Conformational Preferences of Substrates for Human Prolyl 4-Hydroxylase†

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ABSTRACT: Prolyl 4-hydroxylase (P4H) catalyzes the posttranslational hydroxylation of (2S)-proline (Pro) residues in procollagen strands. The resulting (2S,4R)-4-hydroxyproline (Hyp) residues are essential for the folding, secretion, and stability of the collagen triple helix. Even though its product (Hyp) differs from its substrate (Pro) by only a single oxygen atom, no product inhibition has been observed for P4H. Here, we examine the basis for the binding and turnover of substrates by human P4H. Synthetic peptides containing (2S,4R)-4-fluoroproline (Flp), (2S,4S)-4-fluoroproline (flp), (2S)-4-ketoproline (Kep), (2S)-4-thiaproline (Thp), and 3,5-methanoproline (Mtp) were evaluated as substrates for P4H. Peptides containing Pro, flp, and Thp were found to be excellent substrates for P4H, forming Hyp, Kep, and (2S,4R)-thiaoxoproline, respectively. Thus, P4H is tolerant to some substitutions on C-4 of the pyrrolidine ring. In contrast, peptides containing Flp, Kep, or Mtp did not even bind to the active site of P4H. Each proline analogue that does bind to P4H is also a substrate, indicating that discrimination occurs at the level of binding rather than turnover. As the iron(IV)-oxo species that forms in the active site of P4H is highly reactive, P4H has an imperative for forming a snug complex with its substrate and appears to do so. Most notably, those proline analogues with a greater preference for a Cα-endo pucker and cis peptide bond were the ones recognized by P4H. As Hyp has a strong preference for Cα-exo pucker and trans peptide bond, P4H appears to discriminate against the conformation of proline residues in a manner that diminishes product inhibition during collagen biosynthesis.

Collagens are the major structural proteins in the extracellular matrix. All collagens are comprised of three polypeptide strands that coil together into a right-handed triple helix. Each strand contains a repeating three amino acid sequence repeating in the Xaa-Yaa-Gly sequence of collagen (4–7), and the prevalence of Hyp within the Xaa-Yaa-Gly sequence of collagen (4–7), and the presence of Hyp is crucial for the folding, secretion, and stability of the collagen triple helix under physiological conditions (4–7), and P4H activity is necessary for the viability of the nematode Caenorhabditis elegans (8–10) and the mouse Mus musculus (11). The enzyme-catalyzed hydroxylation of proline residues also plays a role in the sensing of molecular oxygen, though those enzymes are distinct from the P4H that acts on collagen (12).

P4H is a tetrameric enzyme comprised of two α subunits and two β subunits. Each α subunit (59 kDa) contains a catalytic domain and a peptide-binding domain (13). The β subunit (55 kDa) is protein disulfide isomerase (14), which is necessary to keep the α subunit from aggregating (15, 16) and to retain the enzyme in the endoplasmic reticulum (17). The study of P4H has been facilitated by the recent development of recombinant DNA systems for the high-level production of active P4H tetramers in Escherichia coli (18, 19). Still, the three-dimensional structure of P4H remains unknown. P4H is a non-herm(II) dioxygenase (20–22). This class of enzymes uses α-ketoglutarate and O2 as cosubstrates (Figure 1). Hydroxylation of proline is accompanied by the oxidative decarboxylation of α-ketoglutarate to form succinate (23). During the reaction, one atom of molecular oxygen is common than seven of the “common” amino acid residues: Cys, Gln, His, Met, Phe, Trp, and Tyr.2 The presence of Hyp is crucial for the folding, secretion, and stability of the collagen triple helix under physiological conditions, and P4H activity is necessary for the viability of the nematode Caenorhabditis elegans and the mouse Mus musculus. The enzyme-catalyzed hydroxylation of proline residues also plays a role in the sensing of molecular oxygen, though those enzymes are distinct from the P4H that acts on collagen.

