Determination of the Secondary Structure of Proteins by Laser Raman Spectroscopy

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Abstract: A technique for determining secondary structural content of proteins from their laser Raman spectra in H_2O and ^2H_2O solutions is proposed. The technique uses, as a model, the Raman spectral intensities of the backbone amide vibrations of poly-L-lysine (PLL) in its α-helical, antiparallel β-pleated sheet, and random coil conformations, evaluated at 1240 cm^{-1} in H_2O (amide III) and at 1632 and 1660 cm^{-1} in ^2H_2O (amide I′), and amide I′ intensities of the proteins, lysozyme and ribonuclease A. It is suggested that the Raman spectral intensities of proteins, evaluated at those same frequencies in H_2O and ^2H_2O relative to the intensity of the 1448-cm^{-1} methylene band, are linear combinations of the intensities due to peptide residues in those three conformations. Using this model, the fraction of residues in the α-helix, β-pleated sheet, and random coil conformations was evaluated for seven additional proteins and found to agree well with determinations by other techniques.

In recent years, the laser Raman spectra of several proteins have been reported,[1-8] both in the solid phase and in solution. Qualitative identification has been made of several amide bands which are conformationally sensitive but it has not been possible to predict quantitatively the detailed secondary structure of globular proteins from their Raman spectra.

Such quantitative information is presently obtained predominantly by x-ray diffraction and by optical rotatory dispersion (ORD) or circular dichroism (CD). However, x-ray diffraction structure determinations are restricted to crystals, whereas ORD and CD spectra can be obtained only in solution. Serious discrepancies between estimates of α-helix, β-sheet, and random coil content by CD as opposed to x-ray diffraction have been reported for several proteins.[9,10] It has not been possible to determine whether these differences are caused by deficiencies in interpretation of the CD spectra or are, in fact, due to conformational changes accompanying dissolution, since no single technique is available to determine secondary structure content of proteins in both the crystal and in solution.

Clearly, a technique which could determine protein structure in a variety of phases would be most valuable. Laser Raman spectroscopy holds promise of being such a technique, since spectra are obtained with equal ease in solids, solutions, and suspensions. In fact, Raman spectra[6,8] of a number of proteins have been found to differ between crystal, lyophilized powder, and aqueous solution in the conformationally sensitive amide III region. Yu[6,8] has interpreted these changes as representing differences between the crystalline and solution conformations of insulin[6] and carboxypeptidase A.[8]

A related problem is the difficulty of determining protein conformations in particulate systems (e.g., cell membrane suspensions), because interpretations of ORD and CD spectra are made difficult by light scattering effects.[11] Raman spectroscopy again holds considerable promise with no real experimental or theoretical difficulties in interpretation of spectra from dispersive samples. Recently, for example, the Raman spectrum of hemoglobin-free erythrocyte membranes[12] has been interpreted in terms of the component protein and phospholipid configurations as containing large amounts of α-helical protein and moderately rigid lipid side chains.

In this paper, we report a technique for estimating the fractions of α-helix, β-sheet, and random coil conformations in proteins, based on an interpretation of their amide I′ and amide III Raman spectral intensities. The technique uses, as its basis, amide intensities due to purely α-helical, β-sheet, and random conformations in proteins. These model intensities are obtained, in turn, from amide intensities of poly-L-lysine in its α-helical, β-sheet, and random conformations[13,14] and of two proteins with established secondary conformations, lysozyme and ribonuclease A. The model has been used to estimate the secondary structure of eight additional proteins, and the estimates agree well with those obtained from x-ray diffraction and circular dichroism for the proteins studied. In addition, the Raman spectra of crystalline insulin and its acid solution are interpreted to quantitate the conformational change which has been reported to take place on dissolution.[6]

Experimental Section

Materials and Methods. Egg white lysozyme (6X crystalline), ribonuclease A (isolated from beef pancreas), and bovine albumin (Pentex) (crystallized from purified bovine albumin, fraction V) were obtained from Miles Research Laboratories. Crystalline α-chymotrypsin (A grade from bovine pancreas), crystalline α-chymotrypsinogen (A grade from bovine pancreas), and insulin (recrystallized from bovine pancreas) were obtained from Calbiochem. Crystalline pepsin and crystalline pepsinogen (from swine stomach mucosa) were purchased from Worthington Biochemical Corp. and concanavalin A (Grade IV from Jack Bean) was obtained from Sigma Chemical Co. All proteins were used without further purification.

