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Stabilization of Collagen Fibrils by Hydroxyproline†

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ABSTRACT: The substitution of hydroxyproline for proline in position Y of the repeating Gly-X-Y tripeptide sequence of collagen-like poly(tripeptide)s (i.e., in the position in which Hyp occurs naturally) is predicted to enhance the stability of aggregates of triple helices, while the substitution of Hyp in position X (where no Hyp occurs naturally) is predicted to decrease the stability of aggregates. Earlier conformational energy computations have indicated that two triple helices composed of poly(Gly-Pro-Pro) polypeptide chains pack preferentially with a nearly parallel orientation of the helix axes [Nemethy, G., & Scheraga, H. A. (1984) *Biopolymers 23*, 2781–2799]. Conformational energy computations reported here indicate that the same packing arrangement is preferred for the packing of two poly(Gly-Pro-Hyp) triple helices. The OH groups of the Hyp residues can be accommodated in the space between the two packed triple helices without any steric hindrance. They actually contribute about 1.9 kcal/mol per Gly-Pro-Hyp tripeptide to the packing energy, as a result of the formation of weak hydrogen bonds and other favorable noncovalent interatomic interactions. On the other hand, the substitution of Hyp in position X weakens the packing by about 1.7 kcal/mol per Gly-Hyp-Pro tripeptide. Numerous published experimental studies have established that Hyp in position Y stabilizes an isolated triple helix relative to dissociated random coils, while Hyp in position X has the opposite effect. We propose that Hyp in position Y also enhances the stability of the *assembly* of collagen into microfibrils while, in position X, it decreases this stability.

We demonstrate that hydroxyproline plays an important role in the stabilization not only of the triple-helical collagen molecule but also of its assembly into microfibrils. The 4-hydroxyprolyl residue occurs frequently in the Y position of the Gly-X-Y repeating tripeptide of vertebrate collagens, but never in the X position. For example, in the best-characterized structure, bovine skin type I collagen, Hyp is found 112 times in the 337 triple-helical Gly-X-Y tripeptides of the α1(I) chain (Bornstein & Traub, 1979). Its stabilizing effect on the triple helix has been recognized for many years. The presence of Hyp in position Y raises the melting temperature, i.e., the temperature of the triple helix-random coil conversion, in synthetic poly(Gly-Pro-Hyp) as compared to poly(Gly-Pro-Pro) and in collagens with increasing amounts of Hyp (Kobayashi et al., 1970; Berg & Prockop, 1973; Jimenez et al., 1973; Rosenbloom et al., 1973; Sakakibara et al., 1973; Ward & Mason, 1973; Fessler & Fessler, 1974; Burjanadze, 1982). On the other hand, substitution of Hyp for Pro in position X decreases the melting temperature (Inouye et al., 1982). It has been shown, however, that no hydrogen bonds can be formed directly between the hydroxyl group of Hyp in either position X or Y and any backbone groups of the same triple helix (Ramachandran et al., 1973; Traub, 1975; Miller et al., 1980). Thus, the stabilizing effect of Hyp on the triple helix must come from some other source. It has been proposed that it is due to interactions with the solvent. Specific hydrogen bonding involving water molecules has been proposed in several models (Ramachandran et al., 1973; Traub, 1974; Bansal et al., 1975; Privalov et al., 1979; Suzuki et al., 1980).

In most studies cited, the thermal stabilities of *isolated triple helices* of poly(tripeptides)s were investigated. Very little information is available on the effect of hydroxyproline on the

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assembly of triple helices into microfibrils. The shrinkage temperature T_s of collagen fibrils increases with increasing Hyp content (Gustavson, 1954a,b). A correlation between T_s and Hyp content is seen in data from various sources, compiled by Burjanadze (1982) [cf. Privalov (1982)]. It is not clear, however, whether changes in the shrinkage temperature merely reflect the stabilizing effect of Hyp on the triple helices themselves or whether Hyp contributes directly to the interactions between triple helices. The latter possibiity has been suggested on geometrical grounds, by Bansal et al. (1975), who pointed out the stereochemical possibility of forming a hydrogen bond between a Hyp OH group on one triple helix and a backbone C=O group on an adjacent triple helix [see also Ramachandran et al. (1975)].

