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INACTIVATION OF PURIFIED PHENYLALANINE HYDROXYLASE BY DITHIOTHREITOL

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Purified rat liver phenylalanine hydroxylase is inactivated *in vitro* by ascorbate and thiol compounds, dithiothreitol being the most effective inhibitor, with a second order rate constant for the inactivation of 0.066 ± 0.002 $mM^{-1} \cdot min^{-1}$ at 20 °C and pH 7.2. Anaerobic conditions and catalase protected the enzyme from inactivation by dithiothreitol. This suggests that hydrogen peroxide, produced by oxidation of the thiol, is involved in the inactivation. The substrate, L-phenylalanine, also partially protected the enzyme from this inactivation. It is shown that incubation of the enzyme with dithiothreitol at aerobic conditions, followed by gel filtration, causes the release of iron from the active site. The inactivation by dithiothreitol was reversed by incubation of the iron-depleted enzyme with Fe(II).

Phenylalanine hydroxylase (PAH, EC 1.14.16.1) is the rate limiting enzyme in the metabolism of L-phenylalanine in the liver (1). The fully active enzyme has non-heme high-spin ferric iron coordinated at the active site, with a stoichiometry of one iron atom/subunit (2). This iron is reduced to the ferrous state by the tetrahydropterin cofactor during catalysis (3,4). We have recently shown that dithiothreitol (DTT) inhibits the activity of PAH and, as seen by EPR spectroscopy, it seems to change the environment of the ferric ion, without affecting its redox state (5). On the other hand, DTT is widely used in the *in vitro* assay of PAH activity to maintain the cofactor in its reduced form (6), and it has been reported to be necessary when the enzyme is reconstituted with iron to its full activity in crude extracts (2). In the present study the inhibitory effect of thiol compounds on the isolated enzyme has therefore been further investigated.

Abbreviations: DTT, dithiothreitol; EPR, electron paramagnetic resonance; GSH, reduced glutathione; GSSG, oxidized glutathione; HPLC, high performance liquid chromatography; oxidized DTT, *trans*-4,5-dihydroxy-1,2-dithiane; PAH, phenylalanine hydroxylase.

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MATERIALS AND METHODS

Preparations of liver extracts and purified PAH. Crude extracts were prepared from rat livers by homogenization and centrifugation as described (7). PAH was purified by the procedure II D of Shiman et al. (7). Homogeneous enzyme preparations were obtained with a specific activity of 7.1 ± 0.5 μmol L-tyrosine· min^{-1}· mg $protein^{-1}$ (U/mg) when assayed at pH 6.8 and 20 °C. The concentration of purified protein was estimated using an extinction coefficient at 280 nm (cm^{-1}) of 1.0 for 1 mg/ml (7).

Measurement of PAH activity. To determine the activity in crude extracts, the enzyme samples were preincubated for 6 min at 25 °C with 0.05 M K-phosphate buffer pH 7.25, 0.1 M KCl, 1.8 mg/ml catalase and 1 mM phenylalanine. Then, 1 mM DTT and 0.2 mM of 6-methyl-5,6,7,8-tetrahydropterin (Calbiochem, San Diego, CA) were added and the incubation continued for 6 min at 25 °C. The reaction was stopped with 1 % (v/v) acetic acid in ethanol. After centrifugation, tyrosine was measured in the supernatant by HPLC (PartiSphere SCX, Whatman, England with 10 mM Na-acetate, pH 3.6 as the solvent) and fluorimetric detection at 304 nm, with excitation at 274 nm. The activity of purified PAH was assayed spectrophotometrically at 20 °C and pH 6.8 as described (7).

Generation of anaerobic conditions. When the glucose oxidase-glucose-catalase system was utilized, all the solutions were made anaerobic as described (8). Dioxygen was effectively eliminated from the solutions by this procedure, as measured by a Clark oxygen electrode (Yellow Springs Instruments Biological Oxygen Monitor). Anaerobic conditions were also created by equilibration of all the incubation mixtures with argon (99.99 %).

