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EFFECTS OF SULFHYDRYL AGENTS ON THE ACTIVATION OF TRYPTOPHAN-5-MONOOXYGENASE FROM BOVINE PINEAL GLANDS

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Summary

Partially purified tryptophan-5-monooxygenase (L-tryptophan, tetrahydropteridine:oxygen oxidoreductase (5-hydroxylating) EC 1.14.16.4) from bovine pineal gland was activated by preincubation with sulfhydryl agents such as dithiothreitol, L-cysteine, cysteamine, L-cysteine ethylester, *N*-acetyl-L-cysteine, 2-mercaptoethanol and reduced glutathione, at alkaline pH (optimum pH = 8.5). Dithiothreitol was the most effective of these, leading to approximately 50-fold activation of the enzyme after preincubation. Fe^{2+} or other reducing agents such as borohydride, dithionite and ascorbate facilitated the velocity of the activation in the presence of sulfhydryl agents. In the absence of sulfhydryl agents, no activation was observed even in the presence of Fe^{2+} or other reducing agents, suggesting an obligatory role or sulfhydryl agents during the activation. The relative velocity and full extent of the activation were dependent on the concentrations of both the sulfhydryl agent and the enzyme in the activation mixture. The kinetic analysis of the activation indicated that the sulfhydryl agent reacts with more than 2 sites in the enzyme; one type of site is reduced by sulfhydryl agents, Fe^{2+} or other reducing agents and the other is specifically modified by a sulfhydryl agent.

The activated enzyme did not require any exogenous Fe^{2+} for its catalytic activity, but some roles of iron maybe exist in its catalytic reaction. The optimum pH for catalytic reaction of the activated enzyme was approximately 6.5. The apparent K_m for L-tryptophan and pteridine cofactor, tetrahydropteridine (2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropterin), of the activated enzyme were 30 and 35 μM respectively.

Abbreviations: [$^{14}C_3$]Trp(5OH), 5-hydroxy-L-[1,2,3-$^{14}C_3$]tryptophan; tetrahydropteridine, 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropterin; PLP, pyridoxal 5'-phosphate; D,L-Trp(5OH), 5-hydroxy-D,L-tryptophan.

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Introduction

L-Tryptophan-5-monooxygenase (L-tryptophan, tetrahydropteridine: oxygen oxidoreductase (5-hydroxylating) EC 1.14.16.4), an enzyme involved in the hydroxylation of L-tryptophan to 5-hydroxy-L-tryptophan, catalyzes the initial reaction in the biosynthesis of melatonin in the pineal gland and presumably the rate-limiting step in the biosynthesis of a putative transmitter, 5-hydroxytryptamine, in the central nervous system [1—3]. It has been reported that the activity of tryptophan-5-monooxygenase is stimulated by the addition of sulfhydryl agents [4,5] such as 2-mercaptoethanol or dithiothreitol, and is inhibited by sulfhydryl-binding agents [6] such as *p*-chloromercuribenzsulfonate or monoidoacetate.

Recently we have found that bovine pineal tryptophan-5-monooxygenase is activated more than 50-fold by pretreatment with Fe^{2+} and high concentration of dithiothreitol [7]. In the present study, an attempt has been made to analyze the role of sulfhydryl agents in the activation of tryptophan-5-monooxygenase.

Materials and Methods

Materials

L-[1-^{14}C]Tryptophan was obtained from New England Nuclear Corp. and purified by the partition chromatography on a Sephadex G-25 (coarse) column [4]. 5-Hydroxy-L-[1,2,3-$^{14}C_3$]tryptophan ([$^{14}C_3$]Trp(5OH)) was prepared from 5-hydroxyindole and L-[U-^{14}C]serine using a partially purified tryptophan synthetase (EC 4.2.1.20) from *Escherichia coli* T3 [4]. Commercial sources of chemicals were as follows; 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropterine (tetrahydropteridine) and dithiothreitol from Calibiochem, Sephadex G-25 (coarse) from Pharmacia Fine Chemicals, beef liver catalase (C-100) and Tris (Trizma base) from Sigma, glucose-6-phosphate dehydrogenase from Boehringer Mannheim Japan and bovine serum albumin (Fraction V) from Armour Pharmaceutical Co. Ltd. Pyridoxal 5'-phosphate (PLP) was a gift from Yamanouchi Pharmaceutical Co. Ltd., Tokyo, Japan. Ferrous ammonium sulfate and other reagents were obtained from Nakarai Chemicals, Tokyo, Japan.

