6.5 eu for the entropy which is lost upon freezing an internal rotation adjacent to the imidazolyl double bond. We also took account of a partial compensation by a favorable enthalpy function change of 0.5 kcal/mol as they did, and obtained 1.44 kcal/mol, corresponding to a rate factor of about 12 at 25 °C. This free energy is comparable to 1.8 kcal/mol (corresponding to a rate factor of 21) which was given by Page and Jencks[13a] for the free rotation about the methylene–carboxyl residue bond. They estimated the free energy change using data of gas-phase unimolecular ester cleavages, but we have no related data about imidazoles. The reasons that the imidazolyl group can be approximated by the carboxyl group are as follows: (1) the reduced barrier to rotation for the former may be comparable to that for the latter (0 kcal/mol for carboxyl and probably ~1 kcal/mol for imidazolyl);[39] (2) the free-rotor partition function for the former must be somewhat larger than that for the latter but it will not cause a significant change in entropy;[13a,39] (3) solvation effects in solution take place for both groups,[13] but the effects must be minor as far as the internal rotation is concerned, although the effects are expected to raise the barrier to rotation through solute–solvent interactions. The thermodynamic quantities cited above were calculated at 25 °C while our experiments were carried out at 50 °C. The temperature dependence of the entropy of internal rotation is negligible for the above temperature difference[40] but the corresponding free energy $T\Delta S$ is 1.08 times larger than $T\Delta S$ for 25 °C. Thus the rate factor at 50 °C should be 14. Considering the uncertainties involved, we prefer the rate factor in the range of 12 to 21.

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Laser-Excited Raman Spectroscopy of Biomolecules. VIII. Conformational Study of Bovine Serum Albumin

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Abstract: From the Raman spectrum of 4% aqueous bovine serum albumin the polypeptide backbone is found to be predominantly α-helical (~60%) as revealed by the distribution of intensity in the amide III region and by the relative sharpness of the amide I line. The remainder of the backbone is random-coil and no evidence for β-pleated-sheet conformation can be detected. The intensity ratio (10:7) of the tyrosine doublet at 852 and 827 cm^{-1} indicates that most of the phenolic hydroxyl groups of the 19 tyrosyl residues are weakly hydrogen bonded. The disulfide line is rather broad and low in frequency (503 cm^{-1}), showing that there is some variation in the local geometries of the disulfide groups. Their conformations, however, appear to be limited to the gauche, gauche, gauche form.

Although the amino acid composition of bovine serum albumin (BSA), a major protein constituent of plasma with a molecular weight of about 67000 daltons, has been known[1] since 1949, the complete sequence has apparently not yet been determined.[2,3] Previous investigations of its conformation include reports on its optical rotatory dispersion,[4,5] infrared absorption,[6] and an earlier study of its Raman spectrum in aqueous solution from this Laboratory.[7] These investigations agree in assigning a large amount of α-helical conformation to the native protein. In the present paper much improved spectra of the protein are reported and since much progress has been made in the interpretation of the Raman spectra of proteins in the past five years, more detailed conclusions about the conformation of BSA can now be drawn, especially about the polypeptide backbone conformation and the state of the tyrosyl residues in the protein.

Experimental Section

BSA of high purity was kindly provided by Dr. E. R. Simons and Professor E. R. Blout of the Harvard Medical School. It was used without further treatment at a concentration of 4% by weight in 0.1 *M* NaCl, pH 6. The solution (3 μl) was placed in a "Kimax" melting point capillary (1 mm i.d.) used as a Raman cell.

Raman spectra were recorded on a Spex Ramalog 4 double-monochromator spectrometer equipped with photon counting electronics, an RCA C31034 photomultiplier, and a Coherent Radiation 52G argon-ion laser tuned to 4880 Å as the exciting line. The scattered radiation was observed at 90° to the incident beam.

Results and Discussion

A representative Raman spectrum of aqueous BSA is shown in Figure 1 as recorded, and the frequencies and relative peak intensities of the Raman lines are listed in Table I. Interpretation of the spectrum begins with those lines that are characteristic of the peptide backbone.

Amide III Region. It has been recognized for some time that the region between 1225 and 1300 cm^{-1} in the Raman spectra of proteins is sensitive to the geometry of the peptide groups making up the backbone of the protein. The amide III vibration has been shown[8-10] to be a mixture of C–N bond stretching and in-plane N–H bond bending, which gives rise to a frequency near 1230–1235 cm^{-1} for

Table I. Raman Spectrum of BSA[a]

Frequency, cm^{-1}	Tentative assignment	Frequency, cm^{-1}	Tentative assignment
422 (0)		1055 (2)	
462 (0)		1083 (2)	
503 (2)	ν(SS)	1105 (2)	ν(CN)
573 (0)		1128 (3)	
621 (2)	Phe	1159 (2)	
642 (1)	Tyr	1177 (3)	Tyr + Phe
672 (1)	ν(CS)	1207 (5)	
697 (0)		1248 (5)	Amide III
750 (1)	Trp	1272 (5)	
804 (0)		1320 (8)	γ(CH_2) (?)
827 (3)	Tyr	1340 (9)	
852 (4)		1415 (3)	ν(CO_2^-)
884 (0)		1449 (10)	
900 (3)		1458 (5 sh)	δ(CH_2)
941 (6)	ν(CC)	1550 (2)	Amide II
960 (4 sh)		1587 (0)	Phe
1004 (10)	Phe	1605 (1)	Tyr + Phe
1032 (2)		1615 (0)	
		1652 (21)	Amide I + H_2O

