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Crystal Structure Analysis of Collagen Model Peptide $(Pro\text{-}Pro\text{-}Gly)_{10}$

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Single crystals of $(Pro\text{-}Pro\text{-}Gly)_{10}$ were grown by the hanging drop method. The crystals diffracted to a resolution of 1.8 Å. In the crystals the polypeptides form triple helices that aggregate end-to-end mediated by the solvent molecules, with the basic repeat being 20 Å along the helical axis. Analysis of the 20 Å structure of $(Pro\text{-}Pro\text{-}Gly)_{10}$ using data up to a resolution of 1.9 Å revealed that the overall structure is in accordance with the 7/2 model proposed for collagen. The three strands are held together by the (Gly) N-H···O (Pro-X) hydrogen bond interactions, and additional stability is provided by the (Pro-Y) C^{α}-H···O (Pro-X) hydrogen bonding interactions.

Key words: collagen, hydration, hydroxyproline, model peptide, triple helix.

Collagen is a major structural protein found in connective tissues, bones, blood vessels, *etc.* At least 19 different collagen types have been found in vertebrates (*1*). The triple helix is a unique structural motif of the fibril-forming collagen and is also found in a range of other extracellular matrix proteins, a series of host defense proteins, and certain membrane proteins (*2*). Collagen is formed by the super coiling of three polypeptide chains in a poly-proline II conformation. The general amino acid sequence repeat of these polypeptides is of the form $(Gly\text{-}X\text{-}Y)_n$, and this extends more than 1,000 residues in the case of fibrous collagen. This strict sequence constraint of Gly at every third position is very much necessary for the close packing of the three strands in order to facilitate the formation of the triple helix. The X and Y positions of the sequence are frequently occupied by imino acids, namely, proline and 4-hydroxyproline, which constitute about 20 percent of all residues in collagen. This high content of imino acids imposes a high degree of sterical restrictions which help in stabilizing the extended nature of the chains. The 4-hydroxyproline (Hyp) at the Y position is a result of the post-translational modification of proline by the enzyme prolyl hydroxylase. This Hyp provides the triple helix additional stability as compared to the proline residue (*3, 4*).

Initial models of the coiled-coil helix for the molecular conformation of collagen were proposed in the mid fifties (*5, 6*), based on model building, on the basis of the X-ray diffraction pattern of collagen and diffraction pattern of synthetic polymers like poly-Gly-II and poly-L-Pro-II. These were later confirmed in the early 1960s by the high angle X-ray fiber diffraction pattern of tendon (*7*), which was further substantiated by the fiber diffraction analysis on the kangaroo tail tendon (*8*). Meanwhile, peptides with Gly at every third position and with a high content of imino acids, including $(Pro\text{-}Pro\text{-}Gly)_{10}$ and $(Pro\text{-}Hyp\text{-}Gly)_{10}$ were synthesized (*4, 9*) and found to exist as triple helices in solution. So these peptides became the target of study for a better understanding of the triple helical collagen. Okuyama *et al.* (*10*) in 1981 elucidated the structure of $(Pro\text{-}Pro\text{-}Gly)_{10}$ based on the data collected from a single crystal and solved by the linked-atom least-squares method (*11*).

Of the several models proposed for the detailed conformation of collagen, two models have been generally accepted. One is the Rich and Crick model (*7*), in which each strand has a 10/1 helical symmetry and a helical pitch of 86 Å. This was proposed based on the fiber diffraction pattern from native collagen. The other is the Okuyama model (*10, 12*), in which each strand has a 7/1-helical symmetry and a 60 Å helical pitch. This model could also explain the fiber diffraction pattern of native collagen (*13*). Recently, the crystal structure analysis of $(Pro\text{-}Hyp\text{-}Gly)_{10}$ in which one of the glycines was changed to alanine was solved, and this structure was found to be in accordance with the Okuyama model except for the region around Ala (*14*). Furthermore, a cylindrical Patterson map (*15*) based on data collected from single crystals of $(Pro\text{-}Hyp\text{-}Gly)_{10}$ showed that the main chain conformation was very similar to that of the homologous model peptide $(Pro\text{-}Pro\text{-}Gly)_{10}$, which again confirms the Okuyama model for collagen. To better understand the collagen structure, we are in the process of studying the crystal structures of collagen model peptides. In this paper, one such triple helix-forming peptide $(Pro\text{-}Pro\text{-}Gly)_{10}$ has been analyzed by the recent methodology.

