spondin (Klar *et al.*, 1992), M-spondin (Umemiya *et al.*, 1997), SCO-spondin (Gobron

et al., 1996), semaphorins F and G (Adams *et al.*, 1996)], proteins involved in angiogenesis [brain specific angiogenin inhibitors BAI-1, -2 and -3 (Nishimori *et al.*, 1997; Shiratsuchi *et al.*, 1997)], metalloproteases [ADAMTS (Kuno *et al.* 1999; Hurskainen *et al.*, 1999), e.g. aggrecanase (Arner *et al.*, 1999)], GON-1 (Blelloch & Kimble, 1999), procollagen I N-proteinase (Colige *et al.*, 1997), lacunin (Nardi *et al.*, 1999), *Plasmodium falciparum* protein TRAP (Robson *et al.*, 1988) and proteins from the complement system [properdin (Goundis & Reid, 1988) and the terminal complement components C6, C7, C8 and C9 (Hobart *et al.*, 1995)]. Part of the TSR (WSXWS, also called "the WS motif") is also present in cytokine receptors (Bazan, 1990) (receptors for: erythropoietin, growth hormone, prolactin, IL-2, -3, -4, -5, -6, -7, -9, -11, -13, GM-CSF, G-CSF, leukemia inhibitory factor, oncostatin-M, ciliary neurotrophic factor, thrombopoietin, calcitonin and leptin).

STILL NEW PUZZLING DISCOVERIES

The terminal components of complement (C6, C7, C8 α , C8 β and C9) were analyzed and found to be C-mannosylated (Hofsteenge *et al.*, 1999). All of them contain one or more TSR motifs, either WXXWXXWXXC, or with one or two tryptophan residues substituted by other amino acids. C-Mannosylation was found on all three, two or one tryptophan in the TSRs. Surprisingly, also the tryptophan residues in the complex motifs which lack tryptophan or another aromatic residue on the +3 position (the second Trp residue in the sequence XXXWXXWXXC, as in the case of C9) or even the single Trp residues, which are not part of a WXXW motif (as in C6 and C7), were C-mannosylated. This suggested that the terminal complement components contain, apart from the WXXW motif, a second signal for C-mannosylation.

This signal could be formed either by the primary structure, or by the three-dimensional

structure of a protein. In both cases, a second C-mannosyltransferase may be involved. To test the first possibility, the 12 amino acid long synthetic peptides containing sequences derived from TSRs from C6 and C9 (LLGDFG-PWSDCD and RMSPWSEWSQCD, respectively) were subjected to the *in vitro* C-mannosylation assay, routinely used for WXXW sequences. After mass spectrometry and Edman degradation, the peptide from C6 was found not to be C-mannosylated, and the one from C9 was C-mannosylated only on the first tryptophan residue. This showed that the amino acids in close proximity to the second C-mannosylated tryptophan do not provide the signal for its C-mannosylation or that another C-mannosyltransferase, which would catalyze that reaction, is not present in the protein source used for the assay (pig liver microsomes). Currently, the recombinant C9 protein as well as the TSR from C9 is being expressed in cell culture to check if there may be a signal for C-mannosylation formed after folding of the protein.

All 24 TSRs examined for the presence of the modification were C-mannosylated. The examples of C-mannosylated proteins which contain multiple TSRs are F-spondin and properdin (Table 1). Properdin is an especially interesting example of multiply C-mannosylated protein, because it has in total 20 Trp residues, 14 of which are C-mannosylated. (Hartmann & Hofsteenge, 2000)

WHAT ABOUT THE FUNCTION?

So far, the function(s) of C-mannosylation remains elusive, although several hypotheses can be put forward. The three-dimensional structure of recombinant RNase 2 from *E. coli* has been determined. The Trp-7 (which is C-mannosylated in the native protein) and Trp-10 residues are located in its N-terminal α -helix (Mosimann *et al.*, 1996). Yet, the presence of C-mannosylation seems not to have an

effect on the enzyme activity (Gläsner & Hofsteenge, unpublished results). NMR studies on RNase 2 have shown that non-covalent interactions between sugar and protein exist (Löffler *et al.*, 1996). RNase 2 contains a large insertion loop (marked in blue in Fig. 2), which is absent in other RNases of this family,

transported to the cell surface, as was found by Hilton *et al.* (1996).

In total, six polypeptides from the complement system (properdin and the terminal complement components) were shown to be C-mannosylated. The complement system, one of the innate systems of immunity in ver-

Table 1. Summary of proteins containing C-mannosylated Trp residues

Protein	TSR ^a	TSR _y ^b modified	(C ² -Man)Trp ^c	References
RNase 2	-	-	1	Hofsteenge <i>et al.</i> , 1994
Interleukin-12	-	-	1 (partially)	Doucey <i>et al.</i> , 1999
C6	3	3	6 (5 partially)	Hofsteenge <i>et al.</i> , 1999
C7	2	2	4 (3 partially)	Hofsteenge <i>et al.</i> , 1999
C8 α	2	2	4	Hofsteenge <i>et al.</i> , 1999
C8 β	2	2	4	Hofsteenge <i>et al.</i> , 1999
C9	1	1	2 (1 partially)	Hofsteenge <i>et al.</i> , 1999
Properdin	6	6	14	Hartmann & Hofsteenge, 2000
Thrombospondin	3	3	4	Hofsteenge <i>et al.</i> (unpublished)
<i>Carausius morosus</i> neuropeptide	-	-	1	Gäde <i>et al.</i> , 1992
F-spondin	5	5	8 (3 partially)	Klein & Hofsteenge (unpublished)
Total	24	24	49 (13 partially)	

