aquation of *trans*-$[Co(trien)Cl_2]^+$.[14] On the other hand, in a rather complex study, the reaction of *trans*-$[Co(en)_2Cl_2]^+$ with ethylenediamine in methanol has been deduced to give the species *cis*-$[Co(en)_2(enH)Cl]^{3+}$, and it was concluded that this species was stable in aqueous solution.[15] We feel, however, that the chemical question is not yet fully resolved.

It is, of course, a possibility that ligand detachment at the point of labilization is followed by rearrangement to an intermediate of trigonal bipyramidal geometry rather than by prompt, stereoretentive coordination of solvent. Subsequent reactions of this intermediate would then give the observed products. We are not able at present to distinguish between these two types of possibilities but prefer the scheme of Figure 6 as being simpler and, so far, adequately predictive. Further, the five-coordinated intermediate seems to be preferred by kineticists,[3] and, if the same were involved for the photochemical reactions, it becomes awkward to explain their different stereochemistries.

Finally, some attempts have been made recently to rationalize the photochemistry of Cr(III) and Co(III) ammines in terms of σ and π bonding changes predicted by simple ligand field theory.[16,17] Zink would, for example, predict (correctly) that chloride aquation would occur on photolysis of *cis*-$[Co(en)_2Cl_2]^+$, due to the breaking of the weakest Co–ligand bond, although the contrast to the predominant photoinduced ammonia aquation in $[Co(NH_3)_5Cl]^{2+}$ requires some additional explanation. Also, his treatment does not make stereochemical predictions. The ligand field analysis of bonding differences between the ground and the excited states of $[Co(en)_2(H_2O)Cl]^{2+}$ becomes yet more complicated since the three pseudooctahedral axes are all different. It would now be necessary to decide that the Co–water bond is the weakest, but it is not clear now to proceed to predict that the cis isomer would be much more photosensitive toward isomerization than the trans one.

Acknowledgment. This investigation was supported in part by Grant 27984X2 between the National Science Foundation and the University of Southern California.

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Laser-Excited Raman Spectroscopy of Biomolecules. V. Conformational Changes Associated with the Chemical Denaturation of Lysozyme

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Abstract: When lysozyme is chemically denatured by reductive cleavage of the S–S bonds with subsequent cyanoethylation of the sulfhydryl groups, its Raman spectrum is drastically changed. The amide III frequency decreases to 1243 cm^{-1} from 1260 in aqueous solution of the native enzyme and intensifies considerably, while the amide I increases to 1672 cm^{-1} from 1660 with sharpening. These changes are to be expected if the protein assumes a random-coil conformation. Similar changes are found when the native enzyme is denatured by 6 *M* LiBr or precipitated from a mixture of 1% SDS–7% lysozyme, but the S–S bonds remain intact though distorted. However, at SDS concentrations of 7% or higher, lysozyme is soluble with little apparent conformational change.

The denaturation of aqueous lysozyme by thermal means has been studied in several investigations by optical rotatory dispersion,[1] pmr spectroscopy,[2,3] and the Raman effect.[4,5] It is of interest to examine the effects of other denaturing agents on this enzyme, both to determine their structural effects as compared with thermal denaturation and also try to learn something about the changes in the spectra of various important constituents of the molecule when their intramolecular environments alter. Since chemical denaturation can be carried out with a variety of agents, some measure of control over the conformational changes can be exerted and the spectroscopic changes produced thereby can be interpreted more precisely.

