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Vibrational circular dichroism spectra of protein films: thermal denaturation of bovine serum albumin

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Abstract

Vibrational circular dichroism (VCD) spectroscopy has been used for the first time to investigate the thermal denaturation of proteins in H_2O solutions. Films prepared from heated aqueous solutions were used for these investigations. A well-known α-helical protein, bovine serum albumin (BSA), is used for this first study. Both VCD and infrared absorption results obtained for BSA films indicate that the heat treatment of BSA induces significant amounts of β-sheet structure and that the denaturation process is irreversible. To verify the irreversible nature of thermal denaturation, optical rotation was also measured as a function of temperature in both heating and cooling cycles. These results also indicate that thermal denaturation of BSA in solution is irreversible. This study establishes the usefulness of films for VCD investigations and offers new avenues for VCD studies on biologically important systems.
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Keywords: Vibrational circular dichroism; Protein film; Bovine serum albumin; α-Helix; β-Sheet; Optical rotation

1. Introduction

Spectroscopic techniques have been potential tools in the secondary structure analysis of proteins and peptides, both qualitatively and quantitatively [1]. Electronic circular dichroism (ECD) [1] and Fourier transform infrared (FTIR) [2] spectroscopy were widely used in protein structural analysis under various conditions. Vibrational circular dichroism (VCD) [3] spectroscopy is a relatively new technique for the study of conformational preference of proteins and peptides. It measures the differential absorbance of left and right circularly polarized light in the infrared region [3] originating from molecular vibrations. VCD can provide important conformational information in cases where ECD is complicated by the overlap of backbone amide and side-chain aromatic chromophores. This feature is particularly relevant for the studies of peptides containing aromatic amino acid residues that contribute significantly to electronic absorption in the near-UV region (190–230 nm), which is generally considered to arise exclusively from the backbone amide contribution. The conformational preferences of proteins in aqueous solutions have been studied using VCD [4–7]. These previous studies have shown that the amide I vibrational bands give characteristic VCD spectra for α-helix [8], β-sheet [8], combination of α and β [8], random coil [9], and β-turn [10] structures in solutions. There are a few VCD studies in thin film state for oligopeptides [11], but VCD studies on protein films were not available.

Recent studies [12] in our laboratory indicated that the VCD spectra of protein films, obtained from aqueous solution, provide the same structural information as that in aqueous solution but with an advantage that the protein sample amount required for film studies (1–5 mg/ml) is approximately two orders of magnitude smaller than that required for aqueous solution VCD studies (100 mg/ml). To advance this discovery, we undertook thermal denaturing studies using VCD for the first time. In this manuscript, we present the thermal behavior of bovine serum albumin (BSA) by investigating the films formed from heated aqueous solutions.

BSA is a well-known globular protein that has the tendency to aggregate into macromolecular assemblies [13]. Its structure is made up of three homologous domains

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(I, II, and III), which are divided into nine loops by 17 disulphide bonds, each one formed by six helices, and its secondary structure is dominated by α-helix [14,15]. X-ray crystallography studies suggested that the native structure of BSA is predominantly α-helical [16]. It has been suggested, based on X-ray scattering studies [17], that thermally denatured BSA also has predominantly α-helical conformation. The optical rotatory dispersion (ORD) and infrared spectral studies concluded that alkali or thermal denaturation caused a partial loss of α-helical structure with no formation of β-sheet [18]. However, their infrared spectra showed the appearance of a shoulder band for BSA heated above 72 °C at 1620 cm^{-1}, which is indicative of β-sheet formation [18]. In another study, β-sheet formation was suggested at higher temperatures and was more pronounced on cooling [19].

In this paper, we demonstrate the use of VCD for deducing the irreversible structural changes involved in thermal denaturation of BSA. For this purpose, aqueous solutions of BSA heated to different temperatures were deposited as films and studied using VCD. The advantages in VCD film studies arise from the fact that the amount of sample required for the film studies (1–5 mg/ml) is approximately two orders of magnitude smaller than the one needed for VCD studies in aqueous solution (100 mg/ml) [8]. Furthermore, since there is no solvent interference in the film VCD studies, more throughput (and hence better signal-to-noise), than that in aqueous solution VCD studies, can be obtained. The present results indicate a significant amount of β-sheet structure for BSA heated above 60 °C. Furthermore, the thermally induced structural changes observed for BSA films are similar to those in solution [19]. To confirm the irreversible nature of thermal denaturation observed for BSA films, temperature-dependent variations in optical rotation of BSA in aqueous solution are also presented and these results further support the observations on BSA films.

