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INACTIVATION OF TYROSINE 3-MONOOXYGENASE BY ACETONE PRECIPITATION AND ITS RESTORATION BY INCUBATION WITH A SULFHYDRYL AGENT AND IRON

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Summary

The acetone precipitation of a partially purified tyrosine 3-monooxygenase (L-tyrosine, tetrahydropteridine: oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2) resulted in the complete loss of enzymatic activity. The enzymatic activity was restored by incubation with iron and dithiothreitol. The restoration of the activity was a pH-, temperature- and time-dependent reaction. Since cobalt, nickel, copper, zinc, manganese, cadmium, magnesium calcium and barium ions were all ineffective in restoring activity, iron ion appeared to be specifically required in the restoration of the enzyme activity Dithiothreitol could be partially replaced in the restoration step by glutathione, 2-mercaptoethanol or cysteine.

Introduction

Tyrosine 3-monooxygenase (L-tyrosine, tetrahydropteridine oxygen oxidoreductase (3-hydroxylating), EC 1 14.16 2) catalyzes, the rate-limiting step in the biosynthesis of catecholamines, the conversion of L-tyrosine to 3,4-dihydroxy-L-phenylalanine requiring tetrahydrobiopterin as an electron donor [1] Although the regulatory mechanism of the enzyme has been extensively studied, the studies of the reaction mechanism and molecular properties of the enzyme have not progressed owing to its instability and paucity The most important question on the reaction mechanism of the enzyme will be whether or not the enzyme contains the iron which is involved in the enzyme reaction. The present study reports that tyrosine 3-monooxygenase partially purified from rat adrenal medulla was completely inactivated by acetone precipitation

and the enzymatic activity was restored by incubation with iron and dithiothreitol. The result that the restoration of the enzyme activity absolutely and specifically required the presence of iron is in accord with recent circumstantial evidence suggesting that tyrosine 3-monooxygenase might possibly be an iron protein [1]

Methods

Tyrosine 3-monooxygenase was partially purified from rat adrenal glands by $(NH_4)_2SO_4$ fractionation, gel filtration on Ultrogel AcA22 (LKB) and acid precipitation. 500 frozen rat adrenal glands were decapsulated and homogenized in 20 vol. 5 mM sodium phosphate buffer, pH 7 5/1 mM EDTA/1 mM phenylmethylsulfonyl fluoride (Sigma) with a Potter-Elvehjem homogenizer The soluble fraction which was obtained from the homogenate by centrifugation at 78 000 × *g* for 1 h was brought to 30% saturation with solid $(NH_4)_2SO_4$ and centrifuged To the supernatant solid $(NH_4)_2SO_4$ was added to a final saturation of 40% The precipitate was collected by centrifugation, dissolved in 1 ml of 5 mM sodium phosphate buffer, pH 7 5/1 mM EDTA and then chromatographed on a Ultrogel AcA22 column (3.6 × 50 cm) equilibrated with the same buffer. The pH of the active fraction, which was pooled, was adjusted to 5.2 by the addition of 0.1 M acetic acid and the resulting precipitate, which was collected by centrifugation, was dissolved in 2 ml 5 mM sodium phosphate buffer, pH 7 5/25% glycerol/0.05% Tween 80/0 1 mM EDTA The enzyme thus obtained was purified about 40-fold over the soluble fraction of decapsulated rat adrenal glands with an approx. 80% yield as summarized in Table I.

Tyrosine 3-monooxygenase was assayed fluorimetrically as described previously [2]. 1 unit of tyrosine 3-monooxygenase activity is defined as the amount which catalyzes the formation of 1 nmol 3,4-dihydroxy-L-phenylalanine/min at 30°C.

Inactivated tyrosine 3-monooxygenase for reactivation study was prepared by acetone precipitation. To the partially purified enzyme solution (approx. 3—8 mg/ml) 4 vol. cold acetone were added (—20°C) and the resulting precipitate was collected by centrifugation and dissolved in 0.2 vol 5 mM sodium phosphate buffer, pH 7.5/25% glycerol/0.05% Tween 80/0.1 mM EDTA

The standard incubation mixture for the reactivation of inactivated tyrosine 3-monooxygenase contained 50 mM sodium phosphate buffer, pH 7.5/25%

TABLE I

SUMMARY OF PURIFICATION OF TYROSINE 3-MONOOXYGENASE FROM 500 RAT ADRENAL GLANDS

Purification step	Protein (mg)	Activity (units)	Specific activity (units/mg)	Yield (%)
Supernatant (78 000 × *g*, 1 h)	495	220	0 444	100
$(NH_4)_2SO_4$	73 8	172	2 33	78
Ultrogel AcA22	27 5	161	5 85	73
Acid treatment	10 4	179	17 2	81

glycerol/0.05% Tween 80/20 mM dithiothreitol/50 μM $FeSO_4(NH_4)_2SO_4$ and a suitable amount (approx. 3—5 mg/ml) of tyrosine 3-monooxygenase. It should be noted that the protein concentration for reactivation of the enzyme was important, since the efficiency of the reactivation which was done at a lower concentration of protein was very low. The reaction was usually started by the addition of 9 vol. mixture for reactivation to 1 vol. of an inactivated enzyme solution and, therefore, the incubation mixture contained 10 μM EDTA. The reaction was carried out at 24°C for 30 min under aerobic conditions, unless otherwise stated.

Ferrous and ferric ions were determined by a *o*-phenanthroline method [3]. To 250 μl of a sample solution were added 125 μl 0.2 M biphthalate buffer, pH 4.0, and 250 μl of a 0.3% *o*-phenanthroline solution. After mixing, the absorbance of the ferrous-*o*-phenanthroline complex was read at 512 nm within 3 min. Ferric ion was determined on a duplicate solution by adding 50 μl 5% $NH_2OH \cdot HCl$ and swirling for 10—20 s before the addition of the *o*-phenanthroline.

Protein concentrations were determined spectrophotometrically from the absorbance at 280 nm and 260 nm [4].

Results

The acetone precipitation of tyrosine 3-monooxygenase resulted in the complete loss of enzymatic activity and the activity was restored by incubation with iron and dithiothreitol. The extent of restoration of the enzymatic activity varied from only 