EXPERIMENTAL DATA AND STRUCTURE DETERMINATION

The peptide $(Pro\text{-}Pro\text{-}Gly)_{10}$ was obtained from Peptide Institute, Inc., Osaka, and was used for the crystallization experiments without further purification. Crystals suitable for the crystallographic analyses were grown at 10°C by the

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Abbreviation: Hyp, 4-hydroxyproline.

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hanging drop vapor diffusion method. Good quality crystals (Fig. 1) were obtained from the drop containing an initial concentration of peptide at 4.5 mg/ml in 10% (v/v) acetic acid, 8% (w/v) PEG 400, and 0.1% sodium azide equilibrated against a reservoir containing 1 ml of 16% (w/v) PEG 400. The crystal used for measurement was sealed in a glass capillary along with the mother liquor and used for the X-ray diffraction using a 4-circle diffractometer (AFC5R, Cu Kα radiation, generated by a Rigaku RU 200 rotating anode at 40 kV and 150 mA). The crystal was found to be orthorhombic with the cell dimensions of $a=26.91(1)$, $b=26.37(1)$, $c=20.28(1)$ Å based on the peak search using 20 peaks. Still and oscillation photographs (Fig. 2) of the crystal taken using an R-Axis-IV imaging plate (with X-ray generated by Rigaku ultraX18 rotating anode generator operated at 50 kV and 250 mA with Cu Kα radiation) clearly indicated the existence of spots spaced around 100 Å apart in reciprocal space, on either side of the spots corresponding to the near 20 Å unit cell. Based on the existence of these satellite spots, it was concluded that the true repeat along the c direction was around 100 Å and the final cell dimensions are $a=26.91(1)$, $b=26.37(1)$, $c=101.40(1)$ Å, volume $=71955(15)$ Å^3.

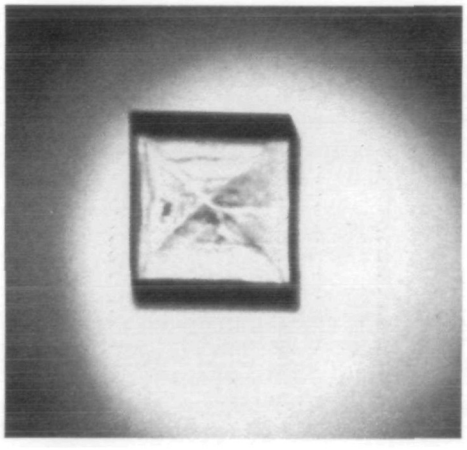

Fig. 1. **A single crystal of (Pro-Pro-Gly)$_{10}$ of size 0.4×0.4×0.3 mm^3.**

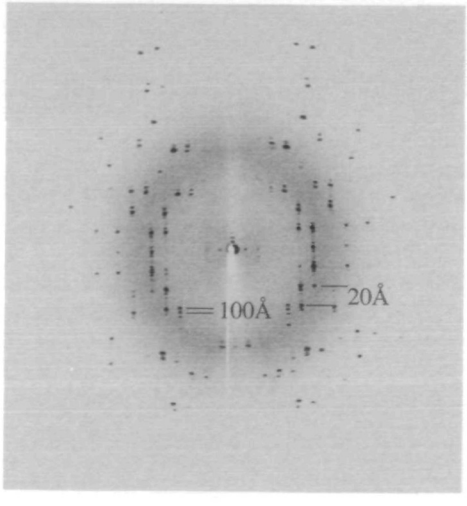

Fig. 2. **Oscillation photograph of (Pro-Pro-Gly)$_{10}$ showing the presence of the satellite spots corresponding to a distance of around 100 Å in reciprocal space along the c-axis.**

Data were collected at 20°C using the 4-circle diffractometer with ω scans. A total of 7,074 reflections were measured, of which 6,924 reflections were found to be unique up to a resolution of 1.82 Å. Intensity measurements were corrected for Lorentz-polarization and absorption effects with the teXsan software of Molecular Structure Inc. (*16*). Decay corrections were also applied, as 15% of decay was observed at the end of the data collection process. The data collection parameters are shown in Table I.

