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Promotion of Polypeptide Folding by Interactions with Asn-Glycans[1]

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We have recently revealed that the intramolecular Asn-glycans promote the refolding of reductively denatured bovine pancreatic RNase B, and that extramolecular Asn-glycans of both high-mannose and complex types also markedly stimulate the oxidative refolding of RNase B and its nonglycosylated form, RNase A [Yamaguchi, H. and Uchida, M. (1996) *J. Biochem.* 120, 474-477; Nishimura *et al.* (1998) *J. Biochem.* 123, 516-520]. The present investigation was undertaken to see whether this function of Asn-glycans is specific to the refolding of pancreatic RNases; *i.e.*, extramolecular Asn-glycans were examined for their effects on the oxidative refolding of hen egg white lysozyme and bovine α-lactalbumin by monitoring changes in activity, dynamic volume, intrinsic fluorescence, and affinity for a fluorescent probe, 1-anilino-8-naphthalenesulfonate. Asn-glycans of both high-mannose and complex types markedly stimulated the oxidative refolding of these proteins, giving similar results to those previously obtained with RNases, though differences attributable to the characteristics of individual proteins were observed in the promotive effects. Thus it seems probable that Asn-glycans generally promote the proper folding of denatured polypeptides.

Key words: Asn-glycan, Asn-glycan function, α-lactalbumin, lysozyme, protein folding.

We have recently revealed that the intramolecular high-mannose type Asn-glycans directly promote the refolding of reductively denatured pancreatic RNase B (*1*), and that extramolecular Asn-glycans of both complex and high-mannose types are effective in promoting the oxidative refolding of RNase B and its nonglycosylated form, RNase A (*1, 2*). We were further interested to see whether this function of Asn-glycans is specific to the oxidative refolding of pancreatic RNases. To answer this question, we chose hen egg white lysozyme [EC 3.2.1.17] and bovine α-lactalbumin (LA), which are both nonglycosylated single polypeptides having four disulfide linkages. We expected these proteins to offer several advantages for this work; *i.e.*, their properties including higher-order structures have been well characterized (*3-7*) and their refolding behaviors have been studied in depth (*8-11*). In this study, extramolecular Asn-glycans with different structures were examined for their effects on the oxidative refolding of these proteins by monitoring changes in activity, dynamic volume, intrinsic fluorescence, and affinity for a fluorescent probe, 1-anilino-8-naphthalenesulfonate (ANS). We describe here that Asn-glycans of both high-mannose and complex types markedly promote the proper folding of reductively denatured lysozyme and LA, giving similar results to those previously obtained with RNases (*1, 2*).

MATERIALS AND METHODS

Materials—Hen egg white lysozyme was obtained from Seikagaku Kogyo. LA (Type III), *Micrococcus lysodeikticus* dried cells, 1-anilino-8-naphthalenesulfonate (ANS), and GlcNAc-Asn were purchased from Sigma. Dextran was a product of Pharmacia, and cyclodextrins were from Wako Pure Chem. Yeast mannan was extracted and purified from baker's yeast (*Saccharomyces cerevisiae*; Oriental Yeast) (*12*). Asn-linked high-mannose type oligosaccharides, 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 lectin, respectively, as described previously (*1, 2*). A mixture of Asn-linked biantennary oligosaccharides, $\pm$NeuAcα2-6$\pm$Galβ1-4GlcNAcβ1-2Manα1-6($\pm$NeuAcα2-6$\pm$Galβ1-4GlcNAcβ1-2Manα1-3)($\pm$GlcNAcβ1-4)Manβ1-4GlcNAcβ1-4($\pm$Fucα1-6)GlcNAc-Asn (CII-Asn), was prepared by repeated pronase digestion of human serum IgG (Sigma), followed by gel-filtration on a Sephadex G-25 (fine grade) column. All of the Asn-glycans and other sugars were freed from metal ions by passing through a Chelex 100 (Bio-Rad) column.

