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Structural Investigations on Poly(4-hydroxy-L-proline). 1. Theoretical Studies[+]

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ABSTRACT: Model building studies on poly(hydroxyproline) indicate that in addition to the well-known helical structure of form A, a left-handed helical structure with trans peptide units and with h = 2.86 Å and n = 2.67 (i.e., 8 residues in 3 turns) is also possible. In this structure which is shown to be in agreement with X-ray data of the form B in the next paper, the γ-hydroxyl group of an $(i + 1)$th Hyp residue is hydrogen bonded to the carbonyl oxygen of an $(i - 1)$th residue. The possibility of a structure with cis peptide units is ruled out. It is shown that both forms A and B are equally favorable from considerations of intramolecular energies. Since form B is further stabilized by intrachain hydrogen bonds, we believe that this is likely to be the ordered conformation for poly(hydroxyproline) in water.

Synthetic polypeptides containing L-proline and 4-hydroxy-L-proline have been extensively studied as models for the fibrous protein collagen, which contains large amounts of these imino acid residues. In spite of the close structural similarity between these residues, there are certain basic differences in their conformational features, as manifested in their homopolymers, viz., polyproline (PP) and poly(hydroxyproline) (PHP). Polyproline undergoes mutarotation in water and acid, from the form I (PP I) structure with all peptide bonds in the cis conformation, to the form II (PP II), in which all peptide bonds are in the trans conformation.[1-3] Similar transformations have also been observed for poly(*o*-acetyl-L-hydroxyproline).[3-5] On the other hand, PHP does not dissolve in most of the organic solvents, except strong acids like dichloroacetic acid.[5] Hence the type of transformations observed for PP in solution have not been observed for PHP. However, X-ray diffraction studies have indicated that PHP exists in two forms, designated as forms A and B.[5] Form A was shown to bear a general resemblance to PP II, but unlike the latter, the PHP structure is stabilized by a series of interchain hydrogen bonds between chains, involving γ-hydroxyl groups.[6] Since the X-ray pattern of form B was rather diffuse, it was not characterized in detail.[5]

[+]Contribution No. 124 from the Molecular Biophysics Unit, Indian Institute of Science, Bangalore, India.

Poly(hydroxyproline) is also more stable in solution than PP II, as indicated by the greater resistance of PHP to disruption by calcium chloride when compared to PP II.[7] The greater stability of an ordered PHP structure in solution can be either an intrinsic property of the isolated helix or the result of stabilizing intermolecular interaction, as in the solid state.[6] Other experiments, including our own, which are discussed in the subsequent paper, indicate that the polymer in solution does not form an aggregated structure but occurs as individual single chain helices. We should therefore be able to account for the extra stability of PHP in terms of intramolecular stabilization offered in some fashion by its hydroxyl group. This, in turn, might also explain the absence of mutarotation in PHP.

Further, we found that, contrary to what has been stated, the presence of the γ-hydroxyl group does not stereochemically hinder the formation of a PP I type structure, with all peptide bonds in the cis orientation. Therefore, the possibility of hydrogen-bond formation involving the γ-hydroxyl group was investigated for structures with all-trans peptide bonds as well as all-cis peptide bonds. Both direct hydrogen bonds and water-bridged hydrogen bonds, as proposed for the collagen structure,[8,9] were explored. A systematic conformational analysis was carried out and the various possible ordered conformations for PHP were, at each stage, checked against not only earlier experimental data but also our own

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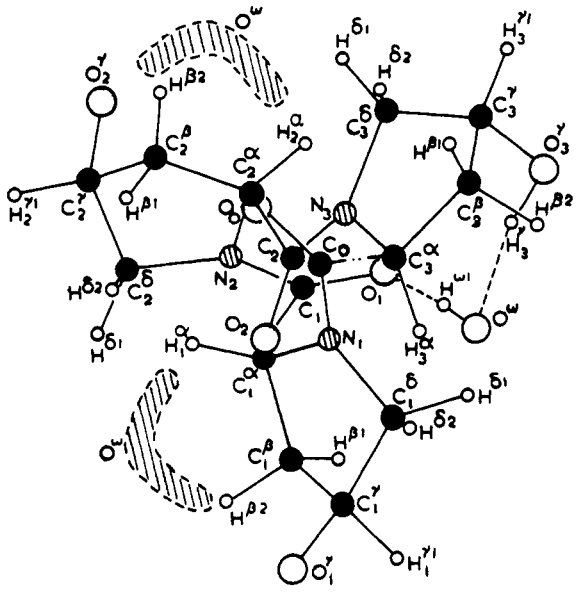

