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Mechanistic Studies on Prolyl-4-Hydroxylase: Demonstration That the Ferryl Intermediate Does Not Exchange with Water

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Prolyl-4-hydroxylase catalyzes the formation of 4-hydroxyproline in collagens. In contrast to deacetoxy/deacetylcephalosporin C synthase, *p*-hydroxyphenylpyruvate hydroxylase, lysyl hydroxylase and α-ketoisocaproate oxygenase, no incorporation of ^{18}O-labeled water into the hydroxylated product was found for the human type I prolyl-4-hydroxylase when *N*-Cbz-Gly-L-Phe-L-Pro-Gly-OEt was used as a substrate. This suggests that the ferryl intermediate for this enzyme is not solvent accessible.

INTRODUCTION

Prolyl-4-hydroxylase belongs to a family of non-heme α-ketoglutarate dependent dioxygenases and catalyzes the hydroxylation of proline residues in a peptide sequence of -X-Pro-Gly- **1** (Fig. 1) (*1–4*). During the reaction, one atom of molecular oxygen is incorporated into succinate while the other one appears in the prolyl hydroxyl group of the peptide product.

It is generally assumed that the ferryl intermediate **8** is responsible for the hydroxylation (Fig. 2) (*4–7*). The oxygen atom of this or a closely related intermediate, which is initially derived from molecular oxygen, has been shown to undergo an exchange reaction with the oxygen atom in water in deacetoxy/deacetylcephalosporin C synthase (*8,9*), *p*-hydroxyphenylpyruvate hydroxylase (*10*), α-ketoisocaproate oxygenase (*11*), and lysyl hydroxylase (*12*). The occurrence of this exchange reaction in a subset of the α-ketoglutarate-dependent dioxygenases is surprising and suggests that the ferryl intermediate is solvent accessible in these enzymes and that the exchange reaction is sufficiently rapid that it can compete with the hydroxylation chemistry. The mechanistic significance of the exchange reaction, however, is still unclear. Previous studies demonstrated that the chick embryo prolyl-4-hydroxylase catalyzed the hydroxylation of $(Pro\text{-}Pro\text{-}Gly)_5$ with 13% exchange with water (*12*). In this paper we describe experiments designed to determine if this exchange reaction occurs during the human type I prolyl-4-hydroxylase catalyzed hydroxylation of *N*-Cbz-Gly-L-Phe-L-Pro-Gly-OEt **14**.

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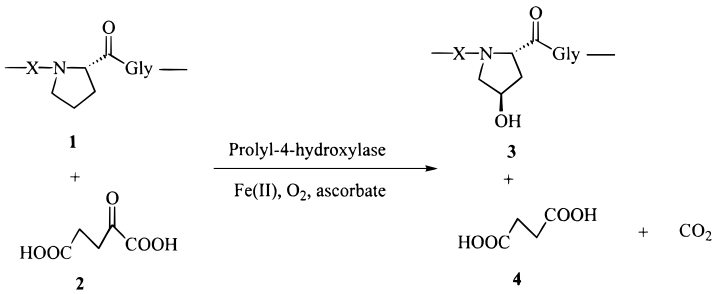

FIG. 1. The prolyl-4-hydroxylase catalyzed reaction.

RESULTS AND DISCUSSION

*Synthesis of N-Cbz-Gly-L-Phe-L-Pro-Gly-OEt **14**.* Type I protocollagen, the natural substrate for prolyl-4-hydroxylase, has a molecular weight of about 150 kDa (*15*). It was therefore necessary to use a small substrate analog for our mechanistic studies (*16*).

The synthesis of **14** is described in Fig. 3. *t*-Boc-L-Pro-COOH **16** was condensed with NH_2-Gly-OEt in the presence of DCC and HOBt to give the dipeptide **17**. This was treated with TFA, without purification, to provide the TFA salt of Pro-Gly-OEt **18** in quantitative yield over the two steps. Coupling of *N*-Cbz-Gly-L-Phe-COOH with **18** in the presence of EDC and HOBt gave the tetrapeptide *N*-Cbz-Gly-L-Phe-L-Pro-Gly-OEt **14** in 80% yield.

