Bioavailable affinity label for collagen prolyl 4-hydroxylase

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Collagen is the most abundant protein in animals. Its prevalent 4-hydroxyproline residues contribute greatly to its conformational stability. The hydroxyl groups arise from a post-translational modification catalyzed by the nonheme iron-dependent enzyme, collagen prolyl 4-hydroxylase (P4H). Here, we report that 4-oxo-5,6-epoxyhexanoate, a mimic of the α-ketoglutarate co-substrate, inactivates human P4H. The inactivation installs a ketone functionality in P4H, providing a handle for proteomic experiments. Caenorhabditis elegans exposed to the esterified epoxy ketone displays the phenotype of a worm lacking P4H. Thus, this affinity label can be used to mediate collagen stability in an animal, as is desirable in the treatment of a variety of fibrotic diseases.

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1. Introduction

Collagen is the most abundant protein in animals, and maintains the structural integrity of various connective tissues. The overproduction of collagen is associated with fibrotic diseases, including myocardial infarction, stroke, peripheral vascular disease, diabetes, and severe anemias. The stability of collagen relies on catalysis by collagen prolyl 4-hydroxylase (P4H). This enzyme is located in the lumen of the endoplasmic reticulum, where it catalyzes a remarkable post-translational modification—the conversion of (2S)-proline residues (Pro) to (2S,4R)-4-hydroxyproline residues (Hyp) in protocollagen strands. The human enzyme consists of an α2β2 tetramer in which each α subunit contains an active site. P4H is essential for animals, as the conformational stability of mature collagen relies on its hydroxylation. Moreover, P4H is a validated target for the treatment of fibrotic diseases.

P4H is a nonheme iron(II) dioxygenase that uses O2 and α-ketoglutarate as co-substrates (Fig. 1A). The three-dimensional structure of mammalian P4H is unknown. We reasoned that an electrophilic analog of α-ketoglutarate could serve as an irreversible inhibitor of the enzyme and, hence, a useful probe for active-site residues. Inspired by natural products such as trapoxin and epolactaene as well as artificial affinity labels, we designed 4-oxo-5,6-epoxyhexanoate (1) for this purpose. Our thought was that the epoxide oxygen would chelate to the active-site iron and thereby be activated for nucleophilic attack (Fig. 1B). We also noted that α-ketoglutarate and epoxy ketone 1 have the same number and a similar arrangement of nonhydrogen atoms. This attribute is important because of the inability of larger α-ketoglutarate analogs to inhibit catalysis by human P4H. Epoxy ketone 1, which also resembles 5-aminolevulinic acid, is a known competitive inhibitor of 5-aminolevulinic acid dehydratase but does not alkylate that enzyme.

Figure 1. (A) Reaction catalyzed by prolyl 4-hydroxylase. (B) Notional image of α-ketoglutarate (left) or epoxy ketone 1 (right) bound in the active site of P4H and interacting with an enzymic base. B. Alkylation is more likely to occur by nucleophilic attack at C6 than C5.
2. Results and discussion

2.1. Effect of epoxy ketone 1 on catalysis by P4H

We produced human P4H by heterologous expression in *Escherichia coli* and we synthesized racemic 1. We assayed the effect of this putative affinity label on the ability of P4H to catalyze the hydroxylation of a fluorescent peptidic substrate, dansyl-Gly–Phe–Pro–Gly-OEt. The data revealed that epoxy ketone 1 elicits irreversible inactivation of P4H, and does so in a concentration-dependent manner (Fig. 2A). ω-Ketoglutarate protected P4H against inactivation (Fig. 2B), suggesting that epoxy ketone 1 competes with this co-substrate for binding to the active site.

2.2. Utility of epoxy ketone 1 as a proteomic probe

Noting that a ketone has chemical reactivity orthogonal to that in natural amino acids, we examined the utility of epoxy ketone 1 in a model proteomic experiment. Epoxy ketone 1 was incubated with P4H in the presence of iron(II). The mixture was treated with biocytin hydrazide and then NaBH₄ to reduce the incipient N-acyl hydrazone (which is unstable). Small molecules were removed by gel-filtration chromatography. The P4H was added to wells of a microplate containing immobilized streptavidin. The wells were washed and exposed to a monoclonal antibody directed against P4H. Visualization with a secondary antibody conjugated to horseradish peroxidase revealed that P4H is associated with streptavidin only in the presence of epoxy ketone 1 (Fig. 2C). The conjugation was confirmed by SDS–PAGE (data not shown). These data indicate that epoxy ketone 1 could serve as a chemo-selective probe for P4H. Thus, epoxy ketone 1 complements a (2S,4S)-4-fluoroproline substrate that spawns a ketone upon P4H catalysis.

