Communications to the Editor

Mechanism-Based Inactivation of the Human Prolyl-4-hydroxylase by 5-Oxaproline-Containing Peptides: Evidence for a Prolyl Radical Intermediate

Min Wu, Hong-Sik Moon, and Tadhg P. Begley*

Department of Chemistry and Chemical Biology
Cornell University, Ithaca, New York 14853

Johanna Myllyharju and Kari I. Kivirikko

Collagen Research Unit
Biocenter and Department of Medical Biochemistry
University of Oulu, Kajaanintie 52A
FIN-90220, Oulu, Finland

Received April 9, 1998

Prolyl-4-hydroxylase catalyzes the hydroxylation of proline residues at X-Pro-Gly sequences in procollagen (Scheme 1). This reaction is an essential step in the biosynthesis of collagen, the major protein component of connective tissue.

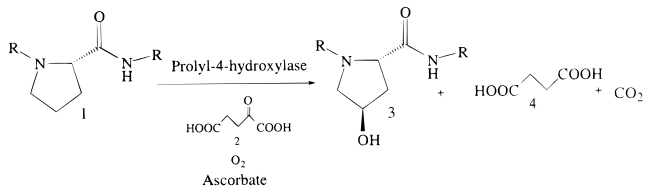
Prolyl-4-hydroxylase (human) is an $\alpha_2\beta_2$ tetramer ($\alpha = 59\,000$ Da, $\beta = 55\,000$ Da) and requires Fe(II), α-ketoglutarate, oxygen, and ascorbate for activity.[1] The genes for the α and β subunits of the human enzyme have been cloned, sequenced, and overexpressed in a baculovirus expression system, and the enzyme can be readily purified in multi-milligram quantities.[2]

5-Oxaproline-containing peptides have been previously identified as mechanism-based inactivating agents for prolyl-4-hydroxylase.[3] The mechanism of this inactivation has not been determined. As a first step toward elucidating this mechanism, we report here the synthesis of a highly fluorescent 5-oxaproline-containing peptide **5** and the identification of its prolyl-4-hydroxylase-catalyzed oxidation product.

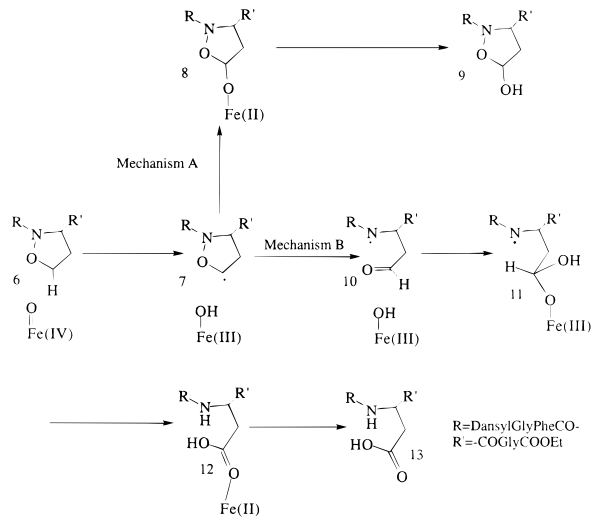
Two mechanisms for the enzymatic oxidation of **5** were considered (Scheme 2). In mechanism A, hydrogen-atom abstraction from the oxaproline by the active site ferryl [$Fe^{IV} = O$] intermediate would give radical **7**. Recombination followed by product dissociation would give the hemiacetal **9**. In mechanism B, β-scission of the weak NO bond of **7** would give **10**. Addition of the iron(III)hydroxide to the aldehyde followed by intramolecular hydrogen-atom transfer[4] and product dissociation would give **13** in which the oxaproline moiety has been oxidized to aspartic acid.

Peptide **5** was synthesized as outlined in Scheme 3.[5,6] This was an efficient suicide substrate for prolyl-4-hydroxylase, covalently labeling the enzyme, and only trace quantities of a polar reaction product could be detected in the reaction mixture.[7,8] This product was purified,[9] and FAB-MS analysis demonstrated that the enzymatic oxidation resulted in the addition of one atom ofoxygen to **5**.[10] This is consistent with the formation of either **9** or **13**.

Scheme 1



Scheme 2



Peptides **9** and **13** were synthesized to differentiate between mechanisms A and B. Chromatographic comparison of these peptides with the enzymatic product demonstrated that **9** was not formed and that the only enzymatic product was **13**. This was confirmed by scale-up of the enzymatic reaction and the isolation of the reaction product in sufficient quantities for complete spectroscopic characterization (^{1}H NMR, COSY, HMQC, HMBC, HRFAB-MS, and MS-MS). The spectra of the enzymatic product and the spectra of peptide **13** were identical.

While the α-ketoglutarate-dependent monooxygenases are not as well-studied as the heme-dependent monooxygenases, several

(1) (a) Kivirikko, K. I.; Pihlajaniemi, T. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1998**, *72*, 325. (b) Kivirikko, K. I.; Myllyla, R.; Pihlajaniemi, T. In *Posttranslational Modification of Proteins*; Harding, J., Crabbe, M. Eds.; CRC Press: Boca Raton, FL, 1992; Chapter 1.