In this paper the effects on the Raman spectrum of aqueous lysozyme produced by lithium bromide (LiBr), by sodium dodecyl sulfate (SDS), and by S–S bond reduction and subsequent blocking of the resultant sulfhydryl groups by S-cyanoethylation are reported. Some of the these results have been published in preliminary form.[6,7]

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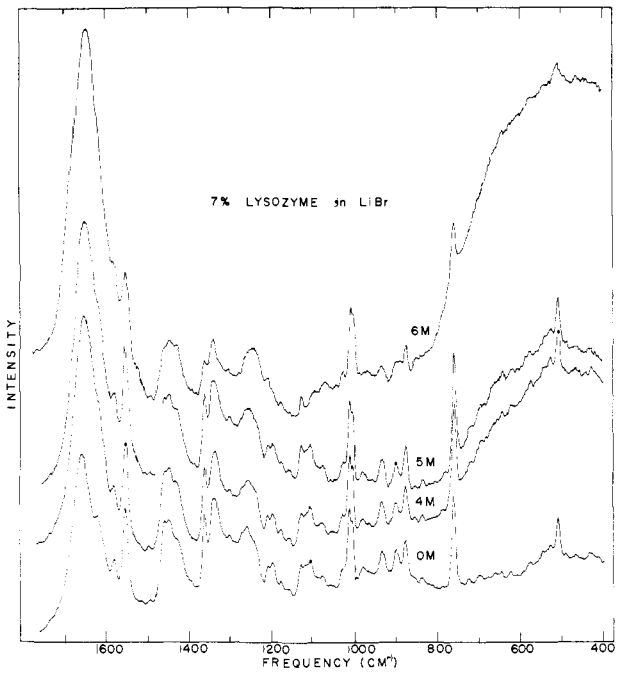

Figure 1. Raman spectra of 7% lysozyme in 0, 4, 5, and 6 *M* LiBr, pH 4.

Experimental Section

Methods. Hen's egg white lysozyme, supplied by Schwarz/Mann as twice-crystallized, salt-free, lyophilized material, was used without further purification. Purified anhydrous LiBr, purchased from City Chemical Corp., was recrystallized from water and dried at 150° for 3 days before usage. SDS, obtained from Fisher Scientific Co. as U.S.P. grade, was recrystallized twice from 95% ethanol.

At 20°, 70 mg of lysozyme dissolves readily in 1 ml of LiBr solution of 2 *M* or higher; for solution of 1 *M* a higher temperature (35°) is needed. The minimum SDS concentration needed to make 7% lysozyme is about 7%, because the enzyme precipitates in more dilute SDS solutions. The range of SDS concentration used in this study was 0.3–15%. The SDS–lysozyme complex was obtained by attempting to make a 7% solution of enzyme in 1% SDS. Approximately 70% of the enzyme and at least 95% of the SDS were precipitated, as determined from the Raman spectrum of the solution after filtration through a 0.05 μm Millipore filter. The SDS-precipitated lysozyme was air-dried. *S*-Cyanoethyl lysozyme was prepared by the procedure reported by Seibles and Weil.[8] Amino acid analyses of SDS–lysozyme and *S*-cyanoethyl lysozyme were consistent with the total amino acid composition of the enzyme.

The sample handling technique and the instrumentation for recording Raman spectra of solutions have been described previously.[5] Spectra of solid samples were recorded with both Cary Model 81 and Jarrell–Ash Model 101 Raman spectrometers, equipped with a Coherent Radiation Model 52G argon-ion laser adjusted to produce approximately 70 mW of power in the 4880-Å exciting line at the sample. Solid samples were packed into a conical depression at the end of a stainless steel rod and scattered radiation was collected at 180 and 90° to the incident beam with Cary and Jarrell–Ash instruments, respectively. Frequency readings are accurate to ±2 cm^{-1}. All spectra were obtained with a spectral slit width of 6 cm^{-1} and each required 1–2 hr for recording.