2.3. Effect of epoxy ketone 1 on an animal

Finally, we determined the effect of epoxy ketone 1 on a live animal. Like all animals (including dinosaurs), *Caenorhabditis elegans* has an abundance of collagen. In wild-type worms, P4H exists largely as a tetramer of two α subunits (DPY-18 and PHY-2, which each contain an active site) and two β subunits. The dpy-18 gene is essential for body morphology, whereas the function of the phy-2 gene is not apparent. The deletion of both genes results in embryos that grow to the twofold stage, but are unable to maintain their shape and consequently explode. These two forms of the α subunit have 56.5% amino-acid sequence identity with each other and ~50% identity with their human homologues. ω-Ketoglutarate
analogs can eliminate the remaining P4H activity in dpy-18 C. elegans at concentrations that do not affect the wild-type worm. This synthetic lethality provided the basis for our assay.

Dpy-18 worms were placed onto solid medium containing known concentrations of esterified epoxy ketone 1. The ester group, which can be hydrolyzed by cellular esterases, served to mask the anionic carboxylate and thus enhance uptake. Concentration-dependent embryonic lethality of dpy-18 C. elegans was observed with LD50 = 0.11 mM (Fig. 3). These data are consistent with lethality being due to P4H inactivation.

3. Conclusions

The need for small-molecule probes and inhibitors of P4H is evident from the lack of structural information about this essential enzyme as well as the list of fibrotic diseases associated with collagen overproduction. The newly discovered role of P4Hs in hypoxia and cancer, and the cardioprotection conferred by P4H inhibitors increase the imperative. Our identification of 4-oxo-5,6-epoxyhexanoate as an affinity label for P4H that is bioavailable upon esterification provides a means to address this need. On-going efforts in our laboratory are directed at identifying the alkylated enzymic residue, improving the potency of the epoxy ketone as an affinity label, and evaluating its selectivity towards other P4Hs.

4. Experimental procedures

4.1. General

BakerDry™ solvents in cyclotainers™ (DMF, ≤20 ppm water; CH2Cl2, ≤30 ppm water; THF, ≤10 ppm water) were from Baker (Phillipsburg, NJ). Unless noted otherwise, all other chemicals were from Aldrich Chemical (Milwaukee, WI), Fisher Scientific (Pittsburgh, PA), or NovaBiochem (San Diego, CA), and were used without further purification.

Human collagen prolyl 4-hydroxylase (P4H) was produced in E. coli and purified as described previously. Dansyl-Gly–Pro–Gly–OEt, a fluorescent P4H substrate, was synthesized as described previously. In the synthetic procedures, the term ‘concentrated under reduced pressure’ refers to the removal of solvents and other volatile materials using a rotary evaporator at water aspirator pressure (<20 torr) while maintaining the water-bath temperature below 50 °C.

4.2. Instrumentation

NMR spectra were obtained with a Bruker AC+ 300 MHz, Bruker DMX 400 WB, or Bruker Avance III 500 spectrometer. Mass spectra were obtained with a Micromass LCT electrospray ionization, time-of-flight analyzer from Waters (Milford, MA). HPLC analyses were preformed with an instrument from Waters controlled with the Millenium (version 3.20) software package and equipped with two 515 pumps, a 717 plus autosampler, and a 996 photodiode array detector.

4.3. Synthesis of 4-oxo-5,6-epoxyhexanoate (1)

Epoxy ketone 1 was synthesized in five steps with an overall yield of 38% by the route shown in Scheme 1 and described below.

4.3.1. Benzyl succinic acid (2)

Benzyl succinic acid was synthesized by following a known procedure. Benzyl 4-chlorooxobutyrate was synthesized by following a known procedure. Benzyl 4-oxo-5-hexenoate (4) was synthesized in 82% yield in benzyl alcohol (31.6 mL, 33 g, 300 mmol), and the resulting solution was heated at reflux for 4 h. The reaction mixture was dissolved in ether (100 mL), and the insoluble succinic acid was removed by filtration. The filtrate was extracted with saturated aqueous Na2CO3 (3 × 100 mL), and the combined aqueous extracts were acidified with 2 M HCl (20 L). The precipitate was collected by filtration and dried under vacuum to give 1 (30 g, 48%).

