*Proc. Natl. Acad. Sci. USA*
Vol. 75, No. 5, pp. 2145–2149, May 1978
Biochemistry

# Prolyl hydroxylase half reaction: Peptidyl prolyl-independent decarboxylation of α-ketoglutarate

(collagen/hydroxyproline/uncoupled decarboxylation/proline,2-oxoglutarate dioxygenase)

DAVID F. COUNTS*, GEORGE J. CARDINALE, AND SIDNEY UDENFRIEND†

Roche Institute of Molecular Biology, Nutley, New Jersey 07110

*Contributed by Sidney Udenfriend, February 9, 1978*

**ABSTRACT** **Prolyl hydroxylase (proline,2-oxoglutarate dioxygenase, EC 1.14.11.2) is a mixed-function oxygenase that hydroxylates peptidyl proline with the simultaneous and stoichiometric decarboxylation of α-ketoglutarate to succinate and $CO_2$. It has been found that highly purified preparations of the enzyme can decarboxylate α-ketoglutarate in the absence of a peptidyl proline substrate. The uncoupled decarboxylation proceeds at only a fraction of the rate of the whole reaction and for study requires substrate quantities of the pure enzyme, as well as oxygen, ferrous ion, and ascorbate. No hydroxyproline is formed under these conditions. Immobilized antiserum to prolyl hydroxylase was found to remove both activities from enzyme preparations. However, addition of free antiserum during incubation inhibits only the complete reaction. Poly(L-proline), a specific inhibitor of prolyl hydroxylation, enhances the uncoupled decarboxylation of α-ketoglutarate without itself being hydroxylated. All of these findings prove that α-ketoglutarate can serve as substrate in the absence of peptidyl proline and is most likely the initial site of attack by oxygen. In the coupled reaction an oxidized form of the keto acid, perhaps a peroxy acid, then attacks prolyl residues in the unhydroxylated substrate.**

Prolyl hydroxylase (proline,2-oxoglutarate dioxygenase, EC 1.14.11.2) catalyzes the hydroxylation of peptidyl proline with the concomitant decarboxylation of α-ketoglutarate. For each mole of proline hydroxylated, one mole of α-ketoglutarate is stoichiometrically decarboxylated to succinate and carbon dioxide. The enzyme also has an absolute requirement for ferrous ion and a reducing agent (1).

The hydroxyl group of the hydroxyproline is derived from molecular oxygen (2, 3). After the specific and absolute requirement of the enzyme for α-ketoglutarate was discovered (1, 4), several other oxygenase systems that require α-ketoglutarate were described (5–9). Lindblad *et al.* (10) reported that $^{18}O$ from molecular oxygen is incorporated not only into carnitine during the γ-butyrobetaine hydroxylase reaction, but also into the succinate derived from α-ketoglutarate. Cardinale *et al.* (11) were able to demonstrate that prolyl hydroxylase catalyzes the incorporation of equal amounts of $^{18}O$ into peptidyl hydroxyproline and into the succinate derived from α-ketoglutarate. On the basis of such findings, Holme *et al.* (12) proposed as a mechanism for the hydroxylation of γ-butyrobetaine that a peroxide bridge is formed between the α-ketoglutarate and γ-butyrobetaine. Such an intermediate could then dissociate to yield the hydroxylated γ-butyrobetaine (carnitine) and succinate. It was proposed (12) that a hydroperoxide is first formed on γ-butyrobetaine; this hydroperoxide could then attack α-ketoglutarate and the intermediate would then decompose to carnitine, succinate, and $CO_2$. An analogous reaction mechanism, involving the initial formation of a hydroperoxide on the number four carbon of the prolyl residue, can be postulated for prolyl hydroxylase (13). Hamilton (14), however, pointed out that because the substrates of all the α-ketoglutarate oxygenases are simple aliphatic compounds of low chemical reactivity, it would be difficult to explain how they would yield peroxides in the first place. He proposed, instead, an initial oxidative attack on the α-ketoglutarate to yield peroxysuccinic acid. Such an intermediate could then attack the other substrate through an oxenoid mechanism, yielding the same type of peroxide bridge that was suggested by the $^{18}O_2$ experiments (10, 11).

