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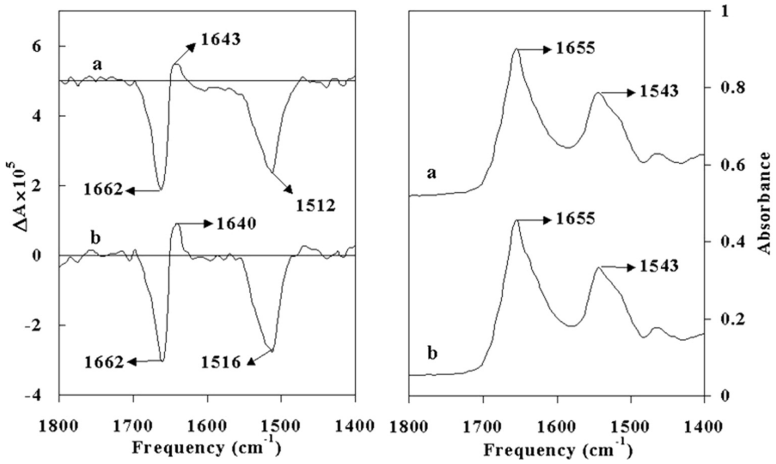

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Vibrational Circular Dichroism of Protein Films

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Abstract: Vibrational circular dichroism (VCD) spectra in the 1800−1400 cm^{-1} region have been measured for the first time for protein films prepared from aqueous buffer solutions. These measurements demonstrate several advantages of significant importance. First, the interference from infrared absorption of water in the amide I region, which is a serious limitation for measurements in water solutions, is eliminated. Second, the amounts of protein samples required for VCD measurements on films are approximately 2 orders of magnitude smaller than those required for the same in water solutions. In addition, the amide I absorption and VCD bands of protein films are found to be independent of film orientation. Furthermore, characteristic VCD patterns have been observed for protein films whose secondary structure is dominated by α-helix, β-sheet, and α + β combinations. These results demonstrate that VCD can be used to study the structure of proteins in the film state.

Introduction

Vibrational circular dichroism (VCD)[1] is a relatively new technique that has been finding increasing applications in conformational analysis of proteins[2] and peptides.[3] VCD is a measurement of the differential absorbance of left and right circularly polarized light in the infrared region resulting from molecular vibrational transitions.[4] VCD can provide important conformational information in cases where electronic circular dichroism (ECD) is complicated by the overlap of transitions from backbone amide and side chain aromatic chromophores. This feature is particularly relevant for the studies of peptides containing Phe, Tyr, and Trp 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 transitions. The conformational preferences of proteins in aqueous solution have been determined using VCD spectroscopy.[2c,5,6] VCD is also widely used to study the conformations of peptides in organic and aqueous solutions.[7−10] Characteristic VCD spectra have been reported for different types of secondary structures, such as α-helices,[11] β-sheets,[11c−e] random coil,[11c−e] β-turns,[12] double helices,[3a,g] etc., for peptides and proteins in solution. A few measurements were also carried out for oligopeptide films.[13] Even though the VCD spectra of oligopeptide films provided a good signal-to-noise ratio, the spectral features did not reveal the structural origin of polypeptides clearly.[13b] Several ECD studies were also reported analyzing the solid-state conformational preference of monodispersed, N- and C-terminal protected, linear homooligopeptides.[14,15] Recently, Hu et al reported the conformational preference of selected protein films using ECD spectroscopy.[16] A few studies were also reported on the conformation of smaller peptide derivatives in the thin film state.[17,18]

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In the present study, we report the VCD spectra of various types of proteins in the film state for the first time. Because there is no solvent interference in the film VCD measurements, the amount of protein sample required for film VCD studies is approximately 2 orders of magnitude smaller than the one used for aqueous solution studies.

Experimental Section

Albumin (bovine pancreas, A-4378), hemoglobin (human, H-7379), pepsin (P-6875), α-chymotrypsin (bovine pancreas, C-4129), ovalbumin (chicken egg, A5503-1G), trypsin (bovine pancreas, T-8253), cytochrome C (horse heart, C-7752), and lysozyme (chicken egg white, L-7651) were purchased from Sigma and used without further purification. For film VCD studies, stock solutions of all of the proteins were prepared to the same concentration (5 mg/mL) in 25 mM tris buffer (pH 7.4) containing sodium chloride (25 mM).

All VCD spectra were recorded on a commercial Chiralir spectrometer (Bomem-Biotools, Canada) modified to minimize the artifacts using the double polarization modulation method.[19] These modifications are as follows: The light from 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 to the 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.

