Comparison of Protein Structure in Crystals and in Solution by Laser Raman Scattering. II. Ribonuclease A and Carboxypeptidase A

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Abstract: The Raman spectra of ribonuclease A in a single crystal, crystalline powder, and lyophilized powder are presented and compared with its solution spectrum at pH 5.71. In the 500–700-cm^{-1} region, where the S–S and C–S stretching and the tyrosyl ring vibrations appear, the spectral features of RNase A single crystal (also crystalline powder) are somewhat similar to that of lyophilized powder but different from that of solution. The spectral differences between crystal and solution phases are interpreted as due to changes in the geometry of the disulfide linkages and the local environment of the "buried" tyrosines upon crystallization. Supporting evidence from the spectra of neurotoxin α and cobramines A and B for our interpretation of the 644-cm^{-1} tyrosyl line intensity increase on crystallization is presented and discussed. On the other hand, in the amide III backbone region (1220–1300 cm^{-1}), a good agreement exists in both frequencies and line widths between the spectra of crystal and solution, indicating that the backbone conformation of RNase A is the same between the two phases. In addition, we compare the Raman spectrum of carboxypeptidase A (Anson) crystals to that of solution and show that there exists a small difference in the line shape of the amide III region. This may be a reflection of the subtle backbone conformational changes when CPDase A is crystallized.

The dramatic success of the X-ray crystallographers[1] in determining the detailed three-dimensional structures of proteins in crystals has raised the question of whether the structures are the same in crystals as in solution. At present, considerable interest exists in the investigation of the effect of crystallization on protein conformation.[2] A comparison of equilibrium and kinetic properties of proteins in crystals and in solution has shown both similarities and differences for a number of proteins.[2] Because of the difficulties of interpretation inherent in most of these measurements, results are often inconclusive.

By assuming that the protein conformations are the same in both phases, biochemists often interpret the functional aspects of a given protein in terms of its structure in the crystalline state. Recently, this assumption was questioned for carboxypeptidase A by Johansen and Vallee.[3] By treating carboxypeptidase crystals with diazotized *p*-arsanilic acid to modify tyrosine-248, an active-site residue of the enzyme, and comparing the visible spectra of crystals and solution, they concluded that the arsanilazocarboxypeptidase enzyme changed the side-chain conformation when it was crystallized.

In a recent communication from this laboratory[4] on the effect of lyophilization on the conformation of ribonuclease A, we have shown that Raman spectroscopy is a sensitive new tool in detecting the conformational differences (both main chain and side chain) of proteins between the solid state and aqueous solution. In that study, significant spectral changes were observed in the amide III backbone region (1200–1300 cm^{-1}) and in the region between 500 and 1000 cm^{-1}, where the tyrosyl ring vibrations, the S–S and C–S stretching, and the C–C–N skeletal stretching vibrations appeared.

In this paper, we wish to report our Raman spectral studies of a single crystal of RNase A and crystalline powder of RNase A and carboxypeptidase A. Comparisons of these spectra to those of their corresponding solutions will be made. Spectral differences between two phases will be interpreted in terms of knowledge obtained from model systems.

Materials and Methods

Crystals of beef pancreas ribonuclease A grown from 55% 2-methyl-2,4-pentanediol (MPD) at pH 5.0[5] were kindly provided to us by Dr. J. Bello of Roswell Park Memorial Institute, Buffalo, N. Y. They were of assorted shapes and sizes. The maximum dimension of some crystals was as large as approximately 3 mm. These crystals were kept in 75% MPD when we received them. Another type of RNase A crystals was prepared from ethanol solution according to the method of McDonald.[6] This preparation only produced a mass of fine fan-shaped rosettes of rectangular or needle-shaped crystals. Crystals of beef pancreas carboxypeptidase A (Anson) were purchased from Worthington Biochemical Corp. (Worthington Lot No. COA-1KB).

The sample of neurotoxin α from Egyptian Cobra (Naja haje annulifera) was a gift from Dr. D. J. Strydom of National Chemical Research Laboratory, Republic of South Africa. Cobramines A and B were kindly supplied by Dr. J. Wolff of National Institutes of Health.

