FTIR observation of compression recovery of the secondary structure of heat denatured ribonuclease A in sucrose solution

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Abstract. We have investigated the effect of pressure on the secondary structure of ribonuclease A using FTIR spectroscopy. In the presence of 47 %w/w sucrose, the intensity of an amide I’ peak assigned to β-sheets of ribonuclease A was slightly recovered by applying pressure up to 200 MPa, while the peak was monotonically decreased with increasing pressure in the absence of sucrose. The present study is the first observation of the compression recovery of β-sheets in a protein by pressure in high concentration of sucrose. This result is consistent with a pressure perturbation calorimetric study (Ravindra et al. 2004 Phys. Chem. Chem. Phys. 6 1952) reported that the sign of volume change of the protein thermal denaturation changed from negative to positive by adding sugars.

1. Introduction
Since coagulation of hen egg white by compression was first observed by Bridgeman [1], pressure has been applied to protein denaturation studies. Pressure denaturation of protein essentially comes from smaller partial molar volume (PMV) of the denatured state compared to that of the native state. The volume change $\Delta V$ for the pressure denaturation is considered to arise from changes in protein internal cavity volume and solvation states of main chain and side chains, and thermal volume contribution [2]. These factors are summed or canceled out each other, as the result, they leads small negative values of $\Delta V$ in the case of globular proteins. The values are ranged from 0 to $-100$ mlmol$^{-1}$ for small globular proteins [3]; the magnitude of $\Delta V$ is generally less than 1 % of the PMV. As the free energy change of protein denaturation is in order of 20 to 40 kJ [4], the pressure denaturation is generally observed under pressure of several hundred MPa.

Assuming the protein denaturation to be the two-state model, the thermal transition midpoints $T_m$ at various pressures can be represented by elliptical temperature-pressure phase diagram. Hawlay [5] reported that the $\Delta V$ of chymotrypsinogen A at pH 2 is positive at $T_m$, from the phase diagram. Recently, this positive $\Delta V$ was directly measured by the pressure perturbation calorimetry (PPC) [6].

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The positive $\Delta V$ should predict pressure-induced recovery of its native structure. However pressure-induced recovery was directly observed only in case of cytochrome $c$ [7, 8], which is an $\alpha$-helix rich protein. On the other hand, in the case of several helical peptides [9-11], folding by pressure was observed.

In this study, we have investigated the effect of pressure on the secondary structure of ribonuclease A (RNase A), whose main secondary structure component is $\beta$-sheet. According to previous PPC studies [12, 13], positive values of $\Delta V$ were observed for RNase A and staphylococcal nuclease at $T_m$ in the presence of high concentration of sugars, while their $\Delta V$ take negative values in the absence of sugars. We demonstrate the pressure-induced recovery of the native state from the heat denatured state of RNase A in high concentration of sucrose solution by FTIR spectrometry.

2. Materials and Methods

2.1. Sample preparation

Bovine pancreatic RNase A was purchased from Sigma. Before sample preparation, RNase A was dissolved in D$_2$O, then incubated at 55 °C for 1 h, and lyophilized to exchange all amide protons to deuteron for the FTIR measurements. All samples were dissolved in 50 mM MES buffer (pD 5.5). The pD values were estimated by adding 0.4 to a value taken from a pH meter. The protein concentration was adjusted to 20 mg/ml, at which we confirmed that the heat-induced aggregation did not occur. Protons from sucrose were also exchanged by dissolving in D$_2$O followed by lyophilization. Sucrose concentration was determined by ATAGO refractometer and refractive index of sucrose-D$_2$O solution was corrected with data from Luten [14].

2.2. FTIR measurements

FTIR spectra were recorded using a JASCO FTIR-680 plus spectrometer equipped with an MCT detector. Temperature-variable measurements under 0.1 MPa were carried out using a CaF$_2$ cell. FTIR measurements under high pressure were carried out using a diamond anvil cell (DAC). The temperature of samples was controlled with circulating thermostatic water around the cell. The standard deviations of measured temperatures were 0.2 °C for all measurements. The pressure inside DAC was calibrated by monitoring the peak shift of 695 cm$^{-1}$, assigned to the photon band of $\alpha$-quartz [15]. Spectral resolution was set to 2 cm$^{-1}$. Each 256 scans of interferograms were collected and Fourier-transformed. Water vapor was purged from sample room by flowing 10-liter of dry N$_2$ gas per minutes, and spectral noise caused by a trace of water vapor was subtracted from the FTIR spectrum of the sample. The second derivative operation was carried out with the Savitzky-Golay method [16] performed by GRAMS™ (Perkin Elmer).