Results and Discussion

1. Denaturation by LiBr. LiBr is well known to be a destabilizer of the structure of biological molecules.[9,10]

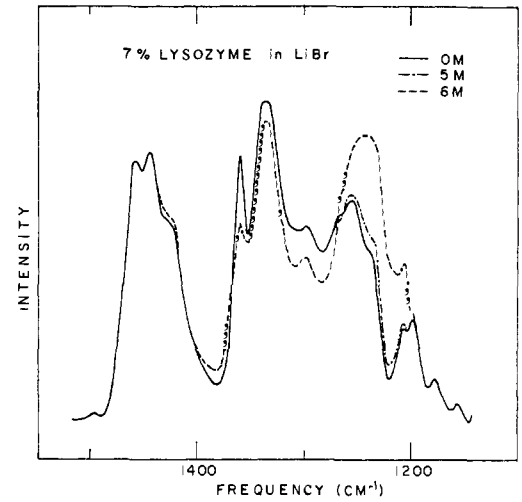

Figure 2. Redrawn Raman spectra (1150–1500 cm^{-1}) of 7% lysozyme in 0, 5, and 6 *M* LiBr, pH 4, normalized to the intensity of the methylene deformation mode at 1448 cm^{-1}, after correction for the water background.

Aqueous LiBr has a Raman spectrum[11] in the 200–2000-cm^{-1} region that is qualitatively much the same as that of pure water except for a substantial intensity increase below 800 cm^{-1} and at 1645 cm^{-1}. Thus the denaturation of lysozyme by LiBr can be followed conveniently by Raman spectroscopy.[6] Figure 1 shows the spectra of 7% lysozyme at LiBr concentrations of 0, 4, 5, and 6 *M*, and Figure 2 compares the four spectra in detail for the amide III region. Since there is essentially no change in the spectrum between 0 and 4 *M*, only a single spectrum is shown for this range.

At 5 *M* LiBr the amide III region starts to change, with the shoulder at 1273 cm^{-1} becoming weaker while the peak at 1260 cm^{-1} and the shoulder at about 1238 cm^{-1} are both elevated as the result of the addition of intensity centered near 1245 cm^{-1}. At 6 *M* LiBr the peak shifts to 1245 cm^{-1} and increases substantially in intensity. The shoulder at 1273 cm^{-1} has almost disappeared while that at 1238 cm^{-1} is no longer visible, either because it is missing or is completely overlapped by the main peak. Our interpretation of these changes is that the protein backbone has been converted mainly to the random-coil form, as was also the case in irreversible thermal denaturation.[5]

Interpretation of the remaining changes is less clear-cut but they are consistent with those seen in the amide III region. The amide I peak is expected to increase from 1660 in the native enzyme to about 1670 cm^{-1} as the amide III frequency decreases, but this expected shift is completely masked by overlap with the intensifying solvent peak at 1645 cm^{-1}; the observed maximum at this point in 6 *M* LiBr is almost entirely due to the solvent. There is a definite broadening and decrease in peak intensity of the disulfide frequency at 509 cm^{-1}, no doubt as the result of several different allowed conformations of the four disulfide groups. The strong solvent background from 6 *M* LiBr makes quantitative assessment quite difficult, but it appears that the half-width of the band has nearly doubled. Its area, however, seems to be unchanged from that of the native enzyme within the rather coarse limit of estimate (±20%).

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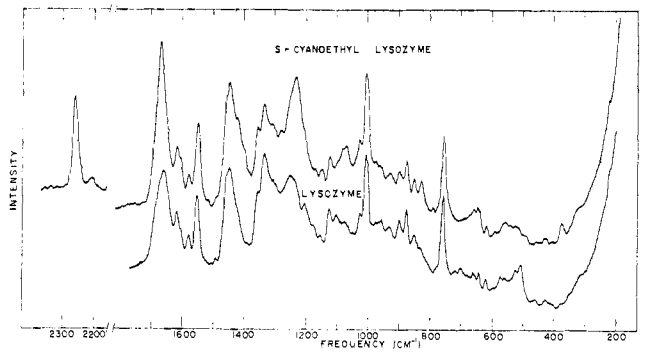

Figure 3. Raman spectra of lysozyme powder and solid *S*-cyanoethyl lysozyme.

Another indication of a random-coil structure is the definite broadening and weakening of the bands at 900, 933, and 1105 cm^{-1} that probably arise from the C–C and C–N stretching vibrations of the protein backbone. These changes are similar to those of irreversibly thermally denatured lysozyme.[5] The weakening and broadening of these bands were predicted by Lord and Yu[12] and can be understood as follows: lysozyme molecules are rigid in their native conformation, so that a side chain buried in a hydrophobic region, for example, has a single definite configuration. Upon denaturation, this configuration goes over into various rotational isomers with slightly different vibrational frequencies, and consequently broadening and weakening of their Raman peaks are expected to occur.

