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Prolyl 4-Hydroxylase: Substrate Isosteres in Which an (*E*)- or (*Z*)-Alkene Replaces the Prolyl Peptide Bond

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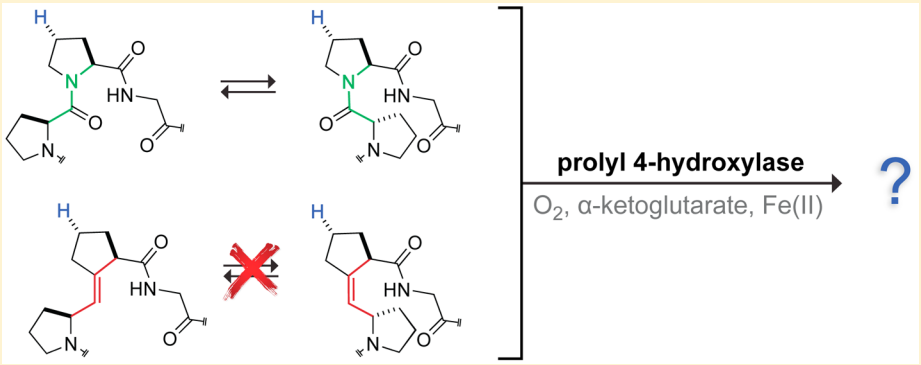
Supporting Information



ABSTRACT: Collagen prolyl 4-hydroxylases (CP4Hs) catalyze a prevalent posttranslational modification, the hydroxylation of (2*S*)-proline residues in protocollagen strands. The ensuing (2*S*,4*R*)-4-hydroxyproline residues are necessary for the conformational stability of the collagen triple helix. Prolyl peptide bonds isomerize between *cis* and *trans* isomers, and the preference of the enzyme is unknown. We synthesized alkene isosteres of the *cis* and *trans* isomers to probe the conformational preferences of human CP4H1. We discovered that the presence of a prolyl peptide bond is necessary for catalysis. The *cis* isostere is, however, an inhibitor with a potency greater than that of the *trans* isostere, suggesting that the *cis* conformation of a prolyl peptide bond is recognized preferentially. Comparative studies with a *Chlamydomonas reinhardtii* P4H, which has a similar catalytic domain but lacks an N-terminal substrate-binding domain, showed a similar preference for the *cis* isostere. These findings support the hypothesis that the catalytic domain of CP4Hs recognizes the *cis* conformation of the prolyl peptide bond and inform the use of alkenes as isosteres for peptide bonds.

Collagen is the principal component of the extracellular matrix, connective tissues, and bone of animals.[1] The overproduction of collagen is associated with a variety of diseases, including fibrotic diseases[2] and cancers.[3−7] The stability of collagen relies on posttranslational modifications.[8] By far, the most common of these modifications is mediated by collagen prolyl 4-hydroxylases (CP4Hs), which are Fe(II)- and α-ketoglutarate-dependent dioxygenases (FAKGDs) that reside in the lumen of the endoplasmic reticulum.[9] Catalysis by CP4Hs converts (2*S*)-proline (Pro) residues in protocollagen strands into (2*S*,4*R*)-4-hydroxyproline (Hyp) residues (Figure 1A), which endow mature collagen triple helices with conformational stability.[10] CP4Hs, while essential for animal life,[11−13] are validated targets for treating fibrotic diseases[14] and metastatic breast cancer.[6]

Catalysis by CP4Hs and other FAKGDs requires Fe(II) and the cosubstrates α-ketoglutarate and molecular oxygen.[15−17] The Fe(II) is bound by a conserved His-X-Asp/Glu···X_n···His motif, and α-ketoglutarate chelates to the enzyme-bound Fe(II) by using its C-1 carboxylate and C-2 keto groups while its C-5 carboxylate group forms hydrogen bonds and engages in Coulombic interactions with a basic residue [often arginine or lysine (Figure 1B)]. All FAKGDs are believed to effect catalysis through a two-stage mechanism in which α-ketoglutarate is decarboxylated oxidatively to generate a highly reactive Fe(IV)═O species that elicits substrate oxidation via a radical rebound process.[15−18]

In vertebrates, CP4Hs exist as $\alpha_2\beta_2$ tetramers. In these tetramers, the α-subunit contains the C-terminal catalytic domain and N-terminal peptide substrate-binding domain (PSB domain). The β-subunit is protein disulfide isomerase, which is a multifunctional protein that maintains the α-subunit in a soluble form and active conformation.[9] Humans have three isoforms of the α-subunit: α(I), α(II), and α(III).[9] All three of these isoforms interact with a single β-subunit to form tetramers, which are known as CP4H1−CP4H3, respectively.

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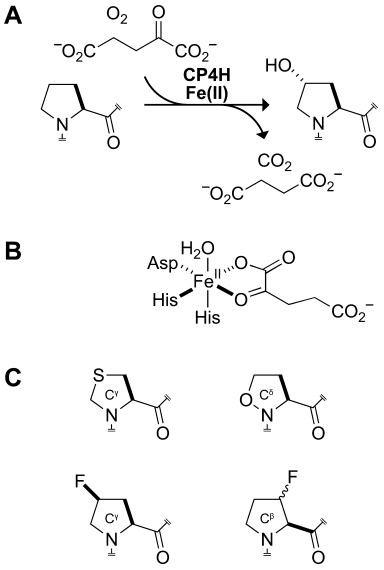

Figure 1. (A) Reaction catalyzed by collagen prolyl 4-hydroxylase (CP4H). (B) Putative enzymic ligands for CP4H.[15−17] (C) Proline analogues that are substrates for CP4H,[30−33] despite substitutions at C^{β}, C^{γ} (i.e., C-4), or C^{δ}.