It should be noted that because the ZnSe optical elements were not antireflection coated, the throughput in our instrument is reduced to ~50%. Because the throughput can be increased to ~80%, with proper antireflection coating, better signal-to-noise, than the one reported here, can be achieved in practice.

All spectra were collected for 3 h at a resolution of 8 cm^{-1}. For the film VCD measurements, about 150 μL of the protein stock solution was cast onto a 2.5 cm diameter CaF_2 window that was placed in a fume hood to provide constant air flow over the sample. The evaporation was continued overnight (16−18 h) at room temperature until a dry thin film formed on the surface of the CaF_2 window. Films were tested for satisfactory VCD characteristics by a comparison of the VCD obtained with the film rotated by 45° around the light beam axis. For all of the VCD data reported here, the VCD band signs were reproduced upon 45° rotation of the film. In separate measurements with 1 h data collection time, we have also tested 90° rotation of the film. VCD bands were found to be unaffected by this rotation as well (data not shown). Baseline corrections were done by subtracting the VCD spectrum of a blank CaF_2 window as obtained under the same conditions as the sample spectrum. Aqueous solution VCD measurements for some of the proteins studied here were reported[2a] by Keiderling and co-workers. Yet due to high absorbance of H_2O in the amide I region, the amounts of protein samples employed earlier[2a] were approximately 2 orders of magnitude larger.

All protein film samples were derived from the stock solution (5 mg/mL). These films had an absorbance between 0.25 and 0.7 in the amide I region. Lower absorbance (i.e., less than 0.2) results in a decreased signal-to-noise ratio, while higher absorbance requires larger film thickness, which can cause artifacts.

We have also carried out measurements on protein films prepared on quartz plate using ECD spectroscopy under identical sample concentrations. These data are not shown here because, in some cases, the film ECD spectra were found to be orientation dependent and contained artifacts, unlike VCD.

For solution-state VCD spectra of pepsin and ovalbumin, ~5 mg of protein sample was dissolved in 100 μL of D_2O (Aldrich) solution. An aliquot of the solution was placed between CaF_2 windows, which were separated by a 25-μm Teflon spacer and held in a standard cell mount. The VCD spectrum of D_2O solvent was subtracted from the VCD spectra of proteins. The spectra were collected for 3 h at a resolution of 8 cm^{-1}.

Results and Discussion

The types of proteins used in the present study were chosen on the basis of their dominant secondary structure in the solution state. The protein samples were selected to represent three different types of protein structures as first classified by Levitt and Chothia.[20] For analyzing the VCD data, we make use of the correlations[2] established by Keiderling and co-workers between the VCD spectral features in protein aqueous solutions and known protein structures of a uniform conformational type in the solution state. The VCD pattern that is characteristic of right-handed α-helices is a positive couplet (broad, weak + ΔA then intense − ΔA, with increase in frequency) at ~1655 cm^{-1}. The characteristic β-sheet VCD spectrum contains a weak negative band between 1615 and 1635 cm^{-1}. The presence of an additional weak negative band at 1690 cm^{-1} is indicative of the presence of antiparallel β-sheet conformation. If both 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, that is, a strong low wavenumber negative band, a weak intermediate positive band, and a strong negative band at higher wavenumber giving a (−, +, −) pattern (referred to as a "W" pattern). However, it is not yet possible to distinguish between (α + β) type and α/β type[21] structures using VCD.

We describe below our film VCD results based on the predominant secondary structures that occur in different types of proteins. The results for individual cases are discussed below under the following headings: "all-α", "all-β", and "α + β" proteins.

"All-α" Proteins. Bovine serum albumin (BSA) and hemoglobin belong to a class of proteins which have predominantly α-helical conformation, denoted here as "all α". Vibrational absorption and VCD spectra in the amide I and II regions of BSA film are shown in Figure 1. The VCD spectrum of BSA film contains weak positive (1643 cm^{-1}) and strong negative (1662 cm^{-1}) bands. These are the characteristic features of proteins adopting α-helical conformation.[2a] Moreover, the corresponding IR absorption spectrum shows an intense band at 1656 cm^{-1} that is characteristic of α-helical conformation.[22] The VCD spectrum of BSA in solution[2c] also indicated α-helical structure. To study the effect of orientation of protein film, the VCD measurement was repeated by rotating the film 45° (Figure 1, dotted line) with respect to the light path axis. The results indicate no change in the pattern of the VCD band signs, suggesting that the VCD band signs of these proteins in thin film states are orientation independent. Because the spectra were unaltered, upon 45° rotation, in sign, magnitude, and position of VCD bands, the average of the spectra obtained in normal