The systematic extinction of (h00) with h odd and (0k0) with k odd are observed, and all the (00l) reflections were too weak to be observed. Comparison of sections at $w=0$ and $w=1/2$ of the Patterson map indicated the presence of a 2-fold screw symmetry along the c axis. Therefore the space group was concluded to be $P2_12_12_1$. Two strong near meridional reflections, (1 0 35) and (0 1 35), are observed, showing a spacing of 2.86 Å, which agrees with the short axial repeat generally reported for the collagen triple helices and polypeptide models of collagen (*17*).

The unit cell parameters agree with the dimensions of the triple helix with 4 triple helices in a unit cell (based on the space group $P2_12_12_1$, with one triple helix per asymmetric unit) and the helix axis along the direction of the longest axis. All the above observations are similar to those made in the earlier case (*10*) of the crystal structure analysis of (Pro-Pro-Gly)$_{10}$.

Attempts were made to solve the structure of (Pro-Pro-Gly)$_{10}$ by the molecular replacement method. The Oku-

TABLE I. **Crystal data and data collection parameters of (Pro-Pro-Gly)$_{10}$.**

Chemical formula	$C_{360}H_{570}O_{90}N_{90}$
Molecular weight	7,599.01
Space group	$P2_12_12_1$, $Z=4$
Cell dimensions	$a=26.91(1)$, $b=26.37(1)$, $c=101.40(1)$ Å, $\alpha=\beta=\gamma=90°$,
Volume	$V=71955(15)$ Å^3,
Crystal dimensions	0.40×0.40×0.30 mm
Scan mode	Omega
Scan speed	4°/min
Scan width	$(\Delta w)=(1.10+0.3\tan\theta)°$
No. of standard reflections	3
No. of reflections measured	7,074
No. of unique reflections	6,924
Range of h, k, l	0<h<14, 0<k<14, 0<l<55
$2\theta_{max}$	50°

TABLE II. **Average F_o for $l=5n$, $5n+1$, $5n+2$, $5n+3$, $5n+4$ reflections ($n=1$ to 10) in various resolution ranges.**

Resolution range (Å)	Average F_o				
	$5n$	$5n+1$	$5n+2$	$5n+3$	$5n+4$
8.0-6.0	27.64	5.98	4.08	4.30	4.96
6.0-4.5	33.66	10.78	5.44	4.63	7.47
4.5-3.5	32.77	12.46	7.68	6.39	7.71
3.5-2.8	21.23	11.13	6.87	6.72	8.00
2.8-2.3	15.17	10.73	6.99	6.49	7.23
2.3-2.0	12.85	9.85	7.31	6.77	7.34
2.0-1.9	11.83	9.79	7.25	7.39	7.70
8.0-1.9	18.01	10.47	7.00	6.60	7.50
No. of observed reflections ($F_o>\sigma F_o$)					
3,476	1,035	894	506	454	587

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yama model was taken as the probe model for (Pro-Pro-Gly)$_{10}$, which has a 7/2 helical symmetry (*10*) with a pseudo repeat of around 20 Å along the helical axis. Software package X-PLOR (*18*) was used in order to place the model at a unique position in the unit cell. Unique positions along the *a* and *b* directions were found unambiguously and were also verified using *hk*0 reflections, but no unambiguous position could be found along the *c* direction, because the unique helical symmetry of the model combined with the crystallographic symmetry produce about 10 ambiguous positions. Also, since 42% of the data were found to be unobserved ($F_o < \sigma F_o$), it was not practically possible to place the molecule at a unique position in the unit cell. Table II gives the average F_o statistics of the reflections with $F_o > \sigma F_o$ at various resolution ranges.

This situation basically constitutes a fiber like columnar, end-to-end association of the (Pro-Pro-Gly)$_{10}$ mediated through the solvent in the crystal, and hence the subcell

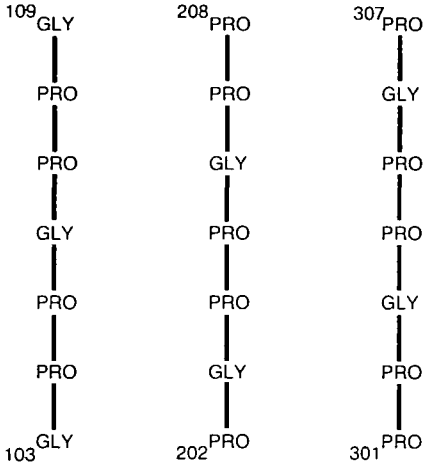

Fig. 3. **Schematic representation of the triple helix used as the probe model.**

TABLE III. **Final refinement statistics for the 20 Å model of (Pro-Pro-Gly)$_{10}$.**

Resolution range	8.0–1.9 Å
Number of reflections used ($F > 2\sigma F$)	945
R factor	21.9%
Free *R* factor	26.6%
Root mean square deviations	
Δbonds	0.016 Å
Δangles	3.46°
Average *B*-values	15.35 Å^2

R factor was based on 867 reflections, and free *R* factor was based on 78 reflections.