Figure 1. A projection of a single helix of poly(hydroxyproline) (form A) with intrachain hydrogen bonds via water molecules. The region in which the water molecules can occur is shown hatched. A representative water molecule O^{w} linking the γ-hydroxyl group of residue 3 to the carbonyl oxygen of residue 1 is shown.

studies, using spectroscopic techniques and X-ray diffraction. For the sake of convenience, the results of the theoretical studies are presented in this paper. The details of the experimental studies are given in the succeeding paper.

Results and Discussion

(i) Intrachain Hydrogen-Bond Scheme in the Structure of Form A of Poly(hydroxyproline). The structure of form A of poly(hydroxyproline) is a left-handed helix with three residues in the basic repeating unit. The individual residues are also related by an approximate helical symmetry. The structure is stabilized in the solid state by the formation of interchain hydrogen bonds linking the γ-hydroxyl group in one chain to a carbonyl oxygen in a neighboring chain.[6] An investigation of the atomic coordinates of this structure revealed that the γ-hydroxyl group cannot form any direct intrachain hydrogen bond. Recent conformational studies on PHP have also indicated that it is not possible for the γ-hydroxyl group of hydroxyproline to form any intrachain hydrogen bond either with a carbonyl oxygen or with a γ-hydroxyl group of a neighboring residue.[10]

The possibility of hydrogen-bond formation via a water molecule as an intermediary was therefore explored. An examination of the detailed atomic coordinates showed that the distance of the γ-hydroxyl group of an $(i + 1)$th residue in the chain to a carbonyl oxygen of the $(i - 1)$th residue is of the correct order for the formation of a water-bridged hydrogen bond. This scheme of intrachain hydrogen-bond formation is similar to that proposed for the collagen structure.[8,9] A projection of the helical structure of poly(hydroxyproline) form A, along with a water molecule, is shown in Figure 1. The possible location for the water molecule was obtained such that it forms good hydrogen bonds with the γ-hydroxyl group as well as the carbonyl oxygen. It was found that the water molecule can occur over a fairly wide region and still serve to link the two groups. The whole region in which it can occur is shown (as cross-hatched) in Figure 1. One typical location of the water molecule is also shown in this figure. The hydrogen-bond parameters and other relevant details of the structure are given in Table I.

Table I
Relevant Data Regarding the Two Possible Ordered Conformations of Poly(hydroxyproline)

	conf A[a]	conf B[b]
unit height, Å	3.05	2.86
unit twist, deg	−120	−135
no. of units/turn	−3.0	−2.67
tilt of the peptide unit,[c] deg	95	60
dihedral angles (ϕ,ψ,ω), deg	−79,151,180	−60,110,180
energy, kcal/(mol of residue) (only nonbonded + electrostatic + backbone torsional)	−7.9	−9.5
hydrogen-bond parameters: (only intrachain H bond)		
length, Å	$O^{\gamma}_{(i+1)}\cdots O^{w}$ = 2.65 $O^{w}\cdots O_{(i-1)}$ = 2.68	$O^{\gamma}_{(i+1)}\cdots O_{(i-1)}$ = 2.86
angle, deg	O^{γ}-$H^{\gamma}\wedge O^{\gamma}\cdots O^{w}$ = 10 O^{w}-$H^{w}\wedge O^{w}\cdots O$ = 10	O^{γ}-$H^{\gamma}\wedge O^{\gamma}\cdots O$ = 7
short contacts (Å)	$H^{\alpha}_{1}\cdots H^{\delta 2}_{2}$ (1.76)	$H^{\alpha}_{1}\cdots H^{\delta 2}_{2}$ (1.83)
unit cell parameters, Å (hexagonal unit cell)	a = 12.3 c = 9.15	a = 15.75 c = 22.88

[a] This is the conformation described by Sasisekharan,[6] in which the repeating unit consists of three hydroxyproline residues related by approximate threefold screw symmetry. The data given are the average for the three residues. [b] This is the new structure obtained from purely theoretical considerations. [c] Angle between the helical axis and the plane of the peptide unit.

