a neighboring residue, is not possible for the triple-helical, coiled-coil, collagen-type structure.

(22) The possibility of a conformation corresponding to $\psi = -60^\circ$ was also considered. Such a conformation cannot have any intramolecular hydrogen bond. Also this structure is not compatible with the X-ray and CD data.[23]

(23) S. K. Brahmachari, M. Bansal, V. S. Ananthanarayanan, and V. Sasisekharan, *Macromolecules*, following paper in this issue.

Structural Investigations on Poly(4-hydroxy-L-proline).

2. Physicochemical Studies[†]

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Received February 21, 1978

ABSTRACT: We report experimental studies which confirm our prediction, namely that the ordered structure of poly(hydroxyproline) in solution corresponds to a left-handed helical structure with intrachain hydrogen bonds. The CD studies show that the poly(hydroxyproline) molecule has essentially the same conformation in aqueous solution and in the film obtained subsequently by evaporation. X-ray diffraction patterns of the sample in this form (B form) have been recorded at different relative humidities. The patterns recorded at relative humidities over 66% can be interpreted in terms of a helical structure with intrachain hydrogen bonds. These results lead us to conclude that the ordered conformation of poly(hydroxyproline) in solution is form B and not form A. This offers a simple explanation for the greater stability of the poly(hydroxyproline) helix in solution as compared to the poly(proline) form II helix and also for the absence of mutarotation for poly(hydroxyproline).

In the preceding paper[1] it has been shown that two ordered structures (A and B) are possible for poly(hydroxyproline) (PHP). Both structures are left-handed helices, with all peptide bonds in the trans conformation. The form A is obtained by precipitation from an aqueous or acidic medium and has been well characterized by X-ray studies.[2,3] In the solid state, form A has been shown to have a regular helical structure with three residues per turn and stabilized by interchain hydrogen bonds involving the γ-hydroxyl groups.[2] In this structure, intrachain hydrogen bonds can only be formed via water molecules as intermediaries.[1] Another form of PHP was obtained by slow evaporation of an aqueous solution, but since it gave a very diffuse X-ray pattern, it was not studied in detail.[3] We have proposed in the previous paper that this B form of PHP has a nonintegral helical structure, with the γ-hydroxyl group directly hydrogen bonded to a carbonyl oxygen in the same chain. We have further suggested that this is also the ordered conformation most likely to exist in solution. PHP is soluble only in aqueous or strongly acidic media and hence physicochemical studies can be carried out only in these solvents. Previous studies have indicated that the PHP molecule has a left-handed helical structure in solution and that only one regular structure is observed, viz., no mutarotation occurs for PHP.[4,5]

In this paper the details of the experimental studies carried out by us are described. We have carried out the following physicochemical studies: (i) measurement of molar ellipticity value at various concentrations, to see whether molecular association or aggregation occurs in solution for PHP; (ii) ORD studies in acid/water mixtures to study the order–disorder transition; (iii) IR studies of dry and wet pastes, to check for the presence of bound water molecules; (iv) CD studies in aqueous solution and films to see whether PHP undergoes any structural change on going from solution to solid state; (v) X-ray diffraction studies to characterize the B form in detail; and (vi) X-ray studies at different conditions of humidity, to understand the structural transformations occurring for PHP, in the solid state.

All these studies confirm our hypothesis that the ordered conformation of PHP in water is form B and not form A.

Experimental Section

Materials and Methods. Poly(4-hydroxy-L-proline) samples of mol wt 17 000 and 27 000 were obtained from Sigma Chemicals Co. The ORD and CD measurements were carried out using a JASCO Model J-20 spectropolarimeter. Films were cast on demountable quartz cells and IR studies were carried out using UR-10 Infrared spectrophotometer with KRS-5 plates. Concentrations of the samples used for CD and ORD studies were varied from 4 to 0.0067 mg/mL. Path lengths varying from 5 cm to 0.1 mm were used. Optical densities were measured by SP-700 spectrophotometer. Ellipticity values for film are expressed in millidegrees. Dichloroacetic acid (DCA) was obtained from Riedel (Germany) and was dried over anhydrous Na_2SO_4 before distillation under vacuum.