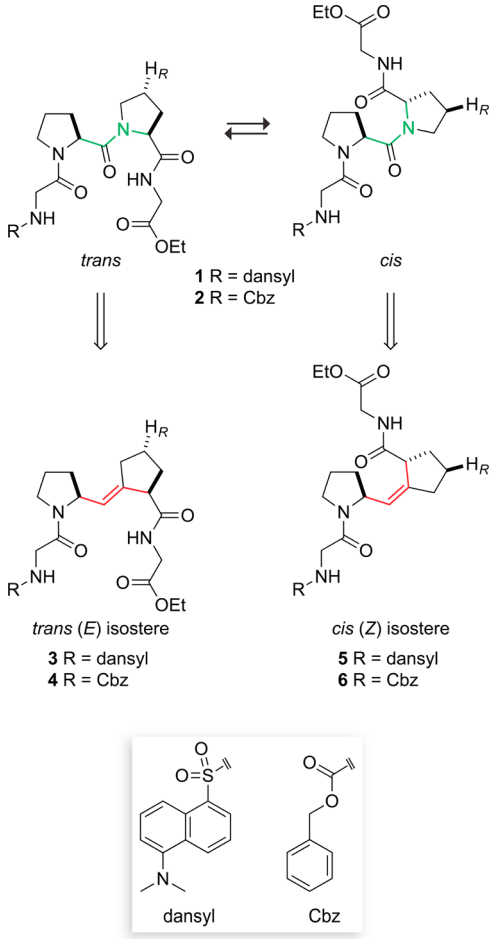

Figure 2. Peptide substrates for CP4H and their alkene isosteres. Tetrapeptide RGlyProProGlyOEt (**1** and **2**) can adopt a *trans* or *cis* conformation in solution. The corresponding alkene isosteres are locked as a *trans* (**3** and **4**) or *cis* (**5** and **6**) isomer.

CP4H1 is the most prevalent of these holoenzymes and has been characterized most extensively. Although the structure of the tetrameric complex is unknown, those of the individual domains of the α(I) subunit have provided insight into the interaction of CP4Hs with the protocollagen substrate, as well as the interaction of two α(I) subunits in the tetramer.[19−22]

Many features of the reaction catalyzed by CP4Hs are well-known.[9] Moreover, the development of CP4H inhibitors is of keen interest.[2,14,23,24] Like others,[25] we have directed our attention toward inhibitors that mimic the α-ketoglutarate cosubstrate.[12,26−28] Because many enzymes use α-ketoglutarate as a substrate or cosubstrate, CP4H inhibitors based upon the protocollagen substrate could offer improved selectivity. CP4H is recalcitrant to substitutions in its Fe(II)-binding motif.[29] The enzyme can, however, tolerate alterations in its substrate.[30−33] For example, CP4H catalyzes the hydroxylation of proline analogues that bear a substitution at C^{β}, C^{γ}, or C^{δ} (Figure 1C).

Little is known about the conformation of the peptide substrate during catalysis. Proline is a secondary amine, and prolyl peptide bonds are tertiary amides. Accordingly, the *cis* isomer of prolyl peptide bonds (Figure 2A) occurs much more frequently than with non-prolyl residues.[34] Biochemical and biophysical investigations have suggested that β-turns are sites of prolyl hydroxylation.[35−37] β-Turns can have *cis* or *trans* prolyl peptide bonds,[38] and the isomerization state of the peptide bond in CP4H substrates is unknown, as is the necessity of even having a peptide bond. Here, we probe this fundamental issue by using alkene isosteres, which delete the peptide bond while preserving its geometric constraints. We describe the synthesis of collagen mimetic peptides in which a prolyl peptide bond is replaced with an (*E*)- or (*Z*)-alkene, which are the smallest isosteres that fix the bond as a *trans* or *cis* isomer, respectively.[39] We find that the presence of a peptide bond is necessary for catalysis by both human CP4H1 and a related P4H from the algae *Chlamydomonas reinhardtii* (CrP4H1[40]). These enzymes prefer to bind to the *cis* isostere relative to the *trans*, and recognition of the *cis* isostere likely occurs in the catalytic domain. These results provide new insight into catalysis by prolyl 4-hydroxylases.

EXPERIMENTAL PROCEDURES

Peptide Synthesis. Detailed procedures for the synthesis of peptides **1** and **2**, *trans* isosteres **3** and **4** (Scheme 1), and *cis* isosteres **5** and **6** (Scheme 2) are reported in the Supporting Information.

Instrumentation. The progress of reactions catalyzed by prolyl 4-hydroxylases was assessed by analytical UPLC using an Acquity UPLC H-Class system from Waters (equipped with a photodiode array detector, a quaternary solvent manager, a sample manager with a flow-through needle, and Empower 3 software). The concentration of dansyl-labeled peptides was determined with a Cary 60 UV−vis spectrometer from Agilent Technologies (Santa Clara, CA) using an extinction coefficient of 3900 M^{-1} cm^{-1}. Solution concentrations of proteins were calculated with the Beer−Lambert law from A_{280} values as measured with a NanoVue Plus spectrophotometer from GE Healthcare (Piscataway, NJ) and extinction coefficients of 290000 M^{-1} cm^{-1} for human CP4H1[41] and 44000 M^{-1} cm^{-1} for CrP4H-1 (which was estimated with EXPASY software by assuming that all cysteine residues form disulfide bonds). Values of IC_{50} were calculated from experimental data with Prism version 6.0 from GraphPad Software (La Jolla, CA).

