Purification and initial characterization of proline 4-hydroxylase from *Streptomyces griseoviridus* P8648: a 2-oxoacid, ferrous-dependent dioxygenase involved in etamycin biosynthesis

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Proline 4-hydroxylase is a 2-oxoacid, ferrous-ion-dependent dioxygenase involved in the biosynthesis of the secondary metabolite etamycin. The purification, in low yield, of proline 4-hydroxylase from *Streptomyces griseoviridus* P8648 to near apparent homogeneity and its initial characterization are reported. In most respects proline 4-hydroxylase is a typical member of the 2-oxoacid-dependent dioxygenase family. It is monomeric (M_r approx. 38000) (by gel filtration on Superdex-G75) and has typically strict requirements for ferrous ion and 2-oxoglutarate. The enzyme was inhibited by aromatic analogues of 2-oxoglutarate. L-Proline-uncoupled turnover of 2-oxoglutarate to succinate and CO_2 was observed. The addition of L-ascorbate did not stimulate L-proline-coupled turnover of 2-oxoglutarate, but did stimulate L-proline-uncoupled turnover. L-Ascorbate caused a time-dependent inhibition of L-proline hydroxylation. The enzyme was completely inactivated by preincubation with diethyl pyrocarbonate under histidine-modifying conditions. This inactivation could be partially prevented by the inclusion of L-proline and 2-oxoglutarate in the preincubation mixture, suggesting the presence of histidine residue(s) at the active site.

INTRODUCTION

Three of the four diastereoisomers of the non-proteinogenic amino acid 4-hydroxyproline occur naturally [1]. Of these *trans*-4-hydroxy-L-proline is most abundant, being found in secondary metabolites such as the echinocandins [2] and actinomycins [3], as well as being an important constituent of collagen. L-Proline has been identified as the precursor of the hydroxylated residues in each of these cases [4], and the protocollagen L-prolyl-hydroxylating system has been extensively investigated (for a review see [5]).

Mammalian prolyl 4-hydroxylase (P4H; EC 1.14.11.2) catalyses the post-translational hydroxylation of specific L-prolyl residues in the polypeptide precursor of collagen to *trans*-4-hydroxy-L-prolyl residues [5]. Free L-proline is not accepted as a hydroxylation substrate. P4H is a member of the 2-oxoacid and ferrous-ion-dependent dioxygenase family (for reviews see [6,7]) and is an $\alpha_2\beta_2$ tetramer in which the dioxygenase activity is located in the α-subunit (M_r 64000) [5]. The β-subunit has been shown to be identical with protein disulphide-isomerase, and its catalytic role, if any, remains unknown. Mechanistic and inhibition studies have been carried out on P4H because of its potential as a therapeutic target [8]. Interpretations of these studies have been hampered by the polymeric nature of the substrate and the tetrameric structure of the enzyme.

In contrast, hydroxylation of free L-proline to free *trans*-4-hydroxy-L-proline has been demonstrated in the biosynthesis of actinomycin I by *Streptomyces antibioticus* 3720 [9], and of etamycin by *Streptomyces griseoviridus* P8648 [10]. In the latter case, a crude cell-free extract containing L-proline-hydroxylating activity was obtained, and a requirement for 2-oxoglutarate and ferrous ion was demonstrated (Figure 1).

Recently we reported on the stereospecificity of the P4H reaction, which occurred with retention of stereochemistry at C-4 of L-proline, in accordance with observations for other enzymes in the family catalysing hydroxylation reactions [11]. In the present paper we report the purification of bacterial P4H to near-

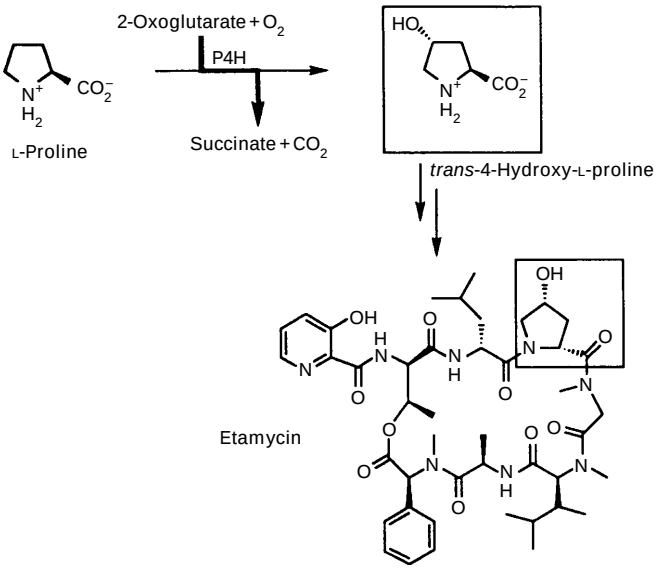

Figure 1 The P4H reaction

Abbreviations used: P4H, prolyl 4-hydroxylase; DTT, dithiothreitol; DEPC, diethyl pyrocarbonate.

