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Chaperone-Like Functions of High-Mannose Type and Complex-Type *N*-Glycans and Their Molecular Basis[1]

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It has recently become apparent that high-mannose type *N*-glycans directly promote protein folding, whereas complex-type ones play a crucial role in the stabilization of protein functional conformations through hydrophobic interactions with the hydrophobic protein surfaces. Here an attempt was made to understand more deeply the molecular basis of these chaperone-like functions with the aid of information obtained from spacefill models of *N*-glycans. The promotion of protein folding by high-mannose *N*-glycans seemed to be based on their unique structure, which includes a hydrophobic region similar to the cyclodextrin cavity. The promotive features of high-mannose *N*-glycans newly observed under various conditions furnished strong support for the view that both intra- and extramolecular high-mannose *N*-glycans are directly involved in the promotion of protein folding in the endoplasmic reticulum. Further, it was revealed that the *N*-acetyllactosamine units in complex-type *N*-glycans have an amphiphilic structure and greatly contribute to the formation of extensive hydrophobic surfaces and, consequently, to the *N*-glycan–protein hydrophobic interactions. The processing of high-mannose type *N*-glycans to complex-type ones seems to be an ingenious device to enable the *N*-glycans to perform these two chaperone-like functions.

Key words: glycoprotein, *N*-glycan function, *N*-linked oligosaccharide, protein folding, protein stabilization.

Most secretory proteins become *N*-glycosylated as soon as the growing polypeptide chains enter the endoplasmic reticulum, before their functional conformations are established (*1, 2*). Although much is known about the structure and biosynthesis of *N*-glycans, the central questions of why the transferred *N*-glycans should be processed to high-mannose-type, complex-type, or hybrid-type oligosaccharide structures and how the *N*-glycans interact with the protein moieties are far from being elucidated. With a view to approaching these long-standing questions, we have been studying *in vitro* how the *N*-glycan structures are related to the protein conformations. It has become apparent that high-mannose type *N*-glycans directly promote protein folding (*3–5*), whereas complex-type ones play a crucial role in the stabilization of protein functional conformations (*6–8*). Although such dependence of protein conformation on the *N*-glycans is of decided importance for the acquisition and retention of the biological activities of glycoproteins, our knowledge about the molecular basis for the interactions between *N*-glycans and proteins is still limited. In this study, an attempt was made to understand more deeply the molecular basis of these *N*-glycan functions with the aid of information obtained from spacefill models of *N*-glycans of the high-mannose and complex types.

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Abbreviations: CIV-Asn, (Galβ1-4GlcNAcβ1-3)$_{0\ or\ 1}$Galβ1-4GlcNAcβ1-6[(Galβ1-4GlcNAcβ1-3)$_{0\ or\ 1}$Galβ1-4GlcNAcβ1-2]Manα1-6[(Galβ1-4GlcNAcβ1-3)$_{0\ or\ 1}$Galβ1-4GlcNAcβ1-4(Galβ1-4GlcNAcβ1-2)Manα1-3]Manβ1-4GlcNAcβ1-4(±Fucα1-6)GlcNAc-Asn; CHO-EPO, human erythropoietin produced in Chinese hamster ovary cells; EC-EPO, human erythropoietin produced in *Escherichia coli*; EPO, human erythropoietin; M9-Asn, Manα1-2Manα1-6(Manα1-2Manα1-3)-Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-Asn; RNase B_H, RNase B with a higher oligomannose glycan, $Man_{8\ or\ 9}GlcNAc_2$; RNase B_L, RNase B with a lower oligomannose glycan, $Man_5GlcNAc_2$; SPR, surface plasmon resonance.

MATERIALS AND METHODS

Materials—Bovine pancreatic RNase A (type IIIA) and its *N*-glycosylated form, RNase B (type XII-B), were obtained from Sigma and purified as previously described (*3*). A RNase B species with a higher oligomannose glycan, $Man_{8\ or\ 9}GlcNAc_2$, (RNase B_H) was prepared by affinity fractionation of RNase B on a concanavalin A-Cellulofine column as previously described (*9*). To obtain another RNase B species with a lower oligomannose glycan, $Man_5GlcNAc_2$ (RNase B_L), RNase B was digested with 1,2-α-mannosidase according to the previous procedures (*10*), and the product was purified by gel-filtration on a Sephadex-G50 (fine grade) column. Protein concentrations were calculated using ε_{280} = 9,800 $M^{-1}\cdot cm^{-1}$ (*11*) and ε_{275} = 8,160 $M^{-1}\cdot cm^{-1}$ (*12*) for the native and reductively denatured RNases, respectively. Bovine serum albumin, *Micrococcus lysodeikticus* dried cells, GSH, and GSSG were also obtained from

