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Binding Affinity of *N*-Glycans for Aromatic Amino Acid Residues: Implications for Novel Interactions between *N*-Glycans and Proteins[1]

Haruki Yamaguchi,[2] Tomoko Nishiyama, and Makoto Uchida

Department of Applied Biological Chemistry, College of Agriculture, Osaka Prefecture University, Gakuen-cho 1-1, Sakai, Osaka 599-8531

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This study advances direct evidence of the binding affinity of *N*-glycans for aromatic amino acid residues. The intrinsic fluorescence intensities of bovine pancreatic RNase A, bovine α-lactalbumin, and aromatic amino acids were markedly depressed in solutions (1 mM or so) of free *N*-glycans of both the high-mannose and complex types. In addition, free *N*-glycans inhibited the chemical modifications of the solvent-exposed tyrosine and tryptophan residues of these proteins, confirming the affinity of *N*-glycans for aromatic amino acid residues. The results are discussed in connection with the roles of *N*-glycans in novel interactions between *N*-glycans and proteins.

Key words: aromatic amino acid, glycoprotein, *N*-glycan, *N*-glycan function, *N*-glycan-protein interaction.

The stabilizing effect of *N*-glycosylation on protein structure has been reported for several glycoproteins (*1-5*). On the other hand, we recently revealed that the intramolecular *N*-glycans directly promote the refolding of reductively denatured bovine pancreatic RNase B, and that extramolecular free *N*-glycans of both the high-mannose and complex types also markedly stimulate the oxidative refolding of bovine pancreatic RNase A, hen egg-white lysozyme, and bovine α-lactalbumin (LA) (*6-8*). Although these facts implied some affinity of *N*-glycans for proteins, little is known about the molecular basis for the interaction between them. In the above studies, we noticed that extramolecular free *N*-glycans depress the intrinsic fluorescence intensity of proteins. This finding prompted us to determine whether or not *N*-glycans have any affinity for proteins. This report presents direct evidence that *N*-glycans have affinity for aromatic amino acid residues.

RNase A (type IIIA), LA (type III, Ca^{2+}-depleted), and GlcNAc-Asn were purchased from Sigma, and RNase A was purified as previously described (*6*). *N*-Linked high-mannose type glycans, Manα1-6(Manα1-3)Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-Asn (M5-Asn) and Manα1-2Manα1-6(Manα1-2Manα1-3)Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-Asn (M9-Asn), were obtained from ovalbumin and soybean agglutinin, respectively, as described previously (*6, 9*). A mixture of *N*-linked biantennary glycans, ±NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-6(±NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(±Fucα1-6)GlcNAc-Asn (CII-Asn), was prepared by repeated pronase digestion of human apo-transferrin (Nacalai Tesque), followed by gel-filtration on a Sephadex G-25 column. Another mixture of *N*-linked triantennary glycans, ±NeuAcα2-3Galβ1-4GlcNAcβ1-4(±NeuAcα2-6Galβ1-4GlcNAcβ1-2)Manα1-6(±NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-Asn (CIII-Asn), was prepared from bovine serum fetuin (Seikagaku Kogyo) just as described for CII-Asn after being treated with alkaline borohydride to remove *O*-glycans (*10*). *N*-Glycans devoid of asparagine residues were prepared by pepsin digestion of the glycoproteins followed by glycopeptidase A digestion of the resulting glycopeptides as described previously (*9*). Yeast mannan was extracted and purified from baker's yeast (*Saccharomyces cerevisiae*; Oriental Yeast) (*11*). Dextran was a product of Pharmacia, and α-cyclodextrin, L-tyrosine, L-tryptophan, and L-phenylalanine were from Wako Pure Chem. *N*-Acetylimidazole was purchased from Nacalai Tesque, and *N*-bromosuccinimide was from Aldrich. The protein concentrations of RNase A and LA were calculated using $\varepsilon_{280}=9{,}800\ M^{-1}\cdot cm^{-1}$ (*12*) and $\varepsilon_{280}=28{,}500\ M^{-1}\cdot cm^{-1}$ (*13*), respectively. Fluorescence measurements were performed with a Shimadzu RF-1500 fluorescence spectrophotometer at 25°C. Tyrosyl, tryptophyl, and phenylalanyl fluorescence spectra were recorded with excitation at 268, 280, and 248 nm, respectively.

