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Mechanistic Studies on Prolyl-4-hydroxylase: The Vitamin C Requiring Uncoupled Oxidation

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Abstract—A deuteriated substrate for the human type I prolyl-4-hydroxylase was synthesized and its V/K deuterium isotope effect was determined to be 3.4 ± 0.2. This isotope effect was attributed to the uncoupled oxidation. A dehydroproline containing tetrapeptide was also found to stimulate the uncoupled oxidation.

Prolyl-4-hydroxylase catalyzes the hydroxylation of prolyl 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.[1,2]

The current mechanistic proposal for this enzyme is outlined in Scheme 2.[3–5] and involves the hydroxylation of the substrate by the ferryl intermediate **7**. In addition to this productive reaction, a nonproductive oxidation resulting in enzyme inactivation occurs.[2] The mechanism of this uncoupled oxidation is not understood. One possibility is that the ferryl intermediate **7** oxidizes the enzyme to an inactive form **8**, which requires reduction by ascorbate for reactivation. The uncoupled oxidation is likely to be one of the most important ascorbate requiring enzymatic reactions in humans because the earliest clinical symptoms of scurvy, the vitamin C deficiency disease, are defects in collagen biosynthesis. The function of this inactivation reaction may be to protect the enzyme from a more destructive irreversible oxidation event. Here we describe a deuterium isotope effect and the interaction of a dehydroproline-containing substrate analogue with the enzyme as two possible mechanistic probes for this reaction.

To determine the kinetic significance of the C–H bond cleavage step in the overall reaction Scheme, we have measured the V/K deuterium isotope effect on the reaction using N-Cbz-Gly-Phe-Pro-Gly-OEt as the substrate **20**.[6] Previous research demonstrated that there was no V/K isotope effect on the reaction when procollagen was used as the substrate.[7] We anticipated that it might be possible to detect such an isotope effect with the smaller substrate because of the smaller forward commitment to catalysis for **20** compared to procollagen.

The synthesis of the deuteriated substrate **19** is outlined in Scheme 3. The nondeuteriated substrate **20** was synthesized in an identical manner from N-Boc-Pro. The V/K isotope effect was determined by incubating a mixture of **19** and **20** with the human enzyme,[8] extracting the reaction mixture with dichloromethane and measuring the deuterium content of the extracted substrate and product by FABMS. This mode of analysis was possible because of the deuterium label on the ester of **19**. The V/K isotope effect was then calculated as described by Cleland[9] and found to be 3.4 ± 0.2 (four determinations). When the reaction rate was determined by measuring the rate of carbon dioxide production from α-ketoglutarate, no isotope effect was detected on V/K.

In general, a V/K isotope effect will be observed if the C–H bond cleavage is kinetically significant and occurs at or before the first irreversible step in the reaction sequence. Since the formation of carbon dioxide and the

