Glycoforms Modify the Dynamic Stability and Functional Activity of an Enzyme†

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Received September 20, 1993; Revised Manuscript Received October 29, 1993

ABSTRACT: Glycoproteins generally consist of collections of glycosylated variants (glycoforms) in which an ensemble of different oligosaccharides is associated with each glycosylation site. Bovine pancreatic ribonuclease B occurs naturally as a mixture of five glycoforms in which the same polypeptide sequence is associated with a series of oligomannose sugars attached at the single N-glycosylation site. Individual glycoforms were prepared by exoglycosidase digestions of RNase B and analyzed directly at the protein level by capillary electrophoresis. For the first time, electrophoretically pure single glycoforms have been available to explore the possibility that different sugars might specifically modify the structure, dynamics, stability, and functional properties of the protein to which they are attached. Comparisons of the amide proton exchange rates for individual glycoforms of RNase B and unglycosylated RNase A showed that while the 3D structure was unaffected, glycosylation decreased dynamic fluctuations throughout the molecule. There was individual variation in the NH–ND exchange rates of the same protons in different glycoforms, demonstrating the effects of variable glycosylation on dynamic stability. Consistent with the overall decrease in flexibility, and with the possibility that all of the sugars may afford steric protection to susceptible sites, was the finding that each of the glycoforms tested showed increased resistance to Pronase compared with the unglycosylated protein. In a novel sensitive assay using double-stranded RNA substrate, the different glycoforms showed nearly a 4-fold variation in functional activity; molecular modeling suggested that steric factors may also play a role in modulating this interaction.

Mammalian ribonucleases (RNases)1 regulate many important biological functions by catalyzing the degradation of single- and double-stranded ribonucleic acids (RNAs). It is therefore important to probe variable parameters, such as glycosylation, which may affect the dynamic properties or protease resistance and functional activities of this class of enzymes. Ribonuclease–nucleic acid interactions have been extensively studied, but until now the contribution of the oligosaccharides has not been addressed.

RNase secreted from the pancreas catalytically degrades RNA in the gut and is associated with pancreatic cancer (Reddi & Holland, 1976). RNase is expressed in other normal and tumor cells (osteosarcoma, leiomyosarcoma; P. Fiten et al., unpublished data). RNases and some of their homologues are cytotoxic (D’Allesio et al., 1991; Wool, 1984); moreover, RNases interfere with the double-stranded replicative intermediates of many viruses, with duplex-based latency of Herpes viruses (Stevens et al., 1987), with viral capsid formation (Fisher & Johnson, 1993), and with double-stranded RNA-mediated induction of cytokines such as interferon (Van Damme et al., 1985), IL-6 (Van Damme et al., 1987), and chemokines (Van Damme et al., 1991).

Bovine pancreatic RNase is a mixture of unglycosylated RNase A (13 682 kDa) and a collection of glycoforms (designated RNase B Man-5, -6, -7, -8, and -9) in which the oligomannose series, Man-5 to Man-9, is associated with the single N-glycosylation site at Asn34LeuSer. The significance of such glycoform diversity is currently the subject of much debate (Rademacher et al., 1988); in particular, it is not known whether the particular oligosaccharide structures associated with single glycoform populations can specifically modify the conformation, structure, dynamic fluctuations, stability, and functional activity of the protein so that the observed physical properties and functional activities of a glycoprotein are a combination of a range of individual contributions.

While it is clear that studies comparing glycosylated and unglycosylated variants of proteins have demonstrated many roles for glycosylation and shown the importance of site occupancy, it has not been possible previously to isolate individual glycoforms to address this fundamental question. An important example is tissue plasminogen activator (t-PA). The rate of fibrin-dependent plasminogen activation spans a range which is dependent on the glycosylation site occupancy of both t-PA and its substrate, plasminogen. At the extremes the fibrin-dependent activity for type I1 t-PA (two N-linked glycans) with type I1 plasminogen (one O-linked glycan) is 2–3 times the value for type I t-PA (three N-linked glycans) and type I plasminogen (one N-linked and one O-linked glycan) (Wittwer et al., 1989; Mori et al., unpublished results). The possibility exists that when glycosylated molecules are associated with cascade events (such as t-PA and plasminogen, which are involved in the remodeling of extracellular matrices), activity differences arising from variable site occupancy may be highly amplified. In addition, we would like to know whether, within this framework, glycoform diversity at each individual site can provide a spectrum of activity, allowing the cell a means of achieving a finely regulated response to its environment. Such studies have not been possible because the technology required to separate
individual glycoforms of a glycoprotein has not previously been available. However, the development of capillary electrophoresis to resolve the five glycoforms of bovine pancreatic RNase B (Rudd et al., 1992) has allowed us to prepare and analyze individual pure glycoforms of a glycoprotein for the first time.

