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Vibrational Circular Dichroism of Proteins in H₂O Solution

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Abstract: Most vibrational circular dichroism (VCD) spectra of proteins have been measured in D_2O solution to reduce solvent interference. Here we report the first measurements of VCD spectra in the amide I region for proteins dissolved in H_2O. Such measurements avoid the ambiguities implicit in D_2O solution experiments that arise from incomplete deuteration. Characteristic VCD patterns have been observed for proteins whose secondary structure is dominated by α-helix, β-sheet, $\alpha + \beta$ combinations or coil contributions. In general, the changes in band shape and frequency from the VCD of deuterated proteins reflect expectations based on studies of pure conformations in polypeptide systems. The results for proteins dominated by α or β contributions match previous amide I' (D_2O) studies well, allowing for some intensity variation. The proteins with mixed structures sometimes have amide I VCD different in shape from that of the amide I'. In the cases studied, these changes tend to bring the spectra into better qualitative agreement with expectations based on the protein structure, suggesting that deuteration can affect the amide I' VCD of some proteins in a complex manner. Using both the H_2O and D_2O VCD data together offers promise of obtaining increased insight into protein structure through spectral measurements.

Introduction

Spectroscopic studies have historically been important in protein characterization studies for estimation of average secondary structure. Recently we have extended the set of useful protein techniques to include vibrational circular dichroism (VCD),[1-4] a hybrid of the more established electronic CD and infrared absorption spectroscopies. VCD can sense several different spectrally resolved transitions involving different localized vibrations of the molecule, the most valuable of which for proteins has proven to be the amide I band (primarily due to the C=O stretch on the amide group). Due to interference by water absorption bands, all aqueous-phase VCD studies of the amide I have been done in D_2O solution (there termed the amide I'). This has necessitated subjecting the protein to isotopic exchange either by dissolution and lyophilization or by repeated concentration.[5] The extent of deuteration is a poorly controlled variable in such experiments which leads to an ambiguity in interpretation of the data. Furthermore, lyophilization can be a denaturing process for some proteins. Until now, the effect of deuteration on the aqueous phase VCD spectrum could only be extrapolated by comparison to data for nonaqueous phase polypeptides, which may have other characteristics not replicated in the aqueous phase.

Empirical correlation of the band shape of the spectral features obtained with secondary structure has been shown to be the best method for interpretation of protein VCD. Pancoska et al.[6] have shown that the VCD of the amide I' band of proteins in D_2O solution is quantitatively interpretable in terms of secondary structure through a statistical analysis of the band shape and correlation of the resulting coefficients to X-ray determined structural parameters.[7] Effects of partial deuteration on the band shape can add to the error found in determining the regression relationships used to predict secondary structure from these D_2O-based spectra. We have previously reported that the amide II band (N-H bending and C-N stretch) can be studied for proteins in H_2O solution[8] and have subsequently determined that its shape can also be correlated to secondary structure.[9] The quantitative aspects of such a model are much poorer than those for the amide I', but combining the data from the two spectral regions does lead to an improved fit of spectral to structural parameters.[9]

In this paper we report the first measurement of VCD for the amide I bands of several proteins in H_2O solution. This technical advance is due to a combination of improvements in instrumentation and IR cell design. While these first data to be reported are somewhat noisier than "conventional" amide I' data, they point the way to a new structurally sensitive spectral approach that can utilize multiple bands from a single molecular sample studied in a single experiment and can avoid interpretive ambiguities due to deuteration.

Experimental Section

Poly-L-lysine (P-2636, MW 30000-70000), albumin (bovine, A-0281), α-chymotrypsin (bovine pancreas, C-4129), concanavalin A (jack bean, C-2010), cytochrome *c* (horse heart, C-7752), hemoglobin (human, H-7379), β-lactoglobulin A (bovine milk, L-7880), myoglobin (horse skeletal muscle, M-0630), ribonuclease S (bovine pancreas, R-6000), and triose phosphate isomerase (rabbit muscle, T-6258) were purchased from Sigma and used without further purification. Solutions of all these proteins were prepared to the same concentration (~20 mg/100 μL) in doubly distilled H_2O.