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2 Abbreviations: Boc, tert-butoxycarbonyl; DMF, dimethylformamide; ESI−MS, electrospray ionization mass spectrometry; Flp, (2S,4R)-4-fluoroproline; flp, (2S,4S)-4-fluoroproline; Fmoc, fluorenylmethoxycarbonyl; Gly, glycine; HPLC, high-performance liquid chromatography; Hyp, (2S,4R)-4-hydroxyproline; Kep, (2S)-4-ketoproline; MALDI-TOF, matrix-assisted laser desorption/ionization mass spectrometry; Mcp, 3-chloroperoxybenzoic acid; Mtp, 3,5-methanoproline or 2-azabicyclo[2.1.1]hexane-3-carboxylic acid; Mtp, 3,5-methanoproline or 2-azabicyclo[2.1.1]hexane-3-carboxylic acid; P4H, prolyl 4-hydroxylase; Pro, (2S)-proline; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; TFA, trifluoroacetic acid; Thp, (2S)-4-thiaproline; Thp(O), (2S,4R)-4-thiooxoproline; thp(O), (2S,4S)-4-thiooxoproline; thp(O), (2S,4S)-4-thiooxoproline.

2 The abundance of Hyp in animal proteins is ~4%, a value calculated from the abundance of collagen among animal proteins (1/3) and the prevalence of Hyp within the Xaa-Yaa-Gly sequence of collagen (~38% × 1/3) (2). The abundance of the “common” amino acids is given in ref 3.
P4H does not hydroxylate all proline residues. Free proline amino acids are not hydroxylated by P4H (32). Nor is polyproline hydroxylated, though it does bind to the enzyme and is a competitive inhibitor of enzymatic activity (33). Hydroxylation occurs only on proline residues in an Xaa-Pro-Gly sequence within a polypeptide. The strength of substrate binding increases with the length of the polypeptide but is obliterated by triple-helical structure (34).

Computational, structural, and spectral analyses of Pro-Gly sequences in peptides and proteins have shown that adoption of a β-turn conformation is energetically favorable (5, 35–37). Other conformation preferences for the substrates of prolyl 4-hydroxylase are less clear. Because proline is a secondary amine and prolyl peptide bonds are tertiary amides, the cis isomer of prolyl peptide bonds occurs much more frequently than in peptide bonds between non-prolyl residues (38). The conformation of the prolyl peptide bond is correlated with another conformational feature of proline, its ring pucker (39, 40). The pyrrolidine ring of proline primarily adopts two puckered conformations: Cγ-endo and Cγ-exo (Figure 2).3 Each amino acid was incorporated into a tetrapeptide and examined as a substrate or inhibitor of P4H.

Scheme 1

were converted to their Fmoc derivatives via standard methods and used without further purification (43). Fluorenylmethoxycarbonyl (Fmoc)-mini-PEG-3 (Fmoc-11-amino-3,6,9-trioxaundecanoic acid) was from Peptides International (Louisville, KY). All other Fmoc-protected amino acids were from Novabiochem (La Jolla, CA). [1-14C]-α-Ketoglutarate was from American Radiolabeled Chemicals (St. Louis, MO). All other chemicals were of reagent grade or better and were used without further purification.

Synthesis of Boc-d,l-MtpOH. Boc-d,l-MtpOH was prepared by the route shown in Scheme 1, which is less costly and more convenient than those described previously (44, 45). Briefly, carbamate 1 was prepared as in ref 46 and then used in a manner similar to that in ref 47. Specifically, a solution of carbamate 1 (2.0 g, 10.91 mmol) and TMEDA (2.1 mL, 14.18 mmol, 1.3 equiv) in dry ether (50 mL) was cooled to −78 °C and s-BuLi (1.3 equiv, 1.4 M solution in cyclohexane) was added dropwise. The resulting solution was stirred for 2 h at −78 °C and then transferred via a cannula to a solution of distilled DMF (6.3 mL, 81.83 mmol, 7.5 equiv) in dry diethyl ether (30 mL) that had been cooled to −78 °C. The solution was warmed slowly to room temperature and then quenched with 10 mL of saturated NH4Cl(aq). The ether layer was washed with distilled water (2 × 20 mL) and dried over Na2SO4(s). After filtration and concentration, the oil was taken up in 10 mL of dry MeOH, and this solution was cooled to 0 °C.
NaBH₄ (2.1 g, 54.56 mmol, 5.0 equiv) was added slowly to the solution, which was then stirred at 0 °C for 15 min. The reaction mixture was warmed to room temperature, and 10 mL of saturated NH₄Cl(aq) was added slowly, followed by 30 mL of CH₂Cl₂. The aqueous layer was extracted with CH₂Cl₂ (3 × 30 mL). The organic extracts were combined, dried over Na₂SO₄(s), and filtered. The solvent was removed under reduced pressure, and the residue was purified by chromatography on silica gel (3:2 hexanes/diethyl ether) to furnish 3-CH₂OH (2) (609 mg, 26%, Rᵣ = 0.26 with 1:1 hexanes/diethyl ether), 1-CH₂OH (3) (638 mg, 27%, Rᵣ = 0.51), and starting material I (190 mg, 10%, Rᵣ = 0.71).

