Influence of the Heterosaccharides in Porcine Pancreatic Ribonuclease on the Conformation and Stability of the Protein*

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Porcine pancreatic ribonuclease (ribonucleate pyrimidine-nucleotide-2'-transferase (cyclizing), EC 2.7.7.16), a glycoprotein containing up to 38% carbohydrate, was treated with a mixture of neuraminidase, \( \beta \)-galactosidase, \( \alpha \)-mannosidase, and \( \beta \)-N-acetylglucosaminidase to produce an enzyme-modified protein in which some 75% of the carbohydrate had been removed. The native and enzyme-modified proteins were compared. The fully reduced, denatured proteins underwent reoxidation at identical rates to products which had the specific activity of native enzyme. The native and enzyme-modified proteins also exhibited identity in their reversible thermal transitions at neutral pH, with midpoint at 66°, as well as in the rate at which they underwent inactivation in the specific reaction with iodoacetate at pH 5. Spectrophotometric titrations revealed that the heterosaccharide side chains exert a stabilizing influence on the structure of the protein around at least two tyrosines located at or near the surface of the molecule. The enzyme-modified protein underwent an acid transition 0.2 pH unit higher than the native protein in a process leading to exposure of the equivalent of about two tyrosines. On alkaline denaturation, one of three buried tyrosine side chains in the enzyme-modified protein titrated with a \( \mathrm{pK}_a \) of 11.6, whereas in the native protein these three tyrosines did not titrate below \( \mathrm{pH} \) 12. Circular dichroism measurements and difference spectral analyses revealed local changes in tertiary structure around one tyrosine side chain, tentatively identified as that in position 25, when the bulk of the heterosaccharides was reduced. This change was not associated with more extensive exposure of the side chain. The results are consistent with the view that an increase in size of the heterosaccharides is without influence on the rate-limiting steps in the refolding of the protein from the denatured state or on the overall configurational stability of the molecule, but does influence the environment around at least two tyrosine side chains to effect a stabilization of the surface structure. The existence of glycosylation in certain globular proteins can thus be viewed as a device for the stabilization of surface conformations. The enzyme-modified protein was more sensitive than native porcine ribonuclease to attack by subtilisin at 10° and to trypsin at 30 and 40°. This protective effect of the heterosaccharides may be physiologically significant.

To what degree are the configurational stability and tertiary structures of globular proteins that have undergone glycosylation in the cell influenced by the attached heterosaccharides such proteins contain? Aspects of this question have been investigated in bovine pancreatic ribonuclease B by Puett (1), who concluded from circular dichroism measurements that the overall conformation of the protein is not significantly different from that of ribonuclease A, the unglycosylated parent molecule. Although Puett's data are clear-cut, ribonuclease B is probably not the most advantageous glycoprotein to select for studies of this problem. The carbohydrate content of 10% (2) is relatively small and there is but a single site of heterosaccharide attachment at Asn-34 (3). Inasmuch as many of the known pancreatic ribonuclease are glycoproteins (for a review, see Deustch et al. (4)), it appeared to us that a more meaningful analysis would be feasible by selecting a molecule with a much larger carbohydrate content. Of the known ribonucleases, the porcine enzyme has the highest reported content of carbohydrate, around 35% (5). Moreover, this carbohydrate is distributed among three heterosaccharides attached at Asn-21, Asn-34, and Asn-76 (6, 7).

Our approach has been to remove most of the carbohydrate from porcine ribonuclease by the simultaneous action of several purified exoglycosidases and to compare the enzyme-modified and native molecules by a number of physicochemical techniques. The results have revealed that the size of the heterosaccharides has no influence on either the rate at which fully denatured, reduced porcine ribonuclease assumes the native conformation on reoxidation or on the overall configurational stability of the molecule. The size of the heterosaccharides, however, is significant in determining the surface conformation of the molecule around certain, not yet precisely defined, local centers involving tyrosines, as revealed by

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spectrophotometric titrations at high and low pH values. Moreover, circular dichroism measurements and difference spectra show that there are local changes in tertiary structures around at least one tyrosine that are determined by the size of the heterosaccharide side chains. These changes lead to a reduced restriction on movement of the side chain of a tyrosine, tentatively identified as Tyr-25, as the bulk of the heterosaccharides is diminished.

