Heat-Induced Secondary Structure and Conformation Change of Bovine Serum Albumin Investigated by Fourier Transform Infrared Spectroscopy

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Received May 27, 2004; Revised Manuscript Received June 30, 2004

ABSTRACT: Fourier transform infrared (FT-IR) spectra were measured for an aqueous solution (pD = 5.40) of defatted monomer bovine serum albumin (BSA) over a temperature range of 25−90 °C to investigate temperature-induced secondary structure and conformation changes. The curve fitting method combined with the Fourier self-deconvolution technique allowed us to explore details of the secondary structure and conformation changes in defatted BSA. Particularly striking in the FT-IR spectra was an observation of the formation of an irreversible intermolecular β-sheet of BSA on heating above 70 °C. A band at 1630 cm^{-1} in the spectra was assigned to short-segment chains connecting α-helical segments. The transition temperature for the short-segment chains connecting α-helical segments is lower by 17−18 °C, when compared to those of the α-helix, turn, and intermolecular β-sheet structures of BSA, suggesting that the α-helix and turn structures of BSA are cooperatively denatured on heating. Moreover, the results give an important feature in heat-induced denaturation of BSA that the conformation changes occur twice around both 57 and 75 °C. The appearance of two peaks is interpreted by the collapse of the N-terminal BSA domain due to the crevice in the vicinity between domains I and II at low-temperature transition and by the change in cooperative unit composed of the other two BSA domains at high-temperature transition.

From the point of physiology, serum albumin is the one of the most important blood proteins, and it is a versatile carrier protein, active against a variety of substances with widely differing properties (hydrophobic or hydrophilic, anionic or cationic, etc.) (*1*−*3*). The protein is characterized by an overall oblate shape, and it consists of three domains (I, II, and III), each stabilized by an internal network of disulfide bonds and each bearing a number of ionizable groups with opposite signs (*1*−*3*, *5*). Bovine serum albumin (BSA),[1] molecular mass 66500 Da, is built from 583 amino acid residues containing 20 Tyr (*1*−*5*). The secondary structure of BSA is composed of 67% helix, 10% turn, and 23% extended chain, and no β-sheet is contained (*1*−*3*, *5*−*7*). Modifications in the secondary as well as tertiary structures of BSA occur in dependence of pH, temperature, and various kinds of denaturants. A number of studies have been carried out by use of various spectroscopic techniques to explore heat-induced denaturation of BSA (*8*−*10*). The major conclusions reached from them were as follows: BSA does not denature up to 40 °C. Conformational changes of the BSA molecule are reversible in the temperature range of 42−50 °C, but unfolding of α-helices of BSA is irreversible in the temperature range of 52−60 °C. From 60 °C, the unfolding of BSA progresses and β-aggregation of the BSA molecule begins. Above 70 °C, the gel formation by unfolding of BSA advances further. In addition, like many proteins, BSA forms heat-induced gels in an aqueous environment (*11*−*13*), a property of great interest for protein science (*14*, *15*). Generally, β-aggregation of protein may be defined as one of the association phenomena (*16*−*18*), to which some models of protein association have been proposed (*16*−*18*).

Fourier transform infrared (FT-IR) spectroscopy provides a valuable method for studying protein denaturation because the frequencies, intensities, and bandwidths of characteristic bands in an IR spectrum of a protein are very sensitive to conformational changes in the protein and to microenvironments such as COOH and COO^- groups (*19*−*21*). Therefore, FT-IR spectroscopy can be used to study not only the secondary and tertiary structures but also side chains of proteins in solid, crystal, and solution states for both native and denatured (e.g., thermally, chemically, or mechanically induced aggregates) states (*22*−*27*). Although ultraviolet−visible, NMR, CD, fluorescence, and Raman spectroscopy and X-ray crystallography have often been used for the same purpose, the uniqueness of FT-IR spectroscopy lies in the high-quality spectra that can be obtained with relative ease with very small amounts of protein molecule (10−100 μg) in a variety of environments irrespective of molecular mass.