2. Experimental

Albumin (bovine pancreas, A-4378) was purchased from Sigma and used without further purification. For both VCD and ECD measurements, 5 mg of BSA was dissolved in 1 ml of 25 mM Tris buffer (pH 7.4) solution containing sodium chloride (25 mM).

2.1. VCD measurements

All VCD spectra were recorded on a commercial Chiralir spectrometer (Bomem-Biotools, Canada) modified to minimize the artifacts using double polarization modulation method [20]. The detailed modification of VCD instrument is as follows: the light from the interferometer, brought to an external bench using a BaF_2 lens, is passed through a linear polarizer, photoelastic modulator (PEM), sample, a second PEM, ZnSe-focusing lens, and detector. Both PEMs have ZnSe as the optical element, which does not have antireflection coating. The detector signal is processed by two electronic paths. In one, the low-frequency signal is isolated with a low-pass filter and Fourier-transformed. In the second path, the high-frequency component is isolated with a high-pass filter and analyzed with two lock-in amplifiers. One lock-in amplifier is tuned to the frequency (37.07 kHz) of the first PEM and the second lock-in amplifier is tuned to the frequency (36.95 kHz) of the second PEM. The outputs of these lock-in amplifiers are fed to a low-pass filter, subtracted, and Fourier-transformed.

All spectra were collected for 1 h at a resolution of 8 cm^{-1}. For the temperature-dependent VCD measurements, a 200-μl aliquot of protein stock solution was heated to the desired temperature for 30 min. Then about 150 μl of that heated solution was cast onto a 2.5-cm-diameter CaF_2 window that was placed at a room temperature in a fume hood to provide constant air flow over the sample. The evaporation was continued at room temperature until a thin film formed on the surface of the CaF_2 window. Films were tested for satisfactory VCD characteristics by comparison of the film VCD obtained, with the sample rotating through 90° about the light beam axis. For all the data reported here, the sign and peak-to-peak magnitude were reproduced with rotational position (90°). Baseline corrections were done by subtracting the VCD spectrum of a blank CaF_2 window. BSA protein film samples studied here, which were derived from the aqueous stock solution (5 mg/ml) heated to different temperatures, had an absorbance between 0.3 and 0.6. Lorentzian curves were used for quantitative analysis of both VCD and absorption spectra obtained at different temperatures. Following iterative fitting of Lorentzian curves to the observed bands, the relative amounts of secondary structure were determined from areas under the bands assigned to a particular structure.

2.2. Optical rotation measurements

Optical rotation of BSA (5 mg/ml) in 25 mM Tris buffer solution containing sodium chloride (25 mM) at different temperatures was measured at 589 nm using AUTOPOL IV automatic polarimeter and 0.5 dm jacketed cell. The cell was maintained at a chosen temperature by circulating water using *ISOTEMP 1016S* thermostat (Fischer Scientific). The temperature of the cell was monitored (Omega CN76000) using a thermocouple inserted in the cell body.

3. Results and discussion

To examine the thermal denaturation of BSA, infrared VCD and absorption spectra were obtained for films formed from heated aqueous solutions. For interpreting the VCD data, we make use of correlations established by Pancoska et al. [5], Urbanova et al. [6], and Baumruk and Keiderling [8]

between the VCD features and known protein structures of a uniform conformational type in solution state. The VCD pattern characteristic of right-handed α-helices is a positive couplet at ~1650 cm^{-1} with negative bias [weak broad positive band ($+\Delta A$) in the lower-frequency side followed by strong negative band ($-\Delta A$) at higher-frequency side] in the amide I region. The characteristic β-sheet VCD spectrum contains a single negative band between 1615 and 1635 cm^{-1}. If α and β secondary structures are present in proteins of an intermediate structural type, then the VCD spectra contain a mixture of α and β characteristics, namely, low wavenumber negative band, weak intermediate positive band, and negative band at higher wavenumber, giving a three-peak pattern.

