

# Differences in Conformational Dynamics of Ribonucleases A and S as Observed by Infrared Spectroscopy and Hydrogen–Deuterium Exchange<sup>1</sup>

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Differences in conformational dynamics of bovine pancreatic RNase A and RNase S have been investigated using hydrogen–deuterium (H–D) exchange in conjunction with Fourier transform infrared spectroscopy. Deuteration-induced spectral changes in the amide I and II regions were monitored as a function of time. Second-derivative analysis revealed similar amide I spectral patterns for both proteins in H<sub>2</sub>O as well as fully deuterated in D<sub>2</sub>O. However, the rate of amide proton exchange of RNase S is much faster than that of RNase A at 25°C as determined by changes in the intensity ratio of amide II/amide I bands and frequency red-shifts of amide I components. The frequency red-shifts of the amide I components ascribed to  $\beta$ -sheet,  $\alpha$ -helix, and  $\beta$ -turns are continuous as a function of time, indicating that both proteins are too small to contain isolated secondary structural groups containing only exchanged or unexchanged amide protons in the partially deuterated intermediate states. Despite the dramatic difference in H–D exchange rate, the patterns of spectral changes in the conformation-sensitive amide I regions of RNase A and RNase S are very similar throughout the course of deuteration, indicating a similar pathway of amide proton exchange in both proteins. © 1996 Academic Press, Inc.

**Key Words:** ribonuclease A; ribonuclease S; conformational dynamics; infrared; H–D exchange.

Bovine ribonuclease (RNase)<sup>3</sup> A and RNase S are two important model proteins for the study of protein

structure and function due, in part, to their structural simplicity and commercial availability [for reviews see (1, 2)]. RNase A is a small monomeric enzyme containing 124 amino acid residues and catalyzes the hydrolysis of the phosphodiester linkage of single-stranded RNA leaving terminal 3'-pyrimidine nucleotide monophosphates. RNase S is an enzymatically active derivative of RNase A in which the peptide bond between residues 20 and 21 of RNase A has been cleaved; the resultant RNase S can be separated into two polypeptides referred to as the S-peptide (residues 1–20) and the S-protein (residues 21–124) (3). High-resolution crystal structures determined by X-ray and neutron diffraction (4–6) as well as solution structures determined by nuclear magnetic resonance spectroscopy (7) are available for both RNase A and RNase S. Three-dimensional structures of RNase A and RNase S are kidney-shaped molecules consisting of an N-terminal  $\alpha$ -helix and two other shorter helices packed against a central, twisted antiparallel  $\beta$ -sheet (4–6). The overall structures of the two proteins are very similar except near the cleavage site. However, significant differences in the structural stability of the two proteins have been reported. For example, RNase S was observed to be more susceptible to both further proteolysis (8) and thermally induced denaturation (9, 10) than RNase A.

Despite extensive applications of hydrogen exchange and Fourier transform infrared (FT-IR) spectroscopy in the studies of conformational dynamics [for reviews see (11–13)], structural stability [for review see (14)], and secondary structural compositions [for review see (15–17)] of proteins and polypeptides, the advantages of H–D exchange coupled with FT-IR spectroscopy in comparative studies of two structurally related proteins under the same experimental conditions have not been fully explored (18). For example, only a few investigators have focused their attention on the limited pro-

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<sup>3</sup> Abbreviations used: H–D exchange, hydrogen–deuterium exchange; FT-IR, Fourier transform infrared; RNase, ribonuclease.

teolytic digestion-induced differences in the structural stability of RNase A and RNase S using FT-IR spectroscopy and H-D exchange techniques (10, 19). The lack of more detailed, comparable data on the H-D exchange dynamics of two structurally related proteins in terms of infrared spectroscopic studies is an obvious shortcoming in current studies of structural stability and conformational mobility of proteins. This study is an attempt to bridge the infrared data gap between the initial partially deuterated and fully deuterated species of RNase A and RNase S under identical experimental conditions. Detailed comparisons of the rates of amide proton exchange and the patterns of amide I spectral changes during deuteration of both proteins were carried out at 25 and 50°C, respectively. Results of this study demonstrate that a limited proteolytic digestion can induce changes in the conformational dynamics of proteins and that these changes can be readily monitored by using H-D exchange coupled with FT-IR spectroscopy.