Reductive Denaturation and Oxidative Refolding of Lysozyme—Protein concentration of native lysozyme was determined using A_{280}[1%/cm]=26.3 (*13*). A solution of

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Abbreviations: ANS, 1-anilino-8-naphthalenesulfonate; CII-Asn, $\pm$NeuAcα2-6$\pm$Galβ1-4GlcNAcβ1-2Manα1-6($\pm$NeuAcα2-6$\pm$Galβ1-4GlcNAcβ1-2Manα1-3)($\pm$GlcNAcβ1-4)Manβ1-4GlcNAcβ1-4($\pm$Fucα1-6)GlcNAc-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)Manβ1-4GlcNAc β1-4GlcNAc-Asn.

lysozyme (12 mg/ml) in 0.5 M Tris-HCl (pH 8.5) containing 30 mM DTT and 6 M guanidine hydrochloride was incubated for 2 h at 37°C. This sample was assayed for the number of thiol groups by Ellman's method (*14*) immediately after passage through a Sephadex G-25 column (1× 30 cm) eluted with deaerated 0.1 M acetic acid, showing a content of $8.1 \pm 0.1_{eq}$.

For regeneration, the denatured protein 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 (a) 2 mM EDTA, 4 mM GSH, and 0.2 M NaCl; (b) 2.1 μM $CuSO_4$ and 0.2 M NaCl; or (c) 2 mM EDTA, 2 mM GSH, 0.1 mM GSSG, and 0.2 M NaCl] (2.0 μM final protein concentration). After various times of refolding at 25°C, the solution was analyzed. Asn-glycans were added to the refolding buffer when extramolecular Asn-glycans were assayed for their effect on the refolding of reductively denatured lysozyme.

Reductive Denaturation and Oxidative Refolding of LA—Protein concentrations of the native and reductively denatured LA species were calculated using $\varepsilon_{280} = 28{,}500$ $M^{-1} \cdot cm^{-1}$ (*15*) and $\varepsilon_{280} = 27{,}200$ $M^{-1} \cdot cm^{-1}$ (*15*), respectively. Reductively denatured LA was prepared by incubating the native LA (1 mg/0.15 ml) in 0.1 M Tris-HCl (pH 8.5) containing 1 mM EDTA, 0.14 M DTT, 0.2 M KCl, and 6 M guanidine hydrochloride. Immediately after the treatment at 37°C for 2 h, the denatured protein was quickly desalted by passage through a Sephadex G-25 column (0.78×30 cm) eluted with deaerated 1 M acetic acid and then divided into small parts. The lyophilized products were stored at below −20°C in a desiccator over silica gel under nitrogen gas. This sample was assayed for the number of thiol groups by Ellman's method (*14*), showing a content of $7.7 \pm 0.1_{eq}$, which proved to be stable for at least several weeks.

The refolding of LA from the reductively denatured species was carried out under air-oxidation conditions. A reductively denatured LA was quickly dissolved in 1 M acetic acid (45 μl), and 40 μl of the solution was immediately diluted with the refolding buffer, 0.1 M Tris-acetate buffer (pH 8.5, 25°C, 0.96 ml) containing 4 mM GSH, 10 mM $CaCl_2$, and 0.2 M KCl, with vigorous stirring (2.1 μM final protein concentration, and pH 7.6). After various times of air-oxidation at 25°C, the solution was analyzed. Asn-glycans were added to the refolding buffer when extramolecular Asn-glycans were assayed for their effect on the refolding of reductively denatured LA.

Assay of Enzyme Activity—Lysozyme activity was determined at 25°C by following the decrease in absorbance at 540 nm of a 0.12 mg/ml *Micrococcus lysodeikticus* suspension in 64 mM sodium phosphate buffer (pH 6.2).

Fluorescence Measurements—Fluorescence measurements were performed on a Shimadzu RF-1500 fluorescence spectrophotometer at 25°C. Intrinsic tryptophyl fluorescence spectra from 320 to 400 nm were recorded with excitation at 295 nm. Polypeptide refolding was also monitored by ANS binding in the refolding buffer containing 100 μM ANS. ANS fluorescence spectra from 400 to 560 nm were recorded with excitation at 365 nm.

Size-Exclusion HPLC of the Regeneration Products of Reductively Denatured LA—After quenching the regeneration reactions with 2 mM *N*-ethylmaleimide, 20 μl of each solution was applied to a TSK-GEL G2000SW column (0.75×30 cm) (Tosoh) developed with 50 mM Tris-acetate buffer (pH 7.2) at a flow rate of 0.5 ml/min, and the elution of protein was monitored by measuring A_{225}.