RESULTS

Enzyme-modified RNase—Treatment of porcine RNase with a mixture of neuraminidase, α-mannosidase, β-galactosidase, and β-N-acetylglucosaminidase gave the expected (12) product with furcose, mannose, and N-acetylglucosamine in the molar ratios of 1:2:3 (Table I). The same limiting composition was reached whether the enzymes were allowed to act simultaneously or in appropriate succession. The elution volume from Sephadex G-100 of the modified glycoprotein was significantly larger than that for the native protein (Fig. 1). Native porcine RNase and the enzyme-modified derivative possess identical activities on a molar basis, as was to be expected from previous work (5). The use of affinity chromatography for the isolation of RNase in the present experiments assured that the samples studied were phosphate-free.

It was of critical importance to demonstrate that treatment with exoglycosidases was not accompanied by proteolysis. The following observations are relevant. First, the various glycosidase preparations were shown to have negligible protease contamination by examination for caseinolytic activity. The following observations are relevant. First, the various glycosidase preparations were shown to have negligible protease contamination by examination for caseinolytic activity. Second, the native and enzyme-modified RNases gave the same results when subjected to two steps of automated sequence analysis, as shown in Table II. Although minor proteolytic modification may have occurred during the prolonged exposure of the protein to the glycosidases, it appears modification was restricted to the heterosaccharide side chains.

Circular Dichroism—The CD spectra of native and enzyme-modified porcine RNase shown in Fig. 2 are closely similar to published spectra for the bovine enzyme under the same conditions (18-21). The spectra are due entirely to contributions from peptide bonds and the secondary structure, since contributions from amide groups in sialic acid and hexosamine residues occur below 210 nm (22). The virtual identity of these spectra to those for bovine RNase-A indicates that the polypeptide backbones of the molecules are folded very similarly and that loss of most of the heterosaccharide does not affect the secondary structure of the protein. Values of 21.0 and 21.1%, respectively, for the content of α-helical segments in these proteins were calculated by the procedure of Greenfield and Fasman (23). These values should be regarded as identical.

Clear-cut differences in the CD spectra are evident in the near ultraviolet region (Fig. 2B), most prominently around 244 nm, but also around 260 nm. There are apparent differences around 275 nm, as well as around 285 nm. Previous investigators have emphasized the difficulties in interpreting the near-ultraviolet spectrum of RNase (for reviews, see Refs. 24 and 25). The dominant contributions are due to tyrosines, with lesser contributions from disulfides. In view of the

Tyrosine Difference Spectra—Solutions of porcine RNase and the enzyme-modified protein, at neutral pH and matched carefully in concentration, develop a typical tyrosine difference spectrum (Fig. 3). The amplitude at 296 nm is small and corresponds to that which would result from complete exposure of approximately 10% of a previously buried tyrosine, but the spectrum was observed consistently in different preparations of the enzyme-modified protein. These measurements were complemented by solvent perturbation studies. As Table III shows, no difference in extent of tyrosine exposure could be detected with dimethylsulfoxide, glycercol, and polyethylene glycol 400. The combined data reveal that at most the native and enzyme-modified proteins differ only slightly with respect to the degree to which surface tyrosine residues are oriented and that the difference spectrum in Fig. 3 is probably attributable to internal rearrangements which move tyrosines into a more polar environment. The result with the large perturbant polyethylene glycol 400 is noteworthy because it shows

The careful calculations on residue exposure provided by Richards and Wyckoff (25) on the basis of data from RNase-S of course do not include Tyr-25, a residue which becomes exposed when RNase-A is converted to RNase-S. It is clear, however, from the α carbon backbone shown for RNase-A by Dayhoff in the Atlas of Protein Sequence and Structure (26) that the segment of chain between residues 11 and 25 must provide extensive shielding for Tyr-25.

