THE ACTIVATION OF BOVINE PINEAL TRYPTOPHAN 5-MONOOXYGENASE

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1. Introduction

Tryptophan 5-monooxygenase catalyzes the tetrahydropterin-dependent conversion of L-tryptophan to L-5HTP†, the initial reaction in the biosynthesis of serotonin in the brain and of melatonin in the pineal gland [1–5]. The properties of the enzyme and the mechanism of the reaction have not been fully elucidated simply because this enzyme has not been extensively purified due to low activity in tissue extracts and poor yields upon attempts at purification. In the present communication, we wish to report that tryptophan 5-monooxygenase of bovine pineal gland appears to exist largely as an inactive entity in tissue extracts and is activated approximately 100-fold upon preincubation in the presence of DTT or other sulfhydryl reagents. The velocity of the activation in the presence of DTT was markedly accelerated by Fe^{2+}, borohydride, hydrosulfite or a combination of NADPH, adrenodoxin reductase and adrenodoxin but not by other metal ions such as Hg^{2+}, Cu^{+}, Cu^{2+}, Cd^{2+}, Co^{2+}, Sn^{2+} and Sn^{4+} or excess amounts of catalase. A common property among the activators so far found in their reducing ability suggesting that the activation of bovine pineal tryptophan 5-monooxygenase may represent the reduction of some part(s) of the enzyme protein.

2. Materials and methods

Bovine pineal glands were obtained at a slaughterhouse, transported packed in ice and used immediately or stored frozen at −80°C. All manupulations for the purification of tryptophan 5-monooxygenase were carried out at 0–4°C. The glands (400 g) were homogenized with 1.2 l of 20 mM Tris in a Waring blendor for 8 min††. The homogenate was centrifuged at 30 000 rpm for 60 min. The supernatant (pH 8.1) was adjusted to pH 7.0 and saved. The pellet was suspended in 0.1 M K-phosphate (pH 7.0). To the suspension of pellet (800 ml) were then added 400 ml of 2% digitonin (pH 7.0) and after being stirred for 60 min, the suspension was centrifuged at 30 000 rpm for 30 min. The supernatant and the digitonin extract were combined. The fraction precipitated from the combined supernatant by $(NH_4)_2SO_4$ between 25 and 57% saturation at pH 7.0 was dissolved in 5 mM K-phosphate (pH 7.0) and dialyzed against the same buffer. After the insoluble material was removed centrifugation, the dialyzed enzyme was adsorbed on

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† *Abbreviations used* are: 5HTP, 5-hydroxytryptophan: L-tryptophan-1-^{14}C, L-tryptophan-(side chain-1)-^{14}C; $DMPH_4$, 6,7-dimethyl-5,6,7,8-tetrahydropterin; DDT, dithiothreitol; BSA, bovine serum albumin.

†† All enzyme preparations obtained during the purification procedure were activated to nearly the same extent. The activation was also observed to nearly the same extent when the glands were homogenized in a Potter homogenizer with either 0.25 M sucrose (pH 7), 10 mM Tris–acetate (pH 7.4) or 10 mM Tris–acetate (pH 7.4) containing 10 mM DTT.

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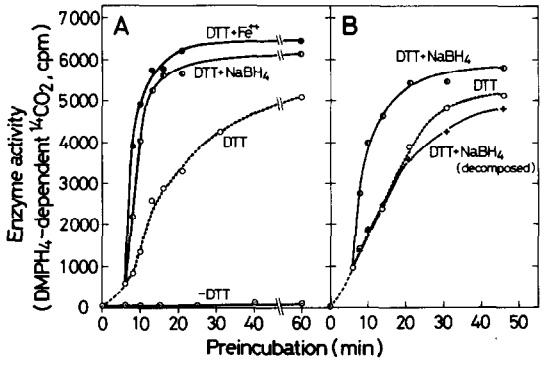