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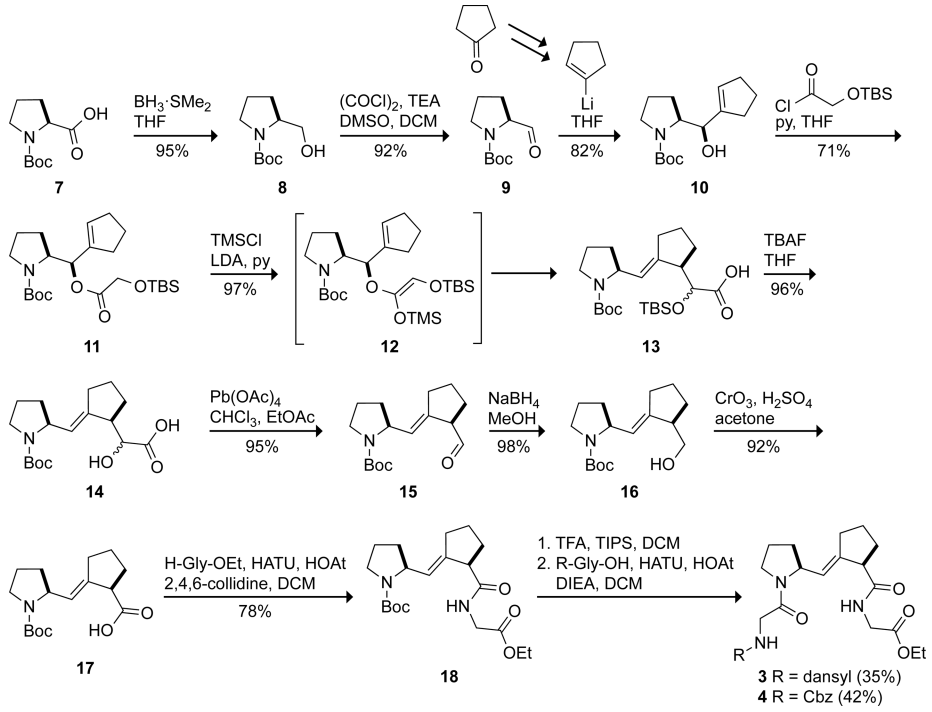
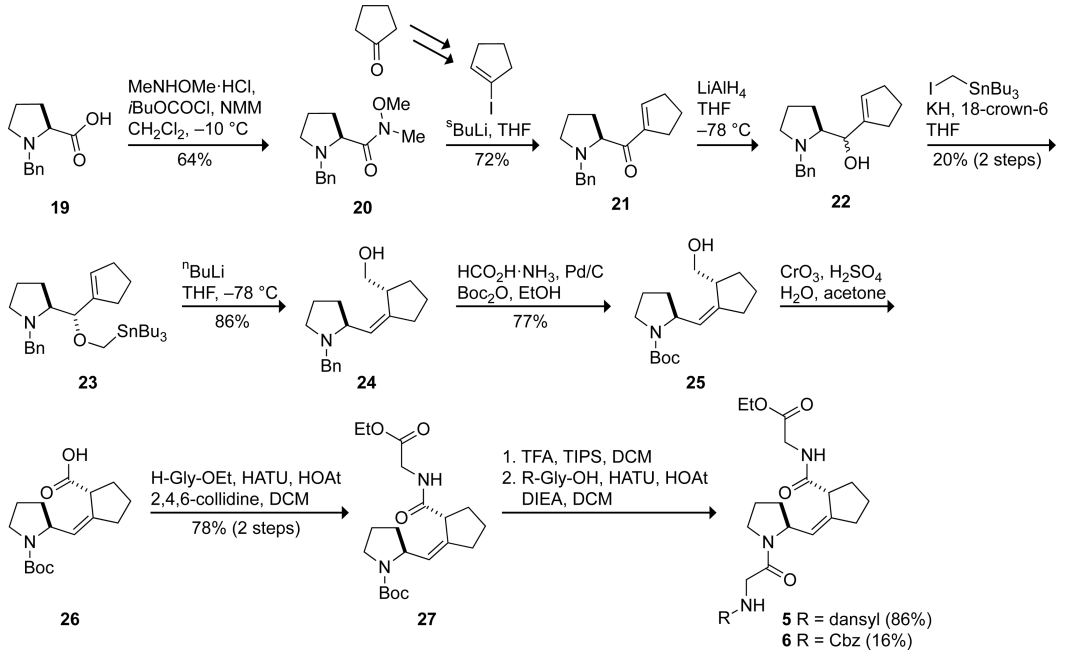
Biochemistry 2017, 56, 219−227

Scheme 1. Synthetic Route to GP*trans*PGOEt *trans* Isosteres 3 and 4



Scheme 2. Synthetic Route to GP*cis*PGOEt *cis* Isosteres 5 and 6



Production of Recombinant Human CP4H1. Human CP4H containing the α(I) isoform was produced heterologously in Origami B(DE3) *Escherichia coli* cells and purified as described previously.[41]

Stock Solutions for Assays. Assay mixtures were made from the following stock solutions. A stock solution of Tris-HCl buffer (10×) was prepared at 500 mM and pH 7.8 in H_2O. A stock solution of α-ketoglutarate (10×) was prepared in H_2O. A stock solution of $FeSO_4$ (10×) was prepared at 500 μM in H_2O. A stock solution of sodium ascorbate (10×) was prepared at 20 mM in H_2O. A stock solution of DTT (10×) was prepared freshly on the day of its use at 1.0 mM in H_2O. A stock solution of catalase (10×) was prepared freshly on the day of its use at 1.0 mg/mL in 50 mM Tris-HCl buffer (pH 7.8). A stock solution of BSA (10 mg/mL) was prepared in 50 mM Tris-HCl buffer (pH 7.8). Concentrated stock solutions of the dansylGPPGOEt substrate and alkene isosteres were prepared in EtOH and diluted to working stock solutions (10×) in H_2O.