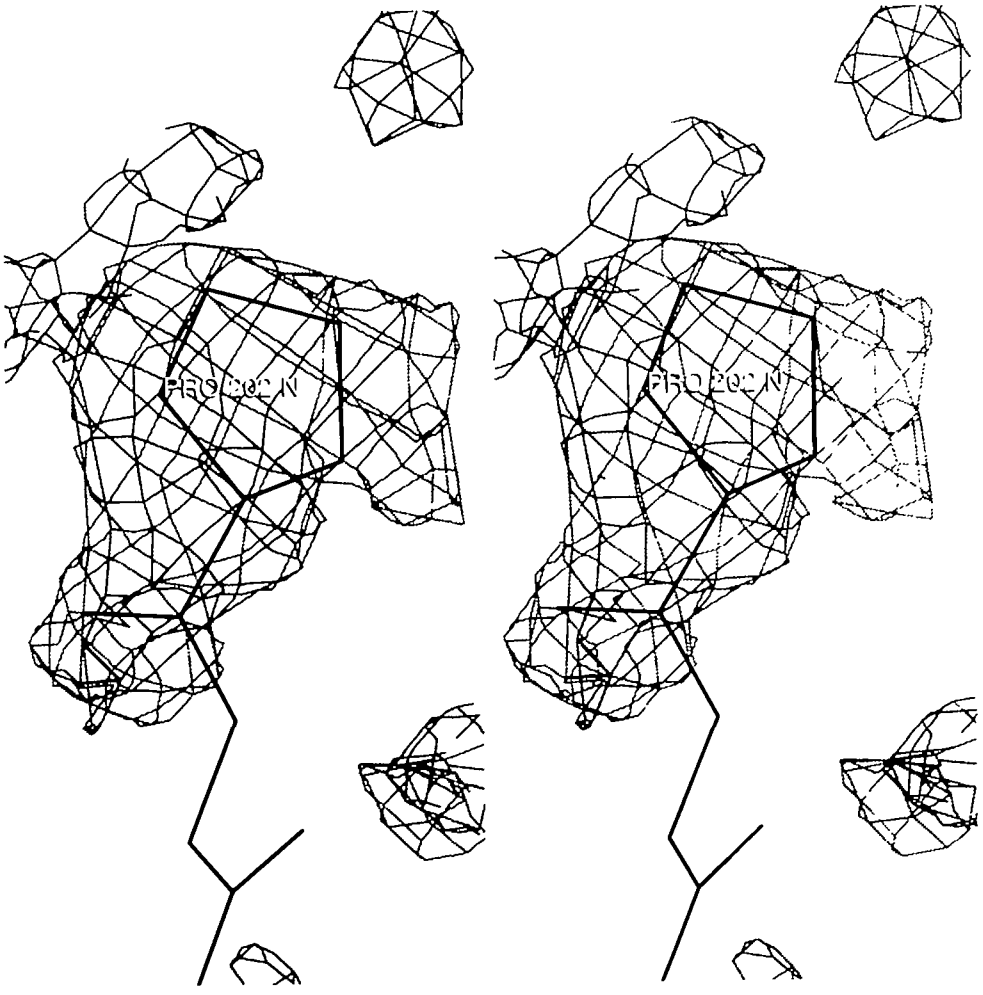

Fig. 4. **$F_o - F_c$ omit map for the residue Pro-202.**

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structure with $c=20.28$ Å was considered to be the basic repeating unit along the l-direction. So only those reflections, namely, $l=5n$, corresponding to the subcell with $c=20.28$ Å along the helical axis were considered for solving the structure.

