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Direct and continuous assay for prolyl 4-hydroxylase

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ABSTRACT

Prolyl 4-hydroxylase (P4H) is a nonheme iron dioxygenase that catalyzes the posttranslational hydroxylation of (2*S*)-proline (Pro) residues in protocollagen strands. The resulting (2*S*,4*R*)-4-hydroxyproline (Hyp) residues are essential for the folding, secretion, and stability of the collagen triple helix. P4H uses α-ketoglutarate and O_2 as cosubstrates, and forms succinate and CO_2 as well as Hyp. Described herein is the first assay for P4H that continuously and directly detects turnover of the proline-containing substrate. This assay is based on (2*S*,4*S*)-4-fluoroproline (flp), a proline analogue that is transformed into (2*S*)-4-ketoproline (Kep) and inorganic fluoride by P4H. The fluoride ion, and thus turnover by P4H, is detected by a fluoride ion-selective electrode. Using this assay, steady-state kinetic parameters for the human P4H-catalyzed turnover of a flp-containing peptide were determined and found to be comparable to those obtained with a discontinuous HPLC-based assay. In addition, this assay can be used to characterize P4H variants, as demonstrated by a comparison of catalysis by D414A P4H and the wild-type enzyme. Finally, the use of the assay to identify small-molecule inhibitors of P4H was verified by an analysis of catalysis in the presence of 2,4-pyridine dicarboxylate, an analogue of α-ketoglutarate. Thus, the assay described herein could facilitate biochemical analyses of this essential enzyme.

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Collagens are the major structural proteins of the extracellular matrix. Collagens undergo a number of posttranslational modifications during biosynthesis. One major modification is the hydroxylation of certain prolyl residues. The repeating amino acid sequence of collagen, wherein every third residue is a glycine (Gly),[1] Xaa–Yaa–Gly, is rich in (2*S*)-proline (Pro). Indeed, Pro is the most common amino acid found in the Xaa position [1]. In the Yaa position, the most common amino acid is (2*S*, 4*R*)-4-hydroxyproline (Hyp). Hyp is formed by a posttranslational modification of Pro catalyzed by prolyl 4-hydroxylase (P4H; EC 1.14.11.2).

Hyp is necessary for the stable formation of the triple-helical structure of collagen. Collagen with decreased levels of Hyp is defective in folding and secretion under physiological conditions [2–4]. P4H activity is required for the viability of the nematode *Caenorhabditis elegans* [5,6] and the mouse *Mus musculus* [7]. In vitro, P4H has been studied by availing enzyme via heterologous expression in insect cells, yeast, and (only recently [8,9]) bacteria.

P4H is a member of the α-ketoglutarate-dependent, nonheme iron(II) dioxygenase family of enzymes [10–12]. These enzymes require iron(II), α-ketoglutarate, and O_2 for catalysis (Fig. 1). To accomplish difficult oxidizing reactions, these enzymes employ a highly reactive iron(IV)-oxo species [13,14]. In P4H, this species abstracts the 4-*proR* hydrogen atom from a Pro substrate [15], and then transfers a hydroxyl radical to form the Hyp product. The key iron(IV)-oxo species is formed by the oxidative decarboxylation of α-ketoglutarate, which also results in the formation of succinate and CO_2 [16]. During the reaction, one atom of molecular oxygen is incorporated into Hyp, and the other into succinate [17]. The turnover of α-ketoglutarate can occur without the formation of Hyp. This uncoupling of α-ketoglutarate decarboxylation from substrate hydroxylation leads to inactivated P4H. Ascorbate can reactivate the enzyme [18–20].

Many nonheme iron dioxygenases catalyze subtle changes to large substrates, making their enzymatic activity difficult to assay. P4H is not an exception. Historically, its activity has been monitored by assays that employ radioactivity. Hydroxylation of the collagen substrate can be monitored directly by the detection of [^{14}C]Hyp formed in collagen containing [^{14}C]Pro after acid hydrolysis of the product [21]. A more rapid assay involves radiolabeling collagen with [3,4-^{3}H]proline and detecting [^{3}H]H_2O after hydroxylation [22]. These assays are, however, not only discontinuous, but also require the time-consuming production of radiolabeled collagen.

