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Oxidative Refolding of Bovine Pancreatic RNases A and B Promoted by Asn-Glycans[1]

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It was previously revealed [Yamaguchi, H. and Uchida, M. (1996) *J. Biochem.* 120, 474-477] that both intra- and extramolecular high-mannose type Asn-glycans promote the renaturation of reductively denatured bovine pancreatic RNases A and B under oxidation conditions. To characterize the conformational changes of the polypeptides during the renaturation promoted by the intramolecular Asn-glycans, RNase B was compared with its nonglycosylated form, RNase A, as to the features of the regeneration from their reductively denatured species under Cu^{2+}-catalyzed oxidation conditions. The refolding intermediates of RNase B, as compared with those of RNase A, seemed to contain much less impaired disulfide linkages. In agreement with this finding, the proper refolding of RNase B was much faster than that of RNase A, as revealed by the intrinsic fluorescence and 1-anilino-8-naphthalenesulfonate binding of the refolding intermediates. Such a promoting effect was also observed for extramolecular Asn-glycans of the complex as well as of the high-mannose type. In contrast, common mono-, oligo-, and polysaccharides, but not yeast mannan, exhibited much lower stimulatory effects on the oxidative refolding of RNase A.

Key words: Asn-glycan, Asn-glycan function, glycoprotein, protein folding, ribonuclease B.

A number of reports have suggested the functions of intramolecular Asn-glycans in the folding of glycopolypeptides (*1-3*). It is well known that Asn-glycans participate in the calnexin-mediated folding of nascent polypeptides (*3*). On the other hand, we recently revealed that not only intramolecular but also extramolecular high-mannose type Asn-glycans directly promote the renaturation of reductively denatured pancreatic RNases A and B [EC 3.1.27.5] (*4*). This finding, although advanced the first time evidence for the immediate function of Asn-glycans in proper folding of polypeptides, was only based on the activity regain observed for the regeneration reaction. In this study, the conformational changes induced by intra- and extramolecular Asn-glycans were monitored by means of intrinsic tyrosine fluorescence. The six tyrosine residues of the native pancreatic RNase differ in their accessibility to a solvent. Three of them are exposed to the solvent and another two are buried in the interior. The remaining one is in a tricky situation and might be exposed to the solvent depending on the conditions (*5-8*). Therefore, intrinsic tyrosine fluorescence spectra obtained during the refolding could be expected to reflect rather precisely the structural features of the refolding intermediates. To corroborate the intrinsic fluorescence result, the refolding was also monitored as to the affinity of a fluorescent hydrophobic probe, 1-anilino-8-naphthalenesulfonate (ANS), for the intermediates. The results obtained here reveal that both intra- and extramolecular Asn-glycans promote polypeptide folding into compact, native-like structures, and that complex-type Asn-glycans are also effective in promotion of the refolding similar to high-mannose type ones.

MATERIALS AND METHODS

Materials—RNase A (type III-A) and RNase B (type XII-B) were obtained from Sigma, and purified as previously described (*4*). Glutathione, ANS, and GlcNAc-Asn were also purchased from Sigma. 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 (*9, 10*). A mixture of Asn-linked complex-type oligosaccharides, $\pm$NeuAcα2-6$\pm$Galβ1-4GlcNAcβ1-2Manα1-6($\pm$NeuAcα2-6$\pm$Galβ1-4GlcNAcβ1-2Manα1-3)($\pm$GlcNAcβ1-4)Manβ1-

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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; 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.

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. Yeast mannan was extracted and purified from baker's yeast (*Saccharomyces cerevisiae*; Oriental Yeast, Tokyo) (*11*). Dextran was a product of Pharmacia, and cyclodextrins were from Wako Pure Chem. Other chemicals used were described in the preceding paper (*4*). All of the Asn-glycans and other sugars were freed from metal ions by passing them through a Chelex 100 (Bio-Rad) column.

