Pressure- and Thermally-Induced Reversible Changes in the Secondary Structure of Ribonuclease A Studied by FT-IR Spectroscopy

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ABSTRACT: Fourier transform infrared (FT-IR) spectroscopy combined with a resolution enhancement technique has been used to characterize pressure and thermal effects on the secondary structure of ribonuclease A. The experiments were performed at pD 7.0 with 50 mg/mL protein solution in D_2O buffer. According to the observed changes in the amide I′ band, secondary structure elements such as α-helices, β-sheets, and turns are cooperatively disrupted by application of either pressures above 570 MPa at 30 °C or temperatures above 60 °C at 0.1 MPa. Pressure- and thermally-denatured ribonuclease A are fully unfolded and do not contain any residual secondary structures. Both the structural changes are intrinsically reversible, although the pressure-induced transition shows a hysteresis. It is found that nonnative turn structures are formed prior to the appearance of the native secondary structure in the folding from the pressure-unfolded state. The structural features upon the pressure-induced unfolding are additionally characterized by the interesting behavior of hydrogen−deuterium exchange at high pressure. Most of the backbone amide protons protected at atmospheric pressure, which are involved in the α-helices and β-sheet, are exchanged with solvent deuterons in the pressure range where the two secondary structural elements are virtually identified as intact. There is a possibility that, for ribonuclease A, application of high pressure up to 570 MPa induces such a partially unfolded state as has native-like secondary structure but permits solvent to be highly accessible to the internal regions.

For globular proteins, a number of thermodynamic studies have revealed that the native states are only marginally stable relative to the denatured states. Structural characterization of the nonnative (partially folded and denatured) states is essential for understanding the mechanisms of protein folding and the principles of structure stabilization (Kim & Baldwin, 1990; Dill & Shortle, 1991; Dobson, 1992). It is well known that the reversible denaturation of globular proteins can be induced by pressure as well as by temperature. Various high pressure experimental techniques have been used: ultraviolet spectroscopy (Brands et al., 1970; Hawley, 1971); fluorescence spectroscopy (Li et al., 1976; Royer et al., 1993); NMR[1] spectroscopy (Samarasinghe et al., 1992; Royer et al., 1993); enzyme activity (Taniguchi & Suzuki, 1983). However, structural features upon the pressure-induced denaturation remain uncertain.

Fourier transform infrared (FT-IR) spectroscopy is one of the most powerful techniques for determining the secondary structure of globular protein in aqueous solution (Byler & Susi, 1986; Surewicz & Mantsch, 1988; Dong et al., 1990). Of all the amide modes of the backbone peptide groups, the most widely used one is the amide I mode (designated amide I′ in the deuterated peptide group). This vibrational mode originates from the C=O stretching vibration of the peptide group, which is weakly coupled with the in-plane N−H bending and the C−N stretching vibration and gives rise to an infrared band in the region between approximately 1600 and 1700 cm^{-1} (Krimm & Bandekar, 1986). However, globular protein usually contains different types of secondary structure such as α-helices, β-sheets, turns, and nonordered structures. Since each of these conformational entities contributes to the infrared spectrum, the observed amide I band contour consists of many overlapping component bands related to different structural elements. The bandwidth of the contributing component bands is usually greater than the separation between the maxima of adjacent peaks. Consequently, in order to extract structural information from the amide I band, extensive mathematical manipulation of the experimentally measured infrared spectra is required. Resolution enhancement techniques such as second-derivative (Savitzky & Golay, 1964; Cameron & Moffatt, 1987) and Fourier self-deconvolution (Kauppinen et al., 1981) can be used to identify the positions of the individual amide I component bands. These techniques provide a sensitive diagnostic tool for monitoring the nature of changes in the conformation of the protein backbone (Surewicz et al., 1993).

FT-IR spectroscopy combined with resolution enhancement techniques has been already employed in the detection of both the pressure- and thermally-induced irreversible changes in the secondary structure of chymotrypsinogen A. The pressure-induced irreversible denaturation starts at 370 MPa and is completed at 760 MPa. The observed changes in the amide I/I′ band suggest that the contributions from the turns and nonordered structures increase in pressure-denatured chymotrypsinogen A, while those from the α-helices and β-sheets decrease dramatically (Wong & Heremans, 1988). On the other hand, for the thermally-induced irreversible denaturation above 50 °C, the contribution from

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[1] Abbreviations: NMR, nuclear magnetic resonance; FT-IR, Fourier transform infrared; pD, glass electrode pH reading in D_2O solution without correction for isotope effects; UV, ultraviolet; CD, circular dichroism; BPTI, bovine pancreatic trypsin inhibitor; ANS, 8-anilino-1-naphthalene sulfonate.

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irreversible hydrogen-bonded extended structure, formed upon the aggregation of the thermally-denatured protein, increases substantially at expense of the native secondary structure elements (Ismail et al., 1992). Such a phenomenon is generally observed in many other thermally-denatured proteins, because FT-IR spectroscopic studies of proteins require the use of relatively high protein concentration (Surewicz et al., 1990; Muga et al., 1991; Naumann et al., 1993). However, irreversible conformational changes prevent us from describing in detail structural features in protein unfolding and refolding. Recently, the thermally-induced reversible changes in the secondary structure of ribonuclease A (Yamamoto & Tasumi, 1991) and ribonuclease T_1 (Fabian et al., 1993) have been successfully characterized by FT-IR spectroscopy.