*Enzyme catalyzed hydroxylation of **14**.* We developed a fast and nonradioactive assay method that involves incubating the peptide substrate with the enzyme for 30 min, extracting the reaction mixture with dichloromethane and analyzing the organic mixture by thin layer chromatography (TLC). *N*-Cbz-Gly-L-Phe-L-Pro-Gly-OEt **14** was found to be a good substrate by this assay with a conversion of about 50% to hydroxylated product *N*-Cbz-Gly-L-Phe-4-L-Hyp-Gly-OEt **15** under our reaction conditions.

The identity of the hydroxylated product *N*-Cbz-Gly-L-Phe-4-L-Hyp-Gly-OEt **15** was confirmed by comigration on the TLC plate with a synthetic sample of **15** and by MS analysis of the dichloromethane extract, which revealed the presence of two molecular ions $[M + H^+]$ of 539.2 and 555.2 corresponding to **14** and **15**, respectively.

*Enzyme catalyzed hydroxylation of **14** in $H_2{}^{16}O/H_2{}^{18}O$.* When compound **14** was incubated with the human type I prolyl-4-hydroxylase in buffer containing $H_2{}^{18}O$ (70% v/v) for 2 h, FAB-MS analysis of the dichloromethane extract demonstrated that no incorporation of oxygen-18 into *N*-Cbz-Gly-L-Phe-4-L-Hyp-Gly-OEt **15** (*m/z* for $[M + H^+]$ 555.2) occurred (Table 1). A control reaction in $H_2{}^{16}O$ containing buffer was run in an identical manner. This demonstrates that the ferryl intermediate **8** of human type I prolyl-4-hydroxylase does not exchange with water.

EXPERIMENTAL

General Methods and Materials

All reagents were commercially obtained and used without purification unless otherwise noted. $H_2{}^{18}O$ (95–98%) was obtained from Cambridge Isotope Laboratory.

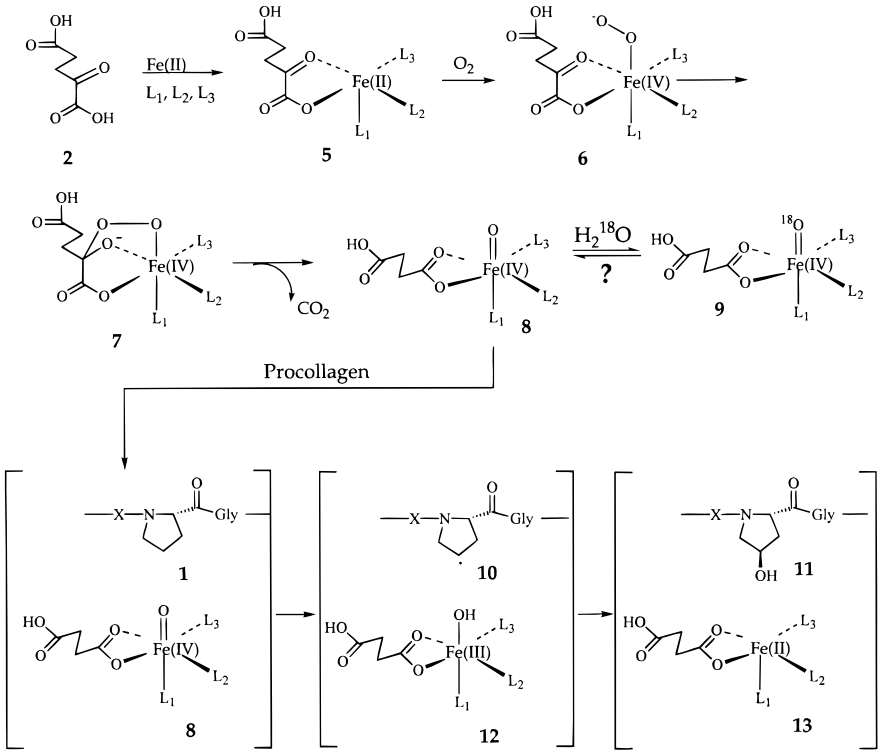

FIG. 2. The current mechanism for the hydroxylation reaction catalyzed by prolyl-4-hydroxylase (His 412, His 483, and Asp 414 are the three enzymatic ligands for the iron) (*13*,*14*).