For Raman scattering experiments, a large single crystal of RNase A was transferred to a 5-mm diameter glass vial filled with 75% MPD. The laser beam was then sent through the crystal in the vial and the scattered light directly from the scattering column inside the crystal was collected by an F/1.1 lens on the entrance slit of a Spex 1401 double monochromator. The shape of the crystal remained unchanged after long exposure (about 18 hr) to the laser beam. After completion of the experiments with the single crystal at various orientations, the crystal was crushed into finely divided particles. The sizes of the crystals were much smaller than the diameter of the focused laser beam (about 50 μ). The Raman spectra were then obtained with the crystalline powder precipitated at the bottom of a 1.0-mm diameter capillary containing 75% MPD.

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(2) J. A. Rupley in "Structure and Stability of Biological Macromolecules," S. N. Timasheff and G. D. Fasman, Ed., Marcel Dekker, New York, N. Y., 1969, pp 291–352.

(3) J. T. Johansen and B. L. Vallee, *Proc. Nat. Acad. Sci. U. S.*, **68**, 2532 (1971).

(4) N. -T. Yu, B. H. Jo, and C. S. Liu, *J. Amer. Chem. Soc.*, **94**, 7572 (1972).

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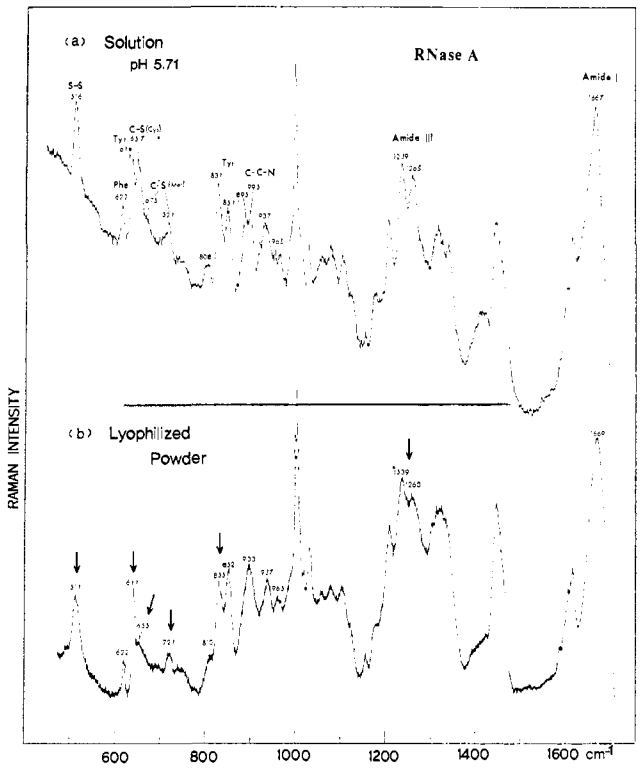

Figure 1. The striking effect of lyophilization on the conformation of RNase A. (a) Raman spectrum of RNase A in aqueous solution: concentration (c), 150 mg/ml; pH 5.71; spectral slit width ($\Delta\sigma$), 4 cm^{-1}; sensitivity (s), 1000 counts/sec full scale; rate of scan (γ), 10 cm^{-1}/min; standard deviation (Sd), 0.7%; laser power (p) at the sample, 100 mW. (b) Raman spectrum of RNase A lyophilized powder at 0% relative humidity: $\Delta\sigma$, 4 cm^{-1}; s, 5000 cps; γ, 10 cm^{-1}/min; Sd, 0.7%; p, 153 mW.

The thickness of the powder layer was about 0.5 mm. This capillary cell was held vertically by a Cary instrument's kinematic base and backing mirror assembly. The laser beam entered the cell from the bottom and traveled through the layer. The light scattered at 90° to the incident beam was then collected and analyzed. For the spectra of RNase A crystalline powder from ethanol solution, the wet powder was packed into a conical depression at the end of an 1/8 in. steel rod. This sample rod was then fastened inside a Thermovac flask equipped with a rubber "O" ring and a vacuum tight stopcock. Several drops of 88% EtOH solution were introduced into the flask. The sample was then equilibrated at room temperature with the vapor of 88% EtOH solution for 12 hr.