RESULTS

Asn-glycans and common carbohydrates with various structures were examined for their promoting effects on the oxidative refolding of lysozyme and LA.

Effects of Asn-Glycans on Refolding of Reductively Denatured Lysozyme—Refolding of reductively denatured lysozyme was performed under nonredox or redox conditions and followed by monitoring activity regain (Fig. 1). The Asn-glycans of both high-mannose and complex types showed remarkable stimulating effects on the oxidative refolding of lysozyme, especially under the nonredox conditions (Fig. 1, A and B), which are unfavorable for the reshuffling of disulfide pairings. It seems probable that larger Asn-glycans of the high-mannose type are more effective than smaller ones, and that complex-type Asn-glycans also exhibit promotive effects. These results are similar to those previously obtained from RNase A refolding with the same Asn-glycans. A distinct depression of protein aggregation, however, was noticed as an initial effect of the Asn-glycans on the oxidative refolding of lysozyme, which readily aggregates in the course of refolding. Further, glutathione tended to raise the refolding yield of lysozyme regardless of the presence of an Asn-glycan in the refolding system, though the role of glutathione in the refolding remains obscure.

To characterize the conformational changes that accompany the regeneration reaction promoted by the Asn-gly-

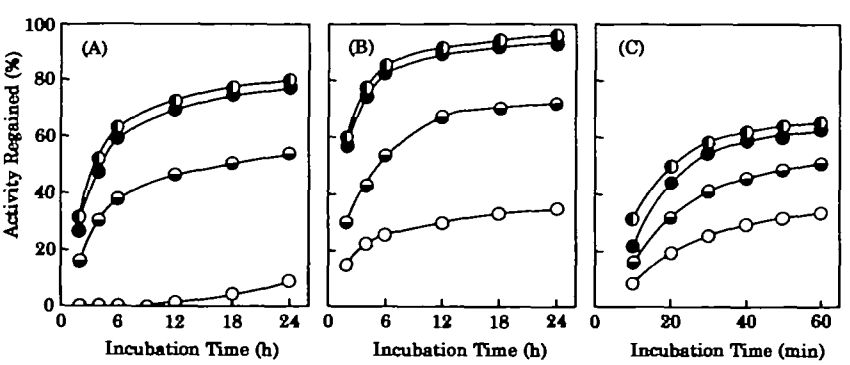

Fig. 1. **Effects of Asn-glycans on the oxidative refolding of reductively denatured lysozyme under various conditions.** Reductively denatured lysozyme was allowed to regenerate with 1 mM concentrations of M5-Asn (◒), M9-Asn (◐), and CII-Asn (●), or without any of these Asn-glycans (○) in 0.1 M Tris-HCl (pH 8.0) containing 2 mM EDTA, 4 mM GSH, and 0.2 M NaCl (A), 2 μM $CuSO_4$, 4 mM GSH, and 0.2 M NaCl (B), or 2 mM GSH, 0.1 M GSSG, and 0.2 M NaCl (C).

cans, the refolding of lysozyme was followed by monitoring the intrinsic fluorescence emission (Fig. 2). The spectral changes are summarized with regard to the fluorescence intensity and wavelength of maximum emission in Fig. 2B. These results strongly suggest that CII-Asn promotes the transformation of unfolded lysozyme molecules into folded species, thus changing the environment of some of the tryptophan residues. Two other Asn-glycans, M5-Asn and M9-Asn, induced similar spectral changes to CII-Asn, though the former was less effective (data not shown), in fair agreement with the observations made for activity regain (Fig. 1A).

These features of refolding were corroborated by using a hydrophobic fluorescent probe, ANS. As revealed earlier, ANS does not normally bind to the fully unfolded state or the native state, but exhibits high affinity for states similar to the "molten globule," a kinetic intermediate appearing in the refolding pathway of proteins (*16*). This state has been defined as a significant secondary structure with moderately high compactness but lacking the rigid tertiary structure of the native protein (*17, 18*). Thus, a decrease in the ANS fluorescence during refolding reflects a decrease in affinity of the protein molecule for the probe and, consequently, the tight packing of the protein tertiary structure. As shown in Fig. 3, the ANS binding reached maximum much more rapidly in the presence of CII-Asn, analogously to the results obtained from intrinsic fluorescence emission. Similar features of ANS binding were also observed for the refolding intermediates formed in the presence of either M5-Asn or M9-Asn (data not shown). It seems probable, therefore, that the extramolecular Asn-glycans of both high-mannose and complex types promote the lysozyme folding into compact, native-like structures.