Aromatic L-amino acid decarboxylase (EC 4.1.1.28) was purifed from hog kidney according to the method of Christenson et al. [8] up to the step of $(NH_4)_2SO_4$ fractionation and was stored frozen at —20 °C without loss of the activity for more than 3 months. Sheep liver dihydropteridine reductase (EC 1.6.99.7) was prepared according to Craine et al. [9] except that calcium phosphate gel treatment was replaced by hydroxyapatite column chromatography.

Enzyme preparation

Bovine glands were obtained from a local slaughterhouse and kept at —80 °C until use. The pineal glands were thawed and trimmed of connective tissue. The glands (120 g) were then cut into small pieces with scissors and homogenized with 4 vol. (v/w) of ice-cold 20 mM Tris (pH 8.5) containing

$5 \cdot 10^{-4}$ M dithiothreitol in a Waring blender for 5 periods of 1 min with 1-min pauses. The homogenate was centrifuged at 77 000 × *g* for 60 min. The precipitate was again homogenized with 240 ml of 20 mM Tris · HCl (pH 8.5) containing $5 \cdot 10^{-4}$ M dithiothreitol in a Waring blender for 5 min as the same above. After centrifugation at 77 000 × *g* for 60 min, the supernatant obtained was combined with the 1st supernatant. Solid $(NH_4)_2SO_4$ (14.4 g/100 ml) was added to the combined extracts (25% saturation) and after being stirring for 60 min the mixture was centrifuged at 5500 × *g* for 30 min. To the supernatant, solid $(NH_4)_2SO_4$ (19.3 g/100 ml) was added (55% saturation) and the mixture was centrifuged as above. The precipitate obtained was dissolved in 24 ml of 5 mM potassium phosphate (pH 6.8) and the solution was dialyzed overnight against 3 l of 5 mM potassium phosphate (pH 6.8). All procedures described above were carried out at 4°C. The enzyme prepared was stored at —80°C without loss of the activity after the activation described below for more than 2 months.

Enzyme assay

Tryptophan-5-monooxygenase was assayed by measurement of $^{14}CO_2$ evolved from 5-hydroxy-L-[1-^{14}C]tryptophan by a modification of the method of Ichiyama et al. [4]. The assay was started by adding 0.01-ml aliquot of an activated enzyme to 0.04 ml of the assay mixture containing 11 nmol of L-[1-^{14}C]tryptophan (spec. act.: 14 100 dpm/nmol), 72.5 nmol of tetrahydropteridine and 10 μmol of potassium phosphate (pH 6.5). Unless otherwise stated, dithiothreitol and ferrous ammonium sulfate were also added to make final concentrations of 20 mM and 50 μM*, respectively. Tetrahydropteridine was added just before starting the reaction by addition of the enzyme. Immediately after the reaction was started, the assay tube was connected to a counting vial through a rubber tube. The vial contained a filter paper strip (17 mm × 80 mm) to which 0.2 ml of 25% 2-phenethylamine in ethanol had been added. After incubation at 25°C for 10 min, the reaction was stopped by immersing the tube into a boiling water bath for 1 min. The decarboxylase mixture (0.95 ml) containing 100 nmol of 5-hydroxy-DL-tryptophan (D,L)-Trp(5OH)), 200 nmol of PLP, 200 nmol of EDTA, 4—7 munits of aromatic L-amino acid decarboxylase and 200 μmol of Tris · HCl (pH 8.8) was injected through the rubber tube into the heated reaction mixture. The mixture was incubated at 37°C for 30 min. As shown by Ichiyama et al. [4], under these conditions 5-hydroxy-L-tryptophan in the assay mixture is stoichiometrically converted to 5-hydroxy-tryptamine and CO_2. The direct decarboxylation of L-[1-^{14}C]tryptophan was less than 0.5% because the concentration of L-tryptophan was diluted out to 11 μM which is far lower than the K_m of the decarboxylase for L-tryptophan. The decarboxylase reaction was stopped by injection of 0.2 ml of 30% $HClO_4$ and assay tubes were incubated at 37°C for an additional 60 min to trap evolved $^{14}CO_2$. The radioactivity was determined by a Packard Tri-Carb liquid spectrophotometer, Model 3320, after addition of 10 ml of a

* The addition of Fe^{2+} was made to give the same experimental conditions in each assay, although exogenous Fe^{2+} was not required for the catalytic reaction of the activated enzyme as described below.

scintillator (PPO 0.55%, dimethyl-POPOP 0.02%, Triton X-100 33% and distilled water 7% in toluene) with about 85% counting efficiency. .