Raman spectra of carboxypeptidase A (Anson) crystals were obtained by the same procedure as that for RNase A crystalline powder from ethanol solution except that 88% EtOH solution was replaced by pure water. Before the experiments, crystals of CPDase A were washed with water three times by centrifugation and decantation of the supernatant solution. Since CPDase A is not soluble at neutral pH and low ionic strength, the solution was prepared by dissolving a 30-mg sample in 0.5 ml of 3 M NaCl solution at pH 7.00.

All the spectra reported here were obtained with the 514.5-nm line of an argon ion laser and at 4-cm^{-1} resolution. The Raman system and spectroscopic methods used in these experiments were similar to those described previously.[7]

Results and Discussion

(a) The Effect of Lyophilization on the Conformation of Ribonuclease A. In Figure 1 we compare the Raman spectrum of RNase A solution to that of lyophilized powder. The effect of lyophilization on the spectrum of RNase solution is quite striking, particularly in the 500–870-cm^{-1} region and the amide III backbone region. The most dramatic change is the intensity increase of the lines at 644 and 854 cm^{-1} relative to the line at 834 cm^{-1}. These three lines have been assigned to the ring vibrations of tyrosines.[4,8] In the S–S and C–S stretching region (500–725 cm^{-1}) the half-width of the S–S line at 516 cm^{-1} increased from 15 to about 20 cm^{-1} and the intensity ratio of the C–S to S–S lines decreased from 0.91 to 0.53, reflecting changes in the local geometry of the disulfide linkages. In the amide III region, the two lines at 1239 (β structure) and 1265 cm^{-1} (α helix) broadened and the latter shifted to 1260 cm^{-1}. Preliminary interpretations of these significant spectral changes have been given earlier.[4]

Lyophilization is a process which removes the unbonded and loosely bonded water molecules from the frozen sample and replaces a certain number of protein–solvent contacts by protein–protein contacts.[4] Since three of the six tyrosines in RNase A have normal pK_a values[9] and presumably are "exposed" to the molecular surface, there is a possibility that the observed intensity changes at 644, 834, and 854 cm^{-1} might be due to the effect of dehydration of these "exposed" tyrosines and not necessarily the result of conformational changes. However, Yu, *et al.*,[4] interpreted the tyrosine intensity changes as due to the changes in the local environment of the three "buried" tyrosines brought about by the conformational changes. This interpretation was primarily based on the fact that the direction of the relative intensity changes at 644, 834, and 854 cm^{-1} upon lyophilization of RNase A was opposite to that observed for amino acid tyrosine on lyophilization or crystallization.[4,10]

Recently, we thought that more convincing evidence might be obtained by examining the effect of lyophilization on the Raman spectrum of a protein containing tyrosine(s) known to be "buried." Now we have found that neurotoxin α is a good model system. This toxin protein from Naja haje venom consists of 61 amino acid residues and four disulfide bridges.[11] Its single tyrosine-24 is known to have an abnormally high pK_a value (*i.e.*, 11.9)[12] and is believed to be "buried." As expected, the line at 644 cm^{-1} did increase its intensity upon lyophilization (Figure 2a). We believe that the intensity increases of the tyrosine lines at 644 and 854 cm^{-1} in RNase A spectra are the manifestation of conformational changes in the vicinity of those "buried" tyrosines (*i.e.*, Tyr 25, 92, and 97).

