Biochimica et Biophysica Acta, 611 (1980) 40—50

BBA 68890

STUDIES ON THE LYSYL HYDROXYLASE REACTION

I. INITIAL VELOCITY KINETICS AND RELATED ASPECTS

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(Received April 17th, 1979)

Key words: Lysyl hydroxylase; Velocity kinetics; Collagen synthesis

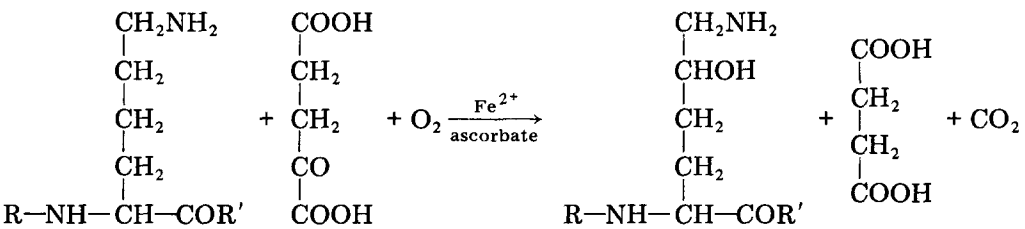
Summary

The kinetics of the lysyl hydroxylase (peptidyllysine, 2-oxoglutarate:oxygen 5-oxidoreductase, EC 1.14.11.4) reaction were studied using enzyme from chick embryos by varying the concentration of one substrate in the presence of different fixed concentrations of the second substrate, while the concentrations of the other substrates were held constant. Intersecting lines were obtained in double-reciprocal plots for all possible pairs involving Fe^{2+}, α-ketoglutarate, O_2 and the peptide substrate, whereas parallel lines were obtained for pairs comprising ascorbate and each of the other substrates. The pair composed of Fe^{2+} and α-ketoglutarate gave an asymmetrical initial velocity pattern, indicating binding of these two reactants in this order, that of Fe^{2+} being at thermodynamic equilibrium. The initial velocity patterns are identical with those reported for prolyl 4-hydroxylase, and the apparent K_m and K_d values calculated from these data are also very similar. The largest difference was found in K_m and K_d for α-ketoglutarate, which were about 4 times the corresponding values for prolyl 4-hydroxylase. Ascorbate was found to be a quite specific requirement for lysyl hydroxylase, but the enzyme catalyzed its reaction for a short time at a high rate in the complete absence of this vitamin, suggesting that the reaction with ascorbate does not occur during each catalytic cycle. Lysyl hydroxylase catalyzed an uncoupled decarboxylation of α-ketoglutarate in the absence of the peptide substrate, the rate being about 4% of that observed in the presence of a saturating concentration of the peptide substrate. This uncoupled decarboxylation required the same cosubstrates as the complete reaction.

Introduction

Lysyl hydroxylase (peptidyllysine; 2-oxoglutarate:oxygen 5-oxidoreductase, EC 1.14.11.4) catalyzes the synthesis of hydroxylysine in collagen by the hydroxylation of the lysyl residues in peptide linkages (for recent reviews, see Refs. 1—3). The enzyme has been purified up to about 3000—4000-fold from chick embryo extract both by conventional procedures [4] and by an affinity column procedure using concanavalin A-agarose [5], and found to have a molecular weight of about 200 000 in gel filtration [5]. The enzyme does not hydroxylate free lysine, and the minimum sequence requirement is probably an -X-Lys-Z- triplet, in which Z can certainly be glycine [6], and possibly also serine or alanine [7]. The hydroxylation of lysyl residues in these triplets is also influenced by the amino acid sequence around the lysyl residue and by the chain length and conformation of the peptide substrate [6—9].