In this paper, we report the pressure- and thermally-induced reversible changes in the secondary structure of ribonuclease A characterized by FT-IR spectroscopy combined with resolution enhancement techniques. We also discuss the fundamental question how similar or different the pressure-induced denaturation is compared with the thermally-induced denaturation. In addition, we examine whether the structural information concerning the nonnative state induced by application of high pressure can be obtained by characterizing in detail the behavior of hydrogen−deuterium exchange.

MATERIALS AND METHODS

Bovine pancreatic ribonuclease A (type XII-A) and poly(L-glutamic acid) sodium salt were purchased from Sigma Chemical Co. and Peptide Institute Inc., respectively, and used without further purification. D_2O (99.8 atom % D) and DCl (99 atom % D) were purchased from CEA-ORIS Bureau des Isotopes Stables and Sigma Chemical Co., respectively.

The sample solution was prepared by dissolving ribonuclease A or poly(L-glutamic acid) sodium salt in 0.05 M Tris-DCl D_2O buffer, pD 7.0, for the pressure experiments, and in 0.02 M sodium phosphate D_2O buffer, pD 7.0, for the thermal ones. The protein or polypeptide concentration was 50 mg/mL. The pD was read directly from a pH meter, and no adjustments were made for isotope effects. All exchangeable backbone amide protons in ribonuclease A were deuterated by means of cooling rapidly in an ice/water bath after incubating the solution at 62 °C for 20 min. The completion of hydrogen−deuterium exchange was confirmed by no further changes in the amide II band. This amide band in the frequency region around 1550 cm^{-1} shifts to around 1450 cm^{-1} as a result of deuteration of the backbone amide protons. All sample solutions were prepared immediately prior to the infrared measurements. It took about 1 h to record the first infrared spectrum after the sample preparation.

For the pressure experiments, the sample solutions were placed together with a small amount of powdered α-quartz in a 1.0 mm diameter hole of a 0.05 mm thick stainless-steel (SUS 304) gasket mounted on a diamond anvil cell. The α-quartz was used as an internal pressure calibrant (Wong et al., 1985). Infrared spectra of the samples were recorded at 30 °C using a Perkin-Elmer 1725X Fourier transform infrared spectrometer equipped with a liquid-nitrogen cooled MCT detector. The infrared beam was condensed by a zinc selenide lens system onto the sample in the diamond anvil cell. For each spectrum, 1000 interferograms were co-added and Fourier transformed to give a spectral resolution of 2 cm^{-1}. Thirty minutes was allowed to equilibrate the sample at the chosen pressure prior to each infrared measurement. The average increasing or decreasing pressure rate was about 150 MPa/h. For the thermal experiments, the sample solutions were placed between a pair of CaF_2 windows with a 0.015 mm stainless-steel (SUS 304) spacer. The windows were mounted in a home-made cell holder. The desired temperature was controlled within 0.2 °C by a circulating water jacket. A DTGS detector was employed. For each spectrum, 80 interferograms were co-added and processed in the same manner as the pressure experiments. Ten minutes was allowed to equilibrate the sample at the chosen temperature prior to each infrared measurement. The average increasing or decreasing temperature rate was 6 °C/h. In order to eliminate spectral contributions of atmospheric water vapor, the spectrometer and sample chamber were continuously purged with dry air.

Reference spectra were recorded in the same cell and under identical conditions with only the medium in which the protein or polypeptide was dissolved. Infrared spectra for ribonuclease A and poly(L-glutamic acid) dissolved in D_2O buffer were obtained by digitally subtracting the appropriate reference spectrum from the spectrum of each sample solution. The bands originating from water vapor in the second-derivative infrared spectrum were subtracted until the absorption-free region of the amide I(I′) band above 1700 cm^{-1} was featureless. Second-derivative spectra were generated by using a nine data-point (9 cm^{-1}) Savitzky−Golay function available from Perkin-Elmer software, IRDM2.

RESULTS

The amide I band contour consists of a number of individual component bands at the frequencies characteristic of specific secondary structure elements. The second-derivative analysis permits the direct separation of the amide I band into its components. Absorbance bands in the original spectrum are revealed as negative bands in the second-derivative spectrum. On the basis of the results previously reported by FT-IR spectroscopic studies (Olinger et al., 1986; Haris et al., 1986; Yamamoto & Tasumi, 1991), the component bands at 1632, 1650, 1663, 1673, and 1681 cm^{-1} for completely deuterated ribonuclease A are due to the amide I′ vibrational mode of peptide group segments in the β-sheet, α-helices, turns, turns, and β-sheet, respectively (Figure 1). Another band at 1609 cm^{-1} is due to the amino acid side-chain absorptions, primarily tyrosine residues (Matsuura et al., 1986). The relative intensity of the component band at 1632 cm^{-1} is the strongest, which suggests that β-sheet structure predominates over other secondary structure elements in ribonuclease A in aqueous solution. This result is agreement with those of both X-ray crystallographic studies [e.g., Wlodawer et al. (1986)] and 2D NMR spectroscopic studies (Robertson et al., 1989; Rico et al., 1989).