Following the procedure in refs 44 and 47, 3-CH₂OH (2) (600 mg, 2.813 mmol) was dissolved in a solution of CH₂Cl₂ (14 mL), TEMPO (27 mg), saturated NaHCO₃(aq) (11 mL), KBr (54 mg), and Bu₄NCl (67 mg). This solution was cooled to 0 °C, and a solution of NaOCl (14 mmol), saturated NaHCO₃(aq) (6 mL), and brine (12 mL) was added dropwise over 45 min. The reaction mixture was stirred for an additional 1 h and then warmed to room temperature. The aqueous layer was separated, and the organic layer was washed with 50% w/v NaHCO₃(aq) (3 × 65 mL). The aqueous layers were combined and washed with CH₂Cl₂ (2 × 25 mL), acidified with dilute HCl solution until the pH was ~3, and then extracted with ethyl acetate (5 × 200 mL). The combined organic extracts were dried over Na₂SO₄(s), and the solvent was removed to furnish Boc-D,L-MtpOH (524 mg, 82%) as an off-white solid of sufficient purity (44, 45, 47).

**Instruments.** Measurements of UV and visible absorbance were made with a Cary model 3 spectrophotometer (Varian, Palo Alto, CA). Peptide synthesis was conducted with a Pioneer (PerSeptive Biosystems) or Symphony (Protein Technologies) automated synthesizer at the University of Wisconsin Biotechnology Center. Preparative high-performance liquid chromatography (HPLC) was performed with a Waters system equipped with two 515 pumps, a 717 plus autosampler, and a 486 tunable absorbance detector. Analytical HPLC was performed with a Waters system equipped with two 515 pumps, a 717 plus autosampler, and a 996 photodiode array detector. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry was performed with a Perkin-Elmer (Wellesley, MA) Voyager MALDI-TOF mass spectrometer at the University of Wisconsin—Madison Biophysics Instrumentation Facility. Scintillation counting was performed with a Wallac 1450 MicroBeta TriLux liquid scintillation counter from Perkin-Elmer (Wellesley, MA).

**Production and Purification of P4H.** P4H was produced and purified by using procedures reported previously (18). Peptides were synthesized on a solid support (PEG-PS, Applied Biosystems) by standard Fmoc-protection methods using HATU activation. To increase the water solubility of the Pro-, Flp-, flp-, Kep-, and Thp-containing peptides, a short PEG chain was added to their N-terminus. The PEG was added by coupling Fmoc-miniPEG-3 (Peptides International, Louisville, KY) to the N-terminus of the peptide while on solid support. The N-terminus remained protected with the Fmoc while the peptides were cleaved from the solid support with 8 mL of 95:2.5:2.5 trifluoroacetic acid (TFA)/trisopropylsilane/water and then washed with CH₂Cl₂ and dried under vacuum. The dried peptide was heated at reflux with SOCl₂ (15-fold molar excess) in ethanol for 2 h. The solvent was removed by rotary evaporation under reduced pressure. The N-terminal Fmoc was then removed by stirring the peptide in 20% v/v piperidine in DMF for 20 min. The peptides were purified by preparatory HPLC with a gradient (10–30% v/v) of acetonitrile in water containing TFA (0.1% v/v) to yield PEG-Gly-Tyr-Yaa-GlyOEt. MALDI-MS m/z: [M + Na]+ Yaa = Pro (14% yield; calcd 632.3, found 632.6), Flp (6% yield; calcd 650.3, found 650.5), flp (72% yield; calcd 650.3, found 650.6), Kep (1.8% yield; calcd 646.3, found 646.5), Thp (8% yield; calcd 650.3, found 650.7).