Despite intensive IR studies of proteins in aqueous solutions (*19*−*27*), several questions remain in exploring protein denaturation by use of IR spectroscopy. For example, the band assignments in the amide I region are not always perfect, and the deconvolution of the amide I band region

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[1] Abbreviations: FT-IR, Fourier transform infrared spectroscopy; BSA, bovine serum albumin.

10.1021/bi0489154 CCC: $27.50
Published on Web 08/19/2004

into component bands is not easy. Thus, to explore the details of the denaturation process, one must investigate further the band assignments and the deconvolution method. It is also of particular important to analyze bands due to C═O stretching modes of COOH and COO^- groups of Asp and Glu and due to a Tyr aromatic ring vibration.

The purpose of the present study is to investigate the heat-induced unfolding process of defatted BSA by use of IR spectroscopy. To extract the detailed information on structural variations from the temperature-dependent IR spectra, we have employed a combined curve fitting and Fourier self-deconvolution method. Our result shows the novel information on the difference in the heat-induced unfolding process of some kinds of secondary structures such as α-helix, turn, intermolecular β-sheet, and short-segment chains connecting the α-helical segment.

MATERIALS AND METHODS

Protein Sample and Purification. Crystallized BSA (lot V73811) was purchased from Reheis Chemical Co. (Phoenix, AZ). All reagents were purchased from Sigma Chemical Co. (St. Louis, MO). TOYOPEARL HW-55F was obtained from Tosoh Corp. (Tokyo, Japan) for gel filtration. Distilled deionized water was used throughout the purification procedure. The protein was defatted by the previously described method (*28*). To obtain the monomer BSA, dimer and higher aggregates were removed by the gel-filtration technique with TOYOPEARL HW-55F (*29*).

Infrared Spectroscopy. Prior to infrared experiments, the protein was dissolved in D_2O at 25 °C for 2 days. The protein sample was lyophilized from D_2O. This procedure was performed twice for each sample. For IR measurement, the sample lyophilized was dissolved in D_2O containing 0.1 M sodium chloride. The pD value of the BSA solution was measured with a standard pH electrode, and the value was corrected according to pD = pH + 0.4 for deuterium isotope effects. The protein concentration was determined by assuming the molar extinction coefficient at 280 nm of 6.67 (28). The final concentration of defatted BSA solution in D_2O was 2.0 wt % with the ionic strength of 0.1 M, and the pD value was 5.40. D_2O solution was used as a buffer in this study.

IR spectra were measured with a Nicolet Magna 760 spectrometer equipped with a liquid nitrogen cooled mercury cadmium telluride (MCT) detector and continuously purged dry air. A protein solution was placed between a pair of CaF_2 windows separated with a 50 μm Teflon spacer, and then the set was inserted in a thermostated cell.

For each measurement, 256 interferograms were co-added and Fourier transformed employing a Happ−Genzel apodization function to generate a spectrum with a spectral resolution of 2 cm^{-1}. The sample temperature was controlled by means of a circulation water system. To obtain spectra at discrete temperatures, the protein solution was heated with an interval of 2 °C. A spectrum at each temperature was obtained by equilibrating the sample for 5 min prior to data collection and was taken to acquire for about 5 min. Therefore, the protein solutions and buffers experienced at least 10 min at each temperature. IR spectra of a buffer solution were measured under identical conditions and subtracted from the spectra of the protein in the relevant solvent and at the corresponding temperature. Minor spectral contributions from residual water vapor were eliminated using a set of water vapor spectra, as followed by Fabian et al. (*30*, *31*).