The peptide bond cleavage between residues 20 and 21 dramatically affects the rate of amide I proton exchange of RNase S in comparison with that of RNase A. However, the sequential changes of spectral patterns in the conformation-sensitive amide I region of RNase S during the course of deuteration resemble those of RNase A, indicating a similar amide proton exchange pathway.

## MATERIALS AND METHODS

**Materials.** Bovine pancreatic ribonuclease A (type XII-A) and ribonuclease S (type XII-S) were purchased from Sigma (St. Louis, MO) and used without further purification. Deuterium oxide (99.9 atom % D) was obtained from Aldrich (Milwaukee, WI).

**Sample preparations.** To prepare the H<sub>2</sub>O solutions, lyophilized proteins were dissolved in 10 mM potassium phosphate, pH 7.0, to give final concentrations of 20 mg/ml. For the D<sub>2</sub>O solutions, lyophilized proteins were dissolved in unbuffered D<sub>2</sub>O to give final concentrations of 5 mg/ml, pH 6.5 (uncorrected). Heat treatment was carried out by incubating protein solutions at 50°C in a water bath for the desired time and then quickly cooling by immersion in another water bath at 25°C to reduce the rate of H-D exchange; the spectrum was measured immediately at room temperature.

**Infrared measurement and spectral analysis.** Infrared spectra were measured at 25 with a Nicolet 730 FT-IR Spectrometer equipped with a dTGS detector and Omnic software. Protein solutions were placed in a CaF<sub>2</sub> cell (P/N 20500, Graseby) with a 6- $\mu$ m spacer for the H<sub>2</sub>O solutions and a 25- $\mu$ m spacer for the D<sub>2</sub>O solutions. For each spectrum, 128-scan (1.5 min, for H-D exchange within 60 min) and 256-scan (3 min, for H-D exchange beyond 60 min) interferograms were collected in a single-beam mode with 4 cm<sup>-1</sup> resolution. Reference spectra were recorded under identical scan conditions with only the corresponding solvent in the cell. Protein spectra were obtained according to previously established criteria (20) and a double-subtraction procedure (21). The resultant spectra were smoothed with a 7-point Savitsky-Golay function to remove white noise. Second-derivative spectra were obtained with the derivative function of the Omnic software. The nonzero baseline correction of the second-derivative spectra in the amide I region was performed as described previously (14).

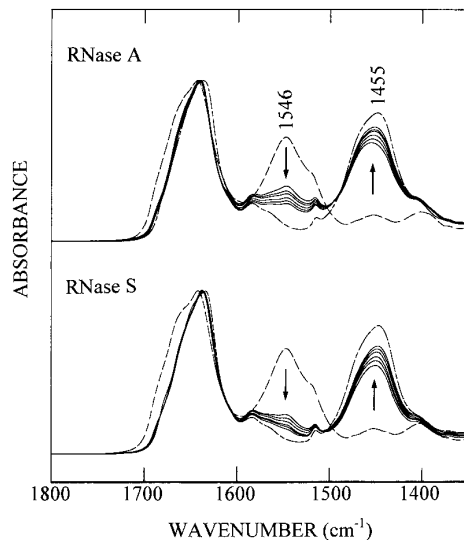


FIG. 1. Infrared spectra of RNase A and RNase S in D<sub>2</sub>O. The spectra were recorded at 1, 4, 10, 30, 60, 120 min (starting time for data collection) after being dissolved in D<sub>2</sub>O at 25°C. Arrows represent the direction of intensity changes. The spectra of the proteins in H<sub>2</sub>O (---) and in D<sub>2</sub>O with complete H-D exchange (—) are included for comparison. The spectral contributions of solvent and atmospheric water vapor have been subtracted from the observed protein spectra as described under Materials and Methods.