The contribution of such an interaction to the energy of packing triple helices has been investigated in the work reported here. We have shown earlier that the most stable manner of packing of poly(Gly-Pro-Pro) triple helices is a near-parallel packing arrangement with an orientation angle of −10° between the helix axes (Némethy, 1983; Némethy & Scheraga, 1984). It will be shown here that the same packing is stabilized even more strongly when Hyp is substituted for Pro in position Y but not in position X.

Methods

The method described earlier for the packing of poly(Gly-Pro-Pro) triple helices was used (Némethy & Scheraga, 1984). The two triple helices were identical, and each of them consisted of three identical CH_3CO–(Gly-L-Pro-L-Hyp)$_5$–$NHCH_3$ polypeptide chains. The bond lengths, bond angles, and backbone dihedral angles of each chain were fixed, with dihedral angle and helical parameters that correspond to the lowest energy computed triple-helical poly(Gly-Pro-Hyp) structure (Miller & Scheraga, 1976; Miller et al., 1980), viz., (ϕ_{Gly},ψ_{Gly},ϕ_{Pro},ψ_{Pro},ϕ_{Hyp},ψ_{Hyp}) = (−74°, 170°, −75°, 167°, −75°, 153°), corresponding to a translational repeat of 8.95 Å and an angular repeat of 44° per tripeptide. All peptide groups were fixed in the planar trans conformation (ω = 180°). Thus, each triple helix was considered as a rigid unit. Only the side-chain dihedral angle $\chi^{3,1}$ describing rotation of the hydroxyl group about the C^γ–O^δ bond, was allowed to vary.

Each triple helix was generated by the method described earlier (Miller & Scheraga, 1976; Miller et al., 1980), by (i) generating one polypeptide chain with the given set of dihedral angles, (ii) determining the helical parameters, i.e., the translational and angular repeat, and computing the location of the helix axis, and (iii) generating the other two strands, assuming that the three chains are conformationally equivalent. The position of the second triple helix, relative to the first one, is described in terms of six external parameters, viz., three Euler angles (α, β, γ) and three components of the translational displacement (t_f, t_g, t_h), as described earlier (Scheraga et al., 1982; Chou et al., 1983; Némethy & Scheraga, 1984).

The residue geometry and the interatomic interaction energies used were those of the revised version, ECEPP/2 (Némethy et al., 1982), of the Empirical Conformational Energy Program for Peptides, ECEPP (Momany et al., 1975). The interaction energy between two triple helices was minimized with respect to the six external variables and the dihedral angle $\chi^{3,1}$ of the Hyp residues. The intrahelix energies were constant, with the exception of a small contribution from the interaction of the hydroxyl H atoms of the Hyp residues with neighboring residues (Miller et al., 1980) because the position of these atoms depends on $\chi^{3,1}$. Only this variable part of the intrahelix energy is included in the energies reported here. The Powell algorithm was used for energy minimization (Powell, 1964). The computations were carried out on a Prime 550 minicomputer with an attached Floating Point Systems AP-120B array processor (Pottle et al., 1980). The standard convention for peptide conformations is used (IUPAC–IUB Commission on Biochemical Nomenclature, 1970).

The relative position and orientation of the axes of the two triple helices can be described in terms of the distance of closest approach, D, between the helix axes and the orientation angle, Ω_0, which is the dihedral angle formed by the two helix axes and a line that connects them and is perpendicular to both of them. The formal definition of these two parameters has been given earlier (Chou et al., 1983; Némethy & Scheraga, 1984). Ω_0 = 0 and ±180° correspond to parallel and antiparallel orientations, respectively, of the triple helices.

