BIOORGANIC CHEMISTRY 8, 219–226 (1979)

α-Ketoglutarate Dependent Dioxygenases: A Mechanism for Prolyl Hydroxylase Action

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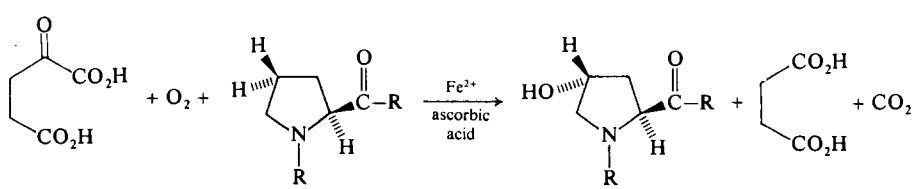
Received August 28, 1978

The enzymatic conversion of proline residues to hydroxyproline residues within collagen peptides is discussed in mechanistic terms. The chemistry of previously suggested reaction mechanisms is reviewed, and a new mechanism which is based on recently published data is proposed. The key issue in the new interpretation is the coupling of the exothermic oxidative decarboxylation of α-ketoglutaric acid to the endothermic production of an oxo-iron species, which then stereospecifically effects an aliphatic hydroxylation of the proline ring.

The study of enzymatic oxidations in which molecular oxygen is used to convert olefinic, aromatic, or aliphatic substrates into the corresponding hydroxylated derivatives has led to current interest in the class of enzymes known as mixed function oxidases (*1*). These metaloenzymes, which effect a wide variety of biochemical oxidations, require, in addition to the substrate of interest, a stoichiometric equivalent of a coreductant. Prolyl hydroxylase was the first enzyme recognized as a member of the group of 2-ketoglutaric acid (αKG) dependent mixed function oxidase enzymes (*2–5*). Other αKG coupled oxygenases include: lysyl hydroxylase, γ-butyrobetaine hydroxylase, thymine-7-hydroxylase, 5-hydroxymethyluracil dioxygenase, 5-formyluracil dioxygenase, and pyrimidine deoxyribonucleoside 2′-hydroxylase. These enzymes are universal and occur in plants and animals, as well as microorganisms.

Prolyl hydroxylase (EC 1.14.11.2) is the enzyme responsible for the biosynthetic conversion of peptidyl bound proline **1** into the *trans*-4-hydroxyprolyl derivative, **2**, as shown below. In this reaction, a stoichiometric amount of both the cosubstrate (αKG) and molecular oxygen are consumed to produce one equivalent each of succinic acid, carbon dioxide, and product **2**. The K_m values for Fe^{2+}, αKG, ascorbic acid, proline substrate, and oxygen for prolyl hydroxylase isolated from chick embryo are 5 μM, 10 μM, 300 μM, 60 μM, and 4.0 vol%, respectively (*2*). Similar values were obtained from rat skin enzyme. Typically prolyl hydroxylase, isolated from various tissues, is a tetramer composed of two nonidentical subunits with molecular weights of approximately 60 000 and 64 000. That the enzyme is truly an oxygenase, has been demonstrated by the fact that labeled oxygen ($^{18}O_2$) is incorporated into **2** (*6*, *7*, *2b*).



1 **2**

R = peptide

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Prolyl hydroxylase requires αKG (*8*, *2b*). The only other cosubstrates that can replace it are also 2-keto acids (*9*). The introduction of glutamate dehydrogenase, NH_4^+, and NADPH to reductively aminate αKG to glutamic acid induces total loss of propyl hydroxylase activity (*2*). Subsequent addition of αKG restores activity. Neither thiamine pyrophosphate nor pyridoxal phosphate is required by the enzyme to effect decarboxylation of αKG to succinic acid.

The iron requirement is specific for ferrous ion; no other metals can replace it. The introduction of iron chelating agents, such as EDTA, bipyridyl, or *o*-phenanthroline, have been reported to both inhibit or have no effect on the hydroxylation (*2*). Thus, how tightly the iron is bound is not yet clear. Spectroscopic studies indicate that after purification the enzyme contains only trace amounts of iron, much less than one equivalent (*10*, *2b*). No heme or cytochrome cofactors are required for activity.

