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PROPERTIES OF PROLYL 4-HYDROXYLASE CONTAINING FIRMLY-BOUND IRON

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Summary

1. Prolyl 4-hydroxylase (prolyl-glycyl-peptide, 2-oxoglutarate:oxygen oxidoreductase, EC 1.14.11.2) was isolated in a form containing iron (0.85—1.1 mol Fe/mol enzyme).

2. The enzyme was pure according to gel electrophoresis and had a high specific activity (1.8—2.6 μmol · min^{-1} · mg^{-1}).

3. Experiments with metal chelators showed this iron to be firmly bound and to be required for catalytic activity.

4. According to EPR spectrometry the bound iron is not part of a [2Fe-2S] or a [4Fe-4S] cluster.

5. The enzyme activity is to a large extent independent of added Fe^{2+}.

6. The enzyme activity is completely dependent on ascorbate.

7. In the absence of ascorbate but the presence of substrates the enzyme is irreversibly inactivated.

8. Continuous measurement of enzyme activity was possible by following oxygen uptake.

Introduction

In collagen synthesis the dioxygenase prolyl 4-hydroxylase (prolyl-glycyl-peptide, 2-oxoglutarate:oxygen oxidoreductase, EC 1.14.11.2) catalyses the post-translational hydroxylation of peptidyl proline, stoicheiometrically coupled to the oxidative decarboxylation of 2-oxoglutarate [1]:

$$\text{-Pro-} + \text{2-oxoglutarate} + O_2 \xrightarrow[\text{ascorbate}]{Fe^{2+}} \text{-Hyp-} + \text{succinate} + CO_2$$

Several groups have proposed a role for iron in the binding of oxygen and

formation of an active intermediate [2,3], in which oxidation of Fe^{2+} to Fe^{3+} by O_2 is also thought to be involved [3—5]. Bhatnagar et al. [6] have invoked a second type of iron with a structural role in addition to that directly involved in the catalysis. Recently, Cumming et al. [7] have suggested that the rat-skin enzyme can bind 4 iron atoms per molecule.

There are conflicting reports in the literature concerning the activity of the enzyme in the absence of added Fe^{2+}. Whereas a number of workers [8—11] find a complete dependence on added Fe^{2+}, others [12—14] find some activity in the absence of added Fe^{2+}, even with a purified preparation [14]. Prockop et al. [14] reasonably attribute these different results to differences in the extent to which the enzyme loses iron during purification. Indeed, Pänkäläinen and Kivirikko [15] found much less than 1 mol Fe/mol enzyme after purification by ion-exchange chromatography.

The role of ascorbate in the reaction is still uncertain. Hobza et al. [3] suggested that it is necessary to regenerate Fe^{2+} after the oxidation of Fe^{2+} to Fe^{3+} by oxygen, but Myllylä et al. [5] have shown that ascorbate is not used stoicheiometrically in the reaction and that the initial phase of the reaction is independent of ascorbate [16].

In the present paper, a preparation of prolyl hydroxylase is described that appears to differ clearly from those previously described, in that it contains 1 mol Fe/mol enzyme and is highly active (50—78% of the maximum) in the absence of added Fe^{2+}. On the other hand, in distinction to the preparation described by Myllylä et al. [16] activity is completely dependent on the addition of ascorbate. In other respects, our preparation closely resembles those described in the literature.

Materials and Methods

Materials

Chick embryos (age 13 days) were obtained from hatchery J. Meereboer (Beets, Holland). Poly-L-proline of different types was from Sigma Chemical Company (St. Louis, MO). $(Pro\text{-}Pro\text{-}Gly)_5 \cdot 4\,H_2O$ was from the Protein Research Foundation (Minoh-Shi, Osaka, Japan). 2-Oxo-[1-^{14}C]glutarate was from New England Nuclear (Boston, MA).