Additional evidence in support of the above interpretation comes from the Raman spectra of cobramines A and B. These are two heat stable, basic proteins from India cobra venom, containing four and three tyrosines, respectively.[13,14] Recent studies[15]

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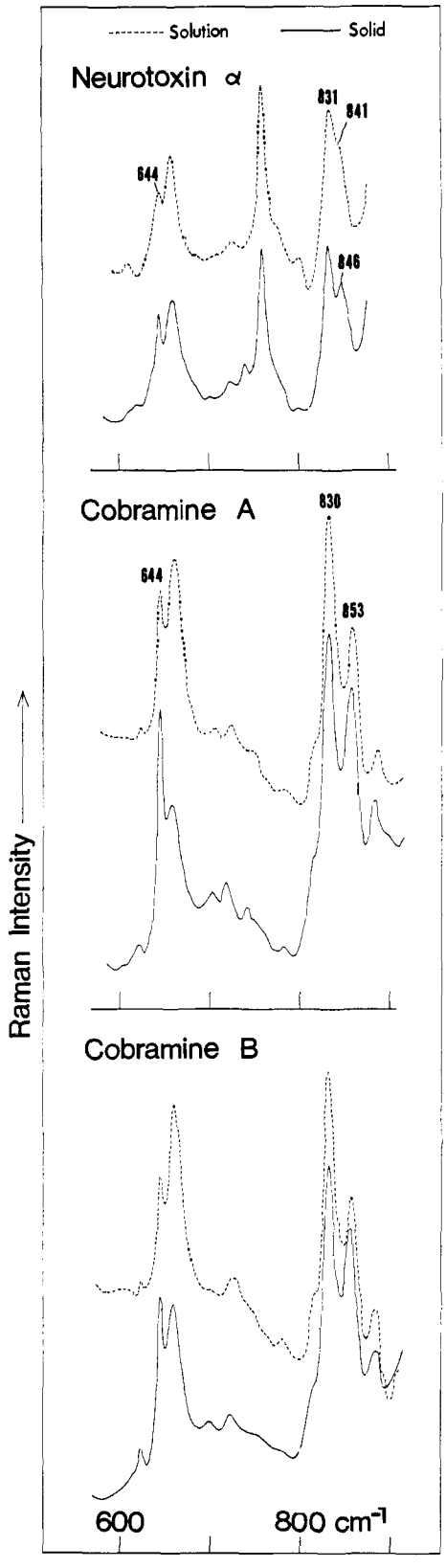

Figure 2. The effect of lyophilization on the scattering intensity of the tyrosyl line at 644 cm^{-1} in the Raman spectra of neurotoxin α, cobramine A, and cobramine B.

on the temperature variation of the intensity ratio of the 644-, 830-, and 853-cm^{-1} lines in cobramine B suggested that all three tyrosines were "buried" and probably involved in interactions similar to those of the three "buried" tyrosines in RNase A. Although,

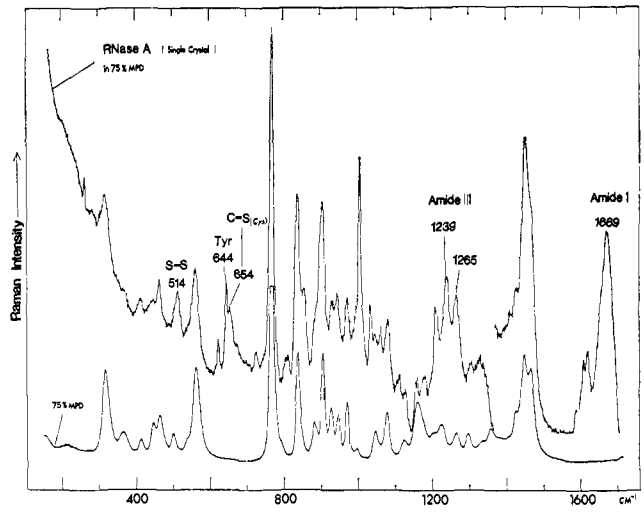

Figure 3. Raman spectra of a single crystal of RNase A (upper) and 75% MPD (2-methyl-2,4-pentanediol) (lower). Conditions for the upper curve: a 3-mm single crystal of RNase A immersed in 75% MPD; $\Delta\sigma$, 4 cm^{-1}; *s*, 1000 cps; γ, 10 cm^{-1}/min; Sd, 0.5%; *p*, 120 mW.

at present, the status of four tyrosines in cobramine A is not known, based on the intensity ratio argument used by Yu, *et al.*,[15] in their studies of cobramine B, it is quite likely that all tyrosines in cobramine A are also "buried." The Raman spectra of these two proteins in the 600–870-cm^{-1} regions are shown in Figures 2b and 2c. The line at 644 cm^{-1} in both cases did increase in scattering intensity when the solutions were freeze-dried, indicating conformational changes.

