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STUDIES ON THE LYSYL HYDROXYLASE REACTION

II. INHIBITION KINETICS AND THE REACTION MECHANISM

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Summary

Product inhibition of lysyl hydroxylase (peptidyllysine, 2-oxoglutarate:oxygen 5-oxidoreductase, EC 1.14.11.4) was studied with succinate, CO_2, dehydroascorbate and hydroxylysine-rich polypeptide chains. The product inhibition patterns and addition data are consistent with a reaction mechanism involving an ordered binding of Fe^{2+}, α-ketoglutarate, O_2 and the peptide substrate to the enzyme in this order, and an ordered release of the hydroxylated peptide, CO_2, succinate and Fe^{2+}, in which Fe^{2+} need not leave the enzyme during each catalytic cycle and in which the order of release of the hydroxylated peptide and CO_2 is uncertain. Ascorbate probably reacts by a substitution mechanism, either after the release of the hydroxylated peptide, CO_2 and succinate or after the release of all products, including Fe^{2+}, and dehydroascorbate is released before the binding of Fe^{2+}. It is suggested that the ascorbate reaction is required to reduce either the enzyme-iron complex or the free enzyme, which may be oxidized by a side-reaction during some catalytic cycles, but not the majority. The mechanisms of the prolyl 4-hydroxylase and lysyl hydroxylase reactions are suggested to be identical.

Zn^{2+}, several citric acid cycle intermediates, nitroblue tetrazolium and homogentisic acid inhibited lysyl hydroxylase competitively with regard to Fe^{2+}, α-ketoglutarate, O_2 and ascorbate respectively, and epinephrine non-competitively with regard to all cosubstrates. Apparent K_i values are given for the product and other inhibitors.

Introduction

Hydroxylysyl residues have two important functions in collagen, serving as sites of attachment for the carbohydrate units and participating in interchain crosslink formation (for reviews, see Refs. 1, 2). The significance of hydroxylysine in stabilizing the crosslinks is demonstrated in one form of the Ehlers-Danlos syndrome, in which a deficiency in lysyl hydroxylase activity leads to a severe connective tissue disorder [3—5]. Thus it seems of importance to elucidate the mechanism of the lysyl hydroxylase reaction and acquire data on its inhibition which might be relevant to the regulation of its activity in certain physiological or pathological conditions. Part I of this study [6] reported initial velocity kinetics and related aspects concerning lysyl hydroxylase. As a further step in determining the mechanism of this reaction, product inhibition patterns have now been analyzed with respect to the hydroxylated peptide, CO_2, succinate and dehydroascorbate. This has in turn yielded information on the order of binding of the peptide substrate and the cosubstrates to the enzyme and the order of release of the products (for reviews, see Refs. 7—9).

Lysyl hydroxylase has recently been reported to be inhibited by several bivalent cations [10] and also by catechol analogs [11] and homogentisic acid [12]. Two of the studies on these inhibitors [11,12], however, were carried out with very crude enzyme preparations, and none of these compounds have been analyzed in terms of detailed kinetics with respect to the peptide substrate and all the cosubstrates. The present work aimed to determine the complete inhibition patterns with respect to Zn^{2+}, epinephrine and homogentisic acid, and also to examine inhibition using several other compounds which have recently been reported to act as inhibitors of prolyl 4-hydroxylase [13].

Materials and Methods

Materials. Lysyl hydroxylase was purified as described in the preceding paper [6], in which the source of the synthetic peptide L-I (Ala-Arg-Gly-Ile-Lys-Gly-Ile-Arg-Gly-Phe-Ser-Gly) and those of the other materials are also given. Additional materials were glomerular basement membrane collagen from pig kidney, from which the sugars were removed by hydrolysis for 3 h at 110°C with 0.2 M HCl [14], and homogentisic acid, nitroblue tetrazolium and epinephrine from Sigma Chemicals Co. (St. Louis, MO).

