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THE ROLE OF ASCORBATE IN THE PROLYL HYDROXYLASE REACTION

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SUMMARY

Ascorbate was not required for the binding of 2-oxoglutarate to pure prolyl hydroxylase, and the enzyme catalyzed hydroxylation in the absence of ascorbate at an essentially maximal rate for 5-10 s, corresponding to 15-30 reaction cycles. After about one min the reaction rate in the absence of ascorbate was very low, even though only 1-2 % of the free bivalent iron had become oxidized. These and additional data indicate that prolyl hydroxylase can catalyze a number of reaction cycles without ascorbate, but at some stage the hydroxylation ceases, probably due to oxidation of the enzyme-bound iron, and ascorbate is then required as a quite specific reductant to re-activate the enzyme.

The hydroxylations in collagen biosynthesis which are catalyzed by prolyl 4-hydroxylase (termed here prolyl hydroxylase), prolyl 3-hydroxylase and lysyl hydroxylase are all dependent on the presence of a reducing agent. This requirement is best fulfilled by ascorbate, and the defective collagen synthesis encountered in scurvy has long been attributed to the need for this vitamin in these reactions (1-4). Several suggestions have been made concerning the role of ascorbate in the prolyl hydroxylase reaction (1-4), but none has so far been proven. Recent studies on the kinetics of the prolyl hydroxylase reaction gave results consistent with an ordered binding of Fe^{2+}, 2-oxoglutarate, O_2 and the polypeptide substrate to the enzyme in this order, the binding of Fe^{2+} being at thermodynamic equilibrium (5). The hydroxylation occurs only after the binding of all four reactants, and the products are then released, possibly in the order: the hydroxylated polypeptide, CO_2 and succinate. Ascorbate was found to react by a substitution mechanism, either before the binding of Fe^{2+} or after the release of one or more of the products (5). It was also found that ascorbate is not stoichiometrically consumed in this hydroxylation (6). We report here data indicating that pure prolyl hydroxylase can catalyze a number of reaction cycles in the absence of ascorbate, but at some stage the hydroxylation ceases, probably due to oxidation of the enzyme-bound Fe^{2+}, and ascorbate is then required as a quite specific compound to re-activate the enzyme.

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MATERIALS AND METHODS

Pure prolyl hydroxylase was isolated from an ammonium sulphate fraction of chick embryo extract by an affinity chromatography procedure using poly(L-proline) (7). The enzyme activity was assayed under standard conditions in a final volume of 1.0 ml which contained 0.1-1.0 µg enzyme, 0.1 mg $(Pro\text{-}Pro\text{-}Gly)_{10}$ · 9 H_2O, 0.025 µmol $FeSO_4$, 0.1 µmol 2-oxo-[1-^{14}C]glutarate (40 000 dpm), 1 µmol ascorbate, 0.1 mg catalase, 1 mg bovine serum albumin and 50 µmol Tris-HCl buffer adjusted to pH 7.8 at 25°C. The samples were incubated at 37°C for 30 min and the $^{14}CO_2$ trapped and counted (see 7,8). The hydroxylation system was modified for certain experiments as described in the legends to the figures and tables.

RESULTS AND DISCUSSION

Binding of 2-oxoglutarate to prolyl hydroxylase in the absence of ascorbate. Prolyl hydroxylase purified by the affinity chromatography procedure (7) used in this study does not retain sufficient iron to catalyze any reaction without the addition of this cation (6). To study whether the binding of 2-oxoglutarate to the enzyme requires prior reduction of the protein by ascorbate, 100 pmol of the enzyme was equilibrium-dialyzed in 10 µl against 1 µM 2-oxo-[1-^{14}C]glutarate (100,000 dpm/nmol) solution containing 20 µM $FeSO_4$. The dialysis was carried out under an N_2 atmosphere, as the enzyme catalyzes an uncoupled decarboxylation of 2-oxoglutarate in the presence of O_2 but the absence of the polypeptide substrate (1,6). The binding of about 10 pmol of 2-oxoglutarate was observed with the native enzyme, it being immaterial whether ascorbate was present or absent in the 2-oxoglutarate solution (Table 1). This binding corresponds to a dissociation constant of about 9 µM (9) assuming the presence of one binding site per enzyme molecule. The value is not accurate, as the measurements were carried out at only one enzyme and 2-oxoglutarate concentration, but it is in reasonable agreement with the dissociation constant of 19 µM obtained from the kinetic data (5). The results indicate that the binding of 2-oxoglutarate to pure prolyl hydroxylase does not require prior reduction of the protein by ascorbate, and thus a reaction with this vitamin is probably not the first event in the hydroxylation cycle.