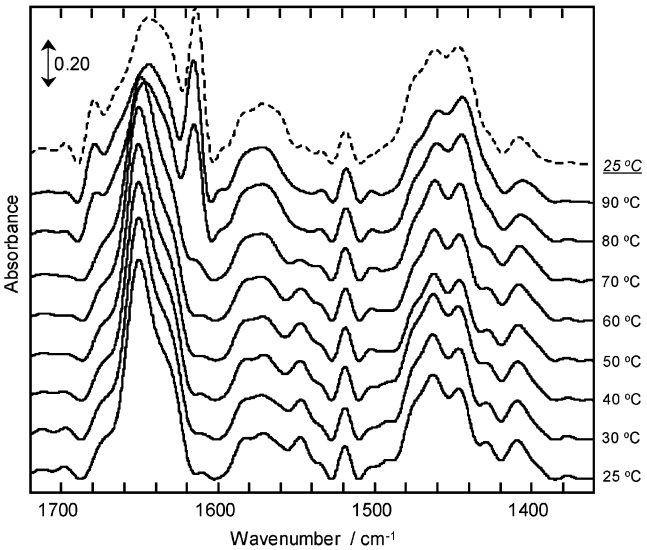

FIGURE 1: Representative deconvolved FT-IR spectra of a BSA solution (pD = 5.40) in the 1720−1380 cm^{-1} region over a temperature range of 25−90 °C (solid lines) and a spectrum of the same solution measured at 25 °C after cooling from 90 °C (dotted line).

Data Analysis for FT-IR Spectra. Contributions due to D_2O to the spectrum of a protein solution were eliminated by subtracting spectra of D_2O at the same temperature as that of the protein solution, in such way that the baseline was flat between 2000 and 1800 cm^{-1}. The final protein spectra were used for further analysis. All illustrated spectra are shown after band-narrowing by Fourier self-deconvolution (FSD) (*32*) to resolve overlapping IR bands using a half-bandwidth of 19.8 cm^{-1} and a band-narrowing factor k = 1.8. Subtraction and FSD calculation were performed by using OMNIC software (Nicolet Instrument Co., Madison, WI).

Deconvolved spectra were fitted with Gaussian band profiles. The number of bands and their positions were taken from the second derivative and the deconvolved spectra. Initial values for the peak heights and widths were estimated from the deconvolved spectra. For the final fits, the positions, heights, and widths of all bands were varied simultaneously. The curve fitting procedure was calculated on GRAMS/386 software (Galactic Inc., Salem, NH).

RESULTS

Heat-Induced FT-IR Spectra of BSA. Figure 1 shows representative deconvolved FT-IR spectra of BSA solution (pD = 5.40) in the 1720−1360 cm^{-1} region measured over a temperature range of 25−90 °C and at 25 °C after cooling. The IR spectrum at 25 °C before heating is dominated by bands due to the amide I′ (mainly C═O stretching vibration), amide II (coupling of the N−H bending and C−N stretching modes), amide II′ (coupling of the N−D bending and C−N stretching modes) modes, and side chain vibrations (*21*−*23*). The amide I′ band becomes broader with temperature, and two additional new bands due to aggregated, intermolecular β-sheets appear clearly at 1685 and 1615 cm^{-1} in

the amide I′ region over 70 °C and at 25 °C after cooling (*10*, *33*−*36*). The appearance of two bands is indicative of aggregation of BSA molecule as will be discussed later. In the spectrum at 25 °C, the amide II band appears near 1550 cm^{-1}, indicating that backbone amide protons are incompletely exchanged (*23*, *37*−*39*). In the spectra above 70 °C and at 25 °C after cooling, the amide II band at 1550 cm^{-1} disappears and the amide II′ band is still observed around 1450 cm^{-1}, suggesting that H−D exchange is completed even for the buried and strongly hydrogen-bonded amide N−H protons (*21*−*23*). Other IR bands observed between 1600 and 1500 cm^{-1} are attributed to amino acid side chains such as Tyr (near 1515 cm^{-1}), Asp (near 1585 cm^{-1}), Glu (near 1570 cm^{-1}), and Arg (near 1586 cm^{-1}), respectively (*21*, *30*, *31*, *40*−*43*). The deconvolved IR spectrum at 25 °C in Figure 1 yields three peaks around 1580 cm^{-1} (Asp, Arg, and Glu), 1570 cm^{-1} (Glu), and 1515 cm^{-1} (Tyr), respectively (*21*, *30*, *31*, *40*−*43*).

