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A Chaperone-Like Function of Intramolecular High-Mannose Chains in the Oxidative Refolding of Bovine Pancreatic RNase B[1]

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This paper describes a chaperone-like function of the intramolecular *N*-glycans of bovine pancreatic RNase B. We studied air-oxidative regeneration from reductively denatured species of RNase B and its nonglycosylated form, RNase A. RNase B was reactivated much faster than RNase A, while RNase A was liable to aggregate during the regeneration. An Asn-linked oligosaccharide, Man_5GlcNA_2Asn, which corresponds to the most predominant sugar chain (*ca.* 60%) of RNase B, enhanced the reactivation of the denatured RNases A and B. The stimulatory effect of this Asn-oligosaccharide revealed that the *N*-glycans of RNase B facilitate the transformation of bulky intermediates into folded, compact species.

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

One of the roles of *N*-linked oligosaccharides of glycoproteins has been supposed to be facilitation of the folding and assembly of nascent polypeptides (*1-3*). However, no direct evidence of this role has thus far been presented, mainly because of the lack of suitable methods to approach this problem. It has recently been expected that *in vitro* studies on the comparison of glycosylated and nonglycosylated variants of proteins as to their behavior under different conditions would provide clear information about the functional role of intramolecular sugar chains. Bovine pancreatic RNase [EC 3.1.4.22], therefore, has been considered appropriate for this purpose, because this enzyme occurs naturally in both the *N*-glycosylated form (RNase B) and the nonglycosylated form (RNase A), which are thought to be structurally identical except for the presence of a single high-mannose type oligosaccharide chain ($Man_{5-9}GlcNAc_2$) linked to Asn-34 of RNase B (*4*). Based on this concept, Grafl *et al.* have shown that the refolding of "nonreductively" denatured RNase B does not depend on the presence of the *N*-glycan chains (*5*). In contrast with their conclusion, Nagai *et al.* recently reported suggestive evidence of the essential role of the intramolecular high-mannose chains in the proper folding of fully unfolded soybean lectin polypeptides (*6*). An attempt, therefore, was made to elucidate the function of the *N*-glycans of RNase B by comparing it with RNase A as to the features of regeneration from "reductively" denatured, *viz.*, fully denatured, species.

RNase A (type III-A) and RNase B (type XII-B) were obtained from Sigma and purified by gel filtration on a Bio-gel P-60 column eluted with 0.1 M ammonium acetate (pH 7.8). RNase B was further purified by affinity chromatography, if necessary, on concanavalin A-Sepharose (*7*). Protein concentrations were calculated using $\varepsilon_{280}=9{,}800\ M^{-1}\cdot cm^{-1}$ (*8*) and $\varepsilon_{275}=8{,}160\ M^{-1}\cdot cm^{-1}$ (*9*) for the native and reductively denatured RNases, respectively. DTT, cytidine 2′:3′-$_c$CMP, trypsin (type XIII), and *N*-ethylmaleimide were also from Sigma. An asparagine-linked high-mannose type oligosaccharide, Manα1-6(Manα1-3)-Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc1-Asn (M5-Asn), was separated from hen ovalbumin as described previously (*10*).

Reductively denatured RNases A and B were prepared by incubating the native enzyme (1 mg/0.1 ml) in 0.5 M Tris-HCl (pH 8.5) containing 2 mM EDTA, 0.13 M DTT, 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 (1×30 cm) eluted with deaerated 0.1 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. These samples were assayed for the number of thiol groups by Ellman's method (*11*), showing a content of $7.8\pm0.1_{eq}$, which proved to be stable for at least several weeks.

The regeneration of RNases A and B from the reductively denatured species was carried out under air-oxidation conditions which, in contrast with redox conditions, had been expected to make the regeneration reaction proceed slowly and hence to allow us to easily observe the regeneration process. The reductively denatured RNase (0.5-10 μM final concentration) was quickly dissolved in 5 mM HCl (0.21 ml), and then most part (0.20 ml) of the solution was immediately diluted into 0.12 M sodium phosphate buffer (pH 7.8, 25°C, 1.00 ml) containing 0.18 M NaCl with vigorous stirring. After various times of air-oxidation reaction at 25°C, the solution was mixed with 0.26 M cytidine 2′:3′-$_c$CMP in 0.10 M sodium phosphate buffer

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Abbreviation: M5-Asn, Manα1-6(Manα1-3)Manα1-6(Manα1-3)-Manβ1-4GlcNAcβ1-4GlcNAc-Asn.

(pH 7.8, 25°C, 20 μl) containing 0.15 M NaCl, and then change in A_{296} was measured at 25°C for 10 min (*12*).