It is now possible to address the fundamental question of the consequences of glycosylation changes at a single site. Single populations of bovine pancreatic RNase B glycoforms were examined in a number of systems designed to compare some aspects of their 3D structure, dynamics (including rigidity, flexibility, and unfolding), stability, and functional activity.

Many physical parameters have been determined for bovine pancreatic RNase; in particular, the crystal structures of both RNase A and RNase B are available (Wlodawer et al., 1988; Williams et al., 1987). The active site, centered around His12, His119, and Lys41, has been well-defined (Shall & Barnard, 1969), as have the anionic binding sites involved in its interaction with RNA (McPherson et al., 1986).

EXPERIMENTAL PROCEDURES

Capillary Electrophoresis of RNase. A 100-cm fused silica capillary, 50-μm i.d., was used; voltage conditions were 1 kV for 1 min, 20 kV for 74 min, 30 °C, wavelength for detection 200 nm, and injection time 1.5 s, in 20 mM sodium phosphate, 50 mM SDS, and 5 mM sodium tetraborate, pH 7.2, on a Beckman P/ACE system with GoldTM Software. HPLC used a Waters system with a TSK 3000 gel filtration column equilibrated in potassium phosphate buffer (0.1 M, pH 7.2).

Enzymatic Modification of RNase B Glycoforms. RNase A and RNase B (Sigma Chemical Co.) were further purified to electrophoretic homogeneity (CE, Figure 1b, f, and SDS-PAGE, data not shown) by concanavalin A-Sepharose affinity chromatography (Pharmacia) and Sephadex (G-50, Pharmacia) gel permeation chromatography.

Three individual glycoforms were prepared by enzymatic modification of RNase B. All the digestions were monitored by capillary electrophoresis; in the case of RNase Man-1, the reaction was carried out in the CE carousel and samples were injected directly into the capillary from the reaction mixtures, eliminating the need to take aliquots and stop the reactions. All peaks were assigned by coinjection of the digestion mixtures with the standard RNase B ladder preparation described in Figure 1a. The glycoforms were purified from exoglycosidase enzymes by gel permeation HPLC before analysis.

The final samples were electrophoretically pure by SDS-PAGE (data not shown) and CE (Figure 1c–e). The molecular weights were determined by mass spectrometry (data not shown), which also confirmed the absence of phosphate adducts in these preparations.

RNase B Man-5. RNase B was digested to a single population of RNase B Man-5 with Aspergillus saitoi α(1–2)-mannosidase at an enzyme:substrate ratio of 25 milliunits:1 mg in 1 mL of 50 mM sodium acetate, pH 5, in 96 h at 37 °C. RNase B Man-1. RNase B was converted to a single population of RNase B Man-1 with jack bean α-mannosidase (5 units:1 mg in 1 mL 10 mM sodium citrate, pH 4.5/0.2 mM zinc acetate) in 95 min at 30 °C. RNase B Man-0. RNase B Man-1 was converted to a single population of RNase B Man-0 with the β(1–4)-mannosidase Helix pomatia in 20 h at 37 °C in 100 mM sodium acetate, pH 4, with an enzyme:substrate ratio of 10 units:50 mg/mL.

Pronase Digestion. In three separate experiments RNase A together with RNase Man-5, RNase Man-1, or RNase Man-0 was incubated with Pronase in the CE carousel at 30 ± 3 °C. The enzyme to substrate ratio was 7.7 units:10 mg, and the buffer was 50 mM Tris-HCl, pH 7.5. Digestions were monitored continually by direct injection into the capillary, and each incubation was repeated at least three times. The data were obtained by integration of the appropriate peaks. Similar digestions of the glycoforms in pairs confirmed that there was no significant difference in their susceptibilities to the protease (data not shown).

The capillary electrophoretic analyses of the digestion mixtures showed no evidence of additional sets of glycoform peaks which could have been aggregated RNase. Structures two or more times larger than monomeric RNase would elute later in the spectrum than either RNase B glycoforms or the peptide fragments.