Methods—The reductive denaturation of RNases A and B was performed exactly as described previously (*4*). The refolding of RNases A and B from the reductively denatured species was carried out under Cu^{2+}-catalyzed oxidation conditions. A reductively denatured RNase (3.3 μM final concentration) was quickly dissolved in 5 mM HCl (0.21 ml), and then most (0.20 ml) of the solution was immediately diluted with the refolding buffer, 0.12 M sodium phosphate buffer (pH 7.8, 25°C, 1.00 ml) containing 60 nM $CuSO_4$ and 0.18 M NaCl, with vigorous stirring. After various times of incubation at 25°C, the solution was submitted to activity estimation as described previously (*4*). Asn-glycans were added to the refolding buffer when extramolecular Asn-glycans were assayed as to their effect on the refolding of reductively denatured polypeptides. Fluorescence measurements were performed with a Shimadzu RF-1500 spectrophotometer at 25°C. Tyrosyl fluorescence spectra, from 290 to 340 nm, were recorded with excitation at 268 nm. Polypeptide refolding was also monitored as to ANS binding in the refolding buffer containing 500 μM ANS. ANS fluorescence spectra, from 450 to 580 nm, were recorded with excitation at 400 nm. Other analytical methods were described previously (*4*).

RESULTS

Refolding of Reductively Denatured RNases A and B—To characterize the function of Asn-glycans in the oxidative refolding of RNase B, an attempt was made to compare RNase B with its nonglycosylated form, RNase A, in the regeneration features of their reductively denatured species under Cu^{2+}-catalyzed oxidation conditions. As shown in Fig. 1, RNase B regained activity much faster than RNase A, although there was no pronounced difference in the oxidation rate of sulfhydryl groups between them. These results suggest that the refolding intermediates of RNase A, as compared with those of RNase B, contain much more impaired disulfide linkages. The addition of a small amount of glutathione to the regeneration solutions, which could be supposed to facilitate the reshuffling of disulfide linkages, preferentially raised the reactivation level of RNase A, as was to be expected (Fig. 2). It seems most likely, therefore, that RNase B, in contrast to RNase A, easily adopts a native-like conformation in which correct disulfide pairings are efficiently allowed.

To relate the regeneration features of RNases A and B to their conformational changes, the refolding of RNases A and B was monitored as to decrease in tyrosine fluorescence which occurs upon refolding. Figure 3 shows that the environment of the tyrosine residues of RNase B greatly changed within the first 1 h of the regeneration, whereas the tyrosine residues of RNase A were still mostly exposed to the solvent at this time of refolding. After 2 h regeneration, the three tyrosine residues of RNase B, in contrast to those of RNase A, seem to have almost been completely buried in the interior. These results strongly suggest that the folding rate of RNase B is rapid compared to that of RNase A, in agreement with the reactivation features observed above.

To confirm the refolding aspects thus revealed, an additional attempt was made to determine the conformations of the folding intermediates using the fluorescent hydrophobic probe, ANS. It is well known that ANS does not bind to the fully unfolded state and normally not to the native state either, but exhibits high affinity for partially folded, compact intermediates (*12*). As shown in Fig. 4, the ANS binding became maximum shortly after the start of the refolding of RNase B, whereas its binding to RNase A intermediates required about 2 h for the maximum to be

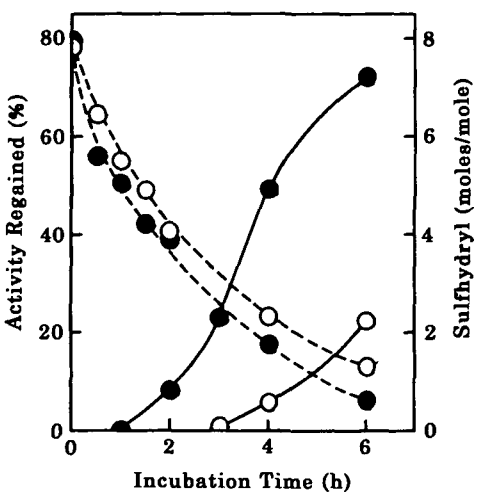

Fig. 1. **Assaying of the oxidative refolding of reductively denatured RNases A and B.** The refolding of reductively denatured RNases A (○) and B (●) was assayed as to the activity regain (solid lines) and sulfhydryl content (dashed lines).