Hydroxylation of proline in the absence of ascorbate. Previous kinetic studies suggest that the reaction with ascorbate occurs by a substitution mechanism either before the binding of Fe^{2+} or after the release of one or more of the products (5). It thus seemed possible that the enzyme would catalyze formation of at least one mol of hydroxyproline per mol of enzyme in the absence of ascorbate. This possibility was studied by incubating the enzyme with Fe^{2+}, 2-oxo[1-^{14}C]glutarate, O_2 and the polypeptide substrate at short time intervals, and by measuring the hydroxylation-coupled release of $^{14}CO_2$.

Essentially no differences were found in the rate of hydroxylation during

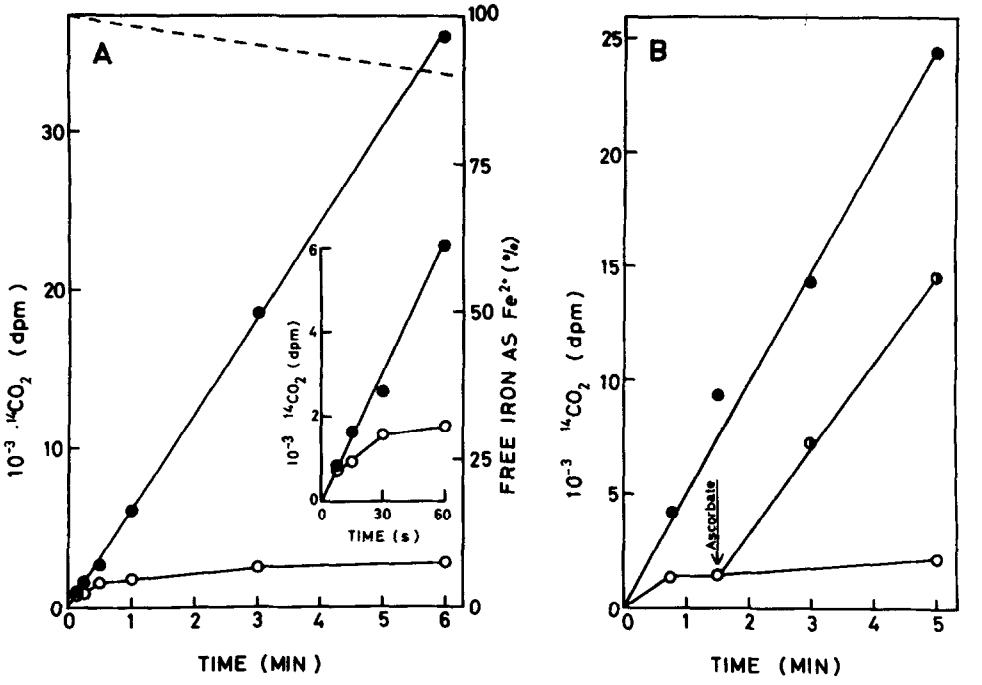

Fig. 1. Hydroxylation-coupled decarboxylation of 2-oxo[1-^{14}C]glutarate in the presence and absence of ascorbate, and the rate of oxidation of free bivalent iron in the reaction mixture. The reaction was carried out in the presence (•) or absence (o) of ascorbate with 4 pmol of the enzyme as described in Methods, except that the concentration of 2-oxo[1-^{14}C]glutarate was 0.04 μmol/ml and its specific activity 7.5 • 10^6 dpm/μmol, the reaction time was varied as indicated, and the temperature was 30°C. As indicated in Methods, the standard incubation mixture did not contain dithiothreitol. In 1B some of the samples incubated without ascorbate received this compound at 90 s (•). The formation of $^{14}CO_2$ in the absence of the enzyme was 60 dpm in 6 min. The rate of oxidation of free bivalent iron (---) was studied by recording the oxygen consumption with a Clark-type oxygen electrode in the standard reaction mixture incubated at 30°C in a closed cell in the absence of ascorbate.

the first 8 s dependent upon whether ascorbate was present or absent in the reaction mixture (Fig. 1A), but $^{14}CO_2$ formation without ascorbate deviated from linearity thereafter, and there was little further hydroxylation after about 1-2 min. The rate of the 2-oxoglutarate decarboxylation in the presence of ascorbate was about 3.4 mol/mol enzyme/s, and that in the absence of ascorbate during the first 8 s about 2.9 mol/mol/s. Accordingly, about 30 mol $^{14}CO_2$ was formed per mol enzyme in 10 s without ascorbate. The experiment shown in Fig. 1A was carried out in the presence of bovine serum albumin and catalase (1,4), but in the absence of dithiothreitol. Identical results were obtained during the first 30-60 s in the absence of bovine serum albumin and

TABLE 1. Binding of 2-oxo[1-^{14}C]glutarate to pure prolyl hydroxylase in equilibrium dialysis

Addition	2-oxo[1-^{14}C]glutarate	
	Found[a] (dpm)	Specific binding (dpm)
Heat-inactivated enzyme	1100	
Enzyme	2230	1130
Enzyme, ascorbate	2140	1040

Prolyl hydroxylase, 24 µg (100 pmol) in 10 µl, was equilibrium-dialyzed against 5 ml of 1 µM 2-oxo[1-^{14}C]glutarate (100,000 dpm/nmol) in 20 µM $FeSO_4$, 0.05 M NaCl, and 0.05 M Hepes, pH 7.5 at 4°C under N_2 for 24 h. The control sample contained 24 µg of heat-inactivated enzyme in 10 µl, and the specific binding was calculated by subtracting this value. The addition of ascorbate (final concentration 1 mM) was made to the 2-oxoglutarate solution.

