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# Conformational implications of enzymatic proline hydroxylation in collagen

(prolyl hydroxylase/β-turn/hydroxyproline/collagen folding)

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ABSTRACT **In 1979 it was proposed that prolyl hydroxylase (prolyl-glycyl-peptide,2-oxoglutarate:oxygen oxidoreductase, EC 1.14.11.2) recognizes the β-turn conformation in nascent procollagen chains and that the hydroxylation process involves a conformational change resulting in "straightening" of the β-turn segments into the linear triple-helical conformation of native collagen. We present experimental data that verify both these postulates. The following peptides were synthesized and studied for their conformation and interaction with prolyl hydroxylase: tBoc-Pro-Gly-Ala-OH, tBoc-Pro-Gly-Val-OH, tBoc-Gly-Val-Pro-Gly-Val-OH, and tBoc-Pro-DAla-Ala-OH. Spectral data showed that these peptides preferred a β-turn conformation. All of them acted as inhibitors of the enzyme; the pentapeptide also acted as a substrate. To mimic the biosynthetic event, a collagen model polypeptide, $(Pro\text{-}Pro\text{-}Gly)_{10}$, was incubated at 37°C with purified prolyl hydroxylase and the necessary cosubstrates and cofactors at pH 7.8. A progressive change from the initially nonhelical to the triple-helical conformation, as monitored by CD spectra and gel filtration, occurred during the course of proline hydroxylation. In addition to leading to increased thermal stability of the triple-helical conformation in $(Pro\text{-}Pro\text{-}Gly)_{10}$ and $(Pro\text{-}Pro\text{-}Gly)_5$, the enzymatic incorporation of the hydroxyproline residues was found to enable these polypeptides to fold into this conformation faster than the unhydroxylated counterparts. These conformational implications of proline hydroxylation in collagen may also be of use in the study of the complement subcomponent Clq and of acetylcholine esterase which contain collagen-like regions in them.**

The post-translational hydroxylation of selected proline residues by prolyl hydroxylase (prolyl-glycyl-peptide,2-oxoglutarate:oxygen oxidoreductase, EC 1.14.11.2) is a crucial event in the biosynthesis of collagen. The resulting hydroxyproline residues are essential for the stability of the triple-helical conformation of collagen at body temperature (1). In regard to the conformational aspects of the enzymatic hydroxylation process, two questions needed to be answered: (*a*) What is the preferred conformation of the peptide substrate? and (*b*) What is the conformational consequence of proline hydroxylation? Based on experimental data and theoretical considerations, it was proposed (2) that prolyl hydroxylase recognizes the β-turn conformation (3) formed at the -Pro-Gly- segments in the nascent procollagen chains and that the hydroxylation process results in "straightening" of these segments into the rigid conformation necessary for the subsequent association of these chains in the triple-helical conformation of native collagen.

In order to test these postulates in a direct manner, we undertook (*a*) the synthesis of simple peptides which were expected to prefer the β-turn conformation to study their interactions with prolyl hydroxylase and (*b*) the study of the conformational change in polypeptide models of collagen during the enzymatic hydroxylation process. The results appear to verify the postulates on the conformational criterion for and the consequence of proline hydroxylation in collagen. Additionally, they indicate an enhanced rate of folding of the hydroxylated polypeptide chains into the collagen-like triple-helix. Preliminary reports of our findings have appeared (4, ‡).

## MATERIALS AND METHODS

**Enzyme.** Prolyl hydroxylase from 13-day chicken embryos was purified to homogeneity by using a recent modification (5) of the affinity chromatography procedure (6). The enzyme activity (expressed in μmol of hydroxyproline produced per mg of protein per hr) ranged from 60 to 90 units.