The backbone conformation of RNase A is best assessed in the amide III vibration region (1220–1300 cm^{-1}). The resolved peaks at 1239 and 1265 cm^{-1} in the spectrum of RNase A solution have been assigned to the antiparallel β-structure and α-helix components of RNase A backbone by Yu, *et al.*[4] This assignment was based on a Raman study of glucagon in various conformational states[16] and the X-ray crystal structure of RNase A.[5] Changes in frequency and line width in this region are considered a reflection of the backbone conformational changes. Upon lyophilization, the two lines at 1239 and 1265 cm^{-1} become broadened, indicating that the β-structure and α-helix parts of the molecule are less uniform in the solid state.

In principle, the amide I region (1630–1700 cm^{-1}) should carry the same conformational information of a protein backbone. However, due to the fact that this region is less sensitive, the antiparallel β-structure and α-helix components cannot be resolved and thus show up as a broad line at 1667 cm^{-1} in solution and at 1669 cm^{-1} in powder. The difference in the amide I frequency between the spectra of solution and powder is within experimental uncertainty and considered insignificant.

(b) Effect of Crystallization on the Conformation of Ribonuclease A. Here, we report the first Raman spectrum of a protein single crystal, ribonuclease A, in Figure 3. Since the crystal contains about 40% of solvent and 60% protein by weight, the scattering intensities of many Raman lines from MPD are quite

(16) N. -T. Yu and C. S. Liu, *J. Amer. Chem. Soc.*, **94**, 5127 (1972).

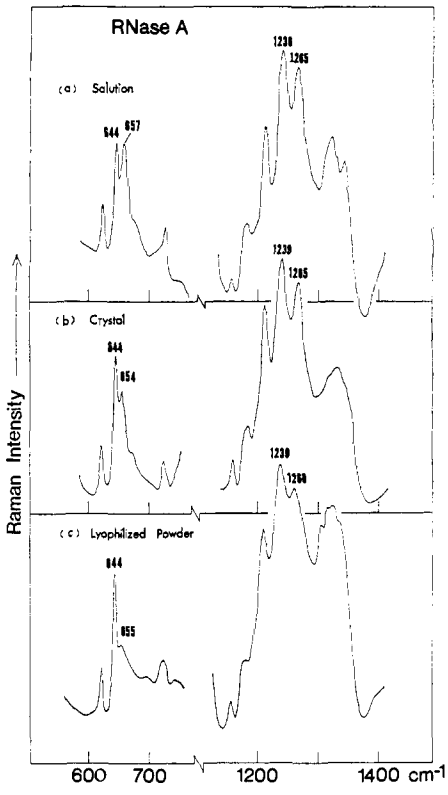

Figure 4. A comparison of the Raman spectra of solution, single crystal, and lyophilized RNase A in the 600–750- and the 1180–1400-cm^{-1} regions. These spectra were redrawn from Figures 1 and 3.