Assay of Human CP4H1 Activity in the Presence of Alkene Isosteres. The catalytic activity of human CP4H1 was assayed as described previously.[41] Briefly, activity assays were

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Biochemistry 2017, 56, 219–227

performed at 30 °C in 100 μL of 50 mM Tris-HCl buffer (pH 7.8) containing human CP4H1 (100 nM), substrate (0−1.86 mM), alkene isostere (0−3.00 mM), α-ketoglutarate (13−1000 μM), $FeSO_4$ (50 μM), sodium ascorbate (2 mM), DTT (100 μM), catalase (0.1 mg/mL to decompose any hydrogen peroxide), and BSA (1 mg/mL). Unless noted otherwise, reaction mixtures were prepared by adding concentrated stock solutions of each component to concentrated assay buffer in this order: $FeSO_4$, DTT, sodium ascorbate, BSA, catalase, CP4H1, peptide substrate, and alkene isostere (or vehicle). Solutions prepared thusly were preincubated at 30 °C for 2 min, after which a reaction was initiated by the addition of α-ketoglutarate. After 15 min, reactions were quenched by boiling for 45 s, and reaction mixtures were subjected to centrifugation at 10000*g*. The supernatant (5−10 μL) was injected into an Acquity UPLC BEH C18 column (2.1 mm × 50 mm, 1.7 μm particle size) from Waters. The column was eluted for 2.9 min at a rate of 0.6 mL/min with a linear gradient of acetonitrile [20 to 68% (v/v)] in water containing TFA [0.1% (v/v)] while monitoring the value of A_{289}. All assays were performed in triplicate. Data are reported as activity relative to control reactions lacking an alkene isostere, where activity is determined from the percent conversion of substrate to product. Dose−response curves were generated for each isostere by plotting the relative activity versus the log of isostere concentration. Values of IC_{50} for each isostere were interpolated from the dose−response curves by nonlinear regression using the sigmoidal dose−response function available in Prism. For substrate inhibition by dansylGlyProProGlyOEt, data were fitted to the substrate inhibition function in Prism.

Production of Recombinant *C. reinhardtii* P4H-1. *C. reinhardtii* $P4H1_{30-245}$ having an N-terminal hexahistidine (His_6) tag ($NHis_6$-CrP4H-1) was produced heterologously in Origami B(DE3) *E. coli* cells and purified as described previously.[42]

Assay of *C. reinhardtii* P4H-1 Activity in the Presence of Alkene Isosteres. The catalytic activity of $NHis_6$-CrP4H-1 was assessed in assays similar to those for human CP4H1 with minor modification. Briefly, activity assays were carried out at 30 °C in 100 μL of 50 mM HEPES-HCl buffer (pH 6.8) containing $NHis_6$-CrP4H-1 (1.2 μM), substrate dansylGlyProProGlyOEt (500 μM), alkene isostere (0−100 μM), α-ketoglutarate (1 mM), $FeSO_4$ (200 μM), sodium ascorbate (2 mM), DTT (100 μM), catalase (0.1 mg/mL), and BSA (1 mg/mL). Reaction mixtures were prepared by adding concentrated stock solutions of each component to concentrated assay buffer in this order: $FeSO_4$, DTT, sodium ascorbate, BSA, catalase, $NHis_6$-CrP4H-1, peptide substrate, and alkene isostere (or vehicle). Solutions prepared thusly were preincubated at 30 °C for 2 min, after which a reaction was initiated by the addition of α-ketoglutarate. After 1 h, reactions were quenched by adding EDTA (1 μL of a 0.5 M solution) and boiling for 45 s. Quenched reaction mixtures were subjected to centrifugation at 10000*g* for 5 min, after which the supernatant (5−10 μL) was analyzed by UPLC as described above.

RESULTS AND DISCUSSION

Synthesis of Alkene Isosteres. The primary substrates for CP4Hs *in vivo* are protocollagens and other large polymers (i.e., elastin).[9] These large protein substrates are not amenable to most chemical analyses. Hence, we chose to employ a model system based upon a small collagen mimetic tetrapeptide, GlyProProGlyOEt (GPPGOEt), with corresponding alkene isosteres GP*trans*PGOEt and GP*cis*PGOEt (Figure 2). Small tetrapeptides of this nature that contain one collagen-like triplet repeat have been found previously to be suitable substrates for CP4Hs.[27,32,33,37,41] We chose to use model peptides capped with an N-terminal dansyl sulfonamide, which are well-known to be substrates for human CP4H,[27,41] as well as peptides capped with an N-terminal Cbz group[30,31] for supplemental analyses (Figure 2). The model substrates dansylGPPGOEt (**1**) and CbzGPPGOEt (**2**) were synthesized by standard solution-phase peptide coupling procedures as described previously for similar peptides.[27]