Lysyl hydroxylase requires ferrous ions, α-ketoglutarate, molecular oxygen and a reducing agent [1—3]. The requirement for the reducing agent is best fulfilled by ascorbate [1—3], but in experiments with crude enzyme preparations, at least, about 70% of the maximal activity could be obtained with dithiothreitol or β-mercaptoethanol, and about 30% with L-cysteine or 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine [10]. Even in the presence of optimal concentrations of all cosubstrates, maximal activity is not found in vitro unless bovine serum albumin, catalase and dithiothreitol are added to the reaction mixture [11—13]. The mechanism of the lysyl hydroxylase reaction and the role of its cosubstrates have not been elucidated, but is has been demonstrated that it involves the stoichiometric decarboxylation of α-ketoglutarate to succinate and CO_2 [6]. The reaction can thus be described by the following equation;



The mechanism of the reaction catalyzed by prolyl 4-hydroxylase, another intracellular enzyme of collagen biosynthesis requiring the same cosubstrates, has recently been subjected to extensive kinetic experiments [14—16]. Similar experiments have now been carried out to analyze the mechanism of the lysyl hydroxylase reaction and the role of its cosubstrates, and the present paper reporting these lays special emphasis on the questions of whether the initial velocity patterns of lysyl hydroxylase and the K_m values for the cosubstrates, as determined from these data, are similar to those reported for prolyl 4-hydroxylase. A possible difference in the latter case was suggested by observations that lysyl hydroxylation in cultured fibroblasts is less readily affected by ascorbate deficiency than is prolyl hydroxylation [17,18], and consequently, special attention was also paid to the specificity of the ascorbate requirement and to other experiments on the role of this vitamin. The question was also

studied of whether lysyl hydroxylase catalyzes any decarboxylation of α-ketoglutarate in the absence of the peptide substrate, such an uncoupled decarboxylation having been found with prolyl 4-hydroxylase [14,19,20]. Studies on the inhibition of the reaction are reported in the second part of this report [21], in which a reaction mechanism is proposed that would be consistent with the data obtained in both parts of the study.

Materials and Methods

Materials. Lysyl hydroxylase was purified from an ammonium sulphate fraction of chick embryo extract by a procedure consisting of affinity chromatography on concanavalin A-agarose and gel filtration [5]. The specific activity of these partially purified enzyme preparations was 2000—3000 times that of the 15 000 × *g* supernatant of the chick embryo homogenate.

The synthetic peptide substrate, L-I (Ala-Arg-Gly-Ile-Lys-Gly-Ile-Arg-Gly-Phe-Ser-Gly) was purchased from the Protein Research Foundation (Minoh, Osaka, Japan). [^{14}C]Lysine-labelled protocollagen substrate was prepared from freshly-isolated chick embryo tendon cells as described previously [22] and aliquots of 120 000 dpm were used in the assays. α-[1-^{14}C]Ketoglutarate was purchased from New England Nuclear Corp. and adjusted to a specific activity of 100 000 dpm/0.1 μmol by mixing with the unlabelled compound. Tetrahydrofolic acid was obtained from Sigma Chemicals Co. (St. Louis, MO), 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine from Regis Chemicals Co. (Morton Grove, IL.), and dehydroascorbic acid from Fluka AG (Buchs, Switzerland). The $Fe_2SO_4 \cdot 7\ H_2O$, a Merck analytical reagent, was further purified with dithizone [15,23].

Assay of lysyl hydroxylase activity. The reaction with lysyl hydroxylase under 'standard conditions' was carried out in a final volume of 1.0 ml containing 0.2—2 μg enzyme, 0.5 mg synthetic peptide L-I, 0.05 μmol Fe_2SO_4, 0.1 μmol α-[1-^{14}C]ketoglutarate (100 000 dpm), 1 μmol ascorbate, 0.1 mg catalase (Calbiochem), 0.1 μmol dithiothreitol, 1 mg bovine serum albumin (Sigma) and 50 μmol Tris-HCl buffer, adjusted to pH 7.8 at 25°C (slightly modified from [6]). The samples were incubated for 40 min at 37°C and the $^{14}CO_2$ was trapped and counted as described previously [6]. The synthetic peptide was thermally denatured for 10 min at 100°C immediately before use [8,9]. As there were slight differences in the specific activities of the various lysyl hydroxylase preparations, the reaction velocities can be compared only within one experiment. The assay system was modified for certain experiments as described in the legends to the figures and tables. The experiments in which the O_2 concentration was varied were carried out as described elsewhere [14]. The experiments with the [^{14}C]lysine-labelled protocollagen substrate were carried out as described previously [11] and in Table III.