It seemed likely that if oxygen initially attacks the α-ketoglutarate, decarboxylation of α-ketoglutarate might occur in the absence of the peptidyl cosubstrate if the oxydation product of the keto acid were unstable. Although no such uncoupling had been seen with γ-butyrobetaine hydroxylase (15), uncoupling was observed with purified rat skin prolyl hydroxylase (13). To rule out the possibility that the findings were due to hydroxylation of traces of underhydroxylated collagen bound to the purified enzyme or hydroxylation of the enzyme itself, it was necessary to utilize substrate quantities of enzyme with α-ketoglutarate and demonstrate that there was no concomitant formation of hydroxyproline. In this paper we present evidence for the catalysis of an uncoupled decarboxylation of α-ketoglutarate by prolyl hydroxylase.

## MATERIALS AND METHODS

**Materials.** Skins of 1-day-old rats were purchased from Pel-Freeze. Poly(L-proline) (type II) and sodium α-ketoglutarate were purchased from Sigma Chemical Co. L-[4-$^3$H]-Proline and α-keto[1-$^{14}$C]glutarate were obtained from New England Nuclear. *p*-Dimethylaminobenzaldehyde was purchased from Fisher Chemical Co. and fluorescamine (Fluram®) was obtained from Hoffmann–La Roche, Inc. Reduced and carboxymethylated collagen (RCM-collagen) from the cuticle of *Ascaris lumbricoides* (16) was purchased from Applied Science Laboratories, Inc. Monoperoxysuccinic acid was synthesized by B. A. Pawson of Hoffmann–La Roche, Inc., Nutley, NJ.

**Measurement of Enzyme Activity.** Prolyl hydroxylase activity was assayed in two ways, by release of tritium from a tritiated preparation of underhydroxylated collagen (4) and by evolution of $^{14}CO_2$ from α-keto[1-$^{14}$C]glutarate (17). For the tritium release assay, the incubation mixture contained the

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviation: RCM-collagen, reduced and carboxymethylated collagen.

* Present address: Washington State University, Department of Veterinary Pathology, Pullman, WA 99163.
† To whom reprint requests should be addressed.

Table 1. Relationship between α-ketoglutarate decarboxylation and hydroxyproline formation

| Reaction | Conditions | $^{14}CO_2$ released, nmol | Hydroxyproline formed, nmol |
|---|---|---|---|
| Chemical blank | Enzyme omitted | 0.8 | Not detectable |
| Uncoupled decarboxylation | Prolyl substrate omitted, 0.27 mg of enzyme (D) | 4.8 | Not detectable |
| Hydroxylation | Complete, 0.02 mg of enzyme (D) | 4.9 | 4.8 |
| Hydroxylation | Complete, 0.27 mg of enzyme (D) | 22.2 | 21.6 |
| Uncoupled decarboxylation | Prolyl substrate omitted, 0.05 mg of enzyme (A) | 4.8 | Not detectable |
| Hydroxylation | Complete, 0.05 mg of enzyme (A) | 8.1 | 8.1 |

Before assay, enzyme solutions were diluted with 0.05 M Tris·HCl buffer, pH 7.35, made 100 μM in dithiothreitol and 10 μM in EDTA, and allowed to stand 30 min prior to incubation at 30° for 120 min in the reaction mixture. An enzyme preparation from DEAE-Sephadex chromatography (D) or affinity chromatography (A) was used in each incubation. The reaction was stopped by adding 0.2 ml of 6 M HCl, and the incubation was continued for 30 min more to release $^{14}CO_2$, which was then trapped on base-impregnated paper. The amount of $^{14}CO_2$ released by the chemical blank has been subtracted from the total amount of $^{14}CO_2$ released by the uncoupled decarboxylation reaction. The amount of $^{14}CO_2$ released by the hydroxylation reaction represents the $^{14}CO_2$ released by the total reaction mixture minus the $^{14}CO_2$ released by the chemical blank plus the uncoupled decarboxylation reaction. After incubation, the reaction mixtures were made 6 M with respect to HCl. The samples were then hydrolyzed for 15 hr at 110° and assayed for hydroxyproline content.

following components in a volume of 1 ml: Tris·HCl buffer, pH 7.2, 50 μmol; α-ketoglutarate, 0.1 μmol; sodium ascorbate, 0.5 μmol; ferrous ammonium sulfate, 0.1 μmol; bovine serum albumin, 2 mg; bovine liver catalase (Boehringer), 400 μg; tritiated substrate (4); and enzyme preparation. The incubation mixture for the decarboxylation assay contained the same components with the following exceptions: 0.1 μmol of α-keto[*1*-$^{14}$C]glutarate (specific activity 0.32 μCi/μmol) replaced nonradioactive α-ketoglutarate, and 100 μg of RCM-collagen replaced the tritiated substrate. Uncoupled decarboxylation of α-ketoglutarate was measured by omitting the RCM-collagen from the assay mixture. All incubations were carried out at 30°. Radioactivity was determined in a liquid scintillation spectrometer, using Bray's solution.