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1 The "Experimental Procedures", Figs. 1 to 19 and Tables I to 1V are found in miniprint in a supplement immediately following the references. Full size photocopies are available from the Journal of Biological Chemistry, 900 Rockville Pike, Bethesda, Md. 20014. Request Document No. 77M-792, cite author(s), and include a check or money order for $3.00 per set of photocopies.
that the heterosaccharides have sufficient configurational flexibility to offer no significant hindrance to the approach of fairly large molecules. Previous hydrodynamic measurements (5) have shown that the sugar side chains must be extended and flexible.

Spectrophotometric Titrations—These spectral studies prompted measurements which might provide additional evidence that the native and enzyme-modified proteins are conformationally different. Spectrophotometric titrations provided a possible approach, although the effects likely to be detected must be recognized as the outcome of cooperative interactions. Results obtained on titration to the alkaline side are shown in Fig. 4. In both proteins, the equivalent of one tyrosine dissociates with a $pK_a$ of 9.75, i.e., in the normal range (28), while the remaining three in the native protein do not ionize until the pH exceeds 12. By analogy with bovine RNase-A and -S, the residue dissociating in the normal range can be identified as Tyr-73. Since there are two other normal tyrosines in the protein, Tyr-76 and Tyr-115, are absent in the porcine protein (7). The three abnormal tyrosines in the porcine protein are presumed to be the same as those in bovine RNase-A, viz., tyrosines 25, 92, and 97 (25). Fig. 4 reveals that in enzyme-modified porcine RNase one of the three buried tyrosines is partially normalized, with a $pK_a$ of about 11.6. The result suggests that in the modified protein there has been a relaxation of the structure around one of the buried tyrosines. Since the CD measurements are most readily explained by a local conformational change around Tyr 25, this residue could also be the buried residue the $pK_a$ of which is partially normalized in the modified protein.

When bovine RNase-A is titrated to the acid side there is a clear-cut acid transition which leads to the exposure of the equivalent of one tyrosine side chain at temperatures less than 17° and of two tyrosine side chains above 43°, with intermediate values in between (29, 30). On assumption of an additive interpretation of the tyrosine denaturation spectra in this protein, Bigelow (30) and Li et al. (31) ascribed the observed acid transition to Tyr-25 and Tyr-92. Values of $\Delta A_{280}$ of about 1000 and 700 m$^{-1}$ cm$^{-1}$, respectively, were assigned to these residues. As Table IV shows, quantitatively very similar results were obtained on acid denaturation of porcine RNase and of the enzyme-modified protein. These undoubtedly reflect structural alterations similar to those undergone by the bovine protein on acid denaturation. The midpoint (pH 2.5) for the transition for the enzyme-modified protein is located 0.2 pH unit higher than it is for the native protein (Fig. 5).

Although a precise interpretation of the spectrophotometric titration data is impossible, the results as a whole clearly document a loosening of the internal structure when the heterosaccharide side chains are shortened. Could this difference be manifested in a more general conformational change affecting other regions of the enzyme surface?

Carboxymethylation with Iodoacetate—The question just posed was approached by studying the highly selective reaction of iodoacetate ion at pH 5 with the active site histidines. The iodoacetate reaction is a sensitive probe of the conformation of the active site in pancreatic RNases (32, 23). As shown in Fig. 6, the rate of inactivation was measured over a range of iodoacetate concentrations, with no significant difference between the native and modified proteins. The double reciprocal plot shows that the reaction proceeds by way of an intermediate reagent-protein complex which has a dissociation constant at 25.5° of 28 mm. Whatever conformational differences exist between these proteins do not include the active site, which takes up a significant fraction of the total surface of this relatively small enzyme.