The ascorbic acid cofactor may in some enzyme preparations be replaced by either dithiothreitol or tetrahydropteridine derivatives with only partial loss of enzyme activity (*8*, *11*, *12*). However, highly purified enzyme appears to strictly require ascorbate, but it is not consumed in stoichiometric proportion to the hydroxylation reaction (*2b*). This nonstoichiometric requirement (and possible lack of specificity) may indicate that ascorbate does not participate in the actual chemistry for the conversion of **1** into **2** (*9*). A possible role for ascorbate is maintenance of cysteine groups in the enzyme at a reduced sulfhydryl state by inhibiting disulfide bond formation. Indeed, sulfhydryl specific reagents, such as *p*-chloromercuribenzoate and *N*-ethylmaleimide inhibit prolyl hydroxylase activity (*13*). Thus, cysteine residues may be at the active site, perhaps coordinated to the iron.

Studies which have employed the vasoactive nonapeptide bradykinin (shown below) and several derivatives, as prolyl hydroxylase substrates, revealed that the relative rate for hydroxylation of the reactive proline depends upon the nature of the neighboring amino acids (*2*, *14*).

Arg-Pro-Pro*-Gly-Phe-Ser-Pro-Phe-Arg

bradykinin

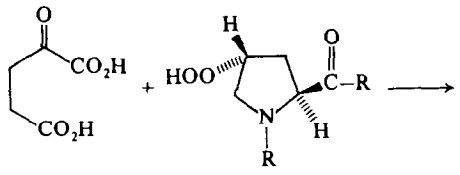
(Only Pro* is hydroxylated)

Whereas replacement of either proline or glycine in the essential sequence -X-Pro-Gly- (X in unsubstituted bradykinin is Pro) by other amino acids completely blocks hydroxylation, replacement of X by alanine increases the hydroxylation rate by a factor of 2.6. Amino acid substitution or addition, remote to the essential tripeptide, had a variable effect on the reaction rate—enhancement and retardation by factors from 3 to 10 were observed (*2a*). Thus, the variation in the amino acid content of collagen may contain a key to determining the extent and efficiency of proline hydroxylation.

Although the mechanism for such hydrocarbon reactions is not well understood and has minimal precedent in organic reactions (*15*), several pertinent facts have emerged from biochemical studies on the enzyme (*2*, *4*). First, if $^{18}O_2$ is used, half of the label is found in the hydroxyl group of hydroxyproline, whilst the other half is found in one of the carboxyl groups of succinic acid. None of the label is found in the water. Furthermore, the decarboxylation reaction may proceed, although at a slower rate, if the proline-containing substrate is absent (*16*). Finally, while basic hydrogen peroxide is

known to readily produce succinic acid from 2-ketoglutaric acid, hydrogen peroxide is not the active oxidizing agent in the enzymatic reaction (*2*).

In a series of papers generally concerned with the role of 2-keto acids in enzymatic oxidations, the suggestion has been made that an initially formed hydroperoxide derivative of the appropriate substrate attacks the 2-ketoglutaric acid and produces the observed products, as shown below for the hydroxylation of proline (*2*, *17*). This



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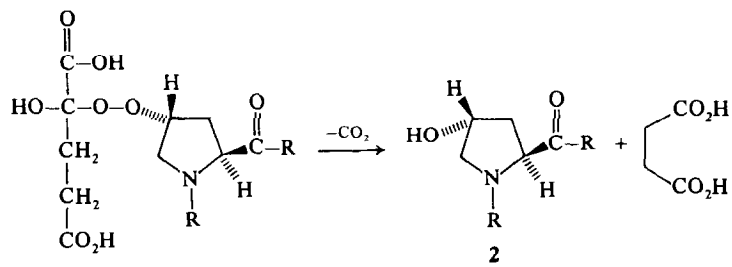
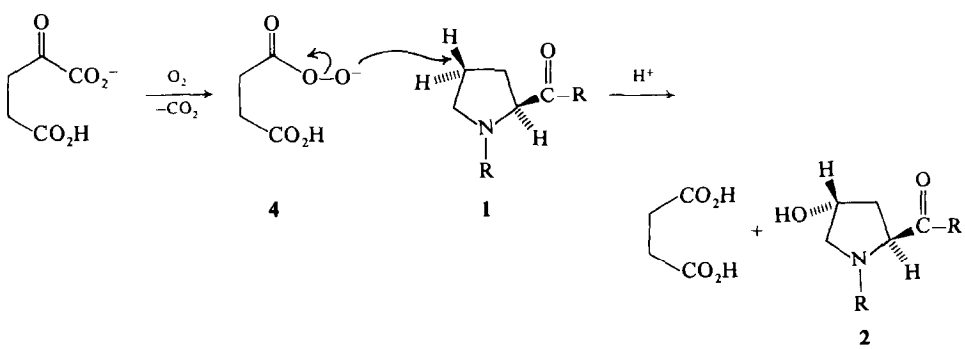


2

mechanism has been criticized by Hamilton, who questioned how the hydroperoxide derivative of an unreactive aliphatic substrate could be initially formed (*18*). Indeed, the reduction of **3** to **2** involves cleavage of an oxygen–oxygen bond, and not a carbon–oxygen bond; thus, the route to produce **3** with the stereochemistry shown would need to be elucidated. In addition, as stated above, prolyl hydroxylase catalyzes the decarboxylation of 2-ketoglutaric acid in the absence of the proline substrate (*2*, *4*, *16*). Thus, **3** is not an obligatory intermediate in the decarboxylation reaction.