Figure 1A shows that RNase B regained activity much faster than RNase A. Since denatured RNases, in contrast to the native ones, are highly sensitive to trypsin digestion (*13*), the regeneration of RNases A and B was compared by SDS-PAGE combined with trypsin treatment, as follows. The same quantities of the RNase A and B regeneration solutions (0.60 ml) were combined, and then mixed with trypsin (20 μg/10 μl of 1 mM HCl) and kept at 25°C for 1 min. The digestion was quenched by adding leupeptin (48 μg/10 μl of water), and then the solution was cooled for 5 min in an ice bath. After being desalted by the method previously reported (*14*), the enzyme protein was heated at 100°C for 5 min in the SDS-PAGE sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 15% glycerol, 0.28 M 2-mercaptoethanol). SDS-PAGE was performed according to Laemmli (*15*) using 13% polyacrylamide slab gels, and protein bands were stained with Coomassie Brilliant Blue R-250. Trypsin-resistant RNase B was clearly detected at 6 h and its level increased markedly after 12 h of regeneration, whereas no distinct band corresponding to the native RNase A has been detected at 6 h as yet (Fig. 1B). It can also be seen in Fig. 1A that the rates of reactivation of both RNase A and B were closely related to the protein concentration during regeneration. However, no significant changes in their reactivation rates were observed at a protein concentration of about 1.0 μM or lower (data not shown), suggesting that the intermolecular interaction is negligible at such a low protein concentration. It is noteworthy that RNase B was reactivated much faster than RNase A under such dilute conditions, and that RNase A, in contrast with RNase B, was liable to aggregate when regenerated at a protein concentration higher than about 50 μM (data not shwon). These results indicate that the intramolecular *N*-glycan chains of RNase B facilitate polypeptide folding through some mechanism, thus depressing the intermolecular interaction which is unfavorable for regeneration.

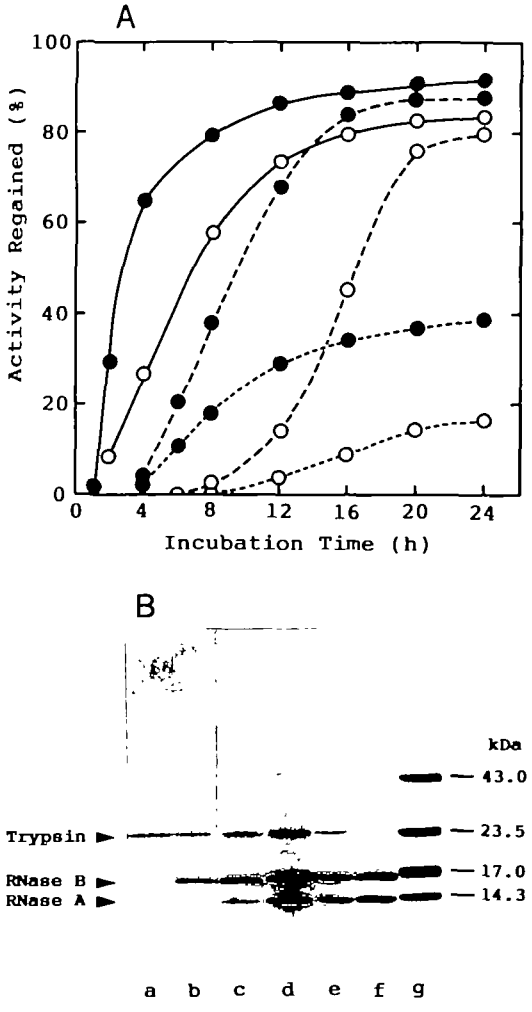

Fig. 1. **Assaying of the oxidative refolding of reductively denatured RNases A and B.** (A) Time courses of the reactivation of reductively denatured RNases A (○) and B (●) at a protein concentration of 0.7 μM (solid line), 3.3 μM (dashed line), or 10 μM (dotted line). (B) SDS-PAGE of trypsin-treated regeneration products. Reductively denatured RNases A and B were separately allowed to regenerate for 0 h (lane a), 6 h (lane b), 12 h (lane c), or 24 h (lanes d and f) at a protein concentration of 3.3 μM, and then combined and proteolyzed at a trypsin to RNase ratio (w/w) of 0.37 (lanes a through d) or 0 (lane f). Lane e contains the native RNases treated with trypsin as just described. A part (corresponding to about 6 μg of the protein submitted to regeneration) of the regeneration products was analyzed by SDS-PAGE. Lane g contains molecular weight standards (hen egg white lysozyme, 14.3 kDa; equine skeletal myoglobin, 17.0 kDa; bovine γ-globulin L chain, 23.5 kDa; and hen ovalbumin, 43.0 kDa).