Sigma. Hen egg white lysozyme was a product of Wako Pure Chem. The protein concentration of lysozyme was determined using A_{280}[1%/cm] = 26.3 (*13*). Dithiothreitol and guanidine hydrochloride were also products of Wako. 1,2-α-Mannosidase (*Aspergillus saitoi*) and β-galactosidase (jack bean) were purchased from Seikagaku Kogyo. Human erythropoietin (EPO) was expressed in and purified from Chinese hamster ovary cells (CHO-EPO) as previously described (*6*). EPO was also produced in *Escherichia coli*, folded, and purified by the method previously described (*6*). The protein concentrations of EPOs were determined using a molar extinction coefficient of 2.26×10^4 $M^{-1} \cdot cm^{-1}$ (*14*). *Ricinus communis* agglutinin I was from Honen Corp. A highly branched complex-type *N*-glycan mixture mostly composed of tetraantennary ones (*15–18*), ±NeuAcα2-3(Galβ1-4GlcNAcβ1-3)$_{0\text{ or }1}$Galβ1-4GlcNAcβ1-6[±NeuAcα2-3(Galβ1-4GlcNAcβ1-3)$_{0\text{ or }1}$Galβ1-4GlcNAcβ1-2]Manα1-6[±NeuAcα2-3(Galβ1-4GlcNAcβ1-3)$_{0\text{ or }1}$Galβ1-4GlcNAcβ1-4(Galβ1-4GlcNAcβ1-2)Manα1-3]Manβ1-4GlcNAcβ1-4(±Fucα1-6)GlcNAc-Asn, was prepared from CHO-EPO as described in the preceding paper (*8*). This *N*-glycan mixture was desialyzed by treatment with 0.1 N H_2SO_4 at 80°C for 1 h (*8*), and is abbreviated as CIV-Asn. An *N*-linked high-mannose type oligosaccharide, Manα1-2Manα1-6(Manα1-2Manα1-3)Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-Asn (M9-Asn), was prepared by repeated pronase digestion of soybean lectin, followed by gel-filtration on a Sephadex G-25 column (*19*). During the preparation of these *N*-glycans, the proteolytic removal of amino acid residues other than the glycosylated asparagine ones was monitored by means of amino acid analysis, as previously described (*20*). Other chemicals used were described in the preceding papers (*7, 8*).

Methods—The reductive denaturation of RNases was performed exactly as described previously (*3*). The refolding features of the denatured RNases were examined under Cu^{2+}-catalyzed oxidation conditions (*4*). The reductive denaturation of lysozyme was also carried out exactly according to the procedures previously described (*5*). For regeneration, the denatured lysozyme solution was first diluted 10-fold with 0.1 M Tris-HCl (pH 8.0) containing 6 M guanidine hydrochloride, then immediately further diluted 20-fold into a refolding buffer [0.1 M Tris-HCl (pH 8.0) containing 2 mM EDTA, 5 mM GSH, 0.5 mM GSSG, and 0.2 M NaCl]. After various times of incubation at 25°C, the refolding was assayed in terms of the activity regained as previously described (*5*). M9-Asn was added to the refolding buffer when the effect of an extramolecular M9-Asn on the refolding of reductively denatured lysozyme was assayed. Protein aggregation was followed by measuring turbidity at 540 nm using a Shimadzu UV-1600 spectrophotometer equipped with a Peltier cell holder. All BIAcore experiments were performed with a BIAcore biosensor X (Pharmacia Biosensor) at 25°C in 20 mM citrate buffer (pH 7.0) containing 0.1 M NaCl. The flow rate was maintained at 5 μl/min. Immobilization of CIV-Asn was performed essentially as reported previously (*8*), resulting in 130 RU of the *N*-glycans being immobilized on the sensor chip. The *N*-glycan immobilization was confirmed by means of a lectin-binding assay involving *R. communis* agglutinin I at different concentrations (5–400 nM) (*21*). Complete β-galactosidase digestion of the immobilized CIV-Asn was also confirmed by the lectin-binding assay. The interactions between the *N*-glycans and EC-EPO were analyzed by injecting EC-EPO at different concentrations (2–15 μM) onto the *N*-glycan–bound surface. Parameter values were calculated by the global fitting procedure (*22*) using the sensorgrams obtained on direct kinetic analysis of EC-EPO. Data analysis was performed with the software of BIAevaluation (Pharmacia Biosensor).

RESULTS

The Role of High-Mannose Type N-Glycans in Protein Folding—High-mannose type *N*-glycans have been supposed to directly promote the folding of nascent polypeptides in the endoplasmic reticulum. Attempts to settle this problem *in vivo*, however, have been thwarted by technical difficulties. On the other hand, an appropriate experimental system for an *in vitro* approach to this problem was provided by the use of bovine pancreatic RNase, which occurs naturally in both *N*-glycosylated form (RNase B) and non-glycosylated form (RNase A), which are thought to be structurally identical except for the presence of a single high-mannose *N*-glycan chain ($Man_{5\text{-}9}GlcNAc_2$) (*23*). Comparison of the refolding features of these two forms revealed that the high-mannose *N*-glycans of RNase B greatly promote the polypeptide folding at an early stage (*3, 4*). This finding, together with the fact that higher oligomannose structures ($Man_{7\text{-}9}GlcNAc_2$) predominate in the nascent glycoproteins in the endoplasmic reticulum (*24, 25*), has aroused interest in the relationship between the size and the promotive effect of *N*-glycans.

It is evident from Fig. 1 that the higher oligomannose chains ($Man_{8\text{ or }9}GlcNAc_2$) promoted the protein folding more strongly than the lower one ($Man_5GlcNAc_2$). This finding is in fair agreement with the previous results that an extramolecular free *N*-glycan, M9-Asn, promoted the refolding of various proteins more strongly than did a smaller one, Manα1-6(Manα1-3)Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-Asn (*4, 5*).

