Comparison of α-Lactalbumin and Lysozyme Using Vibrational Circular Dichroism. Evidence for a Difference in Crystal and Solution Structures†

Marie Urbanova,‡ Rina K. Dukor, Petr Pancoska,‡ Vijai P. Gupta, and Timothy A. Keiderling*

Department of Chemistry, University of Illinois at Chicago, Box 4348, Chicago, Illinois 60680

Received May 20, 1991; Revised Manuscript Received August 7, 1991

ABSTRACT: The conformation of the milk protein α-lactalbumin has been studied using vibrational circular dichroism (VCD) and compared to parallel studies on lysozyme. These proteins have been shown by Acharya et al. [(1989) *J. Mol. Biol. 208*, 99–127] to have very similar three-dimensional crystal structures. However, their VCD spectra in D_2O solution are quite different. The VCD of lysozyme in D_2O more resembles that of α-lactalbumin in 33% propanol/D_2O, under which conditions α-lactalbumin has conformationally transformed to a structure with increased helical fraction. These results can be seen to be consistent with UVCD and resolution-enhanced FTIR spectra of α-lactalbumin and lysozyme in both D_2O and H_2O environments. The solvent sensitivity of the α-lactalbumin spectra and hence of its conformation contrasted with the lack of such sensitivity for lysozyme suggest that the α-lactalbumin crystal structure represents a conformation different from that which is dominant in aqueous solution.

The conformational properties of α-lactalbumin (α-Lac)[1] have been the focus of much recent research (Kronman, 1989). An elegant X-ray crystal structure study has been reported by Acharya et al. (1989) for α-lactalbumin crystallized from baboon milk using data collected at a resolution of 1.7 Å. Acharya et al. (1990) have recently compared this structure in detail to a structure predicted from molecular modeling of bovine α-lactalbumin to show that the proteins from these two sources have essentially the same three-dimensional structure (except for the C-terminal tail). That work further demonstrated the generality and the usefulness of using homologues to postulate three-dimensional structures. Collection and analysis of X-ray data are dependent on the growth of suitable crystals, which is a far from trivial technique. Other methods of getting structural information can be based on spectroscopic methods, which are often less precise in terms of structural dependencies but have the advantage of measuring the properties of the proteins in their native solution state. Of these, NMR, particularly through the multidimensional techniques now available, yields the most detailed structural information, due to its high-resolution possibilities. However, these NMR techniques are currently limited to relatively small proteins and require substantial amounts of purified material.

Some techniques, such as fluorescence and resonance Raman spectroscopies, can give detailed data about specific side chains or subunits in a protein that can be interpreted with respect to the local conformation. Other more traditional studies include ultraviolet circular dichroism (UVCD), infrared (IR), and Raman spectroscopies. Due to their relatively lower resolution, these methods give only averaged information about the entire protein structure, and generally are most sensitive to and are interpreted with respect to the fractional secondary structure composition.

We have shown that the combination of the conformational sensitivity of a measurement dependent on optical activity, such as circular dichroism, with the modestly enhanced resolution of molecular transitions, as seen in the vibrational region of the spectrum, leads to an enhanced sensitivity to protein conformation in solution (Pancoska et al., 1989). The difference in absorption of left and right circularly polarized infrared radiation for vibrational transitions is called vibrational circular dichroism (VCD). Our recent work has demonstrated that VCD-enhanced sensitivity to protein secondary structure can be used to quantitate fractional secondary structure in much the same way as has historically been done with its spectroscopic forerunners (Pancoska et al., 1991). Additionally, coupling such VCD spectral data to UVCD and IR data in a complementary sense can lead to a more reliable determination of the structural characteristics of these proteins, even if only on an averaged basis.

In this paper, we report a preliminary investigation of both bovine α-Lac and HEWL with identical spectroscopic approaches and compare the results to the conclusions derived from the X-ray analyses. Quantitative evaluation of both the VCD and UVCD was also carried out and will be reported in a separate paper addressing the solvent sensitivity of a number of milk proteins (Urbanova et al., unpublished results). In our first report on protein structural stability, we wish to call attention to real differences that are easily detectable on a qualitative level between these proteins as seen in their VCD, UVCD, and FTIR spectra.

Background. α-Lactalbumin (α-Lac) is a Ca^{2+} binding protein found in the whey part of mammalian milk (Broadbeck et al., 1967) and has recently been proposed to be effective for tumor suppression (Bano et al., 1985). The amount of α-helix in bovine α-Lac in phosphate buffer solution at pH 7.6 has been estimated to be ~24% (Clark & Smith, 1989) from UVCD measurements using the analytical method of Provencher and Glöckner (1981). This determination is

† This work was supported by a grant from the National Institutes of Health (GM 30147). Instrumentation purchases were supported in part by grants from the NSF, NIH, and the University of Illinois at Chicago. V.P.G. received a Fulbright travel grant from the Council for International Exchange of Scholars.

* To whom correspondence should be addressed.

‡ Permanent address: Department of Chemical Physics, Charles University, Prague 2, Czechoslovakia.

[1] Abbreviations: FSD, Fourier self-deconvolution; FTIR, Fourier transform infrared; HEWL, hen egg white lysozyme; IR, infrared; α-Lac, α-lactalbumin; UVCD, ultraviolet circular dichroism; VCD, vibrational circular dichroism.