The Raman spectrometer is a SPEX 1403 half-meter double monochromator with a Spectra-Physics 164 Argon ion laser typically operated at 300 mW at 488 nm. A Baird-Atomic interference filter is placed before the sample to remove plasma lines. Most spectra were obtained with an EMI 9502 SA uncooled photomultiplier coupled to a Kiethley picoammeter. Typical dark current was 3×10^{-9} A. Several spectra were obtained with a new detection system consisting of a cooled RCA C31034 photomultiplier coupled to an SSR photon counting system. Spectra were recorded with a spectral slit width of 5 cm^{-1}, typically with a scan speed of 0.2 cm^{-1}/s and a 10-s time constant.

Solutions of the proteins were prepared by dissolving in water or ^2H_2O to obtain concentrations of 50–100 mg/ml. Protein solutions were sealed in melting point capillary tubes and maintained at 4 °C until Raman spectra were obtained, generally 3–6 h after the samples had been brought into solution. Blout et al.[15] have found that deuterium exchange with amide protons reaches an equilibrium of 85–90% exchange after 3 h in ^2H_2O. In the Raman experiments, the sample tubes are held horizontally in a thermostated brass block whose temperature is controlled at 22 ± 1 °C by a circulating water bath. The laser enters the sample vertically; the scattered Raman light is collected at right angles to both the laser axis and the sample tube axis. In no case was any sample deterioration or change in a sequence of two to three repeat spectra observed.

Raman spectral intensities at a particular frequency are measured as the spectral height above a background drawn to represent the combination of broad band luminescence with the Raman spectrum of water or ^2H_2O. This background is usually a straight line between minima in the spectra, except in the region of solvent bands.

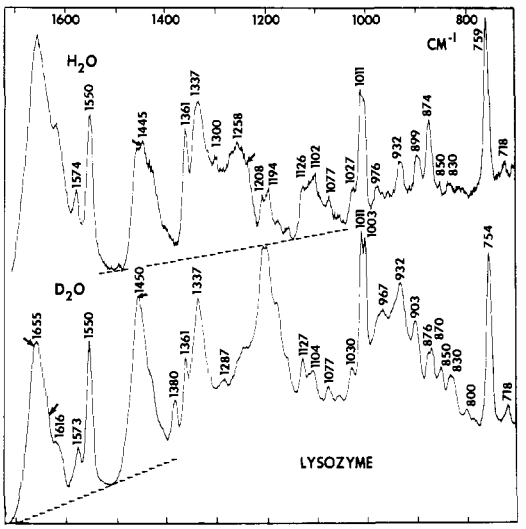

Figure 1. Raman spectra of lysozyme solutions (10%) in H_2O and ^2H_2O. Arrows point to the frequencies whose spectral intensities are used to determine structural content. The dashed baselines were drawn as indicated in the text. Conditions of spectra: spectral slit width ($\Delta\sigma$). 5 cm^{-1}; rate of scan (γ), 0.2 cm^{-1}/s; time constant (τ), 10 s: sensitivity (s) 3×10^{-7} A full scale; laser power (p) 400 mW.

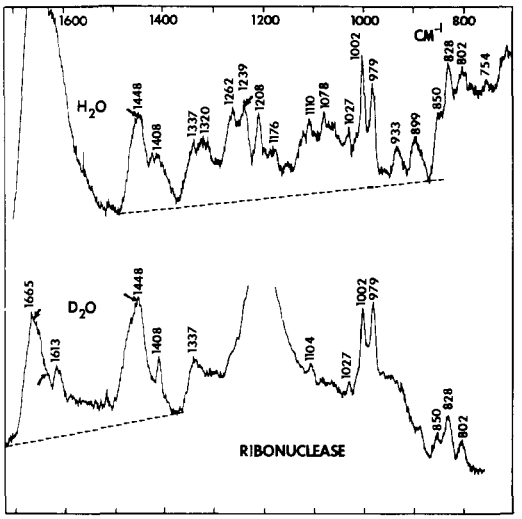

Figure 2. Raman spectra of ribonuclease A solutions (5%) in H_2O and ^2H_2O. Conditions as in Figure 1, except $s = 1 \times 10^{-7}$.