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[1] *Abbreviations used:* Flp, (2*S*,4*R*)-4-fluoroproline; flp, (2*S*,4*S*)-4-fluoroproline; Gly, glycine; HPLC, high-performance liquid chromatography; HIF, hypoxia inducible factor; Hyp, (2*S*,4*R*)-4-hydroxyproline; Kep, (2*S*)-4-ketoproline; PEG, poly(ethylene glycol); P4H, prolyl 4-hydroxylase; PHDs, proline hydroxylase domain proteins; Pro, (2*S*)-proline.

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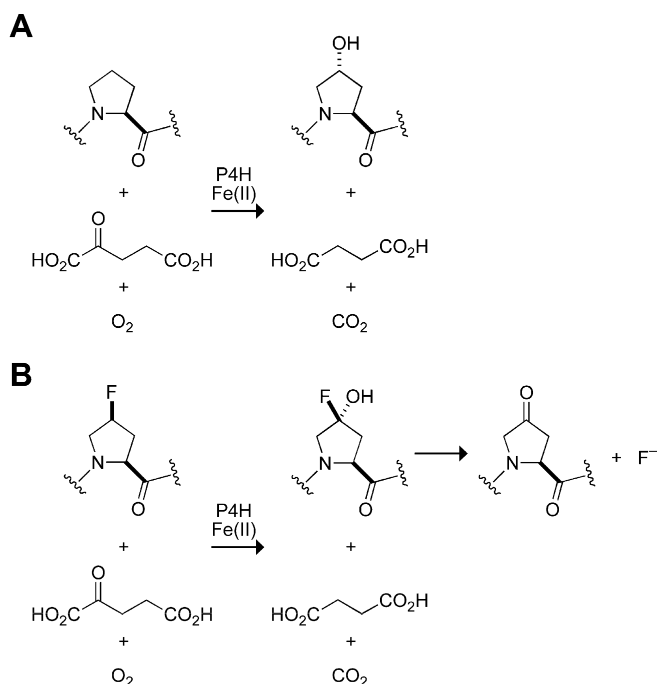

Fig. 1. Reaction catalyzed by prolyl 4-hydroxylase when (A) a Pro-containing peptide or (B) a flp-containing peptide is the substrate.

P4H activity has been measured indirectly by monitoring the turnover of α-ketoglutarate. Assays have been developed to detect residual α-ketoglutarate substrate or the incipient succinate or CO_2 product. Perhaps the most often used P4H assay quantifies the $[^{14}C]CO_2$ product of [α-1-^{14}C]ketoglutarate decarboxylation [16,23]. Trapping of the $[^{14}C]CO_2$ can, however, be inefficient. The turnover of [α-5-^{14}C]ketoglutarate produces [1-^{14}C]succinate, which must be separated from unreacted α-ketoglutarate prior to analysis. Methods of separation by column chromatography [24] and chemical precipitation of α-ketoglutarate [25] have been reported previously.

Assays for P4H activity have also been developed that do not require radioactivity. The consumption of the oxygen cosubstrate has been monitored using an O_2-sensing electrode [26]. An assay developed more recently quantifies unreacted α-ketoglutarate by a postreaction derivitization that forms a fluorescent product [27]. Another assay couples the formation of succinate to that of NAD^+ formation via succinyl-coenzyme A synthetase, pyruvate kinase, and lactate dehydrogenase [28]. Although these assays are of general use for all α-ketoglutarate-dependent dioxygenases, they have the marked disadvantage of being indirect—they do not report on the hydroxylation of a substrate. Direct, but discontinuous, assays that monitor proline hydroxylation without using radioactivity have been developed with peptide substrates. In these assays, the product Hyp-containing peptide is separated from the substrate by thin-layer chromatography [29] or by HPLC [8,30].

Herein, we present the first assay for P4H that is both direct *and* continuous. The assay utilizes an alternative substrate, (2*S*,4*S*)-4-fluoroproline (flp), that on turnover by P4H forms (2*S*)-4-ketoproline (Kep) and releases inorganic fluoride [30]. The rate of substrate turnover is monitored continuously by using a fluoride ion-selective electrode, which has been employed previously in assays for alkaline phosphatase and chondroitin AC lyase [31,32]. Our assay for P4H yields kinetic parameters comparable to those from a discontinuous HPLC-based assay using the same peptide substrate. The assay is also able to reveal the effect of altering an active-site residue and that of a small-molecule inhibitor. Accordingly, the assay is likely to have a substantial impact on biochemical analyses of this essential enzyme.