The water molecule, in the region shown, does not give rise to any steric hindrance with other atoms in the polypeptide chain. According to the hydrogen-bond scheme shown in Figure 1, the water molecule acts as an acceptor for a hydrogen bond from the γ-hydroxyl group and as a donor for the hydrogen bond with the carbonyl oxygen. It may be mentioned that, as in the collagen structure, the water molecule can also act as a donor for both of the above-mentioned hydrogen bonds, leaving the proton of the γ-hydroxyl group free to form an interchain hydrogen bond, as in the solid state.

(ii) Consideration of a Structure with Cis Peptide Units. Conformational analysis of poly(hydroxyproline), taking into consideration the possibility of occurrence of cis peptide units also, was carried out by Hopfinger and Walton.[11] They apparently found that the presence of the γ-hydroxyl group sterically prohibits the formation of the polyproline I type of helix. However, an investigation of the detailed structure proposed for PP I,[12] in which all the peptide bonds are in the cis conformation, revealed that the presence of a γ-hydroxyl group does not cause any steric hindrance to other atoms in the polypeptide chain. The γ-hydroxyl group is oriented away from the core of the helix in this structure, as can be seen from Figure 2, in which a projection of the structure of PP I is shown with a hydroxyl group attached to the γ-carbon atom, in the trans orientation. The puckering of the pyrrolidine ring and the orientation of the hydroxyl group are very similar to that observed for the hydroxyproline residue in the crystal structure of cyclo(Pro-Pro-Hyp), which has all three peptide bonds in the cis conformation.[13] Hence it can be

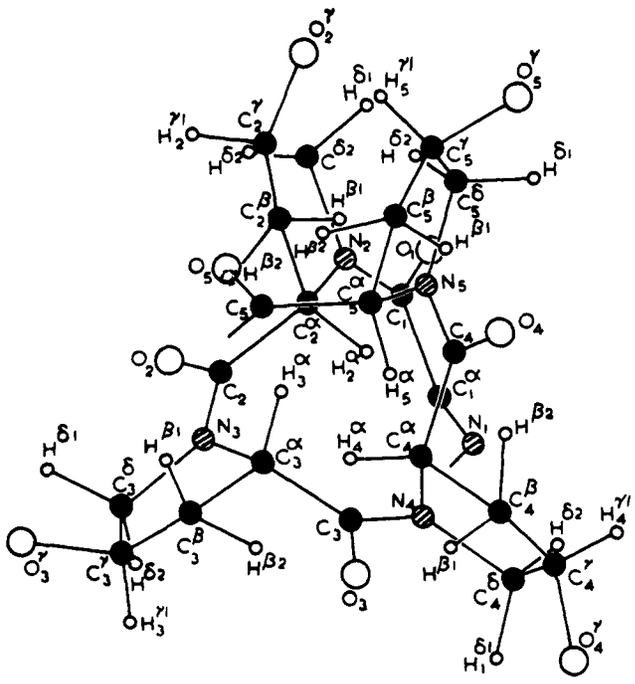

Figure 2. A projection of the polyproline I structure with hydroxyl groups attached to the C^{γ} atoms in a trans orientation. The γ-hydroxyl groups are oriented away from the core of the helix and hence cannot form any intrachain hydrogen bonds.

regarded as a reasonable approximation for the ring geometry in a possible form I type of structure of PHP.

The γ-hydroxyl group in this orientation cannot form any direct intrachain hydrogen bond; therefore the possibility of hydrogen bonding via a water molecule was examined for this structure also. It was found that the distance between the γ-hydroxyl group and a carbonyl oxygen of a hydroxyproline residue two units farther away (e.g., $O^{\gamma}{}_2 \cdots O_4$ in Figure 2) is of the right order for the formation of a hydrogen bond via a water molecule as an intermediary. But if the water molecule is located so as to form good hydrogen bonds with these groups, then it gives rise to severe short contacts with other atoms in the polypeptide chain. Hence, while it is stereochemically possible for PHP to take up a helical structure with cis peptide bonds, such a structure will not be stabilized by any intrachain hydrogen bonds. It is also not possible for systematic interchain hydrogen bonds to be formed in this structure.