**Calculation of amide proton exchange rate.** The fraction of unexchanged amide protons ( $X$ ) was calculated at various times during H-D exchange using the equation (22)

$$X = (A_{II} - A_{II\omega})/A_{I\omega}, \quad [1]$$

where  $A_I$  and  $A_{II}$  are the absorbance maxima of the amide I and amide II bands, respectively,  $A_{II\omega}$  is the amide II absorbance maximum of fully deuterated protein, and  $\omega$  is the ratio of  $A_{II0}/A_{I0}$ , with  $A_{II0}$  and  $A_{I0}$  being the respective absorbance maxima for the amide II and amide I bands of the proteins in H<sub>2</sub>O.

## RESULTS

Figure 1 shows an overlay of the infrared spectra at 25°C of bovine RNase A and RNase S recorded at 1, 4, 10, 30, 60, and 120 min (starting time for data collection) in D<sub>2</sub>O. The spectra of the proteins in H<sub>2</sub>O and completely deuterated in D<sub>2</sub>O are included for comparison. It requires approximately 1.5 min to collect spectral data for each point within 1 h of H-D exchange. The spectra of RNase A and RNase S acquired in H<sub>2</sub>O exhibit the amide I and II band maxima at 1642 and 1546 cm<sup>-1</sup>, respectively, with an amide II/amide I ratio of 0.64. The spectra of the proteins acquired in D<sub>2</sub>O exhibit a time-dependent isotopic shift of the amide II band from 1546 to 1455 cm<sup>-1</sup> due to exchange of hydrogen by deuterium. The amide II band arises mainly from an out-of-phase combination of N-H in-plane bending and C-N stretching vibrations of the protein

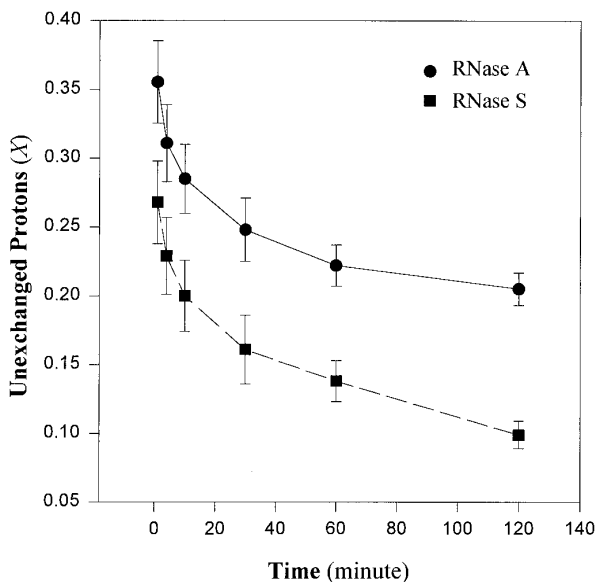


FIG. 2. The fraction of unexchanged amide protons in RNase A and RNase S as a function of time at 25°C. The data were calculated from three separate experiments.

backbone (23). The H–D exchange-induced infrared amide II band red-shift is typical for proteins in D<sub>2</sub>O (15, 16) and has been used to monitor the exchange rate of the protein amide protons for more than three decades (22, 24–26). The spectra of the proteins in D<sub>2</sub>O with complete H–D exchange were obtained by incubating the protein solutions in a 50°C water bath until the red-shift of amide I band was completed. A complete exchange was not achieved at 25°C for either RNase A or RNase S, even after 48 h of incubation. According to the literature, a complete H–D exchange takes up to 8 days for RNase S and even longer for RNase A at room temperature (19). The intensity of the amide II' band near 1450 cm<sup>-1</sup> cannot be used reliably as an indicator of complete H–D exchange due to formation of H–O–D, which exhibits a strong absorption near 1460 cm<sup>-1</sup> overlapping with the amide II' band. Figure 1 shows that the H–D exchange rate of RNase S is much faster than that of RNase A. By calculating the fraction of unexchanged amide protons for three experiments using the Eq. [1] (22) and plotting the fraction as a function of time, the increase in exchange rate of RNase S is obvious (Fig. 2). Two hours after dissolving the protein in D<sub>2</sub>O at 25°C, about 90% of the amide protons in RNase S were exchanged by deuterons, whereas about 80% of amide protons in RNase A were exchanged by deuterons.

