N-Glycosylation Processing and Glycoprotein Folding—Lessons from the Tyrosinase-Related Proteins

Norica Branza-Nichita,[†] Andrei J. Petrescu,[†] Gabriela Negroiu,[†] Raymond A. Dwek,[‡] and Stefana M. Petrescu*[,†]

Institute of Biochemistry, Romanian Academy, Splaiul Independentei 296, 77700 Bucharest 17, Romania, and Oxford Glycobiology Institute, Department of Biochemistry, University of Oxford, South Parks Road OX1 3QU, Oxford, U.K.

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1. Introduction

Protein folding occurs co- and posttranslationally and is usually assisted by chaperones. *N*-Glycosylation of proteins is a co-translational process starting during translocation of the polypeptide chain into the endoplasmic reticulum (ER) lumen. A core glycan unit synthesized in the cytoplasm is transferred *en bloc* to the translocating polypeptide chain and is rapidly trimmed giving rise to several transient glycan structures. These structures are ideal recognition elements for the lectin-based chaperone network operating in the ER. Indeed, it is now widely held that the early *N*-glycan processing events are related to the chaperone-mediated folding of the nascent polypeptide, thus contributing to the quality control mechanism in the ER. The important finding that a lectin-like interaction is involved in promoting efficient folding provides Glycobiology with a fundamental concept for understanding some of the roles of *N*-glycosylation in glycoprotein function.

The task in the ER is to guide the nascent chain through changing conditions in an oxidizing environment and so enable the polypeptide chain to acquire the unique 3-D structure of the native glycoprotein. In many cases, efficient folding is thought to depend on the interaction of the folding polypeptide with ER resident molecular chaperones. There certainly are distinct differences among the ER chaperones in terms of specificity toward the recognition elements exposed by the nascent polypeptide chain. Whereas most of the chaperones identify regions in the polypeptide moiety, two chaperones, calnexin and calreticulin, are known to modulate the folding of the nascent chain through their interaction with the attached *N*-glycans. Calnexin/calreticulin interaction with the nascent glycoprotein is mediated by the monoglucosylated *N*-glycans transiently carried by glycoproteins in the early stages of the *N*-glycan processing. Detailed structural and functional analyses of the process have been performed since the first report on the occurrence of lectin-like chaperones in 1993.[1] Most of this research has been done in vitro, either in translation systems or with purified proteins. Only recently has the work been extended to in vivo systems, and results tend to confirm some of the mechanisms postulated from in vitro studies. One of the best studied examples are the tyrosinases, which are glycoprotein enzymes synthesized in melanocytes and melanoma cells.

Glycoproteins belonging to the tyrosinase family are a good model to study the relation between calnexin interaction and glycoprotein function.[2,3] The enzymatic activity of tyrosinases can be taken as a measure of productive folding. Tyrosinase activity is dependent upon the presence of two copper ions. Misfolding can lead to its inability to bind copper, which results in the inactivation of the enzyme.

Tyrosinase itself is an attractive model because of its long in vivo processing time in the ER.[4] This facilitates investigation of transient interactions during folding. Antigen presentation by the major histocompatibility complex (MHC) is also an ER-dependent event. Tyrosinase-derived antigenic peptides are a model system to study human leukocyte antigen (HLA) loading and presentation mechanisms

* To whom correspondence should be addressed. Phone: 401 223 9069. Fax: 401 223 9068. E-mail: stefana@biochim.ro.
[†] Romanian Academy.
[‡] University of Oxford.

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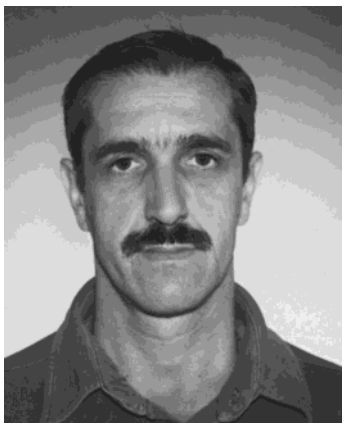
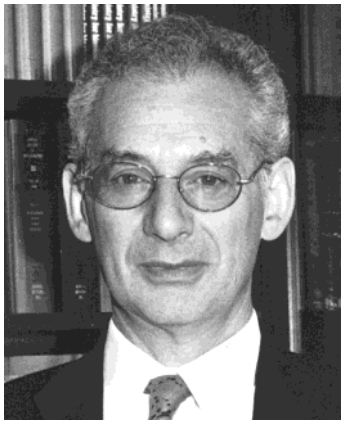
Norica Branza-Nichita, born in April 1970, is a graduate of the University of Bucharest, Faculty of Biochemistry. She obtained her Ph.D. in Biochemistry at the Institute of Biochemistry, Romanian Academy, and currently holds a postdoctoral position at the Glycobiology Institute, University of Oxford. Her current research project includes the investigation of the role of *N*-glycosylation in the biosynthesis, maturation, and transport of endogeneous and viral glycoproteins.



Dr. Andrei J. Petrescu is a graduate in Biophysics from the University of Bucharest and has postdoctoral studies in the Department of Biochemistry, University of Oxford, and at CEA Saclay. He is Head of the Structural Biochemistry Group of the Institute of Biochemistry, Romanian Academy. He has developed physical methods for the study of unfolded states of proteins. He has also determined the structure of glucosylated oligomannose glycans specific to early glycosylation states in ER and has contributed to the characterization of the glycan recognition elements of glycoproteins by lectin-like chaperones during glycoprotein folding and to the characterization of the folding process of tyrosinase-related proteins.

Gabriela Negroiu is a graduate of the University of Bucharest, Faculty of Biochemistry, and Visiting Scientist in the Department of Biochemistry, Rush Medical Center, Chicago. Her previous work was related to the biochemistry of cartilaginous extracellular matrix, and she is currently involved in studies on intracellular processing of the enzymes from the melanosomal complex.



Raymond A. Dwek, FRS, is Professor of Glycobiology and Head of the Department of Biochemistry of Oxford University. He is the founder, since 1991, and Director of the Glycobiology Institute at Oxford University. His current research is dedicated to glycobiology and its applications to medicine, with special emphasis on the role of glycosylation in immunoglobulins, e.g., implications in rheumatoid arthritis, the glycosylation of viruses, the use of glycosylation inhibitors in glycolipid storage disorders and as antivirals, particularly for hepatitis B and C. He has made major contributions in the development of techniques for carbohydrate separation, sequencing, and structure determination.

which take place in the ER. Additionally, the study of the tyrosinase family also allows the later events involved in metal binding into the active site to be probed. In this review we outline the results that have been obtained for tyrosinase and some tyrosinase-related proteins which provide insight into the role of ER chaperones in processes related to glycoprotein folding and degradation in vivo.

2. N-Glycosylation of Glycoproteins

2.1. *N*-Glycan Processing in the ER and Golgi

The nascent polypeptide chain translocated into the ER undergoes co- and posttranslational modifications including *N*-glycosylation, disulfide bond formation, and in the case of multisubunit proteins oligomerization. *N*-Linked glycans are attached co-translationally to Asn residues in Asn-X-Ser/Thr motifs (where X cannot be Pro) while the polypeptides are being translocated into the ER, as a core unit of 14 monosaccharide residues ($Glc_3Man_9GlcNAc_2$).[5] Processing of this glycan in the ER is initiated by the removal of the three glucose residues attached to the terminal mannose D1 on the α1−3 arm of the oligosaccharide by α-glucosidases I and II (Figure 1).[6] The first glucose residue is hydrolyzed within 30 s by α-glucosidase I, a type II transmembrane protein with a lumenal C-terminal catalytic domain,[7] whereas α-glucosidase II hydrolyzes the next Glcα1−3 Glc linkage within 30 min and the final Glc residue at an even slower rate.[8−10] The kinetics of these early steps are closely related with the recognition events involving chaperone-assisted folding of the nascent glycosylated polypeptide chain, which will be discussed in the later sections. Trimming of the deglucosylated glycan continues with the removal of one to four of the α1−2 linked mannose residues by the ER α-mannosidase I.[5] The removal of mannose

Dr. Stefana M. Petrescu is a graduate of the University of Bucharest and had a postdoctoral position in the Department of Biochemistry, University of Oxford. She is Director of the Institute of Biochemistry of the Romanian Academy and Head of the Molecular Glycobiology Laboratory. Her interest in sugar interaction with lectin has recently led to the investigation of glycan-mediated recognition of glycoproteins by chaperone systems and the quality control of glycoprotein folding. As principal investigator of a Wellcome Collaborative grant, she currently works on the *N*-glycosylation and folding of tyrosinase-related proteins as models for the maturation of membrane glycoproteins in the endoplasmic reticulum.

residues on the α1−6 arm may occur before the deglucosylation on the α1−3 arm takes place (Figure 2). This is suggested by the presence of $Glc_3Man_8GlcNAc_2$ and $Glc_3Man_7GlcNAc_2$ glycans in glycoproteins isolated from cells cultivated in the presence of the ER glucosidases inhibitor, *N*-butyl-deoxynojirimicin (NB-DNJ).[11]

N-Glycan processing continues in the Golgi compartment. In the cis Golgi, $Man_6GlcNAc_2$ glycans may be trimmed to $Man_5GlcNAc_2$ (Figure 1) by Golgi mannosidase I.[12] The resulting glycan can be further processed from hybrid to complex structures provided that Golgi mannosidase II removes the remaining terminal mannose residues on the α1−6 arm of the glycan (Figure 2). The product of this reaction is a substrate for *β*-*N*-acetylglucosaminyl transferase II, which adds GlcNAc to the α1−6 arm of the glycan, forming a biantennary glycan. The extent of *N*-glycan processing toward triantennary and tetraantennary structures is limited by glycosidase availability and their accessibility to the processing sites.[13]

Another Golgi enzyme is endo-α-D-mannosidase which is able to cleave the internal linkage between the glucose-substituted mannose and the remainder of the polymannose carbohydrate unit.[14,15] Although it has been reported that this enzyme normally participates in the *N*-glycan processing pathway, it can provide an alternate route for de-glucosylation during a glucosidase blockade[16] (Figure 1).

