J. Biochem. 128, 731–737 (2000)

Stabilization of Human Recombinant Erythropoietin through Interactions with the Highly Branched *N*-Glycans[1]

Teruko Toyoda,[*] Tomokazu Itai,[*] Tsutomu Arakawa,[†] Kenneth H. Aoki,[†] and Haruki Yamaguchi[*,2]

[*]*Course of Applied Biochemistry, Graduate School of Agriculture and Biological Science, Osaka Prefecture University, Sakai, Osaka 599-8531; and* [†]*Amgen Inc., Thousand Oaks, California 91320-1789, USA*

Received June 29, 2000; accepted August 14, 2000

Human erythropoietin (EPO) produced in Chinese hamster ovary cells (CHO-EPO) is a hydrophobic protein stabilized by the highly branched complex-type *N*-glycans. To characterize the stabilizing effect of the *N*-glycans, the properties of enzymatically *N*-glycan–modified CHO-EPO species were compared spectrophotometrically. CD and fluorescence spectra following the protein unfolding induced by guanidine hydrochloride or pH revealed that the inner regions including the galactose residues of the *N*-glycans stabilize the protein conformation. The decrease in the conformational stability caused by enzymatic trimming of the *N*-glycans was associated with the exposure of the hydrophobic protein surface areas accessible to 1-anilino-8-naphthalenesulfonic acid (ANS) binding. Further, the ANS binding and heat denaturation of *Escherichia coli*–expressed EPO (nonglycosylated EPO) were depressed in dilute solutions (1 mM or so) of free *N*-glycans of the complex type. These results, together with the finding that the *N*-glycans of CHO-EPO make little contact with the aromatic amino acid residues exposed on the protein surface, indicate that the inner regions including the galactose residues of the intramolecular *N*-glycans stabilize the protein conformation by clinging to the hydrophobic protein surface areas mainly made up of nonaromatic hydrocarbon groups.

Key words: erythropoietin, glycoprotein, *N*-glycan, *N*-glycan function, *N*-glycan–protein interaction.

In addition to their direct participation in biological activities, *N*-glycans have recently been found to directly promote protein folding (*1–5*) and stabilize protein conformation (*6–10*). Although such a dependence of protein conformation on *N*-glycans is of decided importance in the acquisition and retention of the biological activities of glycoproteins, the mechanisms underlying these functions of *N*-glycans remain unknown.

Erythropoietin is a glycoprotein hormone that regulates the red blood cell level by stimulating the maturation of erythroid precursor cells (*11*, *12*). Human erythropoietin (EPO) is a highly hydrophobic protein stabilized by an approximately equal mass of glycans, *i.e.*, three complex-type *N*-glycan chains mostly having the tetraantennary structure with or without *N*-acetyllactosamine repeating units, and one *O*-glycan composed of sialic acid and Galβ1-3GalNAc (*13–16*). The *O*-glycan, however, does not seem to appreciably contribute to the stabilization of EPO protein, because EPO from which most of the *N*-glycans were enzymatically removed aggregates during incubation under mild conditions (*17*). To reveal the participation of the *N*-glycans in the maintenance of the protein conformation of EPO, Narhi *et al.* compared the stability of fully glycosylated EPO produced in Chinese hamster ovary cells (CHO-EPO) with those of its asialo form and a nonglycosylated EPO expressed in *Escherichia coli* (EC-EPO) (*18*). Monitoring the protein conformation change by CD, they found that the *N*-glycans play an important role in stabilizing the protein molecules to denaturing conditions, and that the terminal sialic acid residues are not involved in the stabilizing function of the *N*-glycans. On the other hand, Tsuda *et al.* reported that the *N*-glycans including the sialic acid residues contribute to the thermal stability of the *in vitro* activity (*16*). In the present study, an attempt was made to get more accurate information about the relationship between the structure and stabilizing effect of the *N*-glycans, and also to get some insight into the interaction between the *N*-glycans and the protein moiety. Spectroscopic examination of the stability and 1-anilino-8-naphthalenesulfonic acid (ANS) binding of EPO species with different *N*-glycan structures revealed that the inner regions including the galactose residues of the *N*-glycans stabilize the protein conformation by clinging to the hydrophobic areas accessible to ANS binding.

[1] This study was supported in part by a Grant-in-Aid for Scientific Research (11480169) from the Ministry of Education, Science, Sports and Culture of Japan.

[2] To whom correspondence should be addressed. E-mail: simplife@biochem.osakafu-u.ac.jp

Abbreviations: ANS, 1-anilino-8-naphthalenesulfonic acid; CIII, ±NeuAcα2-3Galβ1-4GlcNAcβ1-4(±NeuAcα2-6Galβ1-4GlcNAcβ1-2)Manα1-3(±NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc; CHO-EPO, human erythropoietin produced in Chinese hamster ovary cells; Con A, concanavalin A; EC-EPO, human erythropoietin produced in *Escherichia coli*; EPO, human erythropoietin; GS-II, *Griffonia simplicifolia* agglutinin II; RCA-I, *Ricinus communis* agglutinin I.