The amide I and amide II VCD and absorbance spectra in H_2O were measured on a dispersive VCD instrument which has been described in detail elsewhere.[1,3] The key to these experiments was the use of a refillable IR cell (Specac Analytical 1400) consisting of two CaF₂ windows separated by a 6 μm mylar spacer. These spacers proved to be superior in terms of leakage for very long scan times to the more commonly used tin spacers.[10] As has been reported for FTIR-based studies of proteins in H_2O, use of such a short path length dramatically improves S/N.[11,12] The same is

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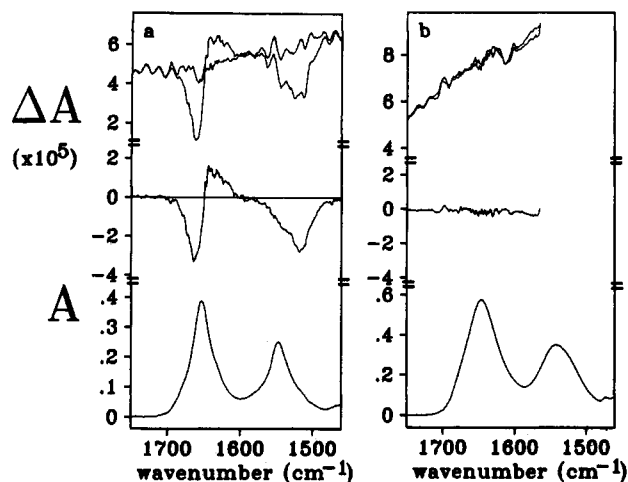(10) Dukor, R. K., personal communication.

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Figure 1. (a) Baseline correction of VCD spectra in amide I and II regions: top, raw baseline (H_2O) and sample (myoglobin in H_2O) VCD spectra (both are an average of 10 consecutive scans); middle, the VCD spectrum of the sample after baseline correction; bottom, the corresponding FTIR absorption spectrum. (b) The same correction as in spectra (a) made for a poly-DL-lysine sample in the amide I region. It is clearly seen that the difference between a VCD spectrum of the sample and the H_2O baseline does not exceed a random noise level.

true for VCD, since in longer path cells (previously we used 15 μm) it was virtually impossible to scan the VCD through the main H_2O absorption band at 1645 cm⁻¹. With a 6 μm path length, the absorbance of H_2O at 1645 cm⁻¹ is less than 1.0. Although the cell has a demountable design, it was assembled before being filled and used as a sealed sample cell but it was cleaned by disassembling. Liquid samples were drawn into the gap through one filling port by syringe suction applied via the opposite port. Initially the cell was filled with doubly distilled water which was used for baseline determination. The water was then pumped out of the cell which was then dried by evacuation. The protein solution sample was then inserted in the cell by following the same procedure.

In most cases, the protein VCD shown here is the result of averaging 10 baseline and 10 sample scans obtained at ~10 cm⁻¹ resolution with a 10 s time constant. The VCD spectra presented here are unsmoothed experimental data but were normalized to a peak absorbance of 1.0 for the amide I band. The VCD intensities were calibrated by using our standard method.[3,13]

Baseline corrections were done by subtracting the VCD spectrum of just the H_2O solvent as obtained under the same conditions as the sample spectrum. Due to the high absorbance of H_2O in the amide I region (in comparison to the absorbance due to the protein itself) this correction is reasonable.[3] There might be concern that measurement of VCD in the presence of such a large absorbance could lead to spectral artifacts. As an example of our relatively high quality baseline, the raw VCD spectrum of myoglobin is illustrated in Figure 1a superimposed on a baseline obtained by measuring the VCD of the solvent (H_2O) itself under the same conditions. The bulk of the VCD fluctuations inside the absorbance band frequency interval are noise, and those outside are reproducible, subtractable features of the baseline as shown. As illustrated in Figure 1b, our tests indicate that the difference between the H_2O baseline and a similarly collected VCD spectrum of a poly-DL-lysine solution which had an absorbance in the amide I region equivalent to that used for our protein samples is not greater than the random noise level.

Absorption spectra of all the polypeptides and proteins studied were also recorded on a Digilab FTS-60 FTIR spectrometer using a cooled MCT detector, 2 cm⁻¹ resolution, and an average of 1024 scans. In order to correct for the water solvent absorption spectrum the automated subtraction method of Dousseau et al.[14] was used. These spectra were

also corrected for residual atmospheric water vapor interference by subtraction of a separately collected water vapor absorbance spectrum as previously described.[8] In most cases the FTIR and dispersive absorbance band shapes are well matched, but the dispersive absorbance spectra usually showed some degree of distortion on the high frequency side of the maximum as a result of incomplete correction for the water absorbance. Therefore FTIR absorbance data were used for normalization of the VCD spectra.

