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THE FUNCTION OF ASCORBATE WITH RESPECT TO PROLYL 4-HYDROXYLASE ACTIVITY

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Summary

1. Incubation in the presence of 2-oxoglutarate and oxygen inactivates prolyl 4-hydroxylase (prolyl-glycyl-peptide, 2-oxoglutarate:oxygen oxidoreductase, EC 1.14.11.2), with a $t_{1/2}$ of 80 s at 37°C. This inactivation is not affected by the presence or absence of the prolyl peptide substrate or added Fe(II).

2. This inactivation can be prevented by either ascorbate or dithiothreitol. It can be reversed by dithiothreitol but not by ascorbate.

3. Although the iron-containing form of prolyl 4-hydroxylase requires ascorbate for activity, ascorbate is not stoicheiometrically consumed in the reaction catalysed by the enzyme. Ascorbate cannot be replaced by alloxan, lactate, NADH plus phenazine methosulphate, dithiothreitol or L-cysteine.

4. Ascorbate has a double function with respect to prolyl 4-hydroxylase activity. On the one hand, it is required to initiate the reaction when the enzyme has become oxidized during isolation. On the other hand it is required for the protection against inactivation induced by 2-oxoglutarate and oxygen, presumably by preventing S-S bridge formation. The latter function may be of physiological importance.

Introduction

Prolyl 4-hydroxylase (prolyl-glycyl-peptide, 2-oxoglutarate:oxygen oxidoreductase, EC 1.14.11.2), a key enzyme in collagen synthesis, catalyses the post-translational hydroxylation of certain proline residues in the collagen peptide chains which is necessary for the triple-helical conformation of this protein (for reviews see Refs. 1—4). It belongs to the group of 2-oxoglutarate-requiring

dioxygenases which catalyse the general reaction

$$SH + O_2 + \text{2-oxoglutarate} \xrightarrow[\text{reductant}]{\text{Fe(II)}} SOH + \text{succinate} + CO_2$$

in which one atom of oxygen is incorporated into the substrate SH with the formation of a hydroxyl group and the other oxygen atom is incorporated into succinate [5]. Ascorbate is by far the best reductant with pure prolyl 4-hydroxylase [6], but may be replaced by other reductors, especially when partly purified enzyme preparations are used [7–10].

The precise role of the reductant is unknown. Lindstedt and co-workers [11] proposed that it is required to keep Fe(II) and -SH groups reduced. Hamilton [12] suggested that in non-enzymatic aromatic hydroxylations ascorbate is involved in the activation of O_2 by the formation of a complex of ascorbate-Fe(II)-O_2. Hobza et al. [13] proposed that enzyme-bound Fe(II) is oxidized to Fe(III) in the O_2-activation reaction and that the Fe(III) is reduced to Fe(II) by ascorbate. Tuderman et al. [14] showed, however, that ascorbate is not used stoicheiometrically during the hydroxylation of peptidyl proline. The possibility that an ascorbate-dehydroascorbate couple functions as an electron carrier was rejected by Rhoads and Udenfriend [10], since dehydroascorbate is ineffective as was also found by Tuderman et al. [14]. Recently Myllylä et al. [6] showed that prolyl 4-hydroxylase functions during 15–30 reaction cycles at an essentially normal rate without ascorbate. After inactivation, activity could be restored by ascorbate, although not completely. They suggested that the reaction is stopped when Fe(II), bound to the enzyme, becomes oxidized in a side reaction and that ascorbate specifically reduces the iron.

We have reported that our preparation of iron-containing prolyl 4-hydroxylase requires ascorbate [15] to start the reaction, suggesting that the iron under our conditions is bound as Fe(III) and has to be reduced before a reaction cycle can start. In the presence of the substrates, but in the absence of ascorbate, the enzyme is inactivated and activity is not restored by addition of ascorbate [15]. This suggests that the inactivation may be prevented but not reversed by ascorbate.

The present work deals with substrate inactivation, the role of ascorbate with respect to enzyme activity and the effect of dithiothreitol on the enzyme.