The fluorescence intensities of RNase A and LA were depressed in 1 mM solutions of free *N*-glycans of both the high-mannose and complex types (Fig. 1). The *N*-glycans of

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

Abbreviations: CII-Asn, ±NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-6(±NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(±Fucα1-6)GlcNAc-Asn; CIII-Asn, ±NeuAcα2-3Galβ1-4GlcNAcβ1-4(±NeuAcα2-6Galβ1-4GlcNAcβ1-2)Manα1-6(±NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-Asn; LA, bovine α-lactalbumin; M5-Asn, Manα1-6(Manα1-3)Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-Asn; M9-Asn, Manα1-2Manα1-6(Manα1-2Manα1-3)Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-Asn.

the complex type, particularly CIII-Asn, markedly affected the fluorescence intensities, and the high-mannose type one, M9-Asn, was less effective. As shown in Fig. 2, the effects of these *N*-glycans increased with their concentrations, and the smallest *N*-glycan, GlcNAc-Asn, seemed slightly effective, whereas sucrose exhibited no detectable effect at all. The *N*-glycans devoid of asparagine residues also depressed the protein fluorescence, similarly to the "normal" *N*-glycans (data not shown). To determine whether or not the depressive effect on protein fluorescence is characteristic of *N*-glycans, some other common carbohydrates were examined as to their effects on the fluorescence of RNase A and LA. As summarized in Fig. 3, the common carbohydrates, but not yeast mannan, had no significant effect on the fluorescence. The considerable effect of yeast mannan is not unexpected because this polysaccharide is a high-mannose type *N*-glycan. These results, together with the facts that three of the six tyrosine residues of RNase A (*14, 15*) and two of the four tryptophan residues of LA (*16*) are accessible to a solvent under native conditions, suggest that the *N*-glycans interact with the solvent-exposed aromatic amino acid residues of RNase A and LA through their affinities attributable to their glycan structures.

To gain more insight into the interaction between the *N*-glycans and the aromatic amino acid residues, the *N*-glycans were examined as to their effects on the fluorescence of free aromatic amino acids. CIII-Asn markedly depressed the fluorescence intensities of the amino acids, whereas CII-Asn and M9-Asn were less effective, and GlcNAc-Asn was only slightly effective, as with RNase A and LA (Fig. 4). Common carbohydrates (see Fig. 3), other than yeast mannan and α-cyclodextrin, had no significant effect on these amino acids (data shown only for sucrose). α-Cyclodextrin was effective for tyrosine and phenylalanine fluorescence, but not for tryptophan fluorescence (data not shown). The indole group of tryptophan appears too large to be included in the cavity of α-cyclodextrin. The lack of a detectable effect of α-cyclodextrin on the tyrosine fluorescence of RNase A could be explained by steric hindrance of the protein moiety as to the complexing of the phenolic groups with α-cyclodextrin molecules. These results obtained for free aromatic amino acids firmly support the idea that the *N*-glycans directly interact with the aromatic amino acid residues of RNase A and LA.

This type of fluorescence quenching, however, does not seem surprising. For example, a fluorescence intensity decrease was previously observed for the complexing of *Rhizopus* glucoamylase with its substrate maltooligosaccharide (*17, 18*). A tryptophan residue located at the catalytic subsite, where substrates tightly complex with the enzyme protein, has been considered responsible for this fluorescence intensity decrease.

Corroborative evidence for the direct interactions between the *N*-glycans and the aromatic side chains came from the influence of the *N*-glycans on the chemical