0006-2960/91/0430-10479$02.50/0

consistent with earlier analyses by Robbins and Holmes (1970) using the Greenfield and Fasman (1969) method. Furthermore, it is known that at low pH α-Lac undergoes a conformational change to the A state (Kronman, 1989) that, in terms of UVCD, appears to involve an increase in the helical fraction (Robbins & Holmes, 1970). In a high propanol concentration (66%), the helical fraction also rises (Clark & Smith, 1989). Some of these spectral changes have been attributed to effects of aromatic side chains on the UVCD (Kronman, 1989), but it is clear that the main chain conformation must change somewhat to encompass the range of these variations.

These UVCD helical predictions can be compared to 31% of the amino acids being involved in α-helices as estimated from the X-ray crystal structure of baboon α-Lac (Acharya et al., 1989). Some of these α-helical segments are short which may affect the UVCD analysis. Since UVCD determination of α-helix content is dependent on the length of the helices (Yang et al., 1986; Chang et al., 1978), the size and direction of the difference between X-ray determination for crystalline α-Lac and the UVCD α-helix prediction for α-Lac in neutral buffer would not be surprising. However, the α-helices are furthermore coupled to a relatively large amount (~21%) of 3_{10}-helix. These two structural types are normally mixed and not distinguishable using either UVCD or IR spectroscopy (Sudha et al., 1983; Richardson, 1981). Considering the 3_{10} contribution, one would expect that the effective amount of helix measured in UVCD should be *higher* than is indicated by the crystal structure determined α-helical value alone. This clearly does not happen.

As regards other aspects of the structure of α-Lac, the fraction of amino acids involved in β-sheets as obtained by X-ray is only 6% in contrast to the estimate of 14% obtained by analysis of the early UVCD spectra (Robbins & Holmes, 1970). The random-coil or "other", i.e., residual, evaluations by UVCD are similarly out of line (~60%). Our previous analyses (Pancoska et al., 1991; Pancoska & Keiderling, 1991) imply that while VCD and UVCD have similar sensitivities to the fraction of α-helical character, in terms of standard deviation from X-ray values of predictions for proteins with known crystal structures, VCD has much better accuracy in determining β-sheet character. Furthermore, VCD appears to have less dependence on helix length than does its more established UVCD analogue (Yasui et al., 1986b; Dukor & Keiderling, 1991). Thus, it is appropriate to determine if VCD can resolve some of the confusion between UVCD solution-phase data and X-ray crystal structure results.

Since hen egg white lysozyme (HEWL) has a high sequence homology to baboon and bovine α-lactalbumin and a very similar crystal structure to baboon α-Lac (Acharya et al., 1989, 1990), we expected that HEWL and bovine α-Lac would have very similar spectra. A recent report by Prestrelski et al. (1991) indicates that the FTIR spectra of these two proteins after deconvolution can be interpreted in a manner that is consistent with the X-ray results. This claim was made despite the fact that the overall band shapes of the two protein amide I′ spectra are noticeably different, particularly in the frequency region normally associated with absorption bands of the α-helices. The fact that these proteins both have a significant fraction of 3_{10}-helix led these workers to assign a new band at ~1639 cm^{-1} to a 3_{10}-helix contribution. Since in nonaqueous solutions VCD is capable of distinguishing 3_{10}-helices from α-helices (Yasui et al., 1986a) and since the 3_{10}-helix amide I band in nonaqueous conditions is higher in frequency than that of the α-helix but would be lower than the α-helix in this assignment (Holloway & Mantsch, 1989; Byler & Susi, 1986; Surewicz & Mantsch, 1988; Mantsch et al., 1986), we felt that a VCD study of these proteins might also verify the nature of this transition.

Experimental Procedures

Materials. α-Lactalbumin from bovine milk (all data presented are for type I, Ca^{2+}-containing) and lysozyme from hen egg white were purchased from Sigma and used without further purification. D_2O and 2-propanol-d_1 were obtained from Aldrich. Before spectroscopic study, the proteins were each dissolved in D_2O and lyophilized twice to achieve hydrogen–deuterium exchange. α-Lac and HEWL were studied in phosphate buffer made up by dissolving the appropriate amounts of sodium phosphate (monobasic and dibasic, Fisher) to 10 mM in D_2O to achieve a pH of 7.5–7.6. Fresh solutions were prepared for each experiment. For a separate set of Ca^{2+} dependence studies, α-Lac was instead dissolved in 20 mM imidazole/D_2O buffer (pH 6.3, Aldrich) to avoid Ca^{2+} precipitation at high excess concentrations where enough 1 M $CaCl_2$ solution was added to achieve 40 mM Ca^{2+}. The results of this study were compared to the results for type I (Ca^{2+}-containing) and for type III (Ca^{2+}-depleted) α-Lac. For mixed solvent studies, α-Lac (type I) was first dissolved in the phosphate buffer; then a concentrated propanol/buffer solution was added to vary the propanol content. Finally, both proteins were studied at several pH values to determine if any pH-dependent factors could be observed with any of the techniques. Freshly prepared NaOD and DCl/D_2O solutions were used for adjustment of pH. Sample preparations using H_2O solutions adjusted to pH ~7 without buffer were done for the amide II and a second set of FTIR studies. The protein concentration was ~5% (w/w) in all samples for the D_2O-based VCD and IR measurements, ~20% for the H_2O-based VCD and FTIR spectra, and ~0.2% for the UVCD measurements.