Theory

Poly-L-lysine is a particularly useful model compound for use in protein structure determinations because it can be obtained as a random coil at low pH, but as the α-helix at high pH and low temperature, and the antiparallel β-sheet conformation at high pH and higher temperature.[16] Recently,[13] Raman spectra have been obtained for all three conformations in aqueous solutions and in ^2H_2O. Indeed, poly-L-lysine is a unique polypeptide because Raman frequency and intensity changes can be observed which accompany conformational changes in aqueous solution without compensation for the effects of differing side chains or solvents. It is for similar reasons that poly-L-lysine has been used for some time as a model for ORD and CD estimates of secondary structural content in proteins,[9,17,18] although modification of some poly-L-lysine CD parameters have been suggested for globular proteins.[10,19]

The laser Raman spectra of poly-L-lysine in its different conformations[13,14] are similar to those of other polypeptides and proteins with the same conformations in the regions of the spectrum that are associated with polypeptide backbone vibrations. These similarities, and the differences between Raman spectra of different polypeptide conformations, are particularly striking in the amide I (1630-1680 cm^{-1}) and amide III (1230-1310 cm^{-1}) regions and in the associated regions for amide deuterated polypeptides, amide I′ (1630-1680 cm^{-1}) and amide III′ (950-1000 cm^{-1}).

For example, amide I vibrations are observed at 1650-1655 cm^{-1} for the α-helical forms of poly-γ-benzyl-L-glutamate, poly-L-leucine, and poly-L-alanine.[1,14] Koenig[1] has reported the amide I Raman maximum for solid α-poly-L-lysine at 1652 cm^{-1}. Unfortunately, in aqueous solutions the amide I vibrations are obscured by the water bending mode centered at 1645 cm^{-1} and so cannot be used for identification of protein structures in solution.

In the amide III region, α-helical poly-γ-benzyl-L-glutamate, poly-L-leucine, and poly-L-alanine[1,14] show rather intense bands at 1294 cm^{-1} and no intensity or only very weak bands between 1230 and 1280 cm^{-1}. Aqueous solutions of α-poly-L-lysine show a strong band at 1311 cm^{-1} but no band in the 1230-1280-cm^{-1} region. On deuteration, the band at 1311 cm^{-1} decreases markedly in intensity, which indicates that intensity at that frequency is predominantly, but not exclusively, due to amide III vibrations. Thus, a protein band at 1300-1320 cm^{-1} may not be used as a unique identification of α-helical content. However, the *absence* of spectral intensity at 1235-1240 cm^{-1} does seem to be diagnostic of helix.

In the amide III region of the Raman spectra of β conformations of polypeptides, a very intense, rather sharp band is observed centered at 1230-1240 cm^{-1}. This band is observed[1] at 1230 cm^{-1} in poly-L-valine, at 1235 cm^{-1} in poly-L-serine, at 1232 cm^{-1} in glucagon fibrils,[6] and at 1240 cm^{-1} in intact calf lens.[6] Poly-L-lysine in the β-sheet conformation has a very intense amide III band at 1240 cm^{-1}. Chen et al.[4] have observed this band as a shoulder at 1238 cm^{-1} in lysozyme which disappears on denaturation. The striking feature of all polypeptides and proteins in the β conformation is the intensity of this amide III band around 1235-1240 cm^{-1} with respect to bands that are not conformationally dependent, e.g., the methylene bending vibrations at 1446-1448 cm^{-1}.

Random coiled poly-L-glutamic acid[21] shows a broad, moderately intense amide III Raman band centered at 1248 cm^{-1}, the same frequency as random coil glucagon.[6] Random poly-L-lysine has a very broad amide III band centered at 1243 cm^{-1}, whose intensity (peak height) is intermediate between those of the α-helix and β conformations, relative to the 1446-1448-cm^{-1} methylene mode. This very broad band overlaps the region of the β-amide III band, exhibiting substantial spectral intensity at 1240 cm^{-1}.

Polypeptides in ^2H_2O solutions have not been so extensively studied by Raman spectroscopy. On deuteration, the amide III′ bands shift to around 950-1110 cm^{-1} but tend to be obscured by side-chain vibrations in that region.[13] Random coil poly-L-glutamic acid has a broad amide I′ band at 1656 cm^{-1},[20] close to that of random poly-L-lysine at 1660 cm^{-1}.[13] Helical poly-L-lysine in ^2H_2O shows a moderately strong amide I′ band at 1632 cm^{-1},[13] while the β conformer has a very intense band at 1658 cm^{-1}. This latter frequency appears to be about 5-7 cm^{-1} lower than the amide I′ band in proteins containing considerable β-sheet.[1] However, all other bands in poly-L-lysine in its various conformations appear at approximately the same frequency and with roughly the same intensity as those in proteins with considerable amounts of a particular conformation.