MATERIALS AND METHODS

Materials—EPO was expressed and purified from Chinese hamster ovary cells as described previously (*18*). EPO was also produced in *E. coli*, folded, and purified by the method previously described (*18*). Spectroscopic examinations showed no appreciable difference in protein conformation between CHO-EPO and EC-EPO as reported previously (*18*). 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 (*19*). Sialidase (*Streptococcus* sp.), β-galactosidase (*Streptococcus* 6646K), and β-*N*-acetylhexosaminidase (jack bean) were purchased from Seikagaku Kogyo. Prestained SDS-PAGE molecular weight standards were obtained from Bio-Rad. Biotinylated *Ricinus communis* agglutinin I (RCA-I) and *Griffonia simplicifolia* agglutinin II (GS-II) were products of Vector Laboratories. Biotinylated concanavalin A (Con A) and horseradish peroxidase–labeled avidin were purchased from Honen Corp. ANS was a product of Sigma, and 3,3′-diaminobenzidine was from Nacalai Tesque. A triantennary *N*-glycan devoid of any amino acid residues, ±NeuAcα2-3Galβ1-4GlcNAcβ1-4(±NeuAcα2-6Galβ1-4GlcNAcβ1-2)Manα1-3(±NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc (CIII), was prepared by pepsin digestion of bovine serum fetuin (Seikagaku Kogyo) followed by glycopeptidase A (Seikagaku Kogyo) digestion of the resulting glycopeptide as described previously (*1*). CIII devoid of sialic acid residues (asialo CIII) was obtained by sialidase digestion of CIII followed by gel filtration on Sephadex G-25.

Enzymatic Modification of N-Glycans of CHO-EPO—Intact CHO-EPO (1 mg) was incubated with sialidase (100 mU) in 10 mM $CaCl_2$/100 mM sodium acetate buffer (pH 6.5) (0.66 ml) at 37°C for 3 h, dialyzed against water, then lyophilized. Part of the digestion product obtained (0.3 mg) was incubated with β-galactosidase (3 mU) in 50 mM sodium phosphate buffer (pH 5.5) (0.6 ml) or with a mixture of β-galactosidase (3 mU) and β-*N*-acetylhexosaminidase (300 mU) in 50 mM sodium phosphate buffer (pH 6.0) (0.6 ml) at 37°C for 16 h, dialyzed against water, then lyophilized.

SDS-PAGE and Dot Lectin Assay—SDS-PAGE was performed by the method of Laemmli (*20*) using 14% acrylamide slab gels. Gels were stained for protein with Coomassie Brilliant Blue R-250. Dot lectin assay was performed essentially as reported previously (*21*), but with some modifications. Briefly, a glycoprotein (0.5 μg) dissolved in 0.15 M NaCl/0.15 M sodium phosphate buffer (pH 7.2) (30 μl) was transferred onto nitrocellulose membrane using a Bio-Dot SF (Bio-Rad). The membrane was blocked with 0.15 M NaCl/0.05% Tween 20/10 mM Tris-HCl (pH 7.4), immersed in a biotinylated lectin solution (10 μg/ml in the blocking buffer), and washed with the blocking buffer. The membrane was treated with a horseradish peroxidase-labeled streptavidin solution (0.3 μg/ml in the blocking buffer), washed with the blocking buffer, then stained with 0.03% 3,3′-diaminobenzidine/0.003% H_2O_2/0.15 M NaCl/0.15 M sodium phosphate buffer (pH 7.2).

Guanidine Hydrochloride–Induced Denaturation of EPOs—A stock solution of EPO in 0.1 M NaCl/20 mM sodium citrate (pH 7.0) was mixed with 7.6 M guanidine hydrochloride in the same buffer, and buffer alone, to obtain solutions containing 12 μM protein along with various concentrations of guanidine hydrochloride. After incubation at 25°C for 24 h, the CD spectra were recorded to monitor the loss of signal at 282 nm. Guanidine hydrochloride-induced denaturation was also determined by following the change in the tryptophyl fluorescence of 4.6 μM protein solutions after incubation at 37°C for 24 h. The spectra, from 290 to 400 nm, were recorded with excitation at 280 nm.

pH Stability of EPOs—The pH of EPO solutions in 35% (w/v) glycerol/0.1 M NaCl/1 mM sodium citrate (pH 7.0) was adjusted by the addition of 1 M citric acid and 1 M sodium citrate in appropriate ratios in a total volume of 20 μl. The final protein concentration of the samples was 45 μM. The CD spectra were recorded to monitor the loss of signal at 282 nm.

Effect of Free N-Glycans on Heat Denaturation of EC-EPO—EC-EPO (0.25 mg/ml) in 0.1 M NaCl/20 mM sodium citrate (pH 7.0) was incubated at 40°C in the presence or absence of an *N*-glycan. Denaturation of EC-EPO was followed by light scattering at 360 nm using a Shimadzu UV-1600 spectrophotometer equipped with a Peltier cell holder.

Spectrophotometry—CD spectra were measured with a JASCO J-720 spectropolarimeter. Mean residue molar ellipticity was calculated using a mean residue weight of 111 for the protein moiety of CHO-EPO. Cuvettes of 1-cm pathlength were used for the near UV CD region. Spectra in the far UV CD were recorded in cells of 0.1-cm pathlength. Fluorescence measurements were performed on a Shimadzu RF-1500 fluorescence spectrophotometer at 25°C. To study the binding of ANS to EPOs, an aliquot (5 μl) of ANS stock solutions (1.5 to 18 mM) was added to a 0.16 mg/ml EPO solution in 0.1 M NaCl/20 mM sodium citrate (pH 7.0), and the fluorescence spectra from 400 to 550 nm were recorded with excitation at 380 nm. Tryptophyl fluorescence spectra of CHO-EPO and EC-EPO (9 μM in 0.1 M NaCl/20 mM sodium citrate, pH 7.0) were recorded with excitation at 280 nm.