Results

Figure 2 compares FTIR absorption and VCD spectra in the amide I and II regions for hemoglobin, α-chymotrypsin, and poly-L-lysine at pH ~ 7 which represent three different dominant secondary structure types, α-helical, β-sheet, and random coil, respectively. The amide II VCD for all these samples adequately reproduces that obtained in previous studies of the amide II of such molecules in H_2O solution.[8]

As in the case of N-protonated α-helical polypeptides in nonaqueous solution,[19,20] those proteins with a high α-helix content such as hemoglobin in H_2O (Figure 2a) give rise to an intense positive couplet amide I VCD with its zero crossing point shifted down in frequency by ~5 cm⁻¹ relative to the absorption maximum. In the amide II region, the VCD spectrum is dominated by an intense negative band on the lower frequency side of the absorbance maximum in agreement with previous protein and model polypeptide studies.[8,20] As shown by the overplotted amide I′ VCD (thin solid line), the magnitude of the α-helical amide I VCD signal (in terms of $\Delta A/A$) is somewhat more intense than that of amide I′. Such a relative magnitude change was also seen in comparative amide I–amide I′ VCD for polypeptides in nonaqueous solutions.[20] Some of this intensity difference may be due to normalization. There is also a significant frequency shift and difference in the band shape on the low-frequency side since no negative band is observed in H_2O. These changes from amide I′ to amide I VCD are common to all the proteins with high α-helical content we have studied.

α-Chymotrypsin (Figure 2b) has a high content of β-sheet which gives rise to a dominant low-energy negative VCD band in the amide I region and a small positive VCD band on the high-frequency side of the absorbance maximum. A medium intensity negative VCD couplet is observed in the amide II region as is characteristic of β-sheets.[8] The change from the amide I′ result was surprisingly small and is, in fact, less than that for other β-sheet proteins (see below).

The amide I VCD of poly-L-lysine (Figure 2c) has the least band shape variation from previous results, being virtually the same as that seen for deuterated random coil polypeptide molecules in D_2O[15,16] or for both isotopomers in nonaqueous solutions.[17] Some intensity difference was evident with the deuterated peptide having more intense VCD.

To ascertain the generality of these observations in Figure 2, we have begun a survey of amide I (H_2O) VCD spectra of proteins. The following example results illustrate our findings and focus on just the amide I spectra since the observation and characterization of amide II VCD have been previously reported.[8] Figure 3 shows a comparison of the conventional normalized amide I absorption spectra and VCD spectra for six more proteins of varying structural type:[18] (a) albumin (all α), (b) concanavalin A (all β), (c) cytochrome c ($\alpha + \beta$), (d) triose phosphate isomerase (α/β), (e) ribonuclease S ($\alpha + \beta$), and (f) β-lactoglobulin A (low α, high β).

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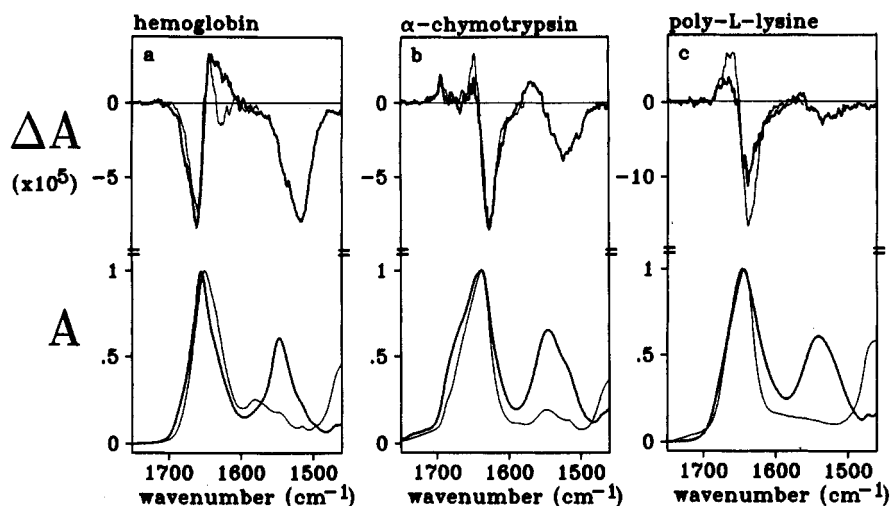(20) Sen, A. C.; Keiderling, T. A. *Biopolymers* **1984**, *23*, 1519–1532.

