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Effects of glycosylation on protein conformation and amide proton exchange rates in RNase B

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Assignment of most of the proton NMR resonances of bovine pancreatic RNase B has been achieved using standard NMR techniques and by comparison with the published assignments for RNase A. A comparison of the NMR spectra of RNase B with RNase A shows that glycosylation of the enzyme has little overall effect on the conformation of the protein in solution. Comparisons of hydrogen–deuterium solvent exchange rates for the NH protons of RNase A and RNase B were made using two-dimensional ^{1}H correlation spectroscopy. In the case of the glycosylated enzyme the exchange rates decreased for the NH protons of residues 9–14, 23–24, 32, 34–35, 39–40, 43–44, 48–49, 60, 71, 75–76, 80, 83–85, 100–101, 107, 111 and 122, relative to the unglycosylated RNase A. These results are consistent with the presence of the oligosaccharide inducing enhanced global dynamic stability and consequent changes to the unfolding equilibrium of the enzyme. The enhanced stability is observed not only for residues in the vicinity of the glycosylation site, asparagine-34, but also at residues remote from this site, as much as 30 Å away.

Hydrogen exchange; NMR; RNase A and B; Effects of glycosylation

1. INTRODUCTION

At present little is understood about the role of carbohydrates on protein structure, function and dynamics in glycoproteins. The measurement of rates of exchange of labile hydrogens in proteins with the hydrogen of bulk water can provide detailed information concerning the conformational and dynamic properties of these molecules [1], since the rate is extremely sensitive to the local chemical environment of the exchange site and to the overall conformational dynamics of the protein. The mechanism of solvent exchange is presumed to occur as a consequence of fluctuations of the structure, enabling even internal hydrogens to make contact with solvent molecules. These fluctuations can range from local fluctuations of limited magnitude to local and global motions associated with co-operative unfolding and refolding events.

Ribonuclease B contains 124 amino acid residues and catalyses the cleavage of single-stranded RNA. This enzyme has five glycoforms consisting of $Man_5GlcNAc_2$ to $Man_9GlcNAc_2$ at the single glycosylation site (asparagine-34) [2,3]. We have used this as a model system to probe by NMR the effects of glycosylation on protein conformation and dynamics.

The main difficulty of this approach when studied by NMR methods is associated with the assignment of observed resonances. The assignment for ^{1}H-NMR resonances of most of the residues of RNase A, a non-glycosylated form of RNase B, have been reported [4–7].

In this paper we demonstrate, from measurements of hydrogen isotope exchange kinetics on RNase A and B, that glycosylation alters the protein's overall conformational dynamics.

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2. MATERIALS AND METHODS

RNase A (Type I-A) and B (Type XII-B) were purchased from Sigma Chemical Co. Analysis by SDS-PAGE revealed that the RNase A contained 10% RNase B and that the RNase B contained 50% RNase A. These preparations were purified by affinity chromatography using Concanavalin A Sepharose and gel filtration (Sephadex G-50F), to give a preparation of RNase A free from RNase B and vice versa.

The enzme was dissolved in 99.9% D_2O or H_2O/10% D_2O to give a concentration of 7–8 mM, with sodium 3-(trimethyl-silyl) propionic-2,2,3,3-d_4 acid as an internal reference at 0.0 ppm. One- and two-dimensional (COSY, NOESY and HOHAHA) ^{1}H-NMR spectra were recorded on a Bruker AM600 spectrometer, at a temperature of 35°C and pH 3.08 and 3.35. For samples made up in H_2O, the jump-and-return sequence for effective water suppression was used in both the HOHAHA and NOESY experiments [8], while the COSY experiments were performed using SCUBA phase cycling [9] to recover resonances under the water peak. pH values are uncorrected meter readings on a radiometer PHM 84 Research pH meter, using an Ingold combination electrode.

Amide NH exchange experiments were carried out at 35°C and pH 3.35. Hydrogen exchange was initiated by dissolution of freeze-dried protein (after 36 h of incubation at 45°C in H_2O) in D_2O to give a protein concentration of 7–8 mM. COSY spectra were recorded at various time intervals over a period of 30 days, with the sample incubating at 35°C between measurements. Data sets comprised 416 × 2048 data points in the frequency domain, with a relaxation delay