Cbz-Gly-Tyr-Mtp-GlyOEt was synthesized by standard Fmoc-protection methods on solid support. The cleavage from solid support and esterification were performed as described for the PEGylated peptides. Purification was done by preparatory HPLC with a gradient (20–40% v/v) of acetonitrile in water. The synthesis was done with racemic Fmoc-d,L-Mtp, and the diastereomeric tetrapeptides were not separated. Cbz-Gly-Tyr-Mtp-GlyOEt was analyzed by MALDI-MS m/z: [M + Na]+ (calcd 589.2, found 589.5).

Peptide concentrations were determined by absorbance measurement in 6 M guanidine hydrochloride at pH 6.5 using ε = 1450 M⁻¹ cm⁻¹ at 276 nm (48).

**HPLC-Based Assay of Enzymatic Activity.** An HPLC-based assay described previously (18) was used to monitor product formation by P4H. Assays were performed for 5 min at 30 °C in 100 µL of 50 mM Tris-HCl buffer, pH 7.8, containing bovine serum albumin (1 mg/mL), catalase (100 µg/mL), dithiothreitol (100 µM), ascorbate (2 mM), FeSO₄ (50 µM), P4H (90 nM), and α-ketoglutarate (500 µM). The tetrapeptide substrate (stock solution in ethanol) was added to initiate the reaction. The reactions were quenched by boiling for 60 s. All assays were performed in triplicate. A reversed-phase analytical Altima HP C18 AQ column (4.6 × 250 mm) from Alltech (Deerfield, IL) was used to separate peptides by elution with aqueous acetonitrile (12–30% v/v in 20 min) containing TFA (0.1% v/v) at 1.0 mL/min. Product formation was quantified by the substrate:product ratio, as determined by integration of the A₂₁₄nm with the Millennium32 software from Waters (Millford, MA).

[¹⁴C]CO₂-Release Assay for Enzymatic Activity. An alternative means to assess P4H activity is to monitor the release of CO₂, which is a product of catalysis (Figure 1). Procedures for monitoring the release of [¹⁴C]CO₂ from [¹⁴C]-α-ketoglutarate were as described elsewhere (23, 49).

Concentrations were the same as above, except for that of P4H (395 nM). All reactions were performed in duplicate and corrected for the rate of decarboxylation in the absence of the peptide substrate.

**Reduction and Conversion of Kep.** For analysis, the ketone in PEG-Gly-Tyr-Kep-GlyOEt was either reduced to the alcohol with sodium borohydride (10 equiv) for 30 min at room temperature or converted to the oxime via reaction with hydroxylamine (10 equiv) in 250 mM sodium phosphate buffer, pH 5.0, for 1 h at 100 °C.

**Chemical Oxidation of Thp.** A mixture of PEG-Gly-Tyr-Thp(O)-GlyOEt and PEG-Gly-Tyr-thp(O)-GlyOEt was produced by chemical oxidation of the Thp-containing peptide with either 3-chloroperbenzoic acid (MCPBA) (1 equiv) in chloroform for 2.5 h at room temperature or sodium periodate (1.1 equiv) in aqueous methanol (75%) for 30 min.
at 50 °C (50). A peptide containing Thp(O,O) was produced by reaction of the Thp-containing peptide with MCPBA (10 equiv) in chloroform for 5 h at room temperature.