To explore the temperature-induced changes in secondary structure of BSA in detail, we concentrated on the amide I′ region and the Tyr band at 1515 cm^{-1} in the IR spectra. The IR spectra in Figure 1 show a broad amide I band contour between 1620 and 1680 cm^{-1} that consists of overlapping component bands arising from different secondary structure elements.

Figure 2A presents the enlarged deconvolved spectra in the amide I′ region of BSA solution (pD = 5.40), and Figure 2B shows their second derivatives. The band assignments in the amide I′ band region can be made by referring to the well-known crystal structure of human serum albumin (*2*, *5*). The second derivative spectra clearly reveal that four bands are present at 1674, 1654, 1630, and 1610 cm^{-1} in the amide I′ region. The band at 1654 cm^{-1} is due to α-helical structures and around 1674 cm^{-1} is associated with turn structures (*21*−*23*). The band at 1630 cm^{-1} is assigned to short-segment chains connecting α-helical segments of BSA. This assignment will be discussed in more detail in the Discussion section. The weak feature at 1610 cm^{-1} at 25 °C is due to the vibrations of Gln and the aromatic side chain (*21*, *42*).

The deconvolved IR spectra and their second derivative of BSA change little in the temperature range of 25−70 °C. Marked spectral changes are noted between 70 and 80 °C. These changes occur sharply at 74−76 °C (data not shown), demonstrating that major structural changes occur in this temperature range. The spectral features below 74 °C are almost identical to those at 25 °C, whereas the spectral patterns above 76 °C and those at 25 °C after cooling are all very similar to each other. The spectra above 74 °C and that at 25 °C after cooling show a broad, nearly featureless amide I′ band contour centered at 1645 cm^{-1} and two sharp bands at 1685 and 1615 cm^{-1}. The band at 1645 cm^{-1} is assigned to disordered structures, indicating the loss or collapse of secondary structures (*27*, *35*, *37*). In addition, the bands at 1685 and 1615 cm^{-1} are associated with aggregated, intermolecular β-sheets (*10*, *33*−*36*). These two bands are diagnostic for inter- and/or intrapeptide chain aggregation leading to antiparallel pleated β-sheet structures (*10*, *33*−*36*). More rigorously, the band at 1685 cm^{-1} is assigned to the antiparallel β-sheet whereas the other band at 1615 cm^{-1} is due to the intermolecular β-sheets resulting from the aggregation. The position at 1615 cm^{-1} and its bandwidth suggest the formation of the β-sheet structures with very strong hydrogen bond interactions leading to irreversible aggregation of the protein. As the result of the formation of the intermolecular β-sheet (β-aggregation), the unfolding of the protein becomes irreversible. The clear appearance of defined bands at 1685 and 1615 cm^{-1} at 74 °C reveals the formation of the significant irreversible β-aggregation of the protein.