Organic solutions were concentrated by rotary evaporation at ~25 mmHg (water aspirator). All reactions were monitored by thin layer chromatography (TLC) on 250 μm EM science silica gel 60 F_{254} using UV light (254 nm) as visualizing agent and 7% ethanolic phosphomolybodic acid or 0.2% ethanolic ninhydrin and heat as developing agents.

Low resolution FAB mass spectra were run on a ZAB mass spectrometer at the University of Illinois using Xe atoms (8 kV) and a matrix of dithiothreitol/dithioerythritol (magic bullet). IR spectra were recorded on a Mattson Galaxy series FT-IR instrument.

*Synthesis of N-Cbz-Gly-*L*-Phe-*L*-Pro-Gly-OEt **14***

t-Boc L*-Pro-Gly-OEt **17*** DCC (623 mg, 3.0 mmol) was added to a stirred solution of *t*-Boc-L-Pro-COOH **16** (500 mg, 2.3 mmol), $^{+}NH_3$-Gly-OEt Cl^{-} (341 mg, 2.4 mmol), HOBt (330 mg, 2.4 mmol), and *N*-ethylmorpholine (309 μl, 2.4 mmol) in anhydrous THF (10 ml) at 0°C. The mixture was allowed to warm to room temperature and stirred for 22 h. The mixture was partitioned between CH_2Cl_2 (50 ml) and saturated $NaHCO_3$ (50 ml). The organic layer was washed with 5% citric acid (50 ml), brine (50 ml), dried ($MgSO_4$), and evaporated under reduced pressure to give the crude

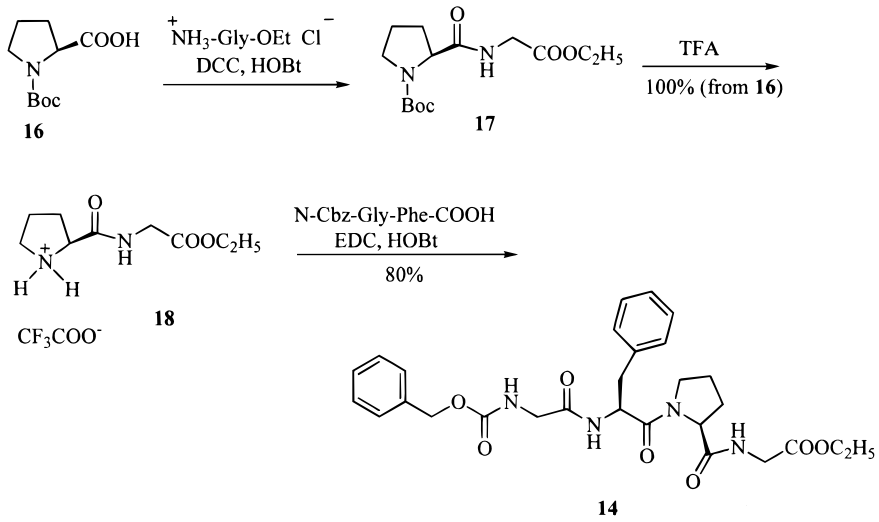

FIG. 3. Synthesis of the prolyl-4-hydroxylase substrate analog *N*-Cbz-Gly-L-Phe-L-Pro-Gly-OEt **14**.

product **17**, which was directly used for the next step: TLC $R_f = 0.40$ (silica, 6% $MeOH/CH_2Cl_2$).

$^+NH_2$-L-Pro-Gly-OEt TFA$^-$ ***18***. Trifluoroacetic acid (7.2 ml, 93 mmol) was added to a stirred solution of **17** (698 mg, 2.3 mmol) in CH_2Cl_2 (21 ml) at 0°C. The mixture was stirred at 0°C for 3 h. The mixture was concentrated under reduced pressure and the residue was purified by flash column chromatography (silica, 8% $MeOH/CH_2Cl_2$) to give the title compound **18** as a yellow solid (812 mg, 100%, two steps): TLC $R_f = 0.09$ (silica, 6% $MeOH/CH_2Cl_2$); ^{1}H NMR (200 MHz, DMSO-d_6) δ 4.20 (m, 1H, Pro-αH), 4.10 (q, 2H, $J = 7.1$ Hz, OCH_2CH_3), 3.89 (m, 2H, Gly-αH), 3.30 (m, 2H, Pro-δH), 2.30 (m, 1H, Pro-βH), 1.90 (m, 1H, Pro-βH; 2H, Pro-γH), 1.20 (t, 3H, $J = 7.1$ Hz, OCH_2CH_3).

