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N-Glycans Stabilize Human Erythropoietin through Hydrophobic Interactions with the Hydrophobic Protein Surface: Studies by Surface Plasmon Resonance Analysis[1]

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Human erythropoietin (EPO) produced in Chinese hamster ovary cells is a hydrophobic protein highly stabilized by multibranched complex-type *N*-glycans. To reveal the molecular basis of the interaction between the *N*-glycans and the EPO protein, complex-type *N*-glycans of different structures were analyzed as to their binding affinity for *Escherichia coli*–expressed EPO by means of the surface plasmon resonance technique. It appears well established that complex-type *N*-glycans, particularly multibranched ones, have hydrophobic regions that extensively stretch across the plane holding acetylamino groups and that *N*-glycan–protein hydrophobic interactions characterized by a slow rate of dissociation stabilize the protein conformation.

Key words: erythropoietin, glycoprotein, *N*-linked oligosaccharide, protein stabilization, surface plasmon resonance.

Protein *N*-glycosylation occurs without exception in integral membrane proteins of higher organisms and is common in secretory proteins. *N*-Glycans were recently found to directly promote protein folding (*1–7*), and to stabilize the protein conformation (*8–13*). It should be emphasized that such a dependence of the protein conformation on *N*-glycans is of decided importance as to the acquisition and retention of the biological activities of glycoproteins.

Although the stabilizing effect of *N*-glycosylation on the protein conformation has been reported for a variety of glycoproteins (*8–13*), little is known about the molecular basis for the interaction between *N*-glycans and proteins. Human erythropoietin (EPO) produced in Chinese hamster ovary cells (CHO-EPO) is appropriate for solving this problem, because its multibranched *N*-glycans play a critical role in stabilizing the extremely unstable protein conformation under denaturing conditions (*8, 14*) To characterize the stabilizing effect of the *N*-glycans, we recently compared the properties of enzymatically *N*-glycan-modified CHO-EPO spectrophotometrically, and found that the inner regions including the galactose residues of the *N*-glycans stabilize the protein conformation by interacting with the hydrophobic protein surface areas accessible to 1-anilino-8-naphthalenesulfonic acid binding (*14*) Although there seems no doubt that the *N*-glycans exhibit binding affinity for the hydrophobic protein surface, we must now consider the following questions: What kind of affinity exists between the *N*-glycans and the protein surface? How are the *N*-glycan structures related to the *N*-glycan–protein interaction? With a view to obtaining further details of the mechanism underlying this stabilizing function of the *N*-glycans, an attempt was made to analyze the interactions between complex-type *N*-glycans and the EPO protein by means of the surface plasmon resonance (SPR) technique. This report presents direct evidence that complex-type *N*-glycans, particularly highly branched ones, have extensively stretched hydrophobic planes and stabilize the protein conformation through hydrophobic interactions with the hydrophobic protein surface.

CHO-EPO was expressed in and purified from Chinese hamster ovary cells as described previously (*8*). EPO was also produced in *Escherichia coli*, folded, and purified by the method previously described (EC-EPO) (*8*). The protein concentrations of EPOs were determined using a molar extinction coefficient of 2.26×10^4 M^{-1} cm^{-1} for all forms of EPO (*15*). β-Galactosidase (jack bean) was purchased from Seikagaku Kogyo. Trimethylamine *N*-oxide was a product of Sigma. A mixture of *N*-linked biantennary glycans, ±NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-6(±NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(±Fucα1-6)GlcNAc-Asn, and another mixture of *N*-linked triantennary glycans, ±NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1-6[±NeuAcα2-3Galβ1-4GlcNAcβ1-4(±NeuAcα2-6Galβ1-4GlcNAcβ1-2)Manα1-3]Manβ1-4GlcNAcβ1-4GlcNAc-Asn, were prepared by repeated pronase digestion