The activity of aromatic L-amino acid decarboxylase was determined by measurement of $^{14}CO_2$ evolved from [$^{14}C_3$]Trp(5OH) as described by Ichiyama et al. [4]. One unit of the enzyme activity was defined as the amount which evolves 1 μmol of CO_2 from L-Trp(5OH) per min at 37 °C.

Protein was determined by the method of Lowry et al. [10] with bovine serum albumin as standard.

Activation of tryptophan-5-monooxygenase

The dialyzed enzyme was incubated with 50 mM dithiothreitol, 50 μM ferrous ammonium sulfate and 150 mM Tris · HCl (pH 8.5) for 60 min at 25 °C under anaerobic conditions just prior to the assay of tryptophan-5-monooxygenase. All reagents were equilibrated with N_2 and stored separately. For this purpose the Thunberg tube was evacuated and then N_2 was introduced; this procedure was repeated 3 times. All pipettes and test tubes (8 mm × 150 mm glass tube) were flushed with N_2 just before use and reagents and the enzyme were mixed anaerobically. Tubes were again flushed with N_2 and then the activation was started by addition of dithiothreitol and Fe^{2+}.

Results

Activation of tryptophan-5-monooxygenase

The enzyme prepared as described under Methods was purified about 5-fold over the crude homogenate with a yield of about 40% (Table I). When the enzyme was assayed without preincubation with dithiothreitol and Fe^{2+}, the activity in the various enzyme preparations was very low, and higher activity was detected at pH 8.0 than at pH 6.5 (Table I). Under these assay conditions, the addition of Fe^{2+} in the presence of dithiothreitol stimulated the

TABLE I

TRYPTOPHAN-5-MONOOXYGENASE ACTIVITY AT VARIOUS PREPARATION STEPS

After activation: The activation and the assay conditions were carried out as described under Methods, except for the used buffer in the assay mixture. The enzyme activity was assayed at 60 min after starting the activation. pH 6.5, KH_2PO_4·KOH buffer, 200 mM; pH 8.0, Tris·HCl buffer, 200 mM. Before activation: The components in the assay mixture were the same above. The assay was started by adding the enzyme (0.015 ml) without preincubation for the activation and the enzyme reaction was done for 60 min. Values are a mean ± S.D. (number of experiments).

	Evolved CO_2		
	pH 6.5		pH 8.0
	After activation (nmol/mg protein in 10 min)	Before activation (nmol/mg protein in 60 min)	Before activation (nmol/mg protein in 60 min)
Homogenate	0.447 ± 0.046 (5)	0.074	0.086
Supernatant	0.832 ± 0.261 (5)	0.027	0.218
$(NH_4)_2SO_4$ (25—55%)	2.290 ± 0.150 (5)	0.034 ± 0.010 (5)	0.534 ± 0.146 (5)