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homogeneity and its initial biochemical characterization, enabling comparison to be made with other dioxygenases in this family, and in particular with mammalian P4H.

MATERIALS AND METHODS

Materials

Streptomyces griseoviridus P8648 was obtained from the American Type Culture Collection (A.T.C.C. 23920). Radiolabelled compounds were obtained from Amersham. Reagents for SDS/PAGE, molecular-mass markers and hydroxyapatite (Bio-Gel HT) were obtained from Bio-Rad Laboratories. Other chromatographic resins were obtained from Pharmacia LKB. *N*-Oxalylglycine was a gift from Zeneca Pharmaceuticals. Other chemicals were obtained from Sigma or Aldrich.

Methods

All protein manipulations, except SDS/PAGE, were carried out at 4 °C. FPLC was performed with a Pharmacia LCC-500 system controller and either P-500 or P-6000 dual pumps. The A_{280} of the eluate was monitored, and fractions were stored immediately at −80 °C until required. SDS/PAGE gels (12.5 %) were performed on a Bio-Rad Mini-Protean II system essentially as described by Laemmli [12].

Cultivation of *Streptomyces griseoviridus* P8648

Streptomyces griseoviridus P8648 was sporulated on slopes of Difco Bacto yeast malt extract [3.8 % (w/v)], 1 % malt extract, 0.4 % D-glucose and 1 % agar, pH 6.8. Seed flasks (500 ml) containing 100 ml of medium [0.5 % (w/v) maltose, 0.5 % (w/v) yeast extract, 0.5 % (w/v) malt extract; pH 7.5] were inoculated and incubated at 28 °C and 250 rev./min on a gyratory shaker with an eccentric throw of 2.5 cm for 48 h. Erlenmeyer flasks (500 ml) containing 100 ml of growth medium containing 0.39 % (w/v) yeast extract, 0.5 % (w/v) peptone, 0.3 % (w/v) malt extract, 34 % (w/v) sucrose, with 1 % (w/v) glucose, and 0.5 % (w/v) $MgCl_2$, added after separate sterilization, were then inoculated (5 % potency) and incubated similarly. Mycelial development was monitored by measuring the D_{550}. Cells were harvested by centrifugation (14000 ***g***, 20 min), washed with 0.9 % (w/v) NaCl, collected on a Büchner funnel and stored at −80 °C until required. No significant loss of P4H activity was observed after 6 months' storage at −80 °C.

Purification of P4H

The following buffers are referred to: A, 50 mM Hepes containing 10 % (w/v) glycerol, 8 mM L-dithiothreitol (DTT), 2 mM benzamidine, 2 mM PMSF, and 1 mg/ml each of aprotinin, leupeptin, pepstatin A, trypsin inhibitor from soya bean and DNase I at pH 7.5; B, 25 mM Hepes containing 10 % (w/v) glycerol and 4 mM DTT at pH 7.5; C, as for buffer B, except for the addition of 1 M NaCl; D, as buffer B, except for the addition of 1 M $(NH_4)_2SO_4$; E, as for buffer B, except for the addition of 150 mM sodium phosphate; F, 200 mM Hepes containing 10 % (w/v) glycerol and 4 mM DTT at pH 7.5. PMSF was added by first dissolving it in a small volume of *N*,*N*-dimethylformamide (< 0.5 % final volume), followed by dropwise addition, with stirring, to the buffer. Buffers B, C, D, E and F were filtered through a 0.2 μm filter, degassed under vacuum (10 min), sparged with nitrogen (100 ml/min; 10 min) and equilibrated at 4 °C before use. Sonication was performed in 30 s bursts using a Heat Systems-Ultrasonics W-380 sonicator fitted with a 1.2 cm horn at power setting 5 at 0–4 °C. Successive bursts were interrupted with at least 30 s of manual stirring to dissipate heat. Total sonic disintegration time was 150 s.

Cells (90 g) were resuspended in buffer A (240 ml) and subjected to 5 × 30 s bursts of sonication. After collection of cell debris by centrifugation (29000 ***g***, 10 min), the protein fraction was made to a final concentration of 0.1 % (w/v) polyethyleneimine by using 5 % (w/v) stock solution and stirred at 4 °C for 10 min. The protein solution was centrifuged (29000 ***g***, 30 min) and the supernatant loaded on to a DEAE-Sepharose Fast Flow (50 mm × 11 cm) column pre-equilibrated in buffer B. After elution of unbound protein with buffer B at 25 ml/min, a 0–40 % linear gradient of buffer C was applied over 1300 ml, followed by a 40–100 % step to buffer C. Fractions of volume 20 ml were collected. P4H activity was eluted between 140 and 220 mM NaCl.