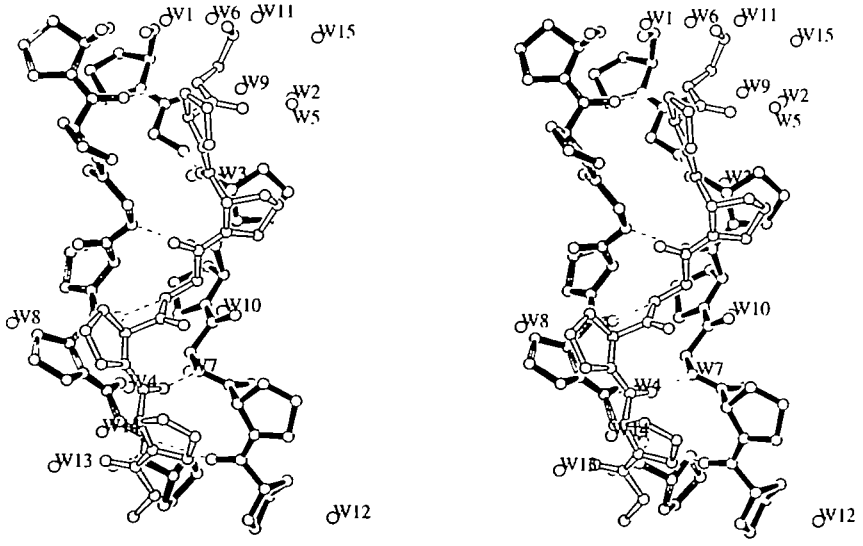
This situation is essentially similar to the assumption made in the earlier case (*10*) of crystal structure analysis of $(Pro\text{-}Pro\text{-}Gly)_{10}$. In the earlier study, however, a single tripeptide unit with standard bond length, bond angles and rotational freedom about only six torsion angles was considered to be the basic repeating unit, and the structure was solved by the linked-atom least-squares method (*11*). In the present study, the structure is analyzed as a single crystal with restraints applied to only bond lengths and bond angles as necessitated in SHELX-97 (*19*), while no torsion angle restraints were applied.

The probe model is again the model proposed by Okuyama, consisting of three strands in a 7/2 helical symmetry with each chain consisting of 7 amino acids, and as a whole consisting of 7 tripeptide units forming a near 20 Å repeat. A schematic representation of the probe model is shown in Fig. 3. The probe model was placed at an appropriate position in the unit cell by the trial and error method using X-PLOR.

The refinement of the structure was done using SHELX-97. A total of 867 reflections in the resolution range 8.0 to 1.9 Å were used for refinement, while 78 reflections were used to monitor the free R. The restraints necessary for refinement of the protein structure as described in the program SHELX-97 were applied and refined by the conjugate gradient method. Constraints were also applied so as to hold the triple helices of the adjacent unit cells at a covalent bonding distance from that of the triple helix under consideration along the helical axis, in order to mimic a continuous triple helix. The model was refined and corrections to the model were made based on the $2F_o-F_c$ electron density map with the molecular graphics program Xfit of the software package XtalView (*20*). After the necessary corrections were made to the model, water molecules were chosen based on the F_o-F_c electron density map. In all, the structure consists of 21 amino acids and 15 water molecules, showing reasonable agreement with the X-ray data and stereochemical restraints. The final R-factor converged to a value of 0.219 for the 867 reflections used in the refinement procedure, while the free R was at 0.266 for 78 reflections. The final refinement statistics are detailed in Table III. The final model including water molecules was investigated by the omit map, which was calculated by the following procedure. The occupancy factors of one residue or five water molecules were set to zero, and the (F_o-F_c) electron density was calculated after 10 cycles of positional refinement to avoid the model bias. One such (F_o-F_c) omit map calculated by omitting the residue Pro-202 is shown in Fig. 4. The agreement between the observed and calculated structure amplitudes ($R=0.219$, free $R=0.266$) is reasonable, if one takes into account the assumption that the structure is an infinite fiber.

RESULTS AND DISCUSSION

Overall Structure—The 20 Å structure of the collagen model peptide $(Pro\text{-}Pro\text{-}Gly)_{10}$ along with the 15 water molecules is shown in Fig. 5. The triple helical structure consists of three polypeptide strands making a diameter of around 10 Å. Each strand consists of seven amino acids and has a left-handed helical conformation (minor helix). Each strand is staggered by one amino acid residue along the helical axis, with the average staggering being 2.86 Å. The three strands coil about a common axis in a right-handed



strand 1 running from residue 103 to 109
strand 2 running from residue 202 to 208
strand 3 running from residue 301 to 307

Fig. 5. Stereo view of the 20 Å model of triple strand along with the 15 water molecules. The interchain N...O hydrogen bonds are indicated by dotted lines.