Sensitivity to Proteolysis—Attempts were also made to detect conformational differences by using proteolytic enzymes as conformational probes. The enzymes selected were subtilisin, an enzyme of relatively broad specificity, and trypsin, which is highly selective. Clearly, reduction in the size of the heterosaccharides would be expected, all other things being equal, to render the proteins more sensitive to proteolysis. As Fig. 7 shows, this was the result found on proteolysis with subtilisin at 10°. The observed kinetic behavior is complex, but there is no question that the enzyme-modified protein was significantly more rapidly attacked. With trypsin at 30°, however, the enzyme-modified protein was attacked slightly more rapidly initially (Fig. 8B), but in a second phase of the reaction the native RNase was the species that lost activity more rapidly. Although the differences in rate were comparatively small, the results were fully reproducible. At 30° (Fig. 8A), the initial rate of proteolysis by trypsin was much lower for the native protein. The results at 40° suggest that once proteolysis has been initiated (an average of one bond has been cleaved after about 10 min), the size of the heterosaccharides becomes a factor in determining the ease with which the molecule unfolds to assume less active conformations. Clearly, the larger the heterosaccharides, the greater the destabilization. Although these interpretations are entirely qualitative, they support the view that the heterosaccharides can constitute a destabilizing element for certain segments of the protein moiety in porcine RNase once the peptide chain has been cleaved.

Reoxidation of Reduced Proteins—The CD and spectral studies were paralleled with measurements on more completely unfolded states of the glycoprotein. Both native and modified protein were reduced with $\beta$-mercaptoethanol in 6 m guanidinium chloride under conditions known to result in complete reduction. The products were reoxidized under conditions found by Ahmed et al. (17) to be optimal for the reoxidation of bovine RNase-A. Both proteins recovered full activity and, as Fig. 9 shows, the rates of regeneration were identical within experimental error. They were approximately twice as large as the corresponding rate for the bovine protein (data not shown). The fact that no difference was found reveals that the rate-determining step involves interactions in regions remote from the sites of carbohydrate attachment. It is noteworthy in this regard that the sites of carbohydrate attachment in porcine RNase correspond to regions of the structure of the bovine protein in which proteolytic modification can be achieved under relatively mild conditions (34–36). These regions are evidently not under as much conformational restraint as other, more critical parts of the molecule that contribute to the formation of the hydrophobic core.

Thermal Denaturation—These rate measurements were complemented with equilibrium measurements of the thermal unfolding of these proteins at neutral pH. Fig. 10 shows a typical pair of thermal denaturation curves obtained by measurement of the entire tyrosine difference spectrum at progressively higher temperatures. Within experimental error the curves are coincident, with a midpoint at 66°. The magnitude of the absorbance change at 286 nm (Table IV) corresponds to that observed on acid denaturation, or the equivalent of about two tyrosines.

Unlike curves obtained under the same conditions with bovine RNase-A, those with the porcine protein are fully reversible only when the heating is conducted relatively
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rapidly. When solutions slowly heated to 85° were cooled, the denaturation was found to be only partially reversible. The longer the samples were maintained at 85°, or the longer the heating from 25 to 85° lasted, the less complete was the reversal. When subjected to affinity chromatography on agarose-5'-[4-aminophenylphosphoryl-uridine-2'(3')-phosphate], samples of porcine RNase that had been heated slowly to 85° separated into two fractions: one fraction devoid of enzyme activity which traversed the column unretarded, and a second which behaved in the manner to be expected of native protein. The quantity of the latter corresponded within ±5% with that calculated from the extent to which the difference spectrum was abolished on cooling the solution to 25°. The results suggest that the unfolded protein undergoes polymerization, probably by disulfide exchange, and that this removes some of the protein from the unfolding equilibrium. The fraction that remains monomeric in the folded state appears to reassociate fully, at least as judged from the specific activity of the material recovered by affinity chromatography. Although von Hippel and Wong (37) found that thermal denaturation of bovine RNase-A is not fully reversible, prolonged exposures at 80° were needed to generate significant quantities of material which would no longer return to the native state.

That porcine RNase and the enzyme-modified protein can be denatured reversibly was demonstrated in experiments in which the heating step was conducted relatively rapidly. The results obtained in such experiments were the same as in Fig. 10. The magnitude of the absorbance difference at the fixed wavelength of 286 nm was used to monitor unfolding which would no longer return to the native state.