2.2 Early Stages of *N*-Glycan Processing Involved in Protein Folding

The three glucose residues of the core glycan unit ($Glc_3Man_9GlcNAc_2$) are situated at the end of the α1−3 arm of the oligosaccharide core (Figure 2). The overall structure of this arm is extended and the number of conformers reduced as revealed by the

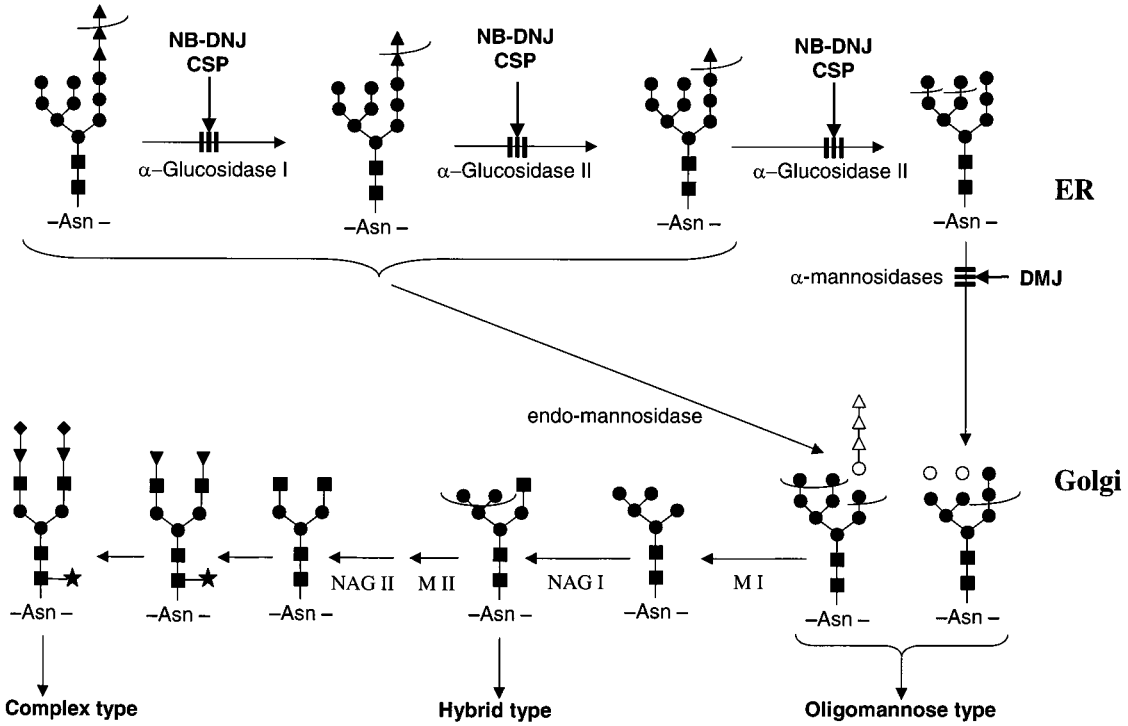


Figure 1. Schematic diagrams of the early stages of *N*-glycan biosynthesis. In the ER, the precursor $Glc_3Man_9GlcNAc_2$ is trimmed sequentially by α-glucosidase I and α-glucosidase II. Both steps are inhibited by castanospermine (CSP) and deoxynojirimicin derivatives, such as *N*-butyl-deoxynojirimicin (NB-DNJ). The Man_9 oligomannose glycoform is trimmed by ER α-mannosidases I and II and transported to the Golgi, where the glycans may be processed to hybrid and complex-type sugars. These steps are inhibited by deoxi-mannojirimicin (DMJ). The mannosidase step may be bypassed in the Golgi by an endo-mannosidase that removes the three Glc residues (△) and one Man residue (○), allowing the further processing of the sugar: (M I) Golgi α-mannosidase I; (NAG I) *β*-*N*-acetyl-glucosaminyl transferase I; (NAG II) *β*-*N*-acetyl-glucosaminyl transferase II; (M II) Golgi α-mannosidase II; (▲) glucose; (●) mannose; (■) *N*-acetyl-glucosamine; (▼) galactose; (◆) sialic acid; (★) fucose.

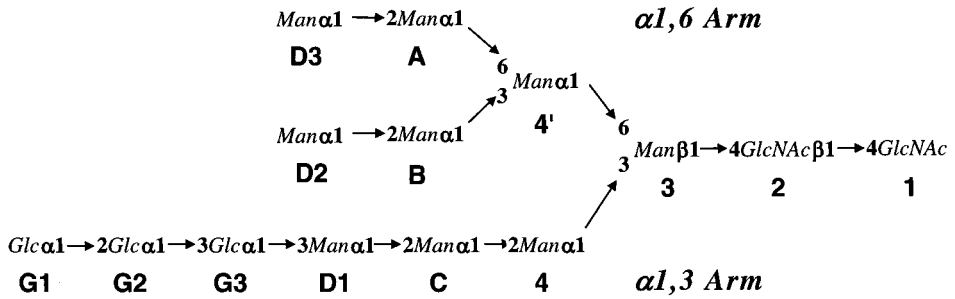


Figure 2. Schematic representation of the $Glc_3Man_9GlcNAc_2$ structure showing the primary sequence and the residue numbering scheme.

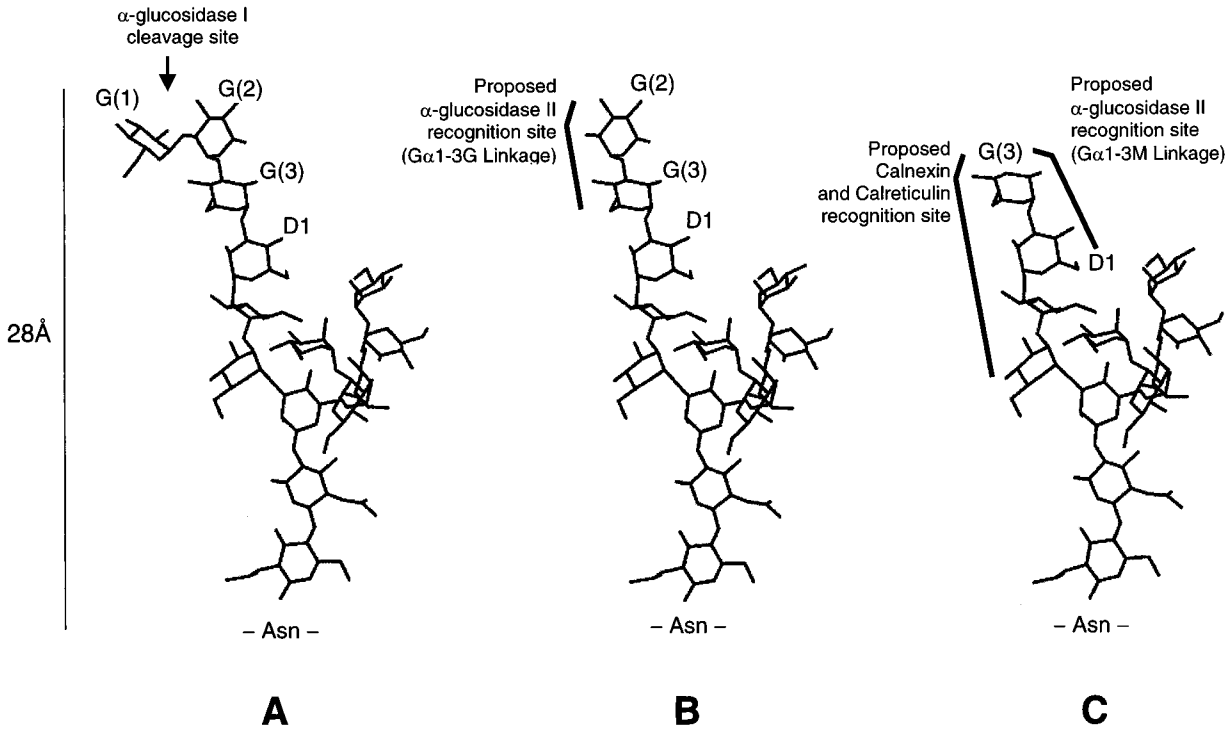


Figure 3. Molecular model of $Glc_3Man_9GlcNAc_2$ and the proposed recognition sites of α-glucosidase I for $Glc_3Man_9GlcNAc_2$ (A), α-glucosidase II for $Glc_2Man_9GlcNAc_2$ (B), and calnexin, calreticulin, and α-glucosidase II for $Glc_1Man_9GlcNAc_2$ (C).[11]

statistical analysis of glycosidic linkages performed on a large database.[17] Solution NMR studies of $Glc_3Man_7GlcNAc_2$[11] revealed that the triglucosidic cap forms a tight loop that exposes the α 1−2 linkage between the first two glucoses for a rapid trimming by α-glucosidase I (Figure 3A). The α-glucosidase II hydrolyses the next two glycosidic linkages: Glcα1−3Glc and Glcα1−3Man. Configurational analysis of the NMR model indicates that these two linkages can have a common epitope starting from the carbon C6 of the first monosaccharide to the C3 of the second one. However, for the two different linkages this epitope is presented on opposite sides of the oligosaccharide (Figure 3B). By contrast, calnexin and calreticulin recognize specifically the Glcα1−3Man linkage and not the Glcα1−3Glc one. As the only structural difference between the two disaccharides is the epimerization at C2 of the inner residue, it follows that the Man C2 atom is involved in the interaction with the lectin. This implies that glucosidase II and calnexin/calreticulin may approach their common substrate from opposite sides (Figure 3C). This model supports the notion that the nascent glycoprotein can form a transient ternary complex with calnexin/calreticulin and glucosidase II. Since the recognition elements of the oligosaccharides are situated at a distance of 28 Å, well removed from the polypeptide core, this makes an ideal "hook" for the ternary complex. The existence of the ternary complex has been confirmed by other studies.[2]