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helical manner, following a left-handed 7/2 helical symmetry as a whole. This model has a tighter winding than the fiber model (Rich and Crick 10/3 model).

The helical parameters of the individual chains show a broad distribution from one tripeptide unit to another, the average unit height of a tripeptide unit being about 8.79 Å and the average twist per tripeptide unit 50.7°. These values are much closer to the case of the 7/2 model (Okuyama model) (*13*) than those of the 10/3 model (Rich and Crick model) based on native collagen (*7*). The super helical twist of the triple helix has a much broader distribution and is centered around −103.1°. The average unit height or the super helical height is around 2.86 Å, *i.e.*, a tripeptide unit is staggered by a height of 2.86 Å from the corresponding tripeptide unit of the neighboring strand in the clockwise direction. This value is consistent with the observed height in the case of stretched collagen (*8*).

Chain Conformation—The average ϕ, ψ, and ω torsion angles as obtained for the Pro(X), Pro(Y), and Gly are shown in Table IV. The average values are much closer to the values obtained in the case of the 7/2 model than to those of the 10/3 model, which means that the strands are more tightly wound in the case of $(Pro\text{-}Pro\text{-}Gly)_{10}$.

The deviation of the C^γ atom above or below the plane formed by the other four atoms of the proline ring represents the puckering of the proline residue. A Pro-down conformation (X-position) and Hyp-up conformation (Y-position) pattern is reported for collagen (*8, 14*) and in the two-dimensional NMR investigation of the solution structure of $(Pro\text{-}Hyp\text{-}Gly)_{10}$ (*21*). The puckering statistics for the proline residues are given in Table V. A positive value indicates a down conformation, while a negative value indicates an up conformation. It can be seen that 5 out of 7 cases show a down conformation in the case of Pro at the X-position, which indicates that the Pro at the X-position may take a predominantly down conformation. But in the case of Pro at the Y-position, the up and down conformations are nearly equally distributed and hence a definite conclusion cannot be obtained from the present structure.

Interchain Hydrogen Bonding—All the Gly amide and Pro(X) carbonyl groups take part in the interchain hydrogen bonding (N-H···O=C) (Fig. 5) running along the direction from strand 1 to strand 2 to strand 3 to strand 1, following the pattern of model II of Rich and Crick (*6*). In addition, the carbonyl Pro(X) is also at hydrogen bonding distance from the C^α of Pro(Y) (C^α-H···O=C), running along the same direction as the N-H···O=C. Therefore, bifurcated hydrogen bonds are present, similar to those found in the case of proteins (*22, 23*) and studied in detail in the case of a collagen model peptide (*24*). The hydrogen bonding parameters are tabulated in Table VI. The hydrogen atoms were fixed geometrically for the amide group of Gly and the C^α atom of the preceding proline (Pro-Y) riding over the parent atom and were not part of the refinement procedure.

TABLE IV. **Average main chain conformation angles.**

Torsion angle	Present study	Okuyama 7/2 helix	Rich & Crick 10/3 helix
ϕ Pro(X)	−77.1°±7.5°	−75.5°	−72.1°
ψ Pro(X)	163.8°±4.7°	152.0°	164.3°
ω Pro(X)	179.1°±1.3°	−176.8°	180.0°
ϕ Pro(Y)	−62.4°±7.9°	−62.6°	−75.0°
ψ Pro(Y)	154.0°±8.8°	147.2°	155.8°
ω Pro(Y)	177.6°±3.9°	−172.8°	180.0°
ϕ Gly	−76.7°±6.6°	−70.2°	−67.6°
ψ Gly	176.6°±7.1°	175.4°	151.4°
ω Gly	179.0°±1.8°	178.2°	180.0°

TABLE V. **Puckering statistics of the proline residues at X and Y positions.**

Proline at X position	
Residue No.	Deviation of C^γ atom[a]
104	(+)0.2310
107	(+)0.4981
204	(−)0.2171
207	(−)0.3587
301	(+)0.5301
304	(+)0.5234
307	(+)0.4051
Proline at Y position	
Residue No.	Deviation of C^γ atom[a]
105	(−)0.1484
108	(+)0.4008
202	(−)0.4327
205	(+)0.3804
208	(−)0.2200
302	(+)0.3951
305	(−)0.3701

[a](+) indicates down conformation, (−) indicates up conformation.