**Preparation of Prolyl Hydroxylase.** Prolyl hydroxylase was purified through the DEAE-Sephadex chromatography step as described by Rhoads and Udenfriend (17), using Tris·HCl buffer, pH 7.35, instead of sodium cacodylate, pH 7.0. The fractions obtained from DEAE-Sephadex chromatography that contained prolyl hydroxylase activity were pooled and the protein was precipitated by bringing the solution to 60% saturation with respect to ammonium sulfate. The precipitate was dissolved in 0.5 M Tris·HCl, pH 7.35, containing 100 μM dithiothreitol and 10 μM EDTA and dialyzed against this same buffer to remove ammonium sulfate. The enzyme at this state of purification is approximately 20% pure. The uncoupled reaction has been obtained with enzyme preparations that are essentially homogeneous (13), but the above enzyme preparation was found suitable for most of the experiments reported here.

Prolyl hydroxylase was also purified by affinity column chromatography with an RCM-collagen-linked agarose column (18), using poly(L-proline) as the eluting agent (19). Due to the difficulty of removing all traces of poly(L-proline) from the enzyme, such preparations exhibit somewhat altered reaction properties.

**Gel Electrophoresis.** Electrophoresis was carried out on polyacrylamide gels according to the method of Davis (20), and the gels were stained for protein as described by Weber and Osborn (21). When gels were sliced to detect activity, the protein bands were visualized by fluorescent staining with anilinonaphthalene sulfonate (22) prior to the slicing.

**Hydroxyproline Assay.** Following incubation with enzyme, concentrated HCl was added to each reaction mixture to bring it to 6 M and the sample was hydrolyzed *in vacuo* at 110° for 24 hr. The hydrolysate was mixed with approximately 150 mg of Norit A activated charcoal (Fisher) and then filtered through Whatman no. 1 filter paper. The filtrate was brought to dryness and assayed for hydroxyproline by the method of Prockop and Udenfriend (23) with the following modifications. After oxidation with chloramine-T, the reaction mixture was extracted three times with toluene to remove all the oxidation products of proline. To increase the sensitivity of the assay, the chromophore was extracted into 1 ml of water by the method of LeRoy *et al.* (24).

**Protein Assay.** Protein was measured by dialyzing the solutions exhausitively against 0.05 M sodium phosphate, pH 8.00, and assaying with fluorescamine (25), using bovine serum albumin as a standard.

**Antiserum to Prolyl Hydroxylase.** A monospecific antiserum to rat skin prolyl hydroxylase was prepared as described by Roberts *et al.* (26).

**Affinity Column of Antibody to Rat Skin Prolyl Hydroxylase.** An agarose gel affinity column was made by linking antiserum directed against rat prolyl hydroxylase to Sepharose 4B as described by Primus *et al.* (27).

## RESULTS

When prolyl hydroxylase is incubated with α-ketoglutarate in the absence of a peptidyl prolyl substrate, there is an enzyme-dependent decarboxylation of α-ketoglutarate (Table 1). This reaction occurs with both the DEAE-Sephadex enzyme preparation and the preparation purified by affinity chromatography. To enhance the uncoupled decarboxylation, longer incubation times were used along with large amounts of enzyme. Under such conditions (0.27 mg of DEAE-Sephadex enzyme) prolyl hydroxylation in the coupled reaction was no longer linear. However, the enzyme-catalyzed production of up to 7 nmol of $^{14}CO_2$ in the absence of peptidyl substrate was shown to vary linearly with the time of incubation as well as with the amount of enzyme used (data not shown). Table 1 also shows that the $^{14}CO_2$ released in the absence of added peptidyl substrate was not due to hydroxylation of the enzyme itself or of any traces of peptidyl proline substrate possibly remaining on the purified enzyme. Although the hydroxyproline assay is sensitive to 1 nmol of this imino acid, no hydroxyproline could be detected in the uncoupled reaction even when 4.8 nmol of α-ketoglutarate was decarboxylated. The different ratios of the two activities obtained with the DEAE-Sephadex- and affinity-purified enzymes were found to result from traces of poly(L-proline) present in the affinity-purified preparations. As will be demonstrated below, poly(L-proline) inhibits prolyl hydroxylation and stimulates the uncoupled decarboxylation.