Fig. 1 shows the infrared VCD and absorption spectra obtained for the BSA films prepared from aqueous solutions heated to different temperatures. At 25 °C, the absorption (Fig. 1) spectrum shows a single amide I intense band at 1655 cm^{-1}, which is characteristic of proteins adopting α-helical structure. The corresponding VCD spectrum shows the characteristic positive couplet with negative bias (VCD intensity of the positive band at lower frequency is smaller than the intensity of the band at higher frequency). When the temperature is increased to 50 °C, the VCD and absorption spectra (Fig. 1) are similar to those at 25 °C, suggesting that there is no change in the BSA structure in this temperature range.

When the temperature is increased further to 60 °C, the IR absorption spectrum (Fig. 1) shows the appearance of a weak shoulder band at 1628 cm^{-1}, which is characteristic of β-sheet conformation. At 70 °C, the IR absorption spectrum (Fig. 1) shows a resolved shoulder band at 1628 cm^{-1} along with major α-helical band at 1655 cm^{-1}, suggesting that BSA takes a minor β-sheet structure along with dominant α-helical structure. Literature results based on FTIR measurements in D_2O solution also suggested that BSA takes a partial β-sheet structure above 65 °C [19]. The corresponding amide I VCD spectrum (Fig. 1) of BSA film at 70 °C shows negative peaks at 1662 and 1616 cm^{-1} (weak), with positive band in between at 1643 cm^{-1} no longer seen clearly. The amide I VCD spectra of proteins with contribution from both α-helix and β-sheet conformations are characterized by a three peak pattern (−+−), or "W" shape, which reflects a simple combination of the α-helix and β-sheet amide I band shapes. The presence of high α-helix and low β-sheet content is characterized by the presence of VCD band shapes dominated by an intense negative band on the high-frequency side of the absorption band combined with a weaker low-frequency negative band; the weak positive band in between does not appear in all cases. In the present case, the appearance of strong negative band at 1662 cm^{-1} and weak negative band on the lower frequency side at 1616 cm^{-1} suggests that BSA film has a combination of high α-helix and low β-sheet structure at 70 °C. A similar type of VCD spectrum was observed for cytochrome *C* in aqueous solution where it showed high α-helix and low β-sheet structure [8].

Further increase in temperature (80 and 90 °C) leads to a clearly resolved shoulder band at 1628 cm^{-1} in the absorption spectrum (Fig. 1). The low-frequency amide I band at 1628 cm^{-1} was previously assigned to BSA aggregation [19], which is due to intermolecular hydrogen bond formation. The corresponding VCD spectrum (Fig. 1) gives a VCD pattern that is similar to that at 70 °C, but with increased intensity at 1616 cm^{-1}. This result suggests that BSA takes an increased amount of β-sheet structure at higher temperatures. The increase in β-sheet structure with temperature can be monitored from the relative intensities of bands assigned to β-sheet and α-helix structures. Fig. 2A shows the variation of secondary structure (as β-sheet/α-