To resolve the overlapping band components under the amide I contour, a second-derivative analysis was performed. Figure 3 shows an overlay of second-derivative spectra of RNase A and RNase S in the amide I region in H<sub>2</sub>O and D<sub>2</sub>O. In H<sub>2</sub>O, RNase A and RNase

S exhibit a similar second-derivative amide I spectra. Both proteins show a strong band near 1641 cm<sup>-1</sup> and a weaker band near 1689 cm<sup>-1</sup> assigned to low-wavenumber and high-wavenumber  $\beta$ -sheet structures, respectively. Both proteins also show a weaker band near 1658 cm<sup>-1</sup> attributed to  $\alpha$ -helices and two weaker bands near 1667 and 1682 cm<sup>-1</sup> due to  $\beta$ -turn structures (20, 27). Similar spectra and band assignments have been reported previously (19, 28). After complete H–D exchange, the amide I spectra of RNase A and RNase S again exhibit a high degree of similarity with a strong band near 1631 cm<sup>-1</sup> and a weak band near 1679 cm<sup>-1</sup> assigned to the low-wavenumber and high-wavenumber  $\beta$ -sheet structures, a weaker band near 1649 cm<sup>-1</sup> attributed to  $\alpha$ -helices, and a very weak band near 1661 cm<sup>-1</sup> due to  $\beta$ -turn structures (10, 15, 19, 28). Under the experimental conditions, a high degree of spectral similarity between RNase A and RNase S is expected, since both proteins have identical amino acid sequences with only the peptide linkage between residues 20 and 21 being hydrolyzed in RNase S (3), and both proteins have similar overall three-dimensional structures except near the cleavage site (6, 29–31). However, dramatic differences were observed between the spectra of the two proteins during the course of H–D exchange.

One minute after dissolving the proteins in D<sub>2</sub>O at 25°C, a 4.9 cm<sup>-1</sup> shift (from 1640.5 cm<sup>-1</sup> in H<sub>2</sub>O to 1635.6 cm<sup>-1</sup>) in the low-wavenumber  $\beta$ -sheet component was observed for RNase S, whereas only a 2.4 cm<sup>-1</sup> shift (from 1641.0 cm<sup>-1</sup> in H<sub>2</sub>O to 1638.6 cm<sup>-1</sup>)

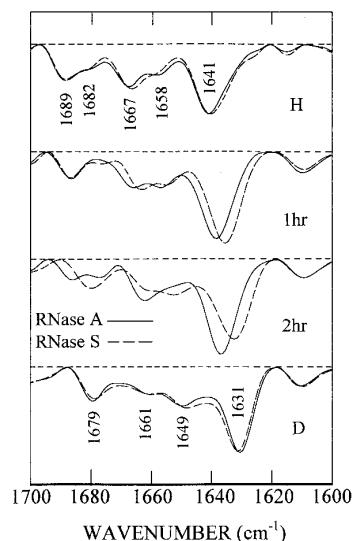


FIG. 3. Comparison of second-derivative spectra of RNase A and RNase S in H<sub>2</sub>O and D<sub>2</sub>O. (Top) Spectra in H<sub>2</sub>O. (Upper middle) Spectra of 1 min H–D exchange at 25°C. (Lower middle) Spectra of 48 h H–D exchange at 25°C. (Bottom) Spectra in D<sub>2</sub>O with complete H–D exchange at 50°C.