(iii) Structure of Poly(hydroxyproline) with Direct Intrachain Hydrogen Bonds. The possibility of poly(hydroxyproline) taking up a helical structure with the γ-hydroxyl group of the imino acid directly hydrogen bonded to a carbonyl oxygen in the same chain was also investigated. A model-building study showed that the only possible intrachain hydrogen bond that can be formed is between the γ-hydroxyl group of an $(i + 1)$th residue and the carbonyl oxygen of the $(i - 1)$th residue, as in the case of the water-bridged hydrogen-bonded structure of form A. Torchia[14] from NMR studies on poly(hydroxyproline) had also suggested that such a hydrogen bond seems to be possible in solution. The approximate ranges of values for the dihedral angles ϕ and ψ were found (from the models) to be $-60 \pm 10°$ for ϕ and $110 \pm 10°$ for ψ. While the value of ϕ is within the generally observed range, the value of ψ is close to the lower limit for this dihedral angle, as evaluated for poly(imino acid) structures from energy calculations.[15,16] Recent theoretical studies in our laboratory,[17] assuming a flexible pyrrolidine ring, have, however, shown that if the prolyl ring has a C^{γ}-exo puckering, then the low-energy conformations for a diprolyl unit occur for $\phi = -55°$ and $\psi = 110°$. Therefore, helical structures were generated with the above-mentioned range of values of the dihedral angles ϕ and ψ for the polypeptide backbone. The geometry of the prolyl ring was taken to be that corresponding to the minimum energy conformation in the diprolyl calculations.[17] A hydroxyl group was fixed in a tetrahedral orientation at the C^{γ} atom, so that it is trans to the carbonyl group. It was found that the distance of the O^{γ} atom of the $(i + 1)$th residue to the carbonyl oxygen of an $(i - 1)$th residue is about 3.1 Å. By varying the ring puckering and slightly shifting the O^{γ} atom from the exact tetrahedral orientation, the distance between the O^{γ} atom and the carbonyl oxygen could be reduced to about 2.85 Å, i.e., within the normally observed range for an O–H···O type of hydrogen bond.[18] The best structure from stereochemical considerations was found to occur for the values of the dihedral angles ϕ and ψ being $-60°$ and $110°$, respectively. The helical parameters for this structure are: unit height (h) = 2.86 Å and unit twist

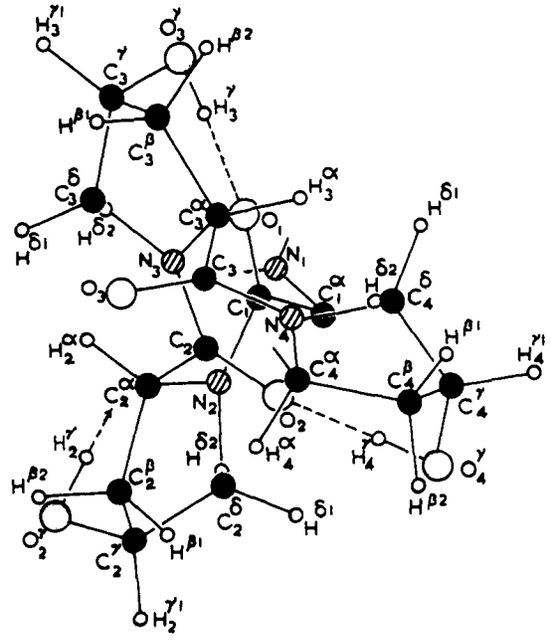

Figure 3. The structure of poly(hydroxyproline) with the γ-hydroxyl group involved in a direct intrachain hydrogen bond with a carbonyl oxygen (conformation B).

Table II
Coordinates of the Atoms in One Residue of 4-Hydroxy-L-proline in Conformation B for Poly(hydroxyproline)[a]

atom	r, Å	ϕ, deg	z, Å	x, Å	y, Å
N	0.93	40.06	−1.17	0.71	0.60
C^{δ}	2.26	47.30	−1.78	1.53	1.66
C^{α}	1.36	0.00	0.00	1.36	0.00
C^{β}	2.79	15.88	0.09	2.68	0.76
C^{γ}	3.22	24.50	−1.27	2.93	1.33
O^{γ}	3.45	6.15	−2.18	3.43	0.37
C	0.51	21.32	1.24	0.48	0.19
O	1.34	76.30	1.74	0.32	1.30
H^{δ_1}	2.20	47.03	−2.88	1.50	1.61
H^{δ_2}	2.92	65.38	−1.45	1.22	2.65
H^{γ_1}	4.24	30.65	−1.17	3.65	2.16
H^{γ}	2.76	1.04	−2.87	2.76	0.05
H^{β_1}	3.04	31.08	0.83	2.60	1.57
H^{β_2}	3.50	1.28	0.37	3.50	0.08
H^{α}	1.86	−35.33	−0.18	1.52	−1.07

[a] The dihedral angles (ϕ,ψ,ω) for the polypeptide backbone conformation have the values −60°, 110°, and 180°, respectively.