(2) Vuori, K.; Pihlajaniemi, T.; Marttila, M.; Kivirikko, K. I. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 7467.

(3) Gunzler, V.; Brocks, D.; Henke, S.; Myllyla, R.; Geiger, R.; Kivirikko, K. I. *J. Biol. Chem.* **1988**, *263*, 19498.

(4) Protein-mediated hydrogen-atom transfer is also possible.

(5) Vasella, A. *Helv. Chim. Acta.* **1977**, *60*, 426.

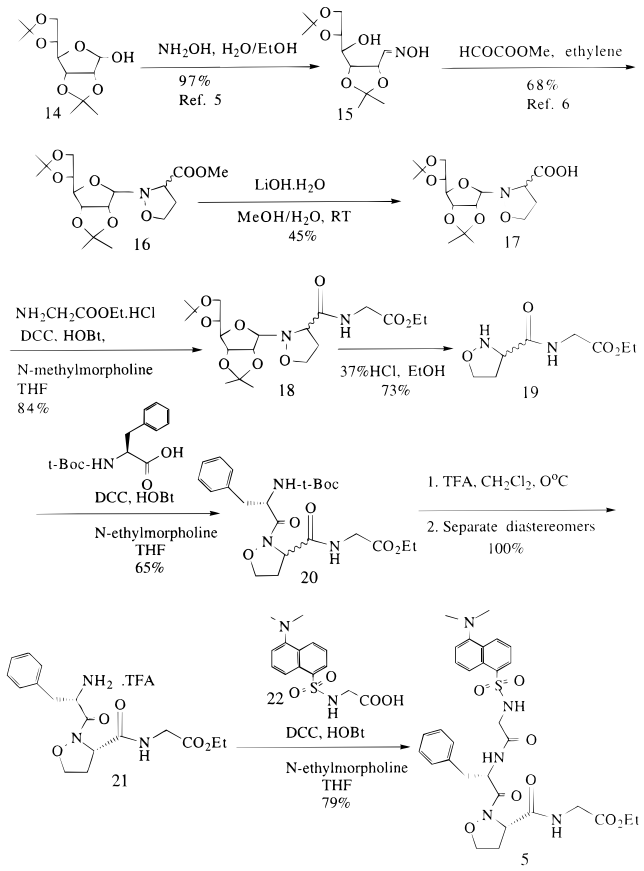
(6) (a) Vasella, A.; Voeffrey, R. *J. Chem. Soc., Chem. Commun.* **1981**, *97*. (b) Vasella, A.; Voeffrey, R.; Pless, J.; Huguenin, R. *Helv. Chim. Acta.* **1983**, *66*, 1241.

(7) The enzymatic reaction mixture consisted of peptide **5** (0.85 mM), $FeSO_4$ (0.05 mM), ascorbic acid (2.0 mM), BSA (1 mg), catalase (0.05 mg), DTT (0.1 mM), α-ketoglutarate (0.5 mM) Tris-HCl (50 mM, pH = 7.8) prolyl-4-hydroxylase (68 μg) in 500 μL. Compound **13** was not formed in control reactions from which the enzyme or α-ketoglutarate were excluded.

(8) The inactivation of prolyl-4-hydroxylase by **5** follows nonpseudo-first-order kinetics due to consumption of the inhibitor. We have estimated the rate constant for the inactivation at several inhibitor concentrations (0, 0.5, 1, 1.5, 2, and 5 μM) by determining the activity remaining after two minutes using the previously described assay procedure.[3] From these data, we determine that $k_{inact} = 0.6$ min^{-1} and $K_I = 1.6$ μM. The enzyme is protected from inactivation by the substrate $(PPG)_{10}$. To demonstrate covalent labeling of the enzyme, we have synthesized an analogue of peptide **5** in which the ethoxy group of the ester has been replaced with biotin hydrazide. This also inactivates the enzyme, and we have demonstrated stable covalent attachment of the label to the enzyme (both subunits) by SDS PAGE followed by western blotting and visualization of biotin-labeled protein with horseradish peroxidase conjugated streptavidin and the Pierce SuperSignal substrate.

10.1021/ja981193h CCC: $18.00
Published on Web 01/06/1999

Scheme 3



examples of this class of enzyme have now been characterized,[11−18] and model studies on the reaction have been described.[19] In each case, the reaction is proposed to proceed via hydrogen-atom abstraction by a ferryl [$Fe^{IV} = O$] intermediate to give a substrate radical. In the systems for which radical probes have been synthesized, experimental evidence in support of such a radical intermediate has been difficult to obtain. The only successful trapping experiment has been described using a cyclopropyl-substituted cephalosporin analogue to trap the radical formed by deacetoxy-deacetylcephalosporin C synthase.[20] The inactivation of γ-butyrobetaine hydroxylase by a cyclopropyl-containing substrate analogue has been reported, but the reaction products have not been characterized.[21] A cyclopropyl-substituted proclavaminic acid analogue was not a substrate for clavaminate synthase, and proline derivatives substituted with radical traps were not substrates for prolyl-4-hydroxylase.[22,23] The experiments described here demonstrate a new strategy for radical-trapping and suggest that the prolyl-4-hydroxylase-catalyzed oxidation of proline residues in procollagen proceeds via a radical intermediate.