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Abbreviations: CII-Asn, Galβ1-4GlcNAcβ1-2Manα1-6(Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(±Fucα1-6)GlcNAc-Asn, CIII-Asn, Galβ1-4GlcNAcβ1-2Manα1-6[Galβ1-4GlcNAcβ1-4(Galβ1-4GlcNAcβ1-2)Manα1-3]Manβ1-4GlcNAcβ1-4GlcNAc-Asn, CIV-Asn, $(Gal\beta1\text{-}4GlcNAc\beta1\text{-}3)_{0\text{ or }1}Gal\beta1\text{-}4GlcNAc\beta1\text{-}6[(Gal\beta1\text{-}4GlcNAc\beta1\text{-}3)_{0\text{ or }1}Gal\beta1\text{-}4GlcNAc\beta1\text{-}2]Man\alpha1\text{-}6[(Gal\beta1\text{-}4GlcNAc\beta1\text{-}3)_{0\text{ or }1}Gal\beta1\text{-}4GlcNAc\beta1\text{-}4(Gal\beta1\text{-}4GlcNAc\beta1\text{-}2)Man\alpha1\text{-}3]Man\beta1\text{-}4GlcNAc\beta1\text{-}4(\pm Fuc\alpha1\text{-}6)GlcNAc$-Asn, CHO-EPO, human erythropoietin produced in Chinese hamster ovary cells, EC-EPO, human erythropoietin produced in *Escherichia coli*, EPO, human erythropoietin, SPR, surface plasmon resonance.

of human apo-transferrin and bovine serum fetuin, respectively, as described in the preceding paper (*16*) A highly branched complex-type *N*-glycan mixture mostly composed of tetraantennary ones (*17–20*), ±NeuAcα2-3(Galβ1-4GlcNAcβ1-3)$_{0\ or\ 1}$Galβ1-4GlcNAcβ1-6[±NeuAcα2-3(Galβ1-4GlcNAcβ1-3)$_{0\ or\ 1}$Galβ1-4GlcNAcβ1-2]Manα1-6[±NeuAcα2-3(Galβ1-4GlcNAcβ1-3)$_{0\ or\ 1}$Galβ1-4GlcNAcβ1-4(Galβ1-4GlcNAcβ1-2)Manα1-3]Manβ1-4GlcNAcβ1-4(±Fucα1-6)GlcNAc-Asn, was also prepared by repeated pronase digestion of CHO-EPO, followed by gel-filtration on a Sephadex G-50 column. 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 (*21*). These bi-, tri-, and tetraantennary *N*-glycans were all desialyzed by treatment with 0 1 N H_2SO_4 at 80°C for 1 h, and are abbreviated as CII-Asn, CIII-Asn, and CIV-Asn, respectively.

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 *N*-glycans was performed essentially according to the manufacturer's standard protocol (Pharmacia Biosensor), but with some modifications. After equilibration of the CM5 sensor surface with 10 mM HEPES (pH 7 4)/150 mM NaCl/0.005% surfactant p-20, the sensor surface was activated by a 14 min injection of 100 mM *N*-hydroxysuccinimide/100 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (70 μl) in water. An *N*-glycan, at a concentration of 15 mg/ml in 10 mM sodium borate (pH 8.5)/1 M NaCl, was injected at the flow rate of 5 μl/min for 20 min. Unused activated carboxymethyl groups on the sensor surface were deactivated by reaction with 1 M ethanolamine (pH 8.5). A second flow cell, which had been exposed to the activation reagents and then deactivated with 1 M ethanolamine, was used to generate a control response. This resulted in 50–130 RU of an *N*-glycan being immobilized on the sensor chip. The immobilization of *N*-glycans was confirmed by means of a lectin binding assay involving *Ricinus communis* agglutinin I at different concentrations (5–400 nM) (*22*). Complete β-galactosidase digestion of CIV-Asn immobilized on a sensor chip was also confirmed by lectin binding assaying as just described.