Materials

Prolyl 4-hydroxylase was isolated from chick embryos by $(NH_4)_2SO_4$ fractionation and affinity chromatography with poly-L-proline, as described previously [15]. The enzyme was pure according to polyacrylamide gel electrophoresis in the absence and the presence of sodium dodecyl sulphate (SDS) [15,16]. $(Pro\text{-}Pro\text{-}Gly)_5 \cdot 4\,H_2O$ was from Protein Research Foundation (Minoh-Shi, Osaka, Japan), 2-oxo[1-^{14}C]glutarate from New England Nuclear (Boston, MA) and horse-heart cytochrome *c* from Sigma Chemical Company (St. Louis, MO).

Methods

Determination of enzyme activity

Method A. The activity of prolyl 4-hydroxylase was determined at 37°C as described previously [15], by measuring the $^{14}CO_2$ production caused by the oxidative decarboxylation of 2-oxo[1-^{14}C]glutarate which takes place stoicheiometrically with the hydroxylation of peptidyl proline [17]. Unless otherwise stated, the reaction mixture contained in a volume of 1 ml, 1 μg enzyme/2 mg serum albumin/0.1 mg bovine liver catalase/0.1 mM dithiothreitol/1 mM ascorbic acid/5 μM $FeSO_4$/0.37 mM (Pro-Pro-Gly)$_5$ · 4 H_2O/0.1 mM 2-oxo[1-^{14}C]-glutarate ($6 \cdot 10^5$ dpm/μmol)/50 mM Tris-HCl buffer. The final pH was 7.4 at 20°C. For special precautions taken with respect to $FeSO_4$ and (Pro-Pro-Gly)$_5$ · 4 H_2O see method B.

Method B. The activity of prolyl 4-hydroxylase was determined essentially as described previously, by measuring the oxygen consumption polarographically [15]. Unless otherwise stated, the prolyl 4-hydroxylase reaction mixture contained, in a volume of 1.5 ml, 3 mg serum albumin/0.15 mg bovine liver catalase/50 mM Tris-HCl buffer/1 mM ascorbate/5 μM $FeSO_4$/37.5 μg enzyme/0.62 mM (Pro-Pro-Gly)$_5$ · 4 H_2O/0.4 mM 2-oxoglutarate. The final pH was 7.4 at 20°C. The $FeSO_4$ was dissolved just before addition to the reaction mixture to avoid hydroxide formation. The solution of (Pro-Pro-Gly)$_5$ · 4 H_2O was boiled for 5 min and cooled in ice just before addition [18]. For experiments at very low oxygen concentration, the oxygen was removed from the test mixture by bubbling with N_2 which contained less than 5 ppm O_2. An immediate rise in the O_2 concentration was achieved by addition of water saturated with O_2.

Ascorbate determination

The ascorbate content of the prolyl 4-hydroxylase reaction mixture, during turnover conditions, was measured spectrophotometrically at 549.5 nm by reduction of cytochrome *c*. At specified times a 50-μl sample of the reaction mixture (see legend to Fig. 2), containing 37.5 μg enzyme, was transferred to a spectrophotometer cuvette (1 cm) that contained 0.195 ml 50 μM cytochrome *c*/100 μM EDTA, to stop the enzymic reaction [15,16,19,20], and 25 mM phosphate buffer (pH 7.5) at 20°C. The ascorbate content was calculated making use of the relation

$A_{549.5nm}$ cytochrome *c* (red-ox) = 21.1 $mM^{-1} \cdot cm^{-1}$

After preincubation for 15 min in an open tube on a shaking water bath at 37°C, the prolyl 4-hydroxylase reaction was started by the addition of 2-oxoglutarate. The method described has the advantage over those making use of the reduction of dichloroindophenol [14,21,22], by the fact that it may be used in the presence of serum albumin and catalase which are necessary for maximum enzyme activity [10,14,15,23,24]. Control experiments showed that there was no interference by the components of the reaction mixture (or by EDTA) with the reduction of cytochrome *c* by ascorbate. Reduction of the ascorbate concentration from 1 mM (optimal) to 0.5 mM, in order to improve the accuracy of the determinations, diminished the enzyme activity by only 2%.

Gel electrophoresis

Electrophoresis on 7.5% polyacrylamide gels was carried out essentially as described by Davis [25] and modified by Knowles and Penefsky [26], but with the omission of sucrose, EDTA and ATP.