It therefore appears that the Raman spectra of proteins (if poly-L-lysine could be considered to be a model) should have three frequencies whose intensities might be used to monitor the conformation of the protein residues in solution: 1240 cm^{-1}, the amide III band in H_2O due to random coil and β-sheet; 1632 cm^{-1}, the amide I′ band in ^2H_2O due to α-helix;

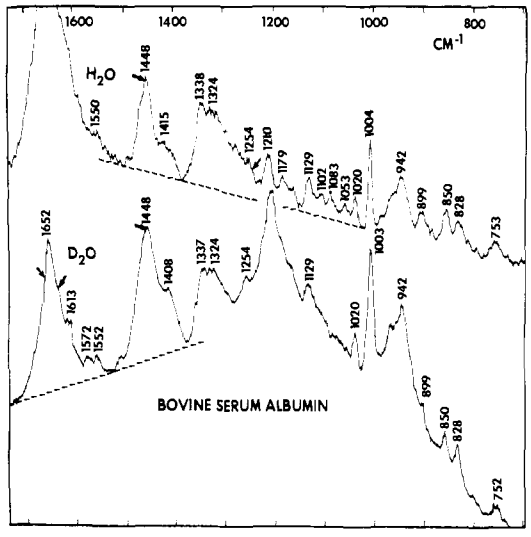

Figure 3. Raman spectra of bovine serum albumin solutions (7%) in H_2O and ^2H_2O. Conditions as in Figure 1.

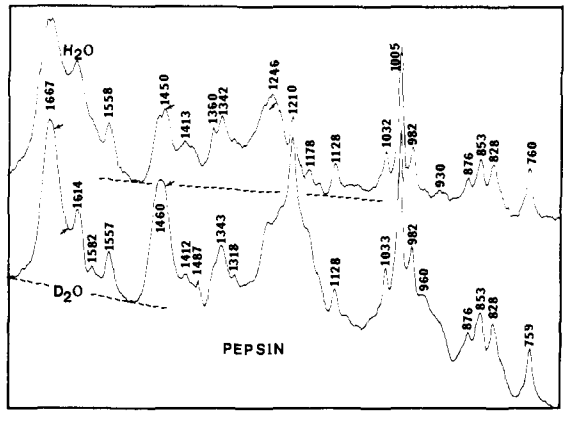

Figure 4. Raman spectra of pepsin solutions (7%) in H_2O and ^2H_2O. These spectra were obtained with photon-counting equipment as outlined in Materials and Methods. Conditions as in Figure 1, except p = 200 mW, s = 3000 counts full scale.

and 1660 cm^{-1}, the amide I′ band in ^2H_2O due to random coil and β-sheet. It is possible that further information could be obtained from the 1311-cm^{-1} amide III band of the α-helix; however, interfering methylene deformation bands of the side chains are also observed at that frequency so that the intensity contribution from the backbone amide III would be difficult to ascertain. We do not further consider this frequency.

In proposing a technique to unravel the conformational information at the frequencies mentioned above, we follow the well-established procedure for protein structural analysis by ORD and CD and propose that the Raman active amide modes of a protein are linear superpositions of bands due to the component conformations. In general, this will lead to severely overlapping bands which are usually not uniquely decomposable. However, if we measure the spectral heights of a protein at the conformationally sensitive frequencies 1240 cm^{-1} in H_2O and 1632 and 1660 cm^{-1} in ^2H_2O relative to some band whose intensity does not depend on conformational content and is not affected by deuteration, say the 1448-cm^{-1} CH_2 bending mode, then these relative spectral intensities will be additive functions of the fractions of α-helix, β-sheet, and random coil present in the protein. If we further make the simplifying assumption that those are the only conformations contained in the protein, then the three relative spectral intensities contain enough information to determine the fractions of each conformation present.

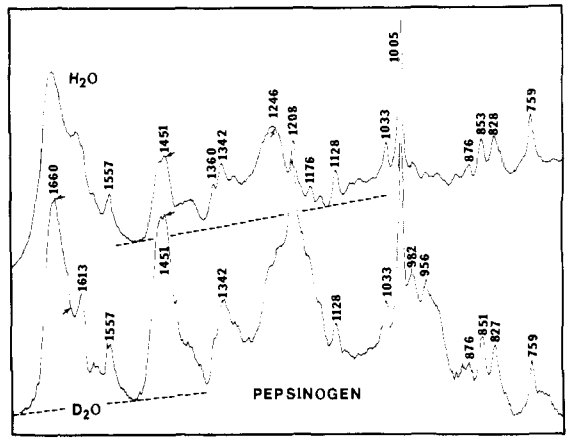

Figure 5. Raman spectra of pepsinogen solutions in H_2O and ^2H_2O. Conditions as in Figure 4.