^aTotal number of TSRs in the protein; ^bnumber of TSRs which contain at least one C-mannosylated Trp residue; ^cnumber of all (C²-Man)-Trp residues in the protein (partially) Trp residue is found in the modified and in the unmodified form.

except for eosinophil cationic protein, its nearest relative (Gleich *et al.*, 1986). Model building shows that the mannosyl residue can fill a cavity formed by main and side chain atoms from this loop (Fig. 2).

In cytokine receptors, for which C-mannosylation remains still to be demonstrated, the recognition sequence for C-mannosylation is present in the form WSXWS, very conserved in the family, and it occurs in the extracellular domain. It was shown for the IL-2 receptor that mutagenesis of either of the tryptophan residues abolishes the binding of the ligand (Miyazaki *et al.*, 1991). It is not clear, though, if the lack of binding is caused by a direct change of the ligand-binding site, or if less receptors are

tebrates, is a system of more than 30 plasma and membrane proteins. It is a very tightly regulated activation cascade, resulting, among others, in the formation of a hydrophilic pore in the membrane of the attacked cell, which leads to cell lysis. There are three pathways of complement activation: classical (antibody-dependent), alternative and lectin (the latter two being antibody-independent) pathways. In the lectin pathway, the main constituent is mannose-binding lectin (MBL), which recognizes terminal mannose residues in glycoproteins. It was shown to bind to the membrane of several pathogens. This leads to the activation of two MBL-associated serine proteases, MASP-1 and MASP-2, which subsequently triggers the complement cascade.

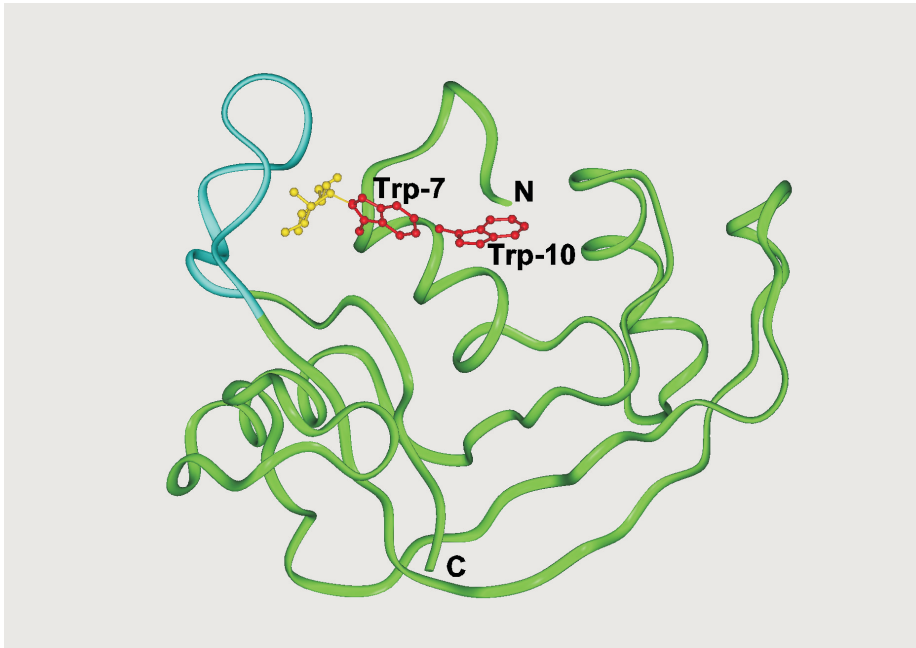


Figure 2. The three-dimensional structure of RNase 2.

Coordinates were from Mosimann *et al.* (1996). The tryptophan residues are shown in red. The mannose moiety was modelled and is shown in yellow. The large insertion loop is shown in blue.

MBL is suspected, however, to play also other roles in host defense (Prodingler *et al.*, 1999). It will be interesting to see whether MBL can bind (C²-Man)-Trp residues in properdin or terminal complement components, and whether this plays a role in the activation or control of complement.

The three-dimensional structure of the TSR or of a protein containing this module has not been determined so far. However, the hydrophilic nature of a mannosyl residue predicts that it is exposed on the outside of the molecule. The modified Trp residues are likely to occur on the surface of the protein, which is consistent with the position of Trp-7 in RNase 2 (Mosimann *et al.*, 1996).

The well-described biological function of complement, as well as the availability of convenient assays (Nilsson & Nilsson, 1984), makes it a good system to study the function of protein C-mannosylation.

The discovery of protein C-mannosylation is a significant contribution to protein glycosylation field. Post-translational modifications are usually essential for the function of a given protein. The ability to reproduce the correct glycosylation pattern is especially important for the study of protein function with the use of recombinant proteins. It is always nec-

essary to know which system allows the most faithful reproduction of glycosylation. The studies of C-mannosylation are a useful complementation of this field.

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