**Peptides and Chemicals.** $(Pro\text{-}Pro\text{-}Gly)_5 \cdot 4H_2O$ and $(Pro\text{-}Pro\text{-}Gly)_5 \cdot 9H_2O$ were obtained from Proteins Research Institute (Osaka, Japan). tBoc-Pro-Gly-Ala-OH, tBoc-Pro-Gly-Val-OH, tBoc-Pro-D-Ala-Ala-OH, and tBoc-Gly-Val-Pro-Gly-Val-OH were synthesized and characterized by using standard procedures (7, 8). Catalase, bovine serum albumin, α-ketoglutaric acid, ferrous sulfate, dithiothreitol, ascorbic acid, and Tris·HCl were obtained from Sigma. α-[1-$^{14}$C]Ketoglutaric acid and scintillation fluids were obtained from New England Nuclear; and Sephadex G-50 was from Pharmacia (Uppsala, Sweden). Deionized water was used throughout. All other chemicals were of analytical grade.

**Enzyme Assay and Proline Hydroxylation.** The enzyme activity was determined by measuring the $^{14}CO_2$ evolution due to the stoichiometric oxidation of labeled α-ketoglutaric acid during proline hydroxylation (6, 9). The synthetic tripeptides and pentapeptides were tested for enzymatic proline hydroxylation by replacing the $(Pro\text{-}Pro\text{-}Gly)_5$ substrate in the procedure for enzyme assay (6) with the respective peptide (35 mM) and using 30 μg of the enzyme. In the inhibition studies, each peptide was used at 5–10 mM and its effect on the hydroxylation of $(Pro\text{-}Pro\text{-}Gly)_5$ was studied at varying concentration of the latter (0.19–1.85 mM in tripeptide units).

**CD Spectral Measurements.** These were made with a Jasco J-20 spectropolarimeter in water-jacketed cells of 1–5 mm path lengths. The conformational change during the enzymatic proline hydroxylation of $(Pro\text{-}Pro\text{-}Gly)_{10}$ was studied as follows. In a conical flask, 2–4 ml of a solution containing polypeptide at 0.5 mg/ml (0.95 mM in tripeptide) previously heated to 100°C for 10 min and chilled in ice, catalase at 0.1 mg/ml, 1.0 mM ascorbic acid, 0.05 mM ferrous sulfate, 0.1 mM dithiothreitol, and prolyl hydroxylase at 30–50 μg/ml in 50 mM Tris·HCl buffer at pH 7.8 was incubated at 37°C. α-Ketoglutaric acid (2.0 mM) containing the $^{14}$C label (200,000 dpm) was then added, the solution was mixed well, and a stopwatch was turned on.

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‡ Chopra, R. K. & Ananthanarayanan, V. S., Twelfth International Congress of Biochemistry, Perth, Australia, Aug. 15–21, 1982.