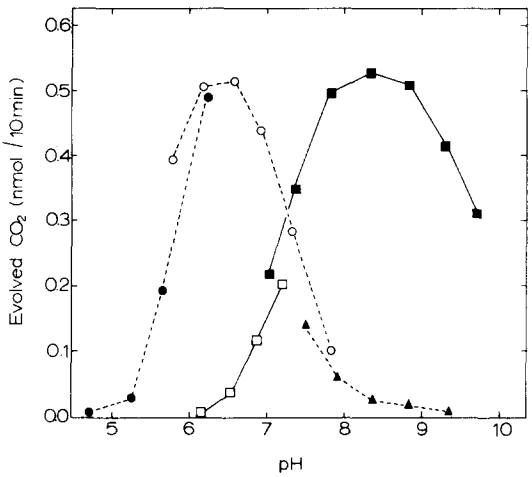

Fig. 1. Effects of pH on the activation and catalytic reaction. The assays were carried out with preincubation as described under Methods except for the following changes. (A) Activation step. The pH-8.5 buffer routinely used for the preincubation step was replaced by equimolar amounts of Tris · HCl buffer (■———■) or KH_2PO_4 ·KOH buffer (□———□) of varying pH. The pH shown was experimentally determined for each specific preincubation mixture (in separately but identically prepared tubes). After preincubation, the assay was started by transfering an aliquot of the activated mixture into the assay mixture containing 200 mM KH_2PO_4 · KOH (pH 6.5). The pH of the reaction mixture was also checked in separately but identically prepared tubes to ascertain that it was very close to 6.5. (B) Monooxygenase reaction step. Activation was carried out using Tris·HCl buffer (pH 8.5) as described under Methods. Following preincubation, an aliquot of the activated mixture was added into the assay mixture containing 200 mM buffer of varying pH. The pH values shown were experimentally determined in complete reaction mixtures (in separately but identically prepared tubes). ●------●, citrate buffer; ○------○, KH_2PO_4 · KOH buffer; ▲------▲, Tris · HCl buffer.

tetrahydropteridine-dependent CO_2 evolved, supporting the finding in pineal glands [5,11]. When the enzyme was preincubated with dithiothreitol and Fe^{2+} at pH 8.5 prior to the assay, activity of the enzyme was stimulated more than 50-fold. The optimum pH for the catalytic reaction of the activated enzyme was observed to be about 6.5, which is different from the optimum pH for the activation of the enzyme (Fig. 1). This optimum pH differs from that previously reported [12] but is almost the same value as the enzyme from mast cells [12]. The kinetic studies of the activated enzyme gave an apparent K_m value for L-tryptophan of 30 μM, which is far lower than the values previously reported for enzymes from some tissues [11—13]. The apparent K_m for the pteridine cofactor was 35 μM, supporting the report by Lovenberg et al. [12].

Requirements for the activation

Dithiothreitol was absolutely required for activation of the enzyme and reducing agents such as Fe^{2+}, borohydride, dithionite and ascorbate have an important role in accelerating the velocity of the activation in the presence of dithiothreitol (Table II). Other sulfhydryl agents such as L-cysteine, cysteamine, L-cysteine ethylester, *N*-acetyl-L-cysteine, 2-mercaptoethanol and reduced glutathione also activated the enzyme but dithiothreitol was the most effective with respect to both the velocity and the full extent of the activation (Table III). It was also revealed that not only the velocity but also the full

TABLE II

REQUIREMENT FOR THE ACTIVATION OF TRYPTOPHAN-5-MONOXYGENASE

The standard activation (Tube 1) and the assay conditions were carried out as described under Methods. Concentration of reducing agents, if used, was 10 mM for $NaBH_4$, and 5 mM for $Na_2S_2O_4$ and ascorbate. The enzyme protein (in the activation mixture, 45.6 mg/ml) was used. Velocity = the enzyme activity at 3 min after starting the activation; full extent = the enzyme activity at 60 min after starting the activation. N.D., not significantly detected the enzyme activity (less than 1% of the standard activation).

Omissions	Addition	% Activity	
		Velocity	Full extent
1 None	None	100	100
2 Fe^{2+}	None	33.1	87.5
3 Dithiothreitol	None	N.D.	N.D.
4 Dithiothreitol, Fe^{2+}	None	N.D.	N.D.
5 Dithiothreitol, Fe^{2+}	$NaBH_4$	N.D.	N.D.
6 Dithiothreitol, Fe^{2+}	$Na_2S_2O_4$	N.D.	N.D.
7 Dithiothreitol, Fe^{2+}	Ascorbate	N.D.	N.D.
8 Fe^{2+}	$NaBH_4$	89.6	87.0
9 Fe^{2+}	$Na_2S_2O_4$	60.3	85.1
10 Fe^{2+}	Ascorbate	56.2	68.2

TABLE III

EFFECT OF VARIOUS SULFHYDRYL AGENTS AND pH ON THE ACTIVATION

The activation and the assay conditions were carried out as described under Methods, except for dithiothreitol and the used buffer in the activation mixture. Enzyme protein (in the activation mixture, 17.2 mg/ml) was used. The enzyme activity was assayed at 60 min after starting the activation. (A) Effect of pH on the activation. Dithiothreitol was substituted by other sulfhydryl agents to make their concentration in the activation mixture 50 mM. The buffer used is described below. pH 6.0 and 7.0: KH_2PO_4.KOH buffer, 150 mM; pH 8.0 and 9.0: Tris·HCl buffer, 150 mM. (B) Effect of the concentration of sulfhydryl agents on the activation. The concentration of sulfhydryl agents in the activation mixture was varied. 150 mM Tris·HCl buffer (pH 8.5) was used.