Table 2. Selective inhibition of the hydroxylation reaction by antiserum directed against prolyl hydroxylase

| Antiserum | nmol $CO_2$ released: Uncoupled decarboxylation | nmol $CO_2$ released: Hydroxylation |
|---|---|---|
| Omitted | 0.18 | 2.75 |
| Added | 0.18 | 0.70 |

DEAE-Sephadex enzyme preparations (0.1 mg/assay) were reacted for 5 min at 30° with sufficient antiserum to give a 75% inhibition of the hydroxylation reaction. These preparations were then assayed for both activities.

Throughout all the enzyme purification procedures used, the hydroxylation and uncoupled decarboxylation activities could not be separated. To further demonstrate that both activities are associated with the same protein, DEAE-Sephadex-purified enzyme was subjected to electrophoresis on polyacrylamide gels. After staining, several protein bands were detected. In replicate gels the bands were visualized fluorometrically with anilinonaphthalene sulfonate (22), cut out, added to the reaction mixture, and assayed for both prolyl hydroxylation and uncoupled decarboxylation of α-ketoglutarate. In all experiments, only one of the protein bands was found to be active and to contain both activities. This band migrated to the same position as homogeneous enzyme preparations prepared by RCM-collagen-linked affinity chromatography (18).

Previous reports of the inhibition of prolyl hydroxylation by antiserum to rat skin prolyl hydroxylase (26) suggested another method of testing the separability of the two activities. When partially purified enzyme preparations were treated with specific antiserum and then assayed, prolyl hydroxylase activity was decreased 75% (Table 2). Surprisingly, uncoupled decarboxylation was unaltered. When the experiment was repeated with homogenous enzyme purified by affinity chromatography, similar results were obtained. On the other hand, antiserum linked to Sepharose in the form of antibody affinity columns removed both activities (Table 3), indicating that both are associated with the same protein. The significance of this apparent inconsistency will be dealt with in the *Discussion*.

Another indication that both activities are properties of the same enzyme is that they have the same temperature optimum (35°) and sensitivity to heat denaturation. When the enzyme was heated at 42.5°, both activities were lost at approximately the same rate (Fig. 1).

The hydroxylation of peptidyl proline has strict cofactor

Table 3. Removal of uncoupled decarboxylation activity and prolyl hydroxylation activity by antibody affinity chromatography

| Enzyme preparation | nmol $CO_2$ released: Uncoupled decarboxylation | nmol $CO_2$ released: Hydroxylation |
|---|---|---|
| Before passage through affinity column | 0.24 | 3.24 |
| After passage through affinity column | 0.08 | 1.13 |

A DEAE-Sephadex enzyme preparation (0.6 mg) in 1.5 ml of 0.05 M Tris·HCl, pH 7.35/100 μM dithiothreitol/10 μM EDTA buffer was passed through a 0.3-cm × 7-cm column filled with 1.5 ml of an affinity gel prepared from an antiserum directed against rat skin prolyl hydroxylase. The column was washed with 2.0 ml of the same buffer. The filtrate was collected and assayed for both activities. Each assay contained 0.15 mg of enzyme preparation and was incubated for 30 min. Reactions were stopped and analyzed as previously described.