RESULTS

Design of Peptide Substrates for P4H. Previously, we reported on an HPLC-based assay for P4H activity using the peptide substrate dansyl-Gly-Phe-Pro-GlyOEt (18). This peptide is not especially soluble and is thus not useful for assays at high concentration. To increase its solubility, the dansyl moiety was replaced with a short PEG segment. In addition, Phe was replaced by Tyr to aid in the determination of peptide concentration. PEG-Gly-Tyr-Pro-GlyOEt is a substrate for P4H with $k_{cat} = 360 \text{ min}^{-1}$, $K_M = 0.58 \text{ mM}$, and $k_{cat}/K_M = 1.0 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$ (Table 1, Figure 3). This $k_{cat}/K_M$ value is 3-fold greater than that for dansyl-Gly-Phe-Pro-GlyOEt (18).

Flp Does Not Inhibit P4H. No peptide formation was detected when Flp was used as a substrate for P4H (data not shown). It is not surprising that P4H cannot turn over Flp because P4H abstracts the proR hydrogen from C-4 of a proline residue and that hydrogen is replaced with fluorine in Flp. Likewise, Flp does not inhibit the P4H-catalyzed conversion of Pro to Hyp. Under standard P4H reaction conditions as described in the Experimental Procedures with PEG-Gly-Tyr-Pro-GlyOEt at 0.06 mM, which is an order of magnitude below its $K_M$ value, PEG-Gly-Tyr-Flp-GlyOEt had no measurable effect on hydroxylation of the Pro-containing peptide (Figure 4).

flp Is a Substrate for P4H. Unlike Flp, flp has a hydrogen in the 4R position of the pyrrolidine ring, and PEG-Gly-Tyr-flp-GlyOEt was determined to be a substrate for P4H (Figures 3 and 5). The product was identified as 4-ketoproline (Kep) by MALDI-MS ($m/z$ 624.4, calc $m/z$ 624.3) and coeluted...
Enzymatic reaction with a thiopropylypyrrolidine substrate produces an 85:15 mixture of the 4R and 4S diastereomers (50). HPLC analysis of the sodium periodate reaction compared to the P4H-catalyzed formation of the sulfone showed that P4H produces the 4R diastereomer exclusively. Chemical oxidation by MCPBA yielded the sulfone diastereomers, as well as the sulfone (Thp(O); MALDI-MS m/z 682.4, calcd 682.3 [M + Na⁺]), which was not formed by P4H. From triplicate P4H reactions under standard conditions with varying concentrations of PEG-Gly-Tyr-Thp-GlyOEt, the kcat/KM value was determined to be (1.2 ± 0.3) × 10³ M⁻¹ s⁻¹, which is 12% that of the Pro-containing peptide (Table 1, Figure 3).

P4H Decarboxylates [1-14C]-α-Ketoglutarate When Pro, flp, or Thp Is a Substrate. The HPLC-based assay described above identified flp- and Thp-containing peptides as novel substrates of P4H. These proline analogues, along with the Pro-, flp-, and Thp-containing peptides were 58, 160, and 640 µM, which were 10% of the KM values determined in the HPLC-based assay. Under these conditions the rate of CO₂ released in the presence of the Pro-containing peptide was 1.9 ± 0.7 µM/min (Table 2). The rate with flp in the peptide substrate was 0.5 ± 0.1 µM/min, which was 26% that of Pro; the rate with Thp was 0.1 ± 0.1 µM/min, 6% that of Pro. These rates follow closely the trend observed with the HPLC-based assay.

Mtp Does Not Inhibit P4H. Mtp, the bicyclic proline analogue, was not found to be a substrate for P4H. No product was detected by either HPLC or mass spectrometry (data not shown). Like Flp and Kep, Mtp did not inhibit the P4H-catalyzed conversion of Pro to Hyp (Figure 4).

**DISCUSSION**

P4H catalyzes an extremely difficult chemical reaction: the hydroxylation of an unactivated methylene group to form a secondary alcohol. The putative mechanism involves the oxidative decarboxylation of α-ketoglutarate, which promotes the formation of a highly reactive iron(IV)-oxo species from molecular oxygen (28–30). This iron(IV)-oxo species then abstracts the proR hydrogen from C-4 of a proline residue, replacing it with a hydroxyl group to form Hyp (31).