Results

Initial velocity patterns of the peptide substrate and the cosubstrates

The hydroxylation-coupled decarboxylation of α-ketoglutarate under the conditions used in this study was linear with time and enzyme concentration,

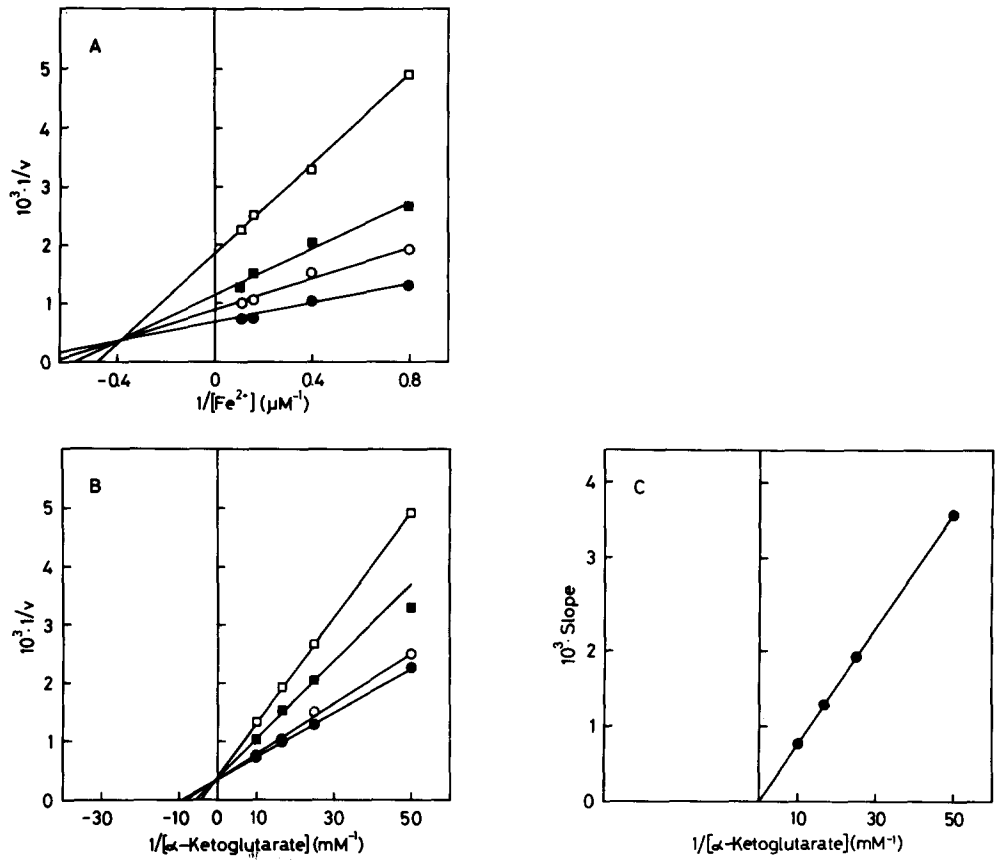


Fig. 1. Effect of Fe^{2+} concentration on the rate of the lysyl hydroxylase reaction at different fixed concentration of α-ketoglutarate (A) and the effect of α-ketoglutarate concentration at different concentrations of Fe^{2+} (B). A secondary plot of the slopes of the lines obtained in A when plotted against [α-ketoglutarate]$^{-1}$ (C). The concentrations of α-ketoglutarate in (A) were: □, 20 μM; ■, 40 μM; ○, 60 μM; ●, 100 μM; and those of Fe^{2+} in (B): □, 1.25 μM; ■, 2.5 μM; ○, 6.25 μM; ●, 8.75 μM. The concentrations of the other components were kept constant: L-I 0.5 mg/ml and ascorbate 1 mM. v was measured in dpm.