Active fractions from DEAE-Sepharose chromatography were pooled, and a saturated solution of $(NH_4)_2SO_4$ (pH 7.5) was added to a final concentration of 70 % satn., together with DTT to 4 mM. The solution was stirred at 4 °C for 10 min, and then centrifuged at 29000 ***g*** for 20 min. The pellet was resuspended in buffer D, filtered through a 0.22 μm-pore membrane, and loaded on to a phenyl-Superose column (10 mm × 10 cm) pre-equilibrated in buffer D. Unbound protein was eluted with the same buffer at 1.0 ml/min, and a 0–100 % gradient into buffer B was then applied over 65 ml. Fractions of volume 3 ml were collected. P4H activity was eluted between 110 and 350 mM $(NH_4)_2SO_4$.

P4H-containing fractions from phenyl-Superose chromatography were pooled, and adjusted to 70 %-satd. $(NH_4)_2SO_4$ by addition of a saturated solution (pH 7.5) together with DTT to 4 mM. The solution was stirred at 4 °C for 10 min and then centrifuged (49000 ***g***, 30 min). The pellet was resuspended in a minimum volume of buffer F, filtered through a 0.2 mm membrane and loaded on to a Superdex G75 HR column (10 mm × 30 cm) and eluted with buffer F at 0.5 ml/min : 0.5 ml fractions were collected. P4H activity was eluted between 9 and 11 ml.

Assays

Protein concentration was determined by the Bradford method [13] with BSA as a standard. Two procedures were routinely used for determination of P4H activities. L-Proline hydroxylation was monitored essentially by the procedure of Onishi et al. [10], except that a higher specific radioactivity of L-[U-^{14}C]proline was used in the assay (206 mCi/mmol; final concn. 13 μM). A concentration of L-proline lower than K_m was used in order to maximize the turnover of proline and hence minimize the quantity of L-[U-^{14}C]proline used. After incubation, L-proline and *trans*-4-hydroxy-L-proline (100 mM solutions) were added to give final concentrations of 17 mM before ion-exchange paper chromatography and analysis as previously described [10]. Incubation mixtures typically contained, in 100 μl: 25 mM Tes or 50 mM Hepes (pH 7.5), 0.5 mM 2-oxoglutarate, 0.5 mM iron(II) ammonium sulphate, 0.33 μCi of L-[U-^{14}C]proline, 0.2 mg/ml bovine liver catalase and 20 μl of assay protein solution. Samples were incubated at 26 °C for 10 min. Under these conditions, the rate of L-proline hydroxylation remained linear with time for at least 10 min. Turnovers and P4H activities were calculated by subtracting background levels of isolated labelled *trans*-4-hydroxy-L-proline found in control samples (containing heat-inactivated enzyme) from the corresponding *trans*-4-hydroxy-L-proline : L-proline ratios found in P4H-containing samples. Specific activities are reported in units of pmol of hydroxyproline formed/min per mg of protein. Errors of within ±5 % were

observed. Apparent K_m values for L-proline and 2-oxoglutarate were determined by use of this assay by incubation for 7 min at 26 °C. For L-proline, standard assay conditions were used, with the concentration of L-proline varied between 20 and 500 μM. In each assay 0.33 μCi of L-[U-^{14}C]proline was diluted with unlabelled L-proline to give the desired concentration (P4H specific activity = 217 pmol/min per mg). For 2-oxoglutarate, the concentration of L-proline was 1 mM (containing 0.33 μCi of L-[U-^{14}C]proline) and the concentration of 2-oxoglutarate varied between 12.5 and 100 μM (P4H specific activity = 180 pmol/min per mg). Assays were carried out at least in triplicate.

Assays for the catalytic decarboxylation of 2-oxoglutarate to succinate were performed essentially as described by Kaule and Günzler [14]. Incubation mixtures typically contained, in 50 μl: 25 mM TES (pH 7.5), 0.1 mM 2-oxoglutarate (sodium salt) containing 0.08 μCi of either 2-oxo[5-^{14}C]glutarate or 2-oxo[U-^{14}C]glutarate (sodium salts) (specific radioactivity approx. 260 mCi/mmol in both cases), 0.2 mg/ml bovine liver catalase, 0.5 mM iron(II) ammonium sulphate, 1 mM L-proline and 10 μl of a protein sample. The mixtures were incubated at 26 °C for 30 min and then processed as described previously [14].

RESULTS AND DISCUSSION

Cultivation of *Streptomyces griseoviridus* P8648

We investigated several media for the cultivation of *S. griseoviridus* P8648 and found that a sucrose–malt-extract–yeast-extract complex medium gave more reproducible cell growth, larger amounts of extractable P4H activity and higher specific activities than the defined medium reported by Onishi et al. [10] for fermentations used for the preparation of crude cell-free extracts. Maximal L-proline hydroxylation activity was obtained from cells grown for approx. 33 h, corresponding to the mid–late exponential phase of growth (Figure 2).

