

A Raman Difference Spectroscopic Investigation of Ovalbumin and S-Ovalbumin

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Synopsis

Raman spectra from 800 to 1850 cm^{-1} of aqueous solutions of ovalbumin and its more heat-stable form, S-ovalbumin, are presented. A Raman difference spectrum (ovalbumin minus S-ovalbumin) shows differences in intensity in the amide I and III regions. These intensity differences lead us to postulate that the conversion of ovalbumin to S-ovalbumin involves a conformation change of a small part ($\sim 3\text{--}4\%$) of the protein from α -helix to anti-parallel β -sheet geometry. This small difference in the three-dimensional arrangement of the peptide chain may contribute to the large difference in the thermodynamic stability between ovalbumin and S-ovalbumin.

INTRODUCTION

The identification of S-ovalbumin, a more heat-stable form of ovalbumin,^{1,2} prompted comparative studies on the properties of the two proteins to find an explanation for their difference in heat stability. Numerous measurements³⁻⁷ have shown little or no difference between the properties of ovalbumin (native) and S-ovalbumin. However, differences are evident under denaturation conditions. Completely converted S-ovalbumin denatures at a slower rate and higher temperature (92.5°C at pH 10) than ovalbumin (84.5°C at pH 10).^{2,3,7} Completely denatured ovalbumin exhibits a faster refolding or aggregation rate than S-ovalbumin.⁴ It has been established by differential scanning calorimetry (DSC)⁷ that S-ovalbumin is not a partially denatured ovalbumin and that an intermediate species is formed during the conversion of ovalbumin to S-ovalbumin. Based on their different aggregation behavior and on the irreversibility of the ovalbumin to S-ovalbumin transformation, a difference in covalent bond structure has been suggested.^{2,3} However, the specific structural difference that would account for the increased stability of S-ovalbumin has not been determined.

Painter and Koenig have reported the Raman spectrum of ovalbumin⁶ and found it indistinguishable from the spectrum of S-ovalbumin. In the following, Raman difference spectroscopy provides evidence for a difference in the conformation of the two proteins.

EXPERIMENTAL

Ovalbumin (a twice recrystallized and lyophilized preparation, lot 564) was purchased from Worthington Biochemical Corporation. An aqueous solution of ovalbumin, adjusted to pH 10, was converted to the S-form by heating at 56°C for 38 hr. Complete conversion to the S-form was indicated by DSC measurements.⁷ Ovalbumin and S-ovalbumin, dissolved in 0.05M KCl, were titrated to pH 8 with 1N NaOH and centrifuged at 36,500 *g* for 30 min in a Spinco model L ultracentrifuge. Protein concentrations were calculated from the absorbance at 280 nm ($E_{280\text{ nm}}^{1\%} = 6.64\text{ g}^{-1}\text{ cm}^2$)⁸ to be ~7%. The clear supernatants were introduced into 1.2-mm (i.d.) quartz capillaries for Raman measurements. The sealed capillaries were positioned at the focus of the incident laser beam and at the focal point of the light-collection optics for the spectrometer. The laser beam was passed into the side of the horizontal capillary, and the total Raman scattering was collected 90° to this incident beam. The 514.5 nm line (600 mW) from a Coherent Radiation CR-3 laser was used for sample excitation. The scattered light was dispersed with a Spex 1401 double monochromator and detected with a cooled RCA C31034A gallium-arsenide photomultiplier. Fixed slits giving a resolution of 4.0 cm^{-1} at the exciting line and 3.3 cm^{-1} at 1850 cm^{-1} were used for all scans. The count time at each 1.0 cm^{-1} data collection increment was 20 sec. A laminar air flow apparatus⁹ was used to adjust and maintain sample temperature at $1 \pm 0.1^\circ\text{C}$. Details of the computer data acquisition and reduction system have been described previously.¹⁰ Care was taken to obtain spectra with high signal-to-noise ratios. Repetitive runs, alternating between ovalbumin and S-ovalbumin, were recorded with identical experimental conditions. The wavelength reproducibility of spectra between like samples was better than 0.1 cm^{-1} .