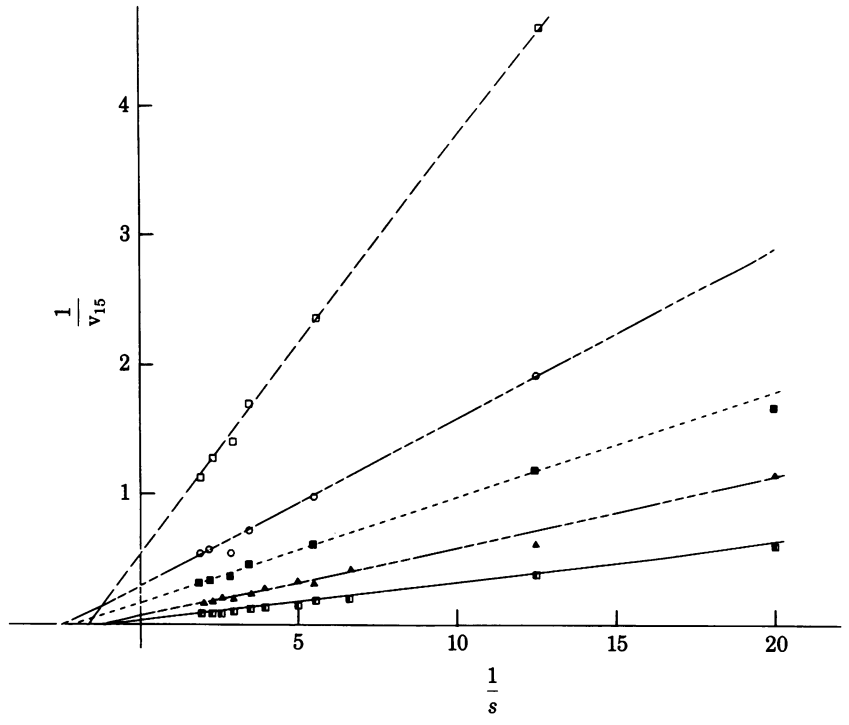


FIG. 1. Lineweaver–Burk plot for the hydroxylation of (Pro-Pro-Gly)$_5$ (0.20–2.0 mM) by prolyl hydroxylase (1 μg) in the absence (▣) and presence of the synthetic peptides tBoc-Pro-DAla-Ala-OH (▲), tBoc-Gly-Val-Pro-Gly-Val-OH (■), tBoc-Pro-Gly-Val-OH (○), and tBoc-Pro-Gly-Ala-OH (□). The rate $v_{15}$ represents mol of hydroxyproline formed in 15 min per mg of the enzyme. The substrate concentration is in mg/ml.

An aliquot (50 μl) of the solution was quickly removed, treated with dilute sulfuric acid, neutralized with alkali, and mixed with scintillation fluid for measurement of $^{14}C$ activity of the unreacted α-ketoglutaric acid. Another aliquot (about 0.2 ml) was injected into the empty CD cell kept at 37°C, the spectrum was recorded (between 220 and 240 nm), and the time was noted when spectral recording at 226 nm was made. The cell was then emptied and refilled with additional aliquots from the original solution and the spectra were recorded as above.

Continuous monitoring of the CD spectrum of the same solution was not feasible because the hydroxylation reaction did not proceed well inside the narrow cell due to insufficient availability of oxygen, even when the cell was not stoppered. The extents of proline hydroxylation at the various time intervals were also estimated in a separate experiment using the $^{14}CO_2$ method (9) and the direct determination of hydroxyproline (10). These were found to correlate well with the estimates obtained by measuring the radioactivity of the unreacted α-ketoglutaric acid.

## RESULTS

**Conformation of Synthetic Peptides and Their Interaction with Prolyl Hydroxylase.** The conformation of the synthetic peptides was determined from CD, IR, and NMR data in aqueous and nonaqueous solvents as in the earlier studies (7, 8). tBoc-Pro-Gly-Ala-OH and tBoc-Pro-Gly-Val-OH were found (§) to adopt the type II β-turn conformation in solvents such as aqueous trifluoroethanol and ethylene glycol, as did their *N*-acetyl derivatives (7). In water, the intramolecular hydrogen bonding weakened, leading to a disordered structure. tBoc-Pro-D-Ala-Ala was found (8) to take up the Venkatachalam type 13 β-turn which was predicted for peptides with the L-D sequence (11). This conformation was found in both nonaqueous solvents and water. The pentapeptide tBoc-Gly-Val-Pro-Gly-Val-OH was found to be similar to the tripeptide tBoc-Pro-Gly-Val-OH and existed predominantly in the type II β-turn conformation in trifluoroethanol.

The interaction of the synthetic peptides with purified prolyl hydroxylase was studied by assessing their susceptibilities to proline hydroxylation and their capacities to inhibit the hydroxylation of the standard synthetic substrate (6), (Pro-Pro-Gly)$_5$. The tripeptides did not undergo any significant hydroxylation; the pentapeptide tBoc-Gly-Val-Pro-Gly-Val-OH was hydroxylated with a $K_m$ of 25 mM (of tripeptide unit) based on a Lineweaver–Burk plot of the kinetic data. This pentapeptide as well as the three tripeptides acted as inhibitors of (Pro-Pro-Gly)$_5$ to varying extents (Fig. 1). The details of the kinetic studies will be given elsewhere.