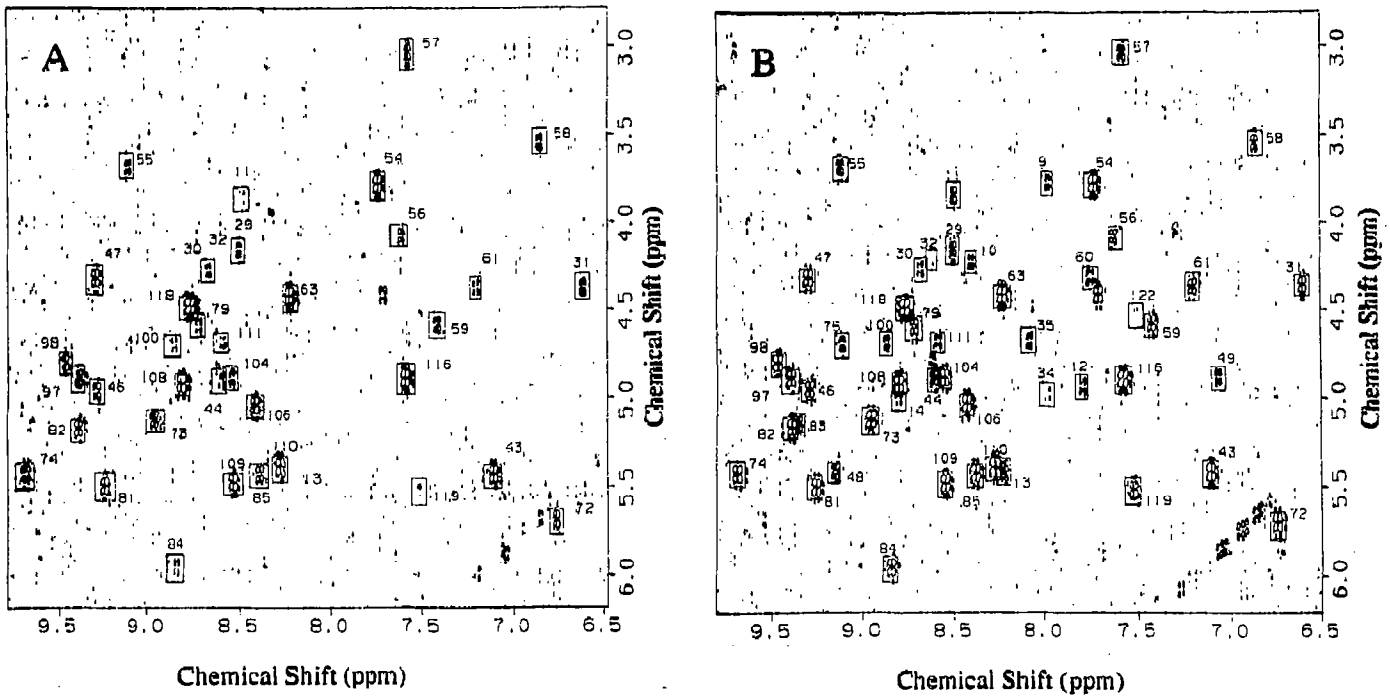

Fig. 1. Fingerprint region of the phase-sensitive COSY spectrum of RNase A (A) and RNase B (B) in D_2O. Spectra were recorded at 600 MHz and 35°C, immediately after dissolving freeze-dried samples in D_2O and adjusting the pH to 3.35.

of 1.0 s. A spectral width of 7042 Hz was used. Each experiment took 7 h to complete. Prior to Fourier transformation the 2D data matrix was multiplied by a double exponential window function in F_1 and a double exponential together with a trapezoidal window function in F_2. The intensity of each NH-CαH cross peak was taken to be the average of the absolute value of the heights of the four components of the cross peak, and were normalised by comparison with the intensities of correlations between non-exchangeable aromatic protons of residues Y25 and Y76.

3. RESULTS AND DISCUSSION

The 1D ^{1}H-NMR spectra together with COSY, NOESY and HOHAHA spectra of RNase A and RNase B were recorded in D_2O and H_2O at pH 3.08 and 3.35, and used in conjunction with the published assignments for RNase A [5–7], to assign most of the resonances of RNase B. A comparison of these spectra for RNase A and B reveals few differences and is consistent with the gross global conformation of ribonuclease being unaffected by glycosylation at asparagine-34. This is in agreement with the results obtained from crystallographic studies which conclude that the structures are essentially the same [10]. Spectral studies on RNase B′ (containing a single oligosaccharide composed of 1 residue of *N*-acetylglucosamine and 3 residues of mannose) showing the circular dichroism (CD) and optical rotatory dispersion (ORD) spectra of this protein to be identical with that of RNase A provides further support for the secondary and tertiary structure being unaffected by glycosylation.