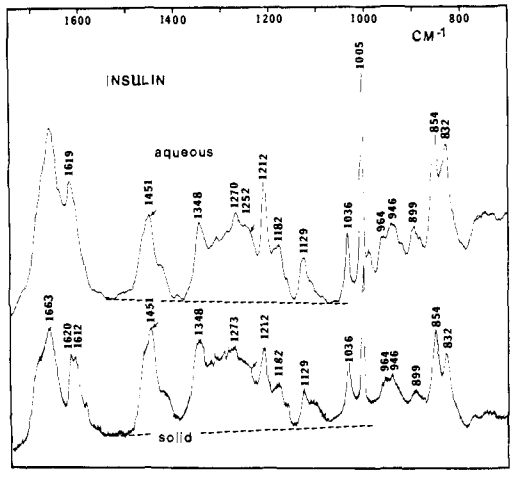

Figure 6. Raman spectra of insulin in aqueous solution (7%) at pH 2.0 and precipitated from water at pH 7.0. Conditions as in Figure 4.

The relationship between conformational content and the relative spectral intensities of the Raman spectrum of a protein is given by four simultaneous equations.

$$C^{\text{protein}} I_{1240}{}^{\text{protein}} = f_\alpha I_{1240}{}^{\alpha} + f_\beta I_{1240}{}^{\beta} + f_R I_{1240}{}^{R} \quad (1)$$

$$C^{\text{protein}} I_{1632}{}^{\text{protein}} = f_\alpha I_{1632}{}^{\alpha} + f_\beta I_{1632}{}^{\beta} + f_R I_{1632}{}^{R} \quad (2)$$

$$C^{\text{protein}} I_{1660}{}^{\text{protein}} = f_\alpha I_{1660}{}^{\alpha} + f_\beta I_{1660}{}^{\beta} + f_R I_{1660}{}^{R} \quad (3)$$

$$1.0 = f_\alpha + f_\beta + f_R \quad (4)$$

In these equations $I_{1240}{}^{\text{protein}}$ is the spectral height of the protein Raman spectrum measured at 1240 cm^{-1} in water relative to the spectral height of the methylene bending mode at 1448 cm^{-1}; $I_{1240}{}^{\alpha}$, that for "pure" α-helical protein (i.e., poly-L-lysine); f_α, f_β, f_R are the fractions of residues in the α-helix, β-sheet, and random coil conformations in the protein; and C^{protein} is a scaling constant (to be determined) for the methylene band intensity relative to the model compound, poly-L-lysine.

Results and Discussion

To test the suggestion that Raman spectra of proteins could be interpreted to determine conformational content, Raman spectra of nine proteins were obtained in water and ^2H_2O solutions: lysozyme, ribonuclease A, α-chymotrypsin, chymotrypsinogen, bovine serum albumin, concanavalin A, pepsin, pepsinogen, and insulin. In addition, spectra of insulin crystallized from water and ^2H_2O at pH 7 were measured. Examples of these spectra are shown in Figures 1-7. Spectra of

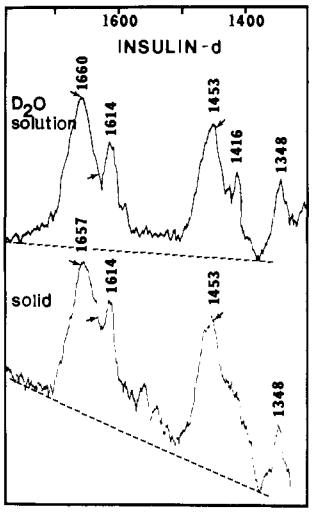

Figure 7. Raman spectra of insulin in ^2H_2O solution at pD 2.0 and precipitated from ^2H_2O at pD 7.0. Conditions as in Figure 4, except s = 1000 counts full scale.

several of these proteins have been reported previously[1-3,5,6] although not in the present detail. The three arrows in each of Figures 1-7 show the spectral intensities which, measured relative to the methylene deformation intensity at 1448 cm^{-1}, are the parameters $I_{1240}^{protein}$, $I_{1632}^{protein}$, and $I_{1660}^{protein}$, required for each protein in eq 1-4. These relative spectral intensities are listed for all proteins in Table I, along with the relative spectral intensities of poly-L-lysine in its three conformations obtained from Yu et al.[13]

It was felt that relative spectral intensities of poly-L-lysine in its various conformations could serve as the model (i.e., the $I_\nu^{configuration}$ in eq 1-3) for the conformational analysis of the Raman spectra of proteins, with the exception of those model intensities in the amide I′ of the β form of polylysine. One reason for this difficulty is apparent in the Raman spectra of proteins containing large fractions of β-sheet structures, e.g., ribonuclease A (Figure 2) and pepsin (Figure 4). In these cases, if poly-L-lysine were to serve as the model for the β component, we would expect a strong band at 1240 cm^{-1} in H_2O as well as a strong band at 1658 cm^{-1} in ^2H_2O. In Figures 2 and 4, the former is present, but the latter is shifted to 1665 cm^{-1}. It is a general feature of proteins with large β contents that amide I′ maxima are observed 5-7 cm^{-1} higher than in β-sheet poly-L-lysine.