X-ray diffraction studies were carried out for unoriented films and pastes. Powder patterns were recorded using a Cu Kα radiation source (40 kV, 20 mA) and a flat plate cassette, the specimen to film distance being varied from 4 to 5 cm. To record patterns at two different humidities (66% and >98%) the PHP sample was kept at one end of a sealed Lindemann tube, with a column of a solution of standard humidity (saturated $NaNO_2$ and distilled water) at the other end. The sample was allowed to equilibrate for several hours before the X-ray pattern was recorded.

Results and Discussion

(i) Absence of Aggregation in Solution. Poly(hydroxyproline) has been found to be more stable than poly(proline) form II in aqueous solution. To verify whether the greater stability of PHP in solution is due to interchain interactions leading to association, as observed for form A in the solid state, the molar ellipticity values were measured at 225 nm, for a wide range of concentration (PHP gives a characteristic positive CD band at this wavelength). There was not very much variation in the molar ellipticity value θ_{225}, which was within the range 6600

[†] Contribution No. 125.

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Table I
Circular Dichroism Data on Poly(hydroxyproline) and Poly(proline) Form II

	poly(hydroxyproline)				poly(proline) form II			
	λ_{max}, nm	λ_{cross}, nm	λ_{min}, nm	ρ^a	λ_{max}, nm	λ_{cross}, nm	λ_{min}, nm	ρ^a
aqueous solution	225	219	205	0.19	228	224	206	0.06
film (from aq soln)	226	219	204	0.21	228	220	206	0.25

[a] $\rho = R_+/R_-$, where R_+ and R_- are the rotational strengths of the positive and negative CD bands, respectively.

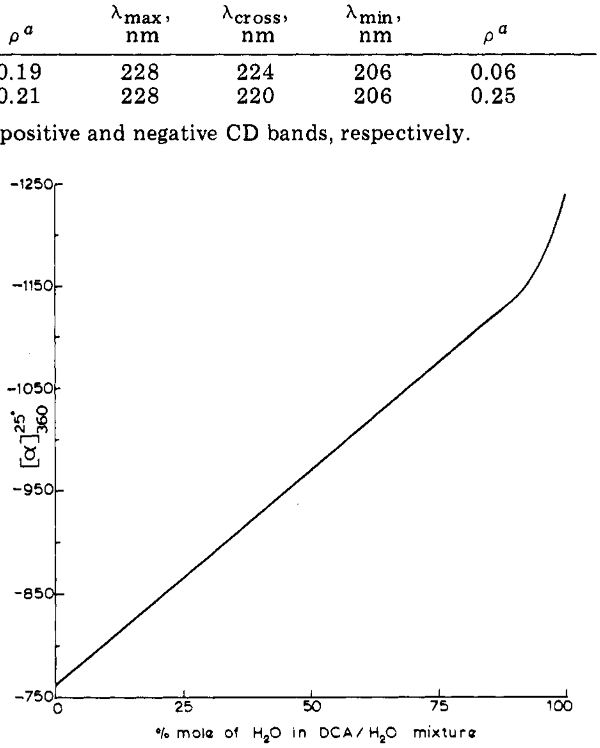

Figure 1. Solvent-induced transition in poly(hydroxyproline). Specific rotation (deg cm^2 g^{-1}) at 360 nm is plotted against solvent composition.

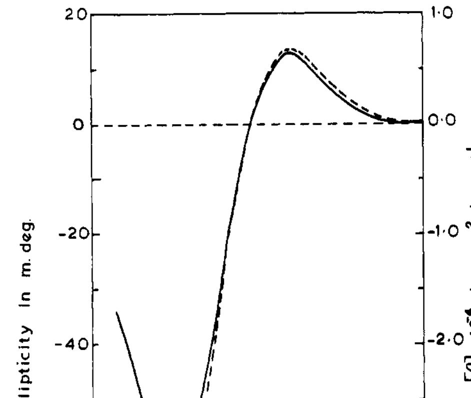

Figure 2. Circular dichroism spectra of poly(hydroxyproline) in solution (---) and (—) for a film, of OD_{230} = 0.37. The ellipticity values are expressed in deg cm^2 dmol^{-1} for the solution spectra and in mdeg for the film.