Starting points for energy minimization correspond to various choices of the relative position and orientation of the two triple helices, following the procedure described earlier for poly(Gly-Pro-Pro) triple helices (Némethy & Scheraga, 1984). Instead of the 240 starting points described in that work, only 108 starting points were used, because many sets of starting points that give high-energy packings for poly(Gly-Pro-Pro) could be omitted. On the other hand, for each of these starting points, three initial values of the dihedral angle $\chi^{3,1}$ were used, corresponding to the optimal staggered t, g^+, and g^- sidechain conformations, determined earlier [Table VII of Miller et al. (1980)].

Regions of contact were defined as before (Chou et al., 1983; Némethy & Scheraga, 1984), by listing pairs of atoms (one each from the two triple helices) that had a separation distance less than 1.1 times the sum of the respective van der Waals radii.

Results and Discussion

All low-energy packing arrangements computed for [CH_3CO–(Gly-Pro-Hyp)$_5$–$NHCH_3$]$_3$ triple helices are the same as those computed earlier (Némethy & Scheraga, 1984) for [CH_3CO–(Gly-Pro-Pro)$_5$–$NHCH_3$]$_3$ triple helices. The orientation angle Ω_0 between the axes of the triple helices was near −10° or 10° in all cases for both polypeptides [cf. Figure 3 of Némethy & Scheraga (1984)], with differences of <±1° in Ω_0 and <0.6 Å in D for corresponding pairs of packing arrangements for the two polypeptides. In particular, the lowest energy packing for the Gly-Pro-Pro triple helices had Ω_0 = −10.4° and D = 12.2 Å, while that for the Gly-Pro-Hyp triple helices had Ω_0 = −10.4° and D = 12.1 Å. The interhelix energy for this packing arrangement was much lower, however, in the case of the Gly-Pro-Hyp sequence, viz., E_{inter} = −45.37 kcal/mol, compared with the value of the E_{inter} = −34.38 kcal/mol computed for the Gly-Pro-Pro sequence (Némethy & Scheraga, 1984). Only this packing arrangement will be considered in the rest of this paper.

The packing is shown in Figure 1, and the interatomic contacts are summarized in Table I. A comparison with Figure 4 and Table III of our paper for [CH_3CO–(Gly-Pro-Pro)$_5$–$NHCH_3$]$_3$ triple helices (Némethy & Scheraga, 1984) shows that the relative disposition of the triple helices is the same and the residues and atoms forming contacts are identical in the two cases, except for the additional contacts formed by the hydroxyl group of the Hyp residues. The most significant new interactions are weak hydrogen bonds formed between Hyp OH groups of one triple helix and backbone C=O groups of a Hyp residue in the other triple helix, listed in Table II. Because of the screw symmetry of the triple helices (Miller & Scheraga, 1976; Miller et al., 1980), the hydrogen bonding pattern is repeated after a translation of 27 Å (i.e., three tripeptide repeat units), except that residues on different chains