RESULTS AND DISCUSSION

Raman spectra from 800 to 1850 cm^{-1} of aqueous solutions of ovalbumin and S-ovalbumin are shown in Fig. 1(A). These represent the sum of seven individual spectra with intensities normalized to the 1450 cm^{-1} methylene/methyl bending mode (taken as internal standard to adjust for small differences in protein concentrations). The difference spectrum in Fig. 1(B) was obtained by subtracting a spectrum of ovalbumin from the spectrum of S-ovalbumin and adding seven such differences. The intensity differences in Fig. 1(B) are not a result of wavelength shifts between scans. We established this by shifting an observed ovalbumin spectrum by 0.1 cm^{-1} and subtracting it from the unshifted spectrum. This difference spectrum, Fig. 2, shows that the sharp phenylalanine band at 1004 cm^{-1} is most sensitive to wavelength shifts. Since the ovalbumin-S-ovalbumin difference spectrum [Fig. 1(B)] shows a much smaller deviation at 1004 cm^{-1} than that in Fig. 2, we conclude that wavelength shifts of the spectrometer between scans were less than 0.1 cm^{-1} . Therefore, the major intensity

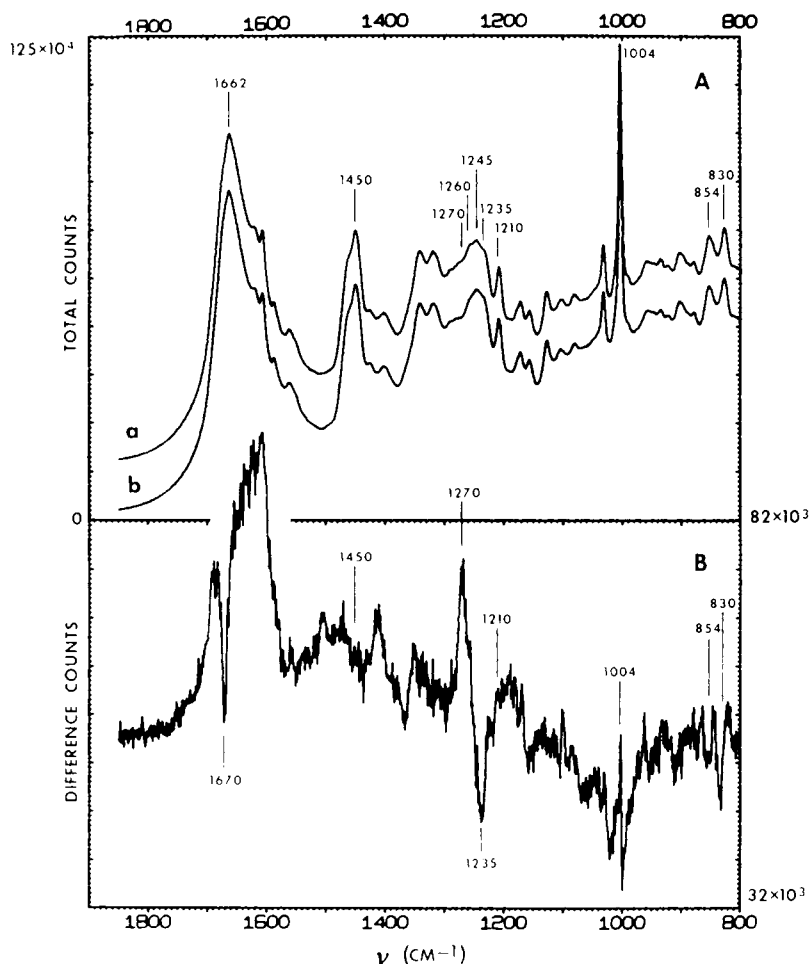


Fig. 1. (A) Raman spectra (sum of seven scans) of $\sim 7\%$ aqueous solutions, pH 8, of ovalbumin (a) and S-ovalbumin (b); (a) displaced by 10×10^4 units from original (a). (B) Raman difference spectrum of ovalbumin minus S-ovalbumin [obtained from data shown in (A)]. Zero displaced since the ovalbumin spectra had a higher background than the S-ovalbumin spectra.

differences in the $1200\text{--}1300\text{ cm}^{-1}$ and $1600\text{--}1700\text{ cm}^{-1}$ regions are experimentally significant.