[a] Solution 4% by weight, pH 6, 0.1 *M* NaCl. Frequencies for sharp lines are accurate to ±1 cm^{-1} and broad lines to ±2 cm^{-1}. Numerical figures in parentheses are relative peak intensities with that of 1449 cm^{-1} taken as 10. sh denotes shoulder. ν means stretching vibration, δ deformation, and γ twisting.

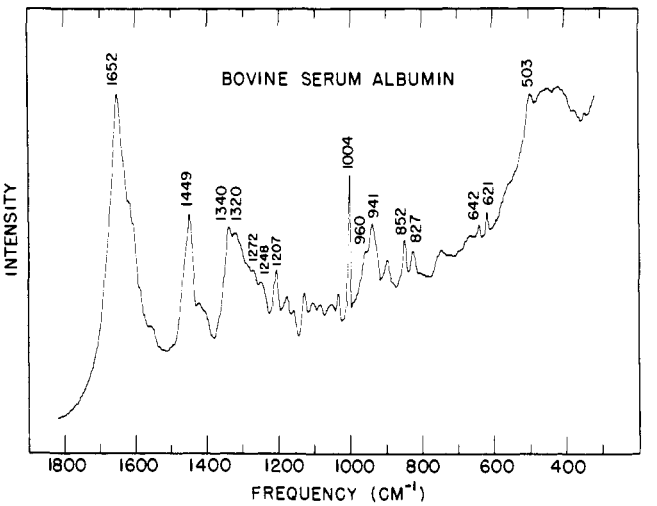

Figure 1. Raman spectrum of 4% BSA as originally recorded: spectral slit width 5 cm^{-1}, time constant 2 sec, scan speed 0.5 cm^{-1}/sec, laser power at the sample approximately 150 mW at 4880 Å.

peptide links in a β-pleated-sheet conformation, near 1240–1250 cm^{-1} for random-coil geometry, and from 1260 to 1300 cm^{-1} for α-helical conformation.

Although the distribution of intensity in the range 1225–1300 cm^{-1} in Figure 1 is warped by the strong lines at 1320 and 1340 cm^{-1} due to the aliphatic side chains, it is still possible to say that there is virtually no β form as indicated by the absence of intensity near 1230–1235 cm^{-1} and that the amide III intensity due to α-helix (1260–1300 cm^{-1}) is substantially larger than that due to random coil (peak at 1248 cm^{-1}). While quantitative assessment is likely to be rather rough, the relative amounts of helical and random-coil residues appear to be 55–60 and 45–40%.

Amide I Region. Usually the intensity distribution in the amide I region in aqueous solution is seriously influenced by the water band at about 1640 cm^{-1}. However, in the spectrum of BSA the amide I frequency at 1652 cm^{-1} is so sharp and strong that the underlying water band has little effect on its shape. This shape is a consequence of the large helical content, whose contribution to the amide I line resembles in sharpness that of a highly ordered homopolypeptide such as the α-helical form of poly-L-alanine.[8,9] The contribution of the random-coil fraction appears as a broadening of the high-frequency side of the line near 1665 cm^{-1}. Thus the amide I line confirms the high helical content indicated by the amide III contour.

These results are in agreement with earlier conclusions of high helical content of BSA from optical rotatory dispersion[4,5] and infrared absorption.[6] It might also be mentioned that the line at 941 cm^{-1} is unusually strong. This line has been associated with the helical conformation by Frushour and Koenig.[9] To be sure, part of the intensity of the line may be due to side-chain frequencies of residues such as glutamic and aspartic acids, leucine, and lysine, but these residues are comparably abundant in β-lactoglobulin, whose spectrum[7] shows much less intensity in this region (900–970 cm^{-1}).

Tyrosine Doublet at 827–852 cm^{-1}. It has been shown earlier[7,11,12] that the intensity ratio of this doublet is determined by the nature of the hydrogen bonding of the phenolic hydroxyl in the tyrosyl residue, which determines the extent of the Fermi resonance between the two components.[12] When the hydroxyl is strongly hydrogen bonded to a negative acceptor such as CO_2^-, the line of higher frequency is weaker than the lower (typical ratio of peak intensities 3:10) whereas the upper becomes more intense if the H bonding is weaker or if an acidic external proton is bonded to the oxygen of the phenolic OH (typical ratio 10:4). There are about 19 tyrosyl residues per BSA molecule and the mean intensity ratio for their doublets (Figure 1) is 10:7. This suggests that a majority of the residues are in the latter category; that is, most of the hydroxyl groups of the tyrosyl residues, whether "buried" or "exposed",[13-16] form weak hydrogen bonds or serve as acceptors to other protons that are acidic donors. Thus in the native protein at pH 6, where probably most of the tyrosyl residues are in α-helical segments, there are few if any available negative binding sites such as CO_2^- for the phenolic OH's.