Assay of lysyl hydroxylase activity. The enzyme activity was assayed as described in the preceding paper [6]. The gas mixtures for those experiments in which the O_2 or CO_2 concentration was varied were prepared and the solutions equilibrated as described elsewhere [13]. Appropriate concentrations of $NaHCO_3$ were also added in the experiments with CO_2 to prevent changes in the pH [15], and in these experiments lysyl hydroxylase activity was assayed with [^{14}C]lysine-labelled protocollagen substrate [6].

Inhibition constants. The apparent K_i values were calculated from the secondary transforms of the primary plots, K_{is} being the K_i calculated from the slopes and K_{ii} that calculated from the intercepts. Unless otherwise noted, the secondary plots were linear within their experimental errors, but even in such cases the data do not exclude a slight non-linearity (see also legend to Table I of part I [6] of this work).

Results

Product inhibition

Succinate. Inhibition by succinate was competitive with respect to α-ketoglutarate (Fig. 1A), non-competitive with respect to Fe^{2+} (not shown), O_2 (Fig. 1B) and the peptide substrate (not shown), and uncompetitive with respect to ascorbate (Fig. 1C). The K_{is} and K_{ii} values ranged between about 5 and 15 mM. The inhibition pattern is identical with that previously found for prolyl 4-hydroxylase except that inhibition with respect to ascorbate was reported to be non-competitive, as the lines obtained were not strictly parallel [16]. Three additional experiments carried out now indicate, however, that the inhibition of prolyl 4-hydroxylase by succinate is in fact likewise uncompetitive with respect to ascorbate (not shown). The pattern of inhibition by succinate with respect to ascorbate did not change from uncompetitive to non-competitive when a fixed level of basement membrane collagen (see below) (30, 60, or 120 μg/ml) or CO_2 (0.48 mM) was added to all tubes (not shown).

Carbon dioxide. CO_2 has previously been shown to be an inhibitor of prolyl 4-hydroxylase, but the mode of inhibition was not studied as this compound