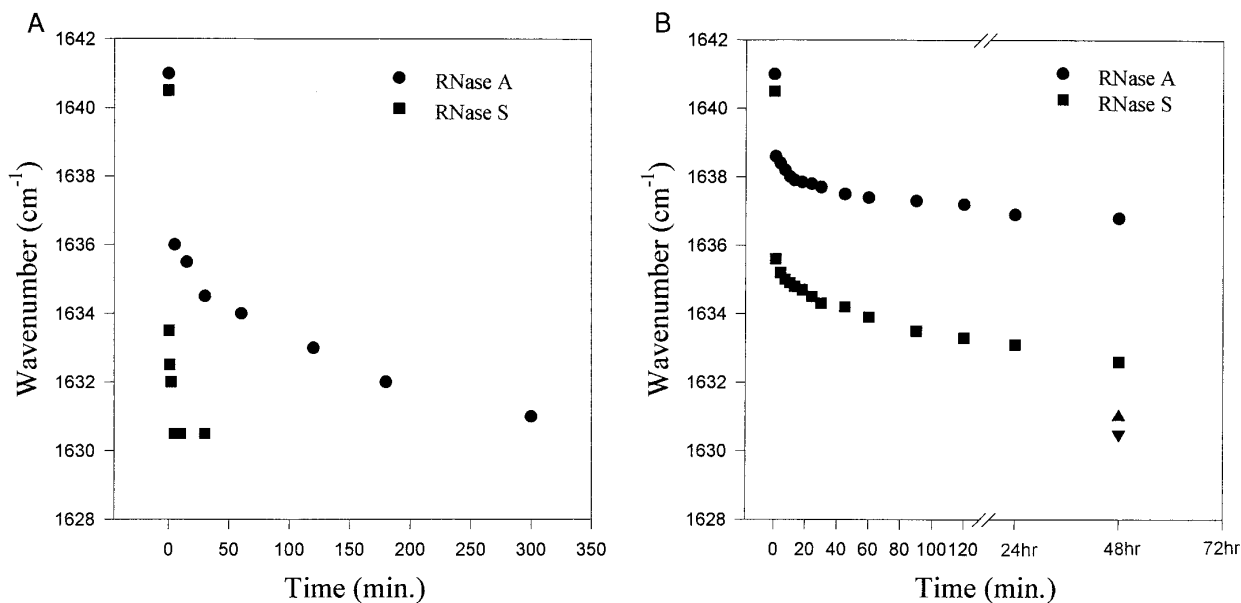


FIG. 4. The plots of frequency of the major  $\beta$ -sheet components of RNase A and RNase S as a function of time at 50 and 25°C. (A) Exchange at 50°C. (B) Exchange at 25°C. ( $\blacktriangle$ ) Fully exchanged RNase A and ( $\blacktriangledown$ ) fully exchanged RNase S, data from (A).

was observed for RNase A. At 48 h, the low-wavenumber  $\beta$ -sheet component was found at 1632.6 cm<sup>-1</sup> for RNase S and at 1636.8 cm<sup>-1</sup> for RNase A. These results confirm the previous report (19) that the amide proton exchanging rate of RNase S is faster than that of RNase A. Figures 4A and 4B show the change in frequency of the major  $\beta$ -sheet components of RNase A and RNase S as a function of time at 50 and 25°C, respectively. Clearly, the amide proton exchange proceeds much slower at 25 than at 50°C. These results are consistent with those reported earlier (19, 28). Complete deuteration can be achieved in about 5 min at 50°C for RNase S, whereas nearly 5 h is needed for RNase A at the same temperature. Earlier studies have shown that the deuteration of RNase A can be completed in 15 to 30 min at 62°C (19, 28, 32).

Figures 5A and 5B present a series of second-derivative spectra in the amide I region recorded during deuteration of RNase A at 50°C and of RNase S at 25°C. In each series, the top spectrum is the protein in H<sub>2</sub>O and the bottom spectrum is the fully deuterated protein. Frequency changes in four representative band components: 1689 and 1641 cm<sup>-1</sup> ( $\beta$ -sheet), 1658 cm<sup>-1</sup> ( $\alpha$ -helix), and 1668 cm<sup>-1</sup> ( $\beta$ -turn) are marked for easier comparison. Despite a large difference in their amide proton exchange rates, the overall spectral changes during deuteration are very similar for both proteins. These results suggest that the H–D exchange pathways of RNase A and RNase S are remarkably similar, if not identical. It should be noted that the frequency change for each individual band component proceeds

gradually. There are no pairs of bands whose absorbances inversely vary as a function of time like those commonly seen in thermally denatured proteins (33–37). In other words, no amide I band component can be identified as an exchanged or unexchanged component during the course of deuteration, especially for the low-wavenumber  $\beta$ -sheet component.