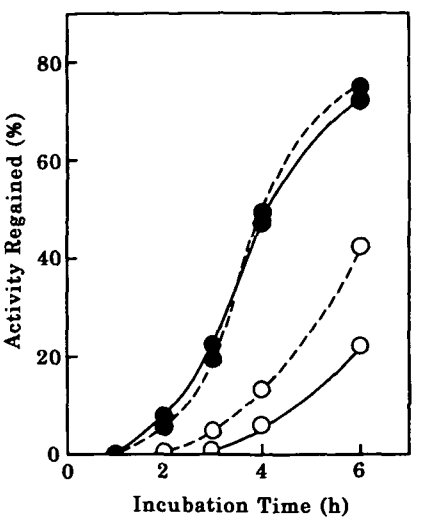

Fig. 2. **Effect of glutathione on oxidative refolding of reductively denatured RNases A and B.** Reductively denatured RNases A (○) and B (●) were allowed to regenerate in the presence (dashed lines) or absence (solid lines) of 1 mM glutathione.

reached, in fair agreement with the results obtained above. It is also clear that the ANS affinity for the intermediates of RNase A is much weaker than that for those of RNase B, suggesting a considerable difference in structure between the RNase A and B intermediates. It seems most likely, therefore, that the reductively denatured RNase B, as compared with RNase A, was readily folded into a compact species having a molten globule-like structure (*13*).

Effect of M5-Asn on Refolding of Reductively Denatured RNase A—The most predominant sugar chain of RNase B is $Man_5GlcNAc_2$, comprising about 60% of the sugar chains of RNase B on a molar basis (*14*). To compare the function of extramolecular M5-Asn with that of the intramolecular Asn-glycans of RNase B, the effect of M5-Asn on the refolding of reductively denatured RNase A was examined under the same Cu^{2+}-catalyzed oxidation conditions (Fig. 5). M5-Asn markedly enhanced the reactivation rate of RNase A as previously observed under air-oxidation conditions (*4*), and the stimulatory effect of M5-Asn increased with its concentration. To characterize the polypeptide refolding promoted by M5-Asn, the renaturation reaction was followed as to the intrinsic fluorescence. As shown in Fig. 6, 5 mM M5-Asn induced a fluorescence change closely similar to that observed for the RNase B refolding. This finding suggests that M5-Asn promoted the transformation of the extended RNase A polypeptides into folded species, thus changing the environment of the three tyrosine residues. Furthermore, this result was found to parallel the ANS-binding features of the refolding intermediates (Fig. 7). Interestingly a higher concentration of M5-Asn produced more intense fluorescence, although the time required to reach the maximum emission was identical. It seems probable, therefore, that M5-Asn, similarly to the intramolecular Asn-glycans of RNase B, promotes the polypeptide folding into compact, native-like structures in which correct disulfide pairings are efficiently allowed.