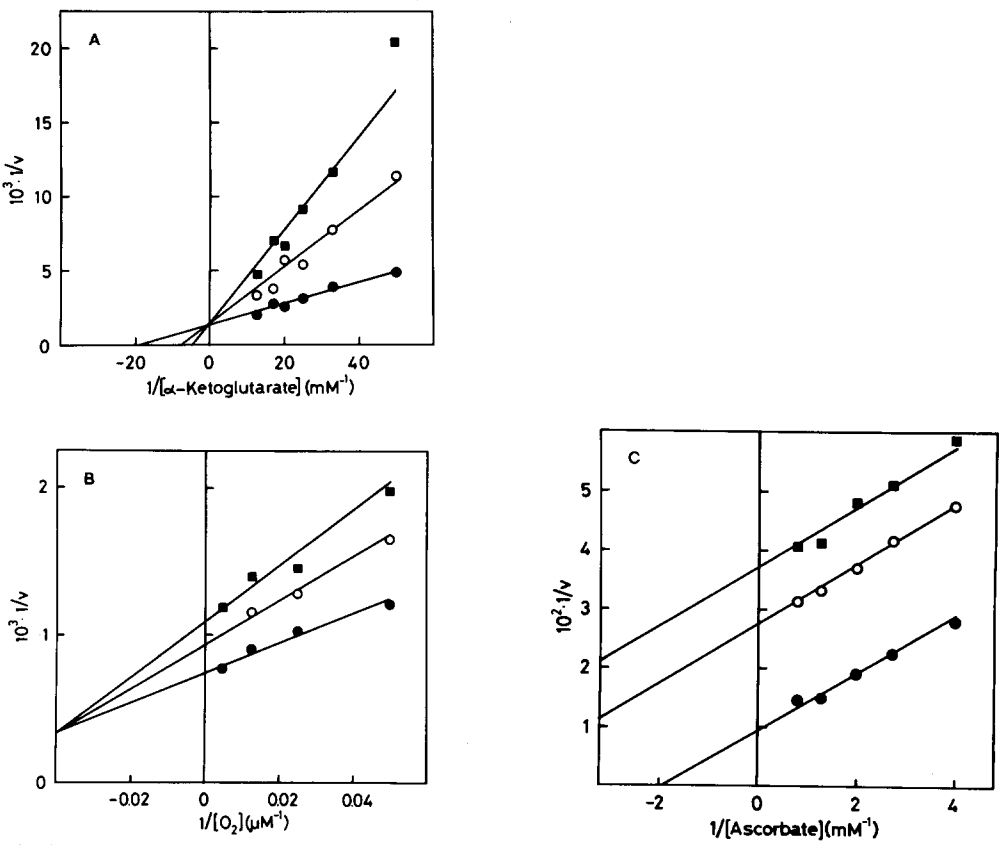


Fig. 1. Inhibition of the lysyl hydroxylase reaction by succinate with respect to α-ketoglutarate (A), O_2 (B) and ascorbate (C). The concentrations of succinate were A: ■, 15 mM, ○, 8 mM, ●, none; B: ■, 7 mM; ○, 5 mM; ● none; C: ■, 10 mM; ○, 5 mM; ●, none.

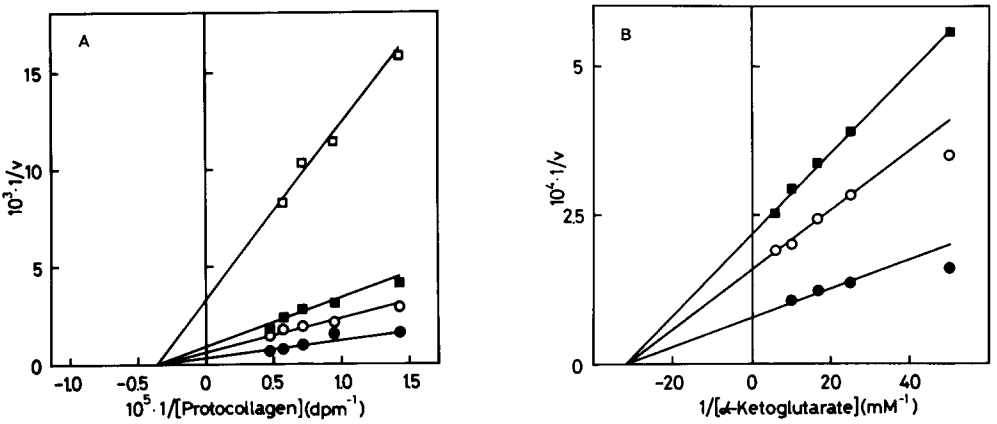


Fig. 2. Inhibition of the lysyl hydroxylase reaction by CO_2 with respect to protocollagen substrate (A) and α-ketoglutarate (B). The concentrations of CO_2 were A: □, 4.78 mM; ■, 2.39 mM; ○, 0.95 mM; ●, none. B: ■, 4.78 mM; ○, 0.95 mM; ●, none.

inhibited the trapping of the $^{14}CO_2$ formed in the reaction, and it was thus not possible to use the assay based on the measurement of the decarboxylation of α-keto[1-^{14}C]glutarate [16]. Since determination of this inhibition pattern seemed important for the elucidation of the reaction sequence, it was decided here to study product inhibition with CO_2 by using [^{14}C]lysine-labelled protocollagen as a substrate and assaying the formation of hydroxy[^{14}C]lysine. CO_2 was found to be a noncompetitive inhibitor with respect to the protocollagen substrate (Fig. 2A), α-ketoglutarate (Fig. 2B), Fe^{2+}, and ascorbate (not shown) with K_{is} and K_{ii} values of about 1—2 mM. Inhibition with respect to O_2 could not be studied because of the technical difficulties involved in varying two gas mixtures in the same experiment.