**Conformational Change During Proline Hydroxylation.** Fig. 2 shows the CD spectra of the incubation mixture at various time intervals. The initial spectrum is mainly due to (Pro-Pro-Gly)$_{10}$ in the nonhelical form (resulting from the prior heating of the polypeptide to 100°C and the subsequent chilling). [Prolyl hydroxylase has a considerable amount (>40%) of the α-helical conformation which contributes to the CD in the experimental wavelength region (unpublished data); however, this contribution as well as contributions from ascorbic and catalase were such that they did not interfere with observation of the changes in the polypeptide CD around 226 nm.] In this form, the polypeptide exhibited a featureless CD spectrum, in contrast to the spectrum of the triple-helical collagen and model polypeptides which show a significant positive CD band around 225 nm arising from the n-π* transition of the peptide chromophore (12). However, this band was quite discernible in the CD of the incubation mixture after about an hour (Fig. 2.). The magnitude of this band grew progressively larger with increas-

§ Ananthanarayanan, V. S. & Shyamasundar, N. S., Seventh International Biophysics Congress and Third Pan-American Biochemistry Congress, Mexico City, Mexico, Aug. 23–28, 1981.

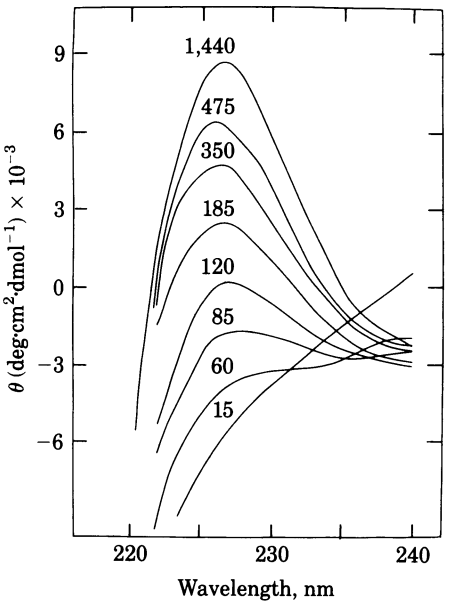


FIG. 2. CD spectral changes due to the enzymatic hydroxylation of $(Pro\text{-}Pro\text{-}Gly)_{10}$ (0.5 mg/ml; pH 7.8; 37°C) at the indicated time intervals (min). Data at other time intervals (used to construct Fig. 3) are not shown. Cell length: 1 mm.