RESULTS

Glycosidase Trimming of the Glycan Chains of CHO-EPO—The *N*-glycans of CHO-EPO are mostly of the tetraantennary type with or without *N*-acetyllactosamine repeating units (*13*, *14*). Figure 1 shows the major structures of the *N*-glycans of CHO-EPO and their changes by glycosidase trimmings. Complete digestions of the substrate glycans were confirmed by SDS-PAGE and by dot-lectin assay (*21*). Apparent molecular weights of the digestion products, which had been treated with different combinations of exoglycosidases, gradually decreased with the enzymatic trimming of their glycan chains (Fig. 2, lanes C, D, and E in the upper panel). One minor band on lane E seemed to correspond to a CHO-EPO species lacking the *O*-glycan (*16*). The newly exposed sugar residues of the digestion products corresponding to lanes C, D, and E, were confirmed by dot-lectin assay, using RCA-I, a galactose-binding lectin (*22*), GS-II, an *N*-acetylglucosamine-binding lectin (*23*), and Con A, a mannose-binding lectin (*24*), respectively (Fig. 2, lower panels). CD and fluorescence measurements showed that those enzymatic modifications caused no significant changes in the conformation of the protein itself (data not shown).

Glycan-Dependent Stability of CHO-EPO—To reveal the

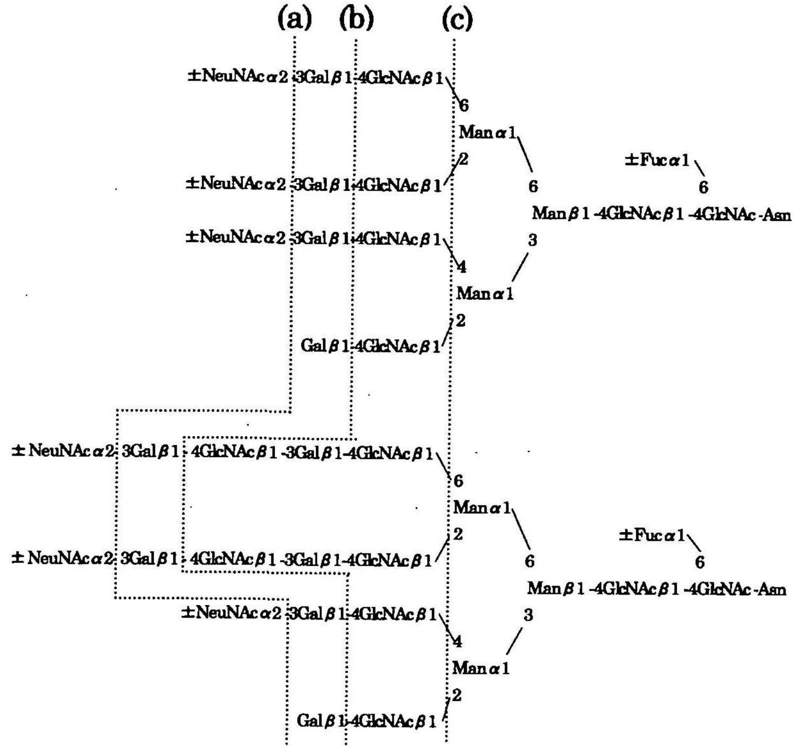

Fig. 1. **Glycosidase trimming of the major *N*-glycans of CHO-EPO.** The productions of asialo, galactose-deficient, and *N*-acetylglucosamine–deficient *N*-glycans are indicated by the expected glycosidase trimmings (dotted lines a, b, and c). The cleavage sites by glycosidases: (a), sialidase; (b), β-galactosidase after sialidase; (c), β-galactosidase/β-*N*-acetylhexosaminidase after sialidase.

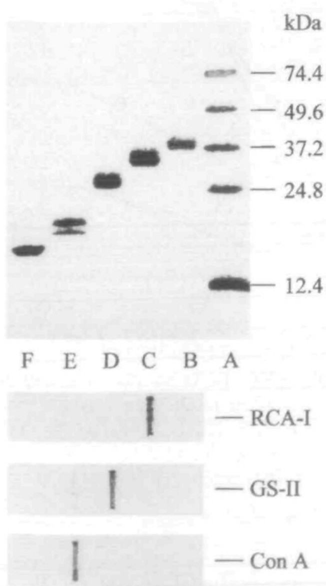

Fig. 2. **Ch... PO species by ...** ... upper panel: SDS-PAGE. Lane A, molecular weight standards; lane B, intact CHO-EPO; lane C, CHO-EPO treated with sialidase; lane D, CHO-EPO treated with β-galactosidase after sialidase digestion; lane E, CHO-EPO treated with β-galactosidase/β-*N*-acetylhexosaminidase after sialidase digestion; lane F, EC-EPO. Lower panels: Each (*ca.* 0.5 μg) of the CHO-EPO species corresponding to lanes B–F was spotted on nitrocellulose membrane and stained with RCA-I, GS-II, and Con A.

effects of the glycan modifications on the stability of CHO-EPO, the unfolding of EPO species with different glycan structures was studied by measuring spectral changes under denaturing conditions. Figure 3 shows the guanidine hydrochloride–induced unfolding of the EPO species monitored by CD at 282 nm and by intrinsic fluorescence. The intact CHO-EPO and the asialo CHO-EPO gave closely similar denaturation curves to each other, confirming the previous results that the stabilizing effect of the glycans of CHO-EPO does not depend on the presence of sialic acid (*18*). Removal of a greater part (*ca.* 80%) of the galactose residues, however, distinctly reduced the stability, and further trimming to expose the mannose residues mostly abolished the stabilizing effect of the glycans. These results suggest that the inner regions including the galactose residues of the glycans stabilize the protein conformation. There was a considerable difference in guanidine hydrochloride concentration between the denaturation curves constructed by CD and by intrinsic fluorescence measurements. It seems likely that the change in the restricted mobility of the aromatic groups precedes that in their environmental hydrophobicity during the guanidine hydrochloride–induced unfolding. The far UV spectra were also recorded for these EPO species under the denaturing conditions. There was no clear correlation, however, between the drop in the helical content and the guanidine hydrochloride concentration (data not shown). This observation might be interpreted as due largely to the accumulation of a non-native α-helical intermediate induced by guanidine hydrochloride, as has been reported with a variety of proteins (*25, 26*).