It was previously revealed that extramolecular free *N*-glycans of both the high-mannose and complex types directly promote the refolding of proteins, whereas common mono-, oligo-, and polysaccharides exhibit much lower, if any, promotive effects (*4, 5*). These results, together with

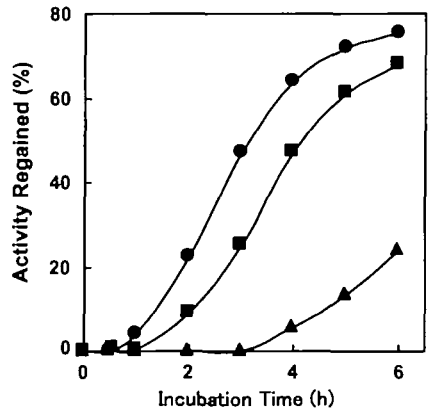
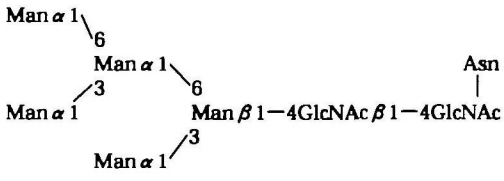


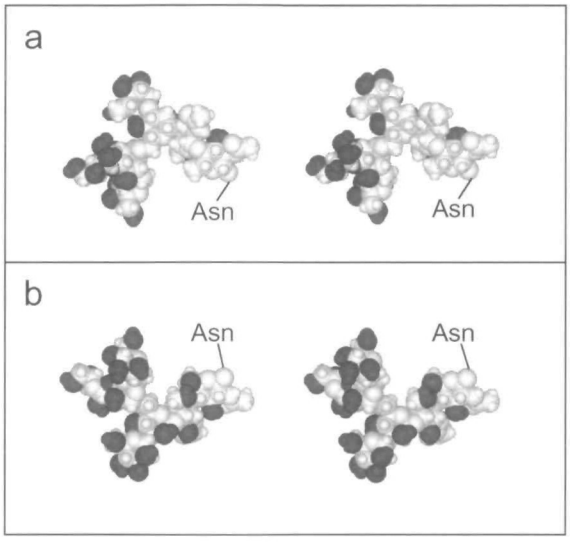
Fig. 1. **Effects of intramolecular high-mannose type *N*-glycans on the refolding of reductively denatured RNases.** The refolding of RNase B_H (●), RNase B_L (■), and RNase A (▲) was assayed in terms of the activity regained. See the text for details.

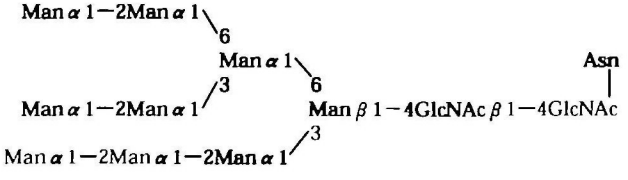
the facts that free *N*-glycans devoid of asparagine residues are also promotive (Yamaguchi, H., unpublished results) and that the smallest *N*-glycan, GlcNAc-Asn, does not show any significant effect (*5*), suggest that the promotive effects of *N*-glycans are attributable to their characteristic sugar compositions and branched structures. It is of interest, therefore, to reveal the oligosaccharide conformations characteristic of *N*-glycans.

Figure 2 gives the stereoviews of the spacefill models of high-mannose *N*-glycans, $Man_5GlcNAc_2$ and $Man_9GlcNAc_2$. It was recently suggested that complex-type *N*-glycans, in contrast to common carbohydrates, have a hydrophobic plane generated through alignment of the hydrophobic patches of carbohydrates (*8*). It can be seen in Fig. 2 that the high-mannose *N*-glycans also have a hydrophobic region surrounded by the pentasaccharide core and three branches in their structures. Although the hydrophobic region of $Man_9GlcNAc_2$ was not entirely visible at any visual angle, survey of the hydrophobic regions visible at various angles convinced us that $Man_9GlcNAc_2$ has a much more extensive hydrophobic region than $Man_5GlcNAc_2$. These hydrophobic regions of high-mannose *N*-glycans seemed to resemble the inner surface of the cyclodextrin cavity, which is known to accommodate aliphatic and aromatic hydrocarbons.