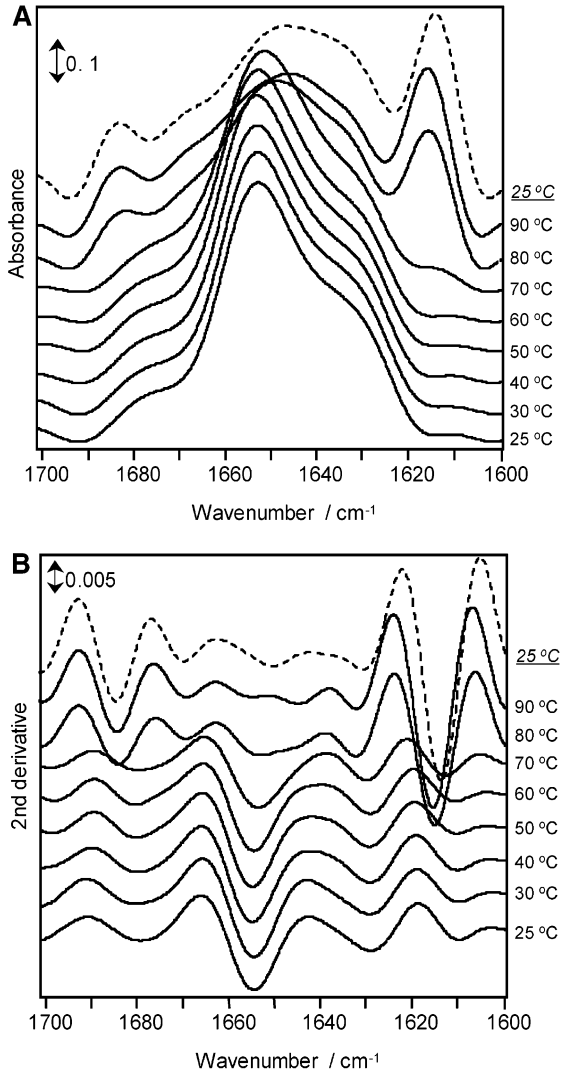

FIGURE 2: (A) Enlarged deconvolved FT-IR spectra of BSA solution. (B) Second derivatives of the spectra shown in (A).

Curve Fitting for Deconvolved IR Spectra of BSA. (i) Secondary Structure Change. The contribution of each component band to the total amide I′ contour can be estimated by curve fitting procedures. The curve fitting can be made either for the original spectra or for the deconvolved spectra (*36*). Panels A, B, and C of Figure 3 show the results of curve fitting for the deconvolved IR spectra of BSA at 30, 74, and 90 °C, respectively. In Figure 3A four bands are identified at 1674, 1652, 1630, and 1610 cm^{-1}, which are assigned to turn structures, α-helical structures, short-segment chains connecting α-helical segments, and the vibration of some amino acid residues. Figure 3B shows six bands at 1685, 1674, 1652, 1630, 1615, and 1610 cm^{-1}. The two bands at 1685 and 1615 cm^{-1} are due to the intermolecular β-sheet structures (*10*, *33*−*36*). In Figure 3C there are also six bands to be considered. Of note is that the visual

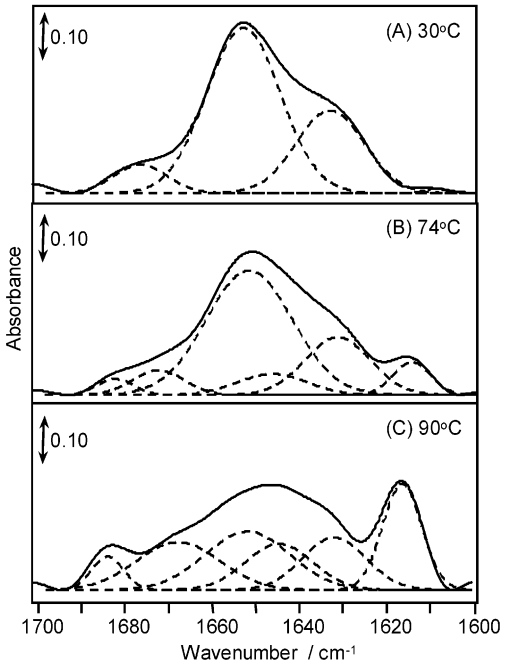

FIGURE 3: Deconvolved FT-IR spectra of the BSA solution (pD = 5.40) in the amide I′ region together with the respective best fitted individual component bands at 30 °C (A), 74 °C (B), and 90 °C (C). Solid and dotted lines indicate the experimental data and individual Gaussian components, respectively.

differences in the curve fitting profiles between 75 and 90 °C are the intensities of six component bands. The bands at 1654 cm^{-1} due to the α-helix and that at 1630 cm^{-1} assignable to the short-segment chains connecting α-helical segments become weaker as temperature increases from 74 to 90 °C, whereas other bands due to the intermolecular β-sheet (1685 and 1615 cm^{-1}) and disordered (1645 cm^{-1}) structures become much stronger at 90 °C (Figure 3B,C). The spectrum obtained after cooling the protein solution from 90 to 25 °C was almost identical to the spectrum at 90 °C (data not shown). These observations indicate that the α-helical structures of BSA are lost at higher temperatures with the formation of intermolecular β-sheets and the increase in the disorder structure.