Methods. VCD and IR absorption spectra were measured on the UIC dispersive spectrophotometer which has been described in detail separately (Keiderling, 1981, 1990). VCD data were obtained over the amide I′ band with ~11 cm^{-1} resolution as the average of four scans and with a time constant of 10 s. The samples were measured in a demountable cell constructed with CaF_2 windows separated by a 25-μm pathlength spacer. Identical spectra measured for racemic poly(DL-lysine), dissolved in the same solvent as was the sample, were used for base-line correction of each VCD spectrum. The absorption maxima for the amide I′ of the protein and polypeptide base-line samples were closely matched to minimize absorption artifacts. The VCD spectra presented were normalized to a maximum absorbance of 1.0 in the amide I′ band for ease of comparision of the data. Actual absorbances were 0.3–0.5.

Identical samples, in the same cell, were measured on a Fourier transform infrared (FTIR) spectrometer (Digilab FTS-60) using a nominal resolution of 4 cm^{-1}. These FTIR spectra were typically obtained as the average of 4096 scans to maximize S/N for Fourier self-deconvolution (FSD) calculations. The spectrometer was continuously purged with dried air, and the resultant spectra were corrected for residual vapor-phase H_2O absorptions by subsequent subtraction to flatten the base line outside of the absorption band. The method of Kauppinen et al. (1981), as implemented in the Digilab software, was used for FSD of the measured FTIR spectra in the carbonyl region with Bessel function apodization, a half-width of 18 cm^{-1}, and an enhancement factor of 2.8 to compare with the results of Prestrelski et al. (1991). However, since an original FTIR resolution of 4 cm^{-1} rather than 2 cm^{-1}

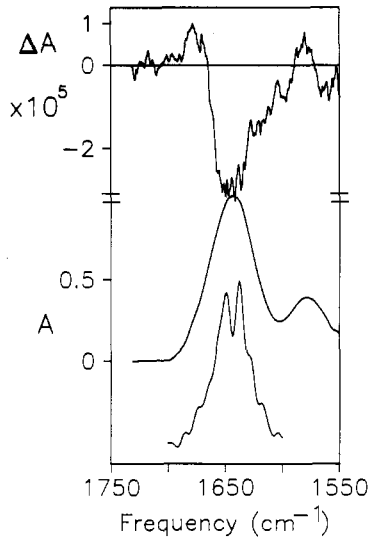

FIGURE 1: VCD (top), IR (middle), and Fourier self-deconvolution (bottom) spectra of bovine α-lactalbumin in phosphate buffer (pH 7.6). The scale for the FSD-FTIR spectrum is arbitrary. The IR and VCD spectra have been normalized to a peak absorbance of A_{max} = 1.0 so that the VCD scale reads directly in $\Delta A/A$ at A_{max}. Protein concentration is ~5%, and path length = 25 μm. The parameters used for deconvolution are a half-width of 18 cm^{-1} and an enhancement factor of 2.8.

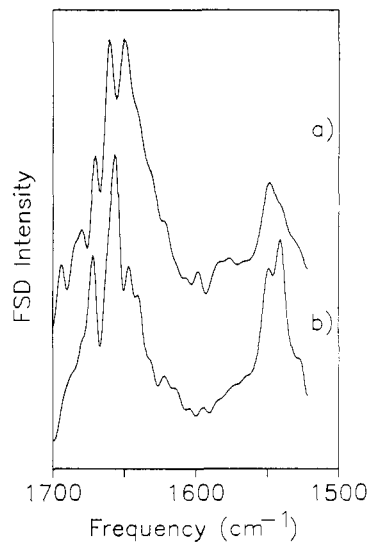

FIGURE 2: Fourier self-deconvolution spectra of (a) bovine α-lactalbumin and (b) hen egg white lysozyme in H_2O (no buffer) over the amide I and II regions. The intensity scale is arbitrary. Protein concentration is ~20% and path length = 15 μm. The same FSD parameters were used as in Figure 1.

was used, the end result of the deconvolution is not quite the same as theirs. The same methods were used for the FTIR studies of H_2O-based samples except that the path length was reduced to 15 μm and the concentration increased to ~20 mg/100 μL.

UVCD spectra were measured with JASCO J-600 dichrometer over the range of 180–260 nm using a strain-free quartz cell of 0.01-cm path length (NSG Precision). The small path length was chosen so that solutions for the UVCD might be closer in concentration to the relatively high values needed for the IR experiments for sake of comparison. A time constant of 2.0 s and a spectral bandwidth of 1 nm were used for our UVCD measurements. The spectra were obtained as the average of three scans and were corrected with a baseline obtained for the solvent in the same cell. The data are presented in terms of molar CD on a per unit amide basis (the average molecular weight of each amino acid residue was assumed to be equal to 113).

Results

α-Lactalbumin. The VCD and IR absorption (measured at low resolution on the dispersive instrument, same instrumental and sample conditions as the VCD spectrum) spectra and the resolution-enhanced FSD-FTIR absorption spectrum of native α-Lac in D_2O/phosphate buffer (pH 7.6) in the amide I′ region are presented in Figure 1. Very similar VCD and FSD-FTIR spectra are obtained in D_2O solution with no buffer at neutral pH, making the buffer effect appear to be negligible. The VCD spectrum is dominated by a negative band centered over the absorbance maximum at 1650 cm^{-1} and has a small positive band to the high-frequency side. This would correspond to a negative-couplet VCD pattern which, aside from frequency, is similar to that seen in our previous studies for α-chymotrypsin or, especially, concanavalin A, which both have high β-sheet contents (Pancoska et al., 1989, 1991).