Inactivation of PAH. The compounds tested, i.e. oxidized and reduced DTT, GSH, GSSG, L-cysteine and ascorbate (Sigma Chemical Co., St. Louis, MO) were freshly prepared in 25 mM K-phosphate, 1 mM EDTA, final pH 7.25. The concentration of the thiols was determined as described (9). The rate of inactivation of PAH by DTT was measured by incubating 0.45-1.0 mg purified rat enzyme at 20 °C with DTT (0-2 mM) in 20 mM K-phosphate pH 7.25, 0.2 M KCl or in the glucose oxidase system prepared in this buffer (8), with and without phenylalanine, $FeCl_2$ or catalase. At the indicated times, 10 μl aliquots were diluted 100-fold into the enzyme assay mixture and the remaining activity was measured spectrophotometrically (7). The pseudo-first order rate constants for the inactivation (k_{obs}) were determined by linear regression analysis of the logarithmic functions.

Metal analysis. Iron content was determined by atomic absorption using a Perkin-Elmer model 402 spectrophotometer equipped with a graphite furnace (type HGA-76B).

RESULTS

Inactivation of rat PAH by thiol compounds and ascorbate. The catalytic activity of preparations of purified PAH was readily inhibited on preincubation with ascorbate and a number of thiol compounds, except for the physiological reductant GSH (Table 1). DTT was found to be the most effective inhibitor and its effect was further studied as a function of time and concentration (Fig. 1A). The inactivation was first order in DTT concentration (Fig. 1B), with a second order rate constant of 0.066 ± 0.002 mM^{-1}· min^{-1} at 20 °C and pH 7.25. In controls, containing no thiol, the enzyme was initially activated at pH 7.25 and 20 °C (Fig. 1A), followed by a slow inactivation.

In crude extracts of rat liver, however, no inhibition of PAH activity by thiols was observed, i.e. the activity was 0.023 ± 0.001 U/mg in control samples and 0.029 ± 0.002 U/mg in samples preincubated for 30 min at 25 °C with 2 mM DTT.

Table 1. Inhibition of rat PAH by thiol compounds and ascorbate

Compound	Concentration (mM)	Activity (%)
None	-	100[a]
DTT	2	6
2-Mercaptoethanol	2	20
L-cysteine	2	58
GSH	2	110
	10	87
Ascorbate	2	22

Rat PAH (0.45 mg/ml) was preincubated with the indicated compounds for 30 min at 20 °C, in 20 K-phosphate buffer pH 7.25, 0.2 M KCl, before the activity was determined. The results are the mean of triplicate determinations. [a]100% activity represents a specific activity of 7.2 ± 0.11 U/mg (mean ± SD).

Effect of disulfides. In order to study if a thiol:disulfide exchange (10) could be involved in the regulation of the catalytic activity of PAH *in vitro*, we examined the effect of disulfides (oxidized DTT and GSSG). No effect on the activity was observed when the disulfides were tested in the range of concentrations from 0.01 to 10 mM and at incubation periods up to 2 h. Moreover, oxidized DTT and GSSG, when added in a molar excess to the DTT-inhibited enzyme, were not able to restore the catalytic activity (data not shown).

Effect of catalase and anaerobiosis on the inactivation by DTT. Catalase significantly prevented the inactivation of PAH by DTT, i.e. the pseudo-first order rate constant for the process with 1 mM DTT at aerobic conditions, i.e. k_{obs} = 0.073 ± 0.002 min^{-1}, decreased to a