To characterize the interaction between the *N*-glycans and the protein moiety of CHO-EPO, EC-EPO and CHO-EPO were injected onto the CIV-bound surface Sensorgrams obtained on direct kinetic analysis of EPOs at different concentrations are presented in Fig. 1A, and the calculated parameter values for these curves are given in Table I. EC-EPO showed an increase in the SPR response, whereas CHO-EPO did not show any significant binding affinity for the CIV chains. This binding affinity of the *N*-glycans for EC-EPO was depressed in the presence of CIV-Asn added to the running buffer, demonstrating the specificity of the interaction between them (Fig. 1B). Higher concentrations of CIV-Asn could not be applied to this test owing to the limited amount of the sample. Interestingly, the binding affinity of the *N*-glycans for EC-EPO is characterized by a slow rate of dissociation for the interaction with the EPO protein. Furthermore, this binding affinity of *N*-glycans for the EPO protein surface remarkably increased under high-salt conditions, showing a hydrophobic interaction between them (Table I). This increase in binding affinity can be exclusively attributed to the enhanced rate of association for the interaction. A similar effect on the rate of association was also observed for an osmolyte, trimethylamine *N*-oxide, which is known to enhance hydrophobicity similarly to high-salt conditions (*23, 24*).

The surface hydrophobicity of a protein has been considered to be a primary factor governing the conformational stability (*25*). It is known that a number of solvent-exposed hydrophobic amino acid residues, such as proline, and aliphatic and aromatic amino acid residues (*26*), make the EPO protein extremely unstable (*8, 14*). In view, however, of the hydrophilic property of glycans, it may sound strange

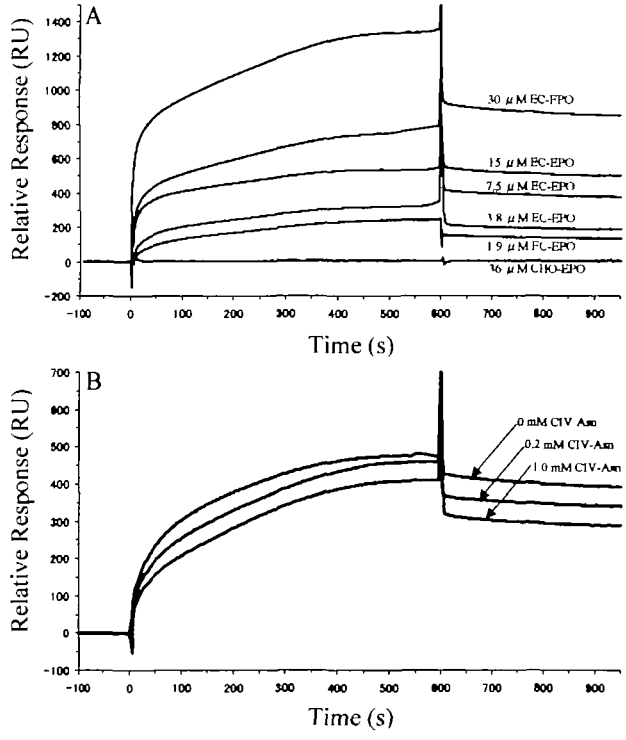

Fig 1 **Sensorgrams showing the interactions between EPOs and surface-bound CIV-Asn.** (A) Kinetics of EPO binding to immobilized CIV-Asn. (B) Competitive inhibition of EC-EPO binding on the addition of free CIV-Asn

TABLE I **Parameters obtained for the interaction analysis between surface-bound *N*-glycans and EC-EPO.**

Additive	*N*-Glycan	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_D (M)	χ^2
No addition	CII-Asn	2.8×10^{-1}	5.2×10^{-4}	1.9×10^{-3}	10 0
No addition	CIII-Asn	3.0×10^{-1}	2.1×10^{-4}	7.0×10^{-4}	2 2
No addition	CIV-Asn	6.3×10^{-1}	2.1×10^{-5}	3.3×10^{-5}	7 1
1 5 M NaCl	CIV-Asn	3.9×10^{3}	6.1×10^{-6}	1.5×10^{-9}	8.7
1 M $(NH_4)_2SO_4$	CIV-Asn	5.2×10^{2}	2.1×10^{-5}	4.0×10^{-8}	1 1
1 M TMAO	CIV-Asn	4.9×10	6.6×10^{-4}	1.3×10^{-5}	9.1

that this unstable protein conformation is stabilized through hydrophobic interactions between the *N*-glycans and the hydrophobic protein surface.