Figure 2. FTIR absorption and dispersive IR VCD spectra of (a) hemoglobin (high α), (b) α-chymotrypsin (high β), and (c) poly-L-lysine at pH 7 (random coil form) in the amide I and II regions. Both absorbance and VCD spectra are normalized to $A_{max} = 1$ for the amide I. Actual peak absorbance of the sample in the maximum of the amide I band was $\sim$0.2-0.4. For comparison, the amide I′ VCD spectra and corresponding FTIR absorption (in D₂O) are overplotted (thin line).
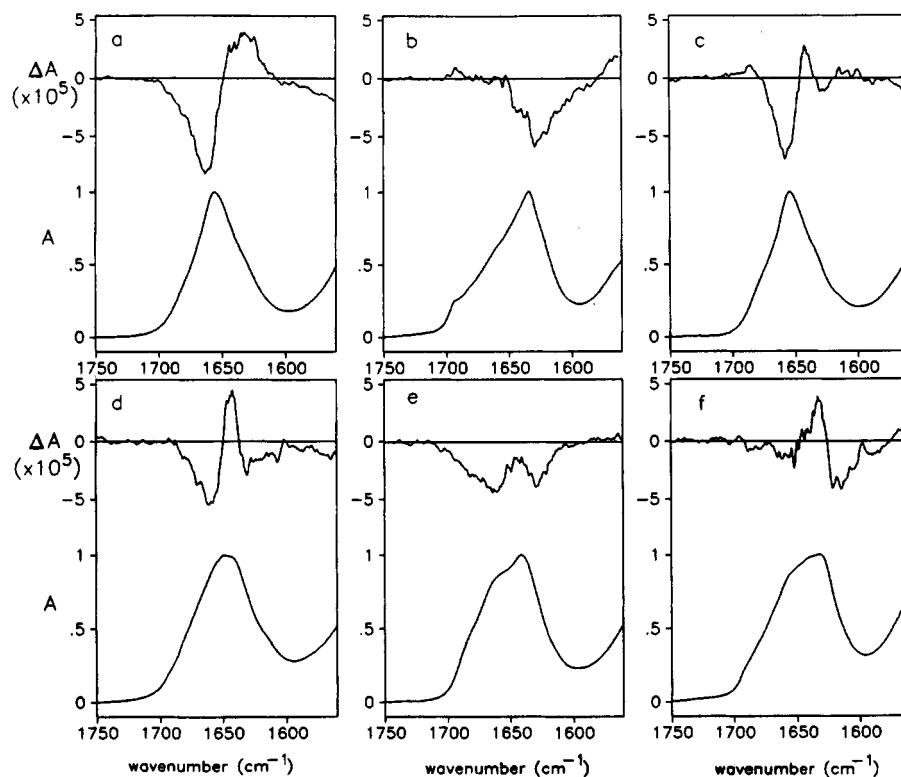

Figure 3. FTIR absorption and dispersive IR VCD spectra of some example proteins in the amide I region: (a) albumin (high α), (b) concanavalin A (high β), (c) cytochrome c (high α + low β), (d) triose phosphate isomerase (α/β), (e) ribonuclease S ($\alpha + \beta$), and (f) β-lactoglobulin A (low α, high β-sheet). Both absorbance and VCD spectra are normalized to $A_{max} = 1$.

Both albumin (Figure 3a) and concanavalin A (Figure 3b) give rise to VCD band shapes that are characteristic of high α-helix and high β-sheet content, respectively, and are in good qualitative agreement with the examples in Figures 1a and 2a (α) and 2b (β). The concanavalin A result (Figure 3b) does deviate more from the deuterated amide I′ result than did the α-chymotrypsin VCD (Figure 2b). As seen in previous studies using amide I′[5,6] and amide II[8] spectra, the VCD band shape of a protein with a dominant form of secondary structure is indicative of that structural type.