Figure 4 shows the temperature-dependent variations of the peak height intensities of the four amide I′ bands at 1674 cm^{-1} (turn), 1652 cm^{-1} (α-helix), 1630 cm^{-1} (short-segment chains connecting α-helical segments), and 1615 cm^{-1} (intermolecular β-sheets). The intensity profile of the band at 1630 cm^{-1} indicates that a slight part of the short-segment chains is gradually lost at intermediate temperature around 57 °C. On the other hand, the intensity changes of the other three bands due to the α-helix, turn, and intermolecular β-sheet structures occur at higher temperatures, and their transitions proceed rapidly. Using the intensity profiles in Figure 4, we determined the transition temperature (T_m) between the folded and unfolded states for each secondary structure (Table 1). The T_m for the three bands due to the α-helix, turn, and intermolecular β-sheet structures are found to be around 75 °C. However, the T_m for the short-segment chains connecting α-helical segments is lower by 17−18 °C, when compared to those of the other bands. The T_m values suggest that the α-helix and turn structures of BSA are cooperatively denatured on heating at nearly the same temperature with the formation of the intermolecular β-sheet

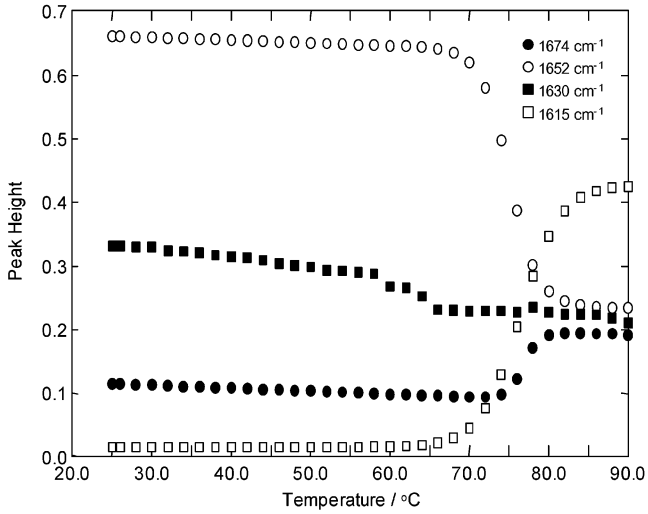

FIGURE 4: Temperature dependences of the peak height intensities of individual Gaussian components associated with the turn (1674 cm^{-1}), α-helix (1652 cm^{-1}), short-segment chains connecting α-helical segments (1630 cm^{-1}), and intermolecular β-sheet structures (1615 cm^{-1}) of BSA, respectively.

Table 1: Denaturation Temperatures (T_m Values) of Secondary Structures of BSA Obtained from This Study and Those from DSC Studies

structure	amide I′ band/cm^{-1}	T_m value/°C
turn	1674	76.8
α-helix	1654	75.4
short-segment chains connecting α-helical segments	1630	61.1
intermolecular β-sheet	1615	76.4
DSC[a]		61.8
DSC[b]		60.9, 66.4

[a] From ref *11*. [b] From ref *40*.

structure. The T_m value obtained from DSC measurement was about 61.8 °C (*11*). This value is very close to that for the short-segment chains connecting α-helical segments (Table 1). It is of note that FT-IR spectra give more detailed information on T_m values.

(ii) Temperature-Dependent Conformational Changes in BSA. The heat-induced unfolding of the protein implicates not only the destruction of the various secondary structure elements but also the structural reorientation of side chain groups. The IR spectra of BSA provide some bands due to side chain vibrations as specific monitors for their microenvironments (*30*, *31*). A band at 1515 cm^{-1} due to Tyr provides a specific local monitor for conformational changes (*30*, *31*). A side chain vibration that can be distinguished particularly well in the spectra of proteins in D_2O is the aromatic ring stretching vibration of Tyr, at 1515 cm^{-1}. According to Fabian et al. (*30*, *31*), an absorbance change in the Tyr band is observed when hydrophobic cores of a protein are formed, as solvent is excluded from the vicinity of Tyr residues that are buried in native protein molecule. Three of the six Tyr residues in BSA are buried in the native molecule whereas the other three are located on the protein surface (*2*). Figure 5 shows a temperature-dependent frequency shift of the Tyr ring vibration at 1515 cm^{-1}. Significant frequency shifts are observed around both 57 and 75 °C (Figure 5A). On the other hand, the peak intensity of the Tyr band is almost constant with temperature increase (Figure 5B). Fabian et al. (*30*, *31*) reported that the frequency