1H NMR (CDCl3, 300 MHz) δ 7.34 (m, 5H), 5.13 (s, 2H), 3.19 (t, J = 7 Hz, 2H). 13C NMR (CDCl3, 75 MHz) δ 173.2, 170.9, 135.6, 128.4, 67.1, 29.5, 28.5.

4.3.2. Benzyl 4-chlorooxobutyrate (3)

Benzyl 4-chlorooxobutyrate was synthesized by following a known procedure. Here, benzyl succinic acid (4.14 g, 20 mmol) was dissolved in CH2Cl2 (150 mL), and the resulting solution was cooled to 0 °C. Oxalyl chloride (13.8 g, 109 mmol) was added slowly, and the reaction mixture was stirred at 0 °C for an additional 30 min. The reaction mixture was allowed to warm to 15 °C and stirred for an additional 30 min. The reaction mixture was then concentrated under reduced pressure, and the residue was dissolved in benzene. The residue was concentrated under reduced pressure to give 3 (4.5 g, 99%) as a clear viscous liquid. 1H NMR (CDCl3, 300 MHz) δ 7.34 (m, 5H), 5.13 (s, 2H), 3.19 (t, J = 7 Hz, 2H), 2.69 (t, J = 7 Hz, 2H). 13C NMR (CDCl3, 75 MHz) δ 173.2, 170.9, 135.6, 128.8, 128.6, 128.4, 67.1, 29.5, 28.5.

4.3.3. Benzyl 4-oxo-5-hexenoate (4)

Benzyl 4-oxo-5-hexenoate (4) was synthesized in 82% yield from benzyl 4-chlorooxobutyrate in the same manner as was ethyl 4-oxo-5-hexenoate (6) from ethyl 4-chlorooxobutyrate, vide infra. 1H NMR (CDCl3, 300 MHz) δ 7.35 (m, 5H), 6.38 (dd, J = 10 and 18 Hz, 1H), 6.26 (dd, J = 1 and 18 Hz, 1H), 5.86 (dd, J = 1 and 10 Hz, 1H), 5.13 (s, 2H), 2.94 (t, J = 7 Hz, 2H), 2.70 (t, J = 7 Hz, 2H).

4.3.4. Benzyl 4-oxo-5,6-epoxyhexanoate (5)

Benzyl 4-oxo-5,6-epoxyhexanoate (5) was synthesized in 98% yield from benzyl 4-oxo-5-hexenoate in the same manner as was...
ethyl 4-oxo-5,6-epoxyhexanoate (6) from ethyl 4-oxo-5-hexenoate (7), vide infra. \(^1\)H NMR (CDCl\(_3\), 300 MHz) \(\delta\) 7.34 (m, 5H), 5.10 (s, 2H), 3.46 (dd, \(J = 2\) and 5 Hz, 1H), 2.99 (dd, \(J = 5\) and 6 Hz, 1H) 2.94 (dd, \(J = 2\) and 6 Hz, 1H), 2.64 (m, 4H). \(^{13}\)C NMR (CDCl\(_3\), 75 MHz) \(\delta\) 206.1, 172.4, 135.9, 128.7, 128.5, 128.4, 66.6, 53.7, 46.3, 31.3, 27.5.

4.3.5. 4-Oxo-5,6-epoxyhexanoic acid (1, free acid)

Benzyl 4-oxo-5,6-epoxyhexanoic acid (340 mg, 1.45 mmol) was dissolved in EtOAc (20 mL). Pd/C (10% w/w, 40 mg) was added, and the reaction mixture was stirred under H\(_2\) for 4 h. The reaction mixture was filtered through Celite\(^\circ\), and concentrated under reduced pressure to give the free acid of epoxy ketone 1 (183 mg, 99%). ESI MS EMM \(m/z\): [M-H] \(143.03\), calculated 143.0344.