Further, the spectral changes associated with pH-induced unfolding of the EPO species were followed by CD at 282 nm. Narhi *et al.* have reported that EC-EPO completely unfolds and precipitates at pH 5.0, and that the presence of 35% glycerol stabilizes EC-EPO, allowing a

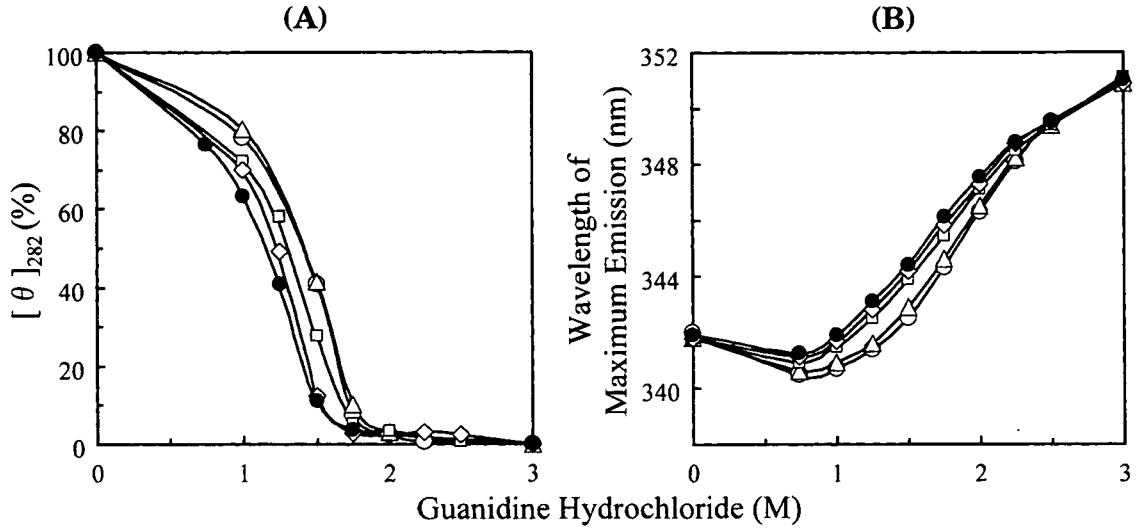

Fig. 3. **Guanidine hydrochloride–induced denaturation of EPO species with different *N*-glycan structures.** The effects of guanidine hydrochloride on the conformations of the intact CHO-EPO (○), asialo CHO-EPO (△), galactose-deficient CHO-EPO (□), and *N*-acetylglucosamine–deficient CHO-EPO (◇), and EC-EPO (●) were determined by monitoring the loss of signal at 282 nm in the CD spectra (A) or the change in the tryptophyl fluorescence spectra (B).

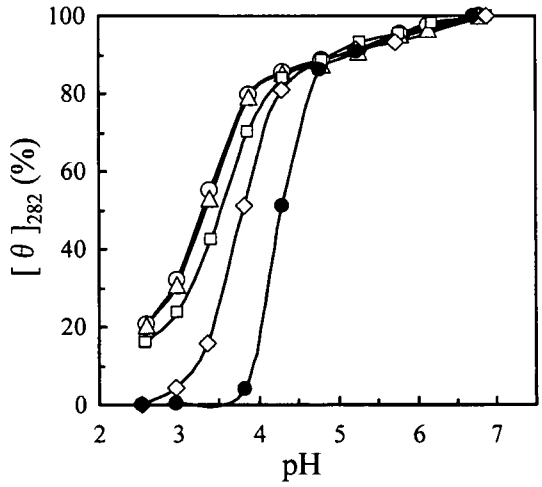

Fig. 4. **pH-induced denaturation of EPO species with different *N*-glycan structures.** The effects of pH on the conformations of the intact CHO-EPO (○), asialo CHO-EPO (△), galactose-deficient CHO-EPO (□), *N*-acetylglucosamine–deficient CHO-EPO (◇), and EC-EPO (●) were determined by monitoring the loss of signal at 282 nm in the CD spectra.

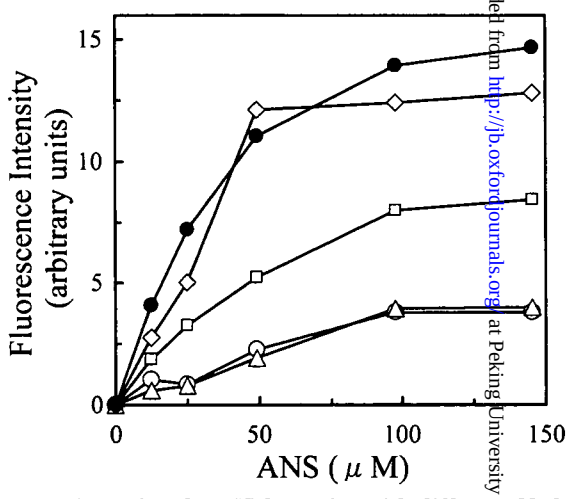

Fig. 5. **Binding of ANS to EPO species with different *N*-glycan structures.** The intensities of the fluorescence peaks for the intact CHO-EPO (○), asialo CHO-EPO (△), galactose-deficient CHO-EPO (□), *N*-acetylglucosamine–deficient CHO-EPO (◇), and EC-EPO (●) were plotted against ANS concentration.