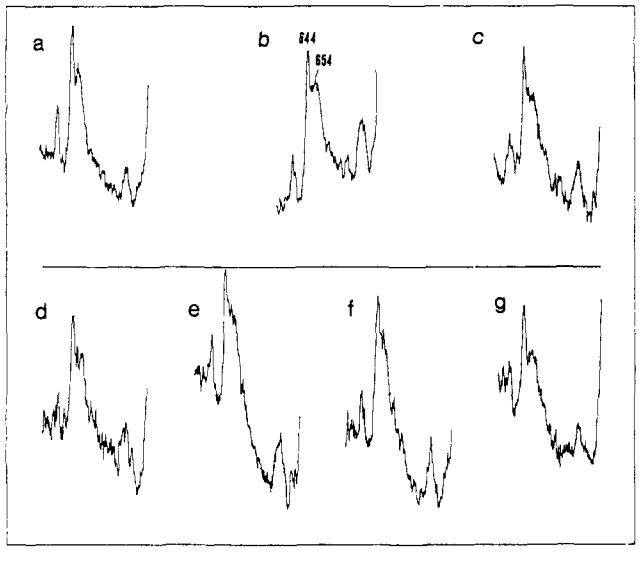

Figure 5. Raman spectra of RNase A in a single crystal at six different orientations (a–f) relative to the fixed laser beam, and in crystalline powder form (g).

strong. However, there are two regions which are relatively clear and appear to be useful for our present studies: *i.e.*, the 600–750 cm^{-1} and the amide III regions. In Figure 4 we compare the spectra of solution, single crystal, and lyophilized powder in these two regions. A comparison between solution and crystal in Figure 4 shows both similarities and differences. In the amide III backbone region, both frequencies and line shapes show a good agreement between two phases. We therefore conclude that there

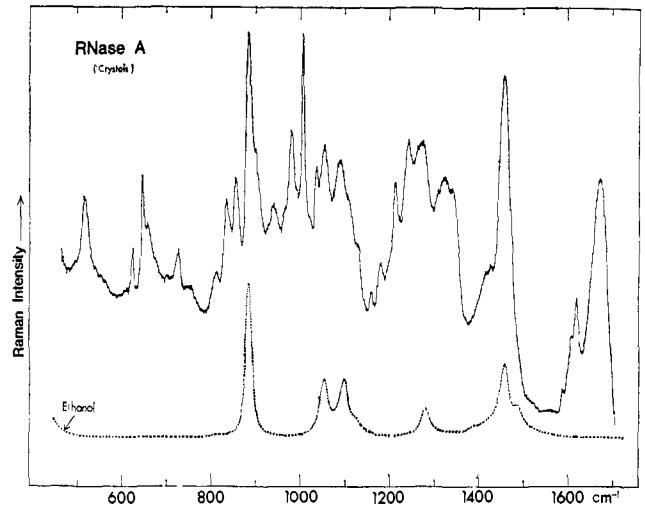

Figure 6. Raman spectra of RNase A crystalline powder (upper) and 88% ethanol solution (lower). Conditions for the upper curve: crystalline powder in equilibrium with the vapor of 88% ethanol solution; $\Delta\sigma$, 4 cm^{-1}; s, 2500 cps; γ, 10 cm^{-1}/min; Sd, 1%; p, 95 mW. The strong line at 983 cm^{-1} is due to the SO_4^{2-} ions in the sample.

is no detectable effect of crystallization on the main-chain conformation of RNase A. On the other hand, in the 600–750-cm^{-1} region, the tyrosyl line at 644 cm^{-1} increased its intensity upon crystallization. The spectral feature in this region of the spectra does not depend significantly on the crystal orientation relative to the laser beam (see Figure 5a–f) and is similar to that of crystalline powder (Figure 5g). Based on the evidence from Raman spectra of neurotoxin α and cobramines A and B as discussed in section a, we believe that the intensity increase of the 644-cm^{-1} line upon crystallization is a reflection of side-chain conformational changes.

An additional Raman spectrum of RNase A crystals is shown in Figure 6. The RNase A crystalline powder was obtained from ethanol solution.[6] The spectral feature in the 600–750-cm^{-1} region is similar to that of RNase A crystals from MPD solution, indicating similar side-chain conformational changes.

(c) Differences between the Backbone Conformation of Carboxypeptidase A in the Crystalline State and in Solution. In the Raman studies of ribonuclease A crystals, we have just shown that there is no detectable difference between the backbone conformation of RNase A in the crystalline state and in solution. Also in the first paper[17] of this series, we have found that in the case of lysozyme the backbone conformation is the same in crystals as in solution. These conclusions have been based on the comparisons of frequencies and line shapes in the amide III region of the Raman spectra. On the contrary, Yu, Liu, and O'Shea,[7] in their earlier studies of insulin crystals, have observed significant spectral differences in the amide III region between the spectra of crystals and solution. These differences were interpreted in terms of backbone conformational differences between two phases.