A series of COSY spectra of ribonuclease A and B were recorded following exchange for various periods of time in D_2O solution at pH 3.35. A comparison of the finger-print region of the COSY spectra of RNase A and B shows that the chemical shifts of all the amide and alpha-carbon protons in RNase B are virtually identical, with no resonance being shifted by more than 0.02 ppm (i.e. no significant shifts), to those observed for RNase A (see Fig. 1). Amide protons not involved in designated hydrogen bonds exhibit rapid hydrogen exchange. 63% and 52% of the cross-peaks in the finger-print regions of the COSY spectra of RNase A and B respectively, are exchanged out of the spectrum within the first 3 hours after the exchange process was initiated. The more slowly exchanging amide protons are protected due to either hydrogen bonding and/or solvent inaccessibility. The fractional exchange of individual hydrogens was determined from the heights of the cross-peaks. In the case of RNase A, protection against exchange (i.e. slower exchange) is observed for residues of α-helices 1 (9, 11–14), 2 (23–24, 29–31), and 3 (54–59); β-sheet residues 43–44, 46–48, 72–76, 79, 81–85, 97–98, 100, 102, 104, 108–111; and β-turn residues 61, 63, 116, and 118–119. These results are consistent with an earlier report on amide exchange studies on RNase A [5]. As noted by Rico et al. [5], neutron diffraction studies [12] on solid RNase A reveal a lower number of protected protons than that observed in solution. In RNase B the above residues are further protected against hydrogen exchange by a factor of 1.5- to 6-fold

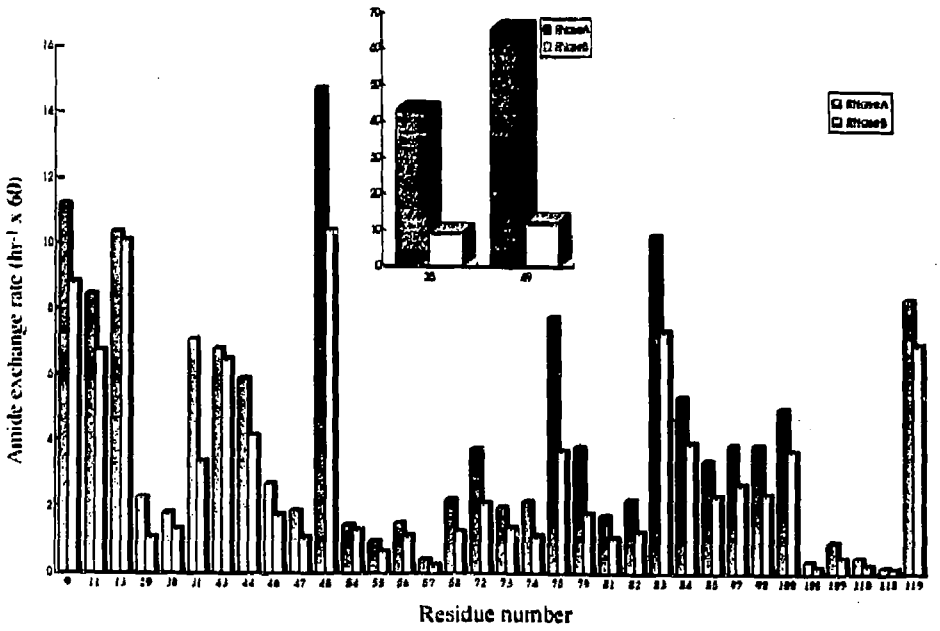

Fig. 2. A comparison of amide hydrogen–deuterium exchange rates for a number of residues of RNases A and B.

(see Fig. 2). Residue 34, at the point of glycosylation in RNase B and residues 32 and 35, in the vicinity of the glycosylation site are additional residues protected from exchange, when compared with the unglycosylated enzyme. Additional residues protected in RNase B relative to RNase A are 10, 23, 39–40, 49, 60, 71, 80, 101, 120 and 122. These residues are exchanged out of the first COSY spectrum obtained in the first 7 hours after the exchange process was initialised for RNase A, while in RNase B a number of these residues are still observable after 48 h. There is a clear general relationship between participation in main-chain hydrogen bonds in β-sheet or α-helical structure and slower hydrogen exchange; 42 of the 46 amide protons of RNase A and 56 of the 60 amide protons of RNase B which are in slower hydrogen exchange are in these types of secondary structure.