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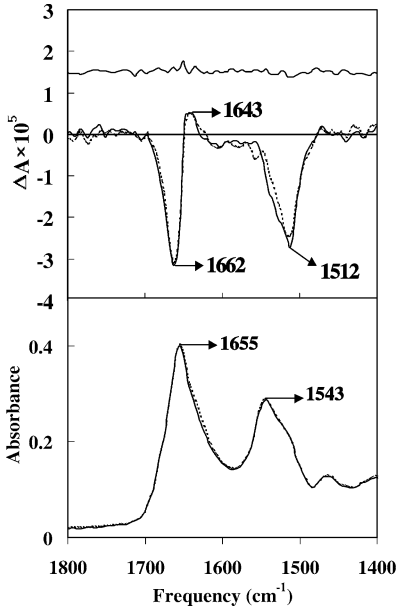

Figure 1. VCD (top) and absorption (bottom) spectrum of bovine serum albumin film. The topmost trace is the noise level in VCD spectrum obtained with 3 h data collection time. The solid and dashed lines indicate the spectra of films at normal and 45° rotation, respectively, with respect to the light beam axis.

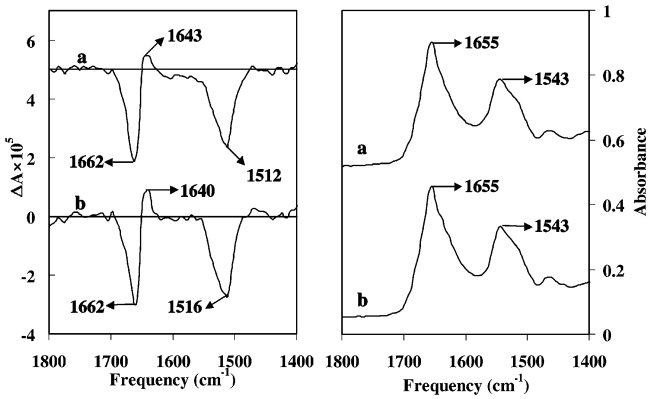

Figure 2. VCD (left panel) and absorption (right panel) spectra for films of "all-α" proteins: bovine serum albumin (a) and hemoglobin (b). The spectra presented here are the average spectra obtained from normal orientation and 45° rotation of the film.

and 45° rotation is presented in Figure 2 and thereafter. The average VCD spectra for BSA (curve a) and hemoglobin (curve b) are shown in Figure 2. Vibrational absorption spectra are also presented as average spectra recorded at normal and 45° rotation. Vibrational absorption and VCD spectra in the amide I and II regions of both BSA (Figure 2, curve a) and hemoglobin (Figure 2, curve b) films show similar spectral features. The VCD spectrum of hemoglobin in aqueous solution was reported in the literature.[2] The VCD spectra of hemoglobin obtained for both film and solution clearly indicate α-helical structure.

"All-*β*" Proteins. Pepsin and α-chymotrypsin belong to a class of proteins which have predominantly *β*-sheet conformation denoted here as "all *β*". Figure 3 shows the VCD and IR absorption spectra of pepsin and α-chymotrypsin films. The VCD spectrum observed for pepsin (Figure 3, curve a) has an intense negative amide I band at 1632 cm^{-1} which is characteristic[2a] of proteins forming *β*-sheet conformation. The corresponding IR absorbance spectrum shows broad amide I and II bands at 1643 and 1528 cm^{-1}, respectively. The

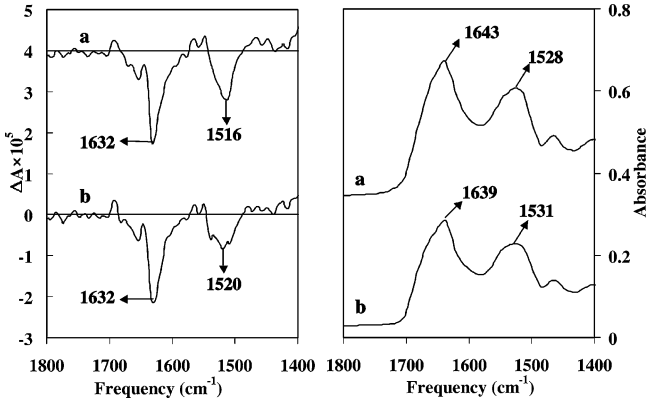

Figure 3. VCD (left panel) and absorption (right panel) spectra for films of "all-*β*" proteins: pepsin (a) and chymotrypsin (b). The spectra presented here are the average spectra obtained from normal orientation and 45° rotation of the film.

