Bioorganic Chemistry **28,** 261–265 (2000)
doi:10.1006/bioo.2000.1182, available online at http://www.idealibrary.com on IDEAL®

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

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Received December 27, 1999

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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0045-2068/00 $35.00

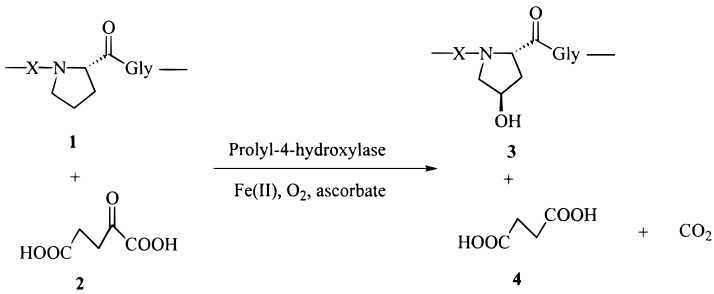

FIG. 1. The prolyl-4-hydroxylase catalyzed reaction.

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.