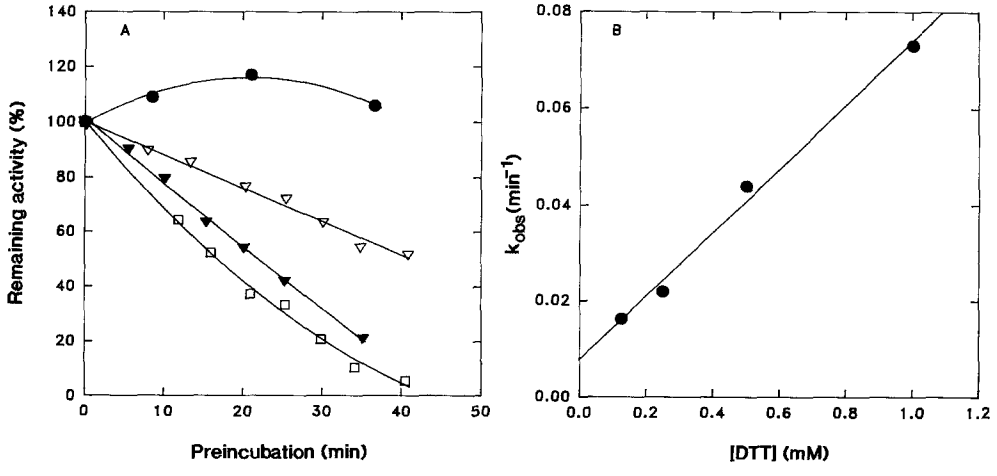

Fig. 1. Inactivation of PAH by DTT. A) Time course for the inactivation. The rat enzyme (0.45 mg/ml) was preincubated with no DTT (●), 0.125 mM DTT (▽), 0.5 mM DTT (▼), and 1 mM DTT (□) at 20 °C in 20 mM K-phosphate buffer pH 7.25, 0.2 M KCl. B) Concentration dependence of the pseudo-first order rate constants for the inactivation.

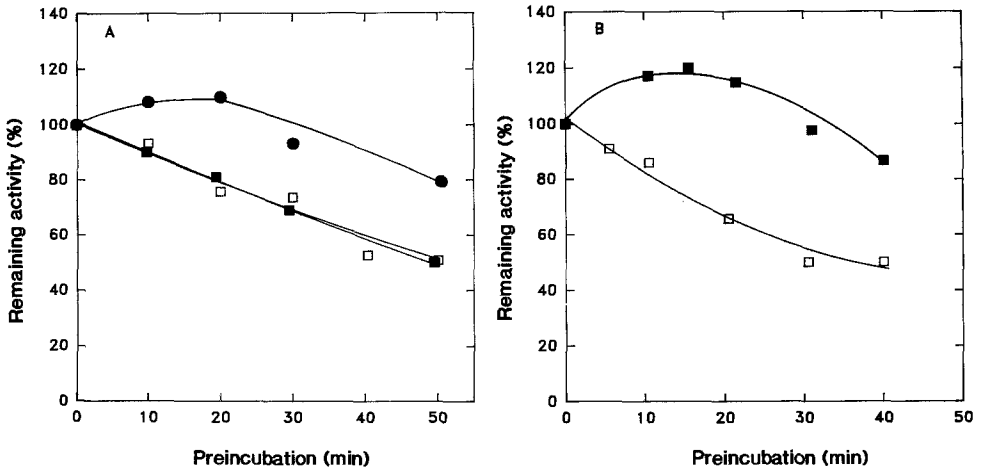

Fig. 2. Effect of catalase and anaerobiosis on the inactivation of PAH by DTT. A) Rat enzyme (1.0 mg/ml) was preincubated with 1 mg/ml catalase (●), with 10 µg/ml catalase and 1 mM DTT (■) and with 1 mg/ml catalase and 1 mM DTT (□) at 20 °C in 20 mM K-phosphate buffer pH 7.25, 0.2 M KCl. B) Rat enzyme (1.0 mg/ml) was preincubated without (■) and with 1 mM DTT (□) at 20 °C in the glucose oxidase system.

value of k_{obs} = 0.0156 ± 0.0013 min^{-1} in the presence of 10 µg/mg catalase (Fig. 2A). Higher concentrations of catalase (1 mg/ml) did not further decrease this value (k_{obs} = 0.0157 ± 0.0013 min^{-1}) (Fig. 2A). Furthermore, when the preincubation with DTT was carried out at anaerobic conditions, generated by the glucose oxidase system (8), the inactivation of PAH was also partially prevented (k_{obs} = 0.025 ± 0.001 min^{-1} for the inactivation with 1 mM DTT) (Fig. 2B). The enzymatic system utilized in order to create anaerobiosis contains 2 µg/ml of catalase, but a similar protection against inactivation by 1 mM DTT was obtained when anaerobiosis was generated by equilibration of the incubation mixture with argon (k_{obs} = 0.026 ± 0.0012 min^{-1}).