Monoglucosylated glycans that bind to calnexin/calreticulin arise not only as a result of glucosidase II digestion, but also by re-glucosylation of completely deglucosylated oligosaccharides by the UDP-Glc: glycoprotein glucosyltransferase (GT).[18] UDP-glucose is transported from the cytosol into the ER[19,20] where it serves as the glucose donor in the re-glucosylation reaction catalyzed by GT.[21] It has been shown that the transferase catalyses the glucosylation of glycans attached to incompletely folded proteins.[22,23] The existence of de- and re-glucosylation reactions indicates that a cycle is involved in the association/dissociation of glycoproteins with chaperones, lasting as long as the glycoproteins are incompletely folded. In this cycle GT acts as the folding sensor. Final release from the calnexin/glucosidase II/glucosyltransferase cycle occurs when the polypeptide has reached a fully folded conformation.[24] Transport from

the ER to Golgi then occurs, being facilitated in many cases by mannose-binding lectins. It has been suggested that ERGIC-53 may function as a receptor mediating the ER to ER-Golgi intermediate compartment (ERGIC) transport of soluble glycoprotein cargo.[25]

There is still debate as to whether calnexin/calreticulin act only as lectins or if they also recognize the polypeptide moiety.[26] It has been recently reported that calnexin and calreticulin function in vitro as true molecular chaperones, interacting with the polypeptide chain of substrate proteins.[27] Using purified components in vitro, calnexin and calreticulin prevented the aggregation of glycoproteins and of nonglycosylated proteins.[27,28] On the basis of this type of assay, it has been proposed that in addition to the lectin binding mode, calnexin/calreticulin possess a polypeptide binding capacity capable of discriminating between conformational states. In contrast, the in vitro experiments performed with monoglucosylated RNaseB as a substrate have shown that calnexin acts exclusively as a lectin, its binding being independent of the protein conformation.[29] To distinguish between the two binding modes, an in vitro system reconstituting the binding reaction has been designed. In this system calnexin binding to RNase B, unfolded or refolded with recombinant protein disulfide isomerase (PDI), and monoglucosylated with purified glucosyl transferase has been studied. The data showed that calnexin binds native as well as denatured RNase B if monoglucosylated, indicating that calnexin acts solely as a lectin in vitro.[29]

A number of studies have investigated the sugar specificity of calnexin/calreticulin. It has been found that Glcα1−3Manα1−2Manα1−2Man tetrasaccharide is a 100-fold more potent competitive inhibitor of calnexin than the Glcα1−3Man disaccharide, suggesting that calnexin recognizes the whole 1,3 arm.[30] These four residues form a continuous molecular surface on $Glc_1Man_9GlcNAc_2$[11] (Figure 3).

2.3. Glycosylation Inhibitors

α-Glucosidases inhibitors, such as castanospermine, deoxynojirimicin (DNJ), and *N*-butyl-deoxynojirimicin (NB-DNJ), have been used extensively to disrupt ER *N*-glycan processing in various systems[31−33] (Figure 1). In the presence of these α-glucosidase I inhibitors, glucose trimming is inhibited.[31] It was predicted that the inhibition of calnexin−glycoprotein interaction would lead to misfolded proteins that would be retained in the ER and degraded.[32] Paradoxically, many α-glucosidase inhibitors have minimal effects on cell viability and secretion,[33] and cell lines deficient in α-glucosidases have a relatively normal phenotype.[34] It therefore seems likely that alternative mechanisms exist within cells to enable the correct folding of many glycoproteins to occur when they are prevented from interacting with their normal chaperones. This may be why the effects of α-glucosidase inhibition on cellular glycoproteins are selective.[35] For example, the transferrin receptor requires correct oligosaccharide processing for cell surface expression, whereas other cell surface glycoproteins in the same cell line are expressed normally in the presence of the inhibitors.[33] The common observation in mammalian systems is that in the presence of inhibitors some glucosylated glycoproteins fold normally (presumably those that are calnexin-independent or utilize other chaperones during their folding), some can be secreted even if they are partially misfolded (gp 120 human immunodeficiency virus), whereas others fail to fold completely and are retained in the ER prior to degradation.[36,37]

3. Tyrosinase and Tyrosinase-Related Proteins—The Regulating Enzymes of Melanogenesis

The melanin synthesis (melanogenesis) in mammalian melanocytes is the result of a complex metabolic pathway in which at least three enzymes known as tyrosinase-related proteins (TRPs) are involved. L-Tyrosine is processed to the final product, melanin, by a series of oxido−reduction and isomerization reactions catalyzed by TRPs, which are localized in the membrane of organelles called "melanosomes"[38] (Figure 4). Two distinct types of melanins can be produced in melanocytes melanosomes, i.e., eumelanin, the black pigment, and pheo-melanin, the yellow−red pigment. Mammalian melanins are usually mixed melanins containing eu- and pheo-melanin in specific ratios that dictate the color of mammalian skin, eyes, and hair (Figure 4).

Tyrosinase and TRP-1 are two proteins of the TRP family which seem to play distinct roles in melanogenesis, despite their high structural homology. Tyrosinase is the crucial enzyme of melanogenesis, catalyzing the rate-limiting step of the pathway, the hydroxylation of tyrosine to dihydroxyphenilalanine (DOPA), and its further oxidation to DOPA-quinone.[39] Although TRP-1 is the most abundant glycoprotein expressed in melanocytes, its role in melanogenesis is controversial. The main function of TRP-1 in melanocytes was thought to be the oxidation of 5,6-dihydroindole-2-carboxilic acid (DHICA) to indole-5,6-quinone-carboxylic acid, an activity confirmed only in mouse but not in human melanocytes.[40] However, TRP-1 from both human and mouse melanocytes was shown to be able to use DOPA as a substrate. Recent studies have suggested that the TRPs function in vivo as a melanogenic complex that may enhance the rate of the pathway and minimize the leakage of potentially toxic melanogenic intermediates.[41] Within the complex, TRP-1 may play an important role in stabilizing tyrosinase, as judged by the increased susceptibility of tyrosinase to premature degradation in cell lines defective of TRP-1.[42]

These melanogenic proteins have common structural properties such as melanocyte specific expression, a transmembrane region, a lumenal domain including similar glycosylation sites, highly conserved cysteine residues, an EGF sequence responsible for protein−protein interaction, and two metal binding regions important to their catalytic functions.[43,44]

Tyrosinase is a type I membrane glycoprotein with 533 amino acids, 4 occupied *N*-glycosylation sites, 17 cysteine residues grouped in two cysteine-rich domains, and two copper binding domains, copper A and copper B[43,44] (Figure 5). Some of the structural