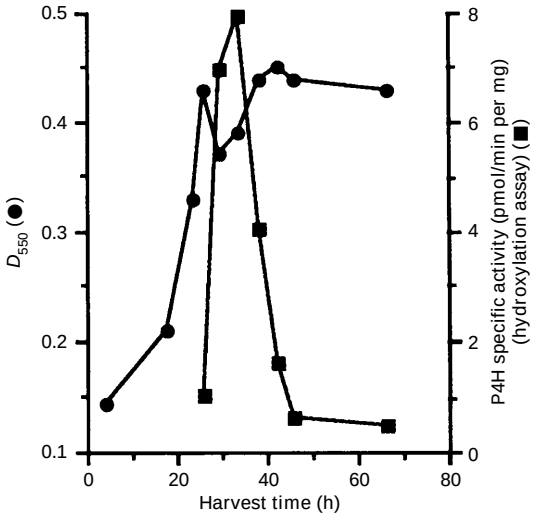

Figure 2 Harvest time of *Streptomyces griseoviridus* P8648 cells for P4H activity

Cells were grown and harvested at the indicated times as described in the Materials and methods section. Cells were resuspended in buffer A and subjected to five 30 s bursts of sonication. Cell debris was collected by centrifugation (49000 ***g***, 20 min) and the supernatant assayed for protein and L-proline hydroxylation activity.

Purification of P4H

Initially we repeated the procedure described by Onishi et al. [10] for the preparation of crude cell-free extracts containing P4H activity [sonication, followed by streptomycin sulphate precipitation and $(NH_4)_2SO_4$ fractionation]. By this protocol a rapid loss of P4H activity was observed, which precluded further purification. The addition of protease inhibitors (PMSF and benzamidine) was found to increase the stability of P4H activity slightly in crude cell-free extracts, whereas the inclusion of glycerol had a more beneficial effect, increasing the rate of L-proline hydroxylation (under standard assay conditions for the turnover of L-proline) by approx. 230 % and the half-life of the P4H activity by approx. 160 %. In an attempt to stabilize the hydroxylation activity further, crude cell lysates obtained after sonication were directly loaded on to an anion-exchange (DEAE-Sepharose FF) resin. In addition to affording a useful purification, this procedure removed both a presumed protease activity, which had previously caused loss of P4H activity upon incubation of the enzyme alone at 4 °C, and other 2-oxoglutarate-utilizing enzyme(s), which, in crude protein samples, rapidly consumed the 2-oxoacid present, invalidating the decarboxylation assay. This procedure also removed ninhydrin-sensitive compounds which caused interference in the P4H hydroxylation assay in crude extracts.

Further purification was achieved by hydrophobic-interaction chromatography on either isobutyl-Sepharose or phenyl-Superose, followed by gel filtration or hydroxyapatite chromatography. The latter preferred three-step procedure (Table 1) gave an approx. 73-fold purification of P4H in low yield, as expected for this class of enzymes from a native bacterial source. No loss of P4H activity was observed after the enzyme had stood for 4–5 h at 4 °C, but significant losses (approx. 30 %) of activity upon freezing and thawing of column fractions was observed; thus the degree of purification based upon specific activity is probably underestimated. SDS/PAGE analysis after gel filtration on Superdex indicated that in separate purifications the most active P4H-containing fraction was in each case associated with a band at M_r approx. 35000 (Figure 3).

Initial characterization of P4H and the influence of ascorbate on the P4H reaction

Gel filtration on Superdex-75 (Figure 4) led to P4H activity being eluted at a volume corresponding to M_r 37700 with reference to protein M_r standards. In this respect, P4H is typical of the majority of enzymes in this family in being a monomer of M_r 30000–40000 [6], although there are exceptions, including mammalian prolyl 4-hydroxylase ($\alpha_2\beta_2$; M_r 240000) [5] and γ-butyrobetaine hydroxylase (α_2; 92000–96000) [15].

A preliminary investigation into the dependence of P4H activity on each of the assay components was carried out. 2-Oxoglutarate was found to be essential for proline hydroxylation, and a modest level of activity (< 10 % of the turnover of L-proline observed in the presence of ferrous iron) observed in the absence of exogenous ferrous iron was probably due to residual enzyme-bound iron remaining from the purification procedure. Similar observations have been reported for other enzymes in this family (see, e.g., [16,17]). In the presence of L-proline no uncoupled turnover of 2-oxoglutarate could be detected (within experimental error), as shown by experiments which revealed the stoichiometry of 2-oxoglutarate:L-proline turnover to be 1:1. Using crude cell-free extracts Onishi et al. [10] found no stimulation of the turnover of L-proline by catalase, but a pronounced activation of P4H by L-ascorbate. In contrast, we

Table 1 Summary of purification of P4H from *Streptomyces griseoviridus* P8648

Step	Total protein (mg)	Specific activity (pmol/min per mg)	Total activity (pmol/min)	Purification (fold)
Sonicated cells	1363	12.2	16629	
DEAE-Sepharose	53*	333	17469	27
Phenyl-Superose	5	452	2260	37
Superdex G75	0.8	907	400	73

* Only approx. 30% of the active protein was used in the phenyl-Sepharose step. Subsequent values have been adjusted to take this into account.