Fig. 1. Effect of dithiothreitol, Fe^{2+} and borohydride on the time course of the activation of tryptophan 5-monooxygenase. The preincubation mixture (0.8 ml) contained 80 μmoles of Tris–acetate (pH 8.1), 12 μl of ethanol, 400 μg of BSA, 25 μg of catalase (Sigma, C-100), 180 μg of metapyrocatechase, 80 nmoles of catechol, 2.4 mg of enzyme and where indicated, 25 μmoles of DTT, 40 nmoles of $Fe(NH_4)_2(SO_4)_2$ or 8 μmoles of $NaBH_4$. Metapyrocatechase and catechol were added to make the preincubation conditions more anaerobic. Enzyme was added to the preincubation mixture in a Thunberg tube at 0-time and the air in the tube was replaced 3 times with N_2 while the tube was immersed in an ice-bath. Catechol was added between second and third replacement. At around 4 min of the preincubation the tube was transferred to a water-bath of 12°C and the preincubation at 12°C was continued for 60 min. $Fe(NH_4)_2(SO_4)_2$ and $NaBH_4$ solutions were separately equilibrated with N_2 and were added to the mixture at 6 min. At various time intervals 50 μl-aliquots of the preincubation mixture were removed and added to 0.1 ml of the assay mixture which contained 15 μmoles of K-phosphate (pH 6.7), 5 μl of ethanol, 0.5 μmole of DTT, 5 nmoles of $Fe(NH_4)_2(SO_4)_2$. 11.25 nmoles of L-tryptophan-1-^{14}C (43 000 cpm) and 125 nmoles of 2-amino-4-hydroxyl-6,7-dimethyltetrahydropteridine ($DMPH_4$). Reactions were carried out at 37°C for 3 min and were stopped by addition of excess *o*-phenanthroline followed by immersion in a boiling water-bath for 1 min. L-5HTP-1-^{14}C formed was then converted almost selectively and stoichiometrically to $^{14}CO_2$ by the action of aromatic L-amino acid decarboxylase and $^{14}CO_2$ evolved was trapped in alkali and counted in a liquid scintillation counter [8]. In all sets of experiment the control incubation containing no tetradropterin was run simultaneously and the value obtained in the absence of reduced pterin cofactor was subtracted from the experimental value. Where indicated a $NaBH_4$ solution was adjusted to pH 4.6 and after H_2 evolution ceased the solution was neutralized and then added to the preincubation mixture. '–DDT' includes addition of Fe^{2+} and $NaBH_4$ and no addition.

a column (2.8 × 59 cm) of hydroxylapatite which had been equilibrated with 5 mM K-phosphate (pH 7.0) containing 20 mM $(NH_4)_2SO_4$, 5% glycerol, 3% ethanol and 0.1 mM L-tryptophan. The column was washed with 75 mM K-phosphate (pH 7.0) containing $(NH_4)_2SO_4$, glycerol, ethanol and L-tryptophan as above and then the enzyme was eluted with a linear gradient between 1 litre each of 75 mM and 350 mM K-phosphate (pH 7.0) both containing $(NH_4)_2SO_4$, glycerol, ethanol and L-tryptophan as above. The enzyme was precipitated from the combined active fractions by $(NH_4)_2SO_4$ (70% saturation), dissolved in a small amount of 5 mM K-phosphate (pH 7.0) and dialyzed against the same buffer. By these procedures, the enzyme was purified approximately 15-fold over the crude homogenate with a yield of 32%. All experiments reported in this paper were carried out with the concentrated hydroxylapatite fraction††. Incubation was carried out with L-tryptophan-1-^{14}C as substrate and the enzyme activity was determined by $^{14}CO_2$ which was liberated from L-5HTP-1-^{14}C by the action of aromatic L-amino acid decarboxylase [4]. Detailed assay conditions are described in the legend to fig. 1. Aromatic L-amino acid decarboxylase was purified from hog kidney essentially according to the method of Christenson et al. [6] up to the step of ammonium sulfate fractionation. Adrenodoxin reductase and adrenodoxin purified from bovine adrenal cortex were kindly donated by Dr. F. Mitani of this department. Metapyrocatechase [7] was a kind gift of Dr. M. Nozaki, Kyoto University. Commercial sources and purification methods of substrates and reagents were previously described [4].