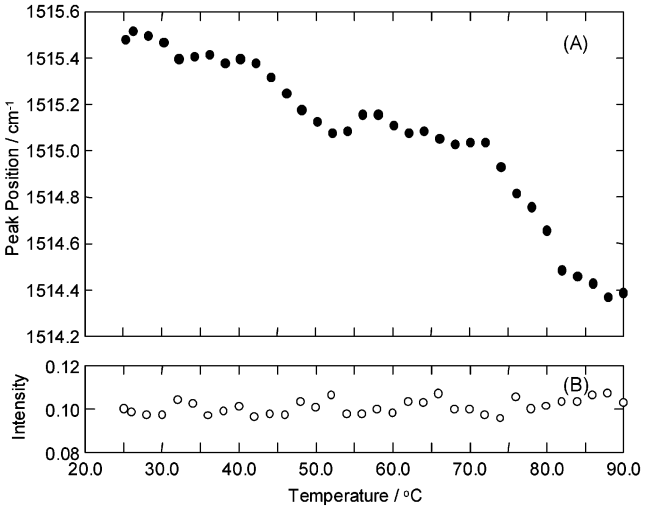

FIGURE 5: (A) Temperature dependence of the peak position of the Tyr band at 1515 cm^{-1}. (B) Corresponding intensity change of the Tyr band.

shift to a higher wavenumber with the constant intensity of the Tyr band during heat-induced denaturation of a protein can be correlated with the strengthening of the OH group of Tyr and with the loosening of hydrogen bonds between the OH group of Tyr and neighboring acceptors. Thus, the results in Figure 5 indicate that the conformation change of BSA occurs twice around both 57 and 75 °C, leading to the loosening and/or destruction of specific hydrogen bonds of Tyr groups inside the protein.

DISCUSSION

Assignment of the Band at 1630 cm^{-1} of BSA. The assignment of the band at 1630 cm^{-1} has been the subject of some controversy. It has often been assigned to an intramolecular β-sheet structure for many proteins (*42*, *44*−*46*). However, the secondary structure of BSA, which is composed of 67% helix, 10% turn, and 23% extended chain, is revealed the absence of β-sheet structure from the X-ray crystallographic study of HSA (*1*−*3*, *5*−*7*). Another possibility of the assignment of this band is an appearance of a band due to dimer formation between proteins. This assignment is dismissed in the present study because the monomer of BSA was taken out and used in this study as described in the Materials and Methods section. Thus, it is reasonable to consider that the IR spectra especially in the lower temperature range are not affected by the dimer of BSA. Moreover, the band at 1630 cm^{-1} is assigned to short-segment chains connecting α-helical segments (*36*, *47*−*50*). This interpretation indicates that the secondary structures of BSA are more flexible and more exposed to solvent. Generally, the hydrogen-bonded secondary structures, such as α-helix and β-sheet, buried in regions inaccessible to solvent are less easily denatured than turns, wide loops, and irregular structures that are exposed to solvent. Therefore, we have assigned the 1630 cm^{-1} band to the short-segment chains connecting α-helical segments of the BSA molecule. This assignment can be verified by taking into account the protein structure revealed by the X-ray crystallographic study (*2*, *5*) and temperature-dependent IR spectra. As shown in the curve fitting results in Figures 3 and 4, the occurrence of intensity variation of 1630 cm^{-1} at lower temperature than those of other bands indicates that the short-segment chains connecting α-helical segments are strongly affected by solvent locating on the protein surface when BSA molecules are thermally denatured.