Other changes in the spectra include the tryptophan peaks at 1338 and 1363 cm^{-1} (Figure 2) which decrease in intensity, while the intensity minimum at 1355 cm^{-1} is almost filled in, all of which indicate a change in environment for the tryptophan residues,[5] possibly due to hydrogen bonding between the indole NH and bromide ion. Certain phenylalanine peaks also increase in intensity, particularly those at 1005 and 1030 cm^{-1}, while the lines at 1199 and 1209 cm^{-1}, which are due to both tyrosine and phenylalanine, decrease in peak intensity.

2. *S*-Cyanoethyl Lysozyme. When the S–S bonds in lysozyme are broken chemically by mercaptoethanol and the resultant free sulfhydryl groups blocked against reoxidation by alkylation with acrylonitrile, drastic changes occur in the Raman spectrum of the enzyme which reflect the conformational changes that take place.[7,13] Spectra of *S*-cyanoethyl lysozyme and lysozyme powder are presented together in Figure 3 and tabulated in Table I. These two spectra, after being redrawn to remove noise and background and normalized to the 1448-cm^{-1} methylene deformation band, are superimposed in Figure 4 for comparison. It can be seen from these figures that alkylation has proceeded as intended; the S–S stretching band at 507 cm^{-1} has decreased greatly in peak intensity along with an increase of the intensity of the C–S stretching band at 660 cm^{-1}, while the C≡N stretching band emerges at 2255 cm^{-1}. This shows that the S–S bonds have been cleaved and new C–S bonds formed with the C≡N groups incorporated into the protein. There might be a small residual amount of S–S bond in the modified sample, since a small band at 515 cm^{-1} can still be observed.

Table I. Raman Frequencies and Intensities of Lysozyme Powder and Solid *S*-Cyanoethyl Lysozyme[a]

Frequency, cm^{-1}		
Lysozyme	*S*-Cyanoethyl lysozyme	Tentative assignment
315 (1)		
333 (0 sh)	333 (1)	
353 (0)		
375 (0)		
	377 (1)	
408 (0 sh)	408 (0)	
429 (1)	429 (1)	
462 (1)	462 (0)	
	492 (1)	
507 (4)		
	515 (1 sh)	ν (S–S)
525 (1 sh)		
543 (0)	543 (0)	Trp
562 (1)	562 (1)	
574 (1)		Trp
598 (0)	598 (0)	
622 (1)	622 (1)	Phe
645 (1)	645 (2)	Tyr
660 (1)	660 (2)	ν (C–S) Cys
700 (1)	700 (1)	ν (C–S) Met
720 (1)		
760 (9)	760 (7)	Trp
	832 (2)	
834 (1 sh)		Tyr
854 (2)	854 (2)	
878 (6)	878 (4)	Trp
900 (5)	900 (3)	
933 (3)	933 (2)	ν (C–C)
960 (3)	960 (2)	
978 (4)	978 (3)	
1005 (5 sh)	1005 (5 sh)	Phe
1012 (11)	1012 (11)	Trp
1030 (1 sh)	1030 (1 sh)	Phe
	1070 (4)	
1076 (3)		
	1079 (4)	
1107 (3)	1107 (0)	ν (C–N)
1129 (2)	1129 (4)	
1154 (1)	1154 (1)	
1176 (1 sh)	1176 (0)	Tyr
1200 (0 sh)	1200 (0 sh)	
1208 (1)		Tyr and Phe
	1223 (0)	
1238 (2 sh)		
	1243 (8)	
1254 (5)		Amide III
	1263 (0)	
1271 (1 sh)		
	1281 (0)	
1300 (0)		
	1308 (1 sh)	
1327 (0 sh)		
1338 (11)	1338 (8)	
1362 (2)	1362 (2)	Trp and δ (C–H)
	1405 (0)	
1427 (1 sh)	1427 (1 sh)	δ (N–H) indole rings
1448 (10)	1448 (10)	
1459 (5 sh)	1459 (5 sh)	δ ($C-H_2$)
1490 (0)	1490 (0)	
1553 (8)	1553 (8)	
1582 (3)	1582 (3)	Trp
1607 (4 sh)	1607 (4 sh)	
1622 (5)	1622 (5)	Trp, Tyr, and Phe
1660 (10)		
	1672 (13)	Amide I