Effects of Asn-Glycans on Refolding of Reductively Denatured LA—Refolding of reductively denatured LA was performed under air-oxidation conditions and examined by means of size-exclusion HPLC. Figure 4 shows the elution patterns of LA refolded in the presence or absence of 1 mM CII-Asn, which include only one protein peak at the position corresponding to the native LA. Small compounds such as GSH and *N*-ethylmaleimide gave peaks near the total available volume of the column. Peaks arising from fully *S*-alkylated LA and the refolding intermediates were not detected. Because of their high hydrophobicity, it seems likely that they were retained on the sample filters and/or HPLC column. It may be said from Fig. 4 that the denatured LA gradually regained its native or native-like conformation, and that CII-Asn greatly enhanced its refolding rate. Under redox conditions, however, the effect of CII-Asn on the LA refolding rate was not detected by size-exclusion HPLC, probably owing mainly to a rapid reshuffling of disulfide pairings of the refolding intermediates. LA, a calcium-binding protein, required calcium for

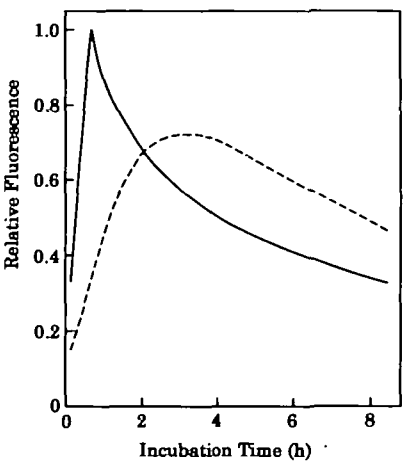

Fig. 3. **Refolding of reductively denatured lysozyme monitored by the change in ANS binding.** Reductively denatured lysozyme was allowed to regenerate with (solid line) or without (dashed line) 1 mM CII-Asn, and then the refolding was followed by monitoring ANS fluorescence emission. The data are expressed as relative fluorescence, where 1 corresponds to the estimated maximum change in fluorescence.