Very similar FTIR results have been reported by Prestrelski et al. (1991) with slightly better resolution for α-Lac at neutral pH with added Ca^{2+}. The spectral shape of the FSD-FTIR is dominated by two strong bands at 1637 and 1649 cm^{-1}. This

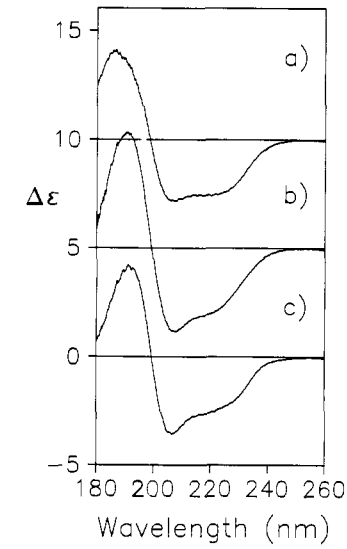

FIGURE 3: UVCD spectra of (a) bovine α-lactalbumin in phosphate buffer, (b) bovine α-lactalbumin in a 33% propanol/phosphate buffer mixture, and (c) hen egg white lysozyme in phosphate buffer. The data are presented in terms of molar CD ($\Delta\epsilon$) on a per unit amide basis. Each spectrum is offset from the one below it by 5 units. The concentration of the protein is ~0.2% and path length = 0.01 cm.

same dominant shape is also seen in the FSD-FTIR of α-Lac in H_2O solution (shown in Figure 2a) where the major peaks are at 1650 and 1660 cm^{-1}. Added Ca^{2+} had no effect either on our measured VCD band shape or on our FTIR spectra, even after FSD, for type I α-Lac. As has been previously reported (Robbins & Holmes, 1970; Clark & Smith, 1989), the UVCD (shown in Figure 3a) of α-Lac in buffer is indicative of a mixed structure even though the band shape is dominated by the α-helical contributions as evidenced in the negative 207/220-nm bands (nearly equal intensity in buffer) and the positive 190-nm band (broadened to shorter wavelength).

The presence of up to 20% propanol in solution does not change any of these spectral band shapes, FSD-FTIR, UVCD, or VCD, but a sudden change in the VCD as well as the FSD-FTIR band shapes (see Figure 4) occurs for α-Lac in a solution with ~33% propanol added. This solvent-induced

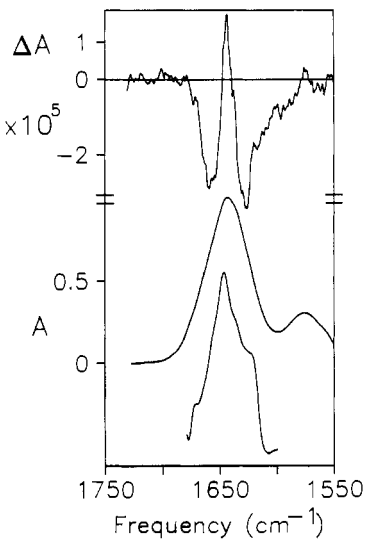

FIGURE 4: VCD (top), IR (middle), and FSD-FTIR (bottom) spectra of bovine α-lactalbumin in 33% propanol/phosphate buffer solution. Scaling and parameters are as in Figure 1.

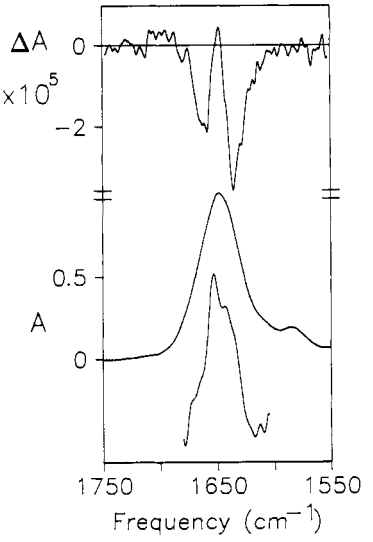

FIGURE 5: VCD (top), IR (middle), and FSD-FTIR (bottom) spectra of hen egg white lysozyme in phosphate buffer/DCl (pH 3.3). Scale and parameters are as in Figure 1.

change is even detectable in UVCD (Figure 3b). We can see from the FTIR-FSD that the major absorption band at 1637 cm^{-1} significantly decreases while that at 1646 cm^{-1}, possibly assignable to α-helical contributions, increases in intensity and shifts to a slightly lower frequency. A new band at ~1621 cm^{-1} also becomes important.

A much more dramatic change is seen in the VCD; upon addition of 33% propanol, the dominant band-shape pattern virtually flips sign. The positive signal at 1675 cm^{-1} disappears, a negative band replaces it, and a new positive band grows in which is correlated to the absorption maximum. The dominant negative VCD is then split into two bands at ~1658 and ~1628 cm^{-1} whose relative intensity changes as the fraction of propanol is increased (an increase in propanol concentration corresponds to an increase in the negative VCD intensity at 1658 cm^{-1}). At even higher propanol concentration (66%), the VCD spectrum changes further to resemble that found in our previous studies of hemoglobin or albumin, which both have high α-helical content (Pancoska et al., 1989, 1991).

The corresponding α-Lac UVCD spectrum at 33% propanol content in solution (Figure 3b) shows that the intensities of both the positive and negative parts of the band shape increase as compared to the band shape for α-Lac in just buffer. Furthermore, the negative 207-nm band increases in intensity relative to the 222-nm band, and the 186-nm band shifts to longer wavelength. These changes are consistent with those seen in previous UVCD studies of the effect of 66% propanol addition to α-Lac (Clark & Smith, 1989). Both observations are qualitatively consistent with standard UVCD estimations of the effect of an increase in α-helical content (Yang et al., 1986).