Effect of phenylalanine and Fe(II) on the inactivation by DTT. The pseudo-first order rate constant for the inactivation by 2 mM DTT at 20 °C was found to decrease from 0.094 ± 0.007 min^{-1} to 0.053 ± 0.005 min^{-1} in the presence of 1 mM phenylalanine, indicating a partial protective effect exerted by the substrate. On the other hand, the rate of inactivation was similar for the enzyme incubated in the presence and absence of added Fe(II) (0.5 mM $FeCl_2$).

Effect of DTT on the iron content of the enzyme. From Table 2 it is seen that the aerobic incubation of the enzyme with DTT facilitates the release of its catalytic iron. Thus, when the enzyme was preincubated with 2 mM DTT and subjected to gel filtration on Sephadex G-25 (Pharmacia, Uppsala, Sweden), it contained only about 30 % of the iron in the enzyme as isolated. Moreover, when noradrenaline, an active site inhibitor (5,11), was present together with DTT, the dissociation of iron was significantly decreased (Table 2). The activity of the iron-depleted enzyme could be recovered upon its incubation with Fe(II). Thus, the addition of 100 µM $FeCl_2$ to DTT-inactivated PAH, after removal of excess DTT by gel filtration,

Table 2. Effect of DTT on the catalytic activity and iron content of PAH

Additions	Specific activity (U/mg)	Fe (atom/mol PAH subunit)
None	6.40 ± 0.28 (90 %)	0.95 ± 0.035 (86 %)
2 mM DTT	0.71 ± 0.11 (10 %)	0.32 ± 0.032 (29 %)
2 mM DTT + 80 µM noradrenaline	1.67 ± 0.27 (23 %)	0.69 ± 0.044 (63 %)

Rat PAH (3.5 mg/ml) was preincubated for 30 min at 20 °C in 15 mM Tris-HCl buffer pH 7.25, 0.2 M KCl, with the indicated additions. Then, the enzyme samples were passed through a Sephadex G-25 column equilibrated with the same buffer, and specific activity and Fe content (expressed as mean ± SD of triplicate determinations) were measured in the collected fractions. In parentheses, the activity and Fe content of the enzyme are expressed as percent of values obtained before preincubation and gel filtration (100 % = 7.1 ± 0.5 U/mg and 1.1 ± 0.067 atom Fe/mol PAH subunit).

instantaneously reconstitutes the enzyme to its full (original) activity, followed by a slow decay of the activity, which was slightly higher than observed for the control enzyme, i.e. not treated with the thiol (Fig. 3). No activity was recovered on incubation of the iron-depleted enzyme with Fe(III) (100 µM $FeCl_3$).

DISCUSSION

We have recently found that incubation of purified rat and bovine PAH with low concentrations (1 mM) of DTT results in an inactivation of the enzyme (5). As shown in the present study (Table 1), other thiols and ascorbate can also inactivate the purified enzyme. From

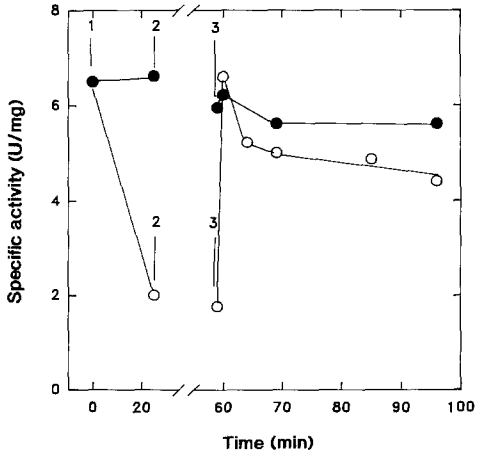

Fig. 3. Reactivation of DTT-inactivated PAH by Fe(II). 10 nmol rat enzyme (5.0 mg/ml) was incubated with 2 mM DTT (○) and without DTT (●) in 15 mM Tris-HCl pH 7.25, 0.2 M NaCl at 20 °C (**1-2**). Then, at (**2**), samples were gel filtrated on a Sephadex G-25 column, equilibrated with the same buffer, and at (**3**) 100 µM $FeCl_2$ was added to the collected fractions, and the activity measured as a function of the incubation time.