± 400 when the concentration was varied from 0.0067 to 3.36 mg/mL. Torchia[7], from proton NMR studies at 220 MHz, also found that there was no change in the proton line widths on changing the concentration from 5 to 25 mg/mL. These studies indicate that no aggregation occurs in solution even at very high polymer concentrations. Hence the greater stability of PHP in aqueous solution is an intrinsic property of the single helix of PHP.

(ii) Order–Disorder Transitions in Solution. As mentioned earlier, PHP is soluble only in aqueous and highly acidic medium. While the optical rotation in aqueous medium corresponds to $[\alpha]_D^{25}$ -400°, the value in a strong helix disrupting solvent like dichloroacetic acid (DCA) is found to be -230°. Kaufman et al.[8] had shown that in 97% formic acid (FA) PHP also has an optical rotation corresponding to $[\alpha]_D^{25}$ -235°, which they attributed to a structure with a few of the peptide bonds in the cis orientation. However, our studies in DCA and the fact that polyproline also gives a value of $[\alpha]_D^{25}$ -250° in a denaturing medium indicate that in a high concentration of formic acid PHP is in a denatured form.

The optical rotation at 360 nm in different H_2O/DCA mixtures is shown in Figure 1. It is seen that with increasing percentage of H_2O the polymer undergoes transition from an unordered to an ordered form. A similar transition was observed in the FA/H_2O mixture.[8] Increase in the ordered structure with increasing water content demonstrates that water promotes the ordering of the polypeptide.

(iii) Absence of Firmly Bound Water Molecules. It has been shown above that water stabilizes the ordered structure of PHP. To see whether water molecules are firmly bound to the polypeptide chain, a paste of the sample was prepared in a minimum quantity of D_2O.[9] The broad band corresponding to O–D stretching frequency, which occurs at 2200–2700 cm^{-1}, was found to be split up around 2350, 2500, and 2650 cm^{-1}. If an excess of D_2O was present, the splitting disappeared. Hence, in the paste form, the splitting of the O–D stretching frequency can be attributed to the occurrence of both hydrogen-bonded and free O–D bonds; its absence at high D_2O content is due to rapid exchange between free and bound D_2O molecules.

It was also found that on drying the moist samples overnight in vacuo, the band due to the O–D stretching frequency completely disappeared. Hence, the D_2O molecules are not strongly bound to the polypeptide chain. This would indicate that even if the form A of PHP has intrachain hydrogen bonds via water molecules (as described in the previous paper), these can be easily disrupted and hence the extra stability of PHP in aqueous solution cannot be explained by the water-bridged form A structure.

(iv) Solution to Solid State Transition. To see whether poly(hydroxyproline) exists in different conformations in solution and the solid form, CD spectra of the polymer were recorded in both states. The films were cast on quartz plates from aqueous solution of the polymer, by drying overnight under vacuum at room temperature, and the CD spectra were recorded the same day. Almost identical CD patterns were obtained, as shown in Figure 2, indicating that the polymer exists in essentially the same conformation both in solution and in film.

The CD band positions and relative band strengths for solution and film of PP-II and PHP are given in Table I. For poly(proline) form II, the positive band of the CD is relatively much stronger in film than in solution and there is a drastic increase in the value of ρ (defined as the ratio

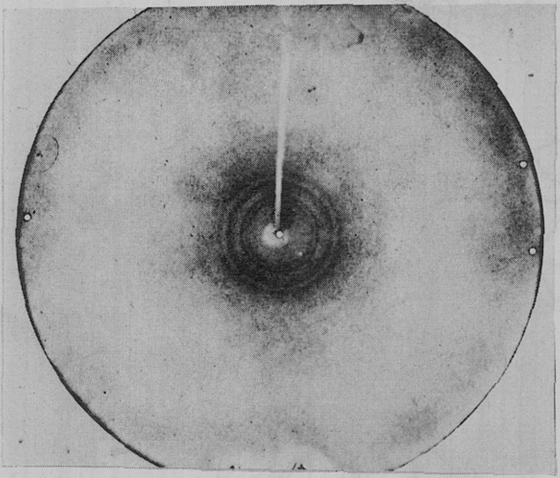

Figure 3. X-ray powder pattern of the B form of poly(hydroxyproline).