## DISCUSSION

*Changes in protein conformational dynamics by limited proteolysis.* It is well-known that the rates of amide proton exchange of proteins are affected dramatically by differences in experimental conditions such as pH, temperature, and pressure (11–13, 32). However, few studies have focused on more detailed comparisons of conformational dynamics of two structurally related proteins using H–D exchange and infrared spectroscopy (18, 19). Results of this study confirm those reported previously by Harris and colleagues (19) that the H–D exchange rate of RNase S is faster than that of RNase A at room temperature. The accelerated amide proton exchange rate of RNase S is apparently a result of greater flexibility (mobility) of the protein conformation. X-ray crystallographic analysis has revealed that the proteolytic cleavage between residues 20 and 21 has disconnected the first  $\alpha$ -helical section (S-peptide) from the remaining protein structure (S-protein) (1, 6). The S-peptide is located over the center and perpendicular to three strands of antiparallel  $\beta$ -sheet structures and remains associated with the S-protein via hydro-

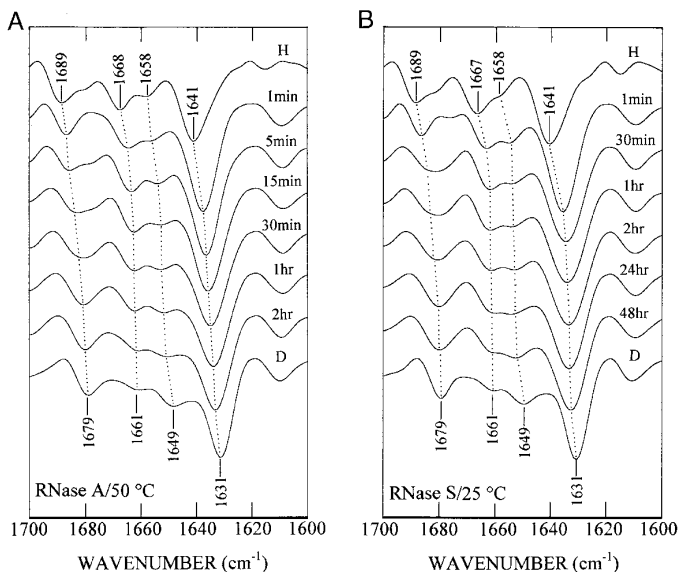


FIG. 5. Second-derivative spectra of RNase A and RNase S recorded during deuteration. (A) Spectra of RNase A at 50°C. (B) Spectra of RNase S at 25°C. For each series, the top spectrum is the protein in H<sub>2</sub>O, and the bottom spectrum is the protein with complete H–D exchange.

gen bonds. The single amide bond cleavage seems to free the RNase S molecule from the conformational restrictions imposed on the RNase A.

Reversible unfolding, local or global, has been proposed to play a major role in H–D exchange mechanisms of proteins (12, 13, 38). Several recent studies using proteins with site-directed mutations provide strong evidence to support this hypothesis (39, 40). Using NMR spectroscopy, Kim and colleagues (39) reported that H–D exchange in bovine pancreatic trypsin inhibitor occurs via two parallel pathways, one involving small noncooperative fluctuations of the native state, and the other involving cooperative global unfolding. Using NMR and differential scanning calorimetry, Perrett and colleagues (40) demonstrated that both local and global unfolding participate in H–D exchange of barnase. In the case of RNase S, the proteolytic cleavage between residues 20 and 21 would allow the protein to undergo faster global unfolding and refolding than in the case of RNase A. Larger differences in the amide proton exchange rates between RNase S and RNase A at 50°C compared with those observed at 25°C are consistent with the above interpretation.

**Continuity of frequency red-shifts of amide I components during deuteration.** The data in Figs. 4 and 5 illustrate the continuity of frequency red-shifts of amide I components during deuteration, especially the major component associated with  $\beta$ -sheet structure. These results disagree with the interpretation pro-