and in all the experiments reported below the formation of $^{14}CO_2$ corresponded to a hydroxylation of less than 5% of the peptide substrate. The kinetics of lysyl hydroxylation were studied by varying the concentration of one substrate in the presence of different fixed concentrations of the second substrate while those of the other substrates were held constant and by analyzing the patterns of the double-reciprocal plots.

Variation of the Fe^{2+} concentration at different fixed concentrations of α-ketoglutarate and at constant concentrations of the other components gave lines that intersected to the left of the ordinate (Fig. 1A). When the data were plotted as (velocity)$^{-1}$ versus [α-ketoglutarate]$^{-1}$ at different fixed concentrations of Fe^{2+}, the lines intersected on the ordinate (Fig. 1B). The slopes of the lines obtained in Fig. 1A, when plotted against [α-ketoglutarate]$^{-1}$, gave a line which passed through the origin (Fig. 1C).

Variation of the Fe^{2+} concentration at different fixed concentrations of O_2

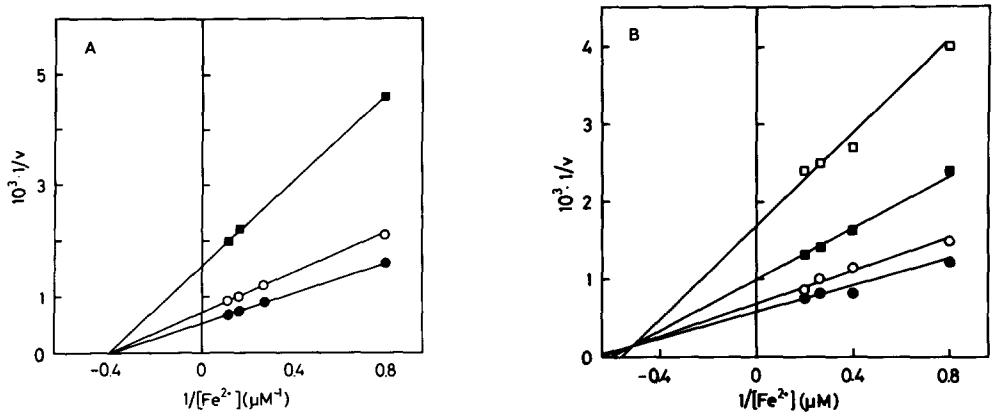


Fig. 2. Effect of Fe^{2+} concentration on the rate of the lysyl hydroxylase reaction at different fixed concentrations of O_2 (A) or the peptide substrate (B). The concentrations of O_2 in (A) were: ■, 10 μM; ○, 50 μM; ●, 200 μM; and those of the peptide substrate in (B): □, 0.05 mg/ml; ■, 0.1 mg/ml; ○, 0.15 mg/ml; ●, 0.25 mg/ml. The concentration of α-ketoglutarate was 0.1 mM and that of ascorbate 1 mM. v was measured in dpm.

(Fig. 2A) or the peptide substrate (Fig. 2B) also gave lines intersecting to the left of the ordinate, but in these cases the lines intersected to the left of the ordinate even when plotted as $(\text{velocity})^{-1}$ versus $[O_2]^{-1}$ or $[\text{peptide}]^{-1}$ at different fixed concentrations of Fe^{2+} (not shown). Lines intersecting to the left of the ordinate were similarly obtained when the α-ketoglutarate concentration was varied at different fixed concentrations of O_2 (not shown) or the peptide substrate (Fig. 3) or when the O_2 or peptide concentration was varied at different fixed concentrations of α-ketoglutarate (not shown). Similar inter-