comparison with the other EPO species to be made under these conditions (*18*). We also examined the unfolding of the EPO species with different glycan structures in the presence of 35% glycerol. It is evident from Fig. 4 that the inner regions including the galactose residues of the *N*-glycans play an important role in stabilizing the EPO protein to acid-induced denaturation, and the sialic acid residues did not seem responsible for the increased stability, confirming the above results observed with guanidine hydrochloride. The changes in pH, however, seemed not to considerably affect the secondary structure estimated by far UV CD. In agreement with the results previously reported (*18*), greater than 70% of the secondary structure was maintained even at pH 2.5 (data not shown).

ANS Binding of EPO Species with Different Glycan Structures—The EPO protein surface is widely occupied by hydrophobic areas mainly made up of hydrophobic amino acid residues such as leucine, isoleucine, valine, proline, and some aromatic amino acids (*27*). With a view to getting some insight into the interaction of the *N*-glycans with the protein surface, the exposure of the hydrophobic areas of the protein surface of the EPO species with different glycan structures was explored by the use of a hydrophobic fluorescent dye, ANS. ANS increases its fluorescence quantum yield upon noncovalent binding to hydrophobic areas of proteins (*28*). Figure 5 shows that the *N*-glycans of CHO-EPO firmly keep the protein surface from the ANS binding, and

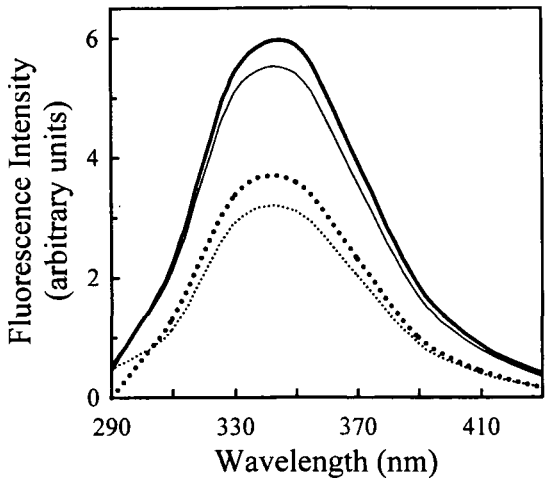

Fig. 6. **Comparison of the intrinsic fluorescence of CHO-EPO and EC-EPO.** Tryptophyl fluorescence spectra of CHO-EPO (thick lines) and EC-EPO (thin lines) were recorded after 1 h of incubation at 25°C with (dotted lines) or without (solid lines) 1 mM asialo CIII.

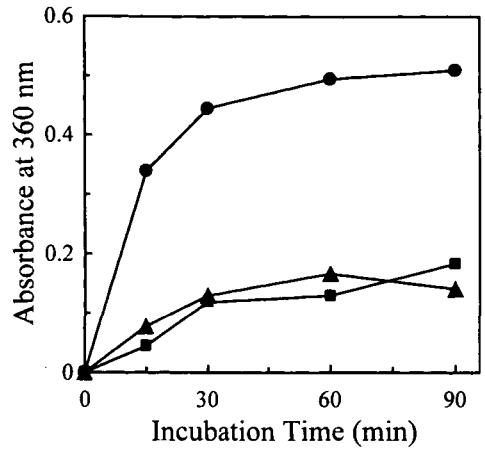

Fig. 7. **Effects of free *N*-glycans on the heat denaturation of EC-EPO monitored by light scattering.** EC-EPO was treated at 40°C with 1 mM concentration of CIII (■), asialo CIII (▲), or without any of these *N*-glycans (●). Each point represents the mean of two independent measurements differing by less than 6%.

that the sialic acid residues of the *N*-glycans are not closely related to the ANS binding, in agreement with the result previously reported (*18*). Removal of the inner sugar residues including galactose, however, resulted in a marked increase in the ANS binding, showing that the inner regions of the *N*-glycans are in contact with the hydrophobic protein surface accessible to ANS binding.

Interactions between N-Glycans and EPO Protein—It is known that the EPO protein molecule has several solvent-exposed aromatic amino acid residues including one or two tryptophan residues (*27*, *29*). On the other hand, it has recently been found that *N*-glycans of both the high-mannose and complex types have binding affinity for aromatic amino acid residues (*30*, *31*), and that *N*-glycans markedly depress the intrinsic fluorescence intensity of proteins by binding to their solvent-exposed aromatic groups (*30*). To see whether the intramolecular *N*-glycan chains of CHO-EPO are in contact with the aromatic amino acid residues exposed on the protein surface, the fluorescence spectra of CHO-EPO were compared with those of EC-EPO (Fig. 6). The tryptophyl fluorescence spectrum of CHO-EPO was similar to that of EC-EPO, and, in addition, the fluorescence intensities of CHO-EPO and EC-EPO were almost equally depressed in solutions of a free triantennary *N*-glycan CIII or its asialo derivative (data shown only for asialo CIII). These results show that the intramolecular *N*-glycans make little contact with the aromatic groups exposed on the protein molecules of CHO-EPO. It seems probable, therefore, that the *N*-glycans of CHO-EPO cling to the hydrophobic areas mainly made up of nonaromatic groups accessible to ANS binding.