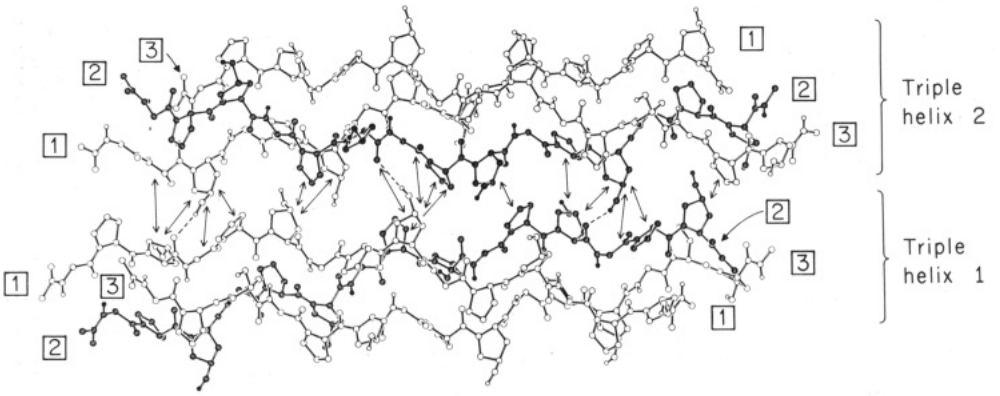

FIGURE 1: Computed lowest energy packing arrangement of two $[CH_3CO–(Gly\text{-}Pro\text{-}Hyp)_5–NHCH_3]_3$ triple helices, showing the near-parallel alignment ($\Omega_0 = -10°$) of the two triple helices and the O–H···O=C hydrogen bonds between the triple helices (shown with dashed lines). The packing of the two triple helices is indicated schematically: double-headed arrows point to residues involved in contacts between the two triple helices. Each arrow corresponds to an entry in Table I. Numbers enclosed in squares at the right and left ends denote the numbering of the strands in each triple helix. Strand 2 of both triple helices is drawn with shaded atoms. Hydrogen atoms are omitted, except for those of OH and NH groups (shown with small circles).

Table I: Contacts between Two $[CH_3CO–(Gly\text{-}Pro\text{-}Hyp)_5–NHCH_3]_3$ Triple Helices in the Lowest Energy Packing Arrangement[a]

			Triple helix 2															
			Strand	1						2								3
Triple helix 1			Residue[c]	2	3	4	5	6	...	5	6	7	8	9	10	11	12	... 14
Strand[b]	Residue[c]			Pro	Hyp	Gly	Pro	Hyp		Pro	Hyp	Gly	Pro	Hyp	Gly	Pro	Hyp	Pro
1	3	Hyp		2	2[d]													
	4	Gly			4													
	5	Pro			10													
	6	Hyp						3		6								
	7	Gly																
	8	Pro																
	9	Hyp									1[d]	1	10					
	...																	
2	8	Pro											1					
	9	Hyp																
	10	Gly																
	11	Pro												7				
	12	Hyp														2	2[d]	
	13	Gly															3	
	14	Pro															10	
	15	Hyp																4

[a] Only the contacts involving heavy atoms are counted. The entries are the numbers of pairwise atomic contacts between the residues indicated at the top and left-hand margins. Each of the 16 entries of the table represents one pair of residues in contact. The total number of pairwise atomic contacts is 68, i.e., the sum of the numbers of the table. [b] No residue of strand 3 of triple helix 1 is involved in any contact in this packing arrangement. [c] Residue number refers to the position along the sequence in each strand. Residues 1, 4, 7, 10, and 13 are Gly, residues 2, 5, 8, 11, and 14 are Pro, and residues 3, 6, 9, 12, and 15 are Hyp. [d] One of the contacts is an elongated $O^{\delta 1}–H^{\delta 1}···O=C$ hydrogen bond between the two triple helices (see Table II).

of the two triple helices are involved (Table I and Figure 1). Thus, there are two such hydrogen bonds in a 9-residue segment, or three hydrogen bonds in the 15-residue segment used in the present work. The hydrogen bonds are weak, because they are about 0.6 Å longer than optimal O–H···O=C bonds. Other contacts involving the Hyp O atom, included in Table I, also contribute to the packing energy because of favorable nonbonded and electrostatic interactions.