posed by Olinger and co-workers (28). They have suggested that the major  $\beta$ -sheet band at 1637 cm<sup>-1</sup> in the spectrum of partially deuterated RNase A is due to the coappearance of the 1641 cm<sup>-1</sup> band as the unexchanged  $\beta$ -sheet component and the 1632 cm<sup>-1</sup> band as the exchanged  $\beta$ -sheet component. If their proposed mechanism is true, the deuteration-induced spectral change at the major  $\beta$ -sheet region should behave like those commonly seen during thermal denaturation of proteins (33–37), that is, an intensity decrease in the original band frequency accompanied by an intensity increase at the new band frequency. This apparently is not the case for either RNase A or RNase S. The frequency red-shifts of the major  $\beta$ -sheet components in both proteins proceed continuously during the course of deuteration, suggesting that RNase A and RNase S are too small to have separate groups of secondary structures with unexchanged and exchanged amide protons. The gradual change of frequency for individual amide I components is most likely associated with the secondary structure containing both exchanged and unexchanged amide protons. A less solvent-accessible core structural element within an ordered secondary structure such as an  $\alpha$ -helix or  $\beta$ -sheet containing only the unexchanged amide protons may not be able to exhibit an absorption frequency independent from those containing already exchanged amide protons under these conditions. The results presented here are consistent with those reported for RNase A by Wlodawer and Sjölin (41) using neutron diffraction and H–D exchange techniques. Twenty-eight of 120 amide protons were found to be at least partially protected from exchange in a single crystal of RNase A. Among them, 12 were found to be completely protected. However, the distribution of protected amide protons in the protein structure is not uniform. While each of the three  $\alpha$ -helices contains two or three partially or completely protected amide protons, the only consecutively protected residues are 11–13 at the carboxyl end of the first  $\alpha$ -helix. Fully protected amide protons are also scattered throughout the  $\beta$ -sheet structure (41).

**Interpretation of amide I spectral data for proteins in D<sub>2</sub>O solution.** Results of this and other previously reported studies (13, 19) give rise to an important question of how to interpret the spectral differences in the amide I region between two proteins in D<sub>2</sub>O. Apparently, proteins with close structural similarity behave significantly differently during the course of deuteration in terms of the amide proton exchange rate, which in turn alters the spectral patterns of these proteins in the amide I region. While these differences can be used advantageously in comparative studies of the conformational mobility of proteins, as in the present study, they can also be misinterpreted as real conformational differences between two proteins. From our experience,

there are three ways to avoid possible misinterpretation of amide I spectral data of proteins. The first is to conduct the infrared study using H<sub>2</sub>O as the protein solvent, if solubilities of the given proteins are above 15 mg/ml. The second is to ensure that comparisons are carried out with spectra recorded after complete H–D exchange. Harris and colleagues (19) have reported that complete deuteration of RNase A was not obtained even after 8 days of prolonged incubation at room temperature. Thus, carrying out protein deuteration at an elevated temperature is highly recommended. The third way is to conduct a parallel study using circular dichroism (CD) spectroscopy. Unlike the infrared amide I spectrum which arises from the C=O stretching vibration of peptide linkages significantly involved with hydrogen bonding (23), the far-uv CD spectrum of a protein arises primarily from the spatial arrangement of peptide amide groups (42–44). Thus, CD spectroscopy is not sensitive to the proton exchange in the protein backbone and can serve as a spectral reference for infrared spectral interpretation. In a previous report, it was demonstrated that the far-uv CD spectra of  $\beta$ -lactoglobulins A and B were nearly identical in H<sub>2</sub>O and D<sub>2</sub>O (18).

Results of the present study are a significant addition to the growing body of knowledge on H–D exchange dynamics of RNase A and RNase S as well as proteins in general. The proteolytic cleavage between residues 20 and 21 in RNase S accelerates dramatically the rate of proton exchange in the protein backbone compared with that of RNase A. However, the exchange pathways of amide protons for both proteins remain very similar, if not identical. These results have further demonstrated the usefulness and advantages of H–D exchange coupled with FT-IR spectroscopy in the studies of conformational dynamics of structurally related proteins, such as those of naturally occurring or site-directed mutagenesis and proteolytic activation of zymogens, perhaps leading to a better understanding of the forces that contribute to the structural mobility of proteins.

*Note added in proof.* In a recent study, a completely deuterated state of RNase A in 10 mM sodium cacodylate/D<sub>2</sub>O buffer, pH 7.0 (uncorrected), was obtained in 20 min at 55°C (45).

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