Interaction between Free N-Glycans and EC-EPO—In connection with the interaction between the *N*-glycans and EPO protein, it might be interesting to see the effect of extramolecular free *N*-glycans on the stability of EC-EPO. Since a sufficient amount of free *N*-glycans was hard to prepare from CHO-EPO owing to the limited availability of this glycoprotein, a triantennary *N*-glycan CIII and its asialo derivative were tested for the effect on heat denaturation of EC-EPO, which is heat-labile and easily aggregates. Interestingly, the heat denaturation of EC-EPO was depressed in 1 mM solutions of CIII or asialo CIII, when monitored by the light-scattering technique (Fig. 7). This result, together with the finding that CIII and asialo CIII also depress the ANS binding of EC-EPO (data not shown), indicates their affinity for the hydrophobic protein surface unfavorable for the protein stability. Further, it is also noteworthy that CIII and asialo CIII were similar to each other in their depressing effects, in agreement with the above observation that the intact CHO-EPO and asialo CHO-EPO gave closely similar denaturation curves (Fig. 3). Unfortunately, no further attempt to probe whether the free *N*-glycans could stabilize the protein conformation could be made because of the small amount of EC-EPO available.

DISCUSSION

Although the stabilizing effect of *N*-glycosylation on protein structure has been reported for a variety of glycoproteins (*6–10*, *18*), little is known about the molecular basis for the interaction between *N*-glycans and proteins. CHO-EPO appeared appropriate for approach to this problem, because the intramolecular *N*-glycans play a critical role in stabilizing the protein conformation to denaturing conditions (*18*, *32*) and, consequently, significant changes in the property of CHO-EPO could be expected even from minor modifications to the *N*-glycan structure. In fact, the results and arguments presented here offer some helpful suggestions for general understanding of the role of *N*-glycans in protein stabilization.

The surface hydrophobicity of proteins has been considered a primary factor governing their conformational stability (*33*). There seems no doubt that a number of the solvent-exposed hydrophobic amino acid residues (*27*), such as proline, and aliphatic and aromatic amino acids, make the EPO protein significantly unstable. It is to be noted that free *N*-glycans of the complex type also inhibit the ANS binding and depress the heat denaturation of EC-EPO. This striking result firmly supports the concept that the intramolecular *N*-glycans stabilize the protein confor-

mation through their interactions with the hydrophobic protein surface. The *N*-glycans, however, seem to require to be linked to the protein moiety for their full stabilizing effects on the protein conformation in view of the highly stabilized conformation of CHO-EPO. The three *N*-glycans, which are similar in structural features to one another (*34*, *35*), are tethered on the opposite end to the EPO receptor binding sites of the elongated protein molecule (*27*), and an extensive hydrophobic protein surface surrounds the *N*-glycosylation sites (*27*). In view of these molecular features of CHO-EPO, it seems unlikely that the stabilizing effect of the *N*-glycosylation on the protein conformation is largely attributable to one or two particular *N*-glycans, although the information at hand is not conclusive.

Polysaccharides can generate areas of hydrophobic surface through alignment of the hydrophobic patch 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 (*36*), and the existence of the hydrophobic acetylamino groups of amino sugar residues. Further, Yano *et al.* revealed that the hydrophobicity of carbohydrates is determined by a set of factors including the CH-dense surface area, degrees of polymerization and branching, molecular planarity, *etc.* (*36*). The hydrophobic surface thus formed is similar to the interior of the cyclodextrin cavity, which is well known to accommodate aliphatic and aromatic hydrocarbons. It seems probable, therefore, that highly branched *N*-glycans have hydrophobic regions extensively stretching across the plane holding acetylamino groups.

The most probable conclusion from the results and arguments presented here is that the inner regions including the galactose residues of the highly branched *N*-glycans stabilize the protein conformation through their interaction with the hydrophobic surface areas mainly made up of non-aromatic hydrocarbon groups. In connection with this conclusion, it is particularly interesting to note the function of the *N*-glycans of human serum IgG. It has been reported that hypogalactosylation of the complex-type *N*-glycans conserved at Asn297 in the CH2 domain of IgG is associated with decline of the IgG function in some intractable diseases (*37*–*39*) and aging (*40*), and that the nonreducing terminal galactose residues of the *N*-glycans play important roles in binding of IgG to C1q and the Fc receptors (*36*). From this information and the results obtained here, it may be said that the galactosyl deficiency weakens the *N*-glycan–protein hydrophobic interaction, resulting in a less stable conformation of Fc portion insufficient to keep its full activity.

It has been revealed that both intra- and extramolecular high-mannose type *N*-glycans directly promote protein folding (*1*–*5*), and that they play critical roles in the subunit assembly and retention of the subunit structure of oligomeric proteins (*1*, *5*, *41*). On the other hand, complex-type *N*-glycans, as described here, seem to stabilize protein conformation through tranquilizing hydrophobic protein surfaces unfavorable for protein stability. Such newly acquired information on the *N*-glycan roles associated with protein structure, together with the recent findings that free oligosaccharides of both the high-mannose and complex types are widespread in animal (*42*–*44*) and plant cells (*45*), indicate the need to consider the influence of *N*-glycans in discussing protein conformation.

We wish to express our gratitude to Ms. C. Yagi for her continued encouragement and support.