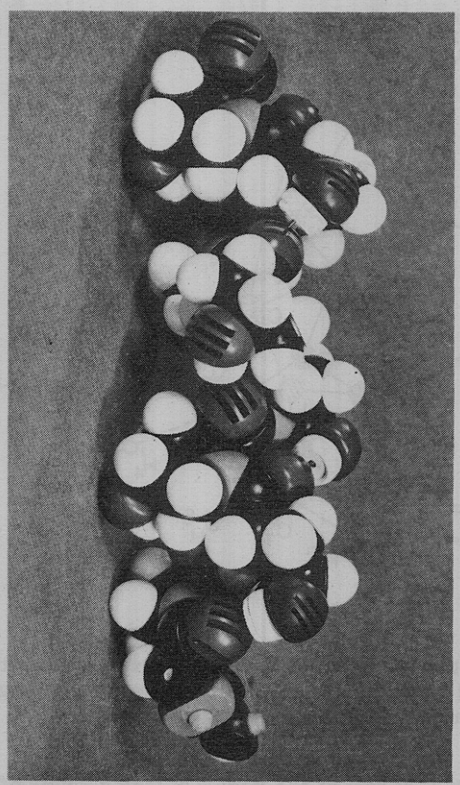

Figure 4. A CPK model of poly(hydroxyproline) form B.

(t) = −135°. A projection of the structure down the helical axis is shown in Figure 3 and a space-filling (CPK) model of the structure is shown in Figure 4. The bond lengths and bond angles, for the polypeptide backbone, correspond to the values for the standard Pauling–Corey geometry. The geometry of the pyrrolidine ring is within the theoretically calculated low-energy region.[19] The helical parameters and other relevant data for this structural model of poly(hydroxyproline) are also summarized in Table I. The coordinates of the atoms in one imino acid residue are given in Table II. We will show in the following paper that this structure is in agreement with the X-ray diffraction data for the form B observed by Sasisekharan[5] and also studied by us in detail. We henceforth refer to this structure as the B form of PHP.

In order to compare the relative stabilities of the PHP chain in forms A and B, energy calculations were carried out, taking into consideration only the intramolecular interactions. The energy values for both the molecular models as well as the short contacts, less than the extreme limit of the contact criteria,[20] are given in Table I. Hydrogen-bond energy has not been included in the energy calculations.

Conclusion

In the form A structure of PHP, intrachain hydrogen bonds can only be formed via water molecules as intermediaries. However, the water molecules occur on the periphery of the helix (as can be seen from Figure 1) and have a free proton pointing away from the core of the helix, so they can be easily disturbed by the solvent medium.

We have also found that a PHP structure with all peptide bonds in the cis conformation is stereochemically possible, but it will not be stabilized by hydrogen bonds of either the intra- or interchain type.

From purely theoretical considerations we have shown that, in addition to the well-characterized form A, PHP can take up another type of left-handed helical conformation, with all peptide bonds remaining in the trans conformation, but with slightly different helical parameters. In this structure the γ-hydroxyl group of an (i + 1)th residue is directly hydrogen bonded to a carbonyl oxygen of the (i − 1)th residue, in the same polypeptide chain.[21] This helical model is in agreement with the X-ray data of form B. This is discussed in the subsequent paper.

It is seen from the energy values given in Table I that the helical conformation for the B form is intrinsically more stable than form A. The slightly higher energy value obtained for form A is due to the H···H short contacts occurring in this structure, and these can be relieved by slight variations in the ring puckering. Hence both structures for PHP are energetically almost equally favorable from intramolecular energy considerations. However, a single chain in the form B conformation will be further stabilized by the formation of the direct intrachain hydrogen bonds. Therefore, we believe this conformation to be likely to be preferred in solution over form A.[22] The detailed physicochemical studies carried out by us to confirm this hypothesis are given in the following paper.[23]

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(21) This type of hydrogen-bonding scheme is also of interest from the point of view of the role of hydroxyproline residues in the collagen structure. However, a preliminary study revealed that the type of intrachain hydrogen bond proposed here, directly linking the γ-OH of hydroxyproline and a carbonyl oxygen in

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

(22) The possibility of a conformation corresponding to $\psi = -60°$ 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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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

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