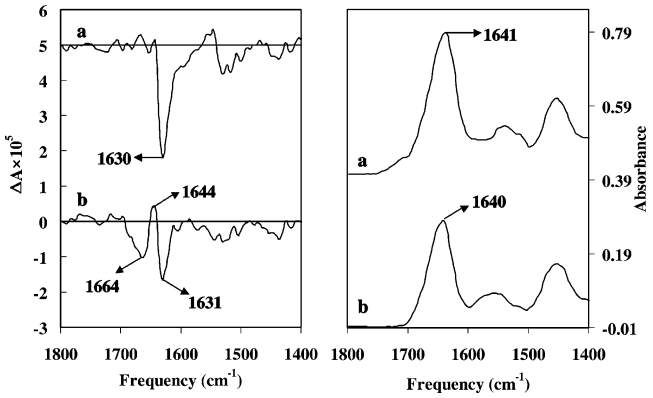

Figure 4. The solution-state VCD (left panel) and absorption (right panel) spectra of pepsin (curve a) and ovalbumin (curve b). The spectra were obtained in D_2O. The concentration of protein sample was ~50 mg/mL, and the path length is 25 μm.

corresponding VCD spectrum (Figure 4, curve a) of pepsin in D_2O solution was also recorded because it was not reported previously. A single negative VCD band at 1630 cm^{-1} suggests that pepsin adopts *β*-sheet structure in solution. This is in good agreement with our VCD film result. It should be noted that in D_2O, the amide II band becomes amide II′ and appears at a lower frequency. For this reason, the amide II VCD band seen at 1516 cm^{-1} (Figure 3a) has no corresponding band in D_2O solution (Figure 4a). In the case of α-chymotrypsin, the film VCD spectrum (Figure 3, curve b) shows a negative amide I band at 1632 cm^{-1} that is indicative of *β*-sheet structure.

We have also measured the IR and VCD spectra of pepsin film obtained from D_2O solution (data not shown). However, because the films were prepared by evaporating the solution on CaF_2 plates in open atmosphere, the deuterium atoms exchange with hydrogens from water in the atmosphere. As a result, the films prepared from D_2O solution showed spectra identical to those obtained from H_2O solutions. Thus, to obtain D_2O film measurements, it is necessary to prepare the films in a N_2 controlled environment.

"α + *β*" Proteins. Ovalbumin and trypsin belong to a class of proteins which have both α-helix and *β*-sheet structural components, denoted here as "α + *β*". Vibrational absorption and VCD spectra of these two proteins in the amide I and II regions are shown in Figure 5. The characteristic VCD feature[2a] of proteins containing both α-helical and *β*-sheet components is a "W" pattern in the amide I region. The VCD spectrum of

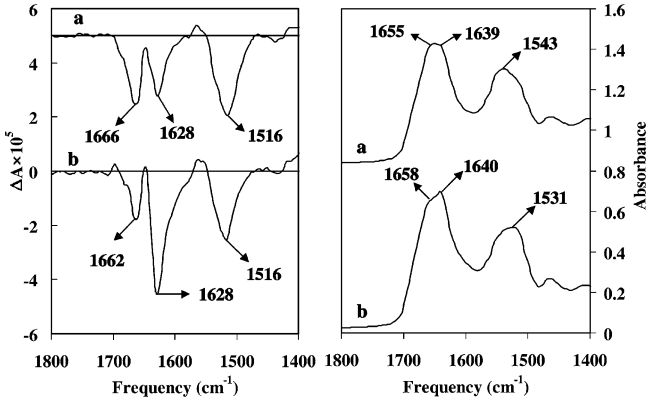

Figure 5. VCD (left panel) and absorption (right panel) spectra for films of "$\alpha + \beta$" proteins: ovalbumin (a) and trypsin (b). The spectra presented here are the average spectra obtained from normal orientation and 45° rotation of the film.