A

	Evolved CO_2 (nmol/10 min)			
	pH 6.0	pH 7.0	pH 8.0	pH 9.0
None	0.009	0.010	0.008	0.009
Dithiothreitol (reduced)	0.010	0.230	0.563	0.495
2-Mercaptoethanol	0.004	0.023	0.325	0.306
N-Acetyl-L-cysteine	0.008	0.046	0.262	0.213
Glutathione (reduced)	0.011	0.037	0.139	0.190

B

	Evolved CO_2 (nmol/10 min)			
	Concentration of SH agent:	5 mM	25 mM	50 mM
Dithiothreitol (reduced)		0.300	0.455	0.535
Dithiothretol (oxidized)		0.011	—	0.009
Cysteamine		0.318	0.365	0.424
2-Mercaptoethanol		0.072	0.343	0.384
L-Cysteine		0.149	0.412	0.474
L-Cysteine ethylester		0.400	0.493	0.444
N-Acetyl-L-cysteine		0.081	0.258	0.328
Glutathione (reduced)		0.058	0.112	0.148
Glutathione (oxidized)		0.010	—	0.008
None	0.006			

TABLE IV

INHIBITION OF TRYPTOPHAN-5-MONOOXYGENASE BY METAL CHELATORS

The activation and the assay conditions were carried out as described under Methods, except that Fe^{2+} was not added to both the activation and the assay mixtures. The assay was started by addition of an aliquot of the activated mixture to the assay mixture containing the metal chelator at 1 h after starting the activation.

Addition		% Activity
1 None		100
2 Fe^{2+} (50 μM)		109.1
3 *o*-Phenanthroline	(0.1 mM)	61.2
4 *o*-Phenanthroline	(0.5 mM)	14.8
5 *m*-Phenanthroline	(0.1 mM)	136.3
6 *m*-Phenanthroline	(0.5 mM)	149.9
7 2,2'-Dipyridyl	(0.1 mM)	99.8
8 2,2'-Dipyridyl	(1.0 mM)	76.2
9 4,4'-Dipyridyl	(0.1 mM)	101.1
10 4,4'-Dipyridyl	(1.0 mM)	96.7

extent of the activation were dependent upon the concentration of those sulfhydryl agents tested.

It had been reported that a sulfhydryl agent stabilizes the tetrahydropterin cofactor and hence can be substituted by a NADPH-dihydropteridine reductase-regenerating system [13]. To ascertain that the activation of the enzyme by sulfhydryl agents was not related to the pteridine cofactor-regenerating system, sulfhydryl agents in the assay mixture were replaced by the tetrahydropterin-generating system containing of 10 nmol of NADHP, 100 nmol of glucose 6-phosphate and the excess amounts of glucose-6-phosphate dehydrogenase and dihydropteridine reductase and then the enzyme activity without preincubation for the activation was determined at pH 7.0 and 8.0. Under these conditions, only negligible activity (less than 0.01 nmol/mg protein in 60 min) was detected at both pH 7.0 and 8.0. Therefore the sulfhydryl agent may act on the enzyme itself in addition to its stabilizing effect on the tetrahydropterin cofactor during the catalytic reaction of the enzyme.

Table IV showed that activity of the activated enzyme was stimulated only slightly (less than 10%) by addition of Fe^{2+}. 2,2'-Dipyridyl did not inhibit the activity of the activated enzyme at 0.1 mM, which is enough to chelate the iron which perhaps exist in the assay mixture, but inhibited (24% inhibition) at 1 mM. 4.4'-Dipyridyl, nonchelating analogue of 2,2'-dipyridyl, neither inhibited nor stimulated. *o*-Phenanthroline inhibited the activity (40 and 85% inhibition at 0.1 and 0.5 mM respectively). *m*-Phenanthroline, nonchelating analogue of *o*-phenanthroline, stimulated the activity of the enzyme. These results suggested that the activated enzyme does not require any exogenous Fe^{2+}.