Amide I VCD spectra of proteins with contributions from both α-helix and β-sheet conformations are characterized by a three-peak pattern (– + –) which reflects a simple combination of the high α and high β amide I band shapes. Both cytochrome c

(Figure 3c) and triose phosphate isomerase (Figure 3d) with high α and low β content have VCD band shapes dominated by an intense positive couplet on the high-frequency side of the absorption band combined with a weaker low-frequency negative band. Ribonuclease S (Figure 3e), a protein with mixed α and β components, gives rise to a VCD pattern with a "W" shape but without a positive component between the two negative features. β-Lactoglobulin A (Figure 3f), which has a substantial amount of β-sheet and a small fraction of α-helix,[21] has an amide I VCD band shape dominated by a low-frequency negative couplet and a weak negative band on the high-frequency side of the absorbance

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band. As a whole, Figure 3 is a good illustration of the consistency of the amide I VCD spectra of globular proteins with various structural patterns.

Discussion

These spectra of the amide I VCD in aqueous solution for polypeptides and proteins represent the first report of protein amide I VCD in H_2O solution. It was necessary to use very high concentrations (but the same as we had used for amide II studies[8]) to get an adequate signal-to-noise ratio in the VCD spectra. Doing experiments in H_2O avoids the exchange problems that arise with D_2O-based studies,[5] but the results in many cases are not drastically different from our previously reported amide I' results.[6] For proteins dominated by single structural type, the protein amide I band shapes that we have observed in H_2O fit our expectations based on previous comparisons of deuterated and nondeuterated amide I' and amide I VCD for polypeptides in nonaqueous solutions.[17,20]

Basically the same (− + −) sign pattern seen here was observed for the amide I' VCD of mixed α and β proteins in D_2O,[6] but there are remarkable differences in relative intensities between amide I and amide I' VCD. Most apparent here is the significant loss of intensity for the lowest energy negative band for both cytochrome c and triose phosphate isomerase in H_2O (relatively high α). On the other hand, the β-lactoglobulin A (relatively high β) VCD is dominated by the low-energy negative band in both solvents, but in H_2O the positive band is more pronounced. These changes can be predicted from the additivity of spectral components corresponding to relatively local structural aspects of the molecule and from changes seen in the VCD of the dominantly α-helical proteins. Since the α-helical amide I VCD no longer has a low-frequency negative band, the mixed-structure proteins will have less negative VCD intensity in that region also due to cancellation between the α-helical and β-sheet contributions.

On the other hand, there is a clear disagreement between the amide I and amide I' VCD[6] band shape of ribonuclease S that does not fit such a simple picture. In this case, the H_2O data closely reflect the spectral properties expected to arise from the protein structure ($\alpha + \beta$). In our opinion, the amide I' band shape (dominated by a low-frequency negative band) does not adequately reflect the relatively high α-helix content of ribonuclease S.[22] The "W" band shape was, in fact, seen for amide I' VCD of other $\alpha + \beta$ proteins such as ribonuclease A or lactoferrin.[6] This may represent a partial denaturization or deuteration effect in the ribonuclease S amide I' VCD determination that calls for further study.

Earlier studies of amide I' VCD of model polypeptides and proteins have demonstrated that these spectra exhibit a high conformational sensitivity to the details of protein secondary structures even on a qualitative level.[5,6] Coupling together the structural dependence of the amide I' VCD with that of the amide II band into a systematic analysis provides a mechanism for quantitative determination of secondary structure whose error limits are low compared to other spectroscopic determinations of structure.[9] It is expected that measurement of these two VCD bands on the same molecule under the same conditions and without the ambiguity of partial deuteration, as made possible by the technique development described here, will help to improve the error limitations of current statistical treatments of VCD spectra in terms of structure. Two reports of statistical analyses of FTIR spectra of proteins in H_2O solution have concluded that simultaneous analysis of amide I and II is superior to analyses of amide I' absorption spectra (D_2O solution) for correlations with protein secondary structure.[11,12] Neural network analyses[23,24] of these spectra (already underway for electronic CD and amide I' + amide II data[25]) will further benefit from consistently determined spectral data.

In summary, this work demonstrates the measurability of the amide I VCD in H_2O solution for polypeptides and proteins and further demonstrates its qualitative correlation with the secondary structural characteristics of these molecules as previously demonstrated for the amide I' in D_2O solution. A previous criticism of the use of VCD for protein study was its reliance on the use of D_2O-based solutions. It is now clear that the effects of deuteration on the amide I VCD are small and systematic, for the most part. In some cases, having the amide I data available has prompted us to reinvestigate and refine our amide I' data set. In general the result was improved consistency with structure. Never-the-less, adding the amide I data to our previously collected amide I' and amide II VCD data sets seems to provide a promising way of further improving the accuracy of protein structure predictions based on empirical spectra–structure correlations.

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