The amide I ($\sim 1645\text{--}1680\text{ cm}^{-1}$) and amide III ($\sim 1230\text{--}1300\text{ cm}^{-1}$)¹¹ regions of polypeptides and proteins are useful for conformational analysis. The spectra-structure correlations of polypeptides with known secondary structures have been well summarized in a recent review.¹² These Raman studies show that the amide I and III band frequencies of the conventionally termed α -helix, antiparallel β -sheet, and random-coil (or disordered) peptide conformations generally occur in regions shown in Table I. The Raman spectra of numerous proteins¹³⁻²⁰ with known conformations agree

TABLE I
Raman-Active Amide III and I Frequencies^a

Conformation	Amide III (approximate cm^{-1})	Amide I (approximate cm^{-1})
α -Helix	1265–1300	1645–1660
Random coil (disordered)	1240–1260	1660–1670
Antiparallel β -sheet	1230–1240	1665–1680

^a Data from Refs. 12–20.

well with these assignments; however, the spectra of some proteins^{21–23} are not completely consistent with Table I. The latter studies show that some caution is required in using the amide III region for detection of α -helical structure, since some side-chain vibrations also occur in this region.

Amide III

The spectra of ovalbumin and S-ovalbumin [Fig. 1(A)] show that the amide III region consists of at least three overlapping bands, centered at 1245 cm^{-1} . Painter and Koenig⁶ assign the central peak (1247 cm^{-1} in their ovalbumin spectrum) to the amide III vibration of parallel β -sheet conformation. They base this assignment by analogy with one made by Yu and Jo²⁴ on the spectrum of carboxypeptidase A. However, Yu and Jo have suggested that a band at 1243 cm^{-1} in the spectrum of carboxypeptidase A could result from amide III vibrations of *both* the random-coil (disor-

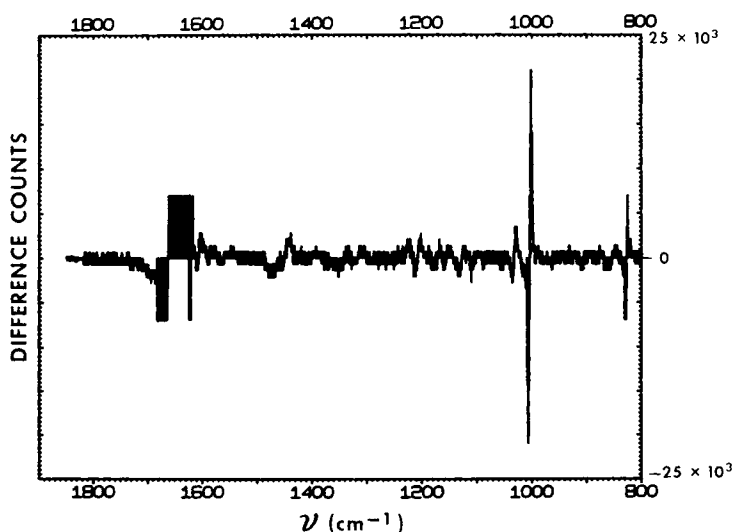


Fig. 2. Effect of shifting an ovalbumin spectrum by 0.1 cm^{-1} and subtracting it from an unshifted ovalbumin spectrum [scale same as Fig. 1(B)].

dered) and parallel β -sheet conformations. Also, additional evidence from Raman spectra of polypeptides^{25,26} and proteins^{13,14} indicates that a band at $\sim 1245\text{ cm}^{-1}$ is more likely associated with the random-coil (or disordered) conformation. We attribute the shoulder at $1230\text{--}1235\text{ cm}^{-1}$ in both spectra [Fig. 1(A)] to a contribution from the antiparallel β -sheet conformation (Table I). A weak shoulder at $\sim 1260\text{ cm}^{-1}$ is possibly due to tyrosine²⁷ and some of the intensity in the $1265\text{--}1300\text{ cm}^{-1}$ region probably results from the amide III vibrations of α -helical structure (Table I).