3. Results and Discussion

3.1. FTIR spectra of the amide I′ band of RNase A and the temperature-variable experiments

Figure 1 shows FTIR spectra of amide I′ band of RNase A at 15 °C and 0.1 MPa. The component bands at 1632, 1651, 1663, 1677 and 1681 cm$^{-1}$ can be seen at second derivative spectra in figure 1 (b). Assignment of each peak was done on the basis of previously reported by FTIR studies [17, 18] and summarized in Table 1. Slight peak shifts by adding sucrose were observed for peaks at 1632 cm$^{-1}$ and 1680 cm$^{-1}$. This suggests that the conformation of RNase A remains intact regardless of the presence of sucrose.

Figure 2 shows FTIR spectra of RNase A at various temperatures, and at 0.1 MPa. Both in the presence and the absence of sucrose, temperature-variable FTIR spectra of RNase A show reversible transition for the heat denaturation. The thermal transition of RNase A was monitored by using the peak intensities at 1632 cm$^{-1}$, indicative of $\beta$-sheets. The native/denatured two-state transition curves are shown in figure. 3. Comparison of the transition curves shows that $T_m$ in the presence of sucrose is
higher by 9.3 °C than $T_m$ in the absence of sucrose. Thus, the addition of sucrose increases the thermal stability of RNase A.

**Figure 1.** FTIR original spectra (a) and their second derivative spectra (b) of amide I’ band of RNase A at 15 °C, pD 5.5. Black and red lines indicate the spectra obtained in the absence and in the presence of 47 %w/w sucrose, respectively.

**Table 1.** Peak assignments of amide I’ band of RNase A.

| Wavenumber /cm$^{-1}$ | - Sucrose | + Sucrose | Assignments |
|-----------------------|-----------|-----------|-------------|
| 1609                  | 1609      | side chain|
| 1632                  | 1633      | $\beta$-sheets |
| 1646                  | 1646      | random coil |
| 1651                  | 1650      | $\alpha$-helices |
| 1663                  | 1663      | turn |
| 1673                  | 1673      | turn |
| 1680                  | 1681      | $\beta$-sheets |

**Figure 2.** Temperature dependence of FTIR spectra of RNase A in the absence (a) and presence (b) of 47 %w/w sucrose.
3.2. Compression recovery of the secondary structure of RNase A around $T_m$

Figure 4 shows pressure-induced change in FTIR spectra of amide I’ band of RNase A in the absence (figures (a), (b)) and the presence (figures (c), (d)) of sucrose around $T_m$. In the absence of sucrose, the peak intensity at 1632 cm$^{-1}$ decreased with increasing pressure, suggesting the pressure-induced denaturation of RNase A. On the other hand, in the presence of 47 %w/w sucrose, the peak intensity increased with increasing pressure up to 220 MPa (curves i in figures (c) and (d)). Further pressurizing up to 900 MPa, decreased the peak intensity (curves ii in figures (c) and (d)).

![Figure 3. Thermal transition curve of RNase A. Solid line and filled circles indicate in the absence of sucrose, and dashed line and open squares indicate in the presence of 47 %w/w sucrose.](image)

![Figure 4. Pressure-variable FTIR spectra of RNase A. Figures (a) and (b) show original spectra and their second derivative spectra in the absence of sucrose at 62.0 °C, respectively. Figures (c) and (d) show original spectra and their second derivative spectra in the presence of 47 %w/w sucrose at 71.5 °C, respectively. Arrows indicate the orientation of compression. For convenience, spectra in (c) and (d) are separated to two series as shown in curves i and ii.](image)

Plots for the peak intensity at 1632 cm$^{-1}$ versus pressure are shown in figure 5 to clarify the pressure dependence in the absence and presence of sucrose. It is obvious that the peak intensity in the presence of sucrose increases with increasing pressure up to ~200 MPa, and that the addition of sucrose increases the denaturation pressure by ~ 300 MPa.
The present FTIR study demonstrated that heat-denatured RNase A recovers β-sheets by pressure in the presence of 47 %w/w sucrose around T_m. This result is in agreement with the previous PPC study [12, 13] which reported that the ΔV for thermal denaturation of the protein change from negative to positive by adding high concentration of sugars.

The present study is the first experimental observation that β-sheets in protein recovers its native structure by pressure in high concentration sucrose solution. To understand the origin of positive ΔV of β-sheets, high-pressure FTIR and PPC studies of model peptides are in progress.

![Figure 5](image)

**Figure 5.** Pressure dependence of the peak intensity at 1632 cm⁻¹ of RNase A around T_m. The peak intensities were normalized to the peak intensity of native state at 15 °C and 0.1 MPa. Filled circles indicate the intensities in the absence of sucrose at 62.0 °C and open circles indicate the intensities in the presence of 47 %w/w sucrose at 71.5 °C.

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