[a] sh denotes a shoulder, ν (A–B) means A–B stretching vibration, δ (C–D) = C–D bending vibration. Numerical figures in parentheses are relative peak intensities with the 1448-cm^{-1} line taken as 10.

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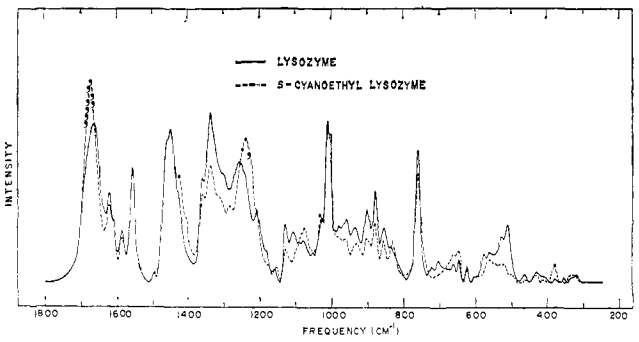

Figure 4. Redrawn Raman spectra of lysozyme powder and *S*-cyanoethyl lysozyme normalized to the intensity of the 1448-cm^{-1} band, after correction for the background.

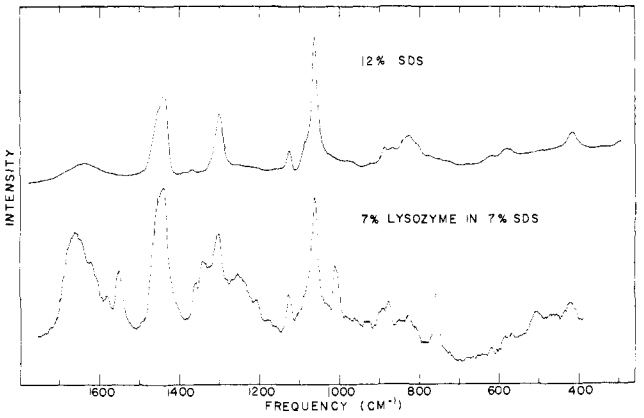

Figure 5. Raman spectra of 7% lysozyme in 7% SDS, pH 5, and of 12% aqueous SDS.

The most striking feature of the spectrum of the S-cyanoethylated enzyme is the substantial intensity increase in the amide I and III bands. Evidence for a random-coil structure for this molecule is provided by the amide III frequency, which has shifted from a moderately intense band centered at 1254 cm^{-1} (in the solid phase) to a stronger band at 1243 cm^{-1}, with shoulders faintly visible on both sides of the peak. The frequency shift in the amide III band is a result of a change in hydrogen bonding of the peptide group; the frequency decreases because a weaker hydrogen bond gives rise to a lower N–H bending frequency.[14] This change is also in accord with the results of denaturation of lysozyme by other agents such as temperature (100°),[5] LiBr, and SDS. Other regions which provide verification of a random-coil conformation are the backbone C–C and C–N stretching bands, which show spectral changes similar to those seen when the enzyme is denatured by other methods.

The amide I band has shifted from 1660 to 1672 cm^{-1} with an intensity increase and sharpening (spectral bandwidth of about 40 *vs.* 50 cm^{-1} in lysozyme powder). This is to be expected if the hydrogen bonding to the amide carbonyl is weakened and more uniform.[7,13,14] The value of the amide I frequency in lysozyme irreversibly denatured by thermal means,[5] which presumably has a random-coil structure, is also 1670 cm^{-1}. It is true that the conversion of an α helix to a β structure also shifts the position of the amide I line in the Raman spectra of certain polypeptides and proteins from 1660 to 1672 cm^{-1}, but in all of these cases the amide III frequency is much lower (~1230 cm^{-1}) and is a more conclusive indicator of β structure.