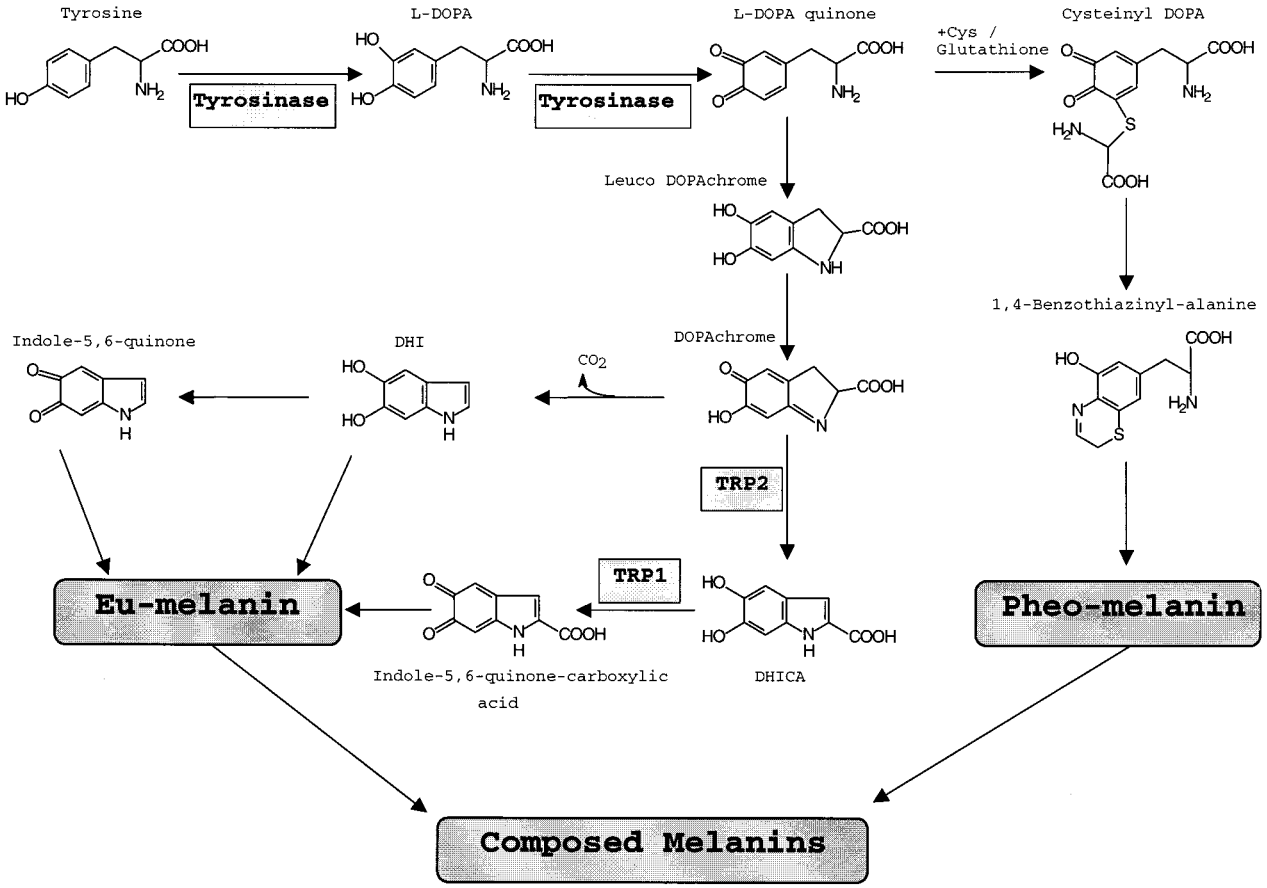


Figure 4. Biosynthetic pathway of melanin synthesis. Melanins are synthesized in melanosomes as high molecular weight biopolymers during melanogenesis. Three enzymes regulate the pathway: Tyrosinase, TRP-1 (tyrosinase-related protein 1), TRP-2 (tyrosinase-related protein 2), DHI (5,6-dihydroxyindole), and DHICA (5,6-dihydroxyindole-2-carboxylic acid).

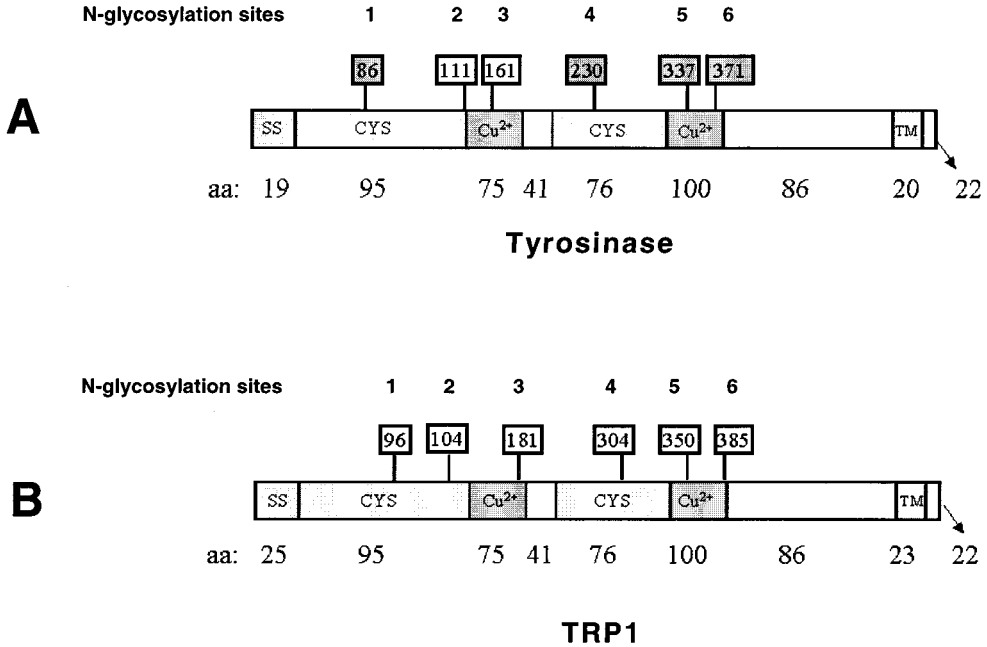


Figure 5. Schematic representation of tyrosinase (A) and TRP-1 (B) structure**,** showing the copper domains (Cu^{2+}), cysteine rich domains (CYS), transmembrane domain (TM), and the six potential *N*-glycosylation sites. Sites 1, 4, 5, and 6, shown in dark, have been reported to be occupied in mouse tyrosinase expressed in CHO cells.[3] Numbering starts from the amino terminus of the polypeptide chain including the signal sequence (**SS**).

motifs found in tyrosinase including the copper B domain appear to be highly conserved not only among tyrosinases from different species, but also among the melanogenic enzymes. For example, tyrosinase and tyrosinase-related protein 1 (TRP-1) share a significant level of homology in several regions including the catalytic domain and the potential *N*-glycosylation sites (Figure 5). The enzymatic activity of tyro-

sinase is dependent upon the binding of two copper atoms in the copper A and copper B binding sites. At each of these sites, three histidine residues coordinate the copper atom and both of the copper atoms coordinate an O_2 molecule.[43] Site-directed mutagenesis studies have revealed that three histidine residues, His 363, 367, and 389, are involved in the coordination of the copper binding in copper B binding domain.[45] Although TRP-1 shares many structural homologies with tyrosinase, including the two metal binding domains, little is known about the copper content of TRP-1. It was found that by comparison with tyrosinase, TRP-1 only bound a small amount of copper in copper incorporation experiments using mouse malignant melanocytes.[46]

Tyrosinase inborn disorders are characterized by the absence of melanin in the skin, hair, and eyes and a series of related abnormalities of the ocular system. The absence of tyrosinase activity is associated with oculocutaneous albinism (OCA) type I in many animal species, including humans.[38,47] Several mutations within the tyrosinase gene of albino individuals have been reported including a substitution in codon 371 causing a putative amino acid change from threonine (ACA) to lysine (AAA) and abolishing a *N*-glycosylation site.[48,49] Expression of the mutant tyrosinase gene results in the synthesis of an immature protein without enzyme activity that blocks the melanin synthesis.[49] Although the mechanism of tyrosinase inactivation in oculocutaneous albinism is not known at present, a role for this particular *N*-glycan site (site 6) (see Figure 5) in the tyrosinase folding in the presence of calnexin has been described recently and will be discussed below.

Like other membrane proteins, tyrosinase is synthesized in the ER where it acquires the correctly folded conformation and then transits to the Golgi and is finally targeted to the melanosomes. The loss of pigmentation in metastatic melanoma cells has been reported to be accompanied by the inhibition of tyrosinase.[50] It is believed that in amelanotic melanoma cells, tyrosinase is retained in the ER and degraded following translocation to proteasomes.[4] In these melanoma cells, the loss of tyrosinase activity is attributed to the misfolding of the nascent chain that cannot reach destinations beyond the ER.

4. Tyrosinase and TRP-1 as Probes of the Role of Calnexin/Calreticulin

4.1. Tyrosinase Folding Is Calnexin Dependent

Previous studies have shown that following the treatment of B16 mouse melanoma cells with NB-DNJ (an α-glucosidase inhibitor), although correctly transported to melanosomes, tyrosinase was inactive.[50] Analysis of mouse tyrosinase oligosaccharide sequences following treatment with NB-DNJ revealed the existence of glucosylated oligosaccharides, including $Glc_3Man_{7-9}GlcNAc_2$. Such *N*-glycan structures have also been found on gp120 expressed in the presence of NB-DNJ.[37]

Tyrosinase does not misfold in the presence of glucosylated *N*-glycans to a degree that causes it to be retained in the ER and degraded. Instead the enzyme is correctly transported to its cellular location, namely, the melanosome, but has virtually no catalytic activity. Therefore, the NB-DNJ-treated melanoma cells cannot support melanogenesis and are profoundly deficient in pigment relative to the normal B16 cells.[50] Similar effects have been observed with human tyrosinase. The enzyme synthesized in the human cell line MM96E in the presence of α-glucosidase inhibitors had no activity, and the cells were nonpigmented.[51]

Triton X-114 extraction of the NB-DNJ-treated cells demonstrated that mouse tyrosinase is correctly incorporated in the melanosomal membrane in an analogous fashion to the untreated tyrosinase.[50] This is an important observation, since melanin synthesis is only initiated after tyrosinase insertion into the melanosomal membrane.[52] The correct insertion of tyrosinase synthesized in the presence of NB-DNJ into the membrane suggests that the de-pigmentation observed in treated cells is due to the tyrosinase inactivity due to its incorrect folding rather than to incorrect transport and localization.