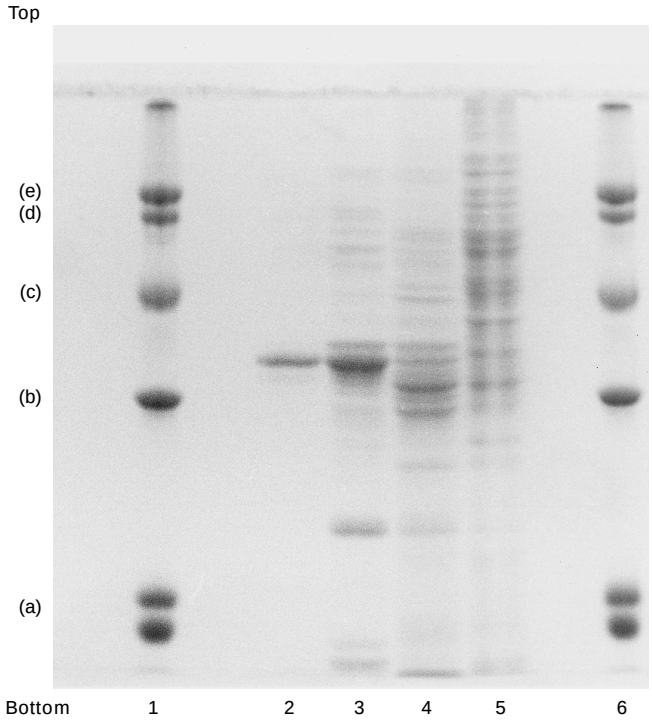

Figure 3 SDS/PAGE analysis of P4H-containing samples

Protein in column fractions was precipitated with trichloroacetic acid to give approx. 10 μg of protein for SDS/PAGE (12.5% acrylamide). Lane 1, M_r markers: (a) 12300, 17200; (b) 30000; (c) 42700; (d) 66300; (e) 76000–78000. Lane 2, pooled Superdex G75 HR fractions. Lane 3, pooled phenyl-Sepharose fractions. Lane 4, pooled DEAE-Sepharose fast-flow fractions. Lane 5, crude cell lysate. Lane 6, M_r markers, as lane 1.

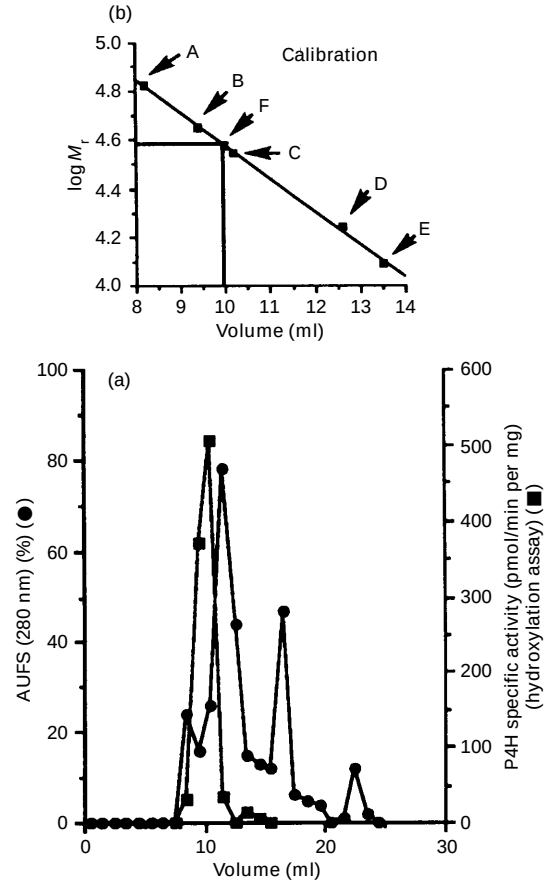

Figure 4 M_r of P4H by Superdex G75 gel filtration

(**a**) A P4H-containing DEAE-Sepharose fraction (3.5 mg) was concentrated by ultrafiltration to 0.5 ml and filtered through a 0.2 μm membrane before loading on to a Superdex-75 column (10 mm × 16 cm) pre-equilibrated in buffer B containing 100 mM NaCl. Protein was eluted with the same buffer at 0.5 ml/min, and 1 ml fractions were collected. Column fractions were assayed for L-proline hydroxylation activity. The activity profile paralleled the specific-activity profile. Determinations were carried out in triplicate. (**b**) shows the calibration of the same column with 100 μl of a protein mixture containing (A) 8 mg/ml BSA, (B) 2.5 mg/ml chicken egg albumin, (C) 2.5 mg/ml β-lactoglobulin, (D) 1 mg/ml myoglobin and (E) 1 mg/ml cytochrome *c*. The same eluting buffer as above was used in this calibration. Point (F) corresponds to elution of P4H. Abbreviation: AUFS, absorbance units full scale (100% = 0.2 A_{280} unit).