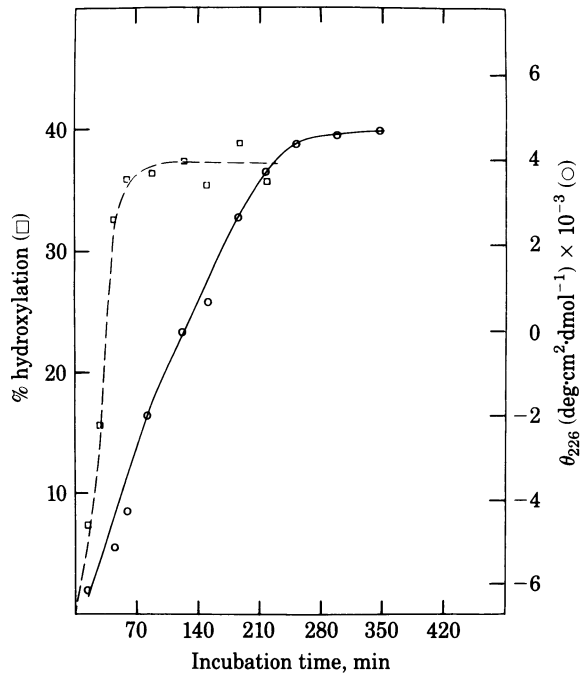


FIG. 3. Changes in the extent of hydroxylation (□) (expressed as percentage of the total of 10 susceptible proline residues) as followed by $^{14}C$ radioactivity of unreacted α-ketoglutaric acid and the ellipticity value at 226 nm (○) as a function of time of incubation of $(Pro\text{-}Pro\text{-}Gly)_{10}$ with prolyl hydroxylase. Experimental conditions were as in Fig. 2.

ing time and, after about 8 hr, the CD spectrum resembled that of native collagen (12).

Simultaneously with the CD measurements the extent of proline hydroxylation was measured (Fig. 3). The CD change lagged behind the hydroxylation. Under comparable conditions, the control solution, which contained $(Pro\text{-}Pro\text{-}Gly)_{10}$ and the other components of the reaction mixture except either α-ketoglutaric acid or prolyl hydroxylase, showed no change in its CD spectrum over the same time period. Observation of a similar conformational change in $(Pro\text{-}Pro\text{-}Gly)_5$ was difficult because of the relatively small change in the CD spectra of the control and hydroxylated samples at the incubation temperature (37°C), part of this change being masked by the contributions from the other components of the reaction mixture. However, precipitation of the protein components by addition of dilute sulfuric acid at the end of the hydroxylation reaction (about 1½ hr), followed by filtration and adjustment of the pH of the filtrate to 7.8, gave a CD spectrum at 0°C which showed the positive CD band at 226 nm characteristic of the triple-helical conformation. Similar treatment of the control solution did not reveal this band. From these data, a conformational change similar to the one observed directly in the case of $(Pro\text{-}Pro\text{-}Gly)_{10}$ is inferred in $(Pro\text{-}Pro\text{-}Gly)_5$ also as a result of the enzymatic proline hydroxylation.

The state of association of $(Pro\text{-}Pro\text{-}Gly)_{10}$ after the enzymatic proline hydroxylation was determined by separating the components of the reaction mixture by gel filtration on Sephadex G-50 at 4°C and comparing the elution profile of the hydroxylated polypeptide with that of the control (13, 14). The data (not shown) revealed that the former had a relatively much larger proportion of the trimeric relative to monomeric species than the latter. Both samples contained only the trimeric species when they were allowed to refold maximally (by keeping at 4°C for several days).

From the characteristics of the CD spectra, from the gel filtration patterns, and from the earlier studies by other workers (13, 14), we conclude that the materials eluting as trimers in the gel filtration are in the triple-helical conformation similar but not necessarily identical to that of native collagen. The possibility of mismatch among the constituent polypeptide chains appears to be small, if not absent (13, 14). The data on the relative stabilities of $(Pro\text{-}Pro\text{-}Gly)_5$ and $(Pro\text{-}Pro\text{-}Gly)_{10}$ and their hydroxylated counterparts are presented in Fig. 4. The extent of proline hydroxylation is different in each of the polypeptides and is not 100% as is the case with synthetic $(Pro\text{-}Hyp\text{-}Gly)_5$ and $(Pro\text{-}Hyp\text{-}Gly)_{10}$ used by others (14) (the data on the melting curves of the polypeptides at other degrees of hydroxylation are not shown in Fig. 4). However, as in the other studies, in each case the hydroxylated polypeptide was thermally more stable than the unhydroxylated counterpart. Also, the ellipticities of the triple-helical conformation of the hydroxylated samples were significantly higher than those of the unhydroxylated ones.

The rate of folding of $(Pro\text{-}Pro\text{-}Gly)_{10}$ at 0.5°C from the initially unfolded state (obtained by heating the control incubation mixture to 70°C for 1 hr and bringing it to 0.5°C) is shown in Fig. 5. The corresponding data for the enzymatically hydroxylated (57%) sample are also shown. The results show a dramatic increase in the rate and extent of refolding of the hydroxylated sample compared with the unhydroxylated counterpart.