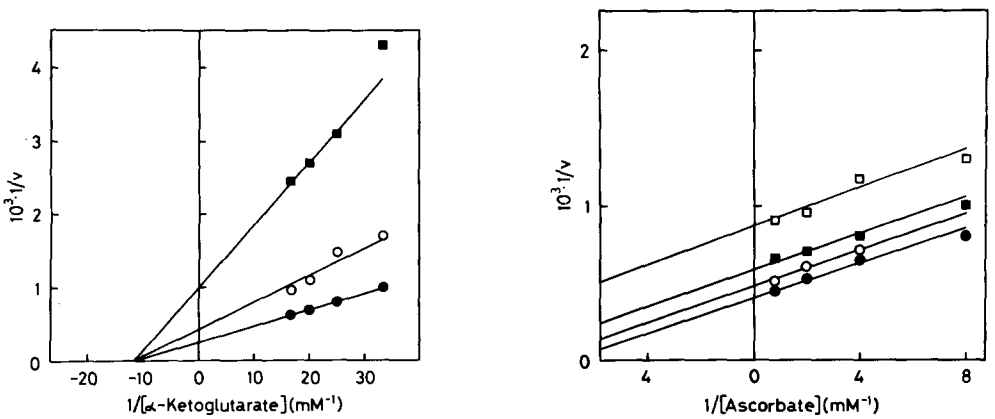


Fig. 3. Effect of α-ketoglutarate concentration on the rate of the lysyl hydroxylase reaction at different fixed concentrations of the peptide substrate. The concentrations of the peptide substrate were: ■, 0.05 mg/ml; ○, 0.15 mg/ml; ●, 0.25 mg/ml. The concentration of Fe^{2+} was 0.05 mM and that of ascorbate 1 mM. v was measured in dpm.

Fig. 4. Effect of ascorbate concentration on the rate of the lysyl hydroxylase reaction at different fixed concentrations of the peptide substrate. The concentrations of the peptide substrate were: □, 0.1 mg/ml; ■, 0.15 mg/ml; ○, 0.2 mg/ml; ●, 0.75 mg/ml. The concentration of Fe^{2+} was 0.05 mM and that of α-ketoglutarate 0.1 mM. v was measured in dpm.

secting patterns were obtained when the peptide concentration was varied at different fixed O_2 concentrations or the O_2 concentration was varied at different fixed concentrations of the peptide (not shown). By contrast, parallel lines were obtained when the ascorbate concentration was varied at different fixed concentrations of Fe^{2+} (not shown), α-ketoglutarate (not shown), O_2 (not shown) or the peptide substrate (Fig. 4), or when the Fe^{2+}, α-ketoglutarate, O_2 or peptide concentration was varied at different fixed ascorbate concentrations (not shown).

The apparent K_m and K_d values for the substrates, as determined from the initial velocity data, are shown in Table I. K_m values for Fe^{2+}, α-ketoglutarate and ascorbate have previously been determined with biologically prepared protocollagen substrate by varying only one component of the reaction mixture [11,24], but K_m has not been determined earlier for O_2, nor has K_d for any of the cosubstrates. The present K_m values are 2—4 times those determined with the protocollagen substrate for the partially purified enzyme from chick embryos [11], whereas they differ much more from the values reported for crude lysyl hydroxylase from human skin fibroblasts [24]. The K_d values differ only slightly from the K_m values (Table I).

Specificity of the ascorbate requirement

The ability of various reductants to replace ascorbate was examined by studying a number of compounds, each at at least 3—4 different concentrations, under standard incubation conditions, except that ascorbate was replaced by the reductant studied and dithiothreitol was omitted. The results obtained in five separate experiments are summarized in Table II, in which the value obtained with the optimal concentration of each reductant is shown as a percentage of the hydroxylation observed in the same experiment with 1 mM ascorbate. The specificity of the ascorbate requirement was found to be quite

