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Substrate Specificity of Proline 4-Hydroxylase: Chemical and Enzymatic Synthesis of 2*S*,3*R*,4*S*-Epoxyproline

Jack E. Baldwin, Robert A. Field, Christopher C. Lawrence, Victor Lee, J. Kenneth Robinson and Christopher J. Schofield

The Dyson Perrins Laboratory and the Oxford Centre for Molecular Sciences, South Parks Road, Oxford OX1 3QY, UK

Abstract: The substrate specificity of *L*-proline 4-hydroxylase, a 2-oxoglutarate dependent dioxygenase from *Streptomyces griseoviridus* P8648, was investigated. Preliminary assays measuring turnover of 2-oxoglutarate indicated 3,4-dehydro-*L*-proline was an efficient substrate. The identity of the product was shown to be 2*S*,3*R*,4*S*-epoxy-*L*-proline by comparison with synthetic 2*S*,3*R*,4*S*- and 2*S*,3*S*,4*R*-epoxy-*L*-prolines.

Three of the four diastereomers of 4-hydroxyproline are naturally occurring.[1] In all cases investigated *L*-proline has been shown to be the precursor of the hydroxylated prolines.[2] 2*S*, 4*R*-Hydroxyproline is the most abundant being both a constituent of collagen[3] and a number of secondary metabolites including the echinocandins and etamycin.[4] The hydroxylation of protocollagen has been extensively investigated and occurs *via* a post-translational modification catalysed by prolyl-4-hydroxylase (EC 1.14.11.2).[3] In contrast the biosynthesis of 2*S*, 4*R*-hydroxyproline in *Streptomyces spp.* occurs by hydroxylation of free *L*-proline[5] and *L*-proline hydroxylase has been purified from *S. griseoviridus* P8648.[6] Both mammalian prolyl hydroxylase and bacterial proline hydroxylase are ferrous ion dependent and utilise dioxygen and 2-oxoglutarate as cosubstrates, hence they belong to the family of 2-oxo acid and related dioxygenases.[7] The stereochemical course of the proline hydroxylation reaction has been shown to occur with retention at C-4 of proline.[8]

A common property of 2-oxoacid dioxygenases is a relatively lax oxidation substrate specificity. As well as alternative hydroxylation substrates having been found in several cases, these enzymes have been shown capable of the epoxidation of alkenes, the sulphoxidation of sulphides and the ketenisation of alkyne functionalties present in unnatural substrates.[3,9] In the case of prolyl 4-hydroxylase a number of proline analogues have been incorporated into suitable peptides and subsequently shown by assays based on the turnover of 2-oxoglutarate to act as inhibitors or apparent substrates.[3] However, because of the polymeric nature of the products in no case were the structures of the products confirmed,[10] and several examples have been reported where substrate analogues apparently increase the rate of turnover of 2-oxoglutarate to succinate and CO_2 without being oxidised.[11]

Herein, we report preliminary studies on the substrate specificity of proline 4-hydroxylase from *S. griseoviridus* P8648, which indicate that this enzyme has potential for the *in vitro* functionalisation of *L*-proline analogues. Since a number of functionalised proline derivatives are natural products[12] or are used as intermediates in the synthesis of pharmaceuticals the development of proline hydroxylase as an *in vitro* reagent may be of some utility.