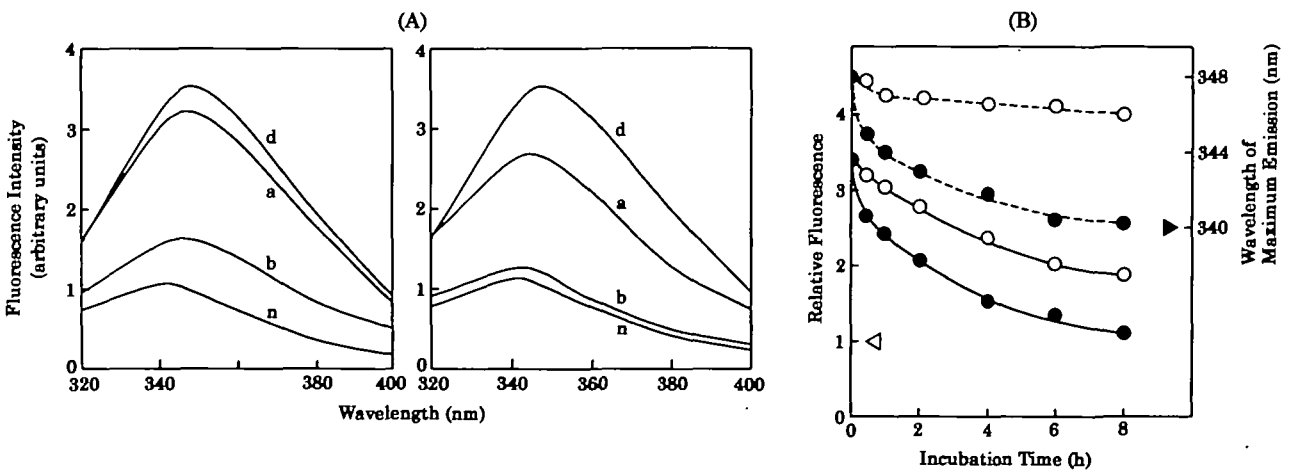

Fig. 2. **Refolding of reductively denatured lysozyme monitored by the change in intrinsic fluorescence.** (A) Reductively denatured lysozyme was allowed to regenerate with (right) or without (left) 1 mM CII-Asn in 0.1 M Tris-HCl (pH 8.0) containing 2 mM EDTA, 4 mM GSH, and 0.2 M NaCl. Spectra were recorded at 0.5 h (a) and 8 h (b) after the start of regeneration. The spectra of the native (n) and fully denatured (d) lysozymes were also recorded. (B) Changes of fluorescence intensity (solid lines) and wavelength of maximum emission (dashed lines) were derived from the spectra obtained with (●) or without (○) 1 mM CII-Asn. The open and filled arrowheads show the fluorescence intensity and wavelength of the maximum emission of the native lysozyme, respectively.

renaturation even in the presence of CII-Asn. Further, glutathione significantly raised the refolding rate of LA, as in the case of the lysozyme refolding.

As shown in Fig. 5, a larger Asn-glycan of the high-mannose type, M9-Asn, markedly promoted the LA refolding similarly to CII-Asn, whereas a smaller one, M5-Asn, was less effective. These promotive properties of the Asn-glycans were similar to those observed with RNase A and lysozyme.

To relate the HPLC profiles of the refolding products to conformational changes of LA, the refolding was followed by monitoring the decrease in tryptophan fluorescence which occurs upon refolding. Three of the four tryptophan residues of the native LA are buried in the interior, and the fourth is exposed to the solvent (*19*). Intrinsic tryptophan fluorescence spectra obtained during the refolding, therefore, should reflect the structural features of the refolding intermediates. Figure 6 demonstrates that the environment of the tryptophan residues of LA changed greatly in the presence of CII-Asn within the first 2 h of the refolding. After 6 h of regeneration, three tryptophan residues of LA seem to have become almost completely buried in the interior, in fair agreement with the refolding features observed by size-exclusion HPLC above.

Further, these results were found to parallel the ANS-binding aspects of the refolding intermediates (Fig. 7). An

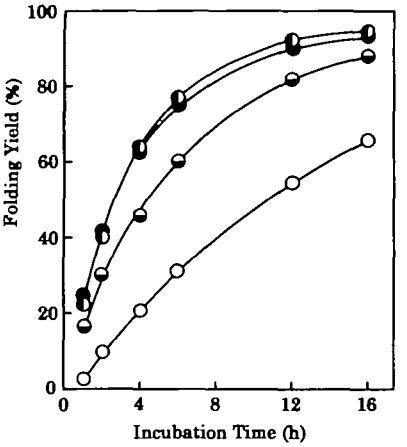

Fig. 5. **Effects of Asn-glycans on the oxidative refolding of reductively denatured LA.** Reductively denatured LA was allowed to regenerate with 1 mM concentrations of M5-Asn (◒), M9-Asn (◐), and CII-Asn (●), or without any of these Asn-glycans (○) under air-oxidation conditions. After quenching the regeneration reactions with 2 mM *N*-ethylmaleimide at the indicated times the products were analyzed by size-exclusion HPLC. Folding yield is expressed as area of the peak at the elution position of the native LA relative to the peak area of the total protein.