3. Results and discussion

Tryptophan 5-monooxygenase activity of bovine pineal gland initially detected in tissue homogenate has been as low as 200 pmoles per min per g of wet tissue. However, it was subsequently found that the enzyme activity increased approximately 100-fold when the enzyme was preincubated with DTT under anaerobic conditions as shown in fig. 1A. In this experiment, the enzyme activity before the preincubation was 50–80 cpm of $DMPH_4$-dependent $^{14}CO_2$

Table 1
Effect of reducing agents on the activation of tryptophan 5-monooxygenase.

Experiment	Additions	DTT	Enzyme activity after 6 min	9 min	46 min *
				$^{14}CO_2$ (cpm)	
I.	None	+	377	1,256	5,561
	NADPH	+		1,020	5,020
	NADPH, adrenodoxin reductase	+		1,161	4,777
	NADPH, adrenodoxin reductase, adrenodoxin	+		2,147	5,712
	$Fe(NH_4)_2(SO_4)_2$	+		3,278	5,549
	None	–	29	19	39
	NADPH, adrenodoxin reductase, adrenodoxin	–		87	34
II.	None	+	667	1,161	5,812
	$Na_2S_2O_4$	+		3,079	6,191
	$Fe(NH_4)_2(SO_4)_2$	+		3,370	6,240
	None	–	68	58	108
	$Na_2S_2O_4$	–		145	374
	$Na_2S_2O_4$, $Fe(NH_4)_2(SO_4)_2$	–		182	229

The preincubation of tryptophan 5-monooxygenase was carried out as described in the legend to fig. 1 except that beef liver glucose dehydrogenase (600 μg) and glucose (52.5 mM) were added to the mixture. Where indicated, DTT (31 mM), adrenodoxin (46 μM) and adrenodoxin reductase (24 munits) were included in the preincubation mixture. NADPH (0.3 mM), $Fe(NH_4)_2(SO_4)_2$ (50 μM) and $Na_2S_2O_4$ (1 mM) were added at 6 min (7.5 min in Experiment II) of the preincubation. The activity of the preincubated enzyme was determined after 6, 9 and 46 min (7.5, 10.5 and 40 min in Experiment II) of the preincubation as described in the legend to fig. 1.

* Enzyme activity at 7.5, 10.5 and 40 min in Experiment II. The increase in enzyme activity after 9 and 46 min over the 6 min-control roughly represents the velocity and extent of the activation, respectively.

evolved. When Fe^{2+} was added to the preincubation mixture, the velocity of the activation in the presence of DTT was markedly accelerated and the effect of Fe^{2+} was substituted by borohydride. When DTT was omitted, no activation occurred even in the presence of both Fe^{2+} and borohydride indicating that DTT is absolutely required for the activation. As shown in fig. 1B, borohydride was effective only when it was freshly prepared. When borohydride was decomposed at pH 4.6 and then added to the preincubation mixture, no acceleratory effect was observed indicating that the effect of borohydride is not due to contaminating iron or other compounds.

This activation of tryptophan 5-monooxygenase was a time-, temperature- and pH-dependent process suggesting that some changes may occur in enzyme protein associated with the activation. When the preincubation was carried out anaerobically in the presence of 30 mM DTT and 50 μM Fe^{2+} at 0°, 12° and 30°C, the time required for the full activation was 60–70, 30–40 and 5–10 min, respectively. The optimum pH for the initial velocity of the activation was found to be around 9, but at this pH the activation was followed by the inactivation of the enzyme. No activation occurred below pH 6. Under aerobic conditions, the activated enzyme appeared to be more unstable than under anaerobic conditions with an approximate half-life of less than 60 min at 0°C and pH 8.1.