TABLE I

APPARENT KINETIC CONSTANTS FOR THE SUBTRATES OF THE LYSYL HYDROXYLASE REACTION

The apparent kinetic constants were determined from data obtained in initial velocity studies and are shown as ranges of values obtained in at least three different experiments. The values are not true constants, as the concentrations of only two substrates were varied in each experiment. K_m is the Michaelis constant and K_d the dissociation constant. The values were determined from primary plots by the equations given in Ref. 25, and from secondary plots of intercepts and slopes of the primary plots versus [fixed substrate]$^{-1}$ [25]. All secondary plots were linear within their experimental errors. It should be noted, however, that due to the limited sensitivity and linearity of the assays, the concentrations of the reactants could be varied only within about one order of magnitude, and hence the data do not exclude a slight non-linearity.

Substrate	Kinetic constant (μM)	
	K_m	K_d
Fe^{2+}	2— 4	2— 3
α-Ketoglutarate	90—120	70—100
O_2	40— 50	30— 50
Ascorbate	190—220	
Peptide substrate	400—500	300—400

TABLE II

MAXIMAL REPLACEMENT OF ASCORBATE BY VARIOUS REDUCTANTS IN THE LYSYL HYDROXYLASE REACTION

Incubation conditions were as described in Methods, except that ascorbate was replaced by various reductants. The reaction rate with a 1 mM concentration of ascorbate was taken as 100%.

Reductant	Optimal concentration * (mM)	Reaction rate (%)
Ascorbate	1	100
Dithiothreitol	3 **	40 **
L-Cysteine	4 **	15 **
β-Mercaptoethanol	5	2
Glutathione	5	0
Tetrahydrofolic acid	3	2
2-Amino-4-hydroxy-6,7-dimethyltetrahydropteridine	5 **	15 **
Dihydroxyfumarate	2	2
Dehydroascorbate	1	0
$NADH_2$	10	0

* Optimal concentration or highest concentration studied, if no replacement was found.
** 1 mM dithiothreitol gave 19%, 1 mM cysteine 9% and 1 mM 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine 5%.

high, dithiothreitol, L-cysteine and 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine being the only reductants that at optimal concentrations gave more than 10% of the activity found with the optimal ascorbate concentration. Only dithiothreitol gave more than 10% at a concentration of 1 mM (Table II). Dehydroascorbate partly replaced ascorbate in the presence of dithiothreitol, but not in its absence (data not shown), apparently by virtue of being reduced to ascorbate [14].

Hydroxylation of lysine in the absence of ascorbate

It has recently been reported that pure prolyl-4-hydroxylase catalyzes its reaction in the absence of ascorbate at an essentially maximal rate for 5—10 s,

TABLE III

HYDROXYLATION OF LYSYL RESIDUES IN [^{14}C]LYSINE-LABELLED PROTOCOLLAGEN IN THE PRESENCE AND ABSENCE OF ASCORBATE

The reaction was carried out with protocollagen substrate as described in Materials and Methods, except that the incubation mixture did not contain dithiothreitol. In order to avoid non-linearity due to the consumption of all the protocollagen substrate, the reaction was carried out for 120 and 180 s in the presence of ascorbate with one-third of the amount enzyme used in the other tubes, and the result was multiplied by three.

Time (s)	—ascorbate (dpm)	+ascorbate (dpm)	$\frac{\text{—ascorbate}}{\text{+ascorbate}}$ (%)
10	790	1 540	51.3
30	730	2 860	25.5
60	1070	9 040	11.8
120	1140	21 170	5.4
180	1150	28 180	4.1

TABLE IV

EFFECT OF VARIOUS COMPONENTS ON THE DECARBOXYLATION OF α-KETOGLUTARATE IN THE LYSYL HYDROXYLASE REACTION

The enzyme reaction was carried out with 3 μg lysyl hydroxylase for 40 min as described in Methods. Only the compounds varied are indicated in the incubation conditions. E = enzyme, S = peptide substrate L-I 0.5 mg/ml.