This particular discrepancy is perhaps not surprising in view of the fact that β-sheet conformations are often not so well developed in proteins as they are in polylysine. Indeed, circular dichroism parameters for β conformations in proteins have been compared with those in poly-L-lysine[10,19] and found to be substantially different. At any rate, it is clear that the parameters (I_ν^β) taken from the Raman spectra of poly-L-lysine in the β-sheet conformation are not applicable to globular proteins in the amide I′ region.

Paralleling the procedure applied by Saxena and Wetlaufer[19] to the determination of model circular dichroism parameters, revised model Raman spectral intensities I_{1632}^β, I_{1660}^β, I_{1632}^R, and I_{1660}^R were obtained by solving eq 1-4 using the x-ray determined structural content of lysozyme and ribonuclease A, without varying the five other $I_\nu^{configuration}$ from those of polylysine. The revised spectral intensities for the model are compiled in Table II. Notice that major changes have occurred in I_{1632}^β and I_{1660}^β, as suggested, while the revised I_{1632}^R and I_{1660}^R are within experimental error of the same parameters of poly-L-lysine.

The parameters from Table II were incorporated into eq 1-4 with the measured Raman spectral intensities of Table I to predict the secondary structure content of the other seven proteins. The conformational contents predicted in this manner from the Raman spectra are compared with those estimated from x-ray diffraction and CD in Table III. The Raman predictions agree well with those predicted by other techniques.

Such agreement suggests that the techniques outlined here may be applied with some confidence (perhaps ±10-15%) to other proteins, to the extent that they contain only α-helical, antiparallel β-sheet, and random coil peptide segments. It is possible that other polypeptide conformations may be incorporated into the formalism developed here, as Raman bands diagnostic of those conformations are assigned in suitable model compounds.

The conformational properties predicted for insulin (see Table III and Figures 6 and 7) are particularly interesting, because this work quantitates, for the first time, a conformational difference between a crystalline protein and its aqueous solution. Yu,[6] on the basis of an earlier Raman study of insulin, had qualitatively suggested that insulin in solution contains considerably less α-helix than does the crystal, and this work confirms that a decrease in helix of 10-17% does accompany dissolution. The lesson here is that solution conformations of proteins should *not* always be assumed to be identical with those determined by x-ray diffraction in crystals, although in many cases they may be. It is our feeling that laser Raman spectroscopy will be a valuable tool in elucidating the extent to which crystal conformations of proteins do differ from those

Table I. Raman Spectral Intensities of Three Conformations of Poly-L-lysine and of Several Proteins Relative to the Spectral Intensity of the 1446-1448-cm^{-1} Band[a]

	H_2O soln, 1240 cm^{-1}	^2H_2O soln	
		1632 cm^{-1}	1660 cm^{-1}
Poly-L-lysine, α helix[b,c]	0.00	0.80	0.55
Poly-L-lysine, β sheet[b,d]	1.20	0.33	1.22
Poly-L-lysine, random coil[b,e]	0.60	0.21	0.70
Lysozyme	0.64	0.50	0.99
Ribonuclease A	0.92	0.52	0.99
α-Chymotrypsin	0.86	0.36	0.88
Chymotrypsinogen	0.78	0.44	0.83
Bovine serum albumin	0.30	0.69	0.86
Concanavalin A	1.39	0.58	1.28
Pepsin	1.20	0.48	1.28
Pepsinogen	1.10	0.56	1.13
Insulin, solution[f]	0.75	0.55	1.10
Insulin, crystalline[g]	0.60	0.65	0.91

[a] Unless otherwise reported, all measurements are of Raman spectra of 5-7% solutions in H_2O or ^2H_2O at pH 7. All values are ±0.05 and are the average of 2-3 measurements. [b] Measured from the spectra of ref 13 and repeated at least twice from spectra obtained for poly-L-lysine solutions prepared as in ref 1. [c] pH or pD 11.8, T = 4 °C. [d] pH or pD 11.8, T = 52 °C or 22 °C after heating to 52 °C for 1 h. [e] pH or pD 3.7, T = 22 °C. [f] pH or pD 2.1, T = 22 °C. [g] Crystallized from H_2O or ^2H_2O at pH 7.1. Spectra were obtained of crystals in contact with supernatant solution.