Glycans can generate areas of hydrophobic surface through alignment of the hydrophobic patches of a carbohydrate, resulting from the disposition of the equatorial and axial hydroxyls to one side of the pyranose ring of a monomer unit, glycosidic linkage formation (*27*), and the existence of the hydrophobic acetylamino groups of amino sugar residues. Furthermore, it has been revealed that the hydrophobicity of carbohydrates is determined by a set of factors including the CH-dense surface area, degrees of polymerization and branching, and molecular planarity (*27*). There seems no doubt, therefore, that complex-type *N*-glycans have hydrophobic regions that extensively stretch across the plane holding acetylamino groups.

Figure 2 shows that complex-type *N*-glycans are amphiphilic, and have both hydrophilic and hydrophobic planes formed in their conformations. It can be seen from the spacefill models that the hydrophobic planes become progressively larger with increasing branching degrees of *N*-glycans. The equatorial disposition of the sparsely distributed hydroxyls seems to make these planes more hydrophobic than they look. In fact, the binding affinity of *N*-glycans for EC-EPO increased with their branching degrees, as estimated by the SPR technique (Table I) These results are consistent with the elution positions of the oligosaccharides corresponding to CII-Asn, CIII-Asn, and CIV-Asn on a HPLC octadecylsilyl column, *i.e.*, 10.2, 12.7, and 14.2–20.8 glucose unit numbers, respectively (*28, 29*). In this connection, it is interesting that the binding affinity (K_D = 3.1 × 10^{-3} M) of a high-mannose *N*-glycan, $Man_9GlcNAc_2$, for EC-EPO is much lower than that of CIV chains. It seems rational, therefore, that the extensively spread hydrophobic surface of the EPO protein is covered mostly with complex-type tetraantennary *N*-glycans.

Enzymatic removal of a greater part (*ca.* 80%) of the galactose residues of the CIV chains immobilized on a sensor chip resulted in a significant decrease in its binding affinity (K_D = 5.4 × 10^{-5} M) for EC-EPO. This finding is in