Effects of dithiothreitol on the activation

When the enzyme was preincubated with various concentrations of dithiothreitol under the standard activation conditions, the activation of the enzyme reached a maximal level after 1 h (Fig. 2). And when dithiothreitol was added to make the final concentration of 50 mM after preincubation of the enzyme for 1 h, the enzyme activity increased and reached a level which was obtained

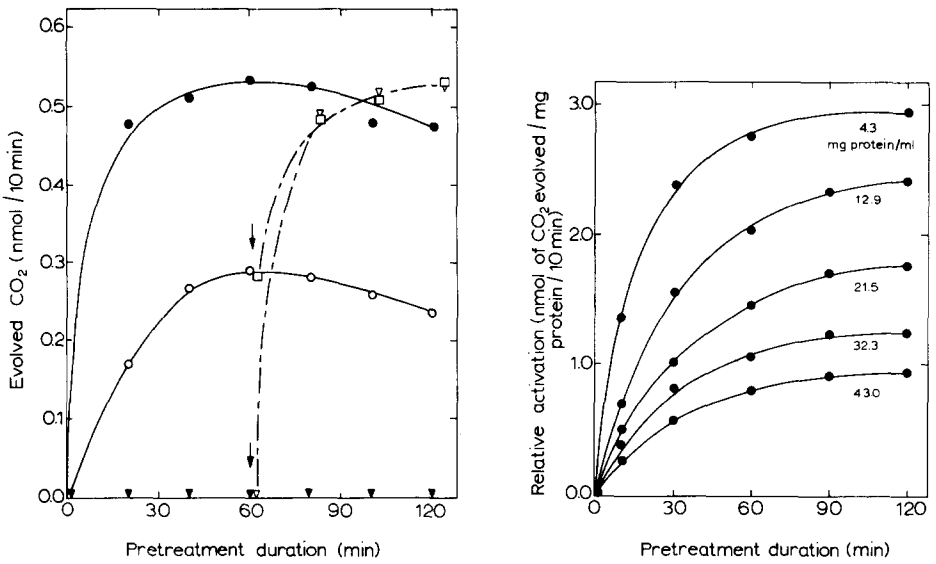

Fig. 2. Effect of dithiothreitol on the activation. The enzyme activity at various times after starting the activation was assayed by transfering a 0.01-ml aliquot of the activated mixture into the assay mixture. The enzyme protein (in the activation mixture, 17.2 mg/ml) was used. ●———●, with addition of 50 mM dithiothreitol at starting the activation; ○———○, with addition of 5 mM dithiothreitol at starting the activation; ▲———▲, without dithiothreitol. At the time indicated by arrows, dithiothreitol was added to make the concentration of 50 mM; △— · — ·△, after preincubation for 1 h without dithiothreitol; □— · — ·□, after preincubation for 1 h with 5 mM dithiothreitol.

Fig. 3. Effect of the enzyme concentration on the activation. The enzyme protein used in the activation mixture was fixed-varied to 4.3, 12.9, 21.5, 32.3 and 43.0 mg protein per ml and dithiothreitol in the activation mixture was fixed to 3 mM. The enzyme activity at various times after starting the activation was assayed by transfering 0.01-ml aliquot of the activation mixture into the assay mixture. Relative activation was the enzyme activity divided by the amount of enzyme protein transfering into the assay mixture.

when 50 mM dithiothreitol was added at the start of the activation. Fig. 3 showed that the relative activation of the enzyme was higher when lower concentrations of the enzyme were subjected to preincubation for the activation. The activation was thus dependent on the concentrations of both dithiothreitol and the enzyme*, suggesting that an apparent equilibrium may exist between dithiothreitol and the enzyme.

Kinetic studies of the activation

The velocity of the activation in the presence of 50 μM Fe^{2+} or its absence was plotted against the concentration of dithiothreitol. As shown in Fig. 4-A, a sigmoidal curve was obtained in the absence of Fe^{2+} and the sigmoidicity was normalized at least in part by addition of Fe^{2+}. The acceleratory effect of Fe^{2+} on the velocity of the activation of the enzyme was thence shown to be much

* The full extent of the activation was shown to be independent on the concentration of dithiothreitol in a range of 5 and 30 mM in a previous communication [7]. In that experiment, however, the concentration of the enzyme in the activation mixture was calculated to be approximately one-fifth of the amount used in the present study.