In an effort to search for other compounds or systems which are effective in place of either DTT or Fe^{2+}, we have found that NADPH-adrenodoxin reductase–adrenodoxin system of adrenal cortex [9] is effective in substituting for Fe^{2+} and increases the velocity of the DTT-dependent activation (table 1). The acceleratory effect of Fe^{2+} was also substituted by 1 mM hydrosulfite (table 1) but not by ascorbate, hydroquinone, $DMPH_4$, reduced pyridine nucleotides or various metals such as Hg^{2+}, Cu^{+}, Cu^{2+}, Cd^{2+}, Co^{2+}, Sn^{2+}, Sn^{4+} and Ni^{2+} under conditions so far

tested. Fe^{3+} was as effective as Fe^{2+} but 65–70% of Fe^{3+} added to the preincubation mixture was rapidly converted to Fe^{2+} under the conditions employed.

DTT could be replaced by other sulfhydryl compounds such as β-mercaptoethanol, α-thioglycerol, GSH, cysteine and *N*-acetylcysteine and by hydrosulfite. DTT was the most effective among them and only very slight activation was observed with hydrosulfite alone or its comination with Fe^{2+} (table 1). When the preincubation was carried out in the presence of 50 μM Fe^{2+} and various concentrations of DTT, 5 mM DTT was found to be almost as effective as 31 mM DTT as far as the extent of the activation is concerned (fig. 2A). However, the velocity of the activation increased significantly at higher concentrations of DTT. The concentration of DTT to give half-maximum velocity of the activation was calculated to be around 23 mM. In order to see whether or not DTT alone is effective in promoting the activation, the effect of metal chelating agents on the time course of the activation was examined. As shown in fig. 2B, low concentrations of α,α′-dipyridyl did not affect seriously the initial velocity of the activation while much lower concentrations of Fe^{2+} increased the velocity. It was suggested that DTT or other sulfhydryl reagents are almost absolutely required for the activation and the velocity of the activation is accelerated by Fe^{2+}, borohydride, hydrosulfite and reduced adrenodoxin; a common property among the activators so far found is their reducing ability.

The stimulatory effect of Fe^{2+} on tryptophan and tyrosine hydroxylase activities has been observed [3, 10]. Recently, Shiman et al. [11] and Friedman et al. [5] have attributed the effect of Fe^{2+} on bovine adrenal tyrosine hydroxylase and rabbit hindbrain tryptophan 5-monooxygenase, respectively, to the known ability of Fe^{2+} to decompose H_2O_2 which is generated during the non-enzymic oxidation of tetrahydropterin and which inactivates the enzymes. Petrack et al. [12] reported that tyrosine hydroxylase of bovine adrenal medulla is activated several fold by preincubation with β-mercaptoethanol and Fe^{2+}. Sulfhydryl reagent and Fe^{2+} thus seem to be involved in the tryptophan and tyrosine hydroxylase reactions as the activator of the enzyme as well as the stabilizer of the cofactor and enzyme itself. Whether or not bovine pineal tryptophan 5-monooxygenase exists as the inactive form in vivo remains to be investigated.

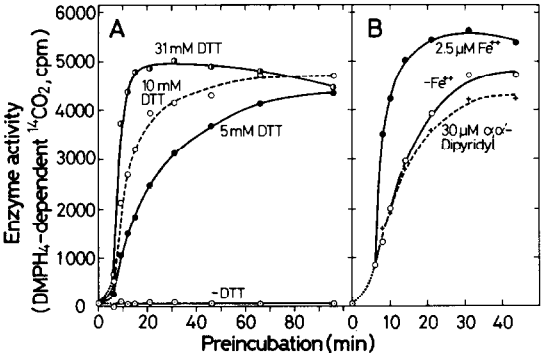

Fig. 2. Effect of various concentrations of dithiothreitol (A) and a low concentration of α,α′-dipydryl (B) on the activation of tryptophan 5-monooxygenase. The preincubation of the enzyme was carried out as described in the legend to fig. 1 except that 50 μM Fe^{2+} and various concentrations of DTT were added (Experiment A) or 31 mM DTT and either 2.5 μM Fe^{2+} or 30 μM α,α′-dipyridyl were added (Experiment B). When the preincubation was carried out in the presence of α,α′-dipyridyl, 3 nmoles more $Fe(NH_4)_2(SO_4)_2$ were added to the assay mixture.

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