TABLE VI. **Interstrand hydrogen bonding parameters observed in the triple helix.**

N-H...O hydrogen bonds involving N of Gly			
Hydrogen bonds	N···O[b]	H···O[b]	<N-H···O[c]
N(203)···O(301)	2.91	2.19	140.3
N(303)···O(104)	2.98	2.16	159.4
N(106)···O(204)	2.91	2.16	145.3
N(206)···O(304)	2.76	1.96	153.2
N(306)···O(107)	2.92	2.17	144.9
N(109)···O(207)	3.03	2.24	152.9
C^α-H···O hydrogen bonds involving C^α of Pro(Y)			
Hydrogen bonds	C^α···O[b]	H···O[b]	<C^α-H···O[c]
C^α(202)···O(301)	3.16	2.35	139.5
C^α(302)···O(104)	3.20	2.33	147.8
C^α(105)···O(204)	3.29	2.45	144.3
C^α(205)···O(304)	3.09	2.30	136.4
C^α(305)···O(107)	3.24	2.41	142.1
C^α(108)···O(207)	3.17	2.34	142.2

[b]Distances in (Å). [c]Angles in (°).

TABLE VII. **Hydrogen bonds involving water molecules.**

Hydrogen bonds	O···Wat/Å	<C=O···Wat/°
O(108)···W(5)	2.74	135.0
O(108)···W(2)	2.41	137.8
O(109)···W(11)	2.60	143.2
O(202)···W(13)	3.02	157.0
O(203)···W(4)	2.70	155.5
O(205)···W(15)#2[d]	2.59	141.0
O(305)···W(3)	3.39	145.6
O(306)···W(9)	2.69	129.9
W(13)···W(14)	3.32	
W(14)···W(4)	2.82	
W(4) ···W(7)	2.82	
W(7) ···W(10)	3.14	
W(5) ···W(15)	3.15	
W(11)···W(15)	2.76	
W(6) ···W(11)	2.96	

[d]Symmetry related water molecule.

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Role of Water Molecules—The water molecules form a cylinder of hydration around the triple helix. Several carbonyl groups of Pro(Y) and Gly are hydrogen bonded to water molecules, and the hydrogen bonding parameters are tabulated in Table VII. A few carbonyl oxygens do not have any water molecule present in their vicinity. This may be due either to the water molecules being highly disordered or to the assumption of a continuous fiber and the structural analysis using the subcell lattice. The hydrogen bonding interactions involving the water molecules are shown in Fig. 6. In the case of the structural study of $(Pro\text{-}Pro\text{-}Gly)_{10}$ by the linked-atom least-squares method (*10*), each tripeptide was associated with three water molecules, and these water molecules were symmetrically present around the triple helix; but in this study only 15 water molecules were found for the 20 Å repeating unit, and the water molecules were not found to be symmetrically dispersed. Only 8 water molecules are at direct hydrogen bonding distance from the triple helix.

Hydrogen bonding interactions between the water molecules have been observed in seven cases (Fig. 6). It is generally accepted that water molecules around the triple helix play a very important role in the aggregation of the triple helices to form the fibril (*3, 9, 25-27*), because direct interaction between the triple helices is not possible owing to the distance between them. Accordingly interactions between the triple helices mediated through water were found at certain places. In one case, two water molecules take part in hydrogen bonding interaction between the carbonyl oxygen of two symmetrically related triple helices (Fig. 6). Even though such interactions are not found in plenty in this structural analysis of $(Pro\text{-}Pro\text{-}Gly)_{10}$, these are the kind of interactions that have been found to be the driving force in keeping the triple helices together.

Packing Structure—The triple helices are packed together in two kinds of clusters, one triangular and the other square (pseudo tetragonal lattice), as shown in Fig. 7, as dictated by the space group $P2_12_12_1$ of the orthorhombic system. The interactions between the triple helices forming the triangular cluster can be clearly seen to be mediated through water molecules, while the wide channels in case of the square cluster presumably contain most of the amorphous water (*10*).