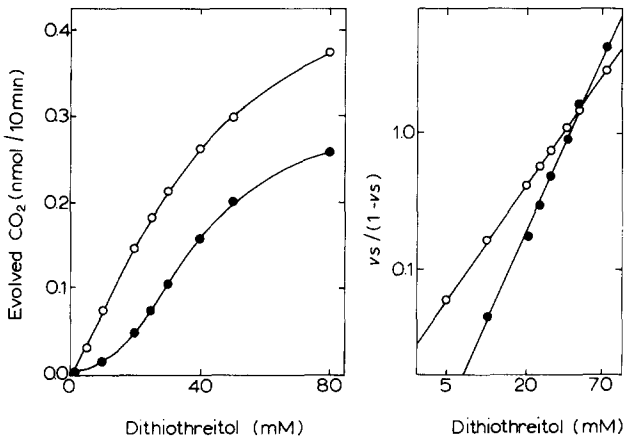

Fig. 4. Effect of dithiothreitol concentration on the velocity of the activation. (A) The enzyme activity was assayed at 3 min after starting the activation. Dithiothreitol concentration in the activation mixture was varied and Fe^{2+} (50 μM) was either added or omitted. The enzyme protein (in the activation mixture, 45.6 mg/ml) was used. ●———●, without Fe^{2+}; ○———○, with Fe^{2+} (B) Hill plots of the data were done according as described in the text.

more remarkable at lower concentrations of dithiothreitol. The plots in Fig. 4-B were rearranged by the modified Hill equation [14]:

$$\log \frac{\bar{v}_s}{1 - \bar{v}_s} = n \log s - \log k$$

where s is the concentration of dithiothreitol, $\bar{v}_s$ is the velocity of the activation of the enzyme preincubated with a given concentration of dithiothreitol divided by the maximum velocity of the activation. Hill coefficient, n, was then evaluated from the slope of the plots to be 2.17 ± 0.14 (5)* and 1.39 ± 0.09 (4) in the absence and presence of Fe^{2+}, respectively. These results suggest that the site of the enzyme which interacts with dithiothreitol may be 2 or more and a function of dithiothreitol was partially substituted by Fe^{2+}.

Discussion

Numerous investigators reported that Fe^{2+} and sulfhydryl group(s) have an important role in the catalytic reactions of oxygenase. Metapyrocatechase was inactivated by various oxidizing agents, such as air or H_2O_2. Nozaki et al. [15] reported that this inactivation is due to oxidation of Fe^{2+} to Fe^{3+}, and the full reactivation is achieved by incubation with Fe^{2+} and a reducing agent such as borohydride, ascorbate and cysteine. Tryptophan-5-monooxygenase in the present study was labile to oxidizing agent such as air and a sulfhydryl agent was essentially required for reactivation. But other reducing agents such as borohydride, dithionite and ascorbate were not an obligatory requirement. Tartaric acid dehydrase [16] was also labile to air and the incubation with both

* The results were expressed in the form of mean ± S.D. of a series (number of experiments).

Fe^{2+} and glutathione or cysteine reactivated the enzyme due to association of smaller protein which is inactive. The sucrose density gradient technique of Martin and Ames [17] was used to determine the effect of the activation on the molecular weight of tryptophan-5-monooxygenase. However no change of the molecular weight ($s_{20,w}$ = 4.50 ± 0.44 (4)*, by using bovine serum albumin as a standard; Hori, S., unpublished) has so far been observed between before and after the activation. Therefore the activation of tryptophan 5-monooxygenase may be mainly due to the modification of some sites in the enzyme.

The present study showed that the activated enzyme does not need any exogenous Fe^{2+} for the catalytic reaction but endogenous iron may be present in the enzyme preparation. It has been reported that homogentisate oxidase [18] and 3,4-dihydroxyphenylacetate-2,3-oxygenase [19] contain one or more ferrous-mercaptan residues in the active centre. Hayaishi et al. [20] proposed that an oxygenase reaction proceeds through forming a ternary complex between the active centre of the enzyme, substrate and oxygen. Recently Fisher et al. [21] reported that phenylalanine monooxygenase highly purified from rat liver contains 1 or 2 mol of iron per mol of the enzyme and this iron plays an important role in the monooxygenase reaction.

Tyrosine monooxygenase, partially purified from bovine adrenal medulla, was shown to be activated by preincubation with 2-mercaptoethanol and Fe^{2+}. In this case, however, 2-mercaptoethanol was specifically required for the activation and could not be replaced by dithiothreitol, cysteine or glutathione [22]. This difference in requirement of sulfhydryl agent for activation between tryptophan and tyrosine monooxygenase may be due to the difference in the structure of the enzyme. The activation energy of tryptophan-5-monooxygenase from bovine pineal glands was calculated to be 19 500 cal/mole from Arrhenius plots (Hori, S., unpublished); the value is comparable to that of tyrosine monooxygenase [22].

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