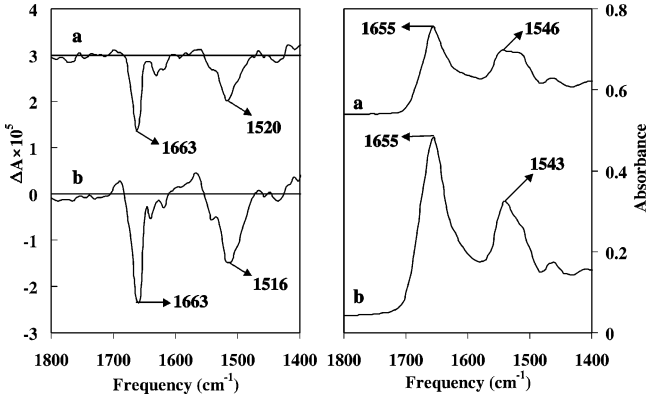

Figure 6. VCD (left panel) and absorption (right panel) spectra for films of "$\alpha + \beta$" proteins: cytochrome C (a) and lysozyme (b). The spectra presented here are the average spectra obtained from normal orientation and 45° rotation of the film.

ovalbumin shows such a pattern (Figure 5, curve a). Similar VCD features were observed for ribonuclease S, which is also an "$\alpha + \beta$" protein, in aqueous solution.[2a] The dominant high- and low-frequency negative VCD bands in ovalbumin are interpreted as ovalbumin having high α-helix and high β-sheet components. It should be noted that this type of mixture of conformations is not evidenced clearly in the normal vibrational absorption spectra. In the present case, the infrared absorption spectrum of ovalbumin film shows broad amide I (1639−1655 cm^{-1}) and amide II (1543 cm^{-1}) bands. A similar amide I absorbance spectrum was observed for ovalbumin in aqueous solution.[23] The solution-state VCD spectra (Figure 4, curve b) of ovalbumin in D_2O were also measured because it was not reported previously. The solution VCD spectrum also suggests that ovalbumin adopts a mixture of α and β structures, characterized by the presence of an intense positive couplet on the higher-frequency side of the absorption band combined with a low-frequency negative band. This observation is in agreement with VCD spectra of ovalbumin film. We have also measured the IR and VCD spectra of ovalbumin film obtained from D_2O solution (data not shown). Here also, as in the pepsin case, the obtained spectra are similar to those of ovalbumin film that was prepared from H_2O solution, due to D−H exchange between D_2O and the H_2O in room atmosphere.

In the case of trypsin, the IR spectrum (Figure 5, curve b) shows an intense amide I band at 1640 cm^{-1} along with a weak shoulder band at 1658 cm^{-1}, which are indicative of predominantly β-sheet conformation with admixture of α-helix. It was also further confirmed by the position of the low-frequency broad amide II band at 1531 cm^{-1}, which is characteristic of predominantly β-sheet conformation. The corresponding VCD spectrum (Figure 5, curve b) of trypsin has contributions from both α-helix and β-sheet conformations [characterized by a three peak pattern (− + −)]. However, the presence of an intense lower-frequency negative VCD band at 1628 cm^{-1} and a weak higher-frequency negative VCD band at 1662 cm^{-1} suggest that trypsin has more β-sheet than α-helix component.

In the case of cytochrome C, the film VCD spectrum (Figure 6, curve a) contains a higher-frequency negative band at 1663 cm^{-1}. A typical α-helical structure should have an accompanying weak positive VCD on the lower-frequency side, but this feature would not be seen if a negative VCD band from a minor β-sheet component occurs at the same position. Although not certain, this is probably the case here. The normal IR spectrum (Figure 6, curve a) shows characteristic intense amide I and II bands for α-helix conformation at 1655 and 1546 cm^{-1}, respectively. The VCD spectrum (Figure 6, curve b) of lysozyme film is similar to that of cytochrome C with a single intense negative band at 1663 cm^{-1}. Here again, it is possible that the weak positive VCD associated with α-helix is not seen due to its overlap with negative VCD from a minor β-sheet component. The predominant α-helical conformation is supported by the existence of characteristic α-helical amide I and II bands at 1655 and 1543 cm^{-1}, respectively, in the normal IR absorption spectrum (Figure 6, curve b).

Conclusion

The present results demonstrate that VCD can provide new insights into the conformations of protein films. It is also clear from the results presented here that the VCD band signs for protein films studied here are orientation independent. We have also done measurements (data not shown) on protein films using ECD spectroscopy under identical conditions. Yet, in some cases, the film ECD spectra were found to be orientation dependent and contained artifacts, unlike VCD. For VCD studies, film-state measurements are advantageous over solution-state measurements because the amount of protein sample required for VCD film studies is approximately 2 orders of magnitude smaller than that for corresponding VCD studies in aqueous solution. Furthermore, because interfering water absorption is absent in the VCD film measurements, better signal-to-noise ratios can be obtained for film VCD measurements. The conformational preferences of several proteins studied here are the same in both film and solution state, but this property need not be universal. Further work in this direction to study the conformational preferences of protein films formed under different conditions (temperature and pH) and to study the protein folding−unfolding process in films is in progress.

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