As a test for structural consequences of our deuterium exchange protocol, the VCD spectra of α-Lac were run immediately after fresh protein samples were dissolved directly in D_2O without previous exchange. A similar VCD band shape resulted as shown in Figure 1, but the noise level was higher due to the HOD absorption. The balance of the various subbands in the FSD-FTIR spectrum did change, with the 1660 cm^{-1} band notably losing intensity as deuteration progressed.

It can be noted here that as a function of pH, the α-Lac spectra we have measured change less than shown here for propanol addition (Urbanova et al., unpublished results). At high pH (~12), little change is evident in the VCD, but the FSD-FTIR exhibits a new peak at ~1643 cm^{-1} which may correlate to unordered segments of the peptide chain. At low pH (~1.4), larger changes are seen in both VCD and FSD-FTIR than at high pH. Again the FSD-FTIR spectrum exhibits a new feature indicative of an increase in the amount of unordered structure. The resultant VCD spectra are somewhat intermediate in shape between the α-Lac/buffer spectra in Figure 1 and the α-Lac/propanol spectra in Figure 4. Under these low-pH conditions, α-Lac should be in its A form, sometimes termed the "molten globule" state (Dolgikh et al., 1985, 1981; Kronman, 1989).

Lysozyme. While HEWL has a very comparable X-ray crystal structure to that of α-Lac, its VCD and FSD-FTIR spectra, shown in Figure 5 as measured at pH 3.3 to better replicate the crystallization conditions, indicate clear differences from the α-Lac results in D_2O-based solution. HEWL has an FSD-FTIR dominated by a peak at ~1653 cm^{-1} with a distinct shoulder at 1642 cm^{-1}. In H_2O, its FSD-FTIR is also dominated by a peak at 1657 cm^{-1} with two principal side lobes at 1672 and 1647 cm^{-1} (Figure 2b).

The lysozyme VCD spectrum is dominated by two negative bands at ~1663 and ~1635 cm^{-1} that overlap a narrower positive band which again is correlated with the absorbance maximum. Although they are not identical in shape, the VCD of HEWL and the VCD of α-Lac in 33% propanol solution have significant qualitative similarities. The lysozyme VCD spectra in D_2O, buffer, or 33% propanol are all about the same as that shown in Figure 5, indicating considerable structural stability with respect to solvent variation. Change of pH also does not lead to a qualitative change in the HEWL VCD spectrum, nor does incomplete, partial deuteration.

As was noted by Clark and Smith (1989), this pattern of HEWL stability with regard to solvent change is also evident in its UVCD spectrum (Figure 3c). As in VCD, the UVCD of HEWL is more similar in its intensity pattern to that of α-Lac in 33% propanol (Figure 3b) than to that of α-Lac in buffer (Figure 3a). The differences between α-Lac and HEWL as seen in UVCD are much smaller than seen in VCD, or even in FTIR, presumably due to the dominance of the α-helical contribution to the observed UVCD band shape (Yang et al., 1986).

The amide II VCD spectra of HEWL and α-Lac are similar, both being negative bands shifted to low frequency from

the absorbance maximum. These amide II VCD differ in intensity, measured as $\Delta A/A$ and indicating HEWL < α-Lac, but this could be due to absorbance variations due to overlapping side chain modes (Venyaminov & Kalnin, 1990). The frequency of the negative maximum also differs by ~5 cm^{-1}, with HEWL > α-Lac. Such amide II band shapes and small intensity and frequency variations are typical of amide II VCD (Gupta & Keiderling, 1991) for α + β proteins (Levitt & Chothia, 1976).

Discussion

Using several different techniques, VCD, UVCD, and FSD-FTIR in both H_2O and D_2O solution, we have above made three general spectroscopic observations: α-Lac and HEWL have different spectra under "normal" conditions, α-Lac has spectra that are quite solvent-sensitive, and α-Lac and HEWL are most similar under conditions of 33% propanol. The parallel of the α-Lac and HEWL crystal structures (Acharya et al., 1989) has led to the assumption that such similarities exist in solution also. *Surprisingly, the degree of crystal structure similarity between these two proteins is not paralleled in any of these spectra.* However, it is well-known that α-Lac is conformationally sensitive as regards environmental change (Kronman, 1989; Clark & Smith, 1989; Kuwajima, 1977), that it adopts a "molten globule" state under certain conditions (Dolgikh et al., 1985), and that it was more easily denatured than HEWL (Barel et al., 1972). The facile structural variation of α-Lac and its spectroscopic consequences that we have observed upon change of solvent using various techniques coupled to the spectral differences from HEWL using those same techniques prompt one to question how well the reported α-Lac crystal structure reflects that of α-Lac in solution under physiological conditions.

The observed IR absorption spectrum in the amide I′ region is an envelope over a heterogeneously broadened band composed of characteristic amide carbonyl vibrations whose frequencies depend on the stereochemistry and environment of each subunit of the polymer (helical, sheet, etc. plus solvent and environmental effects). This is the basis for IR analysis of secondary structure using resolution-enhanced data (Byler & Susi, 1986; Surewicz & Mantsch, 1988). Due to the inherent resolution of the vibrational spectrum, these frequency shifts also have a large effect on the VCD spectra, but with VCD, each stereochemical component imparts a sign and magnitude character on the band that is, to some order, independent of its frequency. This is the basis for the enhanced sensitivity of VCD over IR spectra for stereochemical analysis. By contrast, UVCD has this same sign advantage but lacks the inherent resolution capabilities of vibrational spectroscopy. In combining the results from all three of these methods, a more complete spectroscopic picture evolves. While some of these data were available earlier for α-Lac and HEWL, the VCD spectra reported here offer the most dramatic evidence for the difference in these two proteins and, consequently, drew our attention to the variations also present in the UVCD and FTIR data. Below are discussed our various spectral observations in more detail.