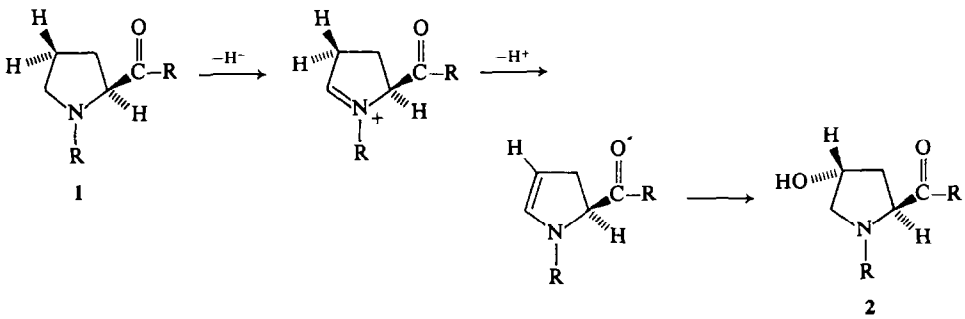
Hamilton, on the other hand, has suggested an oxenoid mechanism, shown below, in which peroxysuccinic acid **4** produced from 2-ketoglutaric acid, is responsible for the hydroxylation (*3*, *18*). However, doubt is cast on this mechanism, since **4** cannot be used



4 **1** **2**

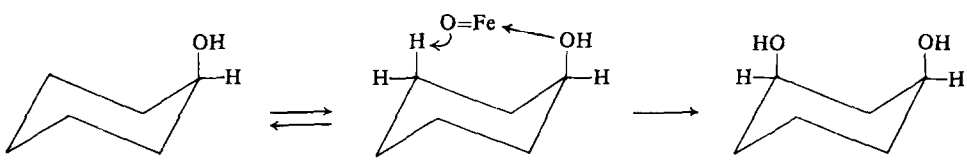
in place of 2-ketoglutaric acid for the enzymatic reaction (2). Moreover, unactivated aliphatic hydrocarbons are normally stable to mild peracids, such as **4**.

An equally unlikely alternative mechanism to those previously suggested involves proline oxidation *via* an initial dehydrogenation reaction (*19a*). Intermediates shown in the following general scheme are analogous to those proposed for the biosynthesis of proline from glutamic acid (*19b*). An advantage to this route would be that it makes use of established enamine chemistry. However, the biosynthetic dehydrogenation utilizes $NADP^+$, a cofactor that is not required by prolyl hydroxylase. Moreover, for peptidyl-bound proline, as opposed to the free amino acid, the cationic nitrogen would be unfavorably adjacent to the carbonyl group of the amide bond to R.