The total energy also contains an intramolecular contribution, arising from nonbonded and electrostatic (but no intramolecular hydrogen bonding) interactions that depend on the dihedral angles $\chi^{3,1}$. The variation of the intrahelix energy as a function of $\chi^{3,1}$ is shown in Figure 2A. It has minima at $\chi^{3,1} = -69°$, $-171°$, and 59°, with relative energies ΔE_h = 0.00, 0.13, and 0.45 kcal/mol, respectively (Miller et al., 1980). The interhelix energy discussed above, obtained by energy

Table II: Interatomic Distances in the Three Hydrogen Bonds of the Lowest Energy Packing Arrangement

$O^{\delta 1}$–$H^{\delta 1}$			O=C			distance[a]	
triple helix	strand[b]	residue	triple helix	strand[b]	residue	$d_{O^{\delta 1}\cdots O}$ (Å)	$d_{H^{\delta 1}\cdots O}$ (Å)
2	1	3	1	1	3	3.35	2.39
1	1	9	2	2	6	3.50	2.81
2	2	12	1	2	12	3.34	2.38

[a] The distances listed are those between the $O^{\delta 1}$ and $H^{\delta 1}$ atoms of a Hyp hydroxyl group and the nearest carbonyl O of the neighboring triple helix. [b] No residue of strand 3 of either triple helix is involved in hydrogen bonding.

minimization with respect to the external variables for various fixed values of $\chi^{3,1}$, is shown in Figure 2B. Its minimum occurs at $\chi^{3,1} = 160°$, corresponding to the optimal O–H···O=C hydrogen-bond arrangement. The total energy is expressed as

$$E_T = E_{inter} + 3\Delta E_h \quad (1)$$

where the factor 3 is included because there are three OH groups that form hydrogen bonds for the length of the triple helices considered here. The other OH groups of the triple helices, not involved in hydrogen bonding, retain the dihedral angles $\chi^{3,1} = -171°$, i.e., are in a staggered conformation of the $C^{\gamma}H$–OH group. Therefore, they do not contribute to the intrahelix energy in eq 1. E_T as a function of $\chi^{3,1}$ is shown in Figure 2C. The minimum of E_T occurs at $\chi^{3,1} = 170°$, with $E_T = -43.85$ kcal/mol, but the energy remains low over a narrow range of this dihedral angle, from about 150° through ±180° to about −160°. The total energy E_T is 9.47 kcal/mol lower than the $E_{inter} = -34.38$ kcal/mol computed (Némethy & Scheraga, 1984) for the lowest energy packing of the corresponding $[CH_3CO–(Gly\text{-}Pro\text{-}Pro)_5–NHCH_3]_3$ triple helices, i.e., about 1.9 kcal/mol lower per Gly-X-Y tripeptide. This energy difference is contributed by the three O–H···O hydrogen bonds and by additional weaker nonbonded interactions of the O–H groups. In the presence of water, the magnitude of this energy difference is smaller. We are currently investigating the effect of hydration on collagen packing.

If Hyp is substituted for Pro in the X position of this lowest energy computed packing arrangement of two poly(Gly-Pro-Pro) triple helices, there are repulsive close contacts involving the Hyp OH groups, raising the energy by 250 kcal/mol. Energy minimization from this starting arrangement as well as from several other starting arrangements gave an interhelix energy of −26.05 kcal/mol for the lowest energy packing arrangement of two $[CH_3CO–(Gly\text{-}Hyp\text{-}Pro)_5–NHCH_3]_3$ triple helices. This value is 8.33 kcal/mol higher than that for the poly(Gly-Pro-Pro) triple helices, i.e., about 1.7 kcal/mol higher per Gly-X-Y tripeptide, indicating a strong destabilization of the packing in the case of poly(Gly-Hyp-Pro).