Protein determination

The concentration of enzyme protein was determined spectrophotometrically, making use of the relation $A_{230nm} = 7.73\ (mg/ml)^{-1} \cdot cm^{-1}$ [27].

Results

As reported previously [15], prolyl 4-hydroxylase is inactivated by incubation in the presence of all its substrates but in the absence of ascorbate (see Table I, lines 1 and 2), with a $t_{1/2}$ for the inactivation of 80 s (cf. Fig. 2, curve A). Preincubation in the absence of 2-oxoglutarate, either in the presence (Table I, line 3) or the absence (line 4) of ascorbate, did not lead to inactivation. Line 5 of Table I demonstrates that the addition of Fe(II) is not required for the inactivation in the absence of ascorbate. Line 6 of Table I shows that preincubation of the enzyme in the absence of $(Pro\text{-}Pro\text{-}Gly)_5$ does not result in inactivation, but that on the contrary a higher activity is found when the reaction is started with $(Pro\text{-}Pro\text{-}Gly)_5$ than when it is started with 2-oxoglutarate (cf. lines 6 and 3). The last line of Table I also shows that the presence of $(Pro\text{-}Pro\text{-}Gly)_5$ is not required for inactivation in the absence of ascorbate. Variation of the moment of ascorbate addition, during the time between the start of the preincubation by the addition of 2-oxoglutarate and the end of the preincubation 5 min later by the addition of $(Pro\text{-}Pro\text{-}Gly)_5$, shows an inactivation with a time-dependency comparable to that shown in Fig. 2A. These results demonstrate that ascorbate must be present when 2-oxoglutarate is added, in order to prevent the inactivation, and that the inactivation cannot be reversed by ascorbate [15].

Gel-electrophoresis experiments showed that the inactivation was not associated with the dissociation of the tetramers to inactive [28] monomers

TABLE I

THE EFFECT OF PREINCUBATION CONDITIONS ON PROLYL 4-HYDROXYLASE ACTIVITY (MEASURED AS O_2 UPTAKE)

16 μg enzyme were used. Concentrations and other assay conditions are as described in Methods. For abbreviations see legend to Fig. 1. The experimental arrangements can be derived from Fig. 1C.

Omission from reaction mixture during preincubation	Preincubation time (min)	Activity ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)
None *	—	0.83
Ascorbate	5	0.12
2-Oxoglutarate	5	0.90
2-Oxoglutarate, ascorbate	5	0.81
Fe(II), ascorbate	3	0.24
$(Pro\text{-}Pro\text{-}Gly)_5$	5	1.23
$(Pro\text{-}Pro\text{-}Gly)_5$, ascorbate	5	0.15

* The reaction was started with ascorbate + 2-oxoglutarate.