N-Cbz-Gly-L-Phe-L-Pro-Gly-OEt ***14***. EDC (631 mg, 3.3 mmole) was added to a stirred solution of **18** (739 mg, 2.4 mmol), *N*-Cbz-Gly-Phe-COOH (922 mg, 2.6 mmol), HOBt (349 mg, 2.6 mmol), and *N*-ethylmorpholine (714 μl, 5.6 mmol) in anhydrous DMF (20 ml) at 0°C. The mixture was allowed to warm to room temperature

TABLE 1

Mass Analysis of the Hydroxylation of *N*-Cbz-Gly-L-Phe-L-Pro-Gly-OEt **14** in $H_2{}^{16}O/H_2{}^{18}O$

	Relative intensity of m/z [M + H$^+$]		
Solvent	555.2	556.2	557.2
$H_2{}^{18}O$	100	34	10
$H_2{}^{16}O$	100	33	9
$H_2{}^{16}O$ (Calculated)	100	33	7

and stirred for 24 h. The reaction was quenched with 10 ml water. The mixture was partitioned between EtOAc (50 ml) and H_2O (10 ml). The organic layer was washed with 5% HCl (50 ml), saturated $NaHCO_3$ (50 ml), brine (50 ml), dried ($MgSO_4$), and evaporated under reduced pressure. The residue was purified by flash column chromatography (silica, 4% MeOH/CH_2Cl_2) to gave the title compound **14** as a white foam (1.01 g, 80%): TLC $R_f = 0.50$ (silica, 6% MeOH/CH_2Cl_2); ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.25 (m, 10H, aromatic-H), 7.00 (t, 1H,-N*H*), 6.70 (m, 1H, -N*H*), 5.30 (m, 1H,-N*H*), 5.12 (s, 2H,-OC*H*$_2$Ph), 4.95 (q, 1H, Phe-αH), 4.55 (m, 1H, Pro-αH), 4.20 (q, 2H, $J = 7.1$ Hz, OC*H*$_2$CH$_3$), 3.90 (d, 2H, $J = 5.4$ Hz, Gly-αH), 3.85 (d, 2H, $J = 5.5$ Hz, Cbz-Gly-αH), 3.50 (m, 2H, Pro-δH), 3.00 (m, 2H, Phe-βH), 2.30 (m, 1H, Pro-βH), 1.90 (m, 1H, Pro-βH; 2H, Pro-γH), 1.20 (t, 3H, $J = 7.1$ Hz, OCH$_2$C*H*$_3$). IR ($CDCl_3$) υ 3300, 3000, 1725, 1675, 1630, 1550, 1475, 1250, 1200 cm^{-1}; LRMS (FAB in magic bullet) m/z[M + H$^+$] 539.1.

*Enzyme catalyzed hydroxylation of **14** in $H_2{}^{18}O/H_2{}^{18}O$.* A solution of compound **14** in methanol (15.5 mM, 60 μl) was added to 450 μl of the assay mixture, which contained human type I prolyl-4-hydroxylase (0.15 mg/ml), a cofactor solution (0.05 mM $FeSO_4.7H_2O$, 2 mM ascorbate, 0.5 mM α-ketoglutarate, 0.1 mM DTT, 2 mg/ml BSA, 0.1 mg/ml catalase, 50 mM Tris (pH 7.8) and 360 μl of $H_2{}^{18}O$ (95–98%)). After reaction at 37°C for 2 h, a 150-μl aliquot was removed and extracted with 150 μl CH_2Cl_2. The organic layer was concentrated *in vacuo* and used for the MS study. The control experiment where $H_2{}^{16}O$ was used instead of $H_2{}^{18}O$ was run in a same fashion.

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