It has become apparent that free oligosaccharides of both the high-mannose and complex types are contained in various organelles including the endoplasmic reticulum in substantial amounts (*26–35*). In view of the promotive effect of these oligosaccharides on protein folding, it would not be surprising if they were involved in the maturation of the nascent proteins in the endoplasmic reticulum. It is known, however, that the concentrations of the nascent proteins in the endoplasmic reticulum are much higher than those







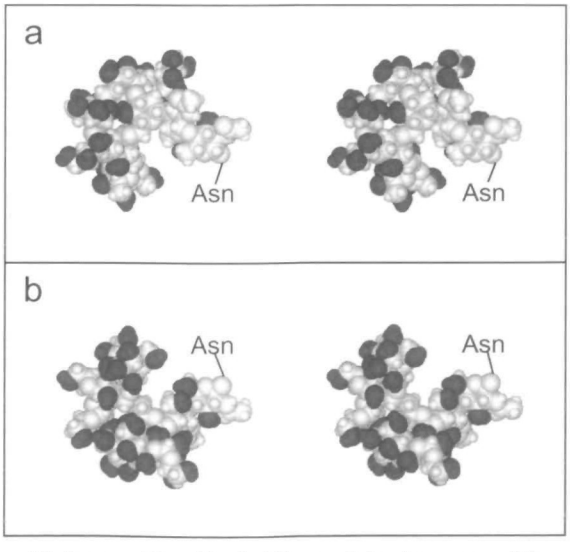


Fig. 2. **Stereoviews of the possible conformations of high-mannose type *N*-glycans.** The sides holding acetylamino groups (a) and the reverse sides (b) of M5-Asn (upper models) and M9-Asn (lower models) are shown as energy-diminished spacefill models. Hydroxyls are shown in black. The figures were made with molecular modeling software Free Wheel (Butch Software Studio, Sagamihara).

commonly found in *in vitro* studies of protein folding. It might be interesting, therefore, to see how the promotive effect of free *N*-glycans on protein folding changes with increasing protein concentration.

To clarify this problem, the refolding of hen egg white lysozyme was performed in the presence of M9-Asn, which is the only high-mannose *N*-glycan available in sufficient quantity. It is known that egg white lysozyme, which is a nonglycosylated polypeptide having four disulfide linkages, cannot be readily refolded from a reductively denatured form *in vitro* and that 1 mM M9-Asn remarkably stimulates its refolding at a low protein concentration (24 μg/ml) (*5*). As shown in Fig. 3, the protein aggregation became increasingly prominent with increasing protein concentration, resulting in a less efficiency of protein refolding. Such limited refolding of lysozyme was not much improved in the presence of 5 mM M9-Asn, suggesting only a low ability of M9-Asn to reduce the protein aggregation. Figure 3 further shows that the protein refolding was considerably depressed even under conditions where no significant protein aggregation was observed, probably due to the formation of soluble aggregates not detectable by A_{540}.

Interestingly, however, the promotive effect of M9-Asn on lysozyme refolding was enhanced in the presence of bovine serum albumin, which is known to depress protein aggregation (*36, 37*) (Fig. 4). Nearly 70% of lysozyme at a concentration of as high as 0.3 mg/ml was refolded in the presence of both 1 mM M9-Asn and 3% (w/w) serum albumin. There seems no doubt that a concerted effect of M9-Asn and serum albumin helped the proper folding of lysozyme. This result seems to afford important information about the role of free *N*-glycans in the protein folding in endoplasmic reticulum, as will be discussed later.

The Role of Complex-Type N-Glycans in the Stabilization of Protein Conformation—CHO-EPO is a hydrophobic protein highly stabilized by multibranched complex-type *N*-glycans (*6, 7*). We recently reported that complex-type *N*-glycans, particularly multibranched ones, have a hydrophobic surface that extensively stretches across the plane holding acetylamino groups and that the *N*-glycans of CHO-EPO stabilize the extremely unstable protein conformation through hydrophobic interactions with the hydrophobic protein surface (*8*). With a view to understanding more deeply the stabilizing function of complex-type *N*-glycans, the spacefill models of complex-type *N*-glycans with different branching degrees were arranged to afford stereoviews of their conformation (Fig. 5). Sialic acid residues were excluded from the *N*-glycan models, because they are not responsible for the stabilization of EPO protein conformation (*7*) and also because the conformations of asialo *N*-glycans seemed independent of the sialic acid residues at the nonreducing ends. It is evident from these models that complex-type *N*-glycans are amphiphilic and have extensive hydrophobic surfaces that become larger with increase in their degree of branching, in fair agreement with their binding affinity for the hydrophobic EPO protein, EC-EPO, estimated by the surface plasmon resonance (SPR) technique (*8*). All of the axial hydroxyls were found on the concave surfaces, whereas the reverse surfaces were mainly made up of CH-dense areas, acetylamino groups, and glycosidic linkages, which are all hydrophobic. The equatorial disposition of the hydroxyls sparsely distributed on the hydrophobic surfaces seems to make them more hydrophobic than they look. Further, it should be emphasized that the complex-type *N*-glycans have much more extensive hydrophobic regions than have the high-mannose type ones. Such extensive hydrophobic planes of complex-type *N*-glycans might be interpreted as being due largely to the presence of *N*-acetyllactosamine units, which are not involved in high-mannose *N*-glycans. To clarify this point, the spacefill models of an *N*-acetyllactosamine unit were

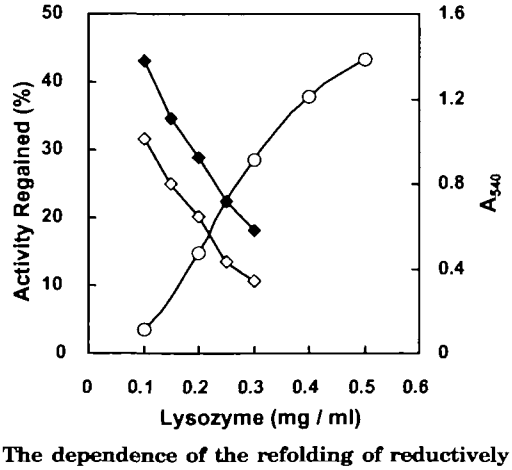


Fig. 3. **The dependence of the refolding of reductively denatured lysozyme on the protein concentrations.** Denatured lysozyme was allowed to regenerate with (◆) or without (◇, ○) 5 mM M9-Asn under redox conditions. The refolding was assayed in terms of the activity regained (◆, ◇) and turbidity at A_{540} (○). See the text for details.