Pressure-Induced Changes in the Secondary Structure of Ribonuclease A. The observed changes in the amide I′ band shown in Figures 2, panels A and B, directly indicate the pressure-induced changes in the secondary structure of ribonuclease A, because the hydrogen−deuterium exchange has been already completed. As pressure is increased, no significant changes in the amide I′ band contour are observed

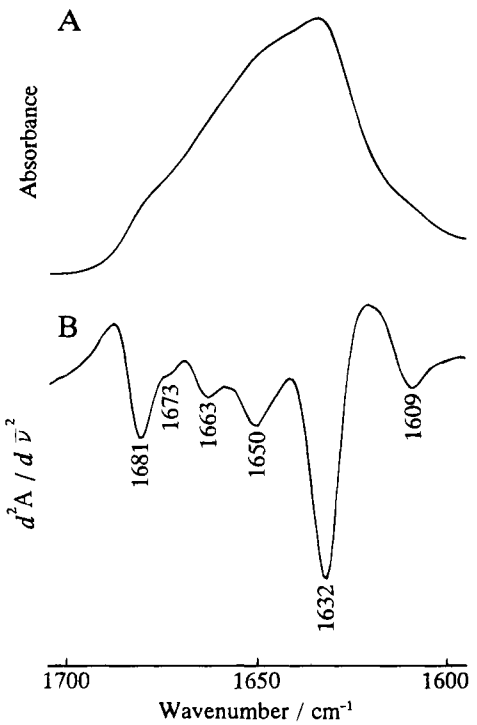

FIGURE 1: Original (A) and second-derivative (B) infrared spectra in the amide I′ region of completely deuterated ribonuclease A at 30 °C and 0.1 MPa. Solution conditions: ribonuclease A is dissolved in 0.05 M Tris-DCl D_2O buffer, pD 7.0; the protein concentration is 50 mg/mL. For this work, band assignments, in cm^{-1}, for completely deuterated ribonuclease A measured in D_2O are as follows: Tyr side-chain, 1609; β-sheet, 1632 and 1681; α-helices, 1650; turns, 1663 and 1673. Other workers (Olinger et al., 1986; Haris et al., 1986; Yamamoto & Tasumi, 1991) have made the following assignments, in cm^{-1}, for completely deuterated ribonuclease A measured in D_2O: Tyr side-chain, 1609−12; β-sheet, 1631−33 and 1678−82; nonordered, 1643−46; α-helices, 1651−53; turns, 1662−63 and 1670−72; undefined, 1618 and 1639.

below 570 MPa. The intensities of the amide I′ component bands characteristic of native ribonuclease A decrease cooperatively over a pressure range between 570 and 1030 MPa. The transition range is much higher than that determined by UV spectroscopic study (Brands et al., 1970). The spectrum at 1030 MPa after the pressure-induced band redistribution is completed exhibits only a broad amide I′ band contour centered at 1639 cm^{-1} with a shoulder around 1670 cm^{-1}. Poly(L-glutamic acid) in neutral pH range is frequently used as a random coil conformation model of polypeptide in aqueous solution (Jackson et al., 1989). The amide I′ band contour shown in Figure 2A at 1030 MPa is well identified with that of poly(L-glutamic acid) shown in Figure 3 at 30 °C and 1080 MPa. Consequently, pressure-denatured ribonuclease A is fully unfolded and does not contain any residual secondary structure elements. As for the frequency shift, the bands at 1632 and 1681 cm^{-1} assigned to the β-sheet structure shift little until the pressure-induced structural changes are completed. The band at 1650 cm^{-1} assigned to the α-helical structure shifts gradually toward high frequency by about 3 cm^{-1}. The band at 1609 cm^{-1} assigned to side-chain absorption of the tyrosine residues splits clearly into the three bands at 1601, 1609, and 1615 cm^{-1} at 770 MPa, although the indication has been already observed from 370 MPa. The newly pressure-induced band at 1601 cm^{-1} may be due to side-chain absorption of the phenylalanine residues (Matsuura et al., 1986). It is possible that the changes in the side-chain absorptions detected by the infrared spectrum correspond to those detected by the ultraviolet spectrum at high pressure (Brands et al., 1970). As soon as pressure is further increased up to 1240 MPa, pressure-unfolded ribonuclease A is precipitated and then gradually forms clear gel within 10 min. Surprisingly, the observed amide I′ band contour is identical with that at 1030 MPa. However, the intensity is considerably reduced, and the broad maximum shifts to 1637 cm^{-1}. The clear gel changes quickly back into solution by a release of pressure up to 1060 MPa, while the polypeptide backbone of ribonuclease A remains fully unfolded. The broad bands at 1667 and 1673 cm^{-1} generally assigned to turn structures are observed at 780 MPa, but it is doubtful whether these bands are similar to those identified in the native state. The dramatic spectral changes occur over the pressure range between about 750 and 450 MPa. The amide I′ band contour after pressure is released up to 0.1 MPa is identical with that shown in Figure 2A at 0.1 MPa, except for the bands at 1601, 1609, and 1661 cm^{-1}. The pressure-induced changes in the secondary structure of ribonuclease A, at least in the α-helices and β-sheet, are completely reversible. On the other hand, the turn structures and the microenvironment of tyrosine residues are different between before pressure is applied and after pressure is released. It is interesting that two of the six tyrosine residues in native ribonuclease A are located in the turn structures.