Materials and methods

Materials

Boc-FlpOH and Boc-flpOH were from OmegaChem (Lévis, Québec). All other reagents were of reagent grade or better and were used without further purification.

Production and purification of P4H

P4H was produced and purified by using procedures reported previously [8]. cDNA encoding the D414A variant of P4H was created by oligonucleotide-mediated site-directed mutagenesis using the pBK1.PDI1.P4H7 plasmid described previously. P4H D414A was produced and purified by the method used for wild-type P4H.

Synthesis of PEGylated peptides

PEG-Gly–Tyr–Yaa–GlyOEt peptides, with Yaa = Flp or Pro, were synthesized as described previously [30]. The peptide with Yaa = flp was also synthesized as described, except for the use of Wang resin (Novabiochem, Gibbstown, NJ).

Fluoride ion-detection assay of enzymatic activity

Assays were performed at room temperature, which was (23 ± 2) °C, in glass vials with stirring. Assay solutions were 0.30 mL of 50 mM Tris–HCl buffer, pH 7.8, containing bovine serum albumin (1.0 mg/mL), catalase (0.10 mg/mL), dithiothreitol (0.10 mM), ascorbate (2.0 mM), $FeSO_4$ (0.050 mM), P4H (90 nM), α-ketoglutarate (0.50 mM), and sodium fluoride (0.040 mM). The reaction mixture was allowed to equilibrate, and the tetrapeptide substrate was added from a stock solution in ethanol to initiate the reaction. The change in the concentration of fluoride ion was monitored with an Orion fluoride ion-selective electrode (Thermo Scientific, Waltham, MA) interfaced with a computer via an electrode amplifier (Vernier, Beaverton, OR). The data were fitted by linear-regression analysis to obtain initial rates. Fluoride ion concentrations were calculated by comparison to a standard curve with sodium fluoride in 50 mM Tris–HCl buffer, pH 7.8, which was found to generate the same signal as sodium fluoride in the assay solution described above.

HPLC-based assay of enzymatic activity

An HPLC-based assay described previously [8] was used to confirm product formation by P4H. Assays were performed for 5 min at room temperature. Assay solutions were 100 μL of 50 mM Tris–HCl buffer, pH 7.8, containing bovine serum albumin (1.0 mg/mL), catalase (0.10 mg/mL), dithiothreitol (0.10 mM), ascorbate (2.0 mM), $FeSO_4$ (0.050 mM), P4H (90 nM), and α-ketoglutarate (0.50 μM).

Results

The P4H-catalyzed turnover of a flp-containing peptide produces a Kep-containing peptide and a fluoride ion (Fig. 1). Hydroxylation of flp by P4H produces an α-fluorohydrin, which is known to fragment in an exothermic reaction [33]. The fluoride ion released, and thus P4H activity, is monitored by utilization of a fluoride ion-selective electrode. Sodium fluoride is added to the reaction mixture to put the initial fluoride concentration within

the linear range of the fluoride ion-selective electrode. As measured by the HPLC-based assay, the enzymatic activity of P4H was found to be unaffected by the presence of salts, including 10 mM sodium fluoride (data not shown).

To assess the substrate specificity of the assay, we compared peptide substrates containing Pro, flp, or (2*S*,4*R*)-4-fluoroproline (Flp). Pro, the natural substrate is turned over, but does not produce fluoride and therefore is not detected by the fluoride ion-selective electrode (data not shown). Flp is a fluoride-containing proline analog that is not a substrate of P4H [30]. Assays in which 0.50 mM PEG-Gly–Tyr–Flp–GlyOEt is added produce no change in signal, as in assays that lack peptide (Fig. 2). In contrast, addition of 0.50 mM of the analogous flp-containing peptide to the assay causes a large increase in signal.

To confirm that the defluorination of flp is due to P4H, the reaction rates were determined at varying P4H concentrations. Duplicate reactions were performed under standard reaction conditions containing 1.0 mM PEG-Gly–Tyr–flp–GlyOEt. With no P4H added to the reaction, there was no increase in fluoride ion concentration. In reactions including 10, 20, 45, or 90 nM P4H, the initial velocity of the reactions correlated linearly with the P4H concentration (Fig. 3).