The aromatic side chains give rise to some very interesting features in the spectra (Figure 4) which reflect changes in the environment of these side chains. The peak intensities of the tyrosine bands at 645 and 834 cm^{-1} increase somewhat while that at 854 cm^{-1} does not seem to change within experimental reproducibility. These changes are analogous to those observed with pH in ribonuclease A.[15] The bands at 1176 and 1208 cm^{-1}, which are due to both phenylalanine and tyrosine, weaken considerably, while that at 1030 cm^{-1}, due to phenylalanine, increases in intensity. The decrease in intensity of 1338 and 1362 cm^{-1}, assigned to tryptophan residues,[12] is really remarkable. Mendelsohn[13] suggested that the amide backbone of the α helix could make some contribution to the intensity of the stronger band at 1338 cm^{-1}, because a similar intensity decrease[12] was observed when lysozyme was dissolved in D_2O.

The change in the environment of the side chains is a consequence of S–S bond rupture, because of which the protein is able to adopt a more expanded conformation. A relationship between conformational changes in cystine 64–80 and tryptophans 62 and 63 has already been suggested.[5] According to the X-ray results on the three-dimensional structure of lysozyme,[16] tyrosine 53, which is located in the β-pleated-sheet region, is hydrogen bonded to the amino group of asparagine 66, which is once removed from cystine 64–80. The other N–H of this asparagine is hydrogen bonded to the O–H of threonine 69. Therefore, distortion or breaking this disulfide bond will certainly change the environment of these neighboring residues, primarily tryptophans 62 and 63 and asparagine 66. As a consequence, the organization of the hydrogen bonds in the β-pleated-sheet region will also be affected. Thus, the rupture of the disulfide bonds may well be associated with the changes in the tyrosine and tryptophan bands.

It is interesting to note that the spectrum of lysozyme powder differs somewhat from that in aqueous solution, as had already been reported by Yu and Jo.[17]

3. Denaturation by SDS. SDS occupies a unique position among denaturants in that only a low concentration is required to induce protein denaturation, in contrast to high concentrations of such agents as LiBr (6 *M*), guanidine hydrochloride (6 *M*), and urea (8 *M*). The spectrum of 7% lysozyme in 7% SDS, pH 5, and that of 12% aqueous SDS are compared in Figure 5. There is a weakening and broadening in peak intensity of the disulfide band at 509 cm^{-1}, compared to that of the spectrum of the native enzyme, but the area under the peak remains unchanged. This means that the four disulfide bonds stay intact but their geometry is somewhat different from that of the native enzyme. The frequencies and intensities of amide I and III do not appear to change. The backbone C–C stretching frequency at 933 cm^{-1}, which is free from SDS interference, decreases markedly. The minimum between the tryptophan lines at 1338 and 1362 cm^{-1} has been

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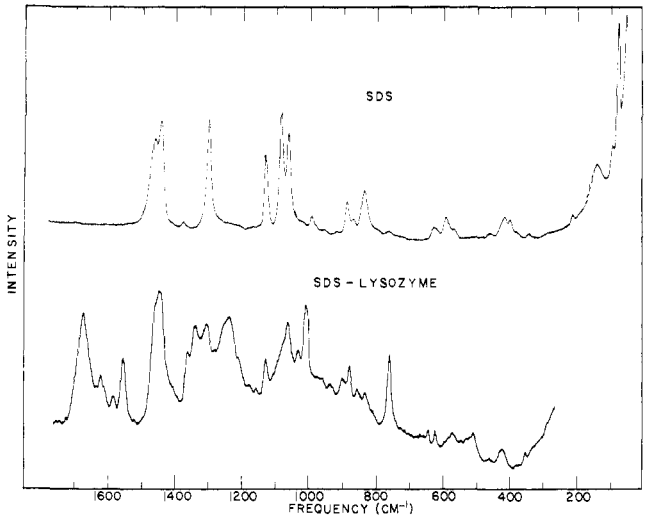