Partial misfolding resulting from the prevention of oligosaccharide trimming has also been observed from studies of the HIV glycoprotein gp 120 expressed in NB-DNJ-treated Chinese hamster ovary cells. These studies showed that although regions of the gp 120 are misfolded, the protein is still correctly transported to the cell surface.[53] The structure of gp120 was probed using a panel of over 40 monoclonal antibodies. It was found that most of the regions of gp120 expressed in the presence of NB-DNJ (gp120+) were indistinguishable from gp120 expressed in the absence of the compound (gp120). However, when the conformations of the V1/V2 loops were investigated, it was found that this region was affected by the retention of glucosylated *N*-glycans. This alteration in antibody recognition was attributed to a change in the conformation of this region of the molecule. It is of interest that gp120+ transits through the ER and Golgi and is secreted from the cell at comparable rates relative to gp120.[53]

To determine whether the lack of complex *N*-glycans was sufficient to render tyrosinase inactive, melanoma cells were treated with deoxynojirimicin (DMJ), an inhibitor of ER and Golgi α-1,2-mannosidases and later glycan processing[50] (Figure 1). There was no effect on tyrosinase activity, suggesting that the absence of complex type *N*-glycan structures is not responsible for the maintenance of tyrosinase in an active form. Furthermore, these data suggest that it is the retention of glucosylated high-mannose structures (due to NB-DNJ treatment) that results in the loss of tyrosinase activity. To investigate whether the glucosylated glycan directly affects tyrosinase activity, the three glucose residues were removed by digestion with α-glucosidases I and II in an in vitro experiment. However, the digestion did not restore the enzymatic activity, showing that the glucosylated *N*-glycans do not interfere sterically with the catalytic site.[50] Thus, the loss of activity may result from partial misfolding of the protein and in particular of the catalytic site, suggesting that folding of this domain is chaperone-dependent.

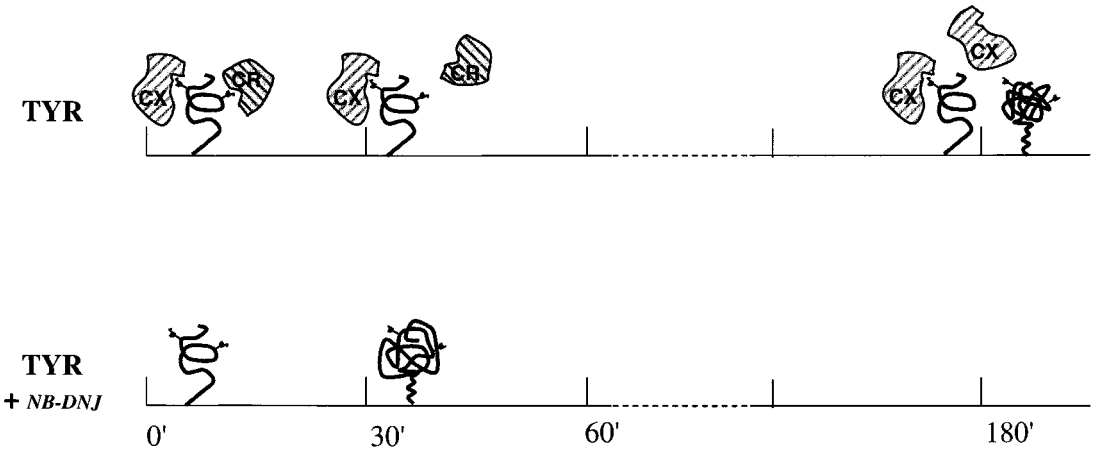


Figure 6. Kinetics of folding and chaperones interaction of tyrosinase in the presence and absence of the glucosidases inhibitor, NB-DNJ: TYR = tyrosinase, CX = calnexin, CR = calreticulin.

Recently, it was shown that human tyrosinase activity expressed in COS cells could be enhanced by co-transfection with the cDNA coding for calnexin. When α-glucosidase activity was inhibited in the same cells by castanospermine treatment, tyrosinase activity was completely abolished.[54] These observations indicate that calnexin/calreticulin may have a role in the correct folding of tyrosinase. This hypothesis has been independently confirmed by the detailed analysis of tyrosinase folding and interaction with calnexin in B16 cells both in the absence or the presence of NB-DNJ.[2] In the absence of *N*-glycan processing inhibitors, tyrosinase takes ~3 h to fold correctly (Figure 6).

The process involves the association of at least two folding intermediates with calnexin in the ER. Calreticulin has also been shown to interact with tyrosinase but only in the first 30 min after synthesis.[3] Analysis of the DOPA-oxidase activity, discriminating native from non-native tyrosinase forms, showed that the calnexin-bound fraction was not active.[2] Therefore, the two observed folding intermediates are both inactive, indicating that activation of tyrosinase requires copper loading which occurs after the calnexin-binding step.

Treating B16 melanoma cells with NB-DNJ prevented tyrosinase from binding to calnexin. As a consequence, tyrosinase folding was accelerated and the resulting protein was more rapidly transported to melanosomes in an inactive copper-free form[2] (Figure 7). Thus, the resulting protein should have a different conformation than that of the wild-type tyrosinase. The difference between the conformation of tyrosinase synthesized in the presence or absence of NB-DNJ must be relatively small as the protein is not retained in the ER and is not subjected to degradation but is correctly trafficked to the melanosomes. Most likely, rapid folding in the absence of calnexin interaction results in a conformation which is unable to accept copper.

Once the tyrosinase−calnexin complex has been formed, the inhibition of α-glucosidase II by NB-DNJ prevented spontaneous release of tyrosinase from the complex. As a result, tyrosinase folding was inhibited and the tyrosinase was degraded. This suggests that α-glucosidase II activity is required to dissociate the protein from calnexin in order to allow folding to occur. This also suggests that a ternary complex between calnexin, glucosidase II, and tyrosinase may occur transiently in living cells. Tyrosinase folding must therefore occur off calnexin, after its release from the complex, as a consequence of α-glucosidase II activity and before rebinding to calnexin, following re-glucosylation by GT. These data also support a mechanism based on lectin-only interactions between tyrosinase and the calnexin/calreticulin cycle in living cells (Figure 7).

Thus, the role of calnexin in the tyrosinase system is to promote efficient folding by slowing it down, retaining the protein in the chaperone/quality control cycle, for as long as it is necessary to acquire the biologically active conformation.

4.2. Kinetics of TRP-1 Folding Is Unchanged in the Presence of Calnexin

Tyrosinase and TRP-1 have been reported to display different maturation times in several cell lines; there are 2−3 h for TRP-1 and 3−6 h for tyrosinase.[4,55] By comparing the kinetics of *N*-glycan processing of the two glycoproteins in the same cell line, it has been found that indeed TRP-1 is processed much faster than tyrosinase. TRP-1 is processed in less than 1 h to its fully glycosylated form, while tyrosinase requires more than 3 h for complete maturation. Within the ER and early Golgi, TRP-1 is detected for ~30 min whereas tyrosinase is present in the same compartments for at least 3 h.[56] The data indicate that differences in the overall processing time of the two glycoproteins are due primarily to their ER residency and hence to their folding process rather than to their transport through the secretory pathway.

As already mentioned, in the case of tyrosinase the kinetics of the folding pathway are changed in the presence of the inhibitor NB-DNJ, which causes it to fold "faster" to an inactive state. Surprisingly, TRP-1 synthesized in B16 melanoma cells treated with NB-DNJ folds with a similar kinetics as in normal conditions[57] (Figure 8). In the presence of this drug, TRP-1 polypeptide is able to fold to an oxidized form stabilized by disulfide bridges. However, the

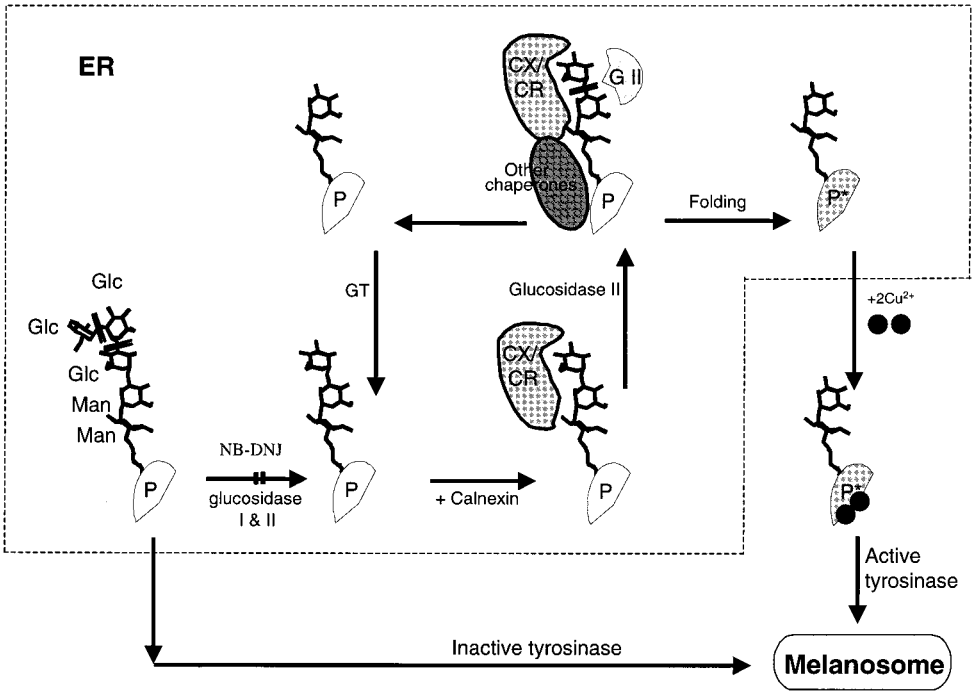


Figure 7. Schematic diagram of the folding and activation pathways of tyrosinase in B16 cells. $Glc_3Man_9GlcNAc_2$ glycans are added to tyrosinase polypeptide co-translationally (represented by a single Glc_3Man_2 arm). Normally, trimming by glucosidases I and II gives $Glc_1Man_9GlcNAc_2$ glycans allowing interaction with calnexin/calreticulin (Cx), which is part of a chaperone network. Further trimming by glucosidases II (G II) enables release of tyrosinase from calnexin and allows folding to occur. The folding state of tyrosinase is probed by UDP: glucose:glycoprotein glucosyl-transferase (GT). Misfolded proteins (P) are reglucosylated and recruited back into the calnexin cycle. Folded proteins (P*) continue their maturation process, which includes acquisition of two Cu^{2+}(•••) and are transported to melanosomes. Pretreatment with NB-DNJ inhibits the first step and results in rapid transport of a misfolded protein (unable to acquire copper) to the melanosomes.