of the maximum positive ellipticity to the maximum negative ellipticity) in films, as compared to the solution.[10] This change has been attributed by Pysh[11] as being either due to a conformational change or due to interactions between the solvent and the peptide carbonyls in solution. Calculations have shown that with increasing solvent–carbonyl interaction, the ρ value decreases.[11]

In PHP there is no significant difference in the ρ value in film form and in solution, and the value is closer to the ρ value for PP-II in a completely desolvated state, as can be seen from Table I. This leads us to deduce that carbonyl oxygens in PHP are not very accessible to solvent molecules and no noticeable structural change takes place in going from solution to film. These results lend support to our hypothesis that the ordered structure in solution is the intramolecularly hydrogen-bonded form B and is observed in both the film and solution CD spectra. We have not been able to obtain films of sufficient transparency, suitable for CD studies, of PHP in A form. To confirm that the ordered structure we observe is the B form and to characterize it in detail, X-ray diffraction studies were also carried out and the results are reported in the next section.

(v) Characterization of the B Form of PHP. The lyophilized sample of poly(hydroxyproline) was dissolved in water and the solution allowed to evaporate to dryness at room temperature (28 °C). Shiny, almost transparent flakes were obtained, which gave a diffuse X-ray pattern, similar to that of the form B reported by Sasisekharan.[3] Since the pattern was found to become more diffuse with prolonged exposure, a moist paste of the sample was sealed in a Lindemann tube and the X-ray pattern recorded. A much more crystalline pattern was now observed and is shown in Figure 3. A systematic study at different conditions of humidity confirmed that, though the basic molecular structure of PHP remains the same, high humidity (>66% RH) is required for the paracrystalline form B to be obtained, indicating that water plays a role only in the packing of PHP molecules in a regular crystalline array within the unit cell.

Since our attempts to obtain oriented films or fibres of PHP in the B form have, so far, been unsuccessful, only a preliminary structure analysis, carried out by using the powder pattern data, is reported here. The unit cell dimensions were calculated from the "d" spacings of the rings observed in the powder pattern. A nonlinear least-squares fitting procedure was used and the cell parameters were refined for several different schemes of indexing, since it was not possible to index the reflections unambiguously, in the beginning. The best fit was obtained for a hexagonal unit cell with a = 15.75 Å and c = 22.88 Å. The observed and calculated values for the "d" spacings are given in Table II along with the indexing scheme.

Table II
Observed and Calculated Spacings in the Powder Pattern of Poly(hydroxyproline) (form B)[a]

h	k	l	d_c	d_o	I_o
1	1	0	7.82	7.82	m
2	0	0	6.79	6.80	m
2	1	0	5.12	5.11	s
3	0	1	4.44	4.43	s
3	1	0	3.76	3.77	w
4	0	1	3.36	3.36	w
0	0	8	2.86	2.86	vw

[a] Indices (hkl) were assigned to the reflections and the spacings calculated (d_c) on the basis of a hexagonal unit cell with $a = b = 15.75$ Å, $c = 22.88$ Å, $\gamma = 120.47°$. Observed intensities (I_o) have been estimated as strong (s), medium (m), weak (w), and very weak (vw).

It is very interesting to note that the value of 22.88 Å for the c-axis repeat corresponds exactly to the pitch of the intrachain hydrogen-bonded structure, which has eight residues in three turns of the helix and a unit height of 2.86 Å. This indicates that the structure proposed by us from theoretical considerations is in fact the B form observed by X-ray diffraction. It may be emphasized that the unit cell parameters were calculated completely independently of the structure and hence the agreement between the two values is particularly significant.

We have also worked out the best possible molecular packing arrangement from stereochemical and hydrogen-bonding considerations. Since, in the B form structure, there are no free polar groups for the formation of direct intermolecular hydrogen bonds, the possibility of hydrogen bond formation with water molecules acting as donors was investigated. We found that if a single molecule constitutes the repeating unit, the a-axis repeat should be of the order of 8.5 Å and no systematic intermolecular hydrogen bonds can be formed. The large observed value of the "a" parameter also indicates that more than one molecule is present in the unit cell. From the geometry of the unit cell it was apparent that three molecules must be present in the unit cell and constitute an asymmetric unit. Since the helix is nonintegral, the only possible space group is P3. Hence various possible orientations about the c axis were tried, for the PHP molecules having the intrachain hydrogen-bonded structure. The stereochemically best possible arrangement was obtained, such that the three molecules in the asymmetric unit were related by a three-fold rotation axis and there were no short contacts, less than the extreme limit criteria of Ramachandran and Sasisekharan,[12] between atoms in neighboring chains. At each stage the possibility of intermolecular hydrogen bond formation via water molecules was also examined.