Effects of Asn-Glycans and Common Sugars with Differ-

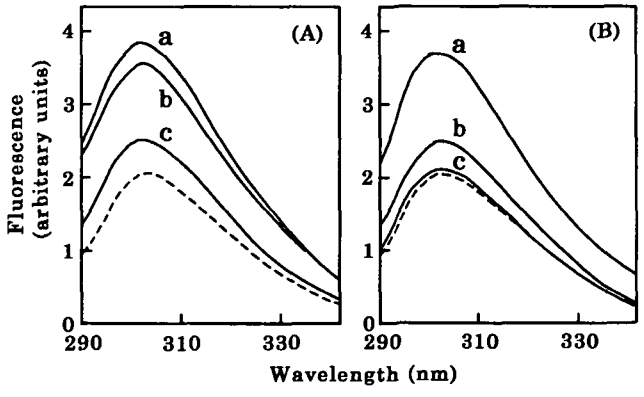

Fig. 3. **Refolding of reductively denatured RNases A and B monitored as to intrinsic fluorescence.** Reductively denatured RNases A (A) and B (B) were allowed to regenerate and then tyrosine fluorescence emission was monitored. Spectra were recorded at 2 min (a), 1 h (b), and 2 h (c) after the start of regeneration. The dashed lines in (A) and (B) show the spectra of the native RNases A and B, respectively.

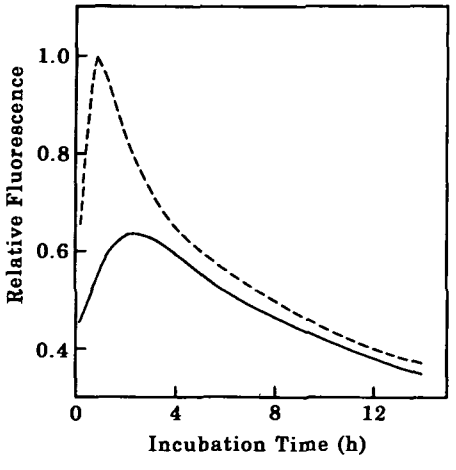

Fig. 4. **Refolding of reductively denatured RNases A and B monitored as to ANS fluorescence.** Reductively denatured RNases A (solid line) and B (dashed line) were allowed to regenerate and then ANS fluorescence emission was monitored. The data are expressed as relative fluorescence, where 1 corresponds to the estimated maximum change in fluorescence.

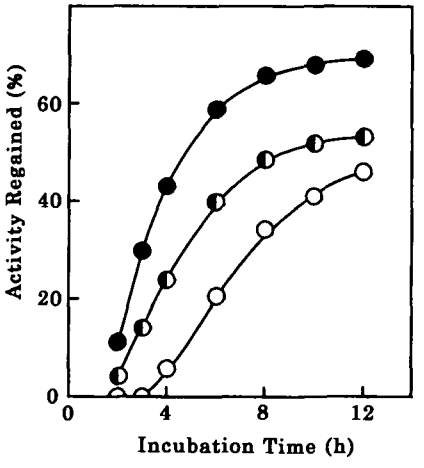

Fig. 5. **Effect of M5-Asn on the oxidative refolding of reductively denatured RNase A.** Reductively denatured RNase A was allowed to regenerate with 0 mM (○), 1 mM (◐), or 5 mM (●) M5-Asn.

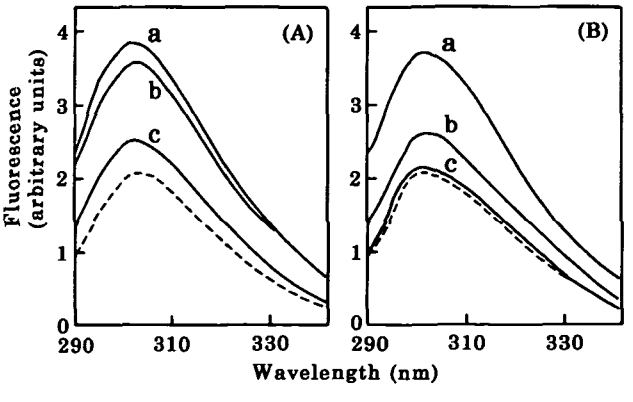

Fig. 6. **Refolding of reductively denatured RNase A monitored as to intrinsic fluorescence.** Reductively denatured RNase A was allowed to regenerate with (B) or without (A) 5 mM M5-Asn, and then tyrosine fluorescence emission was monitored. Spectra were recorded at 2 min (a), 1 h (b), and 2 h (c) after the start of regeneration. The dashed lines show the spectra of the native RNase A.