Methods

Enzyme isolation. The isolation of prolyl hydroxylase was carried out essentially as described by Tuderman et al. [17] with some modifications. After dialysis of the preparation obtained by ammonium sulphate fractionation [17], the solution was centrifuged for 30 min at 40 000 $\times g$, diluted to 10 mg/ml and mixed with the Sepharose 4B to which poly-L-proline (M_r = 50 000) was coupled. The mixture was gently shaken for 3 h at 4°C and the Sepharose was then washed on a glass filter until the red colour had disappeared. The washing was then continued in a column until the effluent had an absorbance less than 0.1 at 230 nm. The bound prolyl hydroxylase was eluted with the prescribed [17] buffer, containing 3 mg/ml poly-L-proline (M_r = 9000). The fractions containing the eluted material were collected until the A_{230nm} fell below 1.5, and the pooled fractions concentrated to about 4 ml in an ultrafiltration cell (Ami-

con) with a PM 30 membrane. The concentrate was centrifuged for 10 min at 100 000 × *g* and the supernatant was applied onto the gel-filtration column. The fractions containing the enzyme were pooled, concentrated in an ultrafiltration cell (Amicon PM 30 membrane) and stored in liquid N_2.

Determination of enzyme activity. Method A. The activity of prolyl hydroxylase was determined with 0.4—9.6 μg enzyme at 37°C by measuring the oxidative decarboxylation of 2-oxo-[1-^{14}C]glutarate which takes place stoicheiometrically with the hydroxylation reaction [1]. Unless otherwise stated, the reaction mixture contained, in a volume of 1 ml, 2 mg serum albumin, 0.1 mg bovine liver catalase, 0.1 mM dithiothreitol, 1 mM ascorbic acid, 5 μM $FeSO_4$, 370 μM (Pro-Pro-Gly)$_5$ · 4 H_2O, 0.1 mM 2-oxo-[1-^{14}C]glutarate (6 · 10^5 dpm/μmol), 50 mM Tris-HCl buffer. 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 to the mixture [17]. After preincubation for 15 min, the reaction was started with 2-oxo-[1-^{14}C]glutarate and stopped 20 min later (unless otherwise stated) with 50 μl 4 M H_2SO_4 [18]. The enzyme activity was constant for up to 40 min. The reaction was carried out in a reaction tube shaken in a water bath. The tube was closed with a rubber stopper to the bottom of which a wire hook was attached, carrying two Whatman filter papers (1 × 2 cm), containing 10 μl 1 M hyamine hydroxide to bind the CO_2. The stoppers were pierced by a small Teflon tube, closed with a pin, through which the H_2SO_4 was injected. Hyamine-bound $^{14}CO_2$ was counted with 80% efficiency in a Triton X-100/toluene mixture (1 : 3) containing 2,5-diphenyloxazol (3 g/l) and 1,4-di-2-(5-phenyloxazolyl)-benzene (0.2 g/l).

Method B. The activity of prolyl hydroxylase was also determined by measuring the oxygen consumption polarographically at 37°C with a Clark-type electrode in a 1.5 ml reaction volume containing 16 μg of enzyme. The other components of the reaction mixture were present in the same concentrations as described above under A, unless otherwise stated.

Gel electrophoresis. Electrophoresis on 7.5% polyacrylamide gels was carried out by the method of Davis [19] as modified by Knowles and Penefsky [20]. Electrophoresis on 13.5% polyacrylamide gels in the presence of sodium dodecyl sulphate was carried out essentially as described by Weber and Osborn [21], using stacking gels as described by Maurer [22].

Iron determination. The iron content of the enzyme preparation was determined as described by Maessen et al. [23,24] for other elements with a Varian Techtron AA_5 spectrophotometer equipped with a carbon rod atomizer Model 61, using mini-Massman type carbon rods (Ringsdorff high purity graphite, grade R.W.I.). A PAR model 120 lock-in amplifier, a storage oscilloscope (Tektronic type 564) and a transient recorder (Biomation model 802) were coupled to the spectrophotometer. The 5-μl sample was dried at 1 V for 16 s and ashed at 6 V for 3 s, and atomization was carried out in the step mode (8 V, 4 s). The absorption was measured at 248.3 nm. The standard $FeSO_4$ solution contained 17.81 μM Fe in 0.02 M HNO_3 supra pure. For a calibration curve four different dilutions of the standard solution were determined at least in quadruplicate.