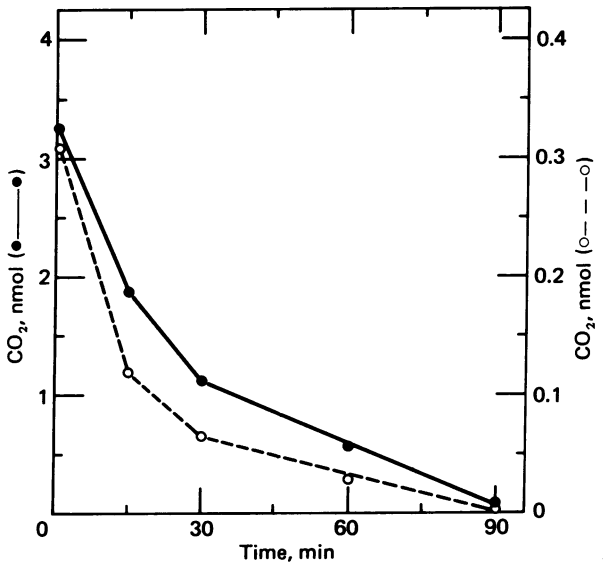


FIG. 1. Thermal denaturation of uncoupled decarboxylation and hydroxylation activity. Enzyme (DEAE-Sephadex, 0.04 mg/assay) was incubated at 42.5° for the time indicated. The enzyme was then added to the reaction mixture and allowed to react for 30 min. ○- - -○ represents the uncoupled decarboxylation and ●—● represents the hydroxylation reaction.

requirements (4). As shown in Table 4, the omission of any of the cofactors of the enzyme resulted in comparable diminution of the uncoupled decarboxylation and hydroxylation. Although there were no differences in cofactor requirements between the two reactions, the pH optimum for prolyl hydroxylation was found to be 7.8 while the uncoupled decarboxylation exhibited no pH dependence between pH 6.7 and pH 7.8 (data not shown).

The apparent $K_m$ for α-ketoglutarate was found to be 28 μM in both the uncoupled decarboxylation reaction and in the overall hydroxylation reaction. Measurements were made with all other cofactors at saturating levels. These $K_m$ values are in good agreement with those previously reported for α-ketoglutarate (10 μM) (16) in the overall hydroxylation reaction. Hutton *et al.* (4) reported that oxaloacetate could substitute, albeit poorly, for α-ketoglutarate in the hydroxylation assay. Similarly, we found that, in the uncoupled decarboxylation assay, nonradioactive oxaloacetate inhibited the decarboxylation of α-ketoglutarate.

An important difference between the two reactions was found when they were compared in the presence and absence of poly(L-proline), a specific, competitive inhibitor of prolyl hydroxylase (28). As shown in Table 5, addition of poly(L-proline) to DEAE-Sephadex-purified enzyme decreased the

Table 4. Cofactor dependence for uncoupled decarboxylation and hydroxylation reactions

| Cofactor omission | nmol $CO_2$ released: Uncoupled decarboxylation | nmol $CO_2$ released: Hydroxylation |
|---|---|---|
| None | 0.14 | 1.83 |
| Ascorbate | 0.03 | 0.20 |
| Ferrous ion | 0.02 | 0.21 |
| $O_2$ | 0.04 | 0.59 |
| $O_2$ returned | 0.14 | 1.83 |

The reaction mixtures were incubated for 30 min in the presence of 0.04 mg of DEAE-Sephadex-purified enzyme per assay.

Table 5. Alteration of uncoupled decarboxylation and prolyl hydroxylation by poly(L-proline)

| Poly(L-proline), nM | nmol $CO_2$ released | |
|---|---|---|
| | Uncoupled decarboxylation | Hydroxylation |
| 0.0 | 1.57 | 24.1 |
| 1.75 | 2.60 | 16.3 |
| 17.5 | 2.51 | 11.2 |

In each incubation 1.14 mg of DEAE-Sephadex-purified enzyme was added in the presence of the indicated concentration of poly(L-proline). The reaction mixtures were incubated for 30 min at 30° and assayed as previously described.

overall hydroxylation reaction as had been shown previously. However, it did not inhibit and, in fact, slightly increased the uncoupled decarboxylation (Table 5). The possibility remained that with such large amounts of enzyme some of the prolyl residues in the poly(L-proline) were hydroxylated. However, no hydroxyproline could be detected in reaction mixtures that contained poly(L-proline) but no peptidyl substrate.

One of the possible intermediates that can be produced by oxidation of α-ketoglutarate is peroxysuccinic acid. Unlabeled peroxysuccinate was substituted for α-ketoglutarate in an assay mixture containing dialyzed prolyl hydroxylase and tritium-labeled chick embryo substrate (4). At concentrations both above and below those found to be optimal for α-ketoglutarate, no evidence of hydroxylation was observed. Abbott and Udenfriend (29) have also reported the inability of peroxysuccinate to replace α-ketoglutarate in the pyridine deoxyribonucleoside 2′-hydroxylase reaction, which is also an α-ketoglutarate-requiring oxygenase system.