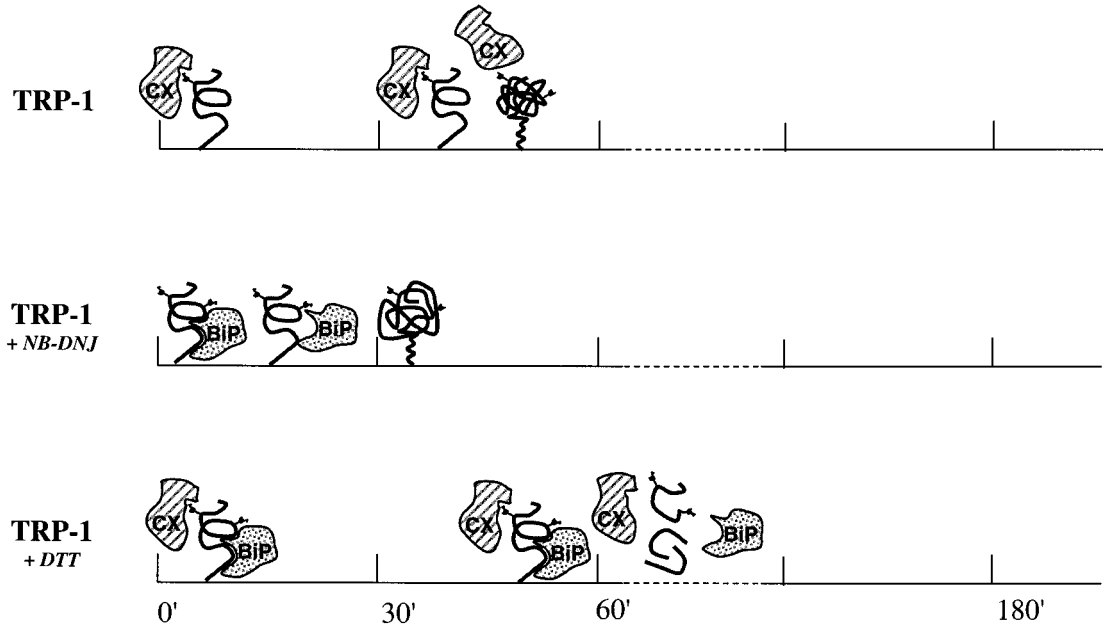


Figure 8. Kinetics of folding and chaperones interaction of TRP-1 in the presence (TRP-1 + NB-DNJ) and absence of the glucosidases inhibitor, NB-DNJ (TRP-1) and in reducing conditions, (TRP-1 +DTT): CX = -calnexin, CR = calreticulin.

conformation of this chain is different from the conformation of normal TRP-1.[57]

In contrast to TRP-1 synthesized in normal conditions, NB-DNJ-treated TRP-1 does not interact with calnexin but with another chaperone, BiP. BiP is a molecular chaperone from the heat-shock family of proteins located in the lumen of the endoplasmic reticulum that binds newly synthesized proteins as they are translocated into the ER and maintains them in a state competent for subsequent folding.[58] BiP is also an essential component of the translocation machinery, as well as playing a role in retrograde transport across the ER membrane of aberrant proteins destined for degradation by the proteasome.[59] It has been recently shown that in NB-DNJ-treated cells, TRP-1 binds avidly to BiP while in normally processed TRP-1 this interaction is hardly detected. It is known that BiP binds to the hydro-

phobic patches which are transiently exposed mainly during the early steps of polypeptide folding,[58] and this was confirmed by the prolonged interaction of BiP with TRP-1 in DTT-treated cells (Figure 8). The poor interaction of TRP-1 with BiP in the untreated cells indicates that TRP-1 hydrophobic patches are rapidly folded into a conformation with very little accessibility to BiP. In contrast, interaction with BiP is dramatically increased in the absence of calnexin or in the presence of denaturing agents. These data indicate that in the TRP-1-folding process, BiP is the "spare" chaperone to be used in the absence of the lectin chaperones calnexin/calreticulin and that under severe stress BiP and calnexin act simultaneously to help the polypeptide chain folding.

It has been found that TRP-1 associates with calnexin for the first 30 min of synthesis until the chain acquires its native conformation.[57] In conditions that maintain the polypeptide in a reduced but glucosylated state, such as in DTT treatment, TRP-1 shows a prolonged interaction with calnexin before being targeted to degradation.[57]

Interestingly, TRP-1 does not seem to have an absolute requirement for calnexin, as its enzymatic activity is not greatly diminished in the presence of glycosylation inhibitors.[56] Moreover, TRP-1 folding is not accelerated in the presence of NB-DNJ, as it has been reported for tyrosinase. However, in the absence of calnexin, the TRP-1 chain adopts a floppy conformation that may influence the overall stability of the glycoprotein.

5. Tyrosinase and TRP-1 Glycosylation Is Protein Specific

5.1. *N*-Glycan Composition

The importance of *N*-glycosylation for proper functioning of tyrosinase has been clearly established by the studies using *N*-glycosylation processing inhibitors and tyrosinase, as discussed above.

A detailed characterization of the tyrosinase *N*-glycan composition and the identification of the site occupancy have been reported recently.[3,56] The expression in CHO cells of mouse tyrosinase mutants lacking single *N*-glycosylation sites showed that sites 1, 4, 5, and 6 at Asn residues 86, 230, 337, and 371 are fully occupied while sites 2 and 3 at Asn residues 111 and 161 are unoccupied[3] (Figure 5). Although the exact location has not been identified, four asparagine-linked oligosaccharides chains per molecule have also been found in hamster tyrosinase.[60]

By sequencing the mouse tyrosinase *N*-glycans and comparing them with the data on hamster tyrosinase *N*-glycans, it was found that both tyrosinases possess similar oligomannosidic series and sialylated complex antennary structures.[56,60] However, the ratio of high-mannose versus complex structures is 1:3 in hamster tyrosinase as compared to 1:1 in mouse tyrosinase. These data, together with the observation that mouse and human tyrosinase share 85% sequence identity and identical potential *N*-glycosylation sites, indicate an interesting and possibly functionally relevant conservation of *N*-glycosylation between tyrosinases from different species.

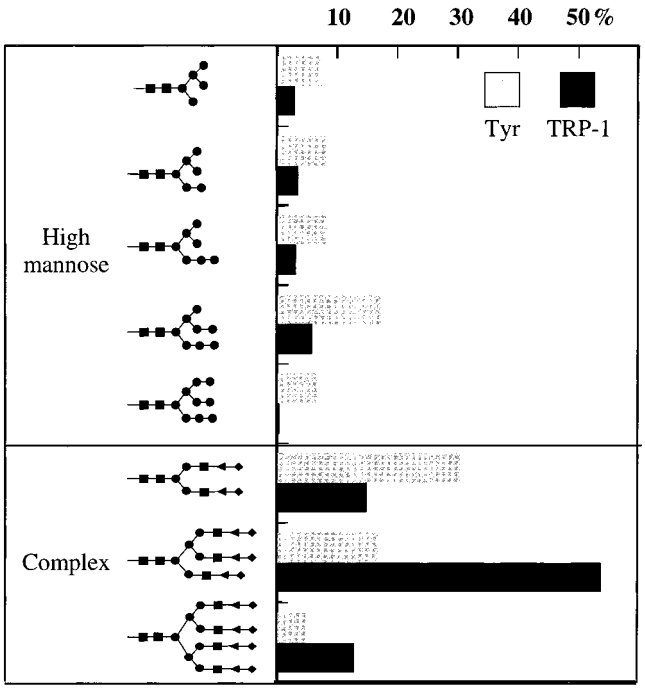


Figure 9. Structure of tyrosinase and TRP-1 *N*-glycans. The percentage molar ratio of total sugar chains released from each enzyme is indicated: (▲) glucose; (●) mannose; (■) *N*-acetyl-glucosamine; (▼) galactose; (◆) sialic acid.