The above procedure was repeated to obtain the stereochemically best arrangement of the triple-helical units within the unit cell. It was found that, for the closest possible stereochemically allowed packing arrangement, the unit cell parameter "a" corresponds to 15.7 Å. This result is in striking agreement with the observed value of 15.75 Å, which again indicates that the structure proposed by us for the B form is correct.

The complete scheme of molecular packing is shown in Figure 4, in which the possible intermolecular hydrogen bonds, via water molecules as intermediaries, are also shown. In the scheme shown, three sets of hydrogen bonds

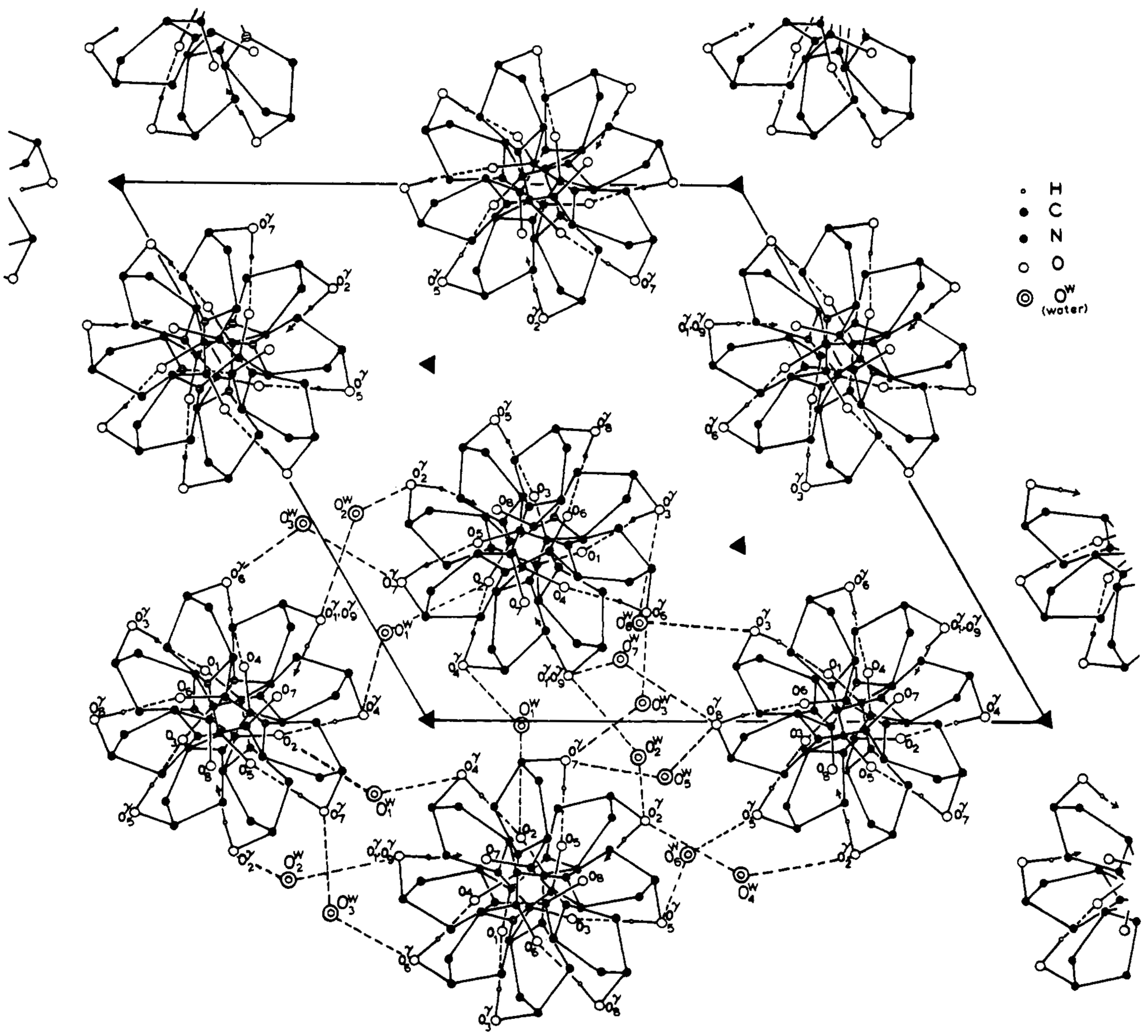

Figure 4. A projection down the *c* axis of the packing of poly(hydroxyproline) molecules (B form) in a hexagonal unit cell. The probable locations of the water molecules involved in intermolecular hydrogen bonds are also shown.

link the PHP chains in each assymetric unit. Additional hydrogen bonds, again via water molecules, link the O^{γ} atoms in neighboring triple-helical units. The atoms of the water molecules, located in the positions shown, do not have any short contacts with atoms in the PHP chains and the hydrogen bond parameters satisfy the criteria required for good O–H···O hydrogen bonds.[13]

Thus, the packing scheme proposed here from stereochemical and hydrogen-bonding considerations is a feasible one and is in agreement with all the data from X-ray diffraction studies, both as regards the unit cell dimensions as well as the observation that the crystallinity of the X-ray pattern of B form depends on the water content of the PHP sample. It is now evident that, since in the B form structure no direct intermolecular hydrogen bonds are possible, in the absence of bridging water molecules, the PHP helices do not pack in a regular crystalline array and so a diffuse X-ray pattern is observed.