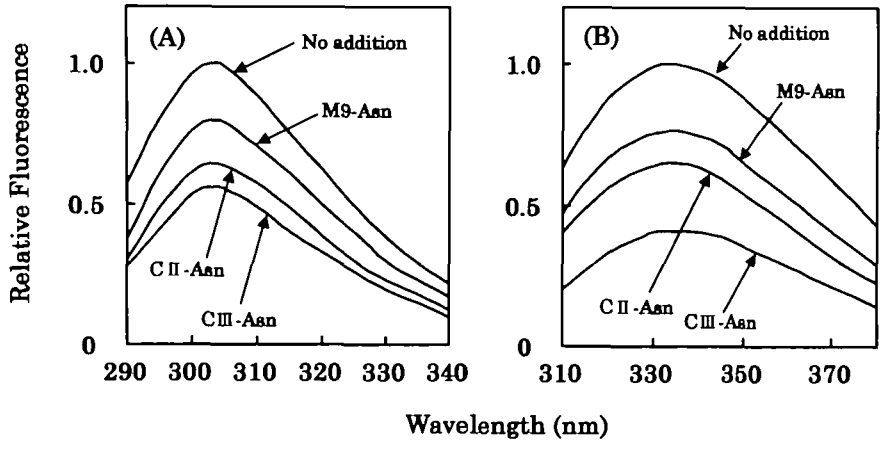

Fig. 1. **Effects of *N*-glycans on the intrinsic fluorescence of RNase A (A) and LA (B).** Spectra of the tyrosyl fluorescence of RNase A (3.3 μM protein concentration) and tryptophyl fluorescence of LA (5.0 μM protein concentration) were recorded after 10 min incubation with 1 mM of an *N*-glycan or without any of the *N*-glycans in 0.1 M Tris-HCl, pH 7.8. The data in (A) and (B) are representative of three to five similar experiments. See the text for more details.

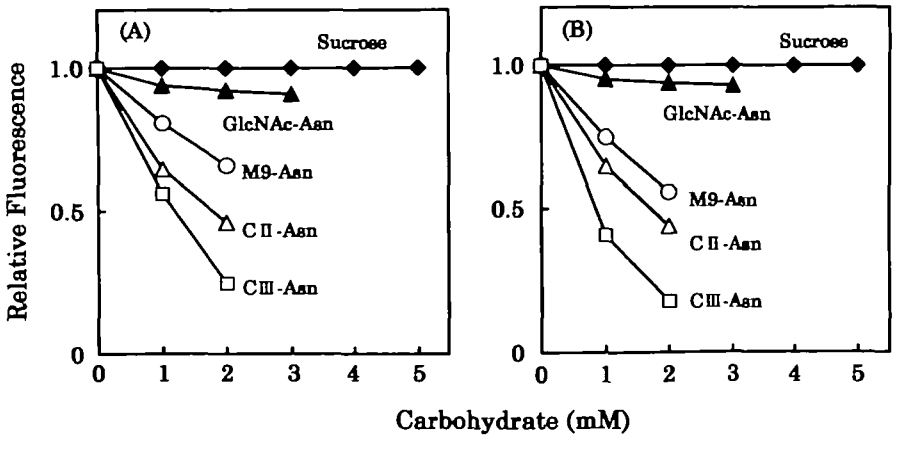

Fig. 2. **The dependence of the fluorescence intensities of RNase A (A) and LA (B) on the carbohydrate concentrations.** Fluorescence measurements were performed with a carbohydrate at the indicated concentrations. Individual points represent the means of at least three independent measurements differing by less than 10%. See the legend to Fig. 1 for more details.

modifications of the tyrosine and tryptophan residues of RNase A and LA, respectively. The earlier studies revealed that three tyrosine residues of RNase A and two tryptophan residues of LA are quantitatively modified with *N*-acetylimidazole and *N*-bromosuccinimide, respectively, under mild conditions (*14, 16*). The *N*-glycans, especially CIII-Asn, distinctly inhibited the chemical modifications of the tyrosine and tryptophan residues, and their inhibitory effects increased with their concentrations (Fig. 5). GlcNAc-Asn showed weak inhibition although it had only a slight effect on protein fluorescence (Fig. 2). Similar effects on the chemical modifications of the tyrosine and trypto-