A comparison of the loss of cross-peak intensities in the fingerprint region of the COSY spectra at different time intervals of exchange for RNase A and RNase B, shows that a large proportion of the amide protons which exhibit slow exchange in RNase A are further protected against hydrogen exchange (by a factor of 1.5–6) in the glycosylated enzyme. This further protection against exchange must arise from the presence of oligomannose oligosaccharides at position 34 of RNase B. Residues which display modified HD exchange behaviour in the presence of the oligosaccharide are highlighted in Fig. 3. The presence of the oligosaccharide is observed to protect not only residues close to the site of glycosylation against hydrogen exchange (see Fig. 3), but a large number of residues throughout the protein. These data can be accounted for by the presence of the oligosaccharide moiety resulting in reduced dynamic fluctuations in the glycosylated enzyme relative to the unglycosylated protein and/or increased stability of the protein in the partially folded states where conformational changes are of primary importance in the mechanism of hydrogen-deuterium solvent exchange. These data contrast with the ^{13}C NMR study of the structure and dynamic behaviour of the oligosaccharide side chain of RNase B [13], where it was suggested that the carbohydrate side chain has a negligible effect (overall or localised) on the conformation or dynamics of the enzyme. Evidence supporting the suggestion that the oligosaccharide in RNase B increases the stability of the protein comes from the results of Puett [11] who showed that although the kinetics of unfolding and refolding in guanidine hydrochloride of RNases A and B′ (containing 1 residue of *N*-acetylglucosamine and 3 residues of mannose) are similar and proceed via a two-state mechanism (indicating that the oligosaccharide has no effect on protein folding under these conditions), the free energy of unfolding of RNase B′ is greater than that of RNase A. This shows that the carbohydrate moiety on RNase B′ has a small stabilizing effect on the protein. Furthermore, the present data shows the amide proton exchange studies to be a highly sensitive technique for probing structural conformation and dynamic changes as a result of glycosylation.

4. CONCLUSIONS

The structure of RNase A in solution, as also reported in the crystallographic studies [8], is essentially unaffected by glycosylation at Asn-34. Glycosylation does, however, alter the global stability/unfolding of the protein, which is seen to be enhanced for RNase B. Experiments are currently underway to measure the ac-

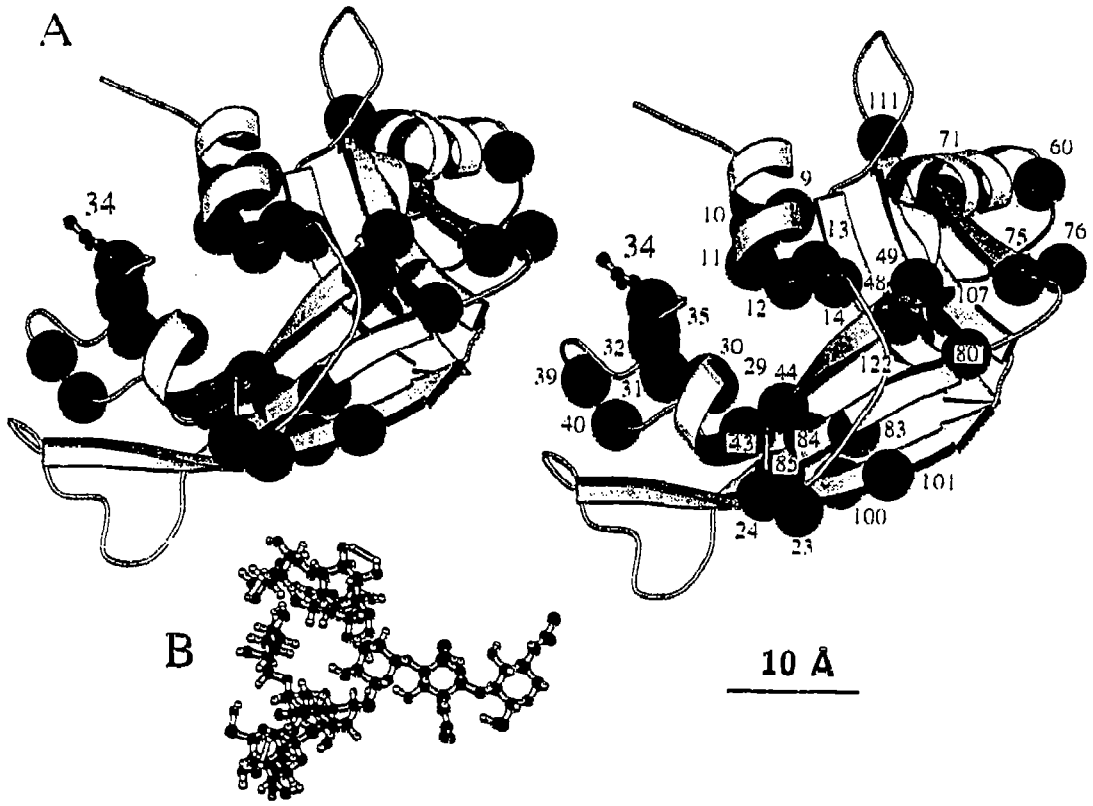

Fig. 3. (A) A schematic representation high-lighting those residues whose amide protons show modified hydrogen–deuterium exchange (shown by the filled balls) in RNase B resulting from glycosylation of the enzyme at Asn-34. The structure of $Man_5GlcNAc_2$ (drawn to the same scale as the protein) is shown in the insert (B).

tivation energy of unfolding of RNase A and RNase B and to investigate in detail the effects of glycosylation on the unfolding pathway.

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