We should note that the members of the TRP family are all glycoproteins containing *N*-linked oligosaccharides. Their polypeptide chains have similar numbers of potential *N*-glycosylation sites, and three of them are in well-conserved positions.[61] For instance, studies on TRP-1 and TRP-2 showed that carbohydrate moiety of TRP-2 seems to be similar to the one of hamster tyrosinase[61] while TRP-1 is differently glycosylated. Both human and murine TRP-1 contain mixtures of high-mannose and complex structures. In contrast to tyrosinase, the carbohydrate analysis of TRP-1 from murine melanoma showed that only 16% of the glycans are of the high-mannose type, 16% are biantennary, while 65% are processed to tri- and tetraantennary structures (Figure 9).[56] This is similar to that reported for TRP-1 from human melanocytes and melanoma cells.[62]

Despite the extended structural homology at the polypeptide level showed by tyrosinase and TRP-1, the structural analysis revealed significant differences in their *N*-glycan pattern (Figure 9). The extent of *N*-glycan processing is limited by glycosidases availability in the Golgi and their accessibility to the processing sites. Clearly the availability is the same for the two glycoproteins, hence the difference in glycosylation indicates different accessibility caused most probably by differences in the 3-D structures of tyrosinase and TRP-1.

5.2. TRP-1 Is a Substrate for Endomannosidase

Tyrosinase and TRP-1 have been recently shown to behave differently in the presence of ER glucosidases inhibitors.[56] Tyrosinase from B16 cells treated with NB-DNJ contains oligosaccharides with $Glc_3Man_{7-9}GlcNAc_2$ structure, which indicates that no further processing of *N*-glycans occurs in the presence

of this inhibitor. By contrast, TRP-1 from the same cell line is able to overcome the inhibitory effect of the ER-glucosidases inhibitors and acquires oligosaccharides of complex type.[56] Similar results were observed with TRP-1 from SK Mel-19, a human melanoma cell line treated with castanospermine or 1-deoxynojirimicin, two other inhibitors of the ER-glucosidases I and II.[62]

The glucosidase blockade can be bypassed by the use of the endo-mannosidase pathway, which acts in the Golgi and trims the three glucose and terminal mannose residues from the oligosaccharide moiety.[63,64] It has been reported that the endo-mannosidase route is cell specific.[14] B16 cells appear to have an active endo-mannosidase, as shown by the processing of TRP-1 in the presence of NB-DNJ. Therefore, these results suggest that tyrosinase is not a substrate for endo-mannosidase whereas TRP-1 is. This raises the interesting and intriguing question as to whether the Golgi endo-mannosidase only acts on those proteins which are already correctly folded. It may well be that the structure of misfolded proteins, such as tyrosinase in the presence of NB-DNJ, is such that the peptide structure around the glycan moiety prohibits the access to the endo-mannosidase.

6. Individual Glycans in Tyrosinase Folding

Although it has been clearly established that *N*-glycosylation is essential for the correct folding of tyrosinase, a role for each individual *N*-linked glycan in this complex process has only recently been studied. This was done by constructing 15 tyrosinase mutants lacking one or more *N*-glycosylation sites using in situ mutagenesis and their transient expression in CHO cells.[3]

The proteins were analyzed with respect to their folding rate, ability to bind calnexin, enzymatic activity, and copper content in order to characterize the folding efficiencies. The number of the *N*-glycans and their location dramatically affect both the calnexin-assisted folding and the final enzymatic activity of the mutants. For instance, when three or all four occupied *N*-glycosylation sites are deleted, tyrosinase cannot be co-immunoprecipitated with calnexin, has no enzymatic activity, and shows an accelerated folding.[3] Similar results have been shown for the B16 tyrosinase biosynthesized in the presence of NB-DNJ.[50]

The presence of any two occupied glycosylation sites is therefore sufficient to produce a percentage of active tyrosinase from the amount of the total synthesized protein. The amount of active tyrosinase depends on the position of the substituted sites on the polypeptide chain. For instance, the mutant Δ(4, 5) (sites 4 and 5 are deleted) has wild-type activity whereas Δ(1, 6) (sites 1 and 6 are deleted) is only 30% active. The presence of a specific pair of *N*-glycans located at sites 1 and 6 is crucial for correct folding of all the remaining mutants and sufficient to reproduce the wild-type activity, regardless of the occupancy of the other *N*-glycosylation sites. This implies that the calnexin pathway does not work on glycoproteins with two *N*-glycosylation sites. Similar

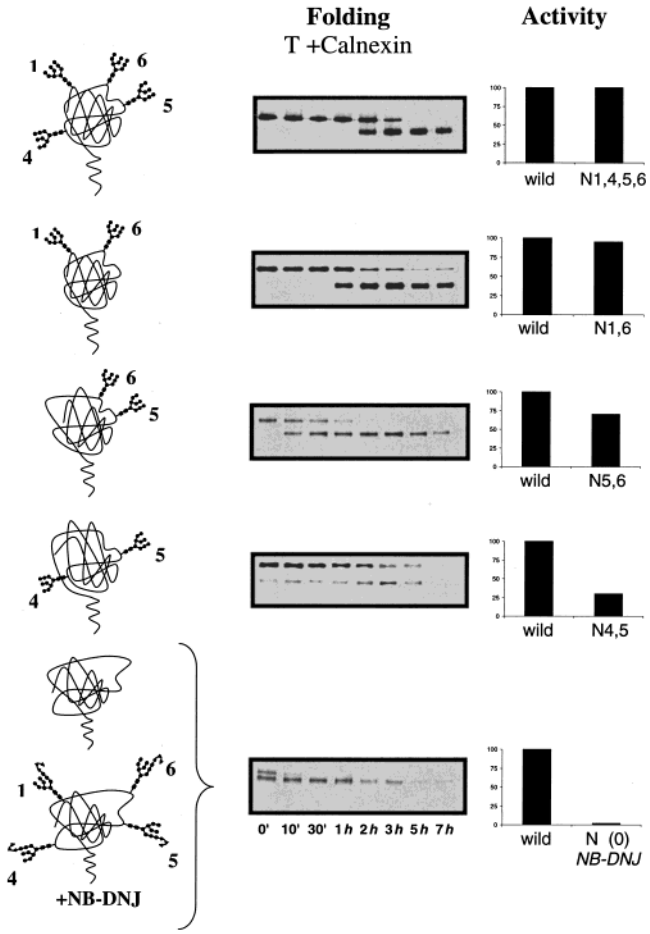


Figure 10. Folding and activity of tyrosinase mutants (N1−6) lacking two or more normally occupied *N*-glycosylation sites. N1,4,5,6 = wild type, N1,6, N4,5, N5,6 = tyrosinase mutants with two occupied *N*-glycosylation sites, N (0) = tyrosinase mutant with all four sites substituted, T = tyrosinase, WT = wild-type tyrosinase (N1,4,5,6). Folding and calnexin interaction with the mutants have been shown by co-immunoprecipitation of calnexin and tyrosinase from pulse-labeled mouse melanoma cells followed by analysis in nonreducing SDS−PAGE (central column).

results were obtained when folding of an artificial double-mutant ribonuclease was analyzed in vitro. There two glycans were needed in order to obtain stable binding of RNase to calnexin.[65]

Removal of one or both of the two glycan sites (1 and 6) results in accelerated folding, shorter interaction time with calnexin, and lower activity of the corresponding mutants, stressing the importance of site-specific glycosylation of tyrosinase (Figure 10).

On the basis of these experiments, a local folding mechanism for tyrosinase polypeptide chain that is regulated by the interaction with the calnexin/calreticulin cycle has been proposed. In this model, individual *N*-glycans play distinctive roles depending on their location on the polypeptide chain. Regions of the protein local to some of the glycan sites may fold rapidly and correctly with limited chaperone assistance. The presence of glycans in regions of the protein structure that spontaneously fold less efficiently may be necessary for the correct function of the GT quality control system and hence may explain the conservation of glycosylation sites in tyrosinase.

It is interesting to note that the removal of site six has the most significant effect on the activity of the expressed enzyme, and this is the site at which mutations have been implicated in OCA.

Copper analysis of tyrosinase mutants with partial activity suggested the presence of populations of both active and inactive tyrosinase in differing amounts. Thus, different *N*-glycosylation mutants show different yields of correctly folded enzyme. The population able to reach the native conformation increases with the time spent by the glycoprotein in the calnexin cycle.[3]

7. Tyrosinase Folding and Copper Loading

Inhibition of *N*-glycan processing and mutations at critical *N*-glycosylation sites have similar consequences on copper incorporation in tyrosinase. In both cases tyrosinase molecules devoided of metal have been reported.[2,3] Apparently there is an absolute requirement for calnexin interaction with tyrosinase during its early stages of folding that guarantees that a conformation is able to bind copper.[2] It has been reported that tyrosinase bound to calnexin is enzymatically inactive and leaves the ER immediately after its dissociation from calnexin.[2] Thus, activation of tyrosinase probably occurs post ER compartment. As tyrosinase activity is dependent upon the presence of copper in its active site, the activation step should include copper loading to the polypeptide chain. Taken together, these observations suggest that the ER folding pathway leads tyrosinase to a nativelike conformational state able to acquire two copper atoms in its active site further along the secretory pathway. Further confirmation to this hypothesis comes from electron microscopy (EM) experiments with intact B16 cells.[66] Cells have been incubated with DOPA in PBS, and the DOPA-reactive organelles have been examined by EM. Tyrosinase activity could be visualized only in the tubular structures specific to the Trans Golgi network (TGN) and in melanosomes. Despite the prolonged DOPA reaction time of EM samples, no trace of activity could be detected in the ER. These data suggest that tyrosinase remains in the ER until it reaches a conformation able to take up the Cu^{2+} ions into its active site. However, the form that leaves the ER is not catalytically active. It then transits the Golgi apparatus where further modifications of the oligosaccharide moiety take place; once in the TGN, the enzyme becomes active by incorporating the metal and it is then further targeted to its destination compartment, the melanosome.