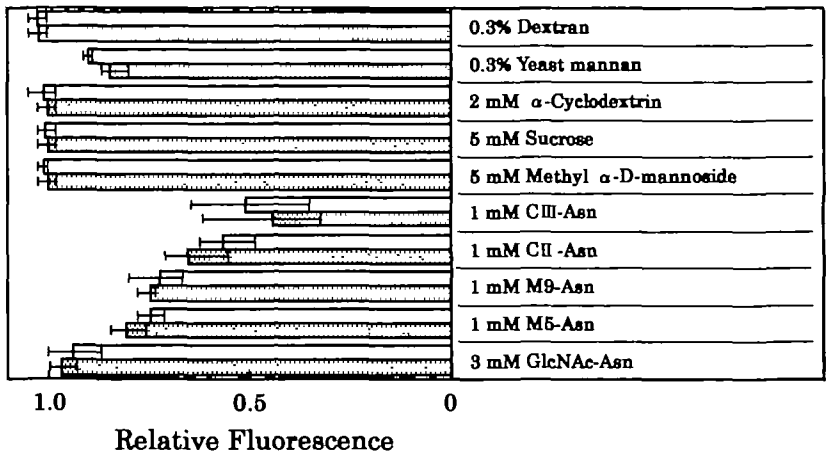

Fig. 3. **Depressing effects of various carbohydrates on the fluorescence intensities of RNase A and LA.** RNase A (open bars) and LA (shaded bars) were incubated for 10 min with a carbohydrate at the indicated concentrations, and then fluorescence spectra were recorded. The fluorescence intensities represent the means of at least three independent measurements, and the data are expressed as relative fluorescence, where 1 corresponds to the fluorescence intensities of RNase A and LA estimated without any of these carbohydrates. Error bars show the maximum and minimum values of the repeated measurements. See the legend to Fig. 1 for more details.

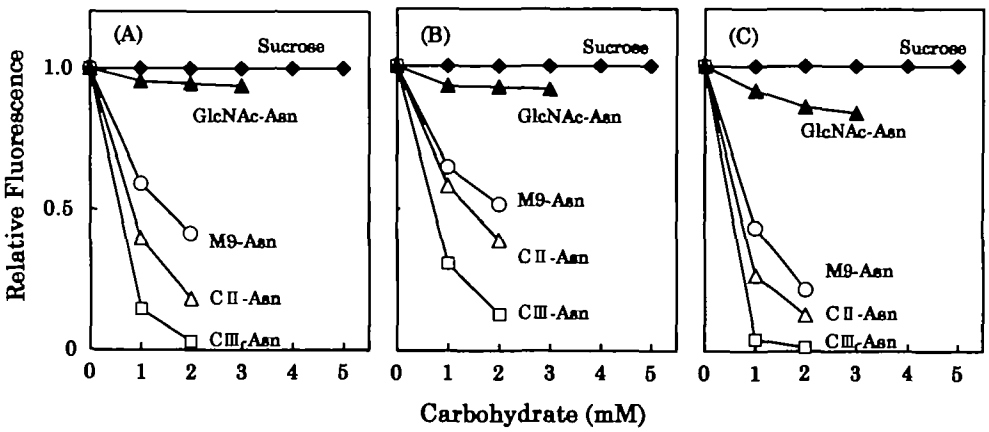

Fig. 4. **The dependence of the fluorescence intensities of aromatic amino acids on the carbohydrate concentrations.** Emission spectra of tyrosine (20 μM) (A), tryptophan (10 μM) (B), and phenylalanine (30 μM) (C) were recorded after 10 min incubation with a carbohydrate at the indicated concentrations. Individual points represent the means of at least three independent measurements differing by less than 8%. See the legend to Fig. 1 for more details.