The fluoride ion-selective electrode assay was compared to the previously developed HPLC-based assay by determining the steady-state kinetic parameters for the P4H-catalyzed turnover of flp. The rate of fluoride ion formation was determined at varying concentrations of the flp-containing peptide (Fig. 4, Table 1). Assays were performed in triplicate under standard conditions, and data were fitted to the Michaelis–Menten equation to obtain kinetic parameters. The k_{cat}/K_M value was determined to be $(9.8 \pm 2.8) \times 10^3\ M^{-1}\ s^{-1}$ (Table 1, Fig. 4). This value is similar to that from the previously developed HPLC-based assay.

A variant of P4H and an inhibitor of P4H were used to probe the utility of this assay. Under standard assay conditions, the D414A variant of P4H showed no detectable catalytic activity at a concentration of 0.90 μM, which is 10-fold greater than normal (Fig. 5A). The effect of the competitive inhibitor 2,4-pyridine dicarboxylate on the rate of reaction was investigated as well. In assays with 0.20 mM flp-containing peptide under standard conditions, 10, 33, and 100 μM concentrations of the inhibitor were shown to inhibit P4H increasingly (Fig. 5B). Considering that the reported K_M value of α-ketoglutarate is 22 μM [34] and its concentration in the assays herein is 0.50 mM, the inhibition determined by the decrease in the rate of fluoride ion production by P4H in the presence of the inhibitor is consistent with the reported K_i value of 2 μM [35,36].

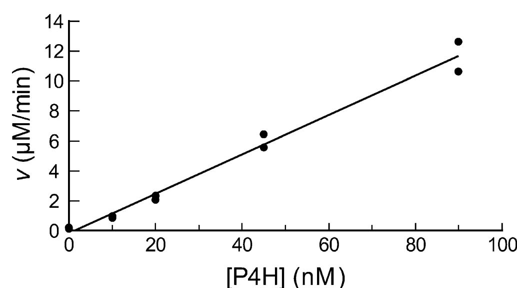

Fig. 3. Dependence of the rate of fluoride-ion release from PEG-Gly–Tyr–flp–GlyOEt (1.0 mM) on the concentration of P4H. Assay conditions are as described under Materials and methods. Reactions were performed in duplicate. Data were fitted by linear-regression analysis. $v = \partial[F^-]/\partial t$.

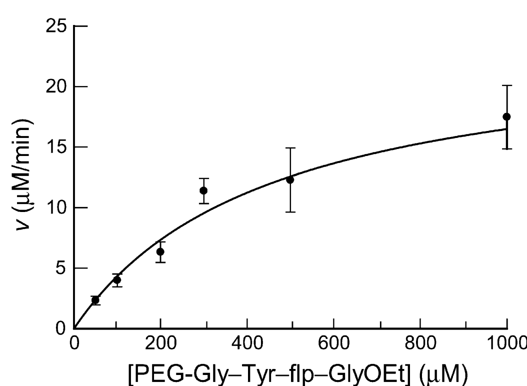

Fig. 4. Catalysis of fluoride ion-release from PEG-Gly–Tyr–flp–GlyOEt by P4H (90 nM). Assay conditions are as described under Materials and methods. Individual points are the average (±SE) of three reactions. Data were fitted to the Michaelis–Menten equation: $v = \partial[F^-]/\partial t = k_{cat}[P4H][peptide]/(K_M + [peptide])$.

Table 1
Comparison of the turnover of PEG-Gly–Tyr–flp–GlyOEt by P4H (90 nM) determined by using the fluoride ion-detection assay or the HPLC-based assay.[a]

Assay	V_{max} (μM min^{-1})	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M (10^3 M^{-1} s^{-1})
Fluoride	24 ± 3	270 ± 33	0.5 ± 0.1	9.8 ± 2.8
HPLC	32 ± 5	360 ± 53	1.2 ± 0.3	5.1 ± 1.4

[a] Reaction components and conditions were as described under Materials and methods. Values represent the mean (±SE) of three replicates.

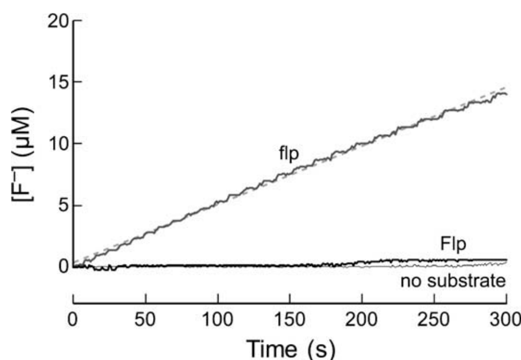

Fig. 2. Fluoride ion-detection assay for P4H (90 nM). Assays were performed in the presence or absence of PEG-Gly–Tyr–Yaa–GlyOEt, where Yaa = flp (0.50 mM) or Flp (0.50 mM). The dashed line shows the fit of the Yaa = flp data by linear-regression analysis. Assay conditions are as described under Materials and methods.