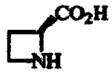
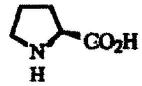
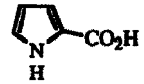
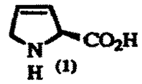
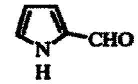
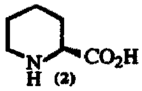
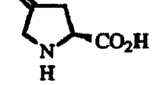
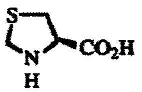
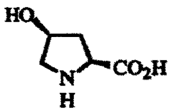
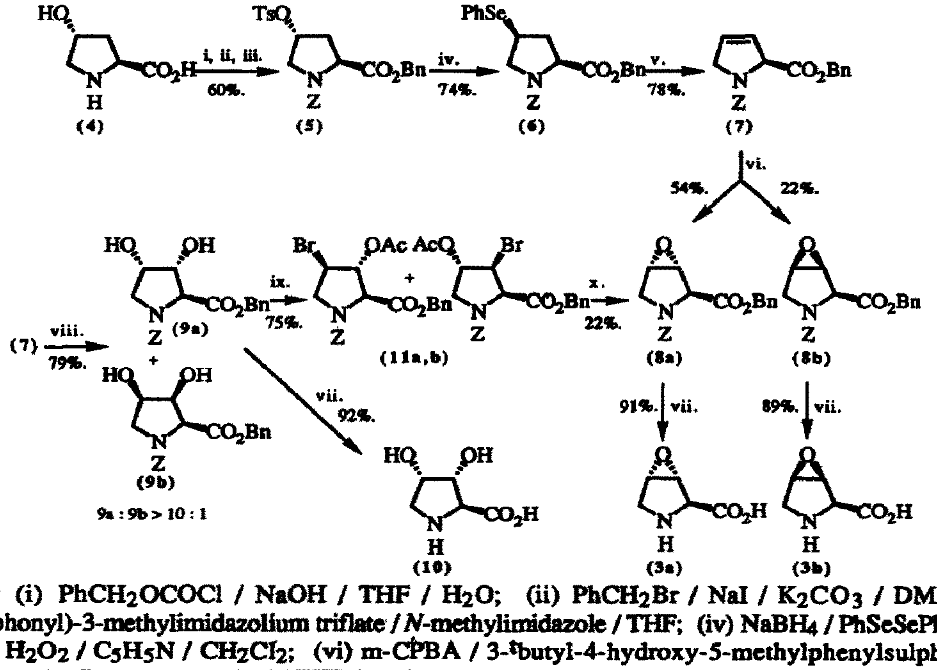
Substrate	2-Oxoglutarate turnover %	Relative activity %	Substrate	2-Oxoglutarate turnover %	Relative activity %
None	3	5		9	17
	51	100		5	10
 (1)	67	134		4	7
 (2)	51	100		15	29
	10	20		1.5	3

Table 1: Proline 4-hydroxylase induced decarboxylation of 2-oxoglutarate in the presence of *L*-proline analogues. Incubations were carried out (at least in triplicate) at 26°C, pH 7.5, with shaking (200rpm) in a total volume of 50 μl of 25 mM TES buffer containing 0.2 $mgml^{-1}$ catalase, 0.5 mM iron (II) ammonium sulphate, 0.1 mM sodium ([^{14}C (U)]-2-oxoglutarate) (0.08 μCi), 1 or 2 mM substrate analogue and 10 μl of proline-4-hydroxylase solution, typically containing 250 μg of proline-4-hydroxylase of activity = 4 $nmolmin^{-1}mg^{-1}$ at [*L*-proline] = 18 μM.

Due to the low levels of protein available to screen analogues as potential substrates we initially used an assay based on the conversion of 2-oxoglutarate to succinate and CO_2.[13] Seven *L*-proline containing peptides [*L*-pro-gly; *L*-pro-gly-gly; *L*-pro-*L*-leu; *L*-pro-*L*-ile; *L*-pro-*L*-leu-gly-NH_2; *L*-pro-*L*-phe-gly-*L*-lys; *L*-pro-2*S*,4*R*-hydroxyproline-*L*-pro] were assayed in this manner, but none showed activity above uncoupled levels, suggesting these compounds were unable to bind to the enzyme active site. Similarly amino and carboxyl derivatives of proline (*e.g.* *L*-prolinamide, *L*-proline napthyl-2-amide, *N*-acetyl *L*-proline, *N*-benzyloxycarbonyl *L*-proline) were inactive.[14] The results for ring modified *L*-proline derivatives were more interesting (**Table 1**), in that increased levels of 2-oxoglutarate decarboxylation were observed suggesting such analogues act as potential unnatural oxidation substrates of proline 4-hydroxylase. In particular, dehydro-*L*-proline **1** stimulated the formation of succinate to a greater extent than the natural substrate *L*-proline. An analogous observation has been reported for hyoscyamine 6β-hydroxylase.[15] Significant turnover of 2-oxoglutarate was also seen in the case of *L*-pipecolic acid **2**. However, stimulation of 2-oxoacid decarboxylation cannot, in itself, be regarded as definitive proof of substrate oxidation. For a number of related enzymes, 'pseudosubstrates' have been identified, which cause a stimulation of 2-oxoglutarate decarboxylation without themselves being turned over.[11] Such reactions would be expected to show a strong dependence on *L*-ascorbate, the proposed alternative reductant participating in such uncoupled cycles.[11] Indeed, inclusion of *L*-ascorbate in the proline 4-hydroxylase reaction mixture was found to stimulate the enzyme catalysed decarboxylation of 2-oxoglutarate both in the presence of **1** (10-15% enhancement) and in the absence of any proline analogue whereas no such enhancement was observed for the coupled hydroxylation of *L*-proline. It was thus important to verify that oxidation of **1** was occurring. The low levels of proline-4-hydroxylase available from the natural source precluded the isolation and direct characterisation of the incubation products resulting from incubation of dehydro-*L*-proline **1**, but phenylisothiocyanate (PITC) derivatisation of the crude product followed by h.p.l.c. analysis suggested the production of a new amino acid. We speculated that an epoxide [*i.e.* **3a** or **3b**] was produced. Authentic standards were therefore required for comparison.