Intracellular copper delivery is under intense investigation. Although some copper 'chaperones' have been identified, there is still little knowledge on how copper transport and delivery take place. There are very few examples of copper chaperones that deliver copper into cytochrome -*c* oxidase, superoxide dismutase, multioxidase Fet3, and ceruloplasmin.[67−69] These copper transporters are localized in the cytosol, in the mitochondria, or in the TGN. In the TGN, only two Cu transporters have been reported so far: the Menkes protein and the Wilson protein.[70] These belong to the P type ATPase family and are cell specific.[71] Another copper enzyme, ceruloplasmin, is believed to take up copper in the TGN of hepatocytes in the presence of the Wilson protein.[71] As the Menkes protein has been identified in the B16 cells, while the Wilson protein was totally absent, it is likely that Cu is delivered to tyrosinase by this transporter, which is a glycoprotein.

Patients with Menkes disease show, besides characteristic neurological features, a peculiar appearance of the hair (pili torti) associated with hypopigmentation, implying a deficiency in the activity of tyrosinase. Interestingly, the Menkes protein has been localized in the TGN, the first compartment of the secretory pathway in melanoma cells where tyrosinase was shown to be active.[4,66] Thus, these findings may further support a model in which copper transfer to tyrosinase and its activation coincide and take place in the TGN, supporting a model for tyrosinase folding occurring in two compartments.

8. Malignant Melanomas and Tyrosinase

The lifetime risk for developing a malignant melanoma in an American in the United States was 1 in 87 in 1996.[72] It is now accepted that T-lymphocytes against melanoma antigens mediate tumor-specific protective immunity.[73] They do this by recognizing peptides which are processed intracellularly from misfolded proteins and are then presented on the cell surface by HLA molecules. In principle, T-lymphocytes are able to monitor and recognize any change or structural alteration occurring in somatic cells. They are particularly important in defense against viral infections. T-cell-defined antigens on human melanomas are divided into three principal categories. These are individually distinct mutated antigens, cancer antigens, and melanocyte differentiation antigens. Cancer and differentiation antigens are not altered or mutated in tumor tissues. For a variety of reasons, tumor cells are not equipped to induce primary T-cell responses since among other factors they also release immunosuppressive molecules. However, tumor cells can be recognized by preactivated cytotoxic T-lymphocytes which have been induced using self-peptides (differentiation antigens) either in an appropriate adjuvant or on an immunomodulatory carrier. This strategy essentially seeks to brake tolerance and is the basis for developing active therapeutic immunization strategies. Since tyrosinase is a key enzyme in melanin synthesis, its normal differentiation antigens could potentially be used in melanoma therapy.

As previously discussed, the loss of tyrosinase activity in some melanoma cells is attributed to the misfolding of the nascent chain that cannot reach destinations beyond the ER. As part of the immune defense, many melanoma epitopes are presented to cytotoxic T-lymphocytes (CTLs) by MHC class I molecules.[74] In general, MHC class-I-associated peptides are derived from intracellular proteins.[75] Interestingly, a tyrosinase peptide, YM**N**GTMSQV (N) corresponding to amino acids 369−377 and including the *N*-linked glycosylation site 6, has been shown to be presented as the converted peptide, YMDGTMSQV (D).[76] This peptide binds to the trans-

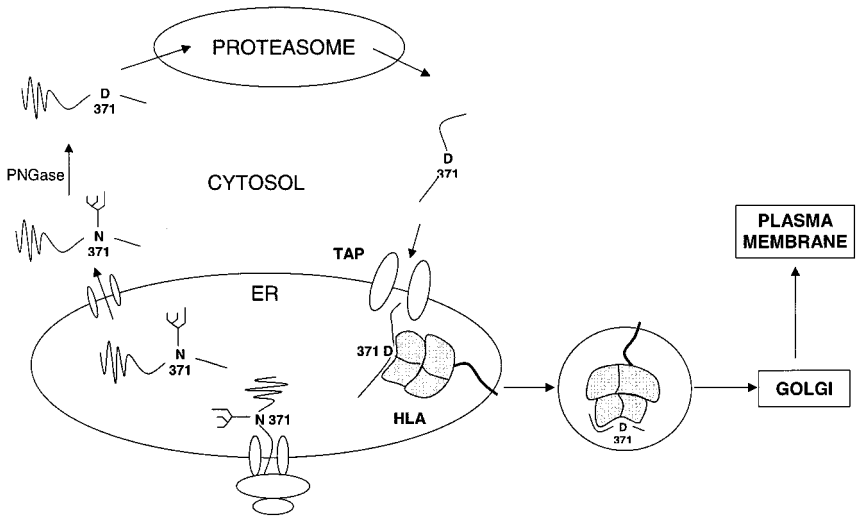


Figure 11. Hypothetical model for the processing and presentation of the tyrosinase antigenic peptide YMDGTMSQV (D) by class I molecules. Misfolded tyrosinase containing the YMNGTMSQV (N) peptide is exported from the ER to the cytosol where it is degraded in proteasomes following deglycosylation in the presence of PNGase, with the simultaneous conversion of Asn to Asp. The resulted peptide is transiently bound by TAP and retranslocated in the ER where it is bound by HLA class I molecule. Upon peptide binding, the loaded class I molecule is released from the loading complex and transported through the Golgi to the cell surface.

porter associated with antigenic processing (TAP) which transports it into the ER. The converted peptide D probably arises as a result of the deglycosylation in the cytosol by the enzyme peptide: *N*-glycanase. The reasons for this are the following. The particular genetic codon of tyrosinase, also in tumors, is transcribed and translated into an asparagine (and not aspartate). The *N*-linked sequon is subsequently glycosylated. Removal of the *N*-linked oligosaccharide by the *N*-glycanase converts the asparagine (N) to an aspartate (D).[77] The D peptide has also been found to be presented by HLA-A0201 on cells expressing full-length tyrosinase. Following binding of the peptide to MHC, the complex is transported through the Golgi where the MHC is further glycosylated and then secreted to the cell surface.

In general, deglycosylation of glycoproteins in the cytosol prior to degradation by the proteasome into peptides may provide a mechanism for limiting the number of peptides that can be presented. Therefore, the deglycosylation enzymes may play a crucial role in limiting the number of T-cell receptors required in the immune system. Studies such as these will allow some insights into the extent of the T-cell receptors repertoire.

Recent research performed using a vaccinia-encoded minigene and a cytosolic-expressed tyrosinase has shown that the degradation of full-length tyrosinase occurs after translation in the ER. Moreover, presentation of the D peptide was TAP and proteasome dependent. Therefore, it has been proposed that processing of grossly misfolded tyrosinase involves translation in the ER, export of full-length tyrosinase to the cytosol, possible deglycosylation, and degradation and then retranslocation of converted peptides by TAP for association with HLA[78] (Figure 11). Interestingly, the NB-DNJ-treated tyrosinase, which has been shown to be only slightly misfolded, is not exported to the cytosol but to the Golgi compartment[50] and therefore not degraded. Even stronger support for this view comes from the investigation of tyrosinase synthesis in amelanotic melanoma cells. Using proteasome inhibitors, it has been shown that in these cells there is an active proteolysis of tyrosinase occurring in proteasomes.[4] Although little is known about the factors regulating the ER-associated degradation (ERAD), it is likely that the chaperones that bind tyrosinase during its folding act in conjunction with some of the ERAD components in specific cellular conditions.[24]

9. Concluding Remarks

In view of the data that we have discussed, the fundamental importance of the *N*-glycosylation in glycoprotein folding is demonstrated by the example of tyrosinase and TRP-1. For tyrosinase, optimal folding is dependent on its association with the chaperones calnexin/calreticulin that recognize tyrosinase *N*-glycans and drive it into an on and off cycle in which α-glucosidase II and GT play important roles. The quality control acts by selecting a conformation of the polypeptide chain able to acquire copper in its active site. Importantly, the quality control system operates locally with individual *N*-glycans having distinctive roles.

During the past few years, it has become more evident that calnexin, calreticulin, α-glucosidase II, and GT are components of a large network of proteins, acting in a coordinated manner and recognizing specific features of the folding polypeptide. The recent data on the interaction of tyrosinase with Erp57 and of TRP-1 with BiP in the absence of calnexin/calreticulin (together with tyrosinase regional folding data) are in favor of a *compass* model operating in the ER. In this model, the folding glycoprotein may be viewed in the center of a magnetic compass surrounded by the network of ER chaperones. The molecular chaperones associate with and dissociate from the nascent polypeptide at specific locations (orientations of the compass) and for specific dura-

tions in order to ensure the correct folding of the chain. This chaperone network includes BiP, calnexin/calreticulin, Erp57, PDI, ERGIC-53, and other components which will no doubt be described in the future with specific roles in the compass model. Some of these proteins may be involved in mechanisms related to the re-translocation of the misfolded glycoprotein in the cytosol. We can understand how they work by analogy with tyrosinase. Thus, cytosolic antigenic peptides derived from tyrosinase are transported back to the ER by TAP where they interact with the class I complex MHC/HLA. The tyrosinase family of proteins is an important example, therefore, which allows us to probe, in detail, the folding process and discover new features of the quality control system. It may also lead to a better understanding of the processing of the (glyco)peptides loading into MHC molecules.

10. Acknowledgments

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