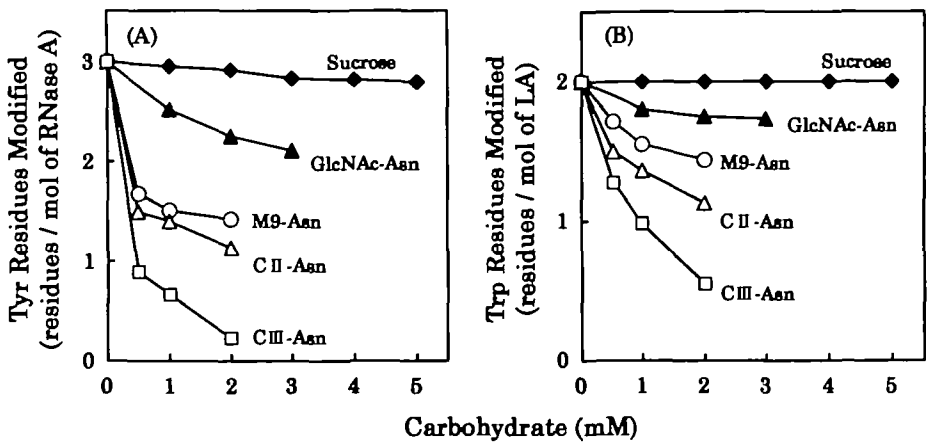

Fig. 5. **Effects of *N*-glycans on the chemical modifications of tyrosine and tryptophan residues.** (A) *O*-Acetylation of tyrosyl residues with *N*-acetylimidazole was performed essentially as described previously (*14*). RNase A (0.33 mM final protein concentration) was incubated with a 180-fold molar excess of *N*-acetylimidazole in 10 mM Tris-HCl (pH 7.5) at 25°C for 1 h, and then the acetylated RNase A was purified on a Sephadex G-25 column (1×28 cm) developed with the same buffer. The amount of tyrosyl residues modified was determined by the method of Balls and Wood (*19*) using 50 mM hydroxylamine hydrochloride at pH 7.5 and 25°C overnight. Individual points represent the means of at least four independent measurements differing by less than 10%. (B) Oxidation of tryptophyl residues with *N*-bromosuccinimide was performed essentially as described previously (*16*). LA (24 μM final protein concentration) was incubated with a 17-fold molar excess of *N*-bromosuccinimide in 0.1 M sodium acetate buffer (pH 6.0) at 25°C for 20 min. The amount of tryptophyl residues modified was estimated from the decrease in absorbance at 280 nm (*20*). Individual points represent the means of at least three independent measurements differing by less than 6%.

phan residues were observed for the *N*-glycans devoid of asparagine residues (data not shown). In contrast, common carbohydrates (see Fig. 3), other than yeast mannan and α-cyclodextrin, had no significant influence on the reactions (data shown only for sucrose). The increasing order of the effects of the *N*-glycans was the same as that found for their depressive effects on the fluorescence intensity.

Thus there seems no doubt that *N*-glycans have affinity for the side chains of aromatic amino acid residues. We must now consider the following questions: What kind of affinity exists between *N*-glycans and aromatic groups? How is the effect of *N*-glycans related to their structures? Are the interactions between *N*-glycans and aromatic groups associated with the folding and stability of proteins? We advance the following arguments regarding these questions, although the data at hand are not sufficient for any definite conclusion.

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, polysaccharides can generate areas of hydrophobic surfaces, *i.e.*, CH-dense surfaces, which are capable of complexing nonpolar solutes (*21*). In fact, both aromatic and aliphatic hydrocarbons are known to have a considerable affinity for water-swollen polysaccharides such as Sephadex, Sepharose, and cellulose (*22, 23*). As revealed by Yano *et al.* (*21*), the polystyrene affinity of carbohydrates is determined by a set of factors including the CH-dense surface area, degree of polymerization (*i.e.*, formation of glycosidic linkages and disappearance of hydroxyl groups), molecular planarity, *etc.* Besides, the hydrophobic acetylamino groups of aminosugar residues also should increase the polystyrene affinity of carbohydrates. On the basis of these lines of evidence, it may well be said that highly branched *N*-glycans have hydrophobic regions similar to the interior of the cyclodextrin cavity, which is known to accomodate aliphatic and aromatic hydrocarbons.

N-Glycans of both the high-mannose and complex types directly promote the refolding of reductively denatured proteins (*6-8*), and of the *N*-glycans tested CIII-Asn is especially effective in promoting protein refolding (Yamaguchi, H. and Kimura, N., unpublished results). Interestingly it seems likely that the increasing order of the affinities of the *N*-glycans for aromatic groups parallels that of their promotive effects on protein refolding. In addition, aromatic amino acid residues have been shown to frequently play crucial roles in protein folding, as exemplified by the studies on RNase A (*14, 24*). Thus it might be possible that *N*-glycans promote protein folding through their interactions with aromatic amino acid residues. Furthermore, the stabilizing effect of *N*-glycans on protein conformation also might be attributed to their affinities for aromatic amino acid residues. Furthermore, *N*-glycans have been shown to be widely involved in various cell-cell interactions including infections (*25*). In many of these cases, however, we are still in the dark as to the mechanisms underlying the roles of the *N*-glycans. It is possible that some of these interactions may be interpreted by the affinity of *N*-glycans for the aromatic amino acid residues of cell surface proteins.

In any event, this study advanced for the first time evidence of the affinity of *N*-glycans for amino acid residues of a particular kind, though it is uncertain whether or not aromatic side chains alone interact with *N*-glycans. A more detailed study including some physicochemical analyses is required for a full understanding of this function of *N*-glycans.