metal analysis and inactivation/reactivation data we have found that the catalytic iron of the enzyme is released from the enzyme as a result of aerobic incubation with DTT and an inactive metal depleted enzyme (apoenzyme) is formed (Table 2). It has previously been reported that DTT (or cysteine) was required for the preparation of the apoenzyme from the holoenzyme (2,12), but the release of the iron from the active site was considered to be due to the presence of a strong metal chelator (*o*-phenanthroline). Here, however, it is shown that DTT alone induces the dissociation of iron from PAH parallel to the loss of its catalytic activity (Table 2). This release of the metal ion from the enzyme can only be partially ascribed to a reductive dissociation of iron, of the type found e.g. on incubation of transferrin with high concentrations of thiol reagents (13) and seems to be a more complicated process. Thus, as shown by EPR spectroscopy, the final state of the iron in PAH after aerobic incubation with 1 mM DTT is largely high-spin (S = 5/2) ferric (5). Moreover, in the Zn(II)-reconstituted tyrosine hydroxylase, a structurally and functionally related enzyme, we have recently found that DTT increases the rate of release of its non-functional metal from the active site (14). Our results on the protective effect exerted by the substrate phenylalanine on the inactivation of PAH by DTT, and the inhibition of iron release when noradrenaline was present together with the thiol, indicates that DTT affects the iron-binding (catalytic) site of the enzyme. Noradrenaline, which coordinates to the catalytic iron by a charge-transfer interaction (11,15), also prevents the release of iron from Fe-reconstituted tyrosine hydroxylase (14).

DTT, and probably ascorbate and the other thiols tested in this study, seem to inactivate PAH mainly by a combined effect of their metal-binding properties (16,17) and by the hydrogen peroxide produced when they undergo metal-ion catalyzed autooxidation (17-19). Both inhibitory mechanisms require a direct interaction of the compounds with the iron at the active site, which could explain the failure of GSH to inactivate the enzyme. GSH has a redox potential similar to that of the other thiols tested, but it has a lower affinity for metals (16) and is a larger molecule, which may represent a steric hindrance for its interaction with the active site metal. Hydrogen peroxide is known to inactivate PAH (3,20) and the large protective effect against inactivation of the enzyme by DTT exerted by catalase and anaerobiosis (Fig. 2) indicates that hydrogen peroxide is involved in the process. Thus, the iron-catalyzed autooxidation of DTT produces hydrogen peroxide which may oxidize residues at or close to the catalytic site, favouring the dissociation of the iron. It has been reported that hydrogen peroxide formed upon aerobic incubation of the enzyme rhodanase with DTT is involved in the formation of an intramolecular disulfide bond resulting from the oxidation of sulfhydryl groups in close proximity at the active site of this enzyme (18). PAH contains five cysteine residues in the catalytic domain, which is highly conserved among the aromatic amino acid hydroxylases (21), with four residues located in the sequences recently proposed to represent the cofactor (and iron) binding sites, i.e. Cys-202

and Cys-216 (22) and Cys-264 and Cys-283 (23). The formation of a disulfide bridge between a pair of Cys-residues on aerobic incubation with thiols could explain why the apoenzyme has been found to have only three free Cys-residues per enzyme subunit, *versus* five in the holoenzyme (12). As shown in this work, it is possible to fully recover the catalytic activity when the DTT-inactivated enzyme is incubated with Fe(II), after DTT has been removed. Further studies are, however, required in order to find out whether this reconstituted enzyme has recovered the five titratable sulfhydryl groups characteristic of the native enzyme.

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