VCD Analyses. The α-Lac VCD band shape does not at all resemble the VCD we have measured for highly helical proteins or even that obtained for α + β proteins, which is quite surprising since in the crystal structure nearly 50% of the structure is helical, if the contributions from the α- and 3_{10}-helices are summed. On the other hand, we have seen evidence of several different extended structures that have negative VCD in the 1620–1650 cm^{-1} region much like that seen for α-Lac in buffer. These have included models of β-sheets (Yasui & Keiderling, 1986; Dukor, 1991) as well as polyproline and related oligomers (Kobrinskaya et al., 1988; Dukor et al., 1991). In fact, the VCD of poly(L-lysine) immediately after pH adjustment to 11.5 (conditions under which a mixture of α-helix, β-sheet, and "random coil" forms are in equilibrium) is somewhat like that of α-lactalbumin in 33% propanol (Yasui & Keiderling, 1986). We have since demonstrated that the "random-coil" form gives a VCD spectrum of the same shape as that of poly(L-proline) II, which is an extended, 3_1-helix of left-handed sense (Dukor & Keiderling, 1991). Perhaps more importantly, the globular proteins whose VCD spectra have the most resemblance to that of α-Lac in D_2O are those dominated by β-sheet and "other" character, and are *not* those having high helical contribution. Hence, our VCD data for α-Lac are more consistent with its having substantial β *or* some other extended form in buffer or D_2O which can then be converted to a helical form when propanol is added.

It should be noted that while we have studied bovine α-Lac, the crystal structure available is for baboon α-Lac. These are highly homologous proteins, but small differences could ensue. Acharya et al. (1990) have recently pointed out that the α-lactalbumins from these two species are likely to have very similar three-dimensional structures. If correct, there is little likelihood that our VCD result would change if we were to have baboon α-Lac available. We have made a preliminary study of Ca^{2+}-containing human α-Lac and found that it gives VCD of a somewhat different band shape than obtained here with bovine α-Lac. But both spectra are predominantly negative and are very different from the HEWL band shape. In light of these solution VCD results and since human α-Lac is even more homologous to both baboon α-Lac and HEWL than to bovine α-Lac (Acharya et al., 1990), the general assumption that the crystal structure of α-Lac is the same as the normal solution structure must be questioned.

Detailed Band Assignments and FTIR-FSD Comparison. Using FSD resolution-enhanced FTIR spectra, peaks over a range of 1649–1659 cm^{-1} have been variously assigned (Byler & Susi, 1986; Yang et al., 1985; Surewicz & Mantsch, 1988) to the amide I′ band of an α-helix. After deconvolution, both α-Lac and HEWL in D_2O have a major absorption band detectable in this range that should correspond to the α-helical fraction. In H_2O, these bands are shifted up in frequency by 11 cm^{-1} for α-Lac and 4 cm^{-1} for HEWL, thereby appearing at 1657–1660 cm^{-1} which can be seen by comparing Figure 2 with Figures 1 and 5.

A previous FSD-FTIR study (Prestrelski et al., 1991) of α-Lac in D_2O/buffer has systematically assigned these major features but in addition detected and assigned, to the same α-helical fraction, a second feature in each protein (~1660 cm^{-1}), which appears as a shoulder in our FSD-FTIR spectra (Figures 1 and 5). In our studies of partial deuteration for both proteins, the intensity of this shoulder decreases with deuteration extent, but the overall similarity of the spectra in D_2O and H_2O for each protein (Figures 1 and 5 compared to Figure 2) suggests that the major peaks are conserved. The same sort of conserved band shape between D_2O- and H_2O-based spectra was seen for cytochrome b_5 (Holloway & Mantsch, 1989). If these two molecules indeed are as different as the spectra force one to believe, it would be reasonable that the shoulder at ~1659 cm^{-1} could arise instead from some other aspect of the structure, for example, turns and bends. Alternatively, the band could represent the undeuterated portion of the α-helices which would be consistent with the quantitative aspects of the Prestrelski et al. (1991) analysis

if not the qualitative aspects. It should be noted that side groups have vibrational transitions of reasonable intensity of both the high- and low-frequency sides of the 1650 cm^{-1} α-helical band (Venyaminov & Kalnin, 1990; Chirgadze et al., 1975). In the H_2O spectra, *both* proteins have resolved side peaks in the deconvolved band shapes at $\sim$1670 cm^{-1} which are most likely not due to helices, and both show little evidence of doubling of the helical band.

The peak located at 1637 cm^{-1} in α-Lac (Figure 1) and at 1642 cm^{-1} in HEWL (Figure 5) has been suggested by Prestrelski et al. (1991) to have its origin in the amide I′ of the 3_{10}-helical components, on the basis of studies of cytochrome b_5 by Holloway and Mantsch (1989). Since both α-Lac and HEWL have a significant amount of 3_{10}-helix in their crystal structures (Acharya et al., 1989), this posed an attractive assignment of an intense band in the α-Lac spectrum. By analogy, since the band-shape patterns are so little affected by deuteration, the H_2O solution spectra would necessarily be assigned so that the corresponding peaks are those falling at 1650 and 1647 cm^{-1}, respectively (Figure 2).