NMR experiments were carried out at millimolar concentrations according to the methods described previously (Joao et al., 1992). The data show comparable line widths of both aromatic and aliphatic protons for both RNase A and RNase B, indicating similar tumbling times in solution. This rules out the possibility of large-scale aggregation.

In Vitro Labeling of ds RNA. A 900 base pair genomic DNA fragment of the murine c-mos oncogene (provided by G. F. Van de Woude, NCI, Frederick, MD) was cloned into pSP64 and pSP65 vectors. Radiolabeled sense and antisense transcripts were generated as follows: 2 μg of linearized plasmid DNA, 20 μmol of [32P]UTP, 30 mmol of the other three nucleotides, 50 units of RNasin (Promega), 10 mmol...
of dithiothreitol, and 10 units of SP6 RNA polymerase were subjected to in vitro transcription for 3–6 h at 37 °C, after which the RNAs were precipitated with ammonium acetate/ethanol. Equimolar quantities of sense and antisense RNA transcripts were mixed and pelleted by centrifugation for 15 min at 15000g. The pellet was washed with 70% cold ethanol and dissolved in 50 μL of a solution containing 80% deionized formamide, 40 mM Pipes, pH 6.7, 400 mM NaCl, and 1 mM EDTA. After 5 min of melting at 95 °C the monomeric RNAs were hybridized at 45 °C for 6 h to form a duplex. The reaction was stopped by the addition of 300 μL of ice-cold 300 mM NaCl, 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 1 mL of ethanol. Precipitates were allowed to form for 30 min at −70 °C or overnight at −20 °C. In particular experiments single-stranded ends were removed from the duplex with RNase T1 (200 units, 30 min, 37 °C), followed by extraction with phenol, gel filtration chromatography on Sephadex G-50, and precipitation. Similar results were obtained with substrate which had not been RNase T1-treated and therefore contained protruding single-stranded ends which were highly susceptible to the enzyme.

Silver and Coomassie blue stained nonreducing SDS–PAGE and Western blots of the glycoform samples at the concentrations used in our work show that over 90% of the glycoproteins are in the monomeric form.

Activity Measurements of RNase with ds RNA. For RNase reactions, 1000 Cerenkov cpm of the radiolabeled duplex RNA was reacted in a total volume of 25 μL for 60 min at 37 °C in 25 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, and 50 mM NaCl. The specific radioactivity of the substrate used was always around 10⁶ cpm/μg. The reaction products were then resuspended in loading buffer (0.025% bromophenol blue, 0.025% xylene cyanol, 0.5% sodium dodecyl sulfate, and 20% sucrose) and separated in nondenaturing gels. Electrophoresis was carried out for 3–4 h at 100 V in 7% polyacrylamide gels in Tris/borate/EDTA buffer. After fixation in 10% acetic acid, the gels were dried and autoradiographed for 12–16 h. For quantitative analysis the autoradiographs were processed in a CAMAG TLC scanner 88 densitometer equipped with a transmission option 76760. Data processing used CAMAG software package version II V 3.11. Protein concentration was determined by standardized HPLC of phenylthiohydantoin-derivatized alanines (residues 4, 5, and 6) during automated Edman degradation on an Applied Biosystems 477A/120A unit. The standard error of the mean molarity of each individual sample was always less than 10%. Activity comparison of individual glycoforms with RNase A and RNase B was done with 1:3 serial dilutions of 100 pmol of each enzyme preparation. The calculated enzyme:substrate ratio was always in excess of 1000 allowing the determination of enzyme activity directly from substrate conversions. The activity was scored by comparing the dilution at which 50% of the substrate was converted.