Thermal Denaturation of the BSA Monomer Studied by FT-IR Spectroscopy. It has been suggested from the results in Figures 4 and 5 that structure changes in BSA occur in two steps in its thermal denaturation process. In the first step, the content in the short-segment chains connecting α-helical segments of BSA decreases with loss of tertiary structure around 57 °C. In the second step, the α-helix, turn, and intermolecular β-sheet structures change around 75 °C. Of note that the denaturation of the turn structure occurs delaying than those of the α-helix and intermolecular β-sheet structures. The change in short-segment chains connecting α-helical segments proceeds at 61.1 °C before the variations in the α-helix, turn, and intermolecular β-sheet structures. Later three secondary structures change in the almost around 75 °C from Table 1.

Several authors investigated the denaturation process of BSA by utilizing differential scanning calorimetry (DSC) measurement (*11*, *51*, *52*). Yamazaki et al. interpreted that the thermodynamically independent two domains owing to the formation of a crevice in the BSA molecule in a particular range of pH and ionic strength give two peaks in the DSC curve (*11*). Kang and Singh described that the DSC profile of intact BSA was best fitted by a non-two-state model with two peaks around 61 and 66 °C (*51*). Giancola et al. reported that the DSC profile of BSA was deconvolved as the sum of two independent two-state transitions (*52*). The following hypothesis was proposed from their observation of DSC profiles on defatted BSA aqueous solution. (i) The peak of low-temperature transition corresponds to the collapse of the N-terminal BSA domain due to the crevice in the vicinity of Trp 212, because of electrostatic repulsive forces particularly in the narrow stretch of the albumin chain between Arg 185 and Arg 217 by heating. (ii) The peak of the high-temperature calorimetric domain is due to the cooperative unit composed of the other two BSA domains, which bares the major unbalanced negative charge.

Therefore, structural changes at the lower temperature region of 57−60 °C observed in this study agree with the temperature of the crevice between domains I and II of the BSA molecule. Especially, the transition at 57 °C may be due to the collapse in the short-segment chains connecting α-helical segments of BSA accompanied with the crevice of domains I and II. On the other hand, the transition temperature around 75 °C by our study is about 10 °C higher than those from DSC studies by other groups. This implies that change in tertiary structure by intermolecular β-sheet formation may occur much lower than changes in secondary structures.

CONCLUSION

Temperature-dependent FT-IR spectra of defatted monomer BSA depending on temperature have been reported in the present study. To explore the secondary structure and conformation changes in defatted BSA, the combined curve fitting method and Fourier self-deconvolution method have been applied to the amide I′ region and the Tyr band around 1515 cm^{-1}. The FT-IR spectra below 74 °C are almost the

same as that at 25 °C, whereas the spectral patterns above 76 °C and that measured at 25 °C after cooling the BSA solution from 90 °C are very similar to each other. This result has revealed that the intermolecular β-sheet formation of BSA is irreversible on heating above 70 °C. In the FT-IR spectra, the band at 1630 cm^{-1} has been assigned to the short-segment chains connecting α-helical segments. The T_m values for three bands due to α-helix, turn, and intermolecular β-sheet structures have been found to be around 75 °C. However, the T_m value for the short-segment chains connecting α-helical segments is lower by 17−18 °C, when compared with those of other secondary structures. The results suggest that short-segment chains connecting the α-helical segment denatures at lower temperature than other secondary structures and that structural changes in α-helix and turn occur on heating at almost the same temperature of 76 °C with the formation of intermolecular β-sheet structure. The conformation changes in BSA occur twice around both 57 and 75 °C. It is interpreted by the collapse of the N-terminal BSA domain due to the crevice in the vicinity between domains I and II at low-temperature transition and by the change in the cooperative unit composed of the other two BSA domains at high-temperature transition.

ACKNOWLEDGMENT

We are indebted to Prof. Y. Ozaki (Kwansei-Gakuin University) for valuable discussions and for FT-IR measurement.

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BI0489154