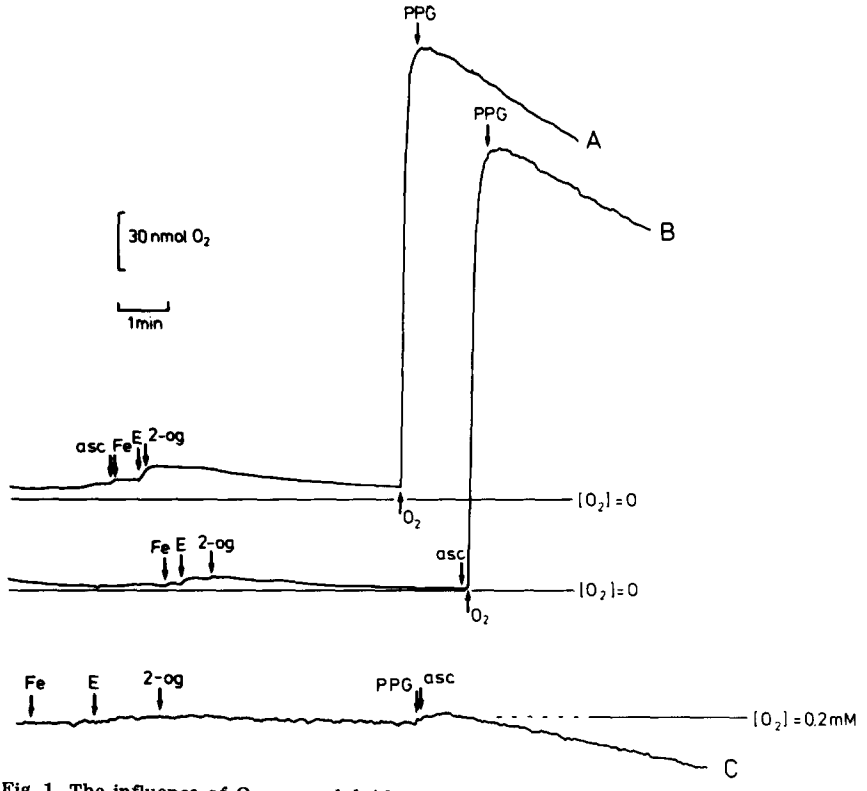

Fig. 1. The influence of O_2 on prolyl 4-hydroxylase activity, incubated with 2-oxoglutarate in the presence and absence of ascorbate. Trace A + B: Removal and addition of O_2 as described in the Methods section, trace C: control in the presence of O_2. Abbreviations: asc, ascorbate; Fe, $FeSO_4$; E, enzyme; PPG (Pro-Pro-Gly)$_5 \cdot 4\ H_2O$; 2-og, 2-oxoglutarate. 15 μg enzyme were used, other concentrations and assay conditions are as described in method B.

(data not shown). Fig. 1 shows that the enzyme is not inactivated when it is preincubated with 2-oxoglutarate in the absence of oxygen, either in the presence or the absence of ascorbate.

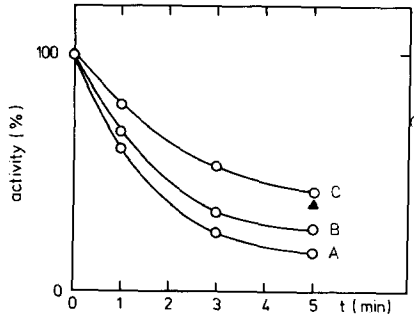

Fig. 2. Inactivation of prolyl 4-hydroxylase after preincubation in the presence of the substrates and in the absence of ascorbate. Activities were measured as O_2 consumption from polarographic traces (see Methods) and corrected for auto-oxidation of ascorbate and dithiothreitol. The preincubation was started by the addition of 2-oxoglutarate. Curve A, no dithiothreitol present; curve B, 0.1 mM dithiothteitol; curve C, 1 mM dithiothreitol; ▲, 2 mM dithiothreitol. 16 μg enzyme were used. Concentrations of other components are as described for method B.

Although our enzyme preparation requires ascorbate for activity [15], no ascorbate consumption could be detected over a non-enzymatic decrease of 1.5 nmol · min^{-1} in a 30 min experiment. The activity of the enzyme was 43 nmol · min^{-1}, measured as O_2 uptake.

Ascorbate could not be replaced as an initiator of the enzyme activity by 1 mM alloxan (cf. Ref. 29), lactate (10 mM) or NADH (0.2 and 0.5 mM) in combination with phenazine methosulphate (0.1 and 5 μM) when the enzyme activity was measured by $^{14}CO_2$ production (data not shown). Neither could it be replaced by dithiothreitol (2 mM) nor L-cysteine (5 mM), reported as optimal concentrations [6].

In these last two experiments the enzyme activity was measured continuously by following the oxygen uptake, since at this concentration dithiothreitol causes enzyme dissociation [28,30].

Although dithiothreitol cannot replace ascorbate as an initiator of the reaction, in concentrations up to 1 mM it is able to partly protect against the inactivation caused by 2-oxoglutarate and oxygen (see Fig. 2). Less protection is obtained with a higher concentration (2 mM, Fig. 2), probably because of dissociation of the enzyme (cf. Refs. 28, 30).