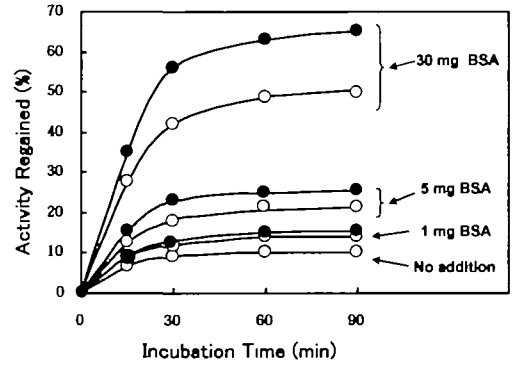
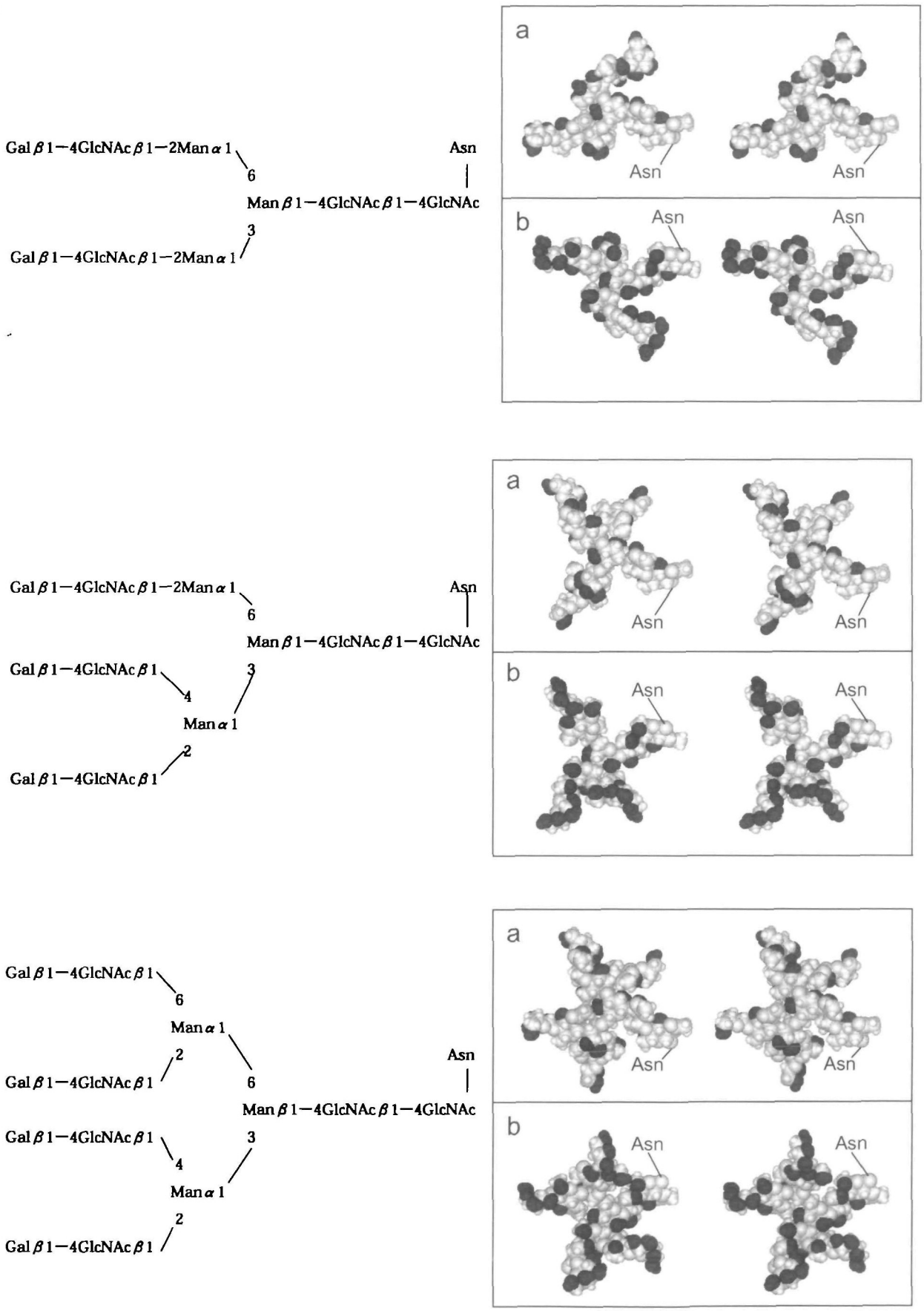


Fig. 4. **Concerted effect of M9-Asn and serum albumin on the refolding of reductively denatured lysozyme.** Denatured lysozyme (0.3 mg/ml) was allowed to regenerate with bovine serum albumin (BSA) at the indicated concentrations in the presence (●) or absence (○) of 1 mM M9-Asn under redox conditions. See the text for details.