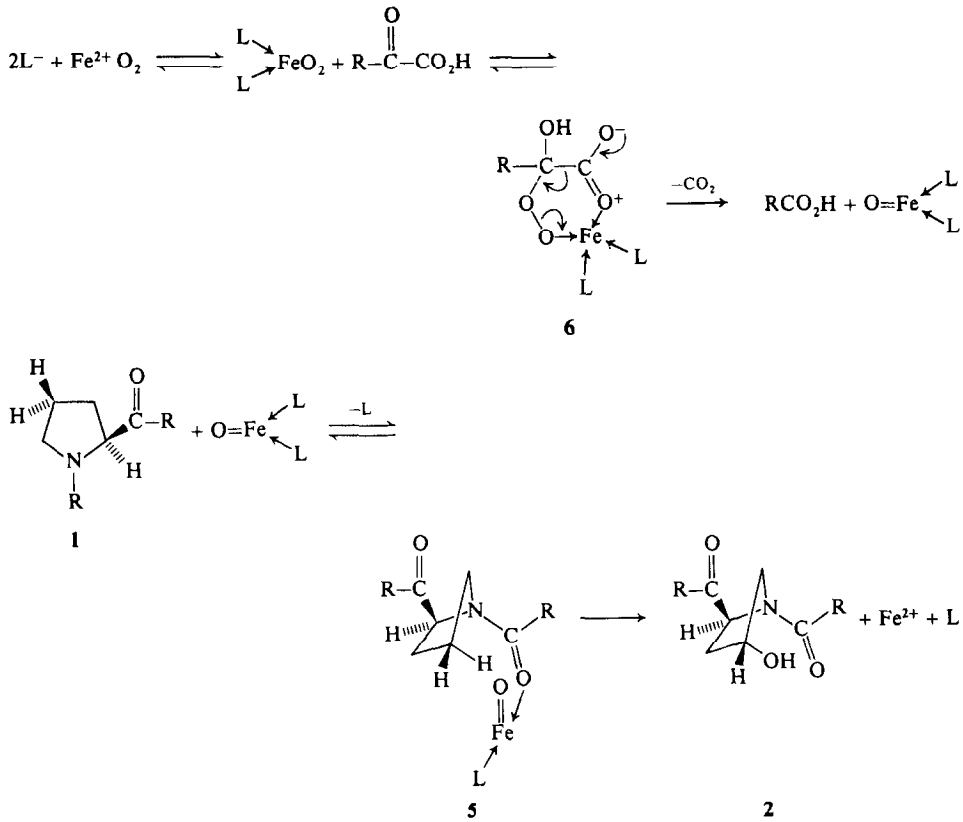
Based on quantum mechanical calculations, Hurych has proposed a synchronous mechanism involving simultaneous decarboxylation and hydroxylation promoted by either superoxide or hydroperoxide anion (*20*). He invokes direct activation of molecular oxygen by iron coordinated to sulfhydryl groups on the enzyme surface. No role is given for iron in the subsequent oxidation of the organic substrates, and no mechanism for formation of products is specified.

In the absence of any enzyme, chemical models for aliphatic hydroxylations are inefficient and have suffered from poor yields. For example, the Undenfriend model, which consists of oxygen, iron, and ascorbic acid, was used by Hamilton to produce cyclohexanol from cyclohexane in yields of the order of 0.1% (*21*). Chvapil and Hurych have also used the Undenfriend reagent to convert proline to hydroxyproline, but here again the yields are on the order of 3% (*22*). Although these products mimic those of enzymatic oxidations, the efficiency is much less than that of an enzyme. More recently, Groves has employed Fenton's reagent (Fe^{2+}/H_2O_2) for the hydroxylation of cyclohexanol, a reaction that gives 96% stereochemical yield of *cis*-1,3-cyclohexanediol, but an overall mass yield of only about 10% (*23*, *24*). An oxo-iron intermediate in which the hydroxyl group of cyclohexanol coordinates to the iron, was proposed to account for the high stereoselectivity of the diol product.



On the basis of available enzymological information and established chemical precedent, the hydroxylation of proline may also be carried out by a high energy oxo-iron intermediate, which is efficiently formed in conjunction with an exothermic

decarboxylation. As shown below, initial attack at the α carbonyl of αKG by an iron–oxygen complex (FeO_2^{2+}) could lead to an intermediate, **6**, which can subsequently decarboxylate. The active iron oxidant may then stereospecifically produce the product, *trans*-4-hydroxyproline, **2**, perhaps through chelation to a proline amide oxygen, as shown in **5** (*25*). In this manner, the exothermic decarboxylation, which should also be

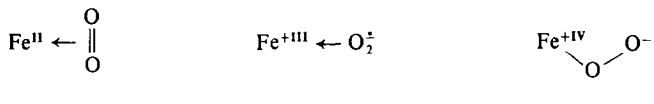


R = petide
L ligand (perhaps ascorbate)

energetically favorable from entropy consideration (formation of two molecules, CO_2 and RCO_2H, from one, $RCOCO_2H$) can provide the driving force required to produce efficiently an active iron oxidant.