found that catalase (0.2–1 mg/ml) caused a significant increase (20–30%) in the turnover of L-proline, whereas ascorbate (1 mM) had a deleterious effect. The stimulation of P4H by catalase is a commonly observed phenomenon for 2-oxoacid-dependent dioxygenase-mediated reactions carried out *in vitro*, and is proposed [18] to occur via scavenging of detrimental peroxide. The low level of decarboxylation of 2-oxoglutarate observed in the absence of substrate reflects uncoupled turnover of P4H, and has been reported for other 2-oxoacid-dependent dioxygenases [5–7]. In the absence of both L-proline and L-ascorbate the rate of uncoupled decarboxylation of 2-oxoglutarate was decreased to about 1% of that in the presence of both.

For many of the 2-oxoacid-dependent dioxygenase family, L-ascorbate is required to maintain a maximal rate of catalysis and, in the case of mammalian prolyl 4-hydroxylase, it has also been shown that L-ascorbate is stoichiometrically oxidized to L-dehydroascorbate [19–21] during uncoupled reaction cycles and has therefore been proposed to play a role in the completion of such cycles by reduction of an enzyme-bound intermediate. Use of the alternative 2-oxoglutarate-decarboxylation assay revealed a similar degree of inhibition (38%) by L-ascorbate in the

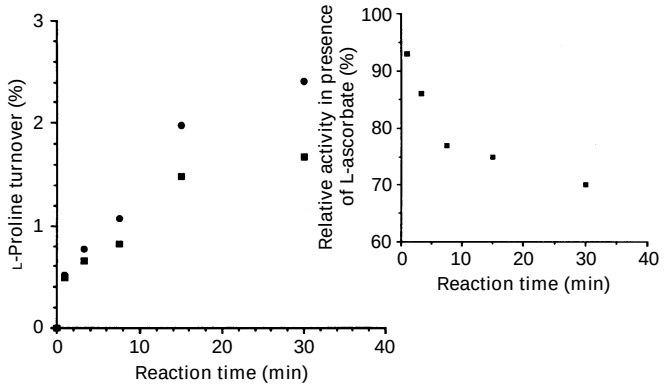

Figure 5 Inhibition of P4H by L-ascorbate

(**a**) P4H activities were determined by the hydroxylation assay with 0.33 μCi of L-[U-^{14}C]proline diluted to 1 mM with unlabelled L-proline under otherwise standard assay conditions as described in the Materials and methods section (P4H specific activity = 1405 pmol/min per mg). Incubations were performed in the absence (●) or presence (■) of 0.5 mM L-ascorbate. Incubations were performed at 26 °C for the indicated time. (**b**) The plot shows the L-proline hydroxylation activity at each time point in the presence of L-ascorbate, expressed as a percentage of the activity found in the absence of L-ascorbate.

presence of L-proline, but a 4-fold stimulation of the decarboxylation reaction in the absence of L-proline. This is an observation consistent with the participation of L-ascorbate in uncoupled decarboxylation cycles. The inhibition of L-proline hydroxylation by L-ascorbate may result from competition for binding at the 2-oxoacid binding site between 2-oxoglutarate and L-ascorbate. Further studies revealed an increase in the degree of inhibition given by L-ascorbate as the reaction time was increased (Figure 5), and a greater stimulation of P4H activity by catalase was observed when L-ascorbate was present in the incubation mixture (results not shown). These results suggest peroxide generation as a major cause of L-ascorbate inhibition under these (i.e. L-proline-coupled) conditions. The different effects of L-ascorbate under complete and partial reaction conditions therefore probably reflect the importance of L-ascorbate to the completion of the catalytic cycle in the latter case.

The completion of uncoupled reaction cycles by L-ascorbate may occur by direct reduction of an enzyme-bound intermediate by L-ascorbate to regenerate active enzyme [19–21]. Alternatively it is possible that, during the uncoupled reaction, oxidation of an appropriately placed 'sacrificial' amino acid side chain (e.g. oxidation of a cysteinyl thiol to a sulphenic acid) occurs, resulting in enzyme inactivation. The oxidized side chain may then be reduced by active-site-bound L-ascorbate. Consistent with this proposal are the observations that activity can be restored to mammalian P4H and lysyl 5-hydroxylase inactivated in the absence of L-ascorbate and substrate by the subsequent addition of L-ascorbate [22,23].