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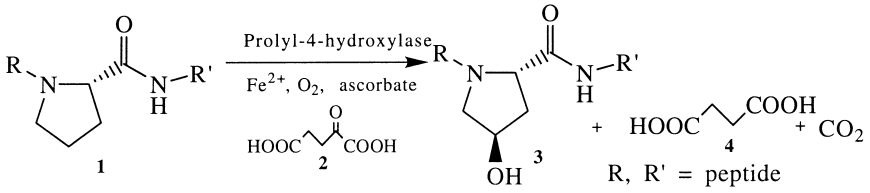
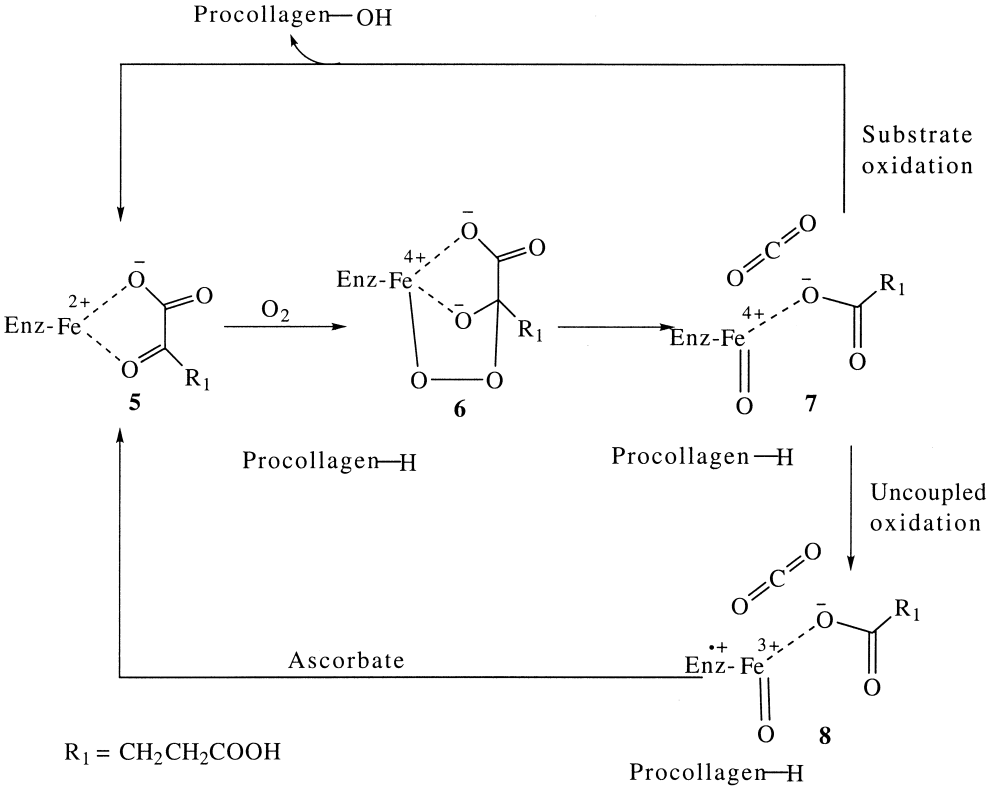


Scheme 1.