Figure 6. Raman spectra of precipitated SDS–lysozyme complex and SDS in the solid phase.

partially filled in, and the weak tyrosine and phenylalanine doublet at ~1205 cm^{-1} has shifted to 1210 cm^{-1} with a decrease in intensity, indicating a change in the environment of these side chains. No further change is observed when lysozyme is dissolved in more concentrated SDS solutions up to 15%, except, of course, stronger SDS lines.

The changes observed in these spectra are somewhat puzzling since they indicate a change in disulfide bonding with no change in protein backbone. However, our current understanding of the relationship between the disulfide bands and the geometry of the group is incomplete[12,18] and further study on this subject should be made to elucidate the character of protein–detergent interaction, which would be useful, among other places, as a model for protein–lipid systems in biological membranes.

When one attempts to dissolve lyophilized lysozyme powder in a solution of 0.3–4.0% SDS, little enzyme dissolution takes place. If an aqueous mixture of 7% lysozyme and 1% SDS is prepared, about 70% of the enzyme is precipitated, and the precipitate consists of a complex of SDS and lysozyme in roughly a 10:1 ratio. The spectra of this precipitate together with that of solid SDS are shown in Figure 6. The spectrum of the precipitate is similar to that of *S*-cyanoethyl lysozyme, except that an S–S stretching band is observed, which indicates that some S–S bonds are still intact. However, the intensity of the C–S stretching band is too weak to make any meaningful conformational assessment. The strong band at 1085 cm^{-1} in the spectrum of SDS has decreased markedly and appears only as a shoulder to the 1064-cm^{-1} band in the spectrum of the complex, showing that the conformation of SDS is different in the pure solid and in the complex.

It has been suggested that the binding of detergent to protein is primarily hydrophobic in nature,[19] by penetration of the hydrocarbon tail into the hydrophobic regions of the protein. Thus, some protein intrachain hydrophobic interactions have been replaced by SDS–protein interactions. On the other hand, electrostatic effects also play an important role here. Since lysozyme is a basic enzyme[20] with an isoionic point at pH 11, it therefore carries positive charge at pH 5, at which the present study was made. As a small amount of SDS is added, the negative charges on the dodecyl sulfate ion will neutralize some of the positive charges on the surface of the enzyme, thus bringing the protein out of the solution. However, when excess SDS ions are present, the negative charges of SDS override those of the protein, so the complex carries a net charge and will therefore be soluble in water.

Conclusions

From the many similarities of the Raman spectra of lysozyme denatured by various agents such as temperature (100°), LiBr, S-cyanoethylation, and SDS, it can be concluded that the denatured protein exists in random-coil conformations. The evidence for the random-coil conformations comes principally from the amide III band but is supported by evidence from the amide I band, from the S–S band in all but *S*-cyanoethyl lysozyme, and from the diffuseness of certain skeletal lines. Some bands due to side chains, especially those of tryptophan, tyrosine, and phenylalanine residues, show very similar qualitative effects from one denaturing agent to another, as a result of the more expanded conformations that the protein adopts. It should be noted, finally, that these random-coil conformations are probably not the same for the various denaturing agents.

Acknowledgment. We thank Professor G. J. Thomas, Jr., of Southeastern Massachusetts University for generously permitting us to use his Raman equipment. Most of the solid spectra were obtained with an instrument loaned to us by the Jarrell-Ash Division, Fisher Scientific Co., for which we thank them. Dr. Noel Relyea prepared one of the samples of *S*-cyanoethyl lysozyme and assisted in the amino acid analyses. We thank Professor E. R. Blout for giving us access to his amino acid analyzer. One of us (M. C. C.) wishes to thank the Massachusetts Institute of Technology for a Sloan Research Traineeship during 1972. This research was generously supported by NIH Grant GM-15310 and NSF Grant GP-13678.

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