2-Oxoacid substrate specificity

The members of the family of 2-oxoacid-dependent dioxygenases that utilize 2-oxoglutarate as a co-substrate are notable for having a lax specificity, but a much stricter requirement for 2-oxoglutarate as the decarboxylation co-substrate. Mammalian prolyl 4-hydroxylase [24] is unusual in that 2-oxoglutarate can apparently be replaced, albeit inefficiently, by an alternative, non-C_5, 2-oxoacid, namely 2-oxoadipate, the homologue of 2-oxoglutarate.

A typically strict requirement for 2-oxoglutarate was found for P4H. Thus replacement of 2-oxoglutarate with 2-oxopentanoate, 2-oxoadipate, pyruvate or 2-oxomalonate (all at 0.5 mM) led to no detectable hydroxylation of L-proline. With L-glutamate a low hydroxylation rate was observed (3% of the 2-oxoglutarate-induced rate), but the low activity observed was probably due to low levels of transamination which produced 2-oxoglutarate from L-glutamate.

The apparent K_m value of 2-oxoglutarate was found to be 32 μM (results not shown), a value comparable with that of many other enzymes in this family, and that of L-proline was 445 μM. High concentrations (> 0.5 mM) of 2-oxoglutarate were found to decrease activity, as has been observed for gibberellin 2β-hydroxylase [25].

IC_{50} values were determined for several known [24,26] inhibitors of prolyl hydroxylase: pyridine-2,4-dicarboxylate (5 μM), pyridine-2,5-dicarboxylate (49 μM), pyridine-2,6-dicarboxylate (which was found to be an activator) and 3,4-dihydroxybenzoate (32 μM). Pyridine-2,4-dicarboxylate was shown to be a competitive inhibitor (results not shown), with a K_i of 5 μM with respect to 2-oxoglutarate. The observation that pyridine-2,4-dicarboxylate and 3,4-dihydroxybenzoate were the most potent aromatic inhibitors found of P4H is consistent with inhibition studies on most other 2-oxoglutarate-dependent dioxygenases studied [26]. Mammalian prolyl 4-hydroxylase is an exception, since, of the pyridinedicarboxylates, pyridine-2,5-dicarboxylate is the most efficient inhibitor, consistent with the observation that 2-oxoadipate can act as a co-substrate for this enzyme. In common with hyoscyamine 6β-hydroxylase [17] and gibberellin 2β-hydroxylase [25], pyridine-2,6-dicarboxylate was found to stimulate P4H activity (maximal enhancement being reached at approx. 100 μM; results not shown). Oxalylglycine, which is an inhibitor of mammalian prolyl hydroxylase [8], was a competitive inhibitor (results not shown) of P4H (K_i = 0.4 μM). No hydroxylation of L-proline was observed when 2-oxoglutarate was replaced with oxalylglycine.

Requirement for ferrous iron

The maximal rate of L-proline hydroxylation was attained at a ferrous iron concentration of approx. 30 μM and proceeded at approx. 8% of the maximal rate in the absence of exogenous iron, further suggesting the presence of residual enzyme-bound iron. No inhibition was observed up to a ferrous iron concentration of 0.5 mM. Inclusion of either cobalt(II) or zinc(II) ions in the P4H incubation mixture, equimolar with iron(II), resulted in complete inhibition of L-proline hydroxylation, whereas manganese(II), copper(II) and mercury(II) ions were somewhat less effective, causing a 20–30% fall in activity. Upon omission of iron(II), only copper(II) ions led to any (10–15%) hydroxylation activity above L-proline-uncoupled levels, but it is possible that the observed activity was due to release of complexed iron from a contaminant rather than utilization of copper(II) itself by P4H.

Inhibition by diethyl pyrocarbonate (DEPC)

Histidine residues have been implicated at the active sites of a number of the 2-oxoacid-dependent [27] and related enzymes, 1-aminocyclopropane-1-carboxylate (ACC) oxidase [28] and isopenicillin N synthase (IPNS) [9], both by sequence comparisons [6,27,29] and by the use of DEPC [27,28], a reportedly selective histidine-modifying reagent [30]. IPNS and ACC oxidase do not utilize a 2-oxoacid cofactor, but are related to the other

Table 2 Inactivation of P4H by DEPC-containing preincubation mixtures

The concentration of a stock solution of DEPC (20 μl) in ethanol (6 ml) was determined as described by Miles [30]. A P4H sample (specific activity 395 pmol/min per mg) was dialysed into 20 mM potassium phosphate (pH 6.8) and incubated at 4 °C with the DEPC solution added to a concentration of 1 mM and containing combinations of the following cofactors: L-Proline (Pro; 1 mM), 2-oxoglutarate (αKG; 0.5 mM), iron(II) ammonium sulphate (Fe^{2+}; 0.5 mM), L-ascorbate (ASC; 1 mM). Control samples contained ethanol in place of DEPC. After preincubation with 1 mM DEPC at 4 °C for 20 min, a 20 μl sample was removed and assayed for L-proline hydroxylation activity. The concentrations of all cofactors etc. in the final assay were adjusted to those of the standard hydroxylation conditions, except for L-proline, which contained 0.33 μCi of L-[U-^{14}C]proline diluted with L-proline to a final concentration of *212.6 μM or † $\sim$ 12.6 μM.