To gain more insight into the function of the *N*-glycan chains of RNase B, the regeneration of reductively denatured RNases A and B was performed using a dilution buffer containing M5-Asn, which corresponds to the most predominant sugar chain (*ca.* 60% on a molar basis) of RNase B (*16*). The presence of 1 mM M5-Asn markedly enhanced the rates of reactivation of both RNase A and B (Fig. 2). Raising the M5-Asn concentration increased their reactivation rates, with a concomitant distinct tendency of depression of the ready aggregation of RNase A (data not shown). It had previously been shown that sugars such as glucose,

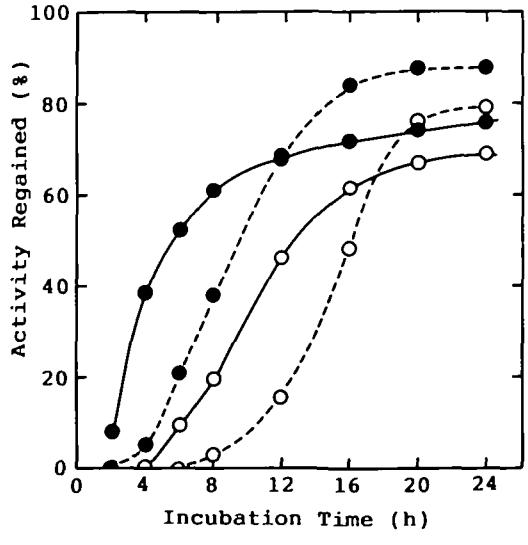

Fig. 2. **Effect of M5-Asn on oxidative refolding of reductively denatured RNases A and B.** Reductively denatured RNases A (○) and B (●) were allowed to regenerate with (solid line) or without (dashed line) 1 mM M5-Asn at a protein concentration of 3.3 μM. See the text for other details.

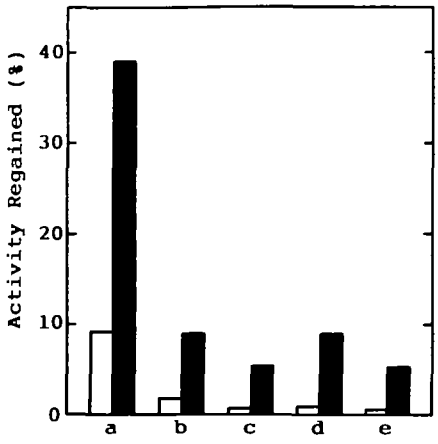

Fig. 3. **Stimulatory effects of various mono- and oligosaccharides on the reactivation of reductively denatured RNases A and B.** Denatured RNases A (open bars) and B (filled bars) were allowed to regenerate for 6 h and 4 h, respectively, at a protein concentration of 3.3 μM. The regeneration solution contained M5-Asn (a), methyl α-D-mannoside (b), glucose (c), lactose (d), or sucrose (e) at a concentration of 1 mM.

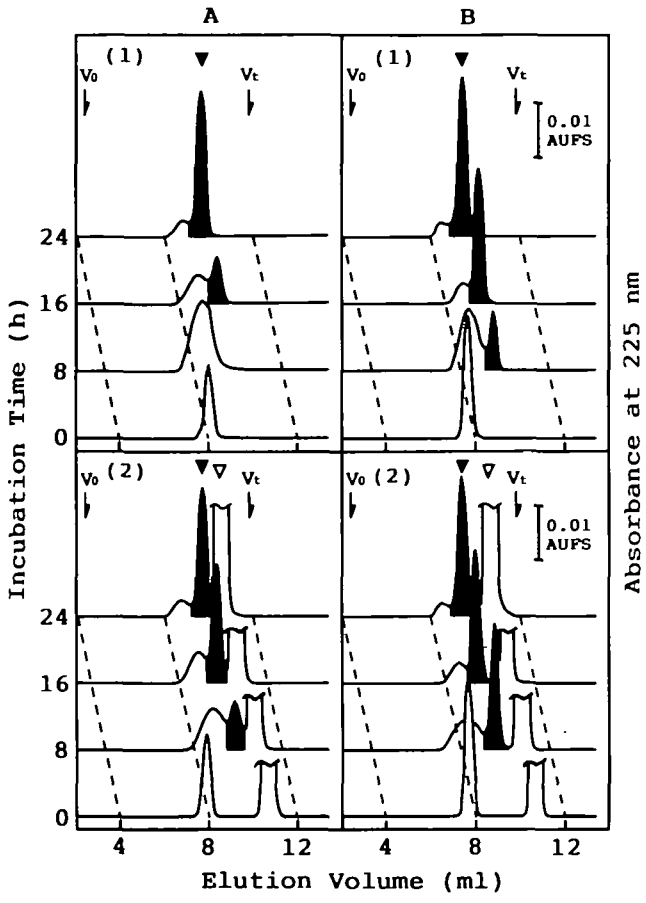

Fig. 4. **Gel-filtration HPLC of the regeneration products of reductively denatured RNases A and B.** Reductively denatured RNases A (A) and B (B) were allowed to regenerate without (A1 and B1) or with (A2 and B2) 1 mM M5-Asn at a protein concentration of 3.3 μM. The enzymatically active fractions are indicated by filled peaks. The filled and open arrows show the elution positions of the native RNases and M5-Asn, respectively. See the text for other details.