trans isosteres dansylGP*trans*PGOEt (**3**) and CbzGP*trans*PGOEt (**4**) were synthesized from Boc-Pro-OH in 11 steps by a route (Scheme 1) adapted from previous reports by Etzkorn, Koert, and co-workers.[43−47] The facial selectivity in the conversion of aldehyde **9** to alcohol **10** is particularly striking. The Boc group in aldehyde **9** blocks the *si* face of the aldehyde, leading to (*S*)-configured alcohol **10**. (The stereochemistry of alcohol **10** was confirmed by X-ray crystallography.) The key step in the route relied on the stereoselective Ireland−Claisen rearrangement of allyl ester **12** to γ,δ-alkenyl acid **13** in nearly quantitative yield.[48] Standard transformations led to acid **17**, which was then coupled with H-Gly-OEt by using a hindered base, 2,4,6-collidine, to prevent isomerization of the olefin. Lastly, deprotection of the N-terminal Boc group of alkene **18** followed by coupling with dansyl-Gly-OH or Cbz-Gly-OH yielded *trans* isostere **3** or **4**, respectively. These isosteres had characteristic alkene ^{1}H chemical shifts at 5−6 ppm, indicating that the alkene did not isomerize to be in conjugation with the amidic carbonyl group.

The corresponding *cis* isosteres dansylGP*cis*PGOEt (**5**) and CbzGP*cis*PGOEt (**6**) were synthesized from Bn-Pro-OH in nine steps by a route (Scheme 2) inspired by the work of Etzkorn and co-workers.[43,49] A variety of reducing agents were evaluated for the stereoselective reduction of ketone **21**, including L-Selectride, K-Selectride, $NaBH_4$, DIBALH, and $LiAlH_4$. The highest yields of alcohol **22** were afforded by $LiAlH_4$, but as a nearly equimolar mixture of diastereomers. Although the desired diastereomer of alcohol **22** was isolable, the separation of diastereomers following alkylation to ether **23** was more facile. (The stereochemistry of the two diastereomers of alcohol **22** was assigned by forming the two oxazolidinones and using ^{1}H NMR spectroscopy.) The key step in the route employed a stereoselective Still−Wittig rearrangement[50] of ether **23** to afford alkene **24** in 86% yield. As with the synthesis of the *trans* isosteres, the coupling of H-Gly-OEt to acid **26** worked well when mediated by 2,4,6-collidine. Lastly, deprotection of the N-terminal Boc group of alkene **27** followed by subsequent coupling of dansyl-Gly-OH or Cbz-Gly-OH yielded *cis* isostere **5** or **6**, respectively. Again, these isosteres had characteristic alkene ^{1}H chemical shifts at 5−6 ppm, indicating that the alkene did not isomerize to be in conjugation with the amidic carbonyl group. Analytical UPLC revealed that compounds **1**−**6** were of high purity.

Interaction of Model Peptides and Alkene Isosteres with Human CP4H. With the model peptides and corresponding alkene isosteres in hand, we set out to probe their interaction with human CP4H. First, we tested the model peptides (**1** and **2**) as substrates for human CP4H by employing a UPLC assay that was adapted from an HPLC assay described previously.[27,32,41] Initial tests confirmed that

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Biochemistry 2017, 56, 219−227

both dansylGPPGOEt and CbzGPPGOEt were substrates for human CP4H1, as expected from previous reports.[27,41] In contrast, neither the *cis* nor the *trans* isosteres (**3**–**6**) served as substrates for the enzyme (data not shown), regardless of concentration (0.1–3.0 mM for dansyl isosteres; 0.1–10 mM for Cbz isosteres).

Although isosteres **3**–**6** did not serve as substrates for human CP4H, we reasoned that they might serve as inhibitors. Using the dansyl model peptides, we found that both dansylGP-*trans*PGOEt and dansylGP*cis*PGOEt were indeed inhibitors of catalysis by human CP4H (Figure 3A), with the *cis* isostere being significantly more potent than the *trans* isostere. In subsequent dose–response experiments, the inhibition curves for both dansyl isosteres were found to be sigmoidal (Figure 3B), with IC_{50} values of (75 ± 6) and (410 ± 30) μM for the *cis* and *trans* isosteres, respectively. Moreover, this same trend in which the *cis* isostere inhibits with greater potency than does the *trans* isostere was also observed with the Cbz-capped isosteres **4** and **6** (Figure 3C), indicating that the trend is independent of the N-terminal group. These data are consistent with a previous correlation indicating that substrates for human CP4H1 tend to have a stronger preference for a *cis* prolyl peptide bond.[32] In contrast to isosteres **3**–**6**, however, none of those previous substrates were fixed as a *cis* (or *trans*) isomer. Because the quantitation and sensitivity were more reliable than those of the Cbz peptides, we proceeded with dansyl isosteres **3** and **5** in subsequent analyses.