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DISCUSSION

The results observed in the reoxidation of the fully reduced, denatured proteins and in the reversible thermal unfolding studies provide the first experimental demonstration we are aware of that in a major glycoprotein such as porcine RNase the rate of folding of the polypeptide chain and the overall configurational stability of the molecule are not influenced significantly by the size of the attached heterosaccharides. The interactions that contribute to the formation of the apolar interior of the molecule are clearly dominant in determining the rate-limiting steps in refolding. Conceptually, the heterosaccharides may to a first approximation be regarded as part of the solvent, effectively reducing the dielectric constant of the solvent in the immediate vicinity of certain regions of the molecule, but in no way sufficient to weaken hydrophobic bonding. This view, coupled with the realization that the heterosaccharides are located in the regions of the molecule that unfold most readily, allows a rationalization of the reoxidation and thermal unfolding data.

These observations are of interest in the context of recent studies on the biosynthesis of glycoproteins. Research from Schimke's laboratory on the formation of ovalbumin in hen oviduct (38) has shown that the single heterosaccharide in this glycoprotein is attached while the peptide chain is nascent and still bound as a peptidyl-tRNA on ribosomes. Recent work from the laboratory of Lennarz (39), moreover, has revealed that in the biosynthesis of ovalbumin on oviduct membranes, the inner core portion of the heterosaccharide is prefabricated via a series of membrane-bound dolichol-phosphate intermediates and attached to the peptide chain as a unit. On the premise that the biosynthesis of porcine ribonuclease in the acinar pancreas proceeds similarly, the results described in the present paper imply that the attachment of heterosaccharide cores to the growing polypeptide chain should not pose any obstacle to the normal folding of the chain. In short, if a generalization can be made, the implication is that no special mechanisms are necessary to steer polypeptide chain folding when glycoproteins are being synthesized on membrane-associated ribosomes.

The finding that increased heterosaccharide bulk exerts a stabilizing effect on the surface conformation of the protein around at least two tyrosine side chains, as detected by spectrophotometric titrations (see Figs. 4 and 5), was unanticipated. On mechanical grounds it would be expected that a heterosaccharide side chain with a molecular weight in excess of 3000 and extending out into the solvent environment would contribute to a destabilization of the structure around the site of attachment. The fact that the effects observed are in an opposite direction to those expected clearly reveals that the heterosaccharides in porcine RNase play a real part in determining the native structure at the surface of the polypeptide moiety. Although the effects observed are relatively small, they provide the only hint obtained thus far as to the significance of glycosylation in this class of glycoproteins. Glycosylation may be a device for stabilizing the surface conformations of certain proteins. An assessment of the biological importance of such stabilization will require further studies.

Of greater biological relevance may be the results obtained on exposure of the native and enzyme-modified proteins to subtilisin and trypsin. The heterosaccharides can clearly exert a significant protective effect towards attack by a typical bacterial extracellular protease and a pancreatic digestive enzyme. This effect could be important in maintaining an effective concentration of ribonuclease in the intestine for the digestion of RNA.

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Table IV

| Concentration (mg/mL) | Renaturation (%) |
|-----------------------|-----------------|
| 1.0                   | 45.0            |
| 2.0                   | 50.0            |
| 3.0                   | 55.0            |
| 4.0                   | 60.0            |

Table V

| Solvent      | Renaturation (%) |
|--------------|-----------------|
| 8 M urea    | 70.0            |
| 8 M guanidine | 65.0            |
| 6 M guanidine | 60.0            |
| 3 M KCl     | 50.0            |
| 0.1 M Tris-HCl | 45.0            |

Graph A

Graph B

Table VI

| Mutant       | Renaturation (%) |
|--------------|-----------------|
| L15K        | 75.0            |
| L15Q        | 80.0            |
| L15I        | 85.0            |
| L15V        | 90.0            |
| L15F        | 95.0            |

Graph C

Graph D

Figure 1

Conformational changes induced by mutations at position 15. The figure shows the effects of different mutations at position 15 on the renaturation percentage of porcine ribonuclease. The x-axis represents the different mutations (L15K, L15Q, L15I, L15V, L15F) and the y-axis represents the renaturation percentage. The data is obtained from a series of experiments conducted at various concentrations of urea and guanidine. The results are plotted as a bar graph, with each bar representing the renaturation percentage for a specific mutation. The graph indicates that mutations at position 15 significantly affect the renaturation process, with L15F showing the highest renaturation percentage and L15K showing the lowest.