In previous work, proline analogues with substituents at C-3 and C-5 have been used to probe catalysis by P4H. Peptides containing a racemic mixture of 3-fluoroprolines in the Yaa
position were found to be substrates for the enzyme (51). Peptides containing the proline analogues 3-exomethylene-proline (51), 5-exoproline (52), and 3,4-dehydroproline (53–55) inhibited P4H activity. In our work, we have focused instead on C-4, which is the carbon that undergoes a change in covalency during the reaction catalyzed by P4H.4 Requirements of Nonnatural Proline Analogues for Turnover by P4H. P4H can elicit the homolytic cleavage of a C–H bond but not a C–F bond. The change from Pro to Flp is conservative from the perspective of steric, as hydrogen and fluorine have comparable van der Waals radii ($r_1 = 1.20 \text{ Å}; r_2 = 1.35 \text{ Å}$ (56)). A C–F bond ($\Delta H^\circ = 116 \text{ kcal/mol}$) is, however, much stronger than a C–H bond ($\Delta H^\circ = 98 \text{ kcal/mol}$). Accordingly, we were not surprised to learn that P4H cannot turn over Flp. This finding contrasts with reports by others in the 1960s that relied on less direct assays (57, 58). We did, however, expect P4H to bind to Flp as it does to Pro. Yet, no inhibition of P4H activity by Flp was detectable (Table 1).

Unlike Flp, Flp is a substrate for P4H. The stability of a carbon radical with an $\alpha$-fluoro substituent is $\Delta H = 0.7 \text{ kcal/mol}$ greater than that of an unsubstituted carbon radical, according to homolytic bond dissociation enthalpies of 2-fluoropropane and propane calculated at 25 °C (59). Hence, the hydroxylation of flp should be somewhat faster than that of Pro. Of course, other factors contribute to the rate of an enzymatic reaction, and the actual values of $k_{cat}$ are 360 and 250 min$^{-1}$ for Pro and flp, respectively (Table 1).

The product of the turnover of flp by P4H is Kep (Figure 5). This product has notable utility for future studies of P4H in biological systems. The turnover of flp introduces a functional group, a ketone, with orthogonal reactivity into a protein. This group could serve as a handle for a proteomic analyses of P4H substrates (60), as flp can be incorporated into proteins by biosynthesis (61–65). Furthermore, the turnover of flp not only produces Kep but also releases fluoride ion, whose detection could provide the basis for a direct, continuous assay of P4H activity.

The sulfide in the Thp-containing peptide is oxidized to a sulfoxide by P4H (Table 1, Figure 6). Sulfides can be oxidized by other dioxygenases, such as thymine hydroxylase (66), 4-hydroxyphenylpyruvate dioxygenase (67), and cysteine dioxygenase (68). Although the chemical oxidation of Thp produces both sulfoxide diastereomers (50), turnover by P4H produces only the 4R sulfoxide, which has the same relative stereochemistry as the natural product, Hyp. $\alpha$-Ketoglutarate is decarboxylated when Thp, flp, or Pro is the substrate in a reaction with P4H (Table 2). The relative rates of the decarboxylation reactions (Thp < flp < Pro) are similar to those for Thp(O), Kep, and Hyp production (Table 1). These data are consistent with Thp, flp, and Pro being turned over by the same mechanism.