[a]The radioactivity of free 2-oxoglutarate, 1000 dpm/10 µl, has not been subtracted from these values.

catalase, but after this the rate of hydroxylation deviated markedly from linearity even in the presence of ascorbate, probably due to rapid inactivation of the enzyme (not shown). The reaction was also performed for 30 s with large amounts of the enzyme, and the formation of hydroxyproline measured by a chemical procedure (10). The results were similar to those obtained in experiments on the measurement of $^{14}CO_2$ formation (not shown). When the enzyme incubated without ascorbate received an addition of this vitamin at 90 s, the rate of hydroxylation was restored (Fig. 1B), indicating that the enzyme did not become irreversibly inactivated.

These experiments indicate that pure prolyl hydroxylase can catalyze the hydroxylation of proline in the absence of ascorbate at an essentially maximal rate for 5-10 s, corresponding to about 15-30 reaction cycles. It seems unlikely that pure prolyl hydroxylase would contain any ascorbate, but even if it contained one mol per mol enzyme, the ascorbate concentration would have been 4 pmol/ml, which is about 10^{-5} times the K_m (1,4,5), and the amount of $^{14}CO_2$ formed in 10 s would have been 30 mol/mol ascorbate. Thus these findings cannot be explained by the possible presence of traces of ascorbate. The enzyme was purified in the presence of 0.1 mM dithiothreitol, but this compound became diluted in the reaction mixture to less than 0.3 µM. Even 1 mM dithiothreitol gives a reaction rate of only about one tenth of that obtained with the optimal ascorbate concentration (see below). Neither can the pres-

ence of this compound, therefore, explain the ability of the enzyme to catalyze the reaction without ascorbate. No such observations have been made previously, apparently because the reaction is usually carried out for 30 min and because the difference between the control samples and those incubated without ascorbate increases rapidly with time (Fig. 1A).

The data also indicate that in the absence of ascorbate the reaction ceases at some stage, probably due to an oxidative side-reaction. The possibility was considered that this might be the oxidation of free Fe^{2+} in the incubation mixture, and for this reason the rate of Fe^{2+} oxidation in the absence of ascorbate, dithiothreitol and the enzyme was studied by measuring the O_2 consumption. The maximal value for the rate of Fe^{2+} oxidation, calculated by assuming that all the O_2 consumed was due to this reaction, was only about 1-2 % of the free Fe^{2+} of the incubation mixture per min (Fig. 1A). Thus the side-reaction prevented by ascorbate is apparently not solely the oxidation of free Fe^{2+}, because ascorbate was clearly required at 15 s, when only 0.25-0.5 % of the free Fe^{2+} had become oxidized, and about 99.5 % or more still remained as Fe^{2+}.

Specificity of the ascorbate requirement. Previous data on this specificity vary considerably. Some studies indicate a high degree of replaceability by certain reduced pteridines (11-13) and thiols (8,12,13), whereas recent work with pure prolyl hydroxylase points to very little replacement by tetrahydrofolic acid or dithiothreitol (6). The ability of various reductants to replace ascorbate was re-investigated by studying a number of compounds, each at at least 3-4 different concentrations (Table 2). Dithiothreitol and L-cysteine were the only ones that gave more than 10 % of the activity found with the optimal ascorbate concentration and even then relatively high concentrations of both these compounds were required and the results obtained were highly variable. Only certain enzyme preparations gave the highest values shown, whereas several others gave much lower degrees of replacement. This variation is apparently attributable to the ability of 1 mM dithiothreitol to completely dissociate the enzyme to inactive subunits in the absence of the substrates (14), rendering the result largely dependent on the degree of dissociation of the enzyme during the experiment.