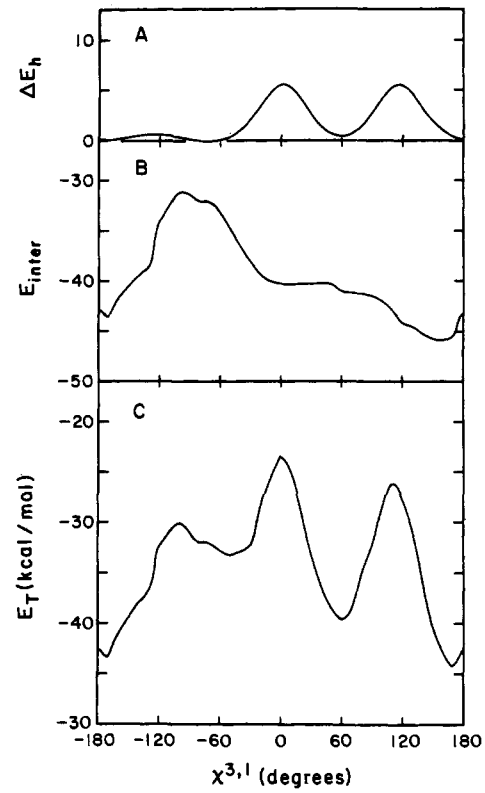

FIGURE 2: Interaction energies as a function of the Hyp C^{γ}–$O^{\delta 1}$ dihedral angle $\chi^{3,1}$. (A) The relative intrahelix energy ΔE_h, expressed per Hyp residue. The value of this energy is the same for one polypeptide chain in the collagen-like conformation and for the collagen-like triple helix because the OH group does not interact significantly with other chains of the triple helix, as shown by Miller et al. (1980). "Disallowed regions" for rotation around the C^{γ}–$O^{\delta 1}$ bond, determined from a hard-sphere model (Bansal et al., 1975), correspond closely to the high-energy parts of this curve. (B) The interhelix energy E_{inter} for the packing of two triple helices of the length considered here, i.e., with five Gly-Pro-Hyp repeating units per polypeptide chain (cf. Figure 1). In the computation of E_{inter}, all dihedral angles $\chi^{3,1}$ in the two triple helices were held fixed at the same value (shown on the abscissa), and the energy of the most favorable packing arrangement was computed and plotted. (C) The total energy E_T for the packing of these two triple helices, computed from eq 1.

CONCLUSIONS

It has been shown earlier (Némethy & Scheraga, 1984) that a near-parallel packing arrangement with an orientation angle of $\Omega_0 = -10°$ is highly favored for long poly(Gly-Pro-Pro) triple helices, with the exclusion of antiparallel and other packings, for reasons discussed earlier. In this study, we have demonstrated that the same packing arrangements are also favored for long poly(Gly-Pro-Hyp) triple helices. The relationship of the computed Ω to the observed alignment of collagen molecules in microfibrils has been discussed earlier (Némethy & Scheraga, 1984).

The presence of the Hyp hydroxyl group strengthens the attractive interaction between the triple helices, because of the formation of hydrogen bonds and additional contributions from noncovalent interactions. This stabilization amounts to 9.47 kcal/mol for triple helices containing five (Gly-Pro-Hyp) repeat units per polypeptide chain, in which three new hydrogen bonds form.

It is significant that the hydroxyl group can be accommodated in the optimal packing arrangement obtained for poly(Gly-Pro-Pro), without perturbing this arrangement. Thus, the OH group is positioned in a location that would correspond to a small unoccupied cavity in the poly(Gly-Pro-Pro) packing. This cavity is filled more efficiently when the OH group is present. In other words, the substitution of Hyp for Pro in position Y results in a better fitting of the triple helices, because the surfaces of the two triple helices exhibit better complementarity. On the other hand, substitution of Hyp for Pro in position X prevents good packing of the triple helices.

In collagen, other residues besides Pro and Hyp occur in positions X and Y. Their presence may affect the packing of

the triple helices, because of the size of side chains and specific interactions between side chains. Water molecules located between the triple helices (Ramachandran et al., 1973; Grigera & Berendsen, 1979) may also influence the packing. Bulky side chains would increase the distance between the axes of triple helices. In such a case, the Hyp hydroxyl groups would have to interact with water molecules located between the triple helices. The effects of various substitutions and of hydration upon packing is being investigated and will be reported later. The significance of the results reported here is the demonstration that the Hyp residue can make a direct contribution to the stabilization of collagen microfibrils in those regions of collagen in which close approach of the triple helices is possible.

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