In the infrared studies reported earlier in this paper, it was seen that for a sample of a moist paste of PHP in D_2O, a splitting occurs in the O–D stretching band, denoting the presence of some hydrogen-bonded D_2O molecules. The removal of these molecules by merely drying overnight in vacuo also indicates that they are not an essential requirement for the molecular structure, but only provide intermolecular linking.

(vi) Structural Transformations in the Solid State. X-ray powder patterns of PHP were recorded at different conditions of humidity. It was found that a wet paste of the sample, equilibrated with water (>98% RH), gave a diffuse B form X-ray pattern. The sample was then equilibrated for several hours in 66% RH and the pattern recorded. A more crystalline B form pattern was now obtained. An interesting feature of the study was that the sample in the B form was found to undergo transformation to the A form on keeping it at room humidity (varying between 30 and 40% RH) for about 8 weeks. A similar transformation was not observed at high humidity (>98% RH). These results are summarized in Figure 5 along with the results from studies described in previous sections.

From these studies it is seen that since the B form → A form transformation occurs in the solid state, it cannot be attributed to a cis → trans isomerization about the peptide bond. This agrees with our theoretical studies which have indicated that both the stable helical structures for PHP have peptide bonds in the trans orientation. As mentioned in the previous section, in the absence of water molecules, the PHP molecules in B form do not pack in