Studies to identify the labeled active-site residues of the inactivated enzyme are currently in progress and will clarify how the chemistry involved in the oxidation of **5** results in enzyme inactivation.

Acknowledgment. We thank Mitch Brenner and Ari Koskinen for helpful discussions and Cathy Lester for assistance in obtaining the 2D NMR spectra. We are grateful to FibroGen, to the Academy of Finland (Research Council for Health), and to the American Heart Association for funding this research.

Supporting Information Available: Reaction schemes for the synthesis of peptides **9** and **13** (2 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

JA981193H

(9) The reaction mixture was extracted with ether to remove unreacted **5**. The reaction product was then extracted into ethyl acetate and purified by HPLC (ODS, 65:35 MeOH/H_2O).

(10) HRFABMS for **5**, $(M + H)^+ = 640.2441$, HRFABMS for the reaction product, $(M + H)^+ = 656.2400$.

(11) Prescott, A. *J. Exp. Bot.* **1993**, *44*, 849.

(12) (a) Baldwin, J.; Adlington, R.; Crouch, N.; Pereira, I. *Tetrahedron* **1993**, *49*, 4907. (b) Baldwin, J.; Adlington, R.; Crouch, N.; Pereira, I. *Tetrahedron*, **1993**, *49*, 7499.

(13) (a) Lawrence, C. C.; Sobey, W. J.; Field, R. A.; Baldwin, J. E.; Schofield, C. J. *Biochem. J.* **1996**, *313*, 185. (b) Baldwin, J.; Field, R.; Lawrence, C.; Merritt, K.; Schofield, C. *Tetrahedron Lett.* **1993**, *34*, 7489. (c) Baldwin, J.; Field, R.; Lawrence, C.; Lee, V.; Robinson, J.; Schofield, C. *Tetrahedron Lett.* **1994**, *35*, 4649.

(14) Whiting, A.; Que, L.; Saari, R.; Hausinger, R.; Fredrick, M.; McCracken, J. *J. Am. Chem. Soc.* **1997**, *119*, 3413.

(15) (a) Pavel, E. G.; Zhou, J.; Busby, R. W.; Gunsior, M.; Townsend, C. A.; Solomon, E. I. *J. Am. Chem. Soc.* **1998**, *120*, 743. (b) Janc, J. W.; Egan, L. A.; Townsend, C. A. *J. Biol. Chem.* **1995**, *270*, 5399. (c) Busby, R. W.; Chang, M. D. T.; Busby, R. C.; Wimp, J.; Townsend, C. A. *J. Biol. Chem.* **1995**, *270*, 4262. (d) Salowe, S. P.; Krol, W. J.; Iwata-Reuyl, K.; Townsend, C. A. *Biochemistry* **1991**, *30*, 2281. (e) Baldwin, J. E.; Adlington, R. M.; Crouch, N. P.; Drake, D. J.; Fujishima, Y.; Elson, S. W.; Baggaley, K. H. *J. Chem. Soc., Chem. Commun.* **1994**, 1133.

(16) Eichhorn, E.; van der Ploeg, J. R.; Kertesz, M.; Leisinger, T. *J. Biol. Chem.* **1997**, *272*, 23031.

(17) (a) Thornburg, L. D.; Lai, M.-T.; Wishnok, J. S.; Stubbe, J. *Biochemistry* **1993**, *32*, 14023. (b) Thornburg, L. D.; Stubbe, J. *Biochemistry* **1993**, *32*, 14034. (c) Lai, M.-T.; Wu, W.; Stubbe, J. *J. Am. Chem. Soc.* **1995**, *117*, 5023.

(18) (a) Holme, E.; Lindstedt, S.; Nordin, I. *Biosci. Reports* **1984**, *4*, 433. (b) Wehbie, R. S.; Punekar, N. S.; Lardy, H. A. *Biochemistry* **1988**, *27*, 2222.

(19) (a) Chiou, Y.-M.; Que, L. *J. Am. Chem. Soc.* **1995**, *117*, 3999. (b) Ha, E. H.; Ho, R. Y. N.; Kisiel, J. F.; Valentine, J. S. *Inorg. Chem.* **1995**, *34*, 2265.

(20) Baldwin, J. E.; Adlington, R. M.; Crouch, N. P.; Keeping, J. W.; Leppard, S. W.; Pitlik, J.; Schofield, C. J.; Sobey, W. J.; Wood, M. E. *J. Chem. Soc., Chem. Commun.* **1991**, 768.

(21) Ziering, D. L.; Pascal, R. A. *J. Am. Chem. Soc.* **1990**, *112*, 834.

(22) Iwata-Reuyl, D.; Basak, A.; Silverman, L. S.; Engle, C. A.; Townsend, C. A. *J. Nat. Prod.* **1993**, *56*, 1373.

(23) Tandon, M.; Wu, M.; Begley, T. P.; Myllyharju, J.; Pirskanen, A.; Kivirikko, K. I. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 1139.