lactose, and sucrose minimize the area of the water-protein interface, and displace the native $\rightleftharpoons$ denatured equilibrium to the left, thus stabilizing the native protein structure (*17, 18*). The stimulatory effect of M5-Asn, therefore, might be explained, at least in part, by such a protein stabilizing action of the high-mannose chains, though any one of these small sugars and methyl α-D-mannoside also showed only a little stimulatory effect, in contrast to M5-Asn, on the regeneration of RNases (Fig. 3). This inference was consistent with the finding that M5-Asn added to the regeneration solution after the folding intermediates had been formed without M5-Asn also greatly enhanced the rates of reactivation of RNases A and B (data not shown). Although RNase B was reactivated much faster than RNase A in the presence of 1 mM M5-Asn as well (Fig. 2), the difference between their reactivation rates decreased with increasing M5-Asn concentration, and no significant difference between them was observed in the presence of about 10 mM M5-Asn (data not shwon). It may well be said, therefore, that the *N*-glycan chains of RNase B facilitate, similarly to M5-Asn, transformation of the folding intermediates into the active conformation, and that the glycan chains attached to Asn-34, compared with free M5-Asn molecules, are highly favorable for facilitating the polypeptide refolding. The stimulatory effect of M5-Asn on the reactivation of denatured RNases A and B was confirmed by SDS-PAGE combined with trypsin treatment (data not shown).

The molecular sizes of the regeneration intermediates are of interest in connection with the stimulatory effect of the sugar chains on the regeneration. The regeneration of RNases A and B, therefore, was examined by means of size-exclusion HPLC. After quenching the regeneration reactions with 1 mM *N*-ethylmaleimide, a part (50 μl) of each reaction solution was applied to a TSK-GEL G3000SW (0.75×30 cm) (Tosoh) developed with 0.1 M sodium acetate buffer (pH 5.0) containing 0.2 mM EDTA, 0.2 M NaCl, and 4 M urea, at the flow rate of 0.4 ml/min, and the elution of protein was monitored by measuring A_{225}. Figure 4 shows that the bulky intermediates of RNases A and B were transformed into the folded, compact species, and that the sugar chains promoted the transformation. The regeneration products represented by the filled peaks proved to be as active as the native enzymes. Confirmatory evidence for such aspects of transformation was provided by SDS-PAGE performed without disulfide reduction (data not shown).

It has been suggested that *N*-glycosylation increases the solubility of folding intermediates so that they can avoid easy aggregation (*1, 3*). However, detailed information about the mechanism underlying this function of *N*-glycans remains to be presented. Although the results of this study do not fully settle this matter, it was clearly demonstrated that the *N*-glycans of RNase B promote polypeptide folding, similarly to chaperones, which prevent other polypeptides from forming nonspecific aggregates and accelerate their proper folding (*19*). It is well known that *N*-glycosylation of proteins occurs at the asparagine residues in the sequence, Asn-Xaa-Thr/Ser, through the action of oligosaccharyltransferase. Recently, a glycosylation-site binding protein, which exhibits specific affinity for this *N*-glycosyl-

ation tripeptide sequence, has been found to be involved in the activity of oligosaccharyltransferase and also to exhibit a high degree of sequence homology with protein disulfide isomerase (*20, 21*). These findings suggest some relationship between the *N*-glycosylation and polypeptide folding, and consequently, it does not look like an accident that the *N*-glycan chains participate in polypeptide folding. We have little doubt that the *N*-glycan chains of RNase B exhibit affinity for some regions of polypeptide chains, thus helping the polypeptides avoid intramolecular interaction which is unfavorable for proper folding.

It is known that the presence of the *N*-glycan chains of RNase B does not affect the folding kinetics after the formation of its four correct disulfide bonds (*5*). Since the initial high-mannose type chains are incorporated into nascent polypeptides before the polypeptide folding and disulfide bond formation are completed (*3, 22*), *N*-glycan chains could function at an early stage of polypeptide maturation in the endoplasmic reticulum. We are interested in the details of the relationship between the function and structure of the *N*-glycan chains, and further work along such lines is in progress.

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