Fig. 5. **Stereoviews of the possible conformations of complex-type *N*-glycans.** The surfaces holding acetylamino groups (a) and the reverse surfaces (b) of a biantennary *N*-glycan, Galβ1-4GlcNAcβ1-2Manα1-6(Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-Asn (top), a triantennary *N*-glycan, Galβ1-4GlcNAcβ1-2Manα1-6[Galβ1-4GlcNAcβ1-4(Galβ1-4GlcNAcβ1-2)Manα1-3]Manβ1-4GlcNAcβ1-4GlcNAc-Asn (middle), and a tetraantennary *N*-glycan, Galβ1-4GlcNAcβ1-6(Galβ1-4GlcNAcβ1-2)Manα1-6[Galβ1-4GlcNAcβ1-4(Galβ1-4GlcNAcβ1-2)Manα1-3]Manβ1-4GlcNAcβ1-4GlcNAc-Asn (bottom) are shown as energy-diminished spacefill models. See the legend in Fig. 2 for more details.

Fig. 5



inspected for their structural features.

From Fig. 6 there seems little doubt that the *N*-acetyllactosamine units in complex-type *N*-glycans have an amphiphilic structure and greatly contribute to the formation of the extensive hydrophobic surfaces of complex-type *N*-glycans. The side holding the acetylamino group, contrary to the reverse side, is almost completely kept away from the hydroxyls. This is true also of the hydrophobic surface of the pentasaccharide core common to all *N*-glycans (Figs. 2 and 5). It seems probable, therefore, that the addition of *N*-acetyllactosamine units to the pentasaccharide core (*i.e.*, the *N*-glycan processing) creates the extensive hydrophobic surface of a complex-type *N*-glycan.

In this connection, it is noteworthy that removal of a greater part of the galactose residues of CHO-EPO significantly reduces the stability of the EPO protein conformation (*7*). We were interested to know how much the galactose residues contribute to the formation of the hydrophobic surface of a multibranched *N*-glycan. It is evident from Fig. 7 that the loss of the galactose residues of *N*-acetyllactosamine units results in a considerable reduction of the hydrophobic surface of a tetraantennary *N*-glycan. It was previously revealed that the binding affinity of *N*-glycans for the hydrophobic EPO protein increases remarkably with the extension of their hydrophobic surfaces (*8*). It may be said, therefore, that removal of the galactose residues of CHO-EPO weakens the hydrophobic interactions between the *N*-glycans and the protein surface. To obtain direct evidence for this view, the interactions between the *N*-glycans (*i.e.*, CIV-Asn), isolated from CHO-EPO and immobilized on a sensor chip, and the EPO potein were analyzed by the surface plasmon resonance (SPR) technique. Before the analysis, the immobilization and complete β-galactosidase digestion of CIV-Asn were confirmed by means of a lectin-binding assay involving a galactose-binding lectin (Fig. 8A). The β-galactosidase digestion of CIV-Asn was regarded as complete, from a comparison with the previous data (*21*). Sensorgrams showing the effect of

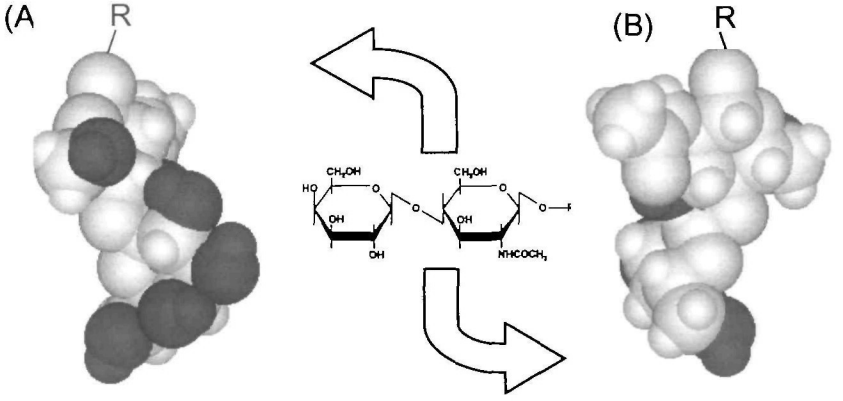


Fig. 6. **Amphiphilic structure of the *N*-acetyllactosamine unit in complex-type *N*-glycans.** The hydrophobic side holding the acetylamino group (B) and the reverse hydrophilic side (A) of an *N*-acetyllactosamine unit are shown as energy-diminished spacefill models. See the legend in Fig. 2 for more details.