The enzyme can be partly reactivated by addition of 1 mM dithiothreitol after inactivation (Fig. 3). Addition of dithiothreitol directly after the addition

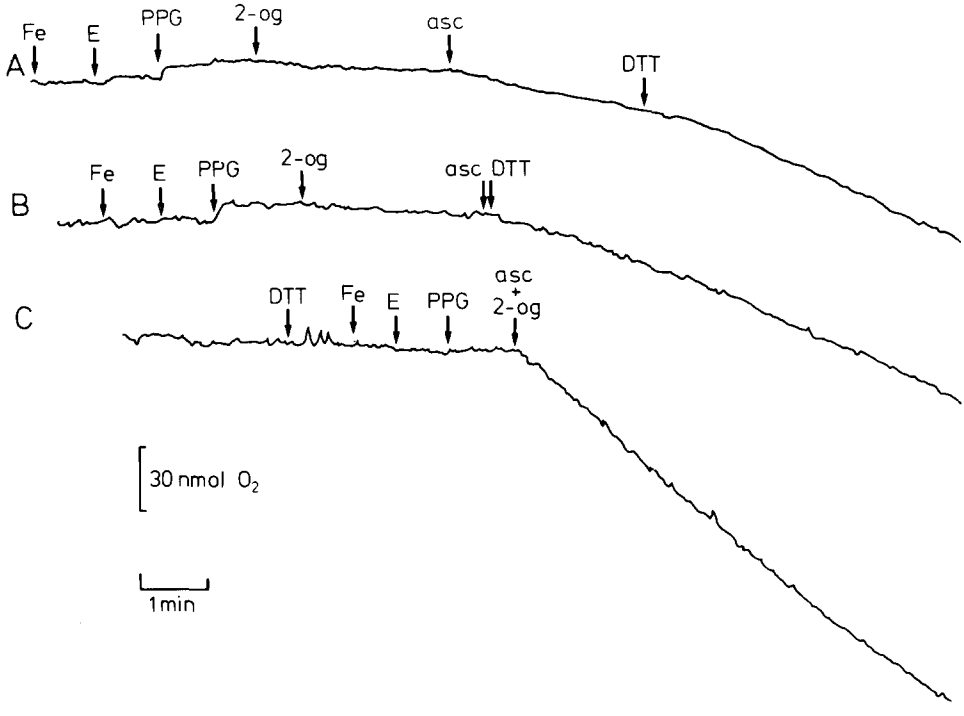

Fig. 3. Reactivation of prolyl 4-hydroxylase by dithiothreitol. 15 μg enzyme/1 mM dithiothreitol. Other assay conditions are as described for Fig. 2. Abbreviations: DTT = dithiothreitol; others are as described for Fig. 1.

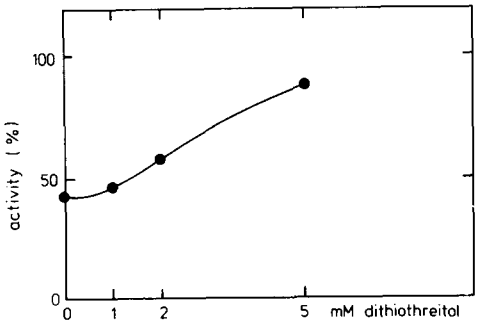

Fig. 4. Reactivation of prolyl 4-hydroxylase by dithiothreitol. After inactivation for 1 min (by omission of ascorbate), dithiothreitol was added together with ascorbate. The activity was measured 1 min later, and expressed as percentage of the activity measured with non-inactivated enzyme that had been in contact with the same dithiothreitol concentration for 1 min. 15 μg enzyme were used per determination. Other assay conditions are as described for Fig. 2.

of ascorbate does not increase the degree of reactivation (curve B). The degree of reactivation was also not increased by addition of an extra 5 μM Fe(II) (data not shown). To study the reactivation at higher dithiothreitol concentrations the simultaneously occurring inactivation, due to dissociation of the tetramer by dithiothreitol (cf. Fig. 2), was accounted for in separate control experiments in which ascorbate was present during the preincubation. The activity after reactivation, expressed as percentage of the activity found after preincubation in the presence of ascorbate and dithiothreitol (Fig. 4), indicates that nearly full reactivation could be obtained with sufficient dithiothreitol.