Frequencies of the Disulfide Bridges. There are about 18 disulfide groups per BSA molecule, which is the same number per unit molecular weight as in lysozyme. The characteristic S–S frequency occurs at a slightly lower value (503 cm^{-1}) in BSA thn in lysozyme (509 cm^{-1}) and with a noticeably wider half-width.[17] Thus we conclude that there is a wider variation in local geometry of the S–S bridges in BSA. Variation in the S–S frequency has been ascribed to changes in the dihedral angle between the C–S–S and S–S–C planes,[17,18] but there is some question about the validity of this interpretation.[19,20] In any case there is little evidence of a shoulder on the high-frequency side of the S–S line due to frequencies in the range 525–545 cm^{-1}. This range has been assigned to the S–S stretching vibration of the -C–S–S–C- groups in gauche,gauche,trans and trans, gauche,trans surroundings in accordance with model compound studies.[21-23] If this assignment is correct, it appears that nearly all (14 or more) of the S–S groups in BSA are not in such conformations but must be in the gauche,gauche,gauche form, whose S–S frequency falls in the range 500–515 cm^{-1}.

There is relatively little methionine in BSA (three residues per molecule), so that most of the Raman intensity in the C–S stretching region (650–750 cm^{-1}) is due to the C–S bond vibrations of the cystine residues. These do not give rise to clear-cut lines in the spectrum because the variation in geometry mentioned above also affects the C–S frequencies and causes a broad distribution of intensity in this range. There are intensity maxima at approximately 672 and 697 cm^{-1}. These frequencies have been ascribed by Miyazawa and Sugeta[23] to conformations in which respectively the hydrogen atom on the α-carbon of cystine or the

amino nitrogen atom is in the trans position across the C^{α}-C^{β} bond from the near sulfur atom. The maximum at 672 cm^{-1} is somewhat more intense, implying more of the first conformation in BSA, but this statement has only qualitative significance under the present circumstances.

Spectrum of Lyophilized Powder BSA. Using the same sample as that of the present study Mendelsohn[24] had earlier obtained the Raman spectrum of the lyophilized powder. This spectrum shows more COOH (1732 cm^{-1}) and less CO_2^- (1415 cm^{-1}) than the aqueous solution at pH 6. In addition, the amide I and amide III lines suggest some decrease in the α-helical content: the former is broader and shifted upward (to 1660 cm^{-1}) and the latter shows more intensity in the neighborhood of 1245 cm^{-1} and less at 1280 cm^{-1}. Other differences include a substantial decrease in the band at 941 cm^{-1} and a slight alteration in the intensity ratio of the tyrosine doublet (to 10:9). All of the observed changes are consistent with some denaturation in the process of lyophilization, a result also observed by Yu et al.[25] for ribonuclease and other proteins.

Conclusions

The presence of a large amount (about 60%) of helical structure in native BSA, previously found by several different techniques, is confirmed by its Raman spectrum. The remainder of the polypeptide backbone was determined to have random-coil conformation and no indication of β-pleated-sheet conformation was observed. The tyrosyl residues are weakly hydrogen bonded, regardless of whether they are "buried" or "exposed" as determined by other methods. The disulfide bridges vary in conformation to a limited extent but not enough to alter their adjacent carbon chains from the gauche,gauche,gauche conformation.

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The Secondary β-Deuterium Isotope Effect in the Formation of Ethyl Radical from Decomposition of Methylethylethyl-2,2,2-d_3-carbinyloxy Radical[1a]

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Abstract: The intramolecular secondary β-deuterium isotope effect in the decomposition of methylethylethyl-2,2,2-d_3-carbinyloxy radical to methyl ethyl ketone and ethyl radical is k_{H_3}/k_{D_3} = 1.25 and is relatively temperature independent between 0 and 80 °C. The comparison of the observed effect with previous theoretical predictions is discussed as well as the predicted temperature dependence.

Several years ago we reported the secondary β-deuterium kinetic isotope effect in the formation of α-phenylethyl-β,β,β-d_3 radical from the concerted decomposition of azobis-α-phenylethane-β,β,β-d_3.[2] The isotope effect, 1.017 per deuterium, is in good agreement with that (k_H/k_D = 1.02) found in the concerted decomposition of *tert*-butyl perhydratropate which was reported by Koenig and Brewer while our previous work was in progress.[3] These studies were carried out to gain insight into the role of hyperconjugation in the stabilization of radicals. Subsequently, the β effects in the formation of *tert*-butyl radical from the concerted decomposition of *tert*-butyl perpivalate (k_H/k_D = 1.02 per D),[4a] the cumyl radical from *tert*-butyl dimethylphenylperacetate (k_H/k_D = 1.02 per D),[4b] the isopropyl radical from