Incubation	$^{14}CO_2$ found (dpm)	$^{14}CO_2$ over negative control (dpm)
E—, S-	100	0
E—, S+	100	0
E+, S—	1 200	1 100
E+, S+	12 200	12 100 *
E—, S—, Fe^{2+}—	80	0
E—, S+, Fe^{2+}—	80	0
E+, S—, Fe^{2+}—	110	30
E+, S+, Fe^{2+}—	430	350
E—, S—, Ascorbate—	90	0
E—, S+, Ascorbate—	90	0
E+, S—, Ascorbate—	100	10
E+, S+, Ascorbate—	220	130

* This value corresponds to about 25 000 dpm with a saturated L-I concentration.

corresponding to 15—30 reaction cycles [16]. An attempt was therefore made to study whether lysyl hydroxylase can similarly catalyze its reaction for a short time in the absence of the vitamin. Biologically prepared [^{14}C]lysine-labelled protocollagen was used as a substrate in these experiments, and the formation of hydroxy[^{14}C]lysine was assayed. The rate of hydroxylation during the first 10 s in the absence of ascorbate and dithiothreitol was about 51% of that found in the presence of the vitamin (Table III), after which the reaction rapidly ceased, there being little additional hydroxylation after 1 min.

Decarboxylation of α-ketoglutarate in the absence of the peptide substrate

Lysyl hydroxylase was found to catalyze an uncoupled decarboxylation of α-ketoglutarate in the absence of the peptide substrate (Table IV), the rate of which was about 9% of that observed with 0.5 mg/ml of the peptide L-I, corresponding to about 4% of that with a saturating L-I concentration. This uncoupled decarboxylation, like the complete reaction, required Fe^{2+} and ascorbate.

Discussion

The intersecting initial velocity patterns of all possible pairs involving Fe^{2+}, α-ketoglutarate, O_2 and the peptide substrate indicate that the binding of these substrates to lysyl hydroxylase occurs by a sequential mechanism (for reviews, see Refs. 25—27). The asymmetrical initial velocity pattern yielded by Fe^{2+} and α-ketoglutarate indicates that Fe^{2+} becomes bound to the enzyme at thermodynamic equilibrium before the binding of α-ketoglutarate and need not leave the enzyme during each catalytic cycle [25—27]. The order of binding of O_2 and the peptide substrate will be discussed in part II of this study [21].

Ascorbate gave parallel lines with each of the other substrates, indicating that this vitamin reacts with the enzyme by a substitution mechanism [25—27] after the release of one or more of the products. These initial velocity patterns are identical with those reported for prolyl 4-hydroxylase [15].

The apparent K_m and K_d values determined from the initial velocity data for Fe^{2+}, O_2 and ascorbate are almost identical with those determined from similar plots for pure chick embryo prolyl 4-hydroxylase [15], whereas the K_m and K_d for α-ketoglutarate are about 4-fold with lysyl hydroxylase. A similar difference between these two enzymes has previously been found with the protocollagen substrate [11,28], and thus lysyl hydroxylase seems to have a somewhat lower affinity for α-ketoglutarate than prolyl 4-hydroxylase has. In the light of the finding that lysyl hydroxylation in cultured fibroblasts is less readily affected by ascorbate deficiency than is prolyl hydroxylation [17], it is of interest that the K_m values of this vitamin for the two enzymes are almost identical. It may be noted that a markedly lower K_m for ascorbate in respect of lysyl hydroxylase from human skin fibroblasts, about 4 μM, has been reported in one study [24] using a very low concentration of the protocollagen substrate. It is not known whether this difference represents in part a true difference between lysyl hydroxylases from these two sources, but another explanation seems more likely. The parallel-line initial velocity pattern between ascorbate and the peptide substrate indicates that the apparent K_m for this vitamin measured at only one low peptide substrate concentration is markedly lower than the true value.