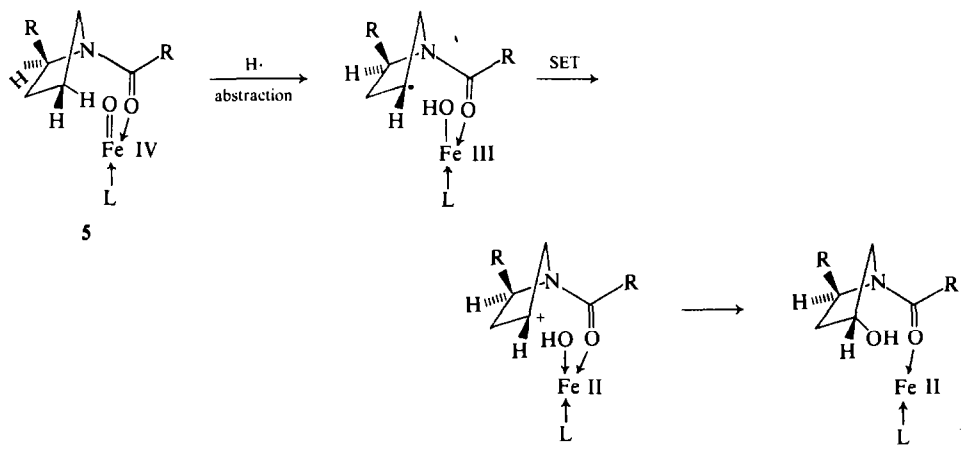
Several points relative to this mechanistic process need to be considered: (i) the likelihood that iron can directly activate molecular oxygen and nucleophilically attack αKG, (ii) the ability of αKG to act as a two-electron reductant and thereby generate an oxo-iron species, (iii) the ease with which an oxo-iron species can stereospecifically insert oxygen into a carbon–hydrogen bond, and (iv) the probability that the oxidative decarboxylation is, in fact, coupled to the hydrocarbon oxidation. Each of these points will be discussed briefly, since we are currently working on chemical models to evaluate the kinetic and thermochemical implications of the proposed mechanism. Further critical analysis must await experimental data.

Iron porphyrins are well-established carriers of activated molecular oxygen in both natural and synthetic systems. In the absence of that ligand system oxygen may coordinate to iron with any of several likely bonding arrangements.



π-complex superoxide complex σ-complex

As the proposed mechanism indicates, the precise oxidation state of iron and the bonding geometry of oxygen are not critical issues at this stage. All three complexes shown would, however, appear to be reasonable reactive intermediates (*1*, *26–28*). Moreover, a highly oxidized iron species has been proposed as the active oxidant for the oxidative decarboxylation of benzoylformic acid in aqueous acid (*29*). The stereochemistry for the conversion of **1** into **2** could be accounted for by the "bicyclic" nature of **5** in which an amide bond coordinates and internally delivers the oxidant (*24*). Although iron and other metal ions in high oxidation states [Cu(III)] are known to coordinate to peptide bonds (*30*, *31*), the proposed mechanism is not limited to such a process (*25*). Indeed, the iron may be bound to ascorbate or to sulfhydryl groups on the enzyme, and could stereoselectively deliver an oxygen to the appropriate carbon–hydrogen bond from that vantage point. A plausible mechanism for the oxygen insertion would involve (A) initial hydrogen atom abstraction by the oxo-iron intermediate to produce a secondary carbon radical and an iron(III) hydroxide complex, (B) a single electron transfer in which ferric ion oxidizes the radical to a carbonium ion, and (C) simple hydroxide collapse onto the cation (*24*, *32*).



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The essential rationale for the suggested mechanism for prolyl hydroxylase centers on the proposal that a highly exothermic decarboxylation can be coupled through an oxo-iron intermediate to an aliphatic oxidation that has a very endothermic activation barrier. The fact that in the enzyme-catalyzed process the oxidative decarboxylation of αKG can reliably be uncoupled from proline hydroxylation (*2*, *16*) provides support for a tandom mechanism. In the absence of substrate **1**, αKG produces succinic acid, but

in the absence of αKG, **1** is not converted into **2**. Hence, decarboxylation must either be synchronous with or precede the hydroxylation. The mechanism proposed herein delineates a sequential process for the consumption of molecular oxygen. Indeed, this suggestion is a subset of the general proposition that potential energy stored in specific chemical bonds of one substance may be made available for use by another species through the energy transfer inherent in a chemical reaction that involves those bonds. Frequently, Nature thermodynamically couples the hydrolysis of ATP to other chemically unrelated processes in order to provide the driving force responsible for enzymatic catalysis. Thus, it seems only reasonable that this same concept, although modified in the spedific manner in which energy is mechanically utilized, would be applied to other enzymatic reaction (*33*).

As another aspect of research in this area, current synthetic organic chemical methods for the controlled oxidation of aliphatics are with only a few exceptions inefficient and unselective (*34*). Thus, the key to this chemistry may likely be found in an examination of models for this class of enzyme-catalyzed reactions.

ACKNOWLEDGMENTS

The author wishes to express sincere gratitude for many fruitful discussions with Professor Richard Johnson of Harvard University. Support for this work from the National Institutes of Health under Grant AM-21975, and the University of Minnesota Graduate School is gratefully acknowledged.

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