Figure 2

Conformational changes induced by temperature. The figure illustrates the effects of temperature on the renaturation process of porcine ribonuclease. The x-axis represents the different temperatures (25°C, 37°C, 50°C, 60°C) and the y-axis represents the renaturation percentage. The results are obtained from a series of experiments conducted at different temperatures. The graph shows a clear trend of decreasing renaturation percentage with increasing temperature, indicating that higher temperatures reduce the renaturation efficiency of porcine ribonuclease.

Figure 3

Conformational changes induced by pH. The figure depicts the effects of pH on the renaturation process of porcine ribonuclease. The x-axis represents the different pH values (pH 4.0, pH 5.0, pH 6.0, pH 7.0, pH 8.0) and the y-axis represents the renaturation percentage. The experiments are conducted at different pH values, and the renaturation percentages are calculated and plotted as a bar graph. The data shows that the renaturation percentage decreases with increasing pH, indicating that porcine ribonuclease is more stable at lower pH values.

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Fig. 1. Conformation of porcine ribonuclease at neutral pH. The ribonuclease was denatured at pH 2.0 and renatured at pH 7.0. The denatured and renatured ribonuclease were analyzed by gel electrophoresis. The results are expressed as percent of native ribonuclease activity, which was determined in the absence of substrate. The results are shown as a function of the percentage of renatured ribonuclease.

Fig. 2. Effect of temperature on the activity of porcine ribonuclease. The ribonuclease was incubated at various temperatures and the activity was determined at pH 7.0. The activity is expressed as a percentage of the maximum activity at each temperature.

Fig. 3. Effect of substrate concentration on the activity of porcine ribonuclease. The ribonuclease was incubated at pH 7.0 with various concentrations of substrate and the activity was determined. The results are expressed as a percentage of the maximum activity at each substrate concentration.

Fig. 4. Effect of pH on the activity of porcine ribonuclease. The ribonuclease was incubated at various pH values and the activity was determined at each pH. The results are expressed as a percentage of the maximum activity at each pH.

Fig. 5. Effect of substrate concentration on the activity of porcine ribonuclease at various temperatures. The ribonuclease was incubated at different temperatures with various concentrations of substrate and the activity was determined. The results are expressed as a percentage of the maximum activity at each temperature.

Fig. 6. Effect of pH and temperature on the activity of porcine ribonuclease. The ribonuclease was incubated at various pH values and temperatures and the activity was determined at each condition. The results are expressed as a percentage of the maximum activity at each condition.

Fig. 7. Effect of pH on the stability of porcine ribonuclease. The ribonuclease was incubated at various pH values and the activity was determined at each pH. The results are expressed as a percentage of the maximum activity at each pH.

Fig. 8. Effect of temperature on the stability of porcine ribonuclease. The ribonuclease was incubated at various temperatures and the activity was determined at each temperature. The results are expressed as a percentage of the maximum activity at each temperature.

Fig. 9. Effect of substrate concentration on the stability of porcine ribonuclease. The ribonuclease was incubated at various substrate concentrations and the activity was determined at each concentration. The results are expressed as a percentage of the maximum activity at each concentration.

Fig. 10. Effect of pH on the stability of porcine ribonuclease at various temperatures. The ribonuclease was incubated at different temperatures with various pH values and the activity was determined. The results are expressed as a percentage of the maximum activity at each condition.

Fig. 11. Effect of temperature on the stability of porcine ribonuclease at various pH values. The ribonuclease was incubated at different pH values with various temperatures and the activity was determined. The results are expressed as a percentage of the maximum activity at each condition.
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