However, there are problems with such an assignment. Since the entire FSD-FTIR band shape shifts as a unit with deuteration (compare Figures 1 and 5 with Figure 2) and since this shift is the same type as seen in cytochrome b_5 (Holloway & Mantsch, 1989), it is reasonable to make a parallel assignment of the H_2O spectrum. This would require the 3_{10}-helix to have a lower frequency than the α-helix in both D_2O and H_2O solution. For protonated peptides in nonaqueous solutions, our earlier studies (Yasui et al., 1986a) of model 3_{10}-helical oligopeptides, based on aminoisobutyric acid (Aib), have shown that, in that environment, the amide I of the 3_{10}-helix absorbs at a higher frequency ($\sim$1665 cm^{-1}) than do short α-helices ($\sim$1654 cm^{-1}) measured under the same conditions (Yasui et al., 1987). Preliminary deuteration studies of these 3_{10}-helical oligopeptides indicate only a small shift of the amide I′ band to 1659 cm^{-1}, which is still higher than the α-helical frequency. On the basis of these observations, the proposal (Prestrelski et al., 1991; Holloway & Mantsch, 1989) of a method for detection of the 3_{10}-helical component of these proteins which is based only on frequency discrimination should be viewed as unsettled.

For protonated oligopeptides, the VCD spectra of these two types of right-handed helices, α and 3_{10}, are very similar, both being describable for the amide I band as a positive couplet centered over the absorption band (Yasui et al., 1986a, 1987; Singh & Keiderling, 1981). Since the frequencies and band shapes of these two VCD contributions are so similar, it is expected that they would overlap and reinforce each other. In oligomers containing mixed 3_{10}- and α-helical structures (nonaqueous solution), we cannot separate the signals from each component, but see only a mixed band shape (Yasui et al., unpublished results). Thus, the presence of a large 3_{10}-helical component in the protein structure cannot explain the observed VCD shape for α-Lac in D_2O/buffer solution. Perhaps more to the point, both α-Lac and HEWL have significant 3_{10}-helical components, yet it is the difference in their spectra that is of interest.

This lack of both VCD and FTIR (frequency) spectral agreement with known 3_{10}-helix characteristics would be consistent with a reassignment of the FSD-FTIR band at 1637 cm^{-1} in α-Lac to the presence of a significant amount of an extended form which may arise from loop unfolding. Such a reassignment is consistent with the sign pattern of the VCD as noted above. However, we must reiterate that uncertainty in assigning a given FTIR band does not impact on the fundamental differences in all of the band shapes of all of the α-Lac and HEWL spectra in solution. The structures are clearly different under these conditions, even if that difference is difficult to assign to a detailed component of the conformation.

α-Lac does have small differences from HEWL even in the crystal structure. The C-terminus chain of α-Lac is more extended than for HEWL, and various loops have different conformations. The helices found in both crystal structures are somewhat short, there is little sheet, and there is a large contribution from turns and "other". Such loops and turns may also be a source of the FSD-FTIR bands anomalously assigned to helices. Furthermore, previous light-scattering experiments (Krigbaum & Kügler, 1970) have demonstrated that the two proteins have significantly different molecular volumes which must arise from differences in their structure. Our proposal of less well-defined loops present in the α-Lac structure may explain both the scattering and the spectroscopic results.

Conclusion

Our spectral data pose a problem: the crystal structure of baboon α-lactalbumin clearly has a significant amount of α- and 3_{10}-helix content. On the other hand, it does not have much β-sheet content. There is a strong parallel between the α-lactalbumin and lysozyme crystal structures, but their spectra in buffer are quite different and only become similar at 33% propanol concentration. The UVCD data support the presence of some helical contribution which dominates the observed spectra. In VCD and FTIR, other structures contribute on an equivalent footing with the α-helix so a different picture develops. This leads us to conclude that the solution structures of α-lactalbumin and lysozyme are not as similar as their crystal structures would lead us to believe, such that the α-lactalbumin solution structure must have significantly less helix than does its crystal structure.

Our results are preliminary in the respect that one can design a wide series of experiments to test the species sensitivity and environmental sensitivity of these spectral band shapes. However, it should be made clear that we have previously demonstrated (Pancoska et al., 1989, 1991; Pancoska & Keiderling, 1991) that our spectra are strongly correlated to secondary structure and that a large number of proteins have been so studied and found to have analyzable spectra. Furthermore, our work points to the danger of interpreting a single, unique piece of spectral data with a heavy reliance on crystal structure results such as the FSD-FTIR study of α-Lac (Prestrelski et al., 1991). FTIR data lack high sensitivity to structural variations since they really can only measure frequency. VCD gives enhancement to structural variation due to its imparting sign characteristics on these same spectral components and to the fact that this sign variation is very specific conformationally since it arises directly from the three-dimensional structure.

Acknowledgments

We thank Drs. Steven Prestrelski and Michael Byler for transmitting copies of their work to us before publication and for comments on our results, Dr. K. Kuwajima for the human α-Lac sample, and Ms. Dongfang Huo for preliminary FTIR results.

Registry No. Lysozyme, 9001-63-2.

References

Acharya, K. R., Stuart, D. I., Walker, N. P. C., Lewis, M., & Phillips, D. C. (1989) *J. Mol. Biol. 208*, 99–127.