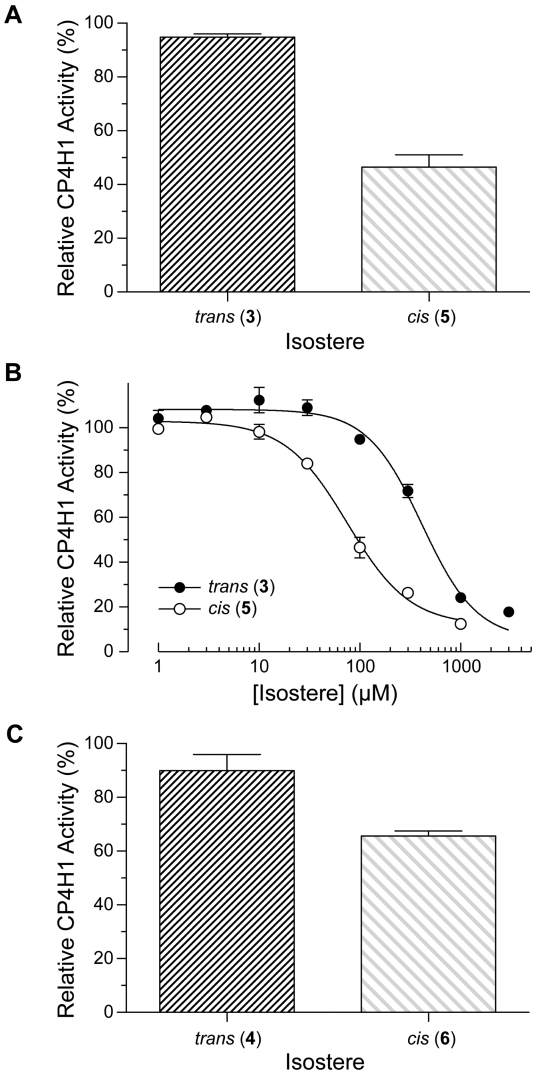

Figure 3. Inhibition of human CP4H1 by alkene isosteres **3**–**6**. (A and B) Assays were performed at pH 7.8 with dansylGPPGOEt (0.50 mM) as the substrate, α-ketoglutarate (1.00 mM), and a dansyl isostere (100 μM in panel A). (C) Assays were performed at pH 7.8 in the presence of a Cbz isostere (1.00 mM in panel C) with CbzGPPGOEt (1.00 mM) as a substrate and α-ketoglutarate (1.00 mM) as a cosubstrate. In all panels, values are the means (±standard deviation) of three independent experiments and represent the ratio of enzymatic activity in the presence and absence of the isostere.

With an interest in characterizing further the inhibition observed for the dansyl isosteres, we next considered their kinetic mechanism. CP4Hs have been shown previously to display an ordered *ter*–*ter* mechanism in which α-ketoglutarate first binds to the CP4H·Fe(II) complex, after which O_2 and the peptide substrate bind in an ordered fashion.[51] We found that turnover of dansylGPPGOEt did not display Michaelis–Menten kinetics, but rather a pattern that could be recognized as substrate inhibition (Figure 4A), which was evident in the

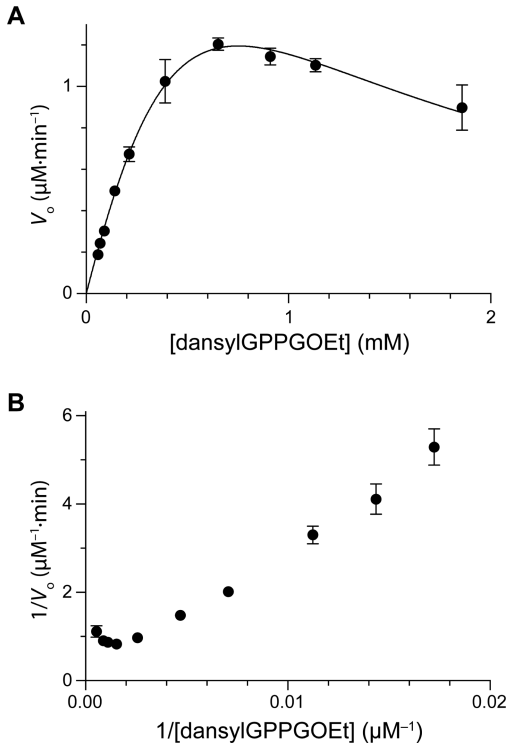

Figure 4. Substrate inhibition of human CP4H1 by dansylGPPGOEt (**1**). (A) Velocity plot. Assays were performed at pH 7.8 with dansylGPPGOEt (58–1860 μM) as the substrate and α-ketoglutarate (1.00 mM) as the cosubstrate. (B) Lineweaver–Burke plot of the data in panel A.

corresponding Lineweaver–Burke plot (Figure 4B). The data in Figure 4A were fitted to the equation for substrate inhibition:

$$v = \frac{V_{max}[S]}{K_M + [S] + \frac{[S]^2}{K_i}}$$

where K_i is the dissociation constant for substrate binding in an inhibitory manner. This fit (R^2 = 0.98) yielded values for V_{max}, K_M, and K_i of (8 ± 3) μM min^{-1}, (2.0 ± 0.8) mM, and (0.3 ± 0.1) μM, respectively.

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Biochemistry 2017, 56, 219–227

Substrate inhibition had been observed previously for a CP4H during turnover of a longer collagen mimetic peptide substrate, $(ProProGly)_{10}$.[51] Moreover, that substrate inhibition was competitive with the cosubstrate α-ketoglutarate. Substrate inhibition by dansylGPPGOEt in our assays was, however, observed even at saturating concentrations of α-ketoglutarate, suggesting that the inhibitory binding mode of dansylGPPGOEt could not be eliminated in this manner.