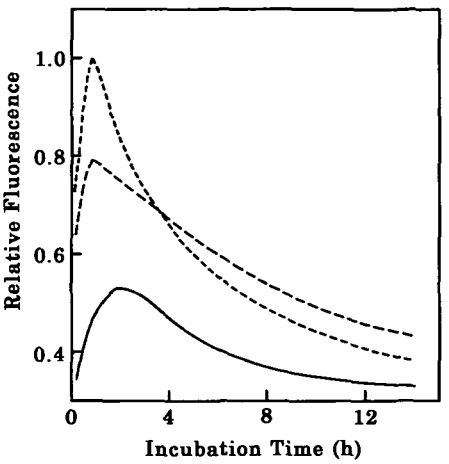

Fig. 7. **Effect of M5-Asn on the oxidative refolding of reductively denatured RNase A.** Reductively denatured RNase A was allowed to regenerate with 0 mM (solid line), 1 mM (dashed line), or 10 mM (dotted line) M5-Asn, and then the refolding was monitored as to ANS fluorescence emission. The data are expressed as relative fluorescence, where 1 corresponds to the estimated maximum change in fluorescence.

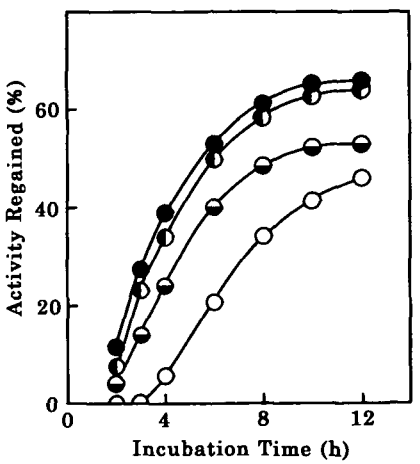

Fig. 8. **Effects of Asn-glycans on the oxidative refolding of reductively denatured RNase A.** Reductively denatured RNase A was allowed to regenerate with 1 mM each of M5-Asn (◒), M9-Asn (◐), and CII-Asn (●), or without any of these Asn-glycans (○).

ent Structures on the Refolding of Reductively Denatured RNase A—It would be very interesting to determine whether or not Asn-glycans differing distinctly from M5-Asn in structure promote polypeptide refolding. It seems probable from Fig. 8 that larger Asn-glycans of the high-mannose type are more effective than smaller ones, and that complex-type Asn-glycans also exhibit promoting effects similar to those of high mannose-type ones, although the relationship between the fine structures and the stimulatory effects of Asn-glycans remains obscure.

Finally, some mono-, oligo-, and polysaccharides were examined as to their effects on the refolding of RNase A under the same regeneration conditions. As shown in Fig. 9, common mono-, oligo-, and polysaccharides, but not yeast mannan, exhibited only slightly stimulatory effects on the oxidative refolding of RNase A at weight concentrations