Acharya, K. R., Stuart, D. I., Phillips, D. C., & Scheraga, H. A. (1990) *J. Protein Chem. 9*, 549–563.
Bano, M., Salomon, D., & Kidwell, W. R. (1985) *J. Biol. Chem. 260*, 5745–5752.
Barel, A. O., Prieels, J. P., Maes, E., Looze, Y., & Lêonis, J. (1972) *Biochim. Biophys. Acta 257*, 288–296.
Broadbeck, U., Denton, W. L., Tanahashi, N., & Ebner, K. E. (1967) *J. Biol. Chem. 242*, 1391–1397.
Byler, D. M., & Susi, H. (1986) *Biopolymers 25*, 469–487.
Chang, C. T., Wu, C.-S. C., & Yang, J. T. (1978) *Anal. Biochem. 91*, 13–31.
Chirgadze, Yu. N., Fedorov, O. V., & Trushina, N. P. (1975) *Biopolymers 14*, 679–694.
Clark, D. C., & Smith, L. J. (1989) *J. Agric. Food Chem. 37*, 627–633.
Dolgikh, D. A., Gilmanshin, R. I., Brazhnikov, E. V., Bychkova, V. E., Semisotnov, G. V., Venyaminov, S. Yu., & Ptitsyn, O. B. (1981) *FEBS Lett. 136*, 311–315.
Dolgikh, D. A., Abaturov, L. V., Bolotina, I. A., Brazhnikov, E. V., Bychkova, V. E., Gilmanshin, R. I., Lebedev, Yu. O., Semisotnov, G. V., Tiktopulo, E. I., & Ptitsyn, O. B. (1985) *Eur. Biophys. J. 13*, 109–121.
Dukor, R. K. (1991) Ph.D. Thesis, University of Illinois at Chicago.
Dukor, R. K., & Keiderling, T. A. (1991) *Biopolymers* (in press).
Dukor, R. K., Keiderling, T. A., & Gut, V. (1991) *Int. J. Pept. Protein Res.* (in press).
Greenfield, N., & Fasman, G. D. (1969) *Biochemistry 8*, 4108–4116.
Gupta, V. P., & Keiderling, T. A. (1991) *Biopolymers* (submitted for publication).
Holloway, P. W., & Mantsch, H. H. (1989) *Biochemistry 28*, 931–935.
Kauppinen, J. K., Moffatt, D. J., Mantsch, H. H., & Cameron, D. G. (1981) *Appl. Spectrosc. 35*, 271–276.
Keiderling, T. A. (1981) *Appl. Spectrosc. Rev. 17*, 189–226.
Keiderling, T. A. (1990) in *Practical Fourier Transform Spectroscopy* (Ferraro, J. R., & Krishnan, K., Eds.) pp 203–284, Academic Press, San Diego, CA.
Kobrinskaya, R., Yasui, S. C., & Keiderling, T. A. (1988) *Proc. Am. Pept. Symp., 10th*, 65–67.
Krigbaum, W. R., & Kügler, F. R. (1970) *Biochemistry 9*, 1216–1223.
Kronman, M. J. (1989) *CRC Crit. Rev. Biochem. Mol. Biol. 24*, 565–667.
Kuwajima, K. (1977) *J. Mol. Biol. 114*, 241–258.
Levitt, M., & Chothia, C. (1976) *Nature 261*, 552–558.
Mantsch, H. H., Casal, H. L., & Jones, R. N. (1986) in *Advances in Spectroscopy* (Clark, R. J. H., & Hester, R. E., Eds.) Vol. 13, pp 1–46, Wiley & Sons, London.
Pancoska, P., & Keiderling, T. A. (1991) *Biochemistry 30*, 6885–6895.
Pancoska, P., Yasui, S. C., & Keiderling, T. A. (1989) *Biochemistry 28*, 5917–5923.
Pancoska, P., Yasui, S. C., & Keiderling, T. A. (1991) *Biochemistry 30*, 5089–5103.
Prestrelski, S. J., Byler, D. M., & Thompson, M. P. (1991) *Int. J. Pept. Protein Res. 37*, 508–512.
Provencher, S. W., & Glöckner, J. (1981) *Biochemistry 20*, 33–37.
Richardson, J. S. (1981) *Adv. Protein Chem. 34*, 167–339.
Robbins, F. M., & Holmes, L. G. (1970) *Biochim. Biophys. Acta 221*, 234–240.
Singh, R. D., & Keiderling, T. A. (1981) *Biopolymers 20*, 237–240.
Sudha, T. S., Vijayakumar, E. K. S., & Balaram, P. (1983) *Int. J. Pept. Protein Res. 22*, 464–468.
Surewicz, W. K., & Mantsch, H. H. (1988) *Biochim. Biophys. Acta 952*, 115–130.
Venyaminov, S. Yu., & Kalnin, N. N. (1990) *Biopolymers 30*, 1243–1257.
Yang, W.-J., Griffiths, P. R., Byler, D. M., & Susi, H. (1985) *Appl. Spectrosc. 39*, 282–287.
Yang, J. T., Wu, C.-S. C., & Martinez, H. M. (1986) *Methods Enzymol. 130*, 208–269.
Yasui, S. C., & Keiderling, T. A., (1986) *J. Am. Chem. Soc. 108*, 5576–5581.
Yasui, S. C., Keiderling, T. A., Bonora, G. M., & Toniolo, C. (1986a) *Biopolymers 25*, 79–89.
Yasui, S. C., Keiderling, T. A., Formaggio, F., Bonora, G. M., & Toniolo, C. (1986b) *J. Am. Chem. Soc. 108*, 4988–4993.
Yasui, S. C., Keiderling, T. A., & Katakai, R. (1987) *Biopolymers 26*, 1407–1412.