All the evidence presented thus far indicates that the decarboxylation of α-ketoglutarate can be catalyzed by prolyl hydroxylase in the absence of a prolyl substrate. However, it was also important to determine whether peptidyl proline could react with oxygen in the absence of α-ketoglutarate. To do so, prolyl hydroxylase preparations that had been dialyzed exhaustively were incubated, in the absence of α-ketoglutarate, with chick embryo substrate (4) containing tritium in the 4-*trans* position of proline. Attack by oxygen on proline would be expected to release the tritium label. With as much as 0.13 mg of DEAE-Sephadex-purified enzyme and a 120-min incubation, no tritium was released (<0.002 nmol). With this amount of enzyme and the same incubation time, 1.2 nmol of $CO_2$ was liberated from α-ketoglutarate in the uncoupled decarboxylation reaction.

## DISCUSSION

In a preliminary report (13) it was found that 9 mol of α-ketoglutarate were decarboxylated for every mol of prolyl hydroxylase in a 30-min incubation. This calculation was based on a molecular weight of 130,000 for the enzyme. If one uses the more recently reported molecular weight of 240,000 (30), the ratio becomes 18 mol of α-ketoglutarate decarboxylated per mole of enzyme. In the present report, various procedures were used and all were unable to separate prolyl hydroxylation from the uncoupled decarboxylation of α-ketoglutarate. Both activities were present at all stages of purification, they coelectrophoresed on acrylamide gels, and they were both removed by antibody affinity chromatography. The two activities were also equally sensitive to heat denaturation and had the same cofactor requirements. All these findings indicate that the activities are related. If the initial step of the overall hydroxylation is an oxidative attack on α-ketoglutarate, then the same $K_m$ for α-ketoglutarate in both reactions is further evidence that one enzyme catalyzes both.

Two differences were, however, observed between the two reactions. Antiserum to prolyl hydroxylase had no effect on the uncoupled decarboxylation, but prolyl hydroxylation was markedly inhibited by it. This finding merely indicates that inhibition of the overall reaction by antibody is competitive with peptidyl substrate and not with α-ketoglutarate.

The second difference between the two activities involved their response to poly(L-proline). Hydroxylation was inhibited, whereas the uncoupled decarboxylation was, if anything, slightly enhanced. Inhibition by poly(L-proline) is known to be competitive with peptidyl substrate (28). The slight stimulation of the uncoupled reaction may result from a conformational change of the enzyme produced by the inhibitor. Because poly(L-proline) both increases the uncoupled decarboxylation and inhibits the hydroxylation reaction, it changes the ratio of these two activities.

The data presented in this paper clearly demonstrate that prolyl hydroxylase can catalyze the decarboxylation of α-ketoglutarate in the absence of a prolyl substrate. This uncoupled decarboxylation lends support to the mechanism of prolyl hydroxylation proposed by Hobza *et al.* (31) and outlined below. The sequence begins with (*i*) orientation of α-ketoglutarate and ferrous ion on the surface of the hydroxylase, (*ii*) activation of molecular oxygen by the formation of a complex with bound $Fe^{2+}$, (*iii*) monoprotonation of oxygen and migration of the OOH group, possibly in the form of an anion, (*iv*) nucleophilic addition of the OOH moiety to the carbon atom of the carbonyl group in α-ketoglutarate, with concomitant weakening of the O—O bond, and (*v*) subsequent interaction of the oxidation product with peptidyl proline. Decay of this intermediate leads to the formation of hydroxyproline and succinate. Had peroxysuccinate proved to be an effective substitute for α-ketoglutarate, one could have concluded that it was the actual intermediate. The fact that it could not substitute for α-ketoglutarate may mean that it was unavailable to the active site or that another intermediate is involved.

Holme (32) has recently reported uncoupled decarboxylation of α-ketoglutarate catalyzed by aged preparations of thymine 7-hydroxylase. The fact that prolyl hydroxylase and thymine 7-hydroxylase, both of which are α-ketoglutarate dioxygenases, exhibit uncoupled decarboxylation suggests that the mechanism proposed may be general for all enzymes of this type.

The authors thank Dr. Selina Chen-Kiang for the antiserum to prolyl hydroxylase and for the antibody affinity column.