Given the complexities in the kinetics with respect to the peptide substrate, we reasoned that, if the kinetic mechanism entailed ordered binding, then the dansyl isosteres would display uncompetitive inhibition versus the α-ketoglutarate cosubstrate. We performed a Lineweaver–Burke analysis using *cis* isostere **5** as a model inhibitor but found an inhibition pattern consistent with noncompetitive inhibition versus α-ketoglutarate (Figure 5A). The slope (Figure 5B) and intercept (Figure 5C) replots for the noncompetitive inhibition were linear, and K_{is} and K_{ii} values of (56 ± 13) and (18 ± 8) μM, respectively, were extracted from the slope (K_{is}) and intercept (K_{ii}) replots by linear regression analysis. This value for K_{is} is indistinguishable from the IC_{50} value of (76 ± 13) μM for *cis* isostere **5**. The observation of noncompetitive inhibition for *cis* isostere **5** versus α-ketoglutarate suggests either that this isostere binds in a CP4H subsite that is independent of the site for hydroxylation or that the binding of the small collagenous tetrapeptides is not strictly ordered relative to the cosubstrates α-ketoglutarate and molecular oxygen. Kinetic experiments cannot distinguish between these two possibilities.

Preliminarily, the inhibition pattern reported above is consistent with the hypothesis that human CP4H preferentially binds to the *cis* conformation compared to the *trans* conformation in collagen-like peptides. The CP4H α-subunit is composed of multiple domains, including a C-terminal catalytic domain and an N-terminal PSB domain,[9,14] both of which likely interact with a typical polymeric substrate. The structure of the PSB domain has been characterized and is known to interact with peptides in a PPII helical conformation, which has *trans* peptide bonds.[20−22] Accordingly, we were not surprised to learn that human CP4H has some affinity for *trans* isostere **3**, which is likely to adopt an extended conformation similar to that of a PPII helix. Given its locked conformation, however, *cis* isostere **5** is less likely to interact favorably with the PSB domain, which raises the issue of whether the C-terminal catalytic domain is responsible for preferential recognition of the *cis* conformation.

To address this issue, we sought a more simple P4H model system. We chose to investigate CrP4H-1, which is an algal P4H.[40] CrP4H-1 is a soluble, 29 kDa monomer with an amino acid sequence 26% identical to that of the catalytic domain of human CP4H1[40] and a known three-dimensional structure.[52,53] FAKGD enzymes are generally conserved at the level of fold,[16] and all essential active-site residues are conserved in the two P4Hs.[40] Moreover, although substrates for algal P4Hs such as CrP4H-1 are typically proline- and serine-rich polymers of the algal cell wall *in vivo*, CrP4H-1 has been observed to turn over collagen-like peptides, albeit with less positional specificity (e.g., CrP4H-1 catalyzes the hydroxylation of proline residues in both position *X* and position *Y*).[40]

As CrP4H-1 can be viewed as a reasonable model system for the catalytic domain of the human CP4H1 α-subunit, we sought to investigate the interaction of dansyl peptides **1**, **3**, and **5** with CrP4H-1. Under an assay condition similar to that used to assay human CP4H1, we found that model peptide **1** served as a substrate for CrP4H-1, albeit with a substrate inhibition phenomenon similar to that observed for human CP4H1 (Figure 6). Again, we did not observe hydroxylation of dansyl isostere **3** or **5** by CrP4H-1 (data not shown). We did find, however, that both dansyl isosteres **3** and **5** served as inhibitors of CrP4H-1. We observed an inhibition pattern virtually identical to that observed for human CP4H1, again with *cis* isostere **5** displaying potency significantly greater than that of *trans* isostere **3** (Figure 7). Similar to human CP4H1, these data suggest that CrP4H-1 preferentially recognizes the *cis* conformation in peptide substrate analogues. Given the similarities between human CP4H1 and algal CrP4H-1, as well as CrP4H-1 being (in essence) a monomeric catalytic domain, our data suggest that the catalytic domains of both