EPR measurements. Electron paramagnetic resonance measurements were

performed at 10—60 K on a Varian E-3 spectrometer adapted for work at low microwave powers. The modulation frequency was 100 kHz.

Protein determination. Protein concentration was determined spectrophotometrically, making use of the relation A_{230nm} equal to 7.73 $(mg/ml)^{-1} \cdot cm^{-1}$ [25].

Results

The enzyme preparations were pure according to gel electrophoresis in the absence and the presence of sodium dodecyl sulphate. The molecular weight of the subunits was 58 000 and 62 000 respectively, which is in good agreement with the reported values [17,26—29].

The rate of the enzyme-catalysed reaction at saturating concentrations of $(Pro\text{-}Pro\text{-}Gly)_5$, calculated by extrapolation of a Lineweaver and Burk plot, varied between 1.8 and 2.6 μmol/min per mg for five different preparations. These values are higher than those reported to date [17]. The turnover number of the enzyme in the uncoupled decarboxylation, i.e. the enzymatic decarboxylation of 2-oxoglutarate that occurs in the absence of peptide substrate [10,11,30,31], ranged from 0.02—0.03 μmol/min per mg, which is close to the value reported by Tuderman et al. [11] but is substantially higher than reported by Counts et al. [10]. Substrate inhibition was found at concentrations of $(Pro\text{-}Pro\text{-}Gly)_5$ higher than 0.56 mM. For the determinations of apparent K_m values, concentrations of the other substrates were as described in methods. The K_m for $(Pro\text{-}Pro\text{-}Gly)_5$ varied from 400 to 500 μM, while Berg et al. [32] reported 300 μM and Myllylä et al. [5] 130—200 μM. The K_m for 2-oxoglutarate was found to be 8 μM, based on a secondary plot from intercepts of Lineweaver and Burk plots, while the ascorbate concentration for $V_{1/2}$ was found to be 250 μM, based on a double reciprocal plot. These values are, respectively, not significantly different or in agreement with values reported in other literature [8,10,33,34]. A pH optimum at 7.4 was found for this pure preparation, which is the same as that reported in 1967 [35] but somewhat lower than the 7.8 reported recently [10].

Table I summarizes the effects on the enzyme activity of omitting different components of the reaction medium. The most striking finding is that without added iron a considerable activity is left (with four preparations ranging from 50—78% of the activity of the complete system). This is in contrast with the findings of others [8—11]. The effects of omitting serum albumin and catalase were also much more pronounced with our preparation than reported before [11,34,35], but the effects of ascorbate and dithiothreitol were comparable [8,10,11,36].

Even after dialysis for 2 h at 4°C against a buffer containing the iron chelators 2 mM EDTA or 2 mM *o*-phenanthroline, followed by extensive dialysis for 20 h to remove the chelators, substantial activity without the addition of iron remained (Table II). The decrease in activity, which was also found in the absence of a chelator, may be due to dissociation of the enzyme during dialysis.

We verified, with flameless atomic absorption spectroscopy (see Methods) that the results could not be explained by contaminating iron (less than or equal to 0.2 μM) in the incubation medium. No non-haem iron was found in

TABLE I

EFFECT OF DIFFERENT COMPONENTS ON PROLYLHYDROXYLASE ACTIVITY

Test conditons were as described in Methods, except that the reaction time in experiment 1 was 10 min, while in experiment 3 50 μM Fe^{2+} and 2 mM ascorbate were used and the reaction time was 15 min. Each value is corrected for the slight $^{14}CO_2$ evolution under the same conditions but in the absence of the enzyme. Expt. 1, 1.17 μg enzyme; Expt. 2, 0.4 μg enzyme; Expt. 3, 0.65 μg enzyme.

Experiment	Component lacking	Activity (μmol/min per mg)
1	None	1.13
	Ascorbate	0.00
	$FeSO_4$	0.75
2	None	1.37
	Catalase	0.13
3	None	0.90
	Serum albumin	0.20
	Dithiothreitol	0.60

the catalase preparation (vide infra).