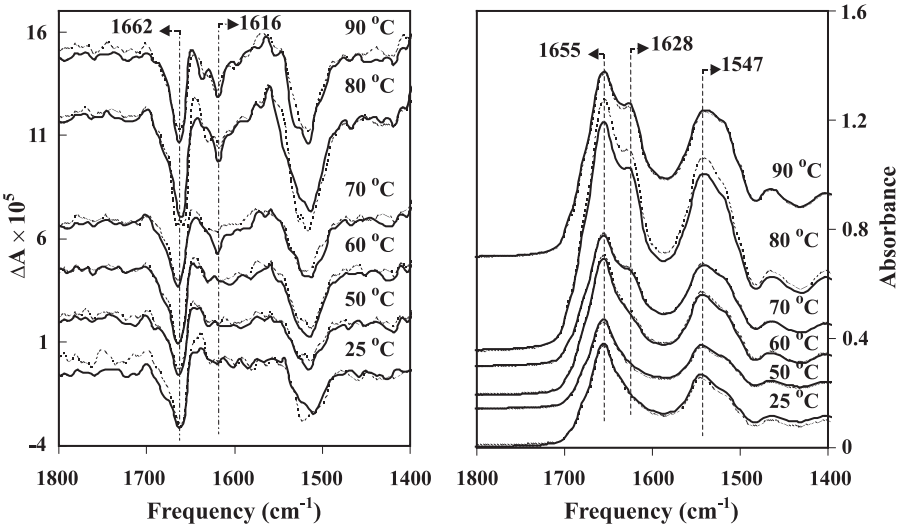

Fig. 1. Thermal behavior of bovine serum albumin films monitored by VCD (left panel) and FTIR absorption (right panel). The spectra were recorded at 8 cm^{-1}. BSA films were obtained from aqueous buffer solutions [25 mM Tris buffer (pH 7.4) containing 25 mM NaCl] heated to the temperature indicated. The solid and dotted lines represent the VCD spectra of BSA film at normal and 90° rotation, respectively.

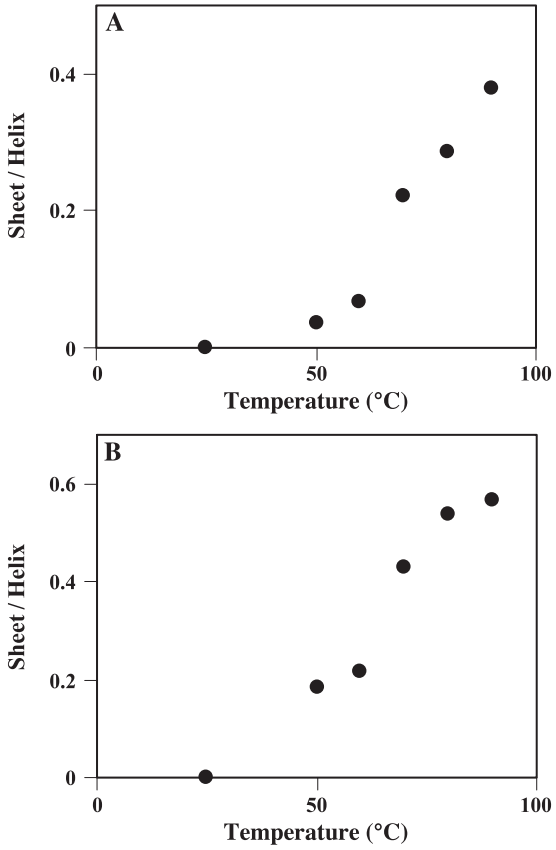

Fig. 2. Variation of β-sheet to α-helix ratio with temperature obtained from the areas of VCD bands (A) and infrared absorption bands (B). Band areas were obtained via Lorentzian curve fitting.

helix ratio) with temperature based on the areas of VCD bands at 1616 and 1662 cm^{-1}. The VCD band areas were obtained, from the average VCD spectrum for normal and 90° rotation, by fitting the Lorentzian band shapes to the observed VCD bands. These data indicate that the ratio between β-sheet and α-helix increases significantly with increasing temperature (Fig. 2A). We have also used curve-fitting of the absorption spectra to determine the ratio between β-sheet and α-helix. Lorentzian bands were fit to the FTIR absorption spectra and the areas of the bands at 1628 and 1655 cm^{-1} were used to determine the β-sheet/α-helix ratio. These results are shown in Fig. 2B. From this figure also, it can be seen that BSA takes a significant amount of β-sheet structure with increasing temperature, and that the VCD and FTIR absorption results are in good qualitative agreement with each other. These results are also in good agreement with previously reported results from FTIR for thermal behavior of BSA in D_2O solution [19]. For comparative studies, we have also carried out temperature-dependent studies on BSA using the films for ECD measurements (data not shown). However, the results indicated that at lower temperature (25–70 °C), ECD results are in good agreement with VCD results, but some artifacts appear to be associated with the ECD spectra of films at higher temperature (80 and 90 °C). Nevertheless, an ECD spectral study in solution on thermally induced structural changes for BSA has been reported by Takeda et al. [21]. Based on the temperature-dependent ECD spectra of BSA solutions, they suggested that the relative amount of α-helix, β-sheet, and disordered forms at 2 °C are 67%, 3%, and 30% respectively. The corresponding proportions at 65 °C were reported to be 44%, 13%, and 43%, respectively. Thus, the observation of a decrease in α-helix and increase in β-sheet, deduced from the literature ECD studies [21] in solution, is in agreement with observations deduced from the current film studies using infrared VCD and absorption. Although Takeda et al. [21] also suggested an increase in the disordered form with temperature, that observation cannot be confirmed by the present studies because the disordered form does not show a VCD spectrum [22].