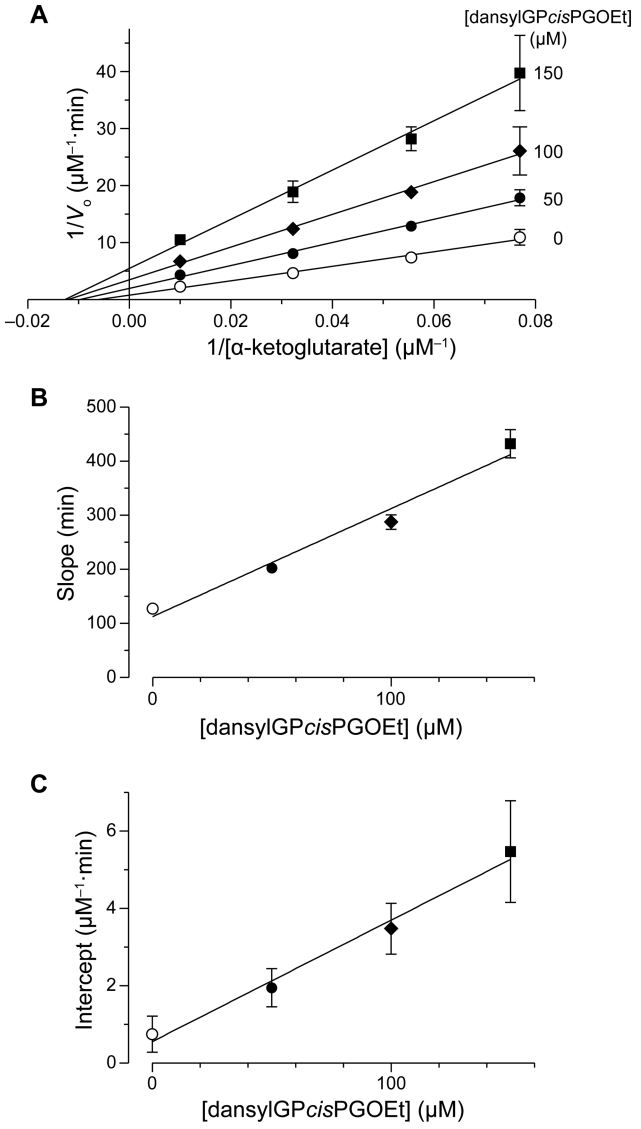

Figure 5. Lineweaver–Burke analysis of inhibition of human CP4H1 by *cis* isostere **5**. (A) A Lineweaver–Burke plot for **5** is indicative of noncompetitive inhibition with respect to α-ketoglutarate, as the intersecting pattern does not converge on the ordinate and the slope (B) and intercept (C) replots are linear. Assays were performed at pH 7.8 with dansylGPPGOEt (500 μM) as the substrate and α-ketoglutarate (13–100 μM) as the cosubstrate in the presence of *cis* isostere **5** (0, 50, 100, or 150 μM). Values are the means (±standard error) of three independent experiments.

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Biochemistry 2017, 56, 219−227

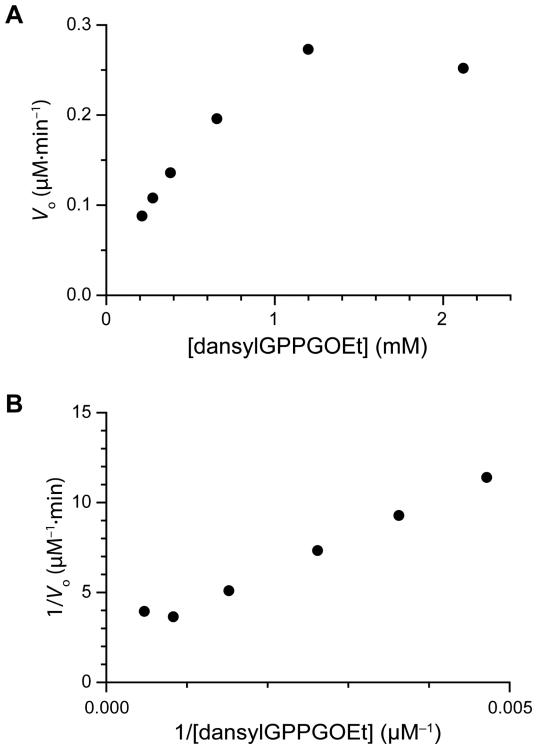

Figure 6. Substrate inhibition of CrP4H-1 by dansylGPPGOEt (**1**). (A) Velocity plot. Assays were performed at pH 7.8 with dansylGPPGOEt (212−2120 μM) as the substrate and α-ketoglutarate (1.00 mM) as the cosubstrate. (B) Lineweaver−Burke plot of the data in panel A.

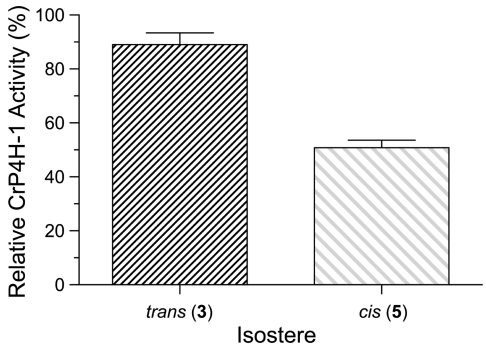

Figure 7. Inhibition of *C. reinhardtii* CrP4H-1 by alkene isosteres **3** and **5**. Assays were performed at pH 7.8 with dansylGPPGOEt (0.50 mM) as the substrate, α-ketoglutarate (1.00 mM), and an alkene isostere (100 μM). Values are the means (±standard deviation) of three independent experiments and represent the ratio of enzymatic activity in the presence and absence of isostere.

P4Hs preferentially recognize the *cis* conformation of prolyl peptide bonds.

Product inhibition is often detrimental to enzyme function.[54−56] This problem could be especially acute with CP4H because of the abundance of collagen strands in the endoplasmic reticulum. The installation of a 4(*R*)-hydroxyl group is known to decrease the fraction of prolyl peptide bonds that reside in the *cis* conformation.[57,58] Taken with our data, this increase in the *trans*:*cis* ratio could slow product inhibition. We note as well that the hydrophilicity conferred by hydroxylation could diminish the affinity for hydrophobic pockets of the PSB domain.[22]

Neither the *cis* nor the *trans* prolyl peptide bond isostere serves as a substrate for CP4H. This result suggests that CP4H interrogates substitutions at the prolyl peptide bond of its substrate more closely than those near C$^{\gamma}$ (Figure 1C). Numerous explanations are possible for this intolerance. The substitution could eliminate a key hydrogen bond between the enzyme and the amidic oxygen (O_{i-1}) of the prolyl peptide bond, perturbing the orientation of the substrate. The substitution could affect the substrate conformation by preventing two potential n → π^* interactions,[46,59−61] one with the C'_{i-1}═O_{i-1} group as a donor and one with the C'_{i-1}═O_{i-1} group as an acceptor. Finally, the conformational mobility of the prolyl peptide bond (even its *cis*−*trans* isomerization) could play an integral role in catalysis by CP4H. These scenarios are the basis for ongoing work in our laboratory.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.6b00976.

Procedures for the synthesis of compounds **1**−**6** (PDF)

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Notes

The authors declare no competing financial interest.

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