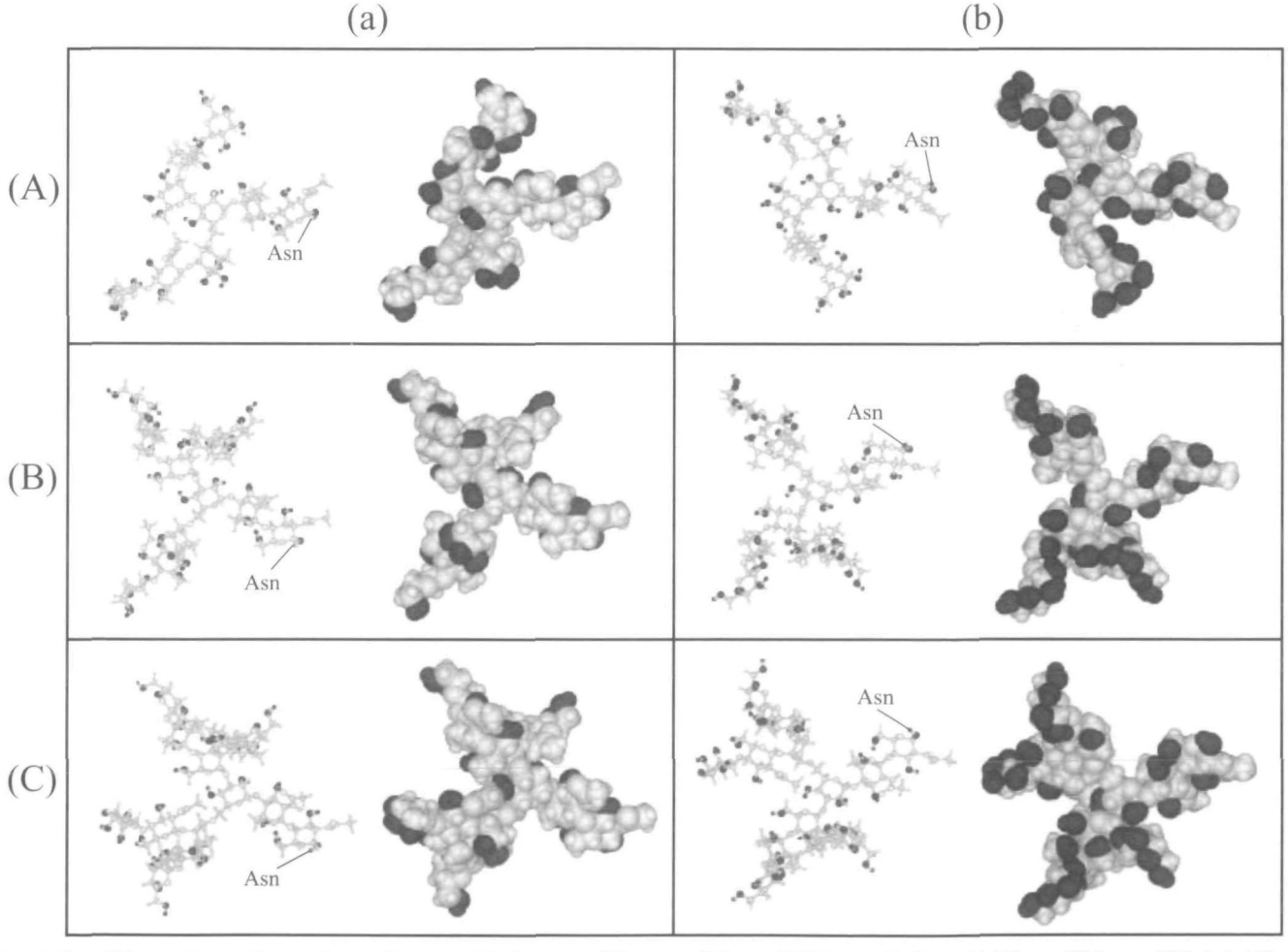

Fig 2. **Possible conformations of complex-type *N*-glycans with different branching degrees.** The planes holding acetylamino groups (a) and the reverse planes (b) of a biantennary *N*-glycan, Galβ1-4GlcNAcβ1-2Manα1-6(Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-Asn (A), a triantennary *N*-glycan, Galβ1-4GlcNAcβ1-2Manα1-6[Galβ1-4GlcNAcβ1-4(Galβ1-4GlcNAcβ1-2)Manα1-3]Manβ1-4GlcNAcβ1-4GlcNAc-Asn (B), 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 (C) are shown as ball and stick models (left), and spacefill models (right) Hydroxyls are shown in black The figures were made with Molecular Modeling Software FREE WHEEL (Butch Software Studio, Sagamihara).

fair agreement with the previous observation that the β-galactosidase trimming of the *N*-glycans of CHO-EPO led to partial exposure of the hydrophobic protein surface accompanied by a decrease in the stability of the protein conformation (*14*). These results, together with the facts that even minor disorders of complex-type *N*-glycan structures would markedly affect the biological functions of glycoproteins (*30–36*) and that high-mannose *N*-glycans seem to interact only weakly with the EPO protein, show that complex-type *N*-glycans are essential for stabilization of the functional conformation of some proteins. On the basis of the results and arguments presented here, it appears well established that an *N*-glycan–protein hydrophobic interaction including binding affinity with a slow rate of dissociation is required to tranquilize the hydrophobic protein surface and maintain the functional conformation of the EPO protein.

The branching degrees of complex-type *N*-glycans of glycoproteins seem to be associated with the extent of their hydrophobic protein surface. The requirement for highly branched *N*-glycans, as revealed for EPO (*14*), could be interpreted as the tranquilization of an extensively stretched hydrophobic protein surface. It thus appears that relatively small mono- and biantennary *N*-glycans could be enough to mask hydrophobic protein patches Furthermore, in view of the hydrophobic patches inherent to the individual pyranose rings (*27*), it may also well be said that the presence of a bisecting GlcNAcβ1-4 residue or an *N*-acetyllactosamine repeating unit in complex-type *N*-glycans would locally enhance their hydrophobicity, and hence strengthen their interactions with the protein surface. This idea is supported by the fact that the presence of a bisecting GlcNAcβ1-4 residue or an *N*-acetyllactosamine repeating unit greatly enhances the affinity of complex-type oligosaccharides for a HPLC octadecylsilyl column (*28, 29*).

It seems probable that the information presented here is essential for a full understanding of diseases associated with aberrant *N*-glycosylation and for the biotechnological production of glycoproteins for therapeutic use.

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