A cursory inspection of the Raman spectra of ovalbumin and S-ovalbumin [Fig. 1(A)] shows no obvious differences. However, the difference spectrum [Fig. 1(B)] reveals significant features which we believe are consistent with a structural difference between the two proteins. Since the direction of subtraction was ovalbumin minus S-ovalbumin, the negative intensity at $\sim 1235\text{ cm}^{-1}$ indicates that S-ovalbumin has more antiparallel β -sheet structure than ovalbumin. There is no difference band at 1245 cm^{-1} ; therefore, the sum-total random-coil (or disordered) conformation is equivalent in the two proteins. Painter and Koenig⁶ suggested that the intensity at 1280 cm^{-1} in the spectrum of ovalbumin is not from an amide III vibration and that the bands at 1260 cm^{-1} in the spectrum of ribonuclease and the one at 1266 cm^{-1} in the spectrum of ovomucoid are due to tyrosine. We believe that the intensity change at 1270 cm^{-1} in our difference spectrum [Fig. 1(B)] results from a higher α -helical content in ovalbumin and not from tyrosine. We arrive at this conclusion by examining other bands due to tyrosine. These bands and their corresponding relative intensities, indicated by (), in a Raman spectrum of tyrosine²⁷ at pH 12.0 are found at 1272 (2), 1210 (6), 854 (7), and 833 cm^{-1} (5). Both the 1272 and 1210 cm^{-1} bands must be the substituent-sensitive a_1 fundamental modes similar to those found in *p*-substituted phenols.^{28,29} The intensity ratio of the 850 to 833 cm^{-1} bands is sensitive to hydrogen bonding (or ionization) of the phenolic hydroxyl group.^{15,30} The ovalbumin-S-ovalbumin difference spectrum shows no wavelength shifts or intensity changes at 1210 , 854 , or 830 cm^{-1} . Therefore, the environment of the tyrosines seems to be unaffected by the conversion of ovalbumin to S-ovalbumin. The expected low-intensity contribution of tyrosine in the 1270 cm^{-1} region (intensity about one-third of the other tyrosine bands), and the lack of change of the other tyrosine bands between ovalbumin and S-ovalbumin, leads us to believe that the positive intensity at 1270 cm^{-1} cannot result from differences in tyrosine residues but instead is due to a higher α -helical conformation in ovalbumin.

Amide I

In Fig. 1(A) the position of the amide I band at 1662 cm^{-1} in the ovalbumin and S-ovalbumin spectra indicates a high random-coil (or disordered) content for both proteins (Table I). The amide I vibrations from α -helix and β -sheet structures most likely contribute intensity to the lower

and higher frequency side of the 1662 cm^{-1} band, respectively. The ν_2 bending mode of water also contributes to the broadening on the low-frequency side ($\sim 1640\text{ cm}^{-1}$) of the amide I band.

In the difference spectrum [Fig. 1(B)], we attribute the negative band at 1670 cm^{-1} to the higher intensity amide I vibration from the antiparallel β -sheet in S-ovalbumin. This is in agreement with the interpretation of the amide III region of the difference spectrum.

The broad positive intensity at $1600\text{--}1650\text{ cm}^{-1}$ is due to a small difference in the amount of water in the two solutions and to any dissimilarity of water associated with the two proteins. Any contribution to the amide I band from the higher α -helical content of ovalbumin should appear as a positive intensity at $\sim 1645\text{ cm}^{-1}$, but this is most likely obscured by the broad residual water band.

A rough estimation of the amount of secondary structure difference between the two solvated proteins was made assuming that the intensity differences of the amide III bands are proportional to the change in conformation. The changes in intensity at ~ 1270 and $\sim 1235\text{ cm}^{-1}$ represent $\sim 3\text{--}4\%$ of the total intensity at the same wavelengths. This small difference in the three-dimensional structure between ovalbumin and S-ovalbumin may be a factor in the stability difference of the two proteins.

SUMMARY

A Raman difference spectrum between ovalbumin and S-ovalbumin provides evidence for a previously undetected structural difference in the three-dimensional conformation of the two proteins. Differences in Raman intensities at 1670 , 1270 , and 1235 cm^{-1} indicate that ovalbumin has approximately $3\text{--}4\%$ more α -helix conformation and that S-ovalbumin has an equivalent amount of increased antiparallel β -sheet geometry.

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