Table II. Relative Raman Spectral Intensities, I_ν^α, I_ν^β, and I_ν^R, for Determination of Conformational Content in Proteins

	H_2O soln, 1240 cm^{-1}	^2H_2O soln	
		1632 cm^{-1}	1660 cm^{-1}
Rel intensity for α helix	0.00	0.80	0.55
Rel intensity for β sheet	1.20	0.72	0.88
Rel intensity for random coil	0.60	0.08	0.78

Table III. Comparison of Secondary Structural Content (%) Estimated by Raman Spectral Analysis and by X-Ray Diffraction and Circular Dichroism

	Ref	Percent of total residues			Scale factor
		α helix	β	Random coil	C
Lysozyme					
Raman	This work[a]	32	9	59	0.72
X-ray diffraction	*b*	29-42	10	48-62	
Circular dichroism	*c-e*	26-29	6-11	55-68	
Ribonuclease A					
Raman	This work[a]	14	35	51	0.79
X-ray diffraction	*f*016-18	36	46-58		
Circular dichroism	*c-e*	9-16	31-34	50-59	
α-Chymotrypsin					
Raman	This work	4	34	62	0.91
X-ray diffraction	*g, h*	3-9	22-50	47-70	
Circular dichroism	*c, g, i*	8-20	10-32	55-82	
Chymotrypsinogen					
Raman	This work	14	37	49	0.95
X-ray diffraction	*j*	6-14	22-50	44-65	
Circular dichroism	*c, g, i*	9-20	10-36	54-70	
Bovine serum albumin					
Raman	This work	60	0 (−2)[r]	40	0.73
Circular dichroism	*k*	54-62	0-3	41-51	
Concanavalin A					
Raman	This work	0 (−1)[r]	48	53	0.65
X-ray diffraction	*l*	3	57	41	
Pepsin					
Raman	This work	4	36	60	0.68
X-ray diffraction	*m*	Small			
Circular dichroism	*n*	5-10	34-58	37-56	
Pepsinogen					
Raman	This work	9	39	52	0.68
Circular dichroism	*o*	5-13	36-49	46-54	
Insulin (pH 2.1)					
Raman	This work	29	11	59	0.66
Circular dichroism	*k*	20-30	10-25	47-55	
Insulin (crystals or supernatant)					
Raman (crystals)	This work	42	19	39	0.77
X-ray diffraction	*p*	52	6	42	
Circular dichroism	*q*	35-45	5-10	40-55	

[a] Structural contents for lysozyme and ribonuclease A are those used to determine amide I′ spectral intensities for the β sheet and random coil conformation of the protein residues as reported in Table II. [b] D. C. Phillips, *Proc. Natl. Acad. Sci. U.S.A.*, **57**, 484 (1967). [c] Reference 17. [d] Reference 18. [e] T. D. Barela and D. W. Darnall, *Biochemistry*, **13**, 1694 (1974). [f] G. Kartha, J. Bello, and D. Harker, *Nature (London)*, **213**, 862 (1967). [g] Reference 19. [h] P. B. Singler, D. M. Blow, B. W. Matthews, and R. Henderson, *J. Mol. Biol.*, **35**, 143 (1968). [i] J. Kraut, *Enzymes*, **3**, 165 (1971). [j] S. T. Freer, J. Kraut, J. D. Robertus, H. T. Wright, and N. H. Xuong, *Biochemistry*, **9**, 1997 (1970). [k] Calculated by the method of ref 19 from S. N. Timasheff, H. Susi, R. Townsend, L. Stevens, M. J. Gorbunoff, and T. F. Kumosinski, "Conformations of Biopolymers", Vol. 1, G. N. Ramachandran, Ed., Academic Press, New York, N.Y., 1967, p 173. [l] K. D. Hardman and C. F. Ainsworth, *Biochemistry*, **11**, 4910 (1972). [m] N. S. Andreeva, V. V. Borosov, N. N. Goborum, V. R. Melikada, V. S. Raiz, V. A. Rostovtski, and N. E. Shutskeva, *Dokl. Akad. Nauk SSR*, **192**, 216 (1970). [n] Calculated by the method of ref 19 from B. Jirgensons, *Biochim. Biophys. Acta*, **200**, 9 (1970). [o] Calculated by the method of ref 19 from K. Grizzuti and G. E. Perlman, *J. Biol. Chem.*, **244**, 1764 (1969). [p] T. Blundell, G. Dodson, D. Hodgkin, and D. Mercola, *Adv. Protein Chem.*, **26**, 279 (1972). [q] Calculated by the method of ref 19 from B. H. Frank and A. J. Veros, *Biochem. Biophys. Res. Commun.*, **32**, 155 (1968). Circular dichroism spectra are reported by them for insulin supernatant at pH 7, the pH at which insulin crystals are precipitated. [r] Small negative values are assumed zero within the precision of the method.