RESULTS

Enzymatic Modification of RNase B To Obtain Individual Glycoforms. Recent advances in capillary electrophoresis have enabled each of the oligomannose glycoforms of RNase B (from Man-0 to Man-9) to be resolved at the protein level in their correct molar proportions for the first time; therefore, it has been possible to analyze population changes directly and to isolate a number of single, electrophoretically pure glycoforms. RNase Man-5, RNase Man-1, and RNase Man-0 were prepared by modification of naturally occurring RNase B (Man-5–9) with exoglycosidase enzymes (Figure 1) and some of their structural and functional properties compared.
FIGURE 2: Effects of glycosylation on the rates of amide H/D exchange for selected residues of RNase. (a) A schematic representation of ribonuclease highlighting those residues whose amide protons (shown by the space-filled balls) show modified hydrogen-deuterium exchange as a result of glycosylation of the enzyme at Asn34. Some of the amide protons of RNase Man-1 and RNase Man-5 which show additional protection from solvent exchange when compared with RNase A are shown. The same residues are also protected in RNase B. However, the dark shaded balls represent amide protons (10, 11, 12, 13, 32, 34) which are less well protected from exchange in RNase B than in either RNase Man-1 or RNase Man-5. The structure of Man$_{5}$GlcNAc$_{2}$ (drawn to the same scale as the protein) is shown attached to Asn34 and extending away from the protein into solution. Residues associated with the active site which also show altered amide proton exchange rates include His12, Lys41, His119, and Asp121. The amide proton exchange rates of Arg39 and Arg85 are also modified. These two residues are involved in the binding of phosphate to the enzyme. (b) A comparison of the amide proton exchange rates for a number of residues which show similar protection from exchange in RNase B, RNase Man-5, and RNase Man-1 compared to RNase A. (c) A bar graph presenting amide-deuterium exchange times for some of those protons highlighted in panel a which show additional protection in RNase Man-1 and RNase Man-5 relative to RNase B. The dark region corresponds to the time range (hours) over which the COSY cross-peak is observable in the NMR spectra. In residues 10, 32, and 34 the cross-peak for RNase A had disappeared at the first time point. (d) Profiles showing typical examples of the results of the Pronase digestion of RNase A together with RNase Man-5, RNase Man-1, or RNase Man-0 and showing the percentage of intact RNase A and either RNase Man-5 (a), RNase Man-1 (b), or RNase Man-0 (c) digested at each time point.

was less than for RNase A; this was consistent with the finding that all of the glycoforms showed increased resistance to Pronase, a broad-spectrum protease which cleaves ribonuclease at many sites. The overall rate of cleavage depends on the dynamic stability of the protein, which includes the rate at which it unfolds. In digestions of mixtures of RNase A and each of the Man-5, Man-1, or Man-0 glycoforms, the glycosylated enzymes were always equally less susceptible to
FIGURE 3: Comparison of the functional activity of RNase A, RNase B, and individual glycoforms of bovine pancreatic ribonuclease in a substrate conversion assay with double-stranded RNA. (left) A representative autoradiogram of residual substrate after digestion with a 1:3 serial dilution of each glycoform. (right) Comparison of the titration curves of the activity of the individual glycoforms (RNase Man-0, RNase Man-1, and RNase Man-5) with RNase A and the natural RNase B mixture toward ds RNA. Each point is the mean of four titrations (two with RNase T1-treated substrate and two with untreated substrate) and the standard errors are shown by the error bars. As dilutions of the enzyme increase from 1:3 to 1:81, the residual substrate increases, which implies that the activity of the enzyme decreases.

FIGURE 4: Two views of the unglycosylated RNase molecule with the Man-9 and Man-5 glycoforms drawn to scale. On the protein (white) the active site is cyan, the glycosylation site (Asn34LeuSer) is green, and the cluster of basic residues (Arg10, Lys31, Lys37, Arg33) in range of all the oligosaccharides is dark blue. These residues provide a strong local anion binding site where the 5'-terminal phosphate of RNA interacts with the enzyme (McPherson et al., 1986). Oxygen atoms are red, sulfur atoms yellow, and nitrogen atoms blue. Two of the oligosaccharides associated with RNase B are shown on the left of the protein: GlcNAc2Man9 (upper) and GlcNAc2Man6 (lower).

the protease than RNase A (Figure 2d). Similar digestions of mixtures of the glycoforms confirmed that all were equally resistant (data not shown).

**RNase Glycoforms Show a Range of Enzymatic Activity toward Double-Stranded RNA.** RNase is an endonuclease which cleaves RNA mainly on the 3'-side of pyrimidine nucleotides, and many RNA molecules have sections of single-stranded RNA interspersed with double-stranded sections. The generation of a well-defined, in vitro labeled double-stranded RNA substrate enabled the functional activities of RNase A, RNase B, RNase Man-5, RNase Man-1, and RNase Man-0 to be compared in a novel sensitive assay system which monitored the rate of cleavage of ds RNA by each glycoform. Activity was scored by comparing the dilution at which 50% of the substrate was converted.