REFERENCES

1. Nagai, K., Shibata, K., and Yamaguchi, H. (1993) Role of intramolecular high-mannose chains in the folding and assembly of soybean (*Glycine max*) lectin polypeptides: Studies by the combined use of spectroscopy and gel-filtration size-analysis. *J. Biochem.* **114**, 830–834
2. Yamaguchi, H. and Uchida, M. (1996) A chaperone-like function of intramolecular high-mannose chains in the oxidative refolding of bovine pancreatic RNase B. *J. Biochem.* **120**, 474–477
3. Nishimura, I., Uchida, M., Inohana, Y., Setoh, K., Daba, K., Nishimura, S., and Yamaguchi, H. (1998) Oxidative refolding of bovine pancreatic RNases A and B promoted by Asn-glycans. *J. Biochem.* **123**, 516–520
4. Kimura, N., Uchida, M., Nishimura, S., and Yamaguchi, H. (1998) Promotion of polypeptide folding by interactions with Asn-glycans. *J. Biochem.* **124**, 857–862
5. Masaoka, H., Shibata, K., and Yamaguchi, H. (1999) Topological and functional characterization of the *N*-glycans of soybean (*Glycine max*) agglutinin. *J. Biochem.* **126**, 212–217
6. Grafl, R., Lang, K., Vogl, H., and Schmidt, F.X. (1987) The mechanism of folding of pancreatic ribonuclease is independent of the presence of covalently linked carbohydrate. *J. Biol. Chem.* **262**, 10624–10629
7. Wyss, D.F., Withka, J.M., Knoppers, M.H., Sterne, K.A., Recny, M.A., and Wagner, G. (1993) ^{1}H resonance assignments and secondary structure of the 13.6 kDa glycosylated adhesion domain of human CD2. *Biochemistry* **32**, 10995–11006
8. Meldgaard, M. and Svendsen, I. (1994) Different effects of *N*-glycosylation on the thermostability of highly homologous bacterial (1,3-1,4)-β-glucanases secreted from yeast. *Microbiology* **140**, 159–166
9. Wyss, D.F., Choi, J.S., Li, J., Knoppers, M.H., Willis, K.J., Arulanandam, A.R.N., Smolyar, A., Reinherz, E.L., and Wagner, G. (1995) Conformation and function of the *N*-linked glycan in the adhesion domain of human CD2. *Science* **269**, 1273–1278
10. Wang, C., Eufemi, M., Turano, C., and Giartosio, A. (1996) Influence of the carbohydrate moiety on the stability of glycoproteins. *Biochemistry* **35**, 7299–7307
11. Graber, S.E. and Krantz, S.B. (1978) Erythropoietin and the control of red cell production. *Annu. Rev. Med.* **29**, 51–66
12. Krantz, S.B. and Goldwasser, E. (1984) Specific binding of erythropoietin to spleen cells infected with the anemia strain of Friend virus. *Proc. Natl. Acad. Sci. USA* **81**, 7574–7578
13. Sasaki, H., Bothner, B., Dell, A., and Fukuda, M. (1987) Carbohydrate structure of erythropoietin expressed in Chinese hamster ovary cells by a human erythropoietin cDNA. *J. Biol. Chem.* **262**, 12059–12076
14. Takeuchi, M., Takasaki, S., Miyazaki, H., Kato, T., Hoshi, S., Kochibe, N., and Kobata, A. (1988) Comparative study of the asparagine-linked sugar chains of human erythropoietins purified from urine and the culture medium of recombinant Chinese hamster ovary cells. *J. Biol. Chem.* **263**, 3657–3663
15. Tsuda, E., Goto, M., Murakami, A., Akai, K., Ueda, M., Kawanishi, G., Takahashi, N., Sasaki, R., Chiba, H., Ishihara, H., Mori, M., Tejima, S., Endo, S., and Arata, Y. (1988) Comparative structural study of *N*-linked oligosaccharides of urinary and recombinant erythropoietins. *Biochemistry* **27**, 5646–5654
16. Tsuda, E., Kawanishi, G., Ueda, M., Masuda, S., and Sasaki, R. (1990) The role of carbohydrate in recombinant human erythropoietin. *Eur. J. Biochem.* **188**, 405–411
17. Dordal, M.S., Wang, F.F., and Goldwasser, E. (1985) The role of carbohydrate in erythropoietin action. *Endocrinology* **116**, 2293–2299
18. Narhi, L.O., Arakawa, T., Aoki, K.H., Elmore, R., Rohde, M.F., Boone, T., and Strickland, T.W. (1991) The effect of carbohydrate on the structure and stability of erythropoietin. *J. Biol. Chem.* **266**, 23022–23026