Carboxypeptidase A is one of the unusual proteins which contains appreciable amounts of parallel β

(17) N. -T. Yu and B. H. Jo, *Arch. Biochem. Biophys.*, in press.

pleated sheet structure.[18,19] Raman spectra of such a protein have not been reported so far in the literature. At present, the Raman spectral characterization of α-helix, random coil, and antiparallel β structure of proteins is well established, but the data for parallel β structure are still lacking.

Raman spectra of carboxypeptidase A in crystals and in solution are compared in Figure 7. In crystals, two frequencies at 1247 and 1270 cm^{-1} may be identified as the amide III vibrations. Upon dissolution, the broader component at 1247 cm^{-1} has become sharpened and the peak maximum was shifted to 1243 cm^{-1}. We believe that the spectral changes observed here are the reflection of backbone conformational changes.

One unusual aspect of the spectral changes is the sharpening of the line at 1247 cm^{-1} on dissolution. Normally the opposite effect is observed with simple peptides.[20] Although the exact nature of this line sharpening is not known, it might mean that the strength of intramolecular hydrogen bonds in solution is more uniform than in the crystalline state.

The assignments of the amide III lines in the spectra of carboxypeptidase A to various structural components of the backbone may be made on the basis of the criteria established by Yu and Liu.[16] In the Raman studies of conformational variations of glucagon, they concluded that the α-helical, random-coiled (H-bonded), and antiparallel β structure of a protein should have the amide III vibrations near 1266, 1248, and 1232 cm^{-1}. The amide III vibrations of random-coiled backbone usually appear as a structureless broad band, and the antiparallel β structure gives rise to a relatively sharp line. Based on this information and our knowledge from the X-ray crytal structure of carboxypeptidase A, we now assign the line at 1270 cm^{-1} to the α-helices and the one at 1247 in crystals and 1243 cm^{-1} in solution to both parallel β pleated sheet and random coil. The small fraction of antiparallel β structure present in the native protein may have been "masked" and did not show up in the spectra. It is possible that the random-coiled backbone of carboxypeptidase A only contributed to the broad background of the component centered at 1243 cm^{-1} and that the sharp peak at 1243 cm^{-1} might be a characteristic frequency for a parallel β sheet structure. In order to confirm this assignment, Raman studies of a model compound known to exist predominantly in a parallel β structure seem desirable.

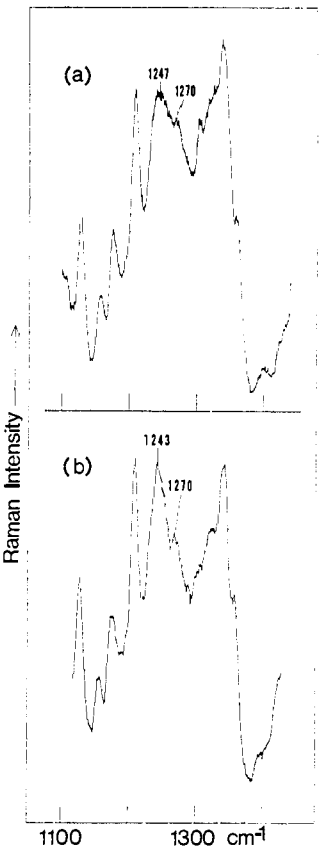

Figure 7. Raman spectra of carboxypeptidase A in the crystalline state (a) and in solution (b). Conditions for a: $\Delta\sigma$, 4 cm^{-1}; s, 2500 cps; γ, 25 cm^{-1}/min; Sd, 1%; p, 80 mW. Conditions for b: pH 7.00; c, 60 mg/ml; ionic strength 3 M NaCl; $\Delta\sigma$, 4 cm^{-1}; s, 1000 cps; γ, 10 cm^{-1}/min; Sd, 0.7%; p, 150 mW.

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