## DISCUSSION

The earlier proposal that β-turns in nascent procollagen are sites of post-translational proline hydroxylation was based on analysis of the conformational features of the -Pro-Gly- segments in linear peptides and globular proteins (2). A recent study (7) of the conformation of simple linear tripeptides of the type Ac-Pro-Gly-X-OH has shown that these adopt, to varying degrees, the β-turn conformation in nonpolar solvents. One of these tripeptides, Ac-Pro-Gly-Phe-OH, was found to exist in the type II β-turn conformation by x-ray crystallography (15). In the light of these data, we decided to test our β-turn hypothesis directly by studying the interaction of such tripeptides with prolyl hy-

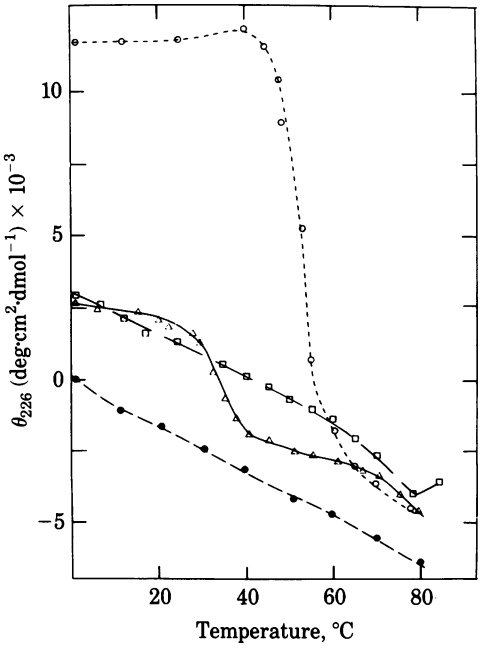


FIG. 4. Melting curves of polypeptide substrates determined by CD measurements at 226 nm, before and after enzymatic proline hydroxylation. In the case of (Pro-Pro-Gly)$_{10}$, the melting was performed in the incubation medium after renaturation of the unhydroxylated control (△) and 64% hydroxylated (○) peptides overnight at 4°C. In the case of (Pro-Pro-Gly)$_5$, the control (●) and 33% hydroxylated (□) peptides were first separated from other components of the incubation mixture by gel filtration, lyophilized, and dissolved in water. Polypeptide concentration in each case was about 0.5 mg/ml and cell length was 1–5 mm.

droxylase. The limited number of earlier studies (16, 17) on the interaction of oligopeptides did not address themselves to the conformation of the peptides used. In the present study, the tripeptides tBoc-Pro-Gly-Ala-OH and tBoc-Pro-Gly-Val-OH were found to exhibit the type II β-turn conformation (§), and tBoc-Pro-DAla-Ala-OH was found (8) to take up the Venkatachalam type 13 (a subclass of the type II) β-turn. The pentapeptide

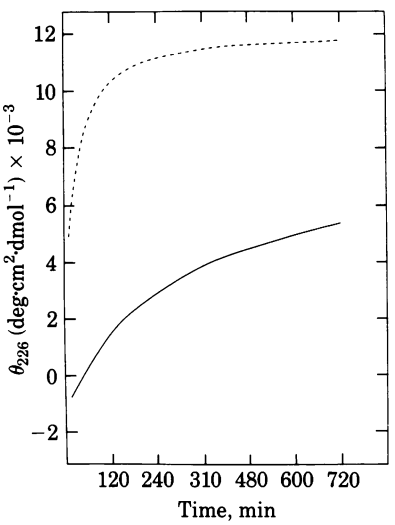


FIG. 5. Time-dependent renaturation of unhydroxylated (——) and 57% hydroxylated (---) samples of (Pro-Pro-Gly)$_{10}$ (0.5 mg/ml), as monitored by ellipticity at 226 nm. After incubation at 37°C for proline hydroxylation, the samples were kept at 70°C for 1 hr and transferred to the CD cell (5 mm) kept at 0.5°C.

tBoc-Gly-Val-Pro-Gly-Val-OH was chosen on the basis of the observed β-turn preferences of similar sequences in tropoelastin model peptides (18). It also served as an extension of the valine-containing tripeptide.