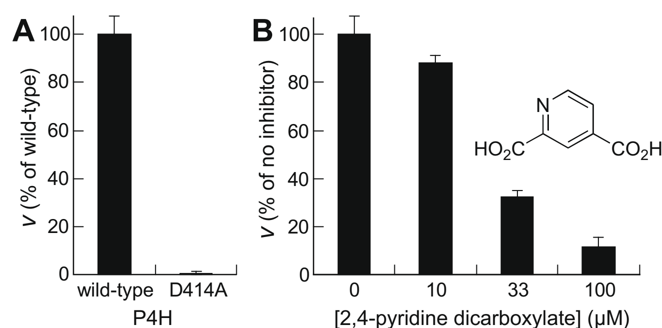

Fig. 5. Catalysis of fluoride ion-release from PEG-Gly–Tyr–flp–GlyOEt (0.20 mM) by a P4H variant or in the presence of a small-molecule inhibitor. (A) Catalysis by wild-type P4H (90 nM) and its D414A variant (0.90 μM). (B) Inhibition of catalysis by 2,4-pyridine dicarboxylate. Assay conditions are as described under Materials and methods. $v = \partial[F^-]/\partial t$.

Discussion

The assay for P4H activity described herein monitors directly and continuously the turnover of a peptide substrate (Figs. 2–4; Table 1). The steady-state kinetic parameters of a peptide substrate containing flp are comparable to those for the analogous peptide containing Pro, the natural substrate, making flp a suitable substrate [30]. The peptide substrate is readily accessible, as flp is available from commercial vendors or accessible by a facile synthetic route [37]. The assay has the advantages of being direct and continuous, in addition to using inexpensive instrumentation, avoiding the use of radioactivity, and not requiring additional enzymes.

Assays of enzymatic activity have identified a number of residues critical for P4H activity. Like most other nonheme iron(II) dioxygenases, the active site of P4H has a two histidine/one carboxylate motif that binds iron. Asp414 has been identified as the source of the carboxylate [38,39]. Recently, a subclass of nonheme iron(II) dioxygenases that perform halogenation reactions, instead of hydroxylations, has been identified [40]. These halogenases lack an active-site carboxylate, containing an alanine residue instead of the canonical aspartate or glutamate. The D414A P4H variant studied herein emulates the active site of a halogenase, though no halogenated product has been detected in a solution of high halide ion concentration (unpublished data). The D414A variant also lacks hydroxylase activity (Fig. 5A), confirming the requirement of the aspartate for hydroxylase activity and validating the competence of the assay for reporting on P4H variants.

P4H plays a major role in the biosynthesis of collagen. Excessive collagen formation causes a number of fibrotic diseases, and P4H has been put forth as a target for beneficial intervention with chemotherapeutic agents [41–43]. Analogs of α-ketoglutarate are known to inhibit P4H competitively with respect to α-ketoglutarate [6,35,36]. The assay is able to reveal inhibition by one such analogue, 2,4-pyridine dicarboxylate (Fig. 5B). We anticipate that the assay could be adapted to a high-throughput format with the use of appropriate fluorescent or colorimetric fluoride-sensing reagents. Such reagents are under development [44].

In addition to stabilizing collagen, the hydroxylation of proline residues also plays a critical role in the sensing of molecular oxygen [43,45]. For example, the formation of Hyp in the transcription factor hypoxia inducible factor (HIF) is catalyzed by the proline hydroxylase domain proteins (PHDs), which are distinct from the collagen P4H. Incorporating flp into a HIF-derived peptide substrate for PHD enzymes could enable the assay described herein to be used in the study of those enzymes as well.

Conclusions

In summary, we have developed a new assay for the activity of P4H that utilizes flp as the substrate. On turnover, the release of fluoride ions is monitored by a fluoride ion-selective electrode. This assay for P4H is the first that is both direct and continuous. We have demonstrated the utility of the assay in characterizing P4H variants and identifying inhibitors. We anticipate the modification of this assay into a high-throughput format suitable for the discovery of novel inhibitors of this important enzyme.

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