	Cofactors etc. present during preincubation	Hydroxyproline (c.p.m.)	L-Proline turnover (%)	Relative activity of DEPC-treated sample (%)
None	DEPC*	520	0.8	5
	Control*	7259	16.3	
	DEPC†	466	0.3	1
	Control†	14087	33.4	
Pro	DEPC*	812	1.6	12
	Control*	5325	12.8	
Fe^{2+}	DEPC†	639	1.0	3
	Control†	13466	31.2	
ASC	DEPC†	638	0.9	3
	Control†	14462	33.4	
αKG, Fe^{2+}	DEPC†	713	9.6	3
	Control†	10602	27.0	
Fe^{2+}, ASC	DEPC†	490	0.7	3
	Control†	11191	25.9	
Pro, Fe^{2+}, ASC	DEPC*	709	1.2	10
	Control*	4932	11.3	
Pro, αKG, ASC	DEPC*	3619	8.9	52
	Control*	7120	17.0	
Fe^{2+}, αKG, ASC	DEPC*	506	0.5	5
	Control*	4474	10.4	
Fe^{2+}, αKG, ASC	DEPC†	963	1.9	8
	Control†	9583	24.3	
Pro, Fe^{2+}, αKG, ASC	DEPC*	3591	9.4	47
	Control*	7699	20.1	

members of the family by sequence similarity and their requirement for a ferrous ion and dioxygen. The DEPC-inhibition studies on P4H, lysyl 5-hydroxylase [27] and ACC oxidase [28] suggest that two or three histidine residues had a ligating role at their iron-binding sites. The recently reported crystal structure of IPNS [29] reveals that the metal-binding ligands of IPNS are provided by an aspartyl, a glutaminyl, two histidinyl and two water molecules. Sequence comparisons in the light of the structure reveal that many of the members of the family are likely to have related structures [29] in which the aspartyl and the two histidinyl ligating residues are conserved.

Upon incubation of P4H with DEPC (1 mM) for 10 min, only 1.8 % of L-proline-hydroxylation activity remained, relative to a control sample, suggesting the presence of histidine residues at the active site of P4H. The effect of including one or more cofactors of P4H in the DEPC preincubation inhibition mixture was investigated (Table 2). The inclusion of both L-proline and 2-oxoglutarate resulted in significant protection against the inactivation process, implying a role for histidine residues at both the L-proline and 2-oxoglutarate binding sites of P4H. These results support the assignment of P4H as a member of the 2-oxoacid and related oxygenase family, but contrast with results obtained for P4H and lysyl 5-hydroxylase, where the polypeptide hydroxylation substrates did not protect against DEPC inactivation [27]. The difference might reflect the difference in the nature of the hydroxylation substrates (polymeric versus monomeric) between the mammalian and bacterial enzymes.

In summary, bacterial P4H has been purified to near-homogeneity from *Streptomyces griseoviridus* P8648 and unequivocally shown to be a member of the 2-oxoacid-dependent dioxygenase family. In contrast with mammalian P4H, it is a monomeric protein and utilizes a monomeric rather than a polymeric substrate. The absolute requirement for 2-oxoglutarate, and the order and magnitude of inhibition by aromatic analogues of the 2-oxoacid, suggest strong similarities between the iron/2-oxoglutarate binding site of P4H and the majority of related dioxygenases, with the exception of mammalian P4H. Inhibition experiments using DEPC have indicated that histidine residues are apparently involved in the binding of iron and 2-oxoglutarate to P4H, in common with prolyl 4-hydroxylase, and possibly in the binding of the hydroxylation substrate, in contrast with the mammalian enzyme.

In the biosynthesis of the 3-hydroxypyridine-2-carboxylate moiety of etamycin (Figure 1) in *S. griseoviridus* P8648, hydroxylation of lysine at C-5 has been implicated as an early step [31]. Thus, *S. griseoviridus* P8648 may therefore also produce a lysine hydroxylase. Thus bacteria may produce dioxygenases which catalyse hydroxylation of both of the monomeric amino acids, L-proline and L-lysine, which are only hydroxylated post-translationally during collagen biosynthesis.

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