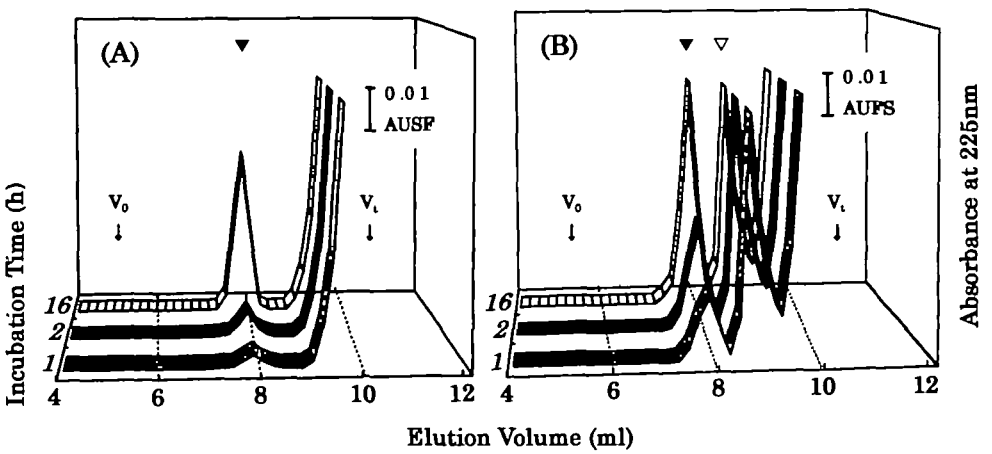

Fig. 4. **Size-exclusion HPLC of the refolding products of reductively denatured LA.** Reductively denatured LA was allowed to regenerate with (B) or without (A) 1 mM CII-Asn under air-oxidation conditions. After quenching the regeneration reactions with 2 mM *N*-ethylmaleimide at the indicated times, the products were analyzed by size-exclusion HPLC. The filled and open arrowheads show the elution positions of the native LA and CII-Asn, respectively.

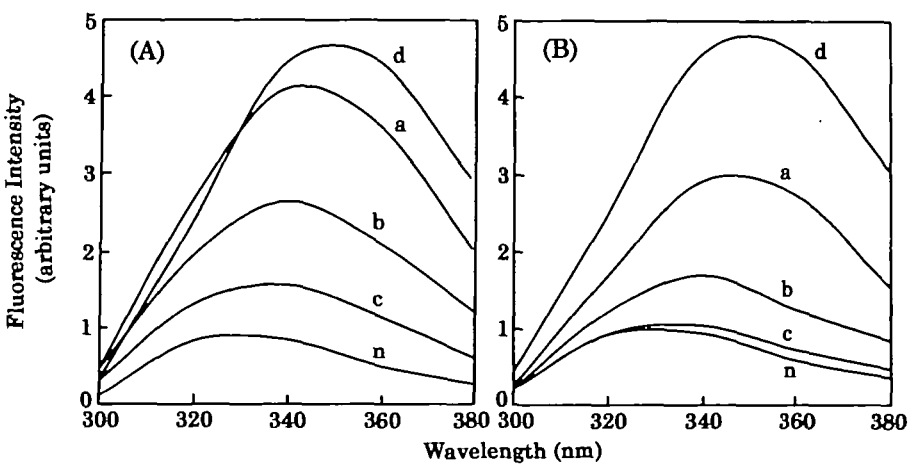

Fig. 6. **Refolding of reductively denatured LA monitored by the change in intrinsic fluorescence.** Reductively denatured LA was allowed to regenerate with (B) or without (A) 1 mM CII-Asn under air-oxidation conditions, and then tryptophyl fluorescence emission was monitored. Spectra were recorded at 1 min (a), 2 h (b), and 6 h (c) after the start of regeneration. The spectra of the native (n) and fully denatured (d) LA species were also recorded.

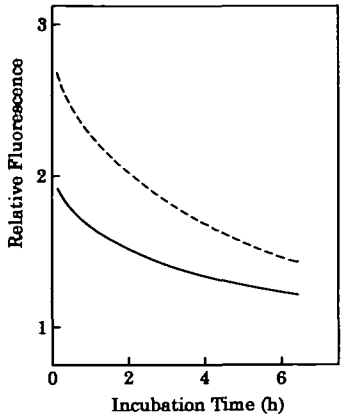

Fig. 7. **Refolding of reductively denatured LA monitored by the change in ANS binding.** Reductively denatured LA was allowed to regenerate with (solid line) or without (dashed line) 1 mM CII-Asn under air-oxidation conditions, and then the refolding was followed by monitoring ANS fluorescence emission. The data are expressed as relative fluorescence, where 1 corresponds to the fluorescence intensity of the refolding buffer containing 100 μM ANS and 2.1 μM native LA.