The effect of ascorbate with respect to the initiation of the reaction and secondly the protection against inactivation due to 2-oxoglutarate and oxygen have about the same half-maximal concentration, 0.1 mM. Prolyl hydroxylase activity was measured after starting the reaction by addition of 2-oxoglutarate, ascorbate being present in concentrations varying from 0 to 1 mM. (First phase). After 1 min the concentration of ascorbate in all incubations was raised to 1 mM. (Second phase). From the reaction rates in the first phase the half-maximal ascorbate concentration for the initiation of the reaction was calculated. For the protective effect this was calculated from the second phase. The difference in rate after raising the ascorbate concentration to 1 mM, stabilizing the enzyme (cf. Fig. 3), and the rate after 1 min preincubation in the absence of ascorbate, was half-maximal when in the first phase the ascorbate concentration was 0.1 mM. The rate after 1 min preincubation in the absence of ascorbate represents the not-inactivated enzyme.

Discussion

Although the iron-containing form of prolyl 4-hydroxylase requires ascorbate for the initiation of the reaction [15], ascorbate is not consumed in the enzyme reaction, which can be reconciled with earlier findings [6,31]. The sensitivity of the ascorbate determination was insufficient to detect a stoicheiometric reduction of the iron present in the amount of enzyme used (0.15 nmol). As initiator of the enzyme reaction, ascorbate, in our hands, could not

be replaced by alloxan, -SH compounds, lactate or NADH plus phenazine methosulphate. The inactivation of the enzyme, brought about by preincubation in the presence of 2-oxoglutarate and oxygen, leaves the enzyme in tetrameric structure but is different from that occurring during isolation of the enzyme since it can be reversed by dithiothreitol but not by ascorbate. Protection against this inactivation can be given by either of these reducing agents. It appears then, that, in the presence of 2-oxoglutarate, oxygen causes not only an oxidation of Fe(II) to Fe(III), which can be reversed by ascorbate, but that with the Fe(III) enzyme an oxidation of (an)other group(s) takes place, which can be reversed by dithiothreitol. It has been suggested on the basis of experiments with -SH group blockers that free -SH groups are essential for enzyme activity and that 2-oxoglutarate provides considerable protection against this blocking [32,33]. Presumably, 2-oxoglutarate induces a conformation change conducive to the oxidation of these -SH groups resulting in S-S bridge formation, independent of the addition of $(Pro\text{-}Pro\text{-}Gly)_5$ or Fe(II) (see Table I). The E'_0 of a disulphide bond is −200 to −400 mV [34] and this may explain why dithiothreitol which has an E'_0 of −332 mV [35], can reactivate the enzyme and ascorbate cannot (E'_0 = +60 mV). At the same time this implies that ascorbate keeps the -SH groups reduced in an indirect way, possibly by prevention of the conformation change necessary for inactivation.

Besides the large effect of preincubation with 2-oxoglutarate on the enzyme, depending on the presence or absence of ascorbate, our results also showed an effect of preincubation with $(Pro\text{-}Pro\text{-}Gly)_5$ (cf. lines 3 and 6 of Table I). The lower activity we found might be explained by the formation of dead-end complexes at the binding of $(Pro\text{-}Pro\text{-}Gly)_5$. Binding to $(Pro\text{-}Pro\text{-}Gly)_n$ has been shown to exist under non-turnover conditions [28] and the formation of dead-end complexes with $(Pro\text{-}Pro\text{-}Gly)_{10}$ has been suggested by Myllylä et al. [31] on kinetic grounds. Ascorbate, however, hardly seems to influence this phenomenon (cf. lines 3 and 4 of Table I).

The picture emerges that an active enzyme, with iron in the Fe(II) form, catalyses the reaction without the need for added iron or ascorbate, as far as the initiation of the reaction is concerned. Iron is required only when it has been lost during the preparation of the enzyme. If the iron has become oxidized during isolation of the enzyme or possibly the protein part of the enzyme has become oxidized, as was recently suggested [36], ascorbate is required to initiate the reaction.

The half-maximal concentration of ascorbate with respect to the initiation of the reaction and the protection against inactivation by 2-oxoglutarate and oxygen is about the same.

Our results also demonstrate that it is necessary for ascorbate to interact with the enzyme before 2-oxoglutarate in order to prevent this inactivation. This requires its permanent presence under turnover conditions and might be an important physiological function of ascorbate.

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