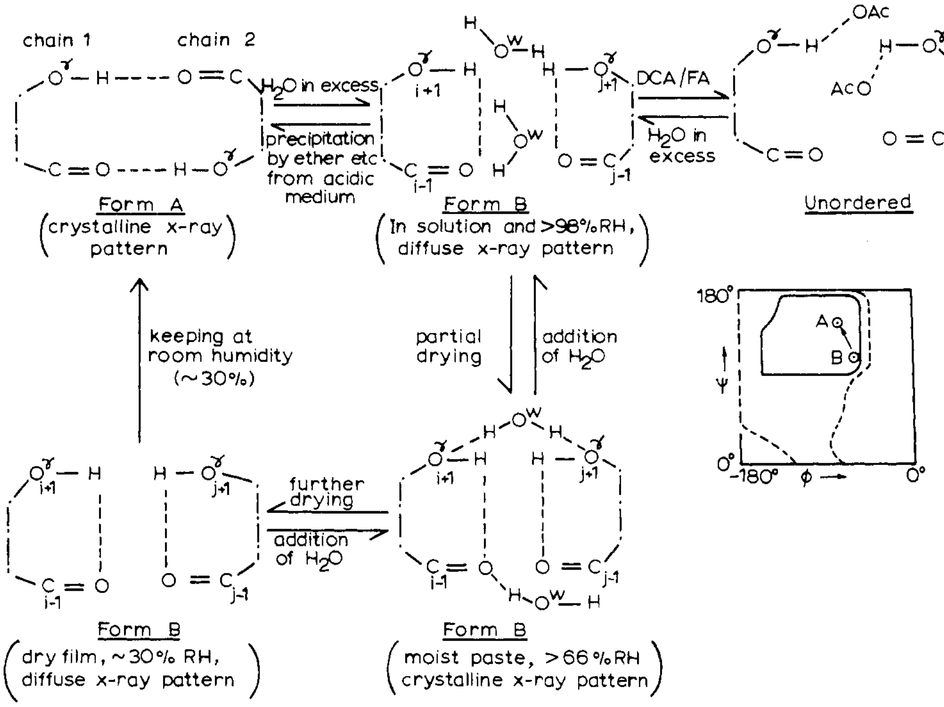

Figure 5. A schematic diagram illustrating the structural transformations in poly(hydroxyproline). The inset shows the small changes required in backbone dihedral angles ϕ and ψ for the B → A transformation to take place.

a crystalline lattice arrangement, since there are no strong intermolecular interactions. Hence at low humidity the polypeptide chains undergo transformation to the form A, which has a threefold screw symmetry and forms an extended lattice structure, stabilized by direct interchain hydrogen bonds involving the γ-hydroxyl groups. As can be seen from Figure 5 only a small change in the backbone dihedral angles ϕ and ψ is required for this transformation to take place, and hence it can occur in the solid state.

The presence of a diffuse X-ray pattern at high humidity (>98%) can also be attributed to the absence of regular lattice packing of PHP helices at high moisture content. However, the fact that it is the B form which is observed at conditions of high humidity and the absence of the B → A transformation under these conditions clearly indicates that the intrachain hydrogen-bonded form B structure is intrinsically very stable and that the ordered structure of PHP in solution is this structure rather than the form A.

Conclusion

Unlike poly(proline) which undergoes mutarotation between the right-handed helical form I, with all peptide bonds in the cis orientation, and the left-handed helical form II, which has all-trans peptide bonds, poly(hydroxyproline) does not mutarotate between two ordered structures in solution. The ordered structure of PHP in solution is not stabilized by any intermolecular interactions, such as those found for PHP in form A in the solid state. The structure is also not stabilized by any firmly bound water molecules, since IR studies in D_2O indicate that the water molecules are easily removable and that rapid exchange occurs between bound and free D_2O molecules, at high humidity. CD studies indicate that PHP does not undergo any structural transformation when cast as a film from an aqueous solution. Our X-ray studies have shown that PHP films, when prepared by slow evaporation from aqueous solution, are in the intrachain hydrogen-bonded form B. Since at high humidity (>66% RH) it is always the B form X-ray pattern which is observed, we conclude from our studies that the ordered structure of PHP in solution is also form B and not form A.

Several previous studies also support our conclusion. It was found by Bensing and Pysh[14] that PHP films, when cast from aqueous solution in the presence of an electric field, show parallel dichroism at 218 and 188 nm, in the ultraviolet absorption spectra. Since the observed spectrum differs from that observed for PP-II, these workers suggested that the PHP has probably undergone mutarotation to a form with cis peptide bonds. However, there are also considerable differences in the absorption bands observed for PP-I and the PHP film. Since in the structure proposed by us for form B of PHP the carbonyl groups are oriented relatively parallel to the helix axis (as seen from the values given for the angles of tilt of the peptide units, in Table I of the preceding paper), it is quite likely that it was the B form which was observed by these workers.

It is worth mentioning here that the hydrodynamic properties of poly(hydroxyproline) as well as poly(proline) apparently exhibit molecular weight dependence. The low molecular weight samples are found to have the conformation of a somewhat flexible rod while the high molecular weight samples behave hydrodynamically like a random coil.[15] However, as pointed out by Tanaka and Scheraga[16] the hydrodynamic behavior of these polypeptides is ill-understood. The available CD data, including our own, do not show any apparent molecular weight dependence over a range from 9000 to 27 000. The ordered conformation of PHP that contributes to this CD is, according to us, form B, both in solution and in the film.