In using the peptides as β-turn models in our study of their interaction with prolyl hydroxylase, we assume that the stereochemistry of the enzyme's active site would be such that only a specific conformation or a limited range of conformations would be recognized for effective interaction. This would be in line with the known stereospecificity of enzymes in general and with a recent stereochemical study (19) of the catalytic mechanism of prolyl hydroxylase which explicitly takes into account the proposal of the β-turn conformation of the substrates. This conformation of the peptide, which in solution would exist in equilibrium with the "open" disordered structure, may be stabilized by specific, possibly nonpolar, interactions at the enzyme active site.

It is interesting to note that all the peptides used in this study acted as inhibitors of prolyl hydroxylase when tested against the standard polypeptide substrate (Pro-Pro-Gly)$_5$. In addition, the pentapeptide tBoc-Gly-Val-Pro-Gly-Val-OH also acted as a substrate but the other peptides did not. The kinetic data shown in Fig. 1 indicate that both the $K_m$ and $V_{max}$ of (Pro-Pro-Gly)$_5$ are affected by the presence of the inhibitors. Because, being a substrate, the pentapeptide may be expected to bind at the same site of the enzyme as the polypeptide substrate, the pattern of inhibition of the penta- and tripeptides (Fig. 1) should be interpreted in terms of a mechanism that involves the binding of these peptides at or near the catalytic site of the enzyme. Although more kinetic data are needed to elucidate the details of this mechanism, the inhibition data shown in Fig. 1 may be taken to indicate that the β-turn conformation of the peptides indeed may be suited for interaction at the active site of prolyl hydroxylase.

It had been suggested that the enzymatic proline hydroxylation would lead to the formation of an intrachain hydrogen bond involving the γ-hydroxyl group of the hydroxyproline residue (2) as in polyhydroxyproline (20). This, in turn, would result in a change in the $\phi$ and $\psi$ angles of the glycine residues from their initial values in the β-turn conformation to those found in the individual (left-handed) helices of collagen; the polypeptide chain can then associate with two other chains the triple-helical conformation through interchain hydrogen bonding (2). The enzymatic hydroxylation process may thus be likened to straightening of previously "kinked" (i.e., folded) chains by passage through a die (21). The data in Figs. 2 and 3 demonstrate the proposed conformational change arising as a direct consequence of the enzymatic proline hydroxylation. Although a conformational change may be inferred from the observed differences in the stabilities of protocollagen and procollagen, at least in part this may arise from several other post-translational modifications besides proline hydroxylation (particularly lysine hydroxylation and disulfide formation in the propeptides) that take place in the elaboration of procollagen (1).

The use of protocollagen was prohibited mainly because of the difficulty of obtaining sufficient amounts of the pure protein. We therefore selected the synthetic polypeptide (Pro-Pro-Gly)$_{10}$ which is a good model for collagen (13, 14, 22) and, in its nonhelical form (obtained by heat denaturation), is a substrate for prolyl hydroxylase (1). The rationale for studying (Pro-Pro-Gly)$_5$ was that such a sequence is found at the COOH terminus of collagen, with four of the prolines hydroxylated (23).