These results indicate a rather high degree of specificity of the ascorbate requirement, and would be consistent with a reaction of this vitamin with some specific site on the enzyme molecule. The ability of some thiols to replace ascorbate to a low extent may explain the low rate of hydroxyproline formation found in cultured cells in the complete absence of this vitamin (13,15,16,17). However, the thiols probably do not react directly with prolyl hydroxylase under normal conditions, as relatively high con-

TABLE 2. Maximal replacement of ascorbate in the prolyl hydroxylase reaction by various reductants

Reductant	Optimal concentration[a] (mM)	Reaction rate (%)
Ascorbate	1	100
Tetrahydrofolic acid	2	3
6,7-Dimethyltetrahydropteridine	2	9
2-Amino-4-hydroxy-6,7-dimethyl-tetrahydropteridine	2	5
Dithiothreitol	2	18[b]
β-Mercaptoethanol	5	1
L-Cysteine	5	16[b]
L-Homocysteine	5	1
Glutathione	5	2
Thioglycolic acid	5	0
Dihydroxyfumarate	2	4
$NADH_2$	10	0
$NADPH_2$	10	0
Dehydroascorbate	5	0

The reaction was carried out as described in Methods, but varying the reductant. Each compound was tested at at least 3-4 different concentrations, and relatively large amounts of enzyme was used, so that about 10,000 dpm were observed in the presence of ascorbate.

[a]Optimal concentration or highest concentration studied, if no replacement was found.

[b]Results highly variable. The values shown are the highest ones observed, some enzyme preparations gave only about 1-5 % (see text).

centrations are required for a low rate of hydroxylation, but they will readily convert dehydroascorbate to ascorbate even at much lower concentrations (see 6).

Inhibition of the reaction by dehydroascorbate. Dehydroascorbate was found to be an inhibitor of the prolyl hydroxylase reaction, the inhibition being non-competitive with respect to 2-oxoglutarate, O_2, the polypeptide substrate and ascorbate (Fig. 2A), but competitive with respect to Fe^{2+} (Fig. 2B). Such a product inhibition pattern and the previous kinetic data (5) would be consistent with an ascorbate reaction by a substitution mechanism after the release of the hydroxylated polypeptide, CO_2 and succinate, but before the

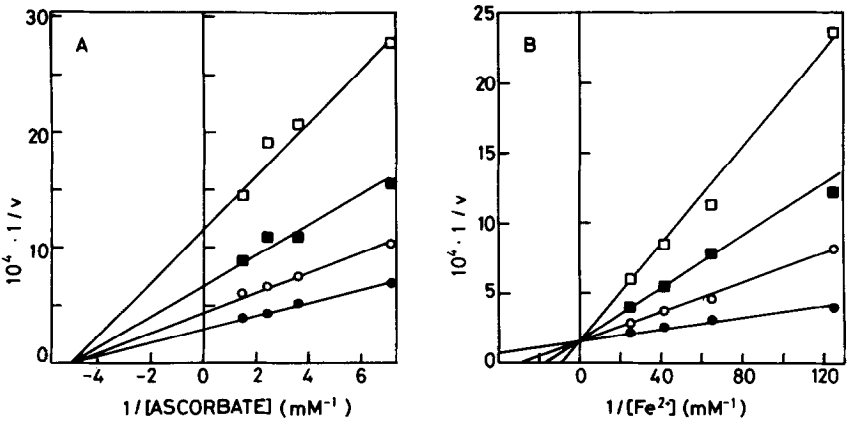

Fig. 2. Inhibition of the prolyl hydroxylase reaction by dehydroascorbate with respect to ascorbate (A) and bivalent iron (B). The concentrations of dehydroascorbate were none (●), 1 mM (○), 2 mM (■) and 3 mM (□). The reaction was carried out under standard conditions, except that the concentration of 2-oxoglutarate was 30 μM and that of ascorbate 0.5 mM (B). The reaction velocity (v) was measured in dpm.

release of Fe^{2+} (18). Accordingly, ascorbate would probably be required for a reaction with the enzyme-bound iron at the end of the catalytic cycle.

Conclusions on the role of ascorbate in the prolyl hydroxylase reaction. The above data demonstrate that the binding of 2-oxoglutarate to pure prolyl hydroxylase does not require any prior reaction with ascorbate, and that the enzyme can catalyze a number of reaction cycles in the absence of this vitamin at an essentially maximal rate. At some stage, however, the reaction ceases, and ascorbate is then required as a quite specific compound to reactivate the system. The reaction which requires ascorbate is apparently not solely the reduction of the free iron of the solution, but it could well be the reduction of the enzyme-bound iron, which may become converted to Fe^{3+} by a side-reaction after the release of the hydroxylated peptide, CO_2 and succinate. The iron is probably bound to an SH group in the enzyme (1,4). An S-Fe^{3+} complex is highly stable, much more so than an S-Fe^{2+} complex (19), and thus Fe^{3+} would probably not dissociate from prolyl hydroxylase (19), and the enzyme would not be available for a new catalytic cycle until the Fe^{3+} became reduced to Fe^{2+}. It seems likely that these conclusions would also hold good for the role of ascorbate in the reactions catalyzed by other 2-oxoglutarate dioxygenases.

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