Since these results suggested that the enzyme preparation contained non-diffusable iron, we determined the iron content of our enzyme preparations by flameless atomic absorption spectroscopy. The reliability of the determinations was tested with two iron-containing proteins: cytochrome *c* and catalase. A cytochrome *c* solution containing 1.75 μM Fe^{2+}, according to the absorbance change at 549.5 nm on reduction, gave by using flameless atomic absorption 1.6—1.9 μM Fe. A catalase solution containing 1.66 μM Fe^{2+} according to absorbance at 277 nm gave 1.47—1.73 μM by flameless atomic absorption. Table III shows that our preparations of prolyl hydroxylase contain 0.85—1.1 mol Fe/mol enzyme (molecular weight 240 000). Preliminary experiments in which the enzyme preparation was mixed with 1 mM EDTA in an ultrafiltration cell followed by diafiltration to remove the EDTA, indicated that the enzyme preparation still contained about 0.6—0.7 mol Fe/mol. As shown in Fig. 1 (Expt. I) maximal activity was found with 5 μM Fe^{2+}, a value much lower than reported in other literature [9,11], while 50 μM gave 40—50%

TABLE II

PROLYLHYDROXYLASE ACTIVITY AFTER DIALYSIS AGAINST IRON CHELATORS

Enzyme samples (0.5 ml, 0.12 mg enzyme) were dialysed for 2 h at 4°C against 250 ml of: (A) 0.2 M NaCl/0.2 M glycine/10 μM dithiothreitol/10 mM Tris-HCl buffer (pH 7.8); (B) idem (A) + 2 mM EDTA; (C) idem (A) + 2 mM *o*-phenanthroline. Subsequently all three preparations were dialysed at 4°C against the buffer used for (A), for 1 h against 2 l, 1 h against 2 l, 2 h against 2 l and 16 h against 2 l. Enzyme activity was determined as described in Methods (9.6 μg enzyme).

Sample	Treatment	Activity (μmol/min per mg)	
		50 μM Fe^{2+}	minus Fe^{2+}
A	Dialysis	0.37	0.27
B	Dialysis + EDTA	0.46	0.31
C	Dialysis + *o*-phenanthroline	0.27	0.25

TABLE III

IRON CONTENT OF PROLYLHYDROXYLASE (M_r = 240 000)

The enzyme was dissolved in isolation buffer: 0.2 M NaCl/0.2 M glycine/10 μM dithiothreitol/10 mM Tris-HCl buffer (pH 7.8 at 4°C). Determinations were as described in Methods.

Preparation	μM Fe	μM enzyme	mol Fe/ mol enzyme
1	1.1	1	1.1
2	2.1	2.5	0.85
3	2.7	2.4	1.1
4	2.6	2.6	1.0
Isolation buffer	<0.15 *		

* Determination limit.

inhibition. The latter concentration is that used by Tuderman et al. [11] to obtain maximal activity.

The addition of 10 μM EDTA or 0.1 mM Tiron (sodium pyrocatechol disulphonate) to the reaction mixture during the measurement of the enzyme activity (iron omitted) caused an inhibition of 98–100%. If in the presence of 10 μM EDTA, iron was added to the test mixture in concentrations up to 15 μM, activity could not be restored to the normal value (Fig. 1, Expts. I and II).

EPR spectroscopy was used to see whether the iron in the enzyme is part of an iron-sulphur cluster and to look for superoxide signals. Enzyme preparations in a concentration of up to 80 μM did not show a signal specific for iron-sulphur proteins, nor could a O_2^- radical signal be detected. Only a weak signal was present at $g = 4.3$ which is generally assigned to high-spin Fe^{3+} in a rhombic environment. This signal was only slightly affected by the addition of 0.2 mM 2-oxoglutarate, 0.6 mM $(Pro\text{-}Pro\text{-}Gly)_5 \cdot 4\,H_2O$ and 2 mM ascorbate in this order.

As stated in Table I ascorbate is necessary in an activity test lasting 20 min. Fig. 2 shows that ascorbate is also necessary in the initial phase of the reaction.