in solution. In that regard, it is interesting that, in the case of insulin (Table III), the conformation appears to be rather more pH dependent than phase dependent. Notice that the circular dichroism of the supernatant from crystals at pH 7 agrees with both the Raman and x-ray diffraction estimates for crystals precipitated at that pH but is very different from the Raman and circular dichroism determined conformations of the solution at pH 2.1.

Notwithstanding the agreement between Raman estimates of conformational content and x-ray diffraction and circular dichroism as shown in Table III, there are certain aspects of the technique proposed here which may add to the uncertainty of the method. The first, and most serious, objection is that the method requires spectral measurements in water and in ^2H_2O. This places two constraints on the model: first, that the amide protons will be completely exchanged in ^2H_2O and, second, that the protein does not change conformation between the two solvents. Blout and co-workers[15] have examined the kinetics of exchange of amide protons in ^2H_2O and have found that after approximately 3 h, exchange of amide protons has reached an equilibrium of approximately 85-90% exchange in globular proteins. Therefore, care must be taken that spectra in ^2H_2O are obtained only after exchange is complete. The question of conformational changes between water and ^2H_2O solutions is not so easily addressed. We can only suggest that the results shown in Table III suggest that *major* conformational changes have not occurred with the proteins studied in this work between the two solvents. Indeed, it may be possible, through a study of suitable model compounds, to employ laser Raman spectroscopy to elucidate the extent of such conformational changes, if any.

A second aspect of the uncertainty which must be ascribed

to the proposed method is that Raman spectral heights are measured, rather than integrated band intensities, and these spectral heights are often measured along the steep sides of bands, rather than at maxima. Indeed, it is our feeling that it is this aspect of the procedure which leads to the differences between Raman estimates of conformational content and those of x-ray diffraction and circular dichroism. But we also feel that the present state of the art of Raman spectroscopy will not allow spectra of such precision that an envelope of overlapping bands, say in the amide I region, can be uniquely decomposed into their components. It is to be anticipated that, as the state of the art improves, such decomposition and subsequent analysis of integrated band intensities may take the place of the approximate method outlined here. Nevertheless, it is our feeling that the technique of conformational analysis by Raman spectroscopy of proteins can be a powerful tool to the understanding of protein conformations in situations that were previously inaccessible, e.g., differences between crystal and solution conformations and the conformations of proteins in highly scattering suspensions.

Finally, we should like to discuss the relative intensity of the methylene deformation observed in proteins at 1448 cm^{-1} in comparison to that in poly-L-lysine. A correlation was attempted between the parameter $C^{protein}$, obtained from solution of eq 1-4, with the number of C-H and $C-H_2$ groups present in the protein, relative to poly-L-lysine. No correlation was obtained. The parameter $C^{protein}$ represents, in the present formulation, the intensity of the 1448-cm^{-1} band in the protein, relative to poly-L-lysine. The band at that frequency has been assigned[4] to a combination of C-H and $C-H_2$ deformations and its intensity would be proportional to the number of C-H and $C-H_2$ groups present only if all such vibrations had the same intensity. For this reason, the lack of correlation is not surprising. It is interesting to notice, however, that for a particular protein, insulin (Table III), the parameter $C^{protein}$ is larger in the crystal than in the solution. We have other, unpublished, Raman spectra of additional proteins, lyophilized, crystalline, and in solution, which also show a tendency for $C^{protein}$ to increase with decreasing amounts of solvent. Although extremely speculative at this time, it may be possible that further investigation of the parameter in proteins may yield information about the interaction between the hydrophobic C-H moiety and its aqueous environment.

In this paper we have outlined a technique for the estimation of secondary structure contents of proteins in aqueous solution, as well as the solid phase, by interpretation of Raman active amide vibrations. This technique may prove particularly useful as a bridge between solid-phase structures of proteins and those in solution because Raman spectra can be obtained and interpreted for both. Furthermore, this technique shows promise of examining protein conformational changes in dispersive systems, particularly the biological membrane, since no compensation is required for Rayleigh scattering in the Raman experiment.

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