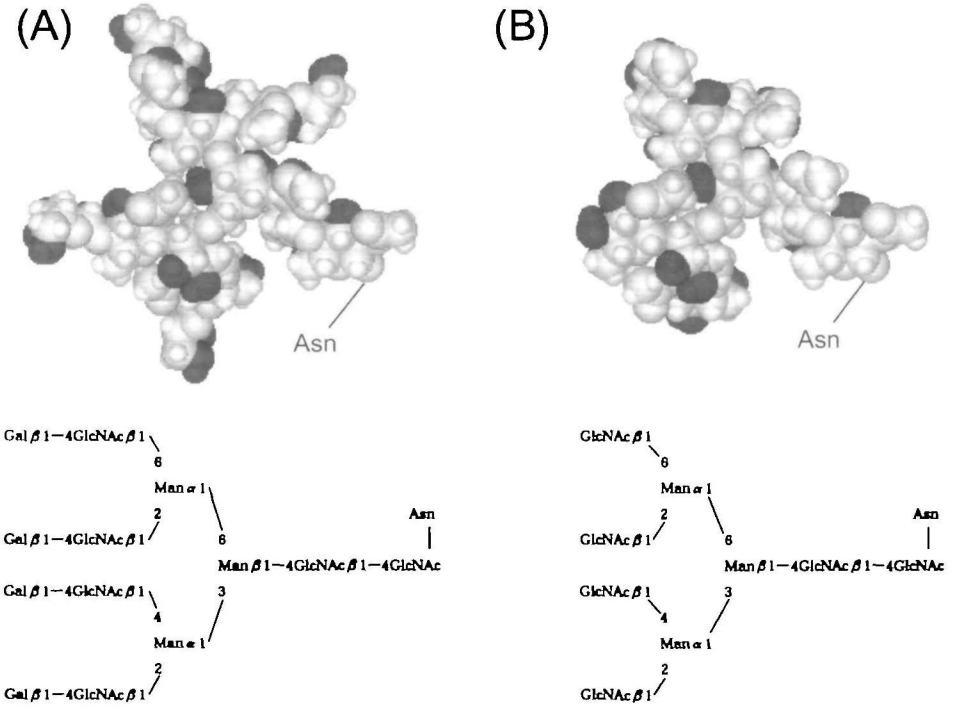


Fig. 7. **Comparison of the hydrophobic surface of a tetraantennary *N*-glycan with that of its degalactosylated form.** The hydrophobic surfaces of a tetraantennary *N*-glycan (A) and its degalactosylated form (B) are shown as energy-diminished spacefill models. See the legend in Fig. 2 for more details.

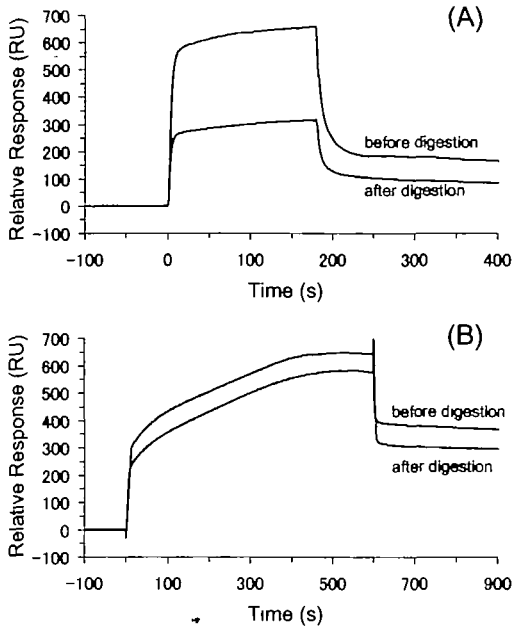


Fig. 8. **Contribution of the galactose residues of CIV-Asn to its binding affinity for EC-EPO.** Sensorgrams show the interactions of surface-bound CIV-Asn with *Ricinus communis* agglutinin I (0.4 μM) (A) and with EC-EPO (15 μM) (B) before and after β-galactosidase digestion. See the text for details.

β-galactosidase digestion of the immobilized CIV-Asn on its binding affinity for EC-EPO are presented in Fig. 8B. Removal of all the terminal galactosyl residues (*ca.* 80% of the total galactose residues) of the immobilized CIV-Asn resulted in a significant decrease in its binding affinity for EC-EPO, *i.e.*, $K_D = 3.4 \times 10^{-5}$ for the intact CIV-Asn and $K_D = 5.9 \times 10^{-6}$ for the β-galactosidase–digested CIV-Asn.

In view of these findings and the previous information (*7, 8*), there seems no doubt that the *N*-acetyllactosamine units in complex-type *N*-glycans greatly contribute to the formation of their extensive hydrophobic surfaces and, consequently, to the *N*-glycan–protein hydrophobic interactions.

DISCUSSION

The results obtained here seem to offer important information about the roles of *N*-glycan in protein folding in the endoplasmic reticulum. In particular, the higher oligomannose chains of RNase B were found to promote the protein folding more strongly than the lower one. Interestingly, this finding is in fair agreement with the previous one that the folding of influenza virus hemagglutinin with a truncated *N*-glycan $Glc_1Man_5GlcNAc_2$ that normally interacts with calnexin is markedly depressed in the endoplasmic reticulum (*38*). These findings, together with the facts that higher oligomannose structures ($Man_{7-9}GlcNAc_2$) predominate in the nascent glycoproteins in the endoplasmic reticulum (*24, 25*) and that the endoplasmic reticulum-resident lectins, calnexin and calreticulin, are unable to directly promote the folding of the nascent glycoproteins (*39–41*), strongly suggest that the high-mannose *N*-glycans play an important role in the promotion of protein folding in the endoplasmic reticulum.