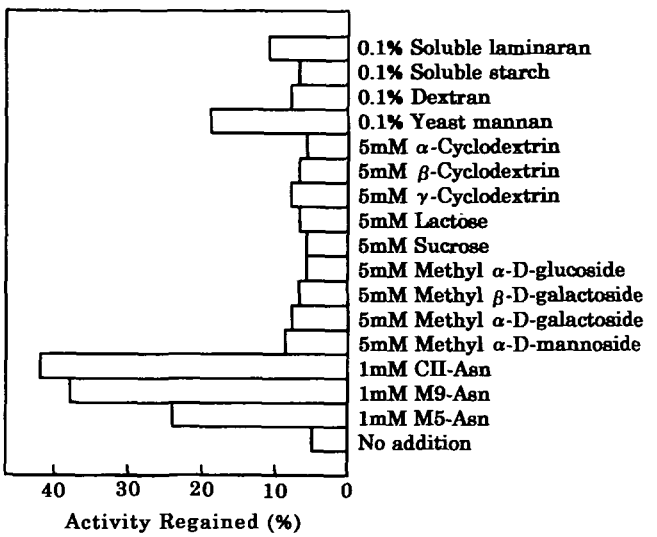

Fig. 9. **Stimulatory effects of various carbohydrates on the reactivation of reductively denatured RNase A.** Reductively denatured RNase A was allowed to regenerate for 4 h with a carbohydrate at the indicated concentration and then examined as to activity regain.

nearly corresponding to 1 mM of an Asn-glycan.

DISCUSSION

In order to characterize the refolding promoted by Asn-glycans, RNases A and B were compared as to the regeneration features observed under nonredox conditions. In contrast with redox conditions, Cu^{2+}-catalyzed oxidation conditions are unfavorable for the reshuffling of disulfide pairings (*15*), and hence were expected to allow us to readily observe the intermediate conformations characteristic of the refolding promoted by Asn-glycans. The most probable conclusion from the evidence at hand is that the intramolecular Asn-glycans of RNase B facilitate the proper folding of reductively denatured RNase B. It is noticeable that extramolecular Asn-glycans exhibited stimulatory effects similar to those of the intramolecular Asn-glycans of RNase B on the polypeptide refolding. This strongly suggests that the intramolecular Asn-glycans fixed on the RNase B polypeptides do not promote the folding merely through a steric effect on the intermolecular interaction, but rather by depressing the intramolecular interactions of the polypeptides unfavorable for their proper folding. However, there seems to be a little difference in the action on the denatured polypeptides between the intra- and extramolecular Asn-glycans. Compared with RNase A regeneration promoted by the extramolecular Asn-glycans, RNase B appears to be regenerated in a high yield without a reactivation plateau (compare Fig. 1 with Figs. 5 and 8), in spite of that it has only one Asn-glycan chain per protein molecule. M5-Asn molecules, which are allowed to move independently in the regeneration solutions, can freely reach any region of the denatured polypeptides and exhibit an indiscriminate action rather an unfavorable one for efficient refolding of the polypeptides. It may well be said, therefore, that Asn34 of RNase is the right position to fix and make the Asn-glycan chains function efficiently.

It is rather surprising that complex-type Asn-glycans, which are considerably different from high-mannose type ones in structure, have strong stimulatory effects on the regeneration of RNase A. This finding has aroused great interest in the relationship between the stimulatory effects and the structures of complex-type Asn-glycans which exhibit greater diversity in structure as compared with high-mannose type ones.

The common mono-, oligo-, and polysaccharides, but not yeast mannan, showed only a little stimulatory effect on RNase A refolding. The significant effect observed for yeast mannan is not strange in view of the fact that this polysaccharide belongs to the high-mannose type Asn-glycans. It has been reported in the last two decades that pretty high concentrations (about 1 M or so) of polyhydric compounds such as glycerol, glucose, lactose, and sucrose, known as "osmolytes," minimize the area of the water-protein interface, and displace the native⇌denatured equilibrium to the left, thus stabilizing the native protein conformations (*16-19*). Even though the function of Asn-glycans is similar to that of osmolytes, there is a distinct difference in the intensity of action between them. It might be possible that Asn-glycans, as compared with osmolytes, promote the refolding through a much stronger action due to their characteristic sugar compositions and highly branched structures, which have been conserved throughout the long evolution of biological species.

This study has raised a number of interesting questions: What kind of interaction exists between Asn-glycans and RNase polypeptides? How is the function of Asn-glycans related to their structures? Do Asn-glycans promote the folding of proteins other than RNases? Is this function of Asn-glycans of physiological significance? The resolution of every one of these issues, although it does not seem easy, is indispensable for a full understanding of the function of Asn-glycans in facilitation of the folding of polypeptides.

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