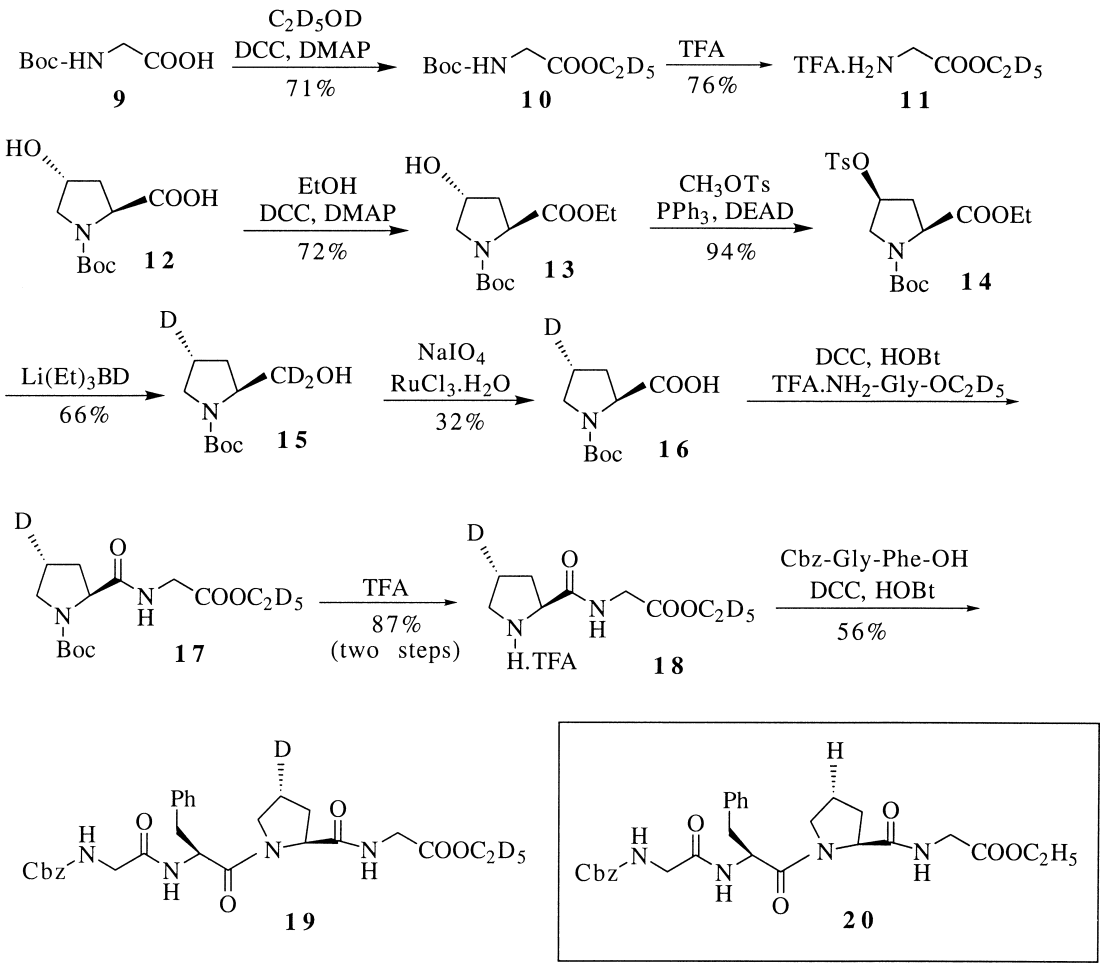


Scheme 2.

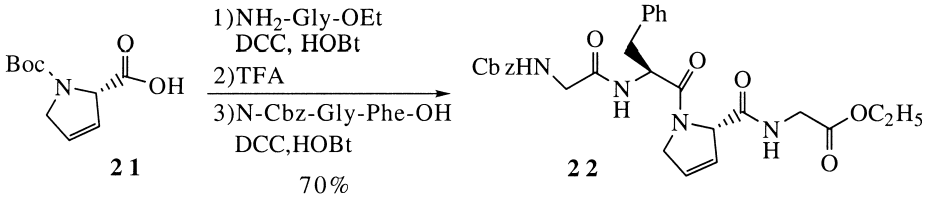
ferryl intermediate **7** is very unlikely to be reversible, it is not surprising that there is no V/K isotope effect on the rate of carbon dioxide formation. The observed V/K isotope effect on the rate of the substrate hydroxylation is likely to result from the uncoupled oxidation which makes it possible for **7** to return to free enzyme and peptide substrate. Thus, with the deuterio substrate, a primary isotope effect on the hydrogen atom abstraction would result in a greater amount of enzyme oxidation to give **8** than would occur with the protio substrate.[10–12] We therefore propose that the V/K isotope effect on prolyl-4-hydroxylase may be a useful mechanistic probe for studying the uncoupled oxidation.

The inhibition of prolyl-4-hydroxylase by dehydroproline has been previously described.[13,14] A reasonable mechanism for this inhibition involves the epoxidation of the double bond followed by alkylation of the enzyme. This proposal is supported by the observation that both proline-3-hydroxylase and proline-4-hydroxylase catalyze the epoxidation of dehydroproline.[15,16] We tested this hypothesis using the small dehydroproline containing peptide **22**, which was synthesized as outlined in Scheme 4.[17]

When **22** was incubated with prolyl-4-hydroxylase under standard assay conditions,[8] no product formation was detected by chromatographic and MS analysis of the dichloromethane extracted peptide. However, when the enzymatic reaction was monitored by measuring the rate of carbon dioxide formation from α-ketoglutarate, **22** appeared to be a very good substrate for the enzyme ($K_m = 200$ μM, $V_{max} = 95\%$, compared to $K_m = 520$ μM, $V_{max} = 30\%$ for N-Cbz-Gly-Phe-Pro-Gly-OEt and $K_m = 200$ μM and $V_{max} = 100\%$ for $(Pro\text{-}Pro\text{-}Gly)_{10}$). This suggests that the uncoupled oxidation is the sole reaction pathway for the dehydroproline containing peptide and that **22** may also be a useful mechanistic probe for this reaction.[18–20]



Scheme 3.



Scheme 4.

Acknowledgements

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