Epoxidation of *N*-benzenesulphonyl-3,4-dehydro-*L*-proline methyl ester with trifluoroperacetic acid has been reported.[16] The resultant epoxides were reported to be chromatograpically inseparable and deprotection to form the free amino acids [*i.e.* **3a** or **3b**] not possible. They were apparently resistant to catalytic hydrogenolysis, hence we investigated the synthesis and deprotection of the *N*-benzyloxycarbonyl-3, 4-epoxy-*L*-proline benzyl esters. The requisite 3,4-dehydro-*L*-proline derivative **7** (**Scheme 1**) was synthesised from 4*R*-hydroxy-*L*-proline **4** using a modified version of the method of Rüeger *et al.*[17] After diprotection, tosylation to give **5** was achieved using 1-(toluenesulphonyl)-3-methylimidazolium triflate.[18] Selenation of **5** to give **6** was carried out in ^{t}BuOH to avoid ester exchange which occurred when ethanol was used as solvent. Selenide **6** was converted to the required dehydro-*L*-proline derivative **7** by oxidative elimination. Treatment of **7** with *m*-CPBA in the presence of a radical inhibitor[19] gave **8a** (54%) and **8b** (22%) which were readily separated by flash chromatography and deprotected to give the desired epoxides **3a** and **3b**. Confirmation of the stereochemical assignments was achieved by synthesis. Thus, reaction of **7** with *N*-methylmorpholine-*N*-oxide and catalytic OsO_4[20] gave diols **9a/b** (**9a**:**9b** > 10:1). Deprotection of **9a** gave the amino acid **10** with analytical data consistent with that previously reported for its enantiomer.[21] Diol **9a** was converted to epoxide **8a** by treatment with acetoxyisobutyryl bromide[22] in acetonitrile to give a mixture of **11a,b**, which when stirred with K_2CO_3 in benzyl alcohol gave epoxide **8a**. (Benzyl-*N*-benzyloxycarbonylpyrrole-2-carboxylate (14%) and unreacted starting material (17%) were also isolated).



Scheme 1: (i) $PhCH_2OCOCl$ / NaOH / THF / H_2O; (ii) $PhCH_2Br$ / NaI / K_2CO_3 / DMF; (iii) 1-(toluenesulphonyl)-3-methylimidazolium triflate / *N*-methylimidazole / THF; (iv) $NaBH_4$ / PhSeSePh / ^{t}BuOH / reflux; (v) H_2O_2 / C_5H_5N / CH_2Cl_2; (vi) m-CPBA / 3-^{t}butyl-4-hydroxy-5-methylphenylsulphide / 1,2-dichloroethane / reflux; (vii) H_2 / Pd / THF / H_2O; (viii) cat. OsO_4 / *N*-methylmorpholine-*N*-oxide / ^{t}BuOH / H_2O; (ix) acetoxyisobutyryl bromide / CH_3CN; (x) K_2CO_3 / $PhCH_2OH$.

H.p.l.c. analysis of PITC-derivatised 3,4-dehydro-*L*-proline **1** incubation mixtures consistently showed a peak with a retention time identical to that of a similarly derivatised sample of synthetic *trans*-3,4-epoxy-*L*-proline **3a**, indicating that proline-4-hydroxylase catalyses epoxidation of 3,4-dehydro-*L*-proline **1** to give *trans*-3,4-epoxy-*L*-proline. No evidence was accrued for the production of *cis*-3,4-epoxy-*L*-proline **3b**. In conclusion we have demonstrated the potential utility of proline hydroxylase for the *in vitro* functionalisation of proline analogues. Current efforts are directed towards the production of the enzyme from a recombinant source to enable preparative scale work to carried out.

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