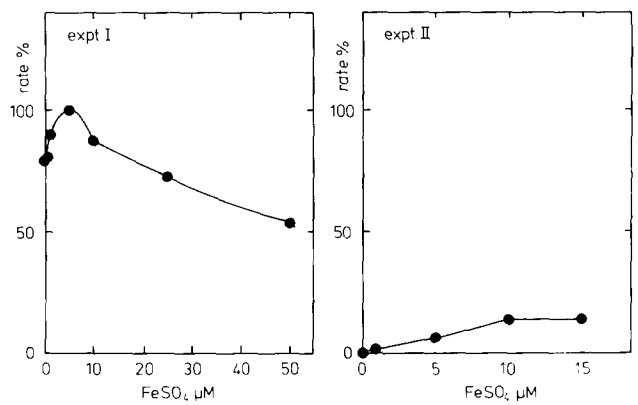

Fig. 1. Prolyl hydroxylase activity at different Fe^{2+} concentrations in the presence and absence of EDTA. Expt. I: no EDTA present, 1 μg enzyme. Expt. II: preincubated (15 min) with 10 μM EDTA, 0.7 μg enzyme. Other conditions as described for measuring $^{14}CO_2$ production.

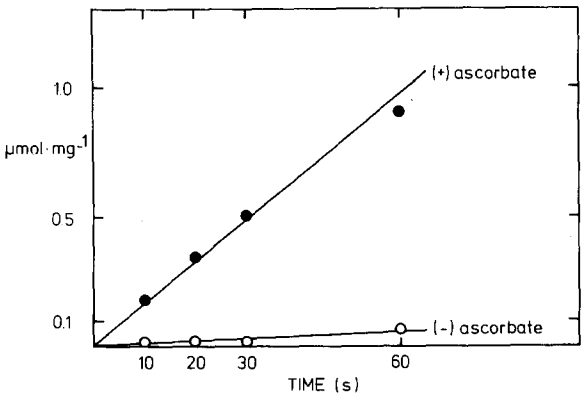

Fig. 2. Effect of ascorbate on prolyl hydroxylase activity. Dithiothreitol was omitted from the reaction mixture, 1.6 μg enzyme; •———•, ascorbate, 1 mM; ○———○, no ascorbate. Other conditions as described for measuring $^{14}CO_2$ production.

This is in contrast to the findings of Myllylä et al. [16] who reported that in the absence of ascorbate the enzyme can undergo 15—30 turnovers at maximal rate. By continuously measuring the oxygen consumption of the reaction it was confirmed that during the initial phase of the reaction ascorbate is required (Fig. 3, trace I). This was also found in the absence of added Fe^{2+} (not shown). Comparison in Fig. 3 of trace I with trace II indicates that when the reaction is started with ascorbate, delaying the addition of ascorbate for 5 min leads to inactivation of the enzyme. The rate decreased from 1.6 μmol/min per mg to 0.5 μmol/min per mg. Fig. 3 trace III shows a control indicating only slight autooxidation of ascorbate.

The rates of oxygen consumption and $^{14}CO_2$ production were in reasonable agreement; e.g. 0.96 μmol $^{14}CO_2$/min per mg and 1.1 μmol O_2/min per mg, respectively, in a typical experiment.