The reasons for choosing this type of assay were the following: first, conventional spectrophotometric assays that monitor the degradation of ss RNA are accurate, but the substrate is a heterogeneous mixture of molecules with different length and partial (and variable) double-stranded structures. Second, we tested radiolabeled single-stranded substrates in our assay and confirmed that the rate of catalysis is much faster than with double-stranded RNA substrate but also that the single-stranded substrate is difficult to handle, less stable, and prone to degradation by the ubiquitous RNases. This results in high interassay variations with ss RNA substrates. The advantage of the ds substrate is that it is defined, that it can be labeled to high specific activities, and that the results of the assays are therefore reproducible.

RNase A was more than three times as active as RNase B, and the individual glycoforms were intermediate in activity in the ratios 1:1.6:2.2:3.6:3.8 (Figure 3):

\[ \text{RNase A} > \text{RNase Man-0} = \text{RNase Man-1} > \text{RNase Man-5} = \text{RNase B} \]

The naturally occurring mixture of RNase glycoforms showed...
no significant decrease in activity compared with Man-5 although it contained 50% of glycoforms larger than the Man-5 structure. It appears that while the activity can be increased by removing mannose residues from Man-5, extending the sugar chain beyond Man-5 does not decrease the functional activity of the enzyme further.

These data demonstrate that the glycosylation of RNase is a factor which modulates the rate at which ds RNA is degraded.

Structure/Function Relationships Probed by Molecular Modeling. The role of the oligosaccharides in the modulation of the activity of RNase was further explored by molecular modeling. Important amino acid residues for the interaction of RNase with (double-stranded) RNA are those within the active site of the enzyme and also a number which form salt bridges with phosphate groups in the backbone of RNA. There are five of these bridges, including one in the active site where cleavage of the phosphodiester bond takes place. In particular, a cluster of residues (Lys 31, Lys 37, Arg 10, and Arg 33) (Figure 4) provides a strong local anion binding site where the 5'-terminal phosphate of RNA interacts with the enzyme (McPherson et al., 1986). The size of all the oligosaccharides associated with RNase is such that they can cover this cationic cluster, thus hindering the formation of the salt bridge and variably reducing the overall fit between the enzyme and substrate.

DISCUSSION

Previous studies of bovine pancreatic RNase A and RNase B have established many physical parameters for the molecules, allowing insight into these data which relate to the effects of single glycosylation changes on RNase B.

Consistent with the X-ray crystallography studies, NMR spectra (data not shown) showed that the overall 3D structure of RNase was unaffected by glycosylation. However, NH-ND exchange rate data showed that the overall dynamic stability of RNase A was increased by glycosylation; i.e., the fluctuations in the structure had decreased. This overall increase in rigidity may contribute to the increased protease resistance and decreased functional activity of the glycosylated variants compared with RNase A. In principle, such dynamic effects may be expected to apply generally to all glycoproteins.

The RNase glycoforms were equally more resistant to Pronase than RNase A, suggesting that, in addition to the decrease in flexibility, a major contributing factor in this interaction may be the steric protection of one or more susceptible sites which are in range of all of the oligosaccharides.

Differences in the dynamic stability of the same amino acid residues in different glycoforms, including ones at the active site and an anionic binding site, may contribute to the 3-fold variation in the functional activity of the glycoforms. In addition, molecular modeling studies suggest that different-sized oligosaccharides may exercise variable steric effects, both at the active site and at a cluster of residues involved in the formation of salt bridges to phosphate in RNA.

The effect of variable glycosylation on the environment of these important regions of the protein is one of the factors by which the activity of RNase is modulated; in this study we have examined only the effect of different oligomannose structures. Interestingly, RNase isolated from other organs and other species is known to be modified with complex, hybrid and larger oligomannose structures (Yamashita et al., 1986; Lawrence et al., 1993a,b). If the results from this study can be generalized, we may expect that glycosylation will increase the dynamic stability and protease resistance of a glycoprotein, although effects may largely be independent of glycoform type. However, for RNase, the size of the attached oligosaccharide is a factor in modulating the interaction between enzyme and substrate. The exact nature of the glycoform may also be an important feature of control for enzymes (such as tissue plasminogen activator) which are multidomain and whose interactions involve both inter- and intramolecular rearrangements.

ACKNOWLEDGMENT

The assistance of Paul Proost in protein purification and microsequencing is greatly appreciated. The authors thank Professor Jo Van Damme and Dr. Mark Wormald for helpful discussions.

REFERENCES