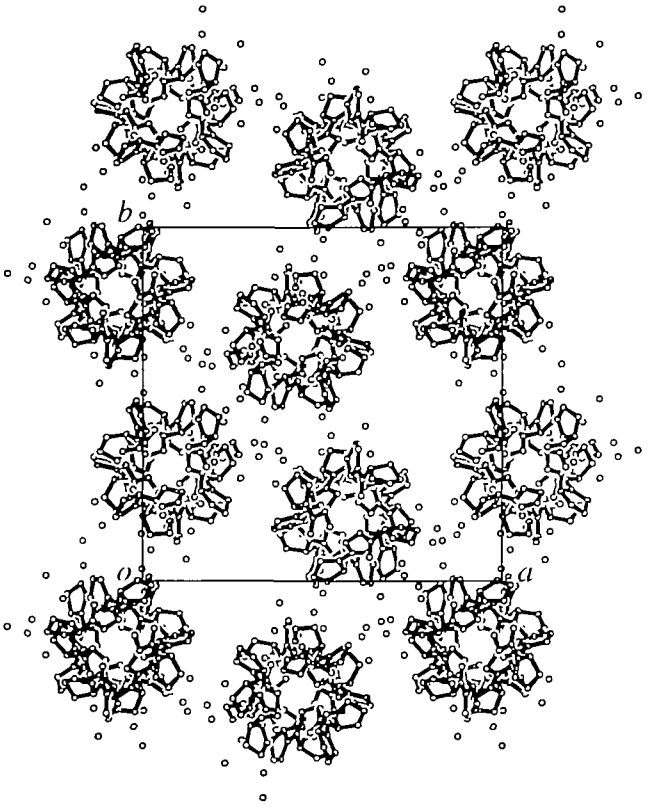

Fig. 7. **Packing structure of triple strands together with the water molecules, viewed along the helix axis.**

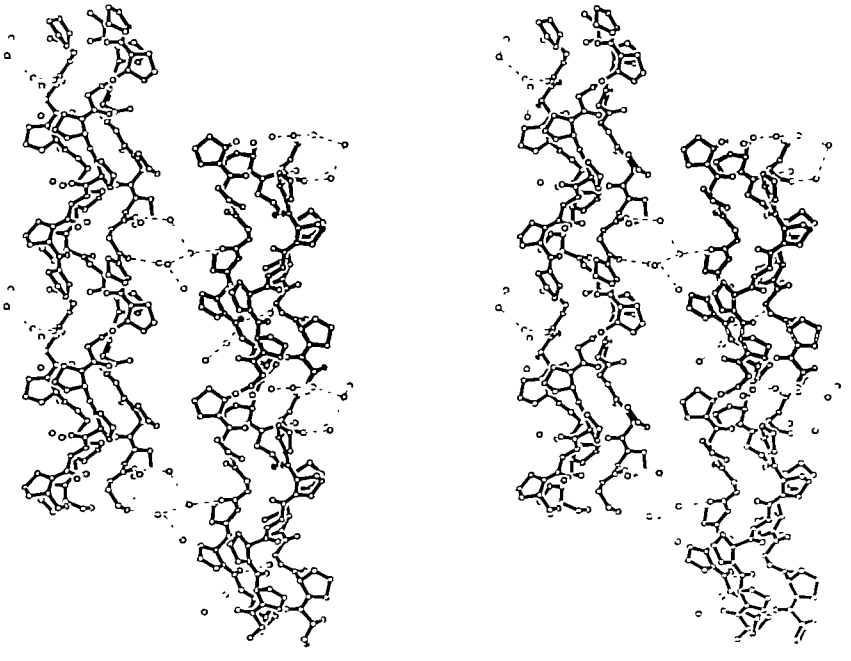

Fig. 6. **Stereo view of the hydrogen bonding network.** The open bonded molecule is the 20 Å model as shown in Fig. 5. Altogether 4 symmetry related molecules and their hydrogen bonding interactions (dotted lines) are shown.

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Implications for Collagen Structure—From the structural analysis of $(Pro\text{-}Pro\text{-}Gly)_n$, we can conclude that the three peptides come together to form a triple helix, the three peptides run in parallel, and each strand is staggered one amino acid residue apart (2.86 Å). The three peptides are held together in a triple helical conformation by the hydrogen bonding between the amide group of Gly and the carboxyl oxygen at the X position of the next strand in the anti-clockwise direction. Also the C^{α}-H···O hydrogen bonds provide extrastability to the triple helical formation. In structural studies involving collagen model peptides with a high content of imino acids, a 7/2 helical twist is generally observed, implying that a similar kind of structure will be observed at least in the imino acid-rich regions of the triple helical collagen.

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