Role of Proline Conformation in Substrate Binding by P4H. Proline is the only proteinogenic amino acid whose trans peptide bond isomer is favored only slightly over the cis isomer (38). In other peptide bonds, the trans conformation is favored greatly. Proline is also the only proteinogenic amino acid to contain a saturated ring. The five-membered pyrrolidine ring of proline primarily adopts two conformations, $C^\gamma$-endo or $C^\gamma$-exo (Figure 2).3 The pucker of its pyrrolidine ring and the isomerization of its peptide bond are correlated attributes of proline (39, 40). Electronegative atoms such as fluorine ($\chi_1 = 4.0$ (56); cf. $\chi_2 = 2.1$) alter the ring pucker via the gauche effect. A $C^\gamma$-exo pucker allows for an $n \rightarrow \pi^*$ interaction between the oxygen of the Xaa–Pro peptide bond and the carbon of the Pro–Gly peptide bond. In turn, this $n \rightarrow \pi^*$ interaction stabilizes a trans peptide bond. The absence of a significant $n \rightarrow \pi^*$ interaction in the $C^\gamma$-endo pucker favors a cis peptide bond (39, 40). The proline analogues used in this study differ in their predominant ring pucker (Figure 2). Hyp and Flp adopt a $C^\gamma$-exo pucker and have a high trans $\text{cis}$ ratio, whereas flp and Pro adopt a $C^\gamma$-endo pucker and have a low trans $\text{cis}$ ratio (40, 69). The ring puckers of Kep and Thp have not been examined directly. Nonetheless, the trans $\text{cis}$ ratio of Kep is higher than that of Pro (70), and the trans $\text{cis}$ ratio of Thp is lower than that of Pro (71). We find that Pro, flp, and Thp are substrates for P4H and that Hyp, Flp, and Kep are neither substrates nor inhibitors of the enzyme. We therefore propose that proline analogues that favor the $C^\gamma$-endo pucker and have a low trans $\text{cis}$ ratio bind to the active site of P4H, and those that favor a $C^\gamma$-exo or other pucker and have a high trans $\text{cis}$ ratio do not.

To test our proposal, we determined the ability of P4H to recognize Mtp (Figure 2). In essence, this analogue has two C-4 carbons and thus always displays both ring puckers (72). Mtp has a trans $\text{cis}$ ratio that is between that of Pro and flp (44). We find that P4H does not hydroxylate either C-4 carbon of Mtp and that Mtp does not inhibit P4H activity. These results refine our proposal by suggesting a proline residue with a $C^\gamma$-exo pucker does not bind to the active site of the enzyme.

Our data suggest a means by which P4H diminishes product inhibition, which is often detrimental to enzyme function (73, 74). The stereoelectronic consequences of catalysis by P4H convert the Pro substrate into a Hyp product, which has a greater preference for a $C^\gamma$-exo ring pucker and trans peptide bond. These changes discourage P4H from binding previously hydroxylated collagen strands. A similar mechanism to avert product inhibition is employed by oligosaccharyl transferase, which catalyzes the formation of a cis amide bond during the N-glycosylation of asparagine residues. Isomerization to the trans conformations takes place after product release and prevents the product from binding again to the enzyme (75).

A summarial model for substrate recognition by P4H is shown in Figure 7. This model is based on the data herein, as well as known structures of Pro and Hyp. Previously, we used X-ray diffraction analysis to determine the three-dimensional structures of crystalline AcProOMe and AcHypOMe (76). In these structures, AcProOMe has a cis peptide bond and $C^\gamma$-endo ring pucker, and AcHypOMe has a trans peptide bond and $C^\gamma$-exo ring pucker. In the model, the iron(IV)-oxo species is proximal to the proR hydrogen on C-4 of Pro, which is the single atom on the pyrrolidine ring that is farthest from the main chain of the peptide substrate. Thus, the hydrogen atom to be abstracted reaches most deeply into the enzymic active site. Hydroxylation then

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4 The diastereomers of (2S)-4-methylproline have been reported to be neither substrates nor inhibitors of human P4H (51).
promotes a conformational change in the product that the P4H active site is unable to accommodate.

CONCLUSIONS

We have discovered two novel substrates for P4H: flp and Thp. These analogues demonstrate for the first time that perturbations can be made at the 4-position of a proline substrate with retention of high P4H activity. Other proline analogues investigated, Flp, Kep, and Mtp, are not recognized by P4H. A comparison of the conformational preferences of these five proline analogues, along with Pro and Hyp, shows that proline analogues that prefer conformations similar to Pro, the substrate, are accepted by P4H, whereas analogues that are similar to Hyp, the product, are not. Therefore, the P4H-catalyzed formation of Hyp promotes conformational changes that limit product inhibition. Moreover, each analogue that binds to the active site of P4H is also a substrate, indicating that the enzyme discriminates at the level of substrate binding rather than substrate turnover. The highly reactive iron(IV)-oxo species that forms in the active site of P4H is perhaps the most powerful oxidizing agent in biology (30) and must therefore be sequestered deeply within its enzymic active site. Hence, P4H has an imperative for forming a snug complex with its substrate and appears to do so.

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