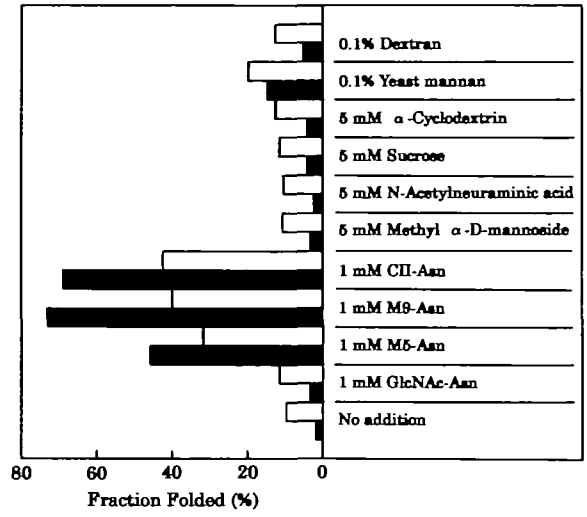

Fig. 8. **Stimulatory effects of various carbohydrates on the refolding of reductively denatured lysozyme and LA.** Reductively denatured lysozyme (filled bars) and LA (open bars) were allowed to regenerate for 12 and 2 h, respectively, with a carbohydrate at the indicated concentration in the refolding buffers containing no oxidizing agents. The refolding of lysozyme and LA was assayed by activity estimation and size-exclusion HPLC, respectively.

initial increase in the ANS fluorescence in the dead time of the mixing was followed by a faster decrease in the fluorescence in the presence of CII-Asn, suggesting the tight packing of the protein structure promoted by CII-Asn.

Comparison of the Promotive Effects of Asn-Glycans with Those of Common Carbohydrates with Different Structures—To determine whether the promotive effect on polypeptide folding is characteristic of Asn-glycans, some mono-, oligo-, and polysaccharides were examined for their effects on the refolding of lysozyme and LA. As summarized in Fig. 8, these common carbohydrates, but not yeast mannan, exhibited much lower promotive effects on the oxidative refolding. The significant effect of yeast mannan is not unexpected because this polysaccharide is a high-mannose type Asn-glycan. These findings, together with the lack of a significant promotive effect by GlcNAc-Asn, indicate that the promotive effect of Asn-glycans is attributable to their glycan structures.

DISCUSSION

The refolding of both lysozyme and LA was markedly promoted by interactions with extramolecular Asn-glycans, as in the case of RNase A (*1, 2*), suggesting that extramolecular Asn-glycans generally promote the proper folding of denatured polypeptides. Differences attributable to the characteristics of individual proteins were, however, observed in these promotive effects. The promotive effect of the Asn-glycans on RNase A refolding was clearly observed only under nonredox conditions, which are unfavorable for the reshuffling of disulfide pairings and hence allow us to easily observe the refolding process. On the other hand, the Asn-glycans distinctly showed their promotive effects on the lysozyme refolding even under redox conditions. It seems likely from these results that RNase A more readily adopts the native conformation on refolding than does lysozyme. Interestingly, the Asn-glycans failed to renature LA in the absence of calcium. LA is known to bind a calcium ion that enhances the stability of the native conformation (*5, 7*). It is also known that LA folds *via* a molten globule intermediate, and that calcium does not bind firmly to the unfolded protein or the molten globule, but binds to the transition state between the molten globule and the native protein (*10, 20*). These facts, together with the conformational changes monitored by means of intrinsic fluorescence and ANS binding, suggest that Asn-glycans promote the transformation of the unfolded polypeptide into the molten globule or the transition state.

In spite of these diverse features of the refolding induced by the Asn-glycans, the relationship between the structure and the degree of the promotive effect of the Asn-glycans was similar for all proteins hitherto examined, supporting the conjecture that extramolecular Asn-glycans generally promote the refolding of polypeptides. These results, combined with those previously obtained with pancreatic RNases (*2*), suggest that intramolecular Asn-glycans also have a general ability to directly promote polypeptide folding, though the mode of action of intramolecular and extramolecular Asn-glycans is expected to differ.

It is interesting that complex-type Asn-glycans, which, contrary to the high-mannose type, are formed on polypeptides after the initial folding of nascent polypeptides, have a strong promotive effect on the refolding of polypeptides. It is generally accepted that newly synthesized proteins are translocated across membranes, during which process they are temporarily subjected to partial unfolding (*21-23*). It appears, therefore, that the intramolecular complex-type Asn-glycans play an important role in maintenance of protein conformation, though it remains unsettled whether they function similarly to extramolecular ones in promoting polypeptide folding. In addition, it is noteworthy that a fairly high concentration of free oligosaccharides of both high-mannose and complex types was recently found in bean hypocotyls (Kimura, Y., personal communication), though their *in vivo* function remains unknown. In view of

the results obtained here, it would not be surprising if they were involved in a novel system associated with protein conformation.

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