4.3.6. Sodium 4-oxo-5,6-epoxyhexanoate (1, sodium salt) 4-Oxo-5,6-epoxyhexanoic acid (206 mg, 1.4 mmol) was dissolved in aqueous NaHCO\(_3\) (3.6 mL, 400 mM), and the resulting solution was stirred for 1 h at 0°C, allowing to warm to room temperature, and then stirred for an additional 3 h. The reaction mixture was diluted with H\(_2\)O (100 mL) and extracted with diethyl ether (3 x 20 mL). A half-saturated solution of KF in MeOH (25 mL) was dissolved in in CH\(_2\)Cl\(_2\) to give 6 (1.04 g, 85%). ESI MS \(m/z\): [M+Na\(^+\)] 195.0, calculated 195.1. \(^1\)H NMR (CDCl\(_3\), 300 MHz) \(\delta\) 4.11 (q, \(J = 7\) Hz, 2H), 3.48 (dd, \(J = 5\) and 5 Hz, 1H), 2.10 (m, 2H), 2.59 (t, \(J = 7\) Hz, 2H), 2.89 (t, \(J = 7\) Hz, 2H), 2.95 (t, \(J = 7\) Hz, 2H), 1.21 (t, \(J = 7\), 3H). \(^{13}\)C NMR (CDCl\(_3\), 75 MHz) \(\delta\) 206.3, 172.6, 60.9, 53.7, 46.3, 31.3, 27.5.

4.3.7. Protection of P4H by \(\alpha\)-ketoglutarate

Epoxy ketone 1 (356 mM), P4H (43 \(\mu\)M), and FeSO\(_4\) (2 mM) were incubated for 5 min with \(\alpha\)-ketoglutarate (0, 15, 30, or 50 mM) in 50 mM Tris–HCl buffer, pH 7.8. BSA (1 mg/mL), catalase

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\text{Scheme 2. Route for the synthesis of 6 (esterified 1).}
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(0.1 mg/mL) ascorbate (2 mM), DTT (0.1 mM), and substrate (dansyl-Gly–Phe–Pro–Gly–OEt) (2.5 mM) were added to initiate the reaction. Aliquots were removed at 15, 60, 120, 180, 240, and 300 s, quenched by boiling for 30 s, and injected onto the C18 reversed-phase HPLC column described above. The column was eluted at 1 mL/min with aqueous acetonitrile (50% v/v), and the absorbance of the eluent was monitored at 337.5 nm. Reaction rates were compared to the reactions without inhibitor.

4.8. Immobilization of P4H

P4H (50 µg) was incubated for 30 min at 37 °C with FeSO4 (0.1 mM), ascorbate (5 mM), and epoxy ketone 1 (13 mM) in 1 mL of PBS. Biocytin hydrazide was added (to 11 mM), and the reaction mixture was incubated for an additional 30 min at 37 °C. NaBH4 (30 mg/mL in 10 mM NaOH) was added to the reaction mixture to reduce the acylhydrazone. The P4H complex was desalted with a NICK™ column from Amersham Pharmacia (Uppsala, Sweden) and incubated on Reacti-Bind™ streptavidin coated microtiter wells to specific to the a-subunit of human P4H (ICN Biomedicals, Costa Mesa, CA) was added to the wells, and incubated for 1 h at 37 °C. The wells were washed (4 ×) with blocking buffer (0.1% w/v BSA and 0.05% v/v Tween 20® in PBS). A mouse monoclonal antibody specific to the a-subunit of human P4H (ICN Biomedicals, Costa Mesa, CA) was added to the wells, and incubated for 1 h at 37 °C. The wells were washed again (4 ×) with blocking buffer. Anti-mouse IgG-peroxidase conjugate from Sigma Chemical (St. Louis, MO) was added to the wells, and the plate was incubated for 1 h at 37 °C. The wells were washed again (4 ×) with blocking buffer. O-Phenylenediamine solution (0.05 M citric acid/0.05 M sodium phosphate buffer, pH 5.0, containing 1 mg/mL O-phenylenediamine and 0.003% v/v H2O2) was added to the wells, and catalysis of O-phenylenediamine oxidation by peroxidase was monitored at 490 nm after quenching the reaction with H2SO4 (2.5 M).

4.9. In vivo assay of epoxy ketone 1

The effect of epoxy ketone 1 on dpy-18 C. elegans was assayed by modification of a known procedure. Here, 6 (esterified 1) in DMSO was added to a plate containing solid growth medium (4 mL), which consisted of agar (17 g/L), Bacto-Peptone (2.5 g/L), NaCl (50 mM), cholesterol (5 mg/L), CaCl2 (1 mM), and MgSO4 (1 mM) in 1 mM potassium phosphate buffer, pH 6.30 The inhibitor was allowed to diffuse throughout the agar for 24 h. Four L4 worms were then added to each plate. After sufficient time for the L4 worms to grow to adult stage and lay eggs (24–48 h), the adult worms were removed to simplify counting. After sufficient time for all live eggs to hatch into worms (24 h), live worms and dead embryos (typically, 40–100) were counted by using a light microscope.

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