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REFERENCES

1. Grafl, R., Lang, K., Vogl, H., and Schmidt, F.X. (1987) The mechanism of folding of pancreatic ribonucleases is independent of the presence of covalently linked carbohydrate. *J. Biol. Chem.* **262**, 10624-10629
2. 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
3. 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
4. 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
5. 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
6. Yamaguchi, H. and Uchida, M. (1996) A chaperone-like function of intramolecular high-mannose type chains in the oxidative refolding of bovine pancreatic RNase B. *J. Biochem.* **120**, 474-477
7. 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
8. Kimura, N., Uchida, M., Nishimura, S., and Yamaguchi, H. (1998) Promotion of polypeptide folding by interactions with Asn-glycans. *J. Biochem.* **124**, 857-862
9. 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
10. Spiro, R.G. and Bhoyroo, V.D. (1974) Structure of the O-glycosidically linked carbohydrate units of fetuin. *J. Biol. Chem.* **249**, 5704-5717
11. Peat, S., Whelan, W.J., and Edwards, T.E. (1961) Polysaccharides of baker's yeast. Part IV. Mannan. *J. Chem. Soc.* **61**, 29-34
12. Tsong, T.Y., Hearn, R.P., Wrathall, D.P., and Sturtevant, J.M. (1970) A calorimetric study of thermally induced conformational transition of ribonuclease A and certain of its derivatives. *Biochemistry* **9**, 2666-2677
13. Ewbank, J.J. and Creighton, T.E. (1993) Structural characterization of the disulfide folding intermediates of bovine α-lactalbumin. *Biochemistry* **32**, 3694-3707
14. Simpson, R.T. and Vallee, B.L. (1966) Side-chain cotton effects of ribonuclease. *Biochemistry* **5**, 2531-2538
15. Egan, W., Shindo, H., and Cohen, J.S. (1978) On the tyrosine residues of ribonuclease A. *J. Biol. Chem.* **253**, 16-17
16. Bell, J.E., Castellino, F.J., Trayer, I.P., and Hill, R.L. (1975) Modification of bovine α-lactalbumin with N-bromosuccinimide and 2-hydroxy-5-nitrobenzylbromide. *J. Biol. Chem.* **250**, 7579-7585
17. Hiromi, K., Tanaka, A., and Ohnishi, M. (1982) Fluorometric studies on the binding of gluconolactone, glucose, and glucosides to the subsites of glucoamylase. *Biochemistry* **21**, 102-107
18. Tanaka, A., Yamashita, T., Ohnishi, M., and Hiromi, K. (1983)

Steady-state and transient kinetic studies on the binding of maltooligosaccharides to glucoamylase. *J. Biochem.* **93**, 1037-1043
19. Balls, A.K. and Wood, H.N. (1956) Acetyl chymotrypsin and its reaction with ethanol. *J. Biol. Chem.* **219**, 245-256
20. Spande, T.F. and Witkop, B. (1963) Determination of the tryptophan content of proteins with N-bromosuccinimide. *Methods Enzymol.* **11**, 498-506
21. Yano, Y., Tanaka, K., Doi, Y., and Janado, M. (1988) The polystyrene affinity of methylglycosides, deoxysugars and glucooligosaccharides. *J. Solution Chem.* **17**, 347-358
22. Fujita, T., Suzuki, Y., Yamauchi, J., Takagahara, I., Fujii, K., Yamashita, J., and Horio, T. (1980) Chromatography in presence of high concentrations of salts on columns of celluloses with and without ion exchange groups (hydrogen bond chromatography). Its application to purification of yeast enzyme. *J. Biochem.* **87**, 89-100
23. Leicht, W. and Pundak, S. (1981) Large-scale purification of halophilic enzymes by salting-out mediated chromatography. *Anal. Biochem.* **114**, 186-192
24. Juminaga, D., Wedemeyer, W.J., Garduno-Juarez, R., McDonald, M.A., and Scheraga, H.A. (1997) Tyrosyl interactions in the folding and unfolding of bovine pancreatic ribonuclease A: A study of tyrosine-to-phenylalanine mutants. *Biochemistry* **36**, 10131-10145
25. Varki, A. (1993) Biological roles of oligosaccharides: all of the theories are correct. *Glycobiology* **3**, 97-130