Dehydroascorbate. Inhibition by dehydroascorbate was competitive with respect to Fe^{2+} (Fig. 3), and noncompetitive with respect to α-ketoglutarate,

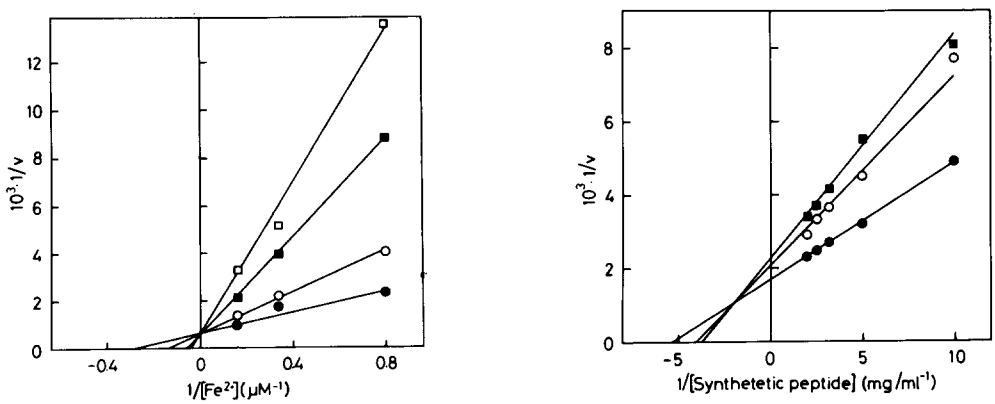


Fig. 3. Inhibition of lysyl hydroxylase reaction by dehydroascorbate with respect to Fe^{2+}. The concentrations of dehydroascorbate were: □, 4.05 mM; ■, 2.7 mM; ○, 1.62 mM; ●, none.

Fig. 4. Inhibition of the lysyl hydroxylase reaction by basement membrane collagen with respect to the peptide substrate (LI). The concentrations of the collagen were: ■, 0.095 mg/ml; ○, 0.068 mg/ml; ●, none.

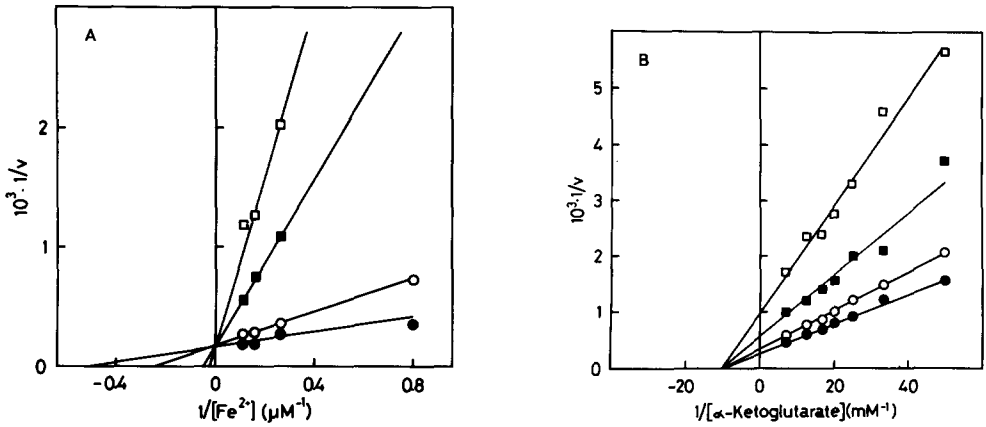


Fig. 5. Inhibition of the lysyl hydroxylase reaction by Zn^{2+} with respect to Fe^{2+} (A) and α-ketoglutarate (B). The concentrations of Zn^{2+} were A: □, 8 μM; ■, 5 μM; ○, 1 μM; ●, none. B: □, 7.9 μM; ■, 4.7 μM; ○, 1.6 μM; ●, none.

ascorbate, O_2 and the peptide substrate (not shown). An inhibition by 50% was obtained with a concentration of about 2 mM. All secondary plots were parabolic.