The specificity of the ascorbate requirement was found to be high, much higher than previously reported for crude lysyl hydroxylase from cultured WI-38 fibroblasts [10], whereas the specificity was very similar to that reported for pure prolyl 4-hydroxylase from chick embryos [16]. The only significant difference between the two hydroxylases is that the optimal dithiothreitol concentration with lysyl hydroxylase was 3 mM and that this concentration gave about 40% of the activity found with 1 mM ascorbate. 1 mM dithiothreitol, however, was about as efficient for lysyl hydroxylase as for prolyl 4-hydroxylase. The difference in the optimal concentration is probably due to the ability of dithiothreitol in low concentrations to dissociate prolyl 4-hydroxylase to its inactive subunits (see Ref. 16).

Lysyl hydroxylase was found to catalyze its reaction for a short time at a high rate in the complete absence of ascorbate. Similar data have previously been reported for prolyl 4-hydroxylase, and have been interpreted as indicating that the reaction with ascorbate does not occur during each catalytic cycle [16]. Thus it seems likely that this conclusion also holds good for the role of ascorbate in the lysyl hydroxylase reaction, but as the enzyme was not pure, it cannot be determined how many moles of hydroxylysine were formed per mol enzyme in 10 s.

On the basis of the present data, the finding that lysyl hydroxylation in cultured fibroblasts is less readily affected by ascorbate deficiency than is prolyl 4-hydroxylation [17] is probably not explicable solely by a different K_m for this vitamin in respect of the two hydroxylases, a difference in the specificity of this vitamin requirement or a difference in whether the vitamin is required for both enzymes during each catalytic cycle. A previous suggestion

that ascorbate may activate an inactive form of prolyl 4-hydroxylase protein [29] has been disproved [30,31]. The formation of a triple-helical procollagen molecules at 37°C requires hydroxylation of at least about 90 prolyl residues per pro-α chain [32,33]. The rate of prolyl hydroxylation apparently decreases in partial ascorbate deficiency, and thus the time required for triple helix formation is prolonged. As lysyl hydroxylase can only react with a non-triple-helical substrate [8,9], the above would allow more time for the hydroxylation of lysyl residues before the triple helix formation prevents any further reaction. The rate of lysyl hydroxylation is obviously also reduced, but as triple helix formation is a complex process, it is possible that in such a situation the extent of lysyl hydroxylation may be reduced less than that of prolyl 4-hydroxylation. If lysyl hydroxylase were present in cells in an excess, such a factor would contribute to the differing sensitivity of the two hydroxylations to ascorbate deficiency. In this connection it is of interest that inhibition of the hydroxylation of lysyl residues in newly-synthesized procollagen in isolated chick embryo tendon cells requires about 10 times higher Zn^{2+} concentrations than does inhibition of the 4-hydroxylation of prolyl residues (Anttinen, H., personal communication), even though the apparent K_i values of Zn^{2+} for lysyl hydroxylase [21] and prolyl 4-hydroxylase [14] are essentially identical.

Lysyl hydroxylase was found to catalyze an uncoupled decarboxylation of α-ketoglutarate in the absence of the peptide substrate at a rate which was about 4% of that found with a saturating L-I concentration. This uncoupled decarboxylation required Fe^{2+} and ascorbate suggesting that it was due to the same enzyme protein as the complete reaction. A similar uncoupled decarboxylation has previously been reported for prolyl 4-hydroxylase [14,19], the rate being about 1.3% of that found with a saturating concentration of the peptide $(Pro\text{-}Pro\text{-}Gly)_{10}$ [14]. These results thus indicate that α-ketoglutarate can be bound to the enzyme and decarboxylated in the absence of the peptide substrate. The intersecting initial velocity patterns, however, exclude [25—27] the release of CO_2 before the binding of the peptide in the complete reaction.

Acknowledgements

The present work was supported in part by a grant from the Medical Research Council of the Academy of Finland. The authors gratefully acknowledge the expert technical assistance of Mrs. Ritva Savilaakso, Mrs. Ritva Pihkakoski and Miss Tuula Räisänen.

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