Since the protein films were made by depositing the heated solutions on room temperature plates, the spectral changes seen in the current film measurements must be arising from thermally induced irreversible structural changes. To verify the irreversible nature of thermal denaturation, we have also measured the optical rotation of BSA in Tris buffer solution (25 mM; 25 mM NaCl) at different temperatures during both heating and cooling cycles. At room temperature, Yang and Doty [23] reported an $[\alpha]_D$ of −63 for BSA in aqueous solution (pH 5), which is close to $[\alpha]_D$=−56 for BSA in Tris buffer (pH 7.4) solution. Fig. 3 shows the variation of optical rotation of BSA at 589 nm with temperature. From this figure, it is clear that BSA does not show significant change in its optical rotation when the temperature is raised from 25 to 45 °C. However, when the temperature is raised above 45 °C, a significant decrease in optical rotation, becoming more negative, was found. The change in optical rotation at 589 nm cannot be unambiguously related to a particular structural change because both random coil and β-sheet structures were stated [23] to have lower optical rotation than α-helix. But our intention in using optical rotation here is only to verify the irreversible nature of thermal denaturation in solution. As can be seen in

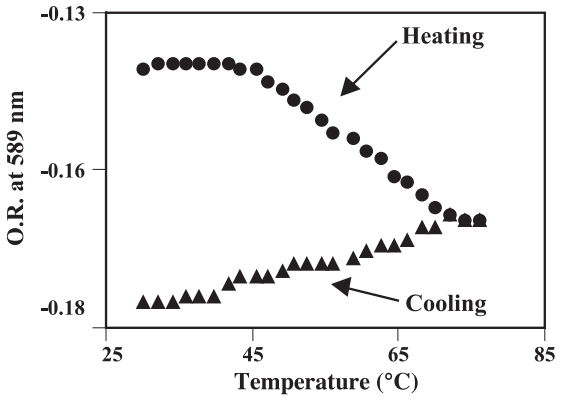

Fig. 3. Thermal behavior of bovine serum albumin in Tris buffer (pH 7.4) solution containing 25 mM NaCl at different temperatures monitored by optical rotation at 589 nm. The data points during heating are represented by circles, while those during cooling are represented by triangles.

Fig. 3, during cooling process, the optical rotation values did not retrace the original values obtained in the heating process; instead, optical rotation continued to become more negative. This observation suggests that heat-induced structural change in BSA solution at 90 °C is irreversible. Wetzel et al. [19] also observed a similar result for BSA in aqueous solutions using ECD and FTIR measurements and for BSA gel [24] using infrared and Raman spectroscopies.

4. Conclusion

The present results demonstrate that thermally induced irreversible structural transitions in protein aqueous solutions can be successfully investigated with VCD by using the films formed from aqueous solutions. VCD film results gave characteristic features at all temperatures studied, for determining the secondary structure. The conclusions obtained from VCD measurements on BSA films, supported by simultaneous infrared absorption measurements, are that the relative amount of β-sheet increases for BSA with temperature and that the thermal denaturation of BSA is irreversible. These conclusions match with those obtained in the literature from both infrared absorption and ECD spectra for BSA solutions. The irreversible nature of thermal denaturation of BSA is also confirmed by optical rotation measurements. This study establishes the usefulness of films for VCD investigations and offers new avenues for VCD studies on biologically important systems.

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