Hydroxylysine-rich polypeptide chains. Since type IV collagen from basement membranes has a high hydroxylysine content [17], this protein, after removal of its hydroxylysing-linked carbohydrate units [14], was studied as an alternative product inhibitor of the reaction. Basement membrane collagen was found to be a noncompetitive inhibitor with respect to the peptide substrate (Fig. 4), O_2, α-ketoglutarate and ascorbate (not shown) with K_{ii} and K_{is} values of about 0.1—0.2 mg/ml.

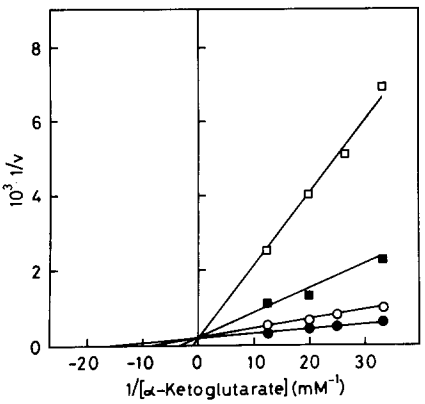
Other inhibitors

Zinc. Inhibition by Zn^{2+} was competitive with respect to Fe^{2+} (Fig. 5A), and non-competitive with respect to α-ketoglutarate (Fig. 5B), the peptide substrate and ascorbate (not shown). The K_{is} and K_{ii} values ranged between about 1 and 2 μM.

Citric acid cycle intermediates. Pyruvate inhibited the reaction competitively with respect to α-ketoglutarate (Fig. 6), noncompetitively with respect to Fe^{2+} and the peptide substrate, and uncompetitively with respect to ascorbate (not shown). A similar inhibition pattern was obtained with oxaloacetate (not shown). Citrate, isocitrate, fumarate and malate were also inhibitors, but the patterns of inhibition were not determined with these compounds.

The K_{is} and K_{ii} values for pyruvate were about 10—20 mM and those for oxaloacetate about 0.4—0.6 mM. The concentrations of the other compounds that gave 50% inhibition under standard incubation conditions were: about 0.3 mM citrate, 2 mM isocitrate, 2 mM fumarate, and 1 mM malate.

Epinephrine. Whereas the inhibition of prolyl 4-hydroxylase by epinephrine was reported to be competitive with respect to Fe^{2+} [16], inhibition of lysyl hydroxylase with this compound was not competitive with respect to Fe^{2+}, although the lines did intersect close to the ordinate (Fig. 7). Two additional



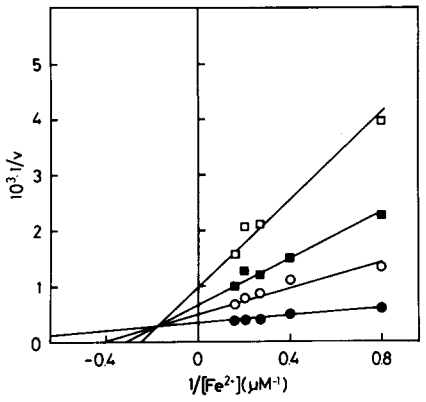
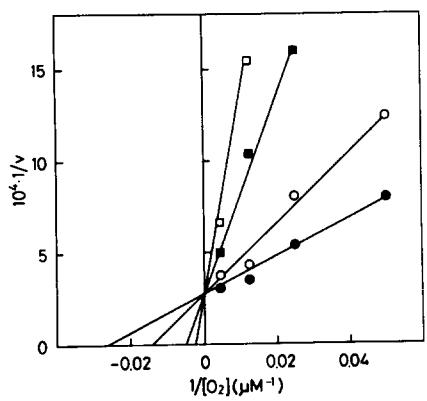


Fig. 6. Inhibition of the lysyl hydroxylase reaction by pyruvate with respect to α-ketoglutarate. The concentrations of pyruvate were: □, 100 mM; ■, 40 mM; ○, 10 mM; ●, none.