19. Philo, J.S., Aoki, K.H., Arakawa, T., Narhi, L.O., and Wen, J. (1996) Dimerization of the extracellular domain of the erythropoietin (EPO) receptor by EPO: One high-affinity and one low-affinity interaction. *Biochemistry* **35**, 1681–1691
20. Laemmli, U.K. (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
21. Chadli, A., Caron, M., Ticha, M., Joubert, R., Bladier, D., and Kocourek, J. (1992) Development of screening methods for detection of carbohydrate-binding proteins by use of soluble glycosylated polyacrylamide-based copolymers. *Anal. Biochem.* **204**, 198–203
22. Baenziger, J.U. and Fiete, D. (1979) Structural determinants of *Ricinus communis* agglutinin and toxin specificity for oligosaccharides. *J. Biol. Chem.* **254**, 9795–9799
23. Goldstein, I.J. and Hayes, C.E. (1978) The lectins: carbohydrate-binding proteins of plants and animals. *Adv. Carbohydr. Chem. Biochem.* **35**, 127–340
24. Poretz, R.D. and Goldstein, I.J. (1970) An examination of the topography of the saccharide binding sites of concanavalin A and of the forces involved in complexation. *Biochemistry* **9**, 2890–2896
25. Hagihara, Y., Aimoto, S., Fink, A.L., and Goto, Y. (1993) Guanidine hydrochloride-induced folding of proteins. *J. Mol. Biol.* **231**, 180–184
26. Hamada, D. and Goto, Y. (1997) The equilibrium intermediate of β-lactoglobulin with non-native α-helical structure. *J. Mol. Biol.* **269**, 479–487
27. Cheetham, J.C., Smith, D.M., Aoki, K.H., Stevenson, J.L., Hoeffel, T.J., Syed, R.S., Egrie, J., and Harvey, T.S. (1998) NMR structure of human erythropoietin and a comparison with its receptor bound conformation. *Nat. Struct. Biol.* **5**, 861–866
28. Stryer, L. (1965) The interaction of a naphthalene dye with apomyoglobin and apohemoglobin. A fluorescent probe of nonpolar binding sites. *J. Mol. Biol.* **13**, 482–495
29. Davis, J.M., Arakawa, T., Strickland, T.W., and Yphantis, D.A. (1987) Characterization of recombinant human erythropoietin produced in Chinese hamster ovary cells. *Biochemistry* **26**, 2633–2638
30. Yamaguchi, H., Nishiyama, T., and Uchida, M. (1999) Binding affinity of *N*-glycans for aromatic amino acid residues: Implications for novel interactions between *N*-glycans and proteins. *J. Biochem.* **126**, 261–265
31. Nishiyama, T., Kimura, N., Jitsuhara, Y., Uchida, M., Ochi, F., and Yamaguchi, H. (2000) *N*-Glycans protect proteins from protease digestions through their binding affinities for aromatic amino acid residues. *J. Biochem.* **127**, 427–433
32. Endo, Y., Nagai, H., Watanabe, Y., Ochi, K., and Takagi, T. (1992) Heat-induced aggregation of recombinant erythropoietin in the intact and deglycosylated states as monitored by gel permeation chromatography combined with a low-angle laser light scattering technique. *J. Biochem.* **112**, 700–706
33. Yue, K. and Dill, K.A. (1995) Forces of tertiary structural organization in globular proteins. *Proc. Natl. Acad. Sci. USA* **92**, 146–150
34. Higuchi, M., Oheda, M., Kuboniwa, H., Tomonoh, K., Shimonaka, Y., and Ochi, N. (1992) Role of sugar chains in the expression of the biological activity of human erythropoietin. *J. Biol. Chem.* **267**, 7703–7709
35. Sasaki, H., Ochi, N., Dell, M., and Fukuda, M. (1988) Site specific glycosylation of human recombinant erythropoietin: analysis of glycopeptides or peptides at each glycosylation site by fast atom bombardment mass spectrometry. *Biochemistry* **27**, 8618–8626
36. Yano, Y., Tanaka, K., Doi, Y., and Janado, M. (1988) The polystyrene affinity of methylglycosides, deoxysugars and glucooligosaccharides. *J. Solution Chem.* **17**, 347–358
37. Parekh, P.B., Dwek, R.A., Sutton, B.J., Fernandes, D.L., Leung, A., Stanworth, D., Rademacher, T.W., Mizuochi, T., Taniguchi, K., Matsuta, K., Takeuchi, Y., Nagano, T., Miyamoto, T., and Kobata, A. (1985) Association of rheumatoid arthritis and primary osteoarthritis with changes in the glycosylation pattern of total serum IgG. *Nature* **316**, 452–457
38. Wormald, M.R., Rudd, P.M., Harvey, D.J., Chang, S.-C., Scragg, I.G., and Dwek, R.A. (1997) Variations in oligosaccharide-protein interactions in immunoglobulin G determine the site-specific glycosylation profiles and modulate the dynamic motion of the Fc oligosaccharides. *Biochemistry* **36**, 1370–1380
39. Tomana, M., Schrohenloher, R.E., Koopman, W.J., Alarcon, G.S., and Paul, W.A. (1988) Abnormal glycosylation of serum IgG from patients with chronic inflammatory diseases. *Arthritis Rheum.* **31**, 333–338
40. Parehk, R., Roitt, I., Isenberg, D., Dwek, R.A., and Rademacher, T. (1988) Age-related galactosylation of the *N*-linked oligosaccharides of human serum IgG. *J. Exp. Med.* **167**, 1731–1736
41. Kimura, Y., Hess, D., and Sturm, A. (1999) The *N*-glycans of jack bean α-mannosidase structure, topology and function. *Eur. J. Biochem.* **264**, 168–175
42. Cacan, R., Dengremont, C., Labiau, O., Kmiecik, D., Mir, A.-M., and Verbert, A. (1996) Occurrence of a cytosolic neutral chitobiase activity involved in oligomannoside degradation: a study with Madin Darby bovine kidney (MDBK) cells. *Biochem. J.* **313**, 597–602
43. Ohashi, S., Iwai, K., Mega, T., and Hase, S. (1999) Quantitation and isomeric structure analysis of free oligosaccharides present in the cytosol fraction of mouse liver: Detection of a free disialo biantennary oligosaccharide and glucosylated oligomannosides. *J. Biochem.* **126**, 852–858
44. Taguchi, T., Kitajima, K., Muto, Y., Inoue, S., Khoo, K.H., Morris, H.R., Dell, A., Wallace, R.A., Selman, K., and Inoue, Y. (1995) Occurrence and structural analysis of highly sulfated multiantennary *N*-linked glycan chains derived from a fertilization-associated carbohydrate rich glycoprotein in unfertilized eggs of *Tribolodon hakonensis*. *Eur. J. Biochem.* **238**, 357–367
45. Kimura, Y. and Matsuo, S. (2000) Free *N*-glycans already occur at an early stage of seed development. *J. Biochem.* **127**, 1013–1019