1. Rhoads, R. & Udenfriend, S. (1968) *Proc. Natl. Acad. Sci. USA* **60**, 1473–1478.
2. Fujimoto, D. & Tamiya, N. (1962) *Biochem. J.* **84**, 333–335.
3. Prockop, D. J., Kaplan, A. & Udenfriend, S. (1962) *Biochem. Biophys. Res. Commun.* **9**, 162–166.
4. Hutton, J. J., Tappel, A. L. & Udenfriend, S. (1966) *Biochem. Biophys. Res. Commun.* **24**, 179–184.
5. Kivirikko, K. I. & Prockop, D. J. (1967) *Proc. Natl. Acad. Sci. USA* **57**, 782–789.
6. Lindstedt, G., Lindstedt, S., Midvedt, T. & Tofft, M. (1967) *Biochem. J.* **103**, 19P.
7. Abbott, M. T., Schandl, E. K., Lee, R. F., Parker, T. S. & Midgett, R. J. (1967) *Biochim. Biophys. Acta* **132**, 525–528.
8. Shaffer, P. M., McCroskey, R. P., Palmatier, R. D., Midgett, R. J. & Abbott, M. T. (1968) *Biochem. Biophys. Res. Commun.* **33**, 806–811.
9. Liu, C. K., Hsu, C. A. & Abbott, M. T. (1973) *Arch. Biochem. Biophys.* **159**, 180–187.
10. Lindblad, B., Lindstedt, G., Tofft, M. & Lindstedt, S. (1969) *J. Am. Chem. Soc.* **91**, 4604–4606.

11. Cardinale, G. J., Rhoads, R. E. & Udenfriend, S. (1971) *Biochem. Biophys. Res. Commun.* **43**, 537–543.
12. Holme, E., Lindstedt, G., Lindstedt, S. & Tofft, M. (1968) *FEBS Lett.* **2**, 29–32.
13. Cardinale, G. J. & Udenfriend, S. (1974) *Adv. Enzymol.* **41**, 245–300.
14. Hamilton, G. A. (1971) in *Progress in Bioorganic Chemistry*, eds. Kaiser, E. T., & Kezdy, F. J. (Wiley, New York), pp. 83–157.
15. Lindstedt, G. & Lindstedt, S. (1970) *J. Biol. Chem.* **245**, 4178–4186.
16. McBride, O. W. & Harrington, W. E. (1967) *Biochemistry* **6**, 1484–1498.
17. Rhoads, R. & Udenfriend, S. (1970) *Arch. Biochem. Biophys.* **139**, 329–339.
18. Berg, R. & Prockop, D. (1973) *J. Biol. Chem.* **248**, 1175–1182.
19. Tuderman, L., Kuutti, E. & Kivirikko, K. (1975) *Eur. J. Biochem.* **52**, 9–16.
20. Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427.
21. Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412.
22. Hartman, B. K. & Udenfriend, S. (1969) *Anal. Biochem.* **30**, 391–394.
23. Prockop, D. J. & Udenfriend, S. (1960) *Anal. Biochem.* **1**, 228–239.
24. LeRoy, E. C., Kaplan, A., Udenfriend, S. & Sjoerdsma, A. (1964) *J. Biol. Chem.* **239**, 3350–3356.
25. Böhlen, P., Stein, S., Dairman, W. & Udenfriend, S. (1973) *Arch. Biochem. Biophys.* **155**, 213–220.
26. Roberts, N. E., McGee, J. O'D. & Udenfriend, S. (1973) *Connect. Tissue Res.* **2**, 65–69.
27. Primus, F. J., Neuman, E. S. & Hansen, H. J. (1971) *J. Immunol.* **118**, 55–61.
28. Prockop, D. J. & Kivirikko, K. (1969) *J. Biol. Chem.* **244**, 4838–4842.
29. Abbott, M. T. & Udenfriend, S. (1974) in *Molecular Mechanisms of Oxygen Activation*, ed. Hayaishi, O. (Academic, New York), p. 205.
30. Risteli, J., Tuderman, L. & Kivirikko, K. (1976) *Biochem. J.* **158**, 369–376.
31. Hobza, P., Hurych, J. & Zahradnik, R. (1973) *Biochim. Biophys. Acta* **304**, 466–472.
32. Holme, E. (1976) *Proceedings of the Tenth International Congress of Biochemistry, Hamburg, Germany*, p. 348.