Fig. 7. Inhibition of the lysyl hydroxylase reaction by epinephrine with respect to Fe^{2+}. The concentrations of epinephrine were: □, 100 μM; ■, 50 μM; ○, 25 μM; ●, none.

experiments gave similar results (not shown). Inhibition was distinctly noncompetitive with respect to α-ketoglutarate, O_2 and the peptide substrate (not shown), and was noncompetitive with respect to ascorbate, although the lines again intersected close to the ordinate (not shown). Epinephrine was thus not competitive with any of the substrates. The K_{is} and K_{ii} values ranged from about 0.2 to 0.5 mM.

Nitroblue tetrazolium. This compound inhibited the reaction competitively with respect to O_2 (Fig. 8), but noncompetitively with respect to Fe^{2+}, α-ketoglutarate and the peptide substrate (not shown). An inhibition by 50% was obtained with a concentration of about 0.02—0.04 mM. All secondary plots were parabolic.



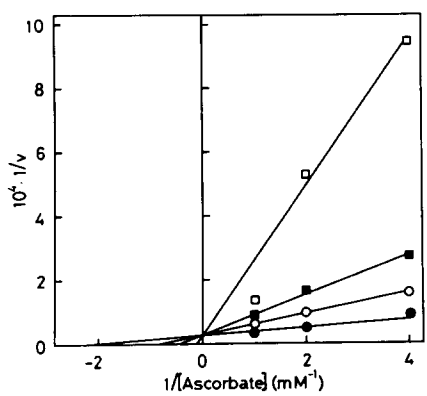


Fig. 8. Inhibition of the lysyl hydroxylase reaction by nitroblue tetrazolium with respect to O_2. The concentrations of nitroblue tetrazolium were: □, 60 μM; ■, 40 μM; ○, 20 μM; ●, none.

Fig. 9. Inhibition of the lysyl hydroxylase reaction by homogentisic acid with respect to ascorbate. The concentrations of homogentisic acid were: □, 4 mM; ■, 2 mM; ○, 0.8 mM; ●, none.

Homogentisic acid. Inhibition with homogentisic acid was competitive with respect to ascorbate (Fig. 9), but noncompetitive with respect to α-ketoglutarate, O_2, the peptide substrate and Fe^{2+} (not shown), an inhibition by 50% being obtained with a concentration of about 0.5—2 mM. All secondary plots were parabolic.

Discussion

Lysyl hydroxylase is one of the α-ketoglutarate dioxygenases, the others of which include prolyl 4-hydroxylase, prolyl 3-hydroxylase, 4-butyrobetaine hydroxylase, thymine 7-hydroxylase, pyrimidine deoxyribonucleoside 2′-hydroxylase and *p*-hydroxyphenylpyruvate hydroxylase (see Refs. 18, 19). The mechanism of prolyl 4-hydroxylase reaction has recently been subjected to extensive kinetic experiments [13,16,20], whereas little is known about the kinetics of the other enzymes. The product inhibition patterns reported here for lysyl hydroxylase are identical with those previously reported for prolyl 4-hydroxylase [16,20], except that some of the experiments were not carried out with the latter enzyme. As the initial velocity patterns of these two hydroxylases are also identical [6,16], it seems very probable that the two reactions are identical in their mechanism. As has been pointed out, the order of release of the products from the enzyme and the stage of the reaction with ascorbate in particular require additional experiments [16,20]. Hence special attention was paid to these questions here, and on the basis of the more complete data some points in the reaction schemes [16,20] reported for prolyl 4-hydroxylase, now receive further elucidation.