Solution studies of PP and PHP using proton NMR,[7,17] ^{13}C NMR, and Raman spectroscopy[18,19] also indicate that there are some marked differences in the structures of the two polymers in solution. In particular, while the pyrrolidine ring is quite flexible in PP-II, it is relatively rigid in PHP, indicating that the interaction of the γ-hydroxyl group with the backbone carbonyl groups renders the ring immobile to some extent. These studies, as well as the observations that the PHP helix is considerably more stable than the PP-II helix in aqueous solution,[6] support our conclusion that the ordered structure of PHP in solution is form B, with the γ-hydroxyl group forming an intrachain hydrogen bond with a backbone carbonyl oxygen. The possibility of such a hydrogen-bond formation was suggested earlier for the polymer (Gly-Hyp)$_n$ by Mattice and Mandelkern,[20] while Torchia[7] from proton NMR studies had proposed a similar scheme for PHP.

From theoretical considerations, it has been shown in the previous paper that, though the presence of the γ-hydroxyl groups does not put any stereochemical restriction on the PHP chain taking up a structure with all-cis units, no intrachain hydrogen bonds, either direct or water-bridged, are possible for this structure. Hence the absence of this structure may be due to the fact that, in solution, once three consecutive units take up trans orientations, a hydrogen bond is formed linking the γ-hydroxyl group of the $(i + 1)$ th residue to the carbonyl oxygen of the $(i - 1)$th residue, and the polypeptide chain is locked in this thermodynamically favorable conformation. Thus, the occurrence of the intrachain hydrogen-bonded B form structure readily explains the absence of mutarotation and the great stability of PHP in solution.

Acknowledgment. We are greatly indebted to Professor G. N. Ramachandran for discussions and suggestions. We are grateful to Mr. I. Sen for the least-squares refinement computer program and other technical help. The work was supported by financial assistance from CSIR and DST (SERC), India, and PL-480-USPHS Grant No. 01-126-N.

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Fourfold Helical Structures for Polypeptides[+]

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Received April 26, 1978

ABSTRACT: Fourfold helical structures for polypeptides and their association in regular lattices with interchain hydrogen bonds were investigated by model building studies. These studies revealed that stereochemically satisfactory fourfold helical sturctures are possible for polyglycine, polyproline, and copolymers of glycine and proline with two and four units in the monomer. In these structures the unit height h for the backbone has been found to be restricted from 2.7 to 3.1 Å with four peptide units per turn of the helix. Energetically both fourfold and threefold helical structures are equally favorable.

It is well known that there are three basic types of polypeptide conformations, namely, the α helix,[1] the extended β form,[2] and the triple helix.[3] Each of these structures can aggregate to form a fibre, the first and the last by close packing of the approximately cylindrical protofibrils and in the β form by stacking of sheets.[4]

The α helix, the β form, and the triple helix represent single, double, and triple chain hydrogen bonded arrangements. The single chain α helix in which we have intrachain hydrogen bonds nearly parallel to the helix axis has been experimentally shown to be right handed. Each chain in the β form is a twofold helix and the chains are aggregated in the form of sheets with hydrogen bonds nearly perpendicular to the chain axis. In polyglycine II[5] and in poly(L-proline) II,[6] each chain is considered to be a threefold helix. In polyglycine II the chains are packed in a hexagonal array, each chain being hydrogen bonded to its six neighbors. These hydrogen bonds lie roughly perpendicular to the helix axis, and run in several directions. This type of triple chain arrangement with interchain hydrogen bond network throughout is possible only for glycyl residue. The three types of regular structures for polypeptides are shown in the (ϕ, ψ)-map in Figure 1. In this figure, the relevant parameters of a helix, n (number of residues per turn) and h (unit height), are also shown as contours, along with the observed (ϕ,ψ) values of glycyl and prolyl residues taken from available single-crystal data on linear peptides.[7] In addition to the above three types of structures it is known that the ω form of poly(β-benzyl L-aspartate) is a fourfold helix.[8] The ω helix like the α helix also has intrachain hydrogen bonds roughly parallel to the helix axis.

To the best of our knowledge there has been no report on fourfold helical structures and their association in a regular lattice with the interchain hydrogen bonding

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[+] Contribution No. 123 from the Molecular Biophysics Unit, Indian Institute of Science, Bangalore.

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