Further, it is to be remembered that free *N*-glycans, which are contained in endoplasmic reticulum (*26, 27*), also promote protein folding (*4, 5*). Surprisingly, jack bean α-mannosidase, deglycosylated and denatured to a species unable to refold, quantitatively regains its functional structure with as low as a concentration 25 μM $Man_9GlcNAc_1$ (*42*). In addition, the present study showed that the promotive effect of M9-Asn on the refolding of lysozyme at a high concentration is enhanced when the protein aggregation is depressed (Fig. 4). These findings, together with the fact that calnexin and calreticulin sequester the nascent glycoproteins by tethering their monoglucosylated high-mannose *N*-glycans, thus depressing their aggregation and raising their folding efficiency (*40, 43*), suggest that free *N*-glycans also play a significant role in the promotion of protein folding in the endoplasmic reticulum.

We must now consider how the structures of high-mannose *N*-glycans are related to their promotive effects on protein folding. In this connection it is necessary to note the following: (i) Complex-type *N*-glycans also have promotive effects that become stronger with the extension of hydrophobic surfaces (*4, 5, 8*). (ii) The increasing order of the binding affinities of *N*-glycans for the hydrophobic regions of proteins parallels that of their promotive effects on protein folding (*5, 8, 44*). (iii) Cyclodextrins have been noted as having promotive effects on protein folding that are attributable to their unique structures, which include a hydrophobic cavity (*45–47*). (iv) It is well known that hydrophobic interactions are predominant in both the proper and the improper folding of proteins. On the basis of the results and arguments presented here, there seems little doubt that the hydrophobic area of high-mannose *N*-glycans is closely associated with their promotive effect on protein folding. Further, it may well be said that glycan-protein hydrophobic interactions play the main role in promoting the protein folding, possibly depressing the hydrophobic interactions unfavorable to the proper folding of proteins. A more detailed study, however, is still required for a full understanding of the mechanism of this *N*-glycan function.

It is known that the intramolecular high-mannose *N*-glycans enhance the dynamic stability and fix the labile surface areas of the protein molecules through hydrogen bonding and van der Waals contact (*48–50*). Such stabilizing effects observed for high-mannose *N*-glycans, however, are not essential for the biological activities of glycoproteins, at least under physiological conditions. To our knowledge, high-mannose *N*-glycans are not closely associated with the protein functional conformations, except in that they play a critical role in the subunit assembly and retention of the subunit structure of oligomeric proteins through their lectin-like interactions with the specific sites of the polypeptides (*9, 51–53*). In agreement with this view, the binding affinity of M9-Asn for the EPO protein is much lower than those of complex-type *N*-glycans, as estimated by the SPR technique (*8*).

Complex-type *N*-glycans, unlike high-mannose type ones, fulfil the critical requirements for the stabilization of the protein functional conformations. In fact, even minor disorders of complex-type *N*-glycan structures would be accompanied by a significant decrease in the conformational stability of the parent proteins (*7, 54, 55*). The results and arguments presented here, together with the fact that the surface hydrophobicity of a protein is a primary factor de-

stabilizing its conformation (*56*), confirm the early suggestions (*7, 8*) that complex-type *N*-glycans have an extensive hydrophobic surface and stabilize the protein conformations through hydrophobic interactions with the hydrophobic protein surfaces that are unfavorable to protein stability. In view of the large contribution of galactose residues to the formation of the hydrophobic surfaces of complex-type *N*-glycans, it seems probable that hypogalactosylation of complex-type *N*-glycans, which is associated with decline of the protein functions in some intractable diseases (*57–59*) and aging (*60*), weakens the *N*-glycan–protein hydrophobic interactions, resulting in a less stable protein functional conformation.

The structures of complex-type *N*-glycans of glycoproteins seem to be closely related to the stability of the parent proteins. That is to say, the high-mannose *N*-glycans of glycoproteins should be processed so that the resulting complex-type ones may cope with the surface properties of the folded proteins. For example, highly branched *N*-glycans should be required for tranquilizing the extensive hydrophobic protein surfaces that are unfavorable to protein stability. The *N*-acetyllactosamine repeating units in complex-type *N*-glycans would extend their hydrophobic areas. Further, in view of the amphiphilic structure of an *N*-acetylglucosamine residue, a bisecting GlcNAcβ1-4 residue should strengthen the hydrophobicity of the pentasaccharide core, ensuring that a local hydrophobic area of the protein surface may be stabilized. These suggestions seem to provide a plausible hypothesis that the surface properties of folded proteins profoundly participate in the processing of their high-mannose *N*-glycans in the Golgi apparatus.

The conclusion reached through this study can be summarized as follows: *N*-Glycans function as "sugar chaperones" in the acquisition and retention of the functional conformations of proteins, and the processing of the high-mannose type to the complex type is an ingenious device to enable the *N*-glycans to perform these two functions. Further details of the molecular basis underlying these functions, however, are essential if solid progress is to be made in medical science and protein engineering.

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