The initial velocity data reported in part I of this study [6] indicate that

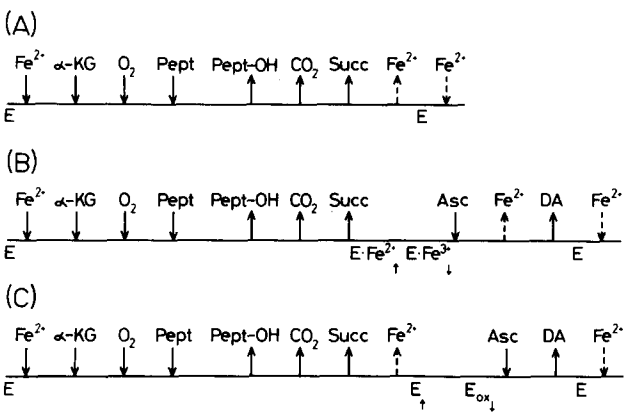


Fig. 10. Schematic representation of the proposed mechanism for the lysyl hydroxylase reaction. It is suggested that the enzyme operates according to the scheme (A) for a number of catalytic cycles, but at some stage the reaction ceases, probably due to oxidation by a side-reaction (as shown by the arrows below the line) of either the enzyme-Fe^{2+} complex (B) or free enzyme (C), and ascorbate is then required to reduce this oxidized form. The order of release of the hydroxylated peptide and CO_2 is uncertain. The dashed lines between the enzyme and Fe^{2+} indicate that the latter need not leave the enzyme during each catalytic cycle. The postulated dead-end complexes (see text) are not shown. Abbreviations: E = enzyme; α—KG = α-ketoglutarate; Pept = peptide substrate; Succ = succinate; Asc = ascorbate; DA = dehydroascorbate.

Fe^{2+} becomes bound to lysyl hydroxylase at thermodynamic equilibrium before the binding of α-ketoglutarate. O_2 can become bound in the absence of the peptide substrate, as lysyl hydroxylase catalyzes an uncoupled decarboxylation of α-ketoglutarate. These findings do not exclude a random binding of O_2 and the peptide substrate, but such a case can apparently be excluded [7—9] on the grounds that product inhibition by the hydroxylate peptide and CO_2 was not competitive with respect to O_2 or the peptide substrate. Furthermore, parallel lines were obtained in the experiments with prolyl 4-hydroxylase when the peptide substrate concentrations were varied at different fixed α-ketoglutarate concentrations in the presence of a saturating concentration of O_2 [16], suggesting [7—9] the binding of α-ketoglutarate, O_2 and the peptide substrate in this order (Fig. 10).

In a reaction mechanism involving an ordered release of the hydroxylated peptide, CO_2 and succinate (Fig. 10, scheme A), as suggested for prolyl 4-hydroxylase [16], the first product should give noncompetitive inhibition respect to Fe^{2+}, α-ketoglutarate, O_2 and the peptide substrate and the second uncompetitive inhibition with respect to all these substrates. The third product should give competitive inhibition with respect to α-ketoglutarate and, as Fe^{2+} is not released during each catalytic cycle, non-competitive inhibition with respect to Fe^{2+}, O_2 and the peptide substrate. Succinate was the only product that gave the last-mentioned pattern and should thus be the third product to be released. Basement membrane collagen and CO_2 both gave the pattern expected for the first product, whereas neither gave the uncompetitive pattern expected for the second. If the second product forms a dead-end complex with the enzyme, however, then noncompetitive inhibition is found instead of uncompetitive [7—9], and in such a case either the hydroxylated peptide or CO_2 can be the second product (Fig. 10A). The data are thus consistent with an ordered release of these three products in which the order of release of the first two is uncertain.

The reaction with ascorbate does not take place during each catalytic cycle [6,16,20], and the enzyme is thus thought to operate according to the scheme A (Fig. 10) for a number of cycles. The initial velocity pattern given by ascorbate and the product inhibition pattern given by dehydroascorbate suggest that ascorbate reacts either after the release of the first three products (Fig. 10, scheme B) or after the release of all products, including Fe^{2+} (Fig. 10, scheme C), and that dehydroascorbate is released before the binding of Fe^{2+}. In both cases product inhibition by dehydroascorbate should be competitive with respect to Fe^{2+} and non-competitive with respect to α-ketoglutarate, O_2 and the peptide substrate, as was indeed the case. The noncompetitive product inhibition by dehydroascorbate and uncompetitive inhibition by succinate with respect to ascorbate are as such consistent with scheme C (Fig. 10), but there are also several possibilities for incorporating this pattern into scheme B. Dehydroascorbate inhibition can be noncompetitive in scheme B, as this product may form a dead-end complex with the postulated enzyme-Fe^{3+} complex (Ref. 20 and below) or else the constant concentration of Fe^{2+} may alter the pattern from uncompetitive to noncompetitive [7—9], or else the release of Fe^{2+} and dehydroascorbate may occur by a random mechanism. Succinate inhibition may be uncompetitive in scheme B, as the nature of the