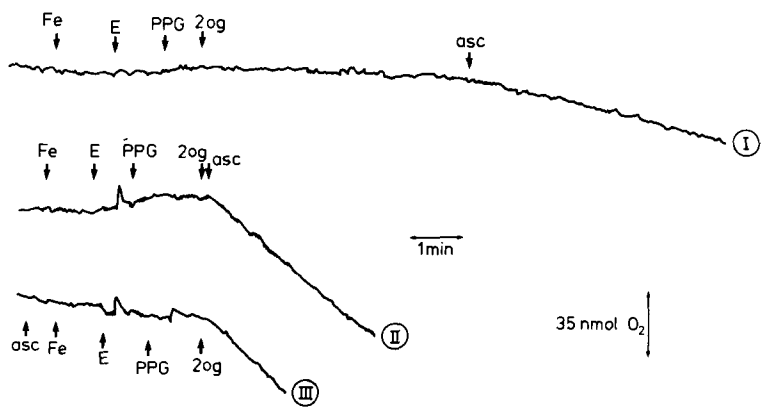

Fig. 3. Inactivation of prolyl hydroxylase in the absence of ascorbate. Dithiothreitol was omitted from the reaction mixture. Additions: asc, ascorbate; Fe, $FeSO_4$; E, 16 μg enzyme, PPG, $(Pro\text{-}Pro\text{-}Gly)_5$; 2 og, 0.4 mM 2-oxoglutarate. Assay conditions were as described in the Methods for measuring O_2 uptake with the exceptions mentioned above.

Discussion

Our enzyme preparations contain 1 atom of firmly bound iron per molecule. The iron is difficult to remove by dialysis against iron chelators.

Our preparations differ from those obtained by Tuderman et al. [11] in that they have a high activity (50—78% of maximum) in the absence of added iron. A simple explanation for the discrepancy would be that iron has been dissociated from the enzyme during its preparation by these workers, perhaps during freezing and thawing after ammonium sulphate fractionation, a step that was omitted in our procedure. A procedure described earlier by this group [15] yielded an enzyme that was indeed free from iron. Also Berg (see ref. 14) has isolated an enzyme with considerable activity in the absence of added iron (40% of maximum) and Prockop et al. [14] suggested that the enzyme may lose iron during isolation. If Fe^{3+} is more firmly bound than Fe^{2+}, as suggested by Myllylä et al. [16], the redox state of the enzyme during isolation may to some extent determine its final iron content.

The fact that our preparations require ascorbate in the initial reaction phase suggests that we isolated the enzyme in a form containing Fe^{3+} which has to be reduced before the reaction can start.

The finding that EDTA, added under conditions in which the enzyme is turning over, strongly inhibits, is also understandable if the iron is reduced during enzyme action, and Fe^{2+} is less firmly bound than Fe^{3+}. The poor restoration of the activity with excess $FeSO_4$ may be due to irreversible inactivation of the enzyme after removal of the iron under turnover conditions. This is in contrast with the restoration of activity by Prockop and Juva [37] but in their embryonic extract the enzyme may have been stabilized by remaining bound to endoplasmic reticulum fragments [38]. The maximal activity of the preparation of Cumming et al. [7], which was isolated in the presence of 10 μM EDTA, was of the same order as that found by us with excess Fe^{2+} in the presence of 10 μM EDTA (Fig. 1, Expt. II).

If after the binding of 2-oxoglutarate to the enzyme, O_2 binds to Fe^{2+} and forms $Fe(III)O_2^-$ as is proposed by Myllylä et al. [16] this may be undetectable in EPR spectrometry because of spin-spin coupling. The lack of response of the EPR signal at $g = 4.3$ to the addition of 2-oxoglutarate may be explained in this way and is in agreement with the absence of an O_2^- radical signal. Based on the finding of Popenoe et al. [39] that a SH group is located near the catalytic site, Hobza et al. [3] suggested that the iron interacts with a SH group. Although non-specifically bound iron also gives a signal at $g = 4.3$ [40] this signal could be caused by a rubredoxin-like structure with four cysteines liganded to Fe^{3+}.

In the view of the stimulation by added iron it seems possible that in comparison with the native enzyme, our preparation has lost part of its iron and that the enzyme can bind more than 1 mol Fe per mol enzyme, as was suggested recently by Cumming et al. [7].

As there are 30 cysteines in the enzyme [26], evenly distributed over the monomers [29], there may be room for more than one rubredoxin-like centre.

It is clearly shown that the enzyme is activated by ascorbate (probably by reduction of Fe^{3+}). The inactivation of the enzyme in the presence of the substrates but the absence of ascorbate suggests a reaction which cannot be

reversed by ascorbate but which is prevented in the presence of this compound. Whether a conformational change in the enzyme due to substrate binding and/or irreversible removal of the iron from the enzyme is involved in the inactivation, is under investigation.

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