The range of conformations available for these polypeptides in the nonhelical form may be expected to be relatively small due to the restricted $\phi_{Pro}$ value ($\approx -60°$). The preferred values of $\psi_{Pro}$ are also limited (2), around either 140° (as in the type

II β-turn) or −30° (as in types I and III β-turns). In view of these observations and the known propensity of -Pro-Gly- segments to take up a folded conformation, we might expect the nonhelical form of $(Pro\text{-}Pro\text{-}Gly)_n$ polypeptides to contain several folded segments that resemble the β-turn (type I, II, or III, depending on the particular sets of $\phi$, $\psi$ angles for the proline and glycine residues); however, such segments cannot be additionally stabilized by the intramolecular hydrogen bonding (due to lack of hydrogen atom on the imide nitrogen). [On the basis of available NMR data (24), the *cis* isomer is unlikely to be significantly populated as a result of heating aqueous solutions of $(Pro\text{-}Pro\text{-}Gly)_{10}$.] The observed CD spectrum of $(Pro\text{-}Pro\text{-}Gly)_{10}$ in the incubation mixture prior to the onset of the hydroxylation process may be taken to correspond to the nonhelical conformation of the polypeptide and may serve as an approximation to that of the unhydroxylated procollagen.

The data presented in Figs. 2 and 3 demonstrate in a dramatic fashion the conformational change that takes place during the enzymatic hydroxylation of $(Pro\text{-}Pro\text{-}Gly)_{10}$ in the presence of the necessary cofactors at 37°C and pH 7.8. Hydroxylation of as little as 5% of the total susceptible proline residues in the polypeptide leads to perceptible conformational change (Fig. 3) in the direction of collagen-like helix formation, as judged from the CD spectral characteristics of native collagen (12). The observed conformational change appears to reflect a two-step process because the CD change continues to occur beyond the completion of proline hydroxylation (Fig. 3). More data are needed for a definite interpretation of the CD spectra at different stages of hydroxylation in terms of single or triple-stranded helical conformation.

The data on the relative stabilities of the unhydroxylated and variously hydroxylated samples of $(Pro\text{-}Pro\text{-}Gly)_5$ and $(Pro\text{-}Pro\text{-}Gly)_{10}$ (Fig. 4) support the well-known effect of hydroxyproline in increasing the thermal stability of collagen (25, 26).

An interesting observation is the remarkably faster rate of refolding of the hydroxylated $(Pro\text{-}Pro\text{-}Gly)_{10}$ compared with the unhydroxylated polypeptide (Fig. 5). This was also observed at other degrees of hydroxylation than the one (57%) shown in Fig. 5. The basis of the rate enhancement should lie in the specific interactions involving the hydroxyl group of hydroxyproline residues that apparently enable the polypeptide chain to overcome the kinetic barrier for triple-helix formation [notably, the *cis/trans* isomerization of peptide bonds (27)] better than the corresponding situation with the proline residue. These interactions may involve the solvent (28) or other parts of the same (20) or different polypeptide chain(s). One possibility hitherto not considered is the significant steric hindrance involving the hydroxyproline residue that would make conformations with low $\psi_{Pro}$ ($\approx -30°$), like the type I or type III β-turn (of the Pro-Gly segments after hydroxylation), unfavorable. This would result in a more extended conformation. If the above results can be extrapolated, it would mean that the enzymatic proline hydroxylation in nascent procollagen may aid the assembly of the individual α chains into the triple-helical conformation at a faster rate than before.

Much work is being carried out toward understanding the exact sequence of events that occur in the assembly of the nascent procollagen chains (29). From the viewpoint of the specific conformational role played by one such event—namely, the enzymatic proline hydroxylation—in the folding and stability of the helical portion of the procollagen molecule, we believe that the results presented here may be of importance. In this context, it is interesting to note that collagen-like sequences including hydroxyproline are found in the complement subcomponent Clq and in acetylcholine esterase. The collagen "tail" in these proteins is known to be important in their respective functions (30, 31). One may thus expect the hydroxyproline residue to play an important role in the biosynthetic assembly of the collagen-like portion of Clq and acetylcholine esterase as well.

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