postulated side-reaction (see below) is not known.

In scheme B, ascorbate might be required to reduce the enzyme-bound iron, which may become converted to Fe^{3+} by an oxidative side-reaction during some catalytic cycles, but not the majority [20]. In scheme C ascorbate reacts only after the release of Fe^{2+}, and in such a case this vitamin would seem to reduce some other group or groups on the enzyme. Such an oxidation of the free enzyme would not occur, however, during the purification procedure, as Fe^{2+} can become bound to the purified enzyme in the complete absence of ascorbate [6,20]. As Fe^{2+} is not released from the enzyme during each catalytic cycle [6,16], scheme C would explain the findings that lysyl hydroxylase can catalyze its reaction for a number of cycles in the complete absence of this vitamin [6], as does also prolyl 4-hydroxylase [20], and also that ascorbate is not stoichiometrically consumed during the prolyl 4-hydroxylase reaction [13].

It should be noted that the noncompetitive product inhibitions by CO_2 and the hydroxylated peptide with respect to ascorbate are not as such consistent with either of the above schemes. Therefore, an additional possibility was considered, in which ascorbate would become bound between the release of the hydroxylated peptide and that of CO_2, and dehydroascorbate would be released before the binding of Fe^{2+}. In this case product inhibition by succinate with respect to ascorbate should be uncompetitive, as found here, but the inhibition should be altered to noncompetitive in the presence of a fixed concentration of either the hydroxylated peptide or CO_2 [7—9], the product which is released between the binding of ascorbate and the release of succinate. Such a change in the inhibition pattern did not take place, however. Furthermore, product inhibition by succinate should not be competitive with respect to α-ketoglutarate in this scheme. For these reasons this possibility was discounted. Noncompetitive product inhibition by the hydroxylated peptide and CO_2 with respect to ascorbate can be explained in schemes B and C by assuming that these products form dead-end complexes either with the enzyme-Fe^{3+} complex (in scheme B) or with the oxidized free enzyme (in scheme C). As formation of dead-end complexes by reaction products is quite a common phenomenon [7—9], all the kinetic data are consistent with both of the two alternative schemes.

The inhibition of lysyl hydroxylase by homogentisic acid was found to be competitive with respect to ascorbate, as previously reported for a crude enzyme preparation [12]. This compound thus appears to react on the enzyme molecule at the same site as ascorbate. The modes of inhibition by Zn^{2+}, citric acid cycle intermediates and nitroblue tetrazolium suggest that these compounds compete with Fe^{2+}, α-ketoglutarate and an activated form of oxygen respectively, these results being similar to those reported for prolyl 4-hydroxylase [13]. Although inhibition of prolyl 4-hydroxylase by epinephrine was reported to be competitive with respect to Fe^{2+} [13], we now find noncompetitive inhibition of lysyl hydroxylase with respect to all reactants. Epinephrine might act in part by binding Fe^{2+}, in part by binding an activated form of oxygen, and perhaps in part by binding to the same site as ascorbate.

The K_i values for the product inhibitors and other inhibitors are very similar to those reported for prolyl 4-hydroxylase [13,16,20]. As mentioned previ-

ously, inhibition by some of these compounds, such as the citric acid cycle intermediates [13], may be important in the regulation of hydroxylation in physiological conditions in vivo, while inhibition by some others, such as homogentisic acid [12], may be significant in pathological states.

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