A convenient empirical parameter for following pressure-induced unfolding of ribonuclease A is the intensity ratio of the amide I′ band at 1632 cm^{-1}. Intensity ratio, R, at 1632 cm^{-1} was determined from the following equation: $R = (I_N - I)/(I_N - I_D)$, where I was the intensity at 1632 cm^{-1} in the transition region, and I_N and I_D were the intensity at 1632 cm^{-1} of the native and completely unfolded state (not gel formed), respectively. Intensity at 1632 cm^{-1} in the second-derivative infrared spectrum was defined as the difference in height between the negative peak at 1632 cm^{-1} and the positive peak around 1620 cm^{-1}. The band at 1632 cm^{-1} assigned to the β-sheet structure exhibits the strongest pressure-dependent intensity change among the different amide I′ component bands and therefore provides a good monitor for the pressure induced unfolding. The results previously described can be more evidently draw from Figure 4. It is interesting that the pressure-induced unfolding of ribonuclease A shows a significant hysteresis.

Thermally-Induced Changes in the Secondary Structure of Ribonuclease A. As shown in Figure 5A, no significant changes in the amide I′ band are observed below 60 °C, except for the band at 1663 cm^{-1} assigned to the turn structures. It would seem that only the amide I′ component band is broadened in a lower temperature range. This result may be correlated with the turn structures being located at the surface of the molecule and being more accessible to solvent compared with the α-helices and β-sheet (Haris et al., 1986; Robertson et al., 1989; Rico et al., 1989). A further increase in temperature dramatically induces the cooperative disappearance of the amide I′ component bands characteristic of native ribonuclease A, which occurs over the relatively narrow temperature range between 60 and 73 °C. This thermally-induced band redistribution is completed at 73 °C and results in a broad maximum observed at 1645 cm^{-1} with a shoulder around 1673 cm^{-1}. The amide I′ band contour shown in Figure 5A at 73 °C is well identified with that of

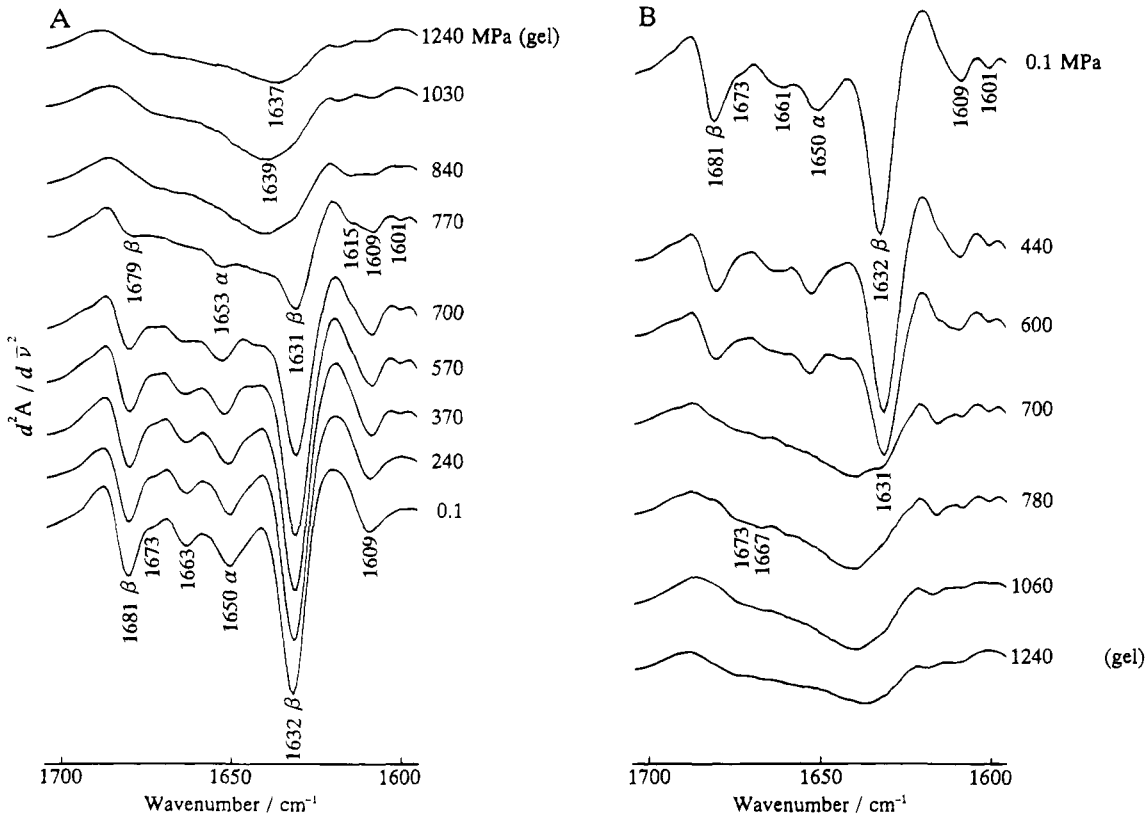

FIGURE 2: Second-derivative infrared spectra in the amide I′ region of completely deuterated ribonuclease A upon increasing (A) and releasing (B) pressure at 30 °C. Solution conditions are as in Figure 1.

poly(L-glutamic acid) shown in Figure 3 at 75 °C and 0.1 MPa. Consequently, thermally-denatured ribonuclease A does not contain such residual secondary structures as are detected by far-UV CD spectroscopic studies (Labhardt, 1982; Robertson & Baldwin, 1991). As for the frequency shift, the bands at 1631 and 1680 cm^{-1} assigned to the β-sheet structure shift little until the thermally-induced structural changes are completed. The band at 1650 cm^{-1} assigned to the α-helical structure shifts toward low frequency by about 3 cm^{-1} above 60 °C. This apparent shift is due to the superimposition of the thermally-induced broad band at around 1645 cm^{-1}. The band at 1609 cm^{-1} assigned to side-chain absorption of the tyrosine residues is broadened without splitting and shifts to 1611 cm^{-1} at 68 °C. The dramatic spectral changes upon decreasing temperature occurs over a slightly lower and wider temperature range than that upon increasing temperature (Figure 5B). The observed amide I′ band contour after temperature is decreased up to 20 °C is nearly identical with that shown in Figure 5A at 20 °C, but the band intensity is considerably different. This intensity reduction depends greatly on the incubating time above 66 °C (data not shown). The conversion of thermally-unfolded ribonuclease A into its irreversible denatured form may increase rapidly at elevated temperature, because the protein concentration used by FT-IR spectroscopy is relatively high compared with other experimental methods [e.g., Brands et al. (1970) and Labhardt (1982)]. However, thermally-induced precipitation or gelation is not observed at 73 °C under the present experimental conditions. From the intensity ratio results at 1632 cm^{-1} (Figure 6), the apparent transition range upon increasing temperature at 0.1 MPa is slightly (about 2 °C) higher than that identified by FT-IR difference spectroscopic study (Yamamoto & Tasumi, 1991). However, the intensity ratio results are quite compatible with this.

Pressure and Thermal Effects on the Hydrogen−Deuterium Exchange of Ribonuclease A. Figure 7 shows the second-derivative infrared spectra of partially deuterated ribonuclease A upon increasing pressure. At 0.1 MPa, the component bands at 1638, 1658, and 1688 cm^{-1} are due to the overlapping of the amide I and amide I′ vibrational mode of peptide group segments in the β-sheet, α-helices, and β-sheet, respectively. These band frequencies are 6~7 cm^{-1} higher than those corresponding to completely deuterated ribonuclease A (see Figure 2A). As determined by 2D NMR spectroscopic studies, the backbone amide protons involved in the α-helices and β-sheet in native ribonuclease A are strongly protected against exchanging with solvent deuterons (Robertson et al., 1989; Rico et al., 1989). Under the present conditions, there is little change in the amide I/I′ bands assigned to the α-helices and β-sheet after incubating at 30 °C and 0.1 MPa for at least 3 days (data not shown). As pressure is increased from 0.1 to 720 MPa, especially between 550 and 630 MPa, dramatic spectral changes are observed. It is noteworthy that the amide I/I′ band contour at 630 MPa is well identified with that shown in Figure 2A at 570 MPa. This result suggests that most of the protected amide protons involved in the α-helices and β-sheet are exchanged with solvent deuterons prior to the pressure-induced transition of the two secondary structure elements. Similarly, as shown in Figure 8, it is evident that the hydrogen−deuterium exchange is dramatically accelerated above 50 °C at 0.1 MPa. The exchange completion occurs at 58 °C prior to the major thermally-induced transition of the secondary structure. However, it would seem likely that the pressure-induced exchange process is different from the

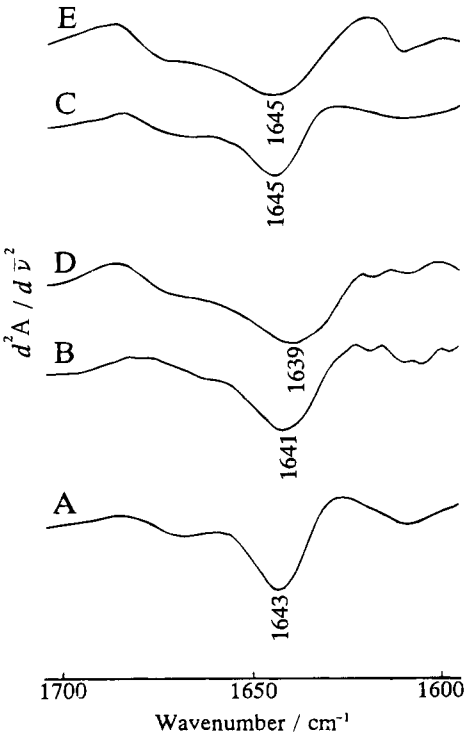

FIGURE 3: Second-derivative infrared spectra in the amide I′ region of poly(L-glutamic acid) (A, B, C) and of completely pressure- (D) and thermally- (E) unfolded ribonuclease A. Spectra A, B, and C were recorded at 30 °C, 0.1 MPa, at 30 °C, 1080 MPa, and at 75 °C, 0.1 MPa, respectively. Spectra D and E are the same as shown in Figure 2A at 1030 MPa and Figure 5A at 73 °C, respectively. Solution conditions: poly(L-glutamic acid) sodium salt was dissolved in 0.05 M Tris-DCl D_2O buffer, pD 7.0, for the pressure experiment, and in 0.02 M sodium phosphate D_2O buffer, pD 7.0, for the thermal experiment; the polypeptide concentration is 50 mg/mL.

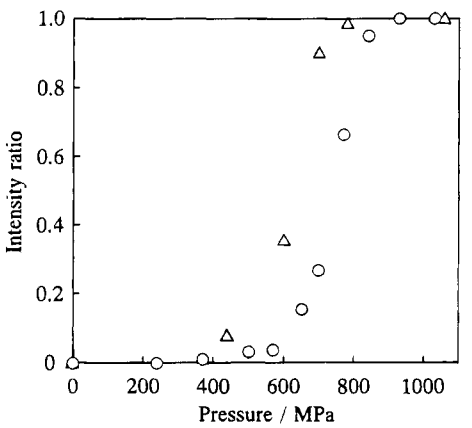

FIGURE 4: Intensity ratio on the amide I′ component band at 1632 cm^{-1} for completely deuterated ribonuclease A upon increasing (○) and releasing (△) pressure shown in Figure 2.

thermally-induced one, especially for the α-helical structures. Until the exchange is completed, the negative peak due to the α-helical structures is distinctly observed below 550 MPa (Figure 7). On the other hand, the obvious negative peak between 1650 and 1660 cm^{-1} is not observed below 53 °C (Figure 8).

DISCUSSION

Comparison between the Pressure- and Thermally-Induced Denaturation for Ribonuclease A. Both pressure- and thermally-denatured ribonuclease A do not have any secondary structure elements such as α-helices, β-sheets, and turns. However, there seems to be a difference in the hydration of the backbone peptide groups between the pressure- and thermally-unfolded state. The amide I(I′) vibrational mode principally originates from the C=O stretching vibration of peptide group (Krimm & Bandekar, 1986). It is expected that the more stable the hydrogen bonding at the C=O site of peptide group is, the lower the observed frequency of the amide I vibrational mode is. The amide I′ bands shown in Figure 2A at 1030 MPa and Figure 4A at 73 °C have a broad maximum at 1639 and 1645 cm^{-1}, respectively. As the result of the hydrogen−deuterium exchange, it is evident that the backbone peptide groups in both the unfolded states are easily accessible to D_2O molecules. The backbone peptide groups of pressure-unfolded ribonuclease A are hydrated in a more stable manner. Interestingly, molecular dynamics simulation study has suggested that the average number of water molecules hydrated to the backbone peptide groups of BPTI increases with increasing pressure, while the average bond length between hydrated water molecules and the backbone peptide groups does not change (Kitchen et al., 1992). This simulation study, however, has not demonstrated the pressure-induced unfolding even at about 1000 MPa. It is doubtful whether structural features of hydrated water molecules for unfolded protein can be straightforwardly applied.

It is noteworthy that the broad bands at 1667 and 1673 cm^{-1} appear suddenly at 780 MPa in the refolding from the pressure-unfolded state (see Figure 2B). Both the bands are generally assigned to turn structures, judging from their band frequencies [e.g., Krimm and Bandekar (1986), Surewicz and Mantsch (1988), and Kennedy et al. (1991)]. They are reproducibly observed prior to the obvious appearance of the bands assigned to other major secondary structure elements such as α-helices or β-sheets. It seems hard that the bands at 1667 and 1673 cm^{-1} are identical with those due to the turn structures in native ribonuclease A. The native turn structures join the α-helix to the β-strand or are located at the hairpin connection between the antiparallel β-strands (Wlodawer et al., 1986; Robertson et al., 1989; Rico et al., 1989). If they were identical, the refolding of not only the turns but also the α-helices and β-sheet would occur cooperatively. In an initial stage during protein folding, "local" interactions should predominate over "nonlocal" ones. (Where "local" interactions are those among chain segments that are connected or near neighboring residues in the sequence. "Nonlocal" refers to interactions among residues that are significantly apart in the sequence.) The use of short linear peptides is reasonable as model systems to determine such local structural elements. It has more recently been identified by 2D NMR spectroscopy that the short linear peptides can form turn-like conformations in equilibrium with the fully extended (unfolded) form in aqueous solution. The following have been concluded: (1) the "nascent" structures develop α-helices, 3_{10}-helices, or turns in protein folding; (2) initial folding events do not necessarily give rise to the structures which are retained in the final folded protein (Dyson et al., 1988; Wright et al., 1988). The broad amide I′ component bands at 1667 and 1673 cm^{-1} shown in Figure 2B at 780 MPa may indicate that there are such "nascent" structures in equilibrium with the fully unfolded state. The formation of local structures

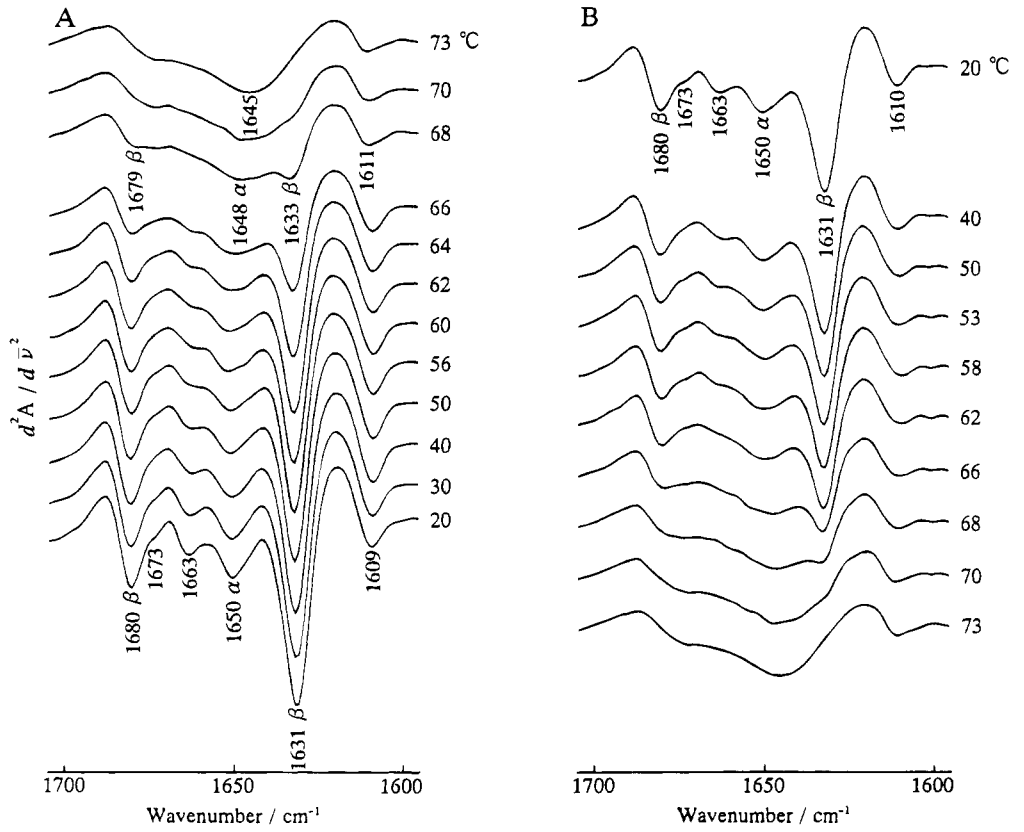

FIGURE 5: Second-derivative infrared spectra in the amide I′ region of completely deuterated ribonuclease A upon increasing (A) and decreasing (B) temperature at 0.1 MPa. Solution conditions: ribonuclease A was dissolved in 0.02 M sodium phosphate D_2O buffer, pD 7.0; the protein concentration is 50 mg/mL.

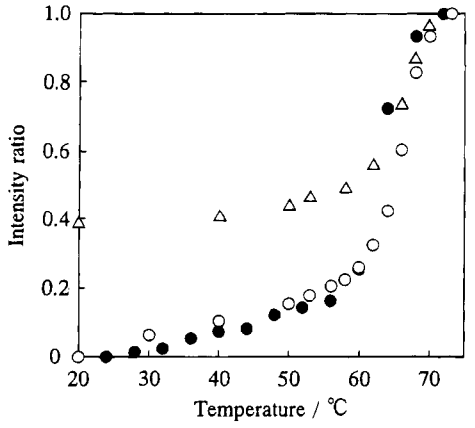

FIGURE 6: Intensity ratio on the amide I′ component band at 1632 cm^{-1} for completely deuterated ribonuclease A upon increasing (○, ●) and decreasing (△) temperature. Open symbols refer to the data shown in Figure 5, and closed symbols refer to the data from Yamamoto and Tasumi (1991).

in certain regions of the polypeptide backbone could become initiating steps in protein folding. Even if such structures are only marginally stable, they would efficiently induce the steric restriction of the polypeptide chain, thus directing the course of protein folding. Indeed, the appearance of the amide I′ component band at 1631 cm^{-1} is induced by a release of pressure from 780 to 700 MPa, which indicates the refolding of the β-sheet structure (see Figure 2B). Structural features of the polypeptide backbone in an initial stage during protein folding might be first characterized by high pressure FT-IR spectroscopy.

Interpretation of Pressure and Thermal Effects on the Hydrogen–Deuterium Exchange. In general, the backbone

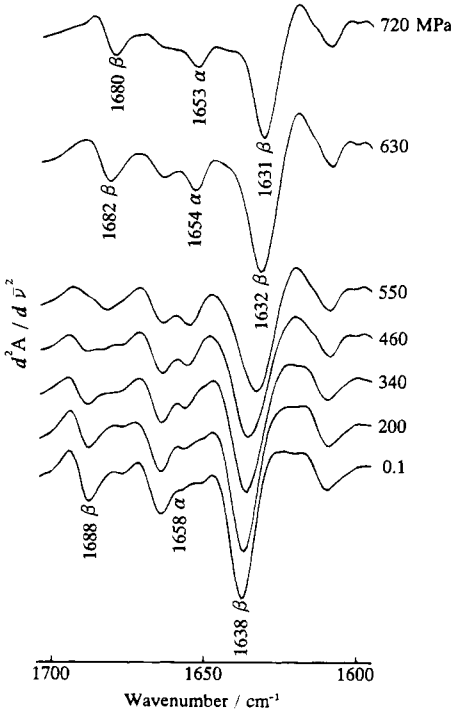

FIGURE 7: Second-derivative infrared spectra in the amide I/I′ region of partially deuterated ribonuclease A upon increasing pressure at 30 °C. It takes about 1 h to record the first infrared spectrum after the sample preparation. Thirty minutes was allowed to equilibrate the sample solution at the chosen pressure prior to each infrared measurement which itself takes 16 min. Solution conditions are as in Figure 1.

amide protons involved in α-helices and β-sheets in native proteins are strongly protected against exchanging with

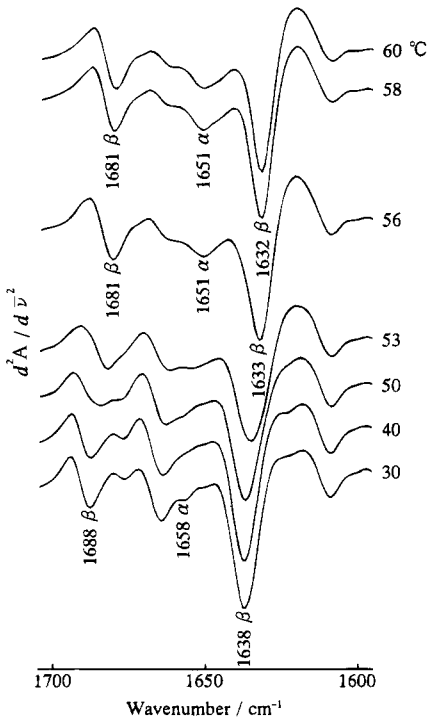

FIGURE 8: Second-derivative infrared spectra in the amideI/I′ region of partially deuterated ribonuclease A upon increasing temperature at 0.1 MPa. It takes about 1 h to record the first infrared spectrum after the sample preparation. Ten minutes was allowed to equilibrate the sample solution at the chosen temperature prior to each infrared measurement which itself takes 11 min. Solution conditions are as in Figure 5.

solvent deuterons. The protection patterns found in α-helices and β-sheets are complex, reflecting tertiary as well as secondary structure. The role of tertiary (side-chain) interactions in stabilizing secondary structure against the exchange is difficult to define, which precludes a rigorous and definitive interpretation of the exchange. The following three exchange mechanisms have been suggested: (1) global unfolding of protein; (2) local unfolding in certain regions of protein; (3) solvent penetration into internal regions. In any mechanism, the backbone amide protons must be transiently exposed to solvent, permitting hydrogen−deuterium exchange to occur (Woodward et al., 1982; Wagner, 1983; Englander & Kallenbach, 1984). These exchange mechanisms depend on the protein stability. Under conditions favoring the denatured state, the dominant mechanism of exchange involves global unfolding. Under predominantly native conditions, more localized effects dominate. Measurements of the hydrogen−deuterium exchange rate have been recently used to detect the persistent secondary structure in unfolded, partially folded, or native protein [e.g., Hughson et al. (1990), Jeng et al. (1990), Robertson and Baldwin (1991), Radford et al. (1992), and Chyan et al. (1993)]. Keeping the above in mind, pressure and thermal effects on the hydrogen−deuterium exchange for ribonuclease A are discussed.

The hydrogen−deuterium exchange is dramatically accelerated above 50 °C and completed at 58 °C prior to the major thermally-induced transition in the secondary structure of ribonuclease A above 60 °C (see Figures 5A and 8). This result suggests that the dominant exchange mechanism is global unfolding with extreme reversibility, since the exchange by localized structural fluctuations is characterized by much lower activation energy than that by global unfolding (Woodward et al., 1982). The exchange at elevated temperature for lysozyme (Radford et al., 1992) and BPTI (Roder et al., 1985; Kim & Woodward, 1993) also occurs primarily via the global unfolding of protein molecule.

An increase in pressure also induces the exchange completion prior to the pressure-induced transition in the secondary structure of ribonuclease A (see Figures 2A and 6). Localized structural fluctuations may be dampened with increasing pressure, which is suggested by the molecular dynamics simulation studies of BPTI (Kitchen et al., 1992; Brunne & Gunsteren, 1993). Consequently, it is expected that the exchange for ribonuclease A at high pressure also occurs primarily via the global unfolding. This interpretation is well applicable to chymotrypsinogen A. The exchange completion for chymotrypsinogen A at high pressure does occur only when the pressure-induced denaturation is completed (Wong & Heremans, 1988). However, the correlation between the hydrogen−deuterium exchange and the secondary structure disruption for ribonuclease A is much poorer at high pressure than that for chymotrypsinogen A. It would seem that considering other contributions is required in order to understand the pressure-induced exchange completion for ribonuclease A. Recently, X-ray crystallographic study at hydrostatic pressure of 100 MPa has reported the nonuniform distribution of compressibility in lysozyme molecule (Kundrot & Richards, 1987). If proteins were nonuniformly compressed in aqueous solution, the application of high pressure where proteins would not be unfolded might be enough to form new sites for either water penetration or local unfolding as the first step in hydrogen−deuterium exchange. Binding of a hydrophobic dye, ANS, to lysozyme, which is associated with the protein unfolding, occurs above 600 MPa, while exposure of the tryptophan residues to aqueous medium is increased at much lower pressure (Li et al., 1976). This result should also implicate such minor conformational changes of protein at high pressure as have been previously described. Moreover, it is very interesting to be compared with structural features in molten globule state recognized as partially folded states of various proteins. The key structural features characterizing molten globule state are the following: (1) compactness close to the native state; (2) presence of native-like secondary structure; (3) little or no evidence for specific tertiary structure (Ptitsyn, 1987; Kuwajima, 1989). In spite of the existence of secondary structure comparable to the native state, the protected amide protons involved in the α-helices and β-sheets in molten globule state are considerably destabilized compared with those in native state [e.g., Hughson et al. (19900, Jeng et al. (1990), and Chyan et al. (1993)]. The drastic destabilization of native tertiary interactions may enable solvent to be highly accessible to the internal regions. Therefore, there is a possibility that, for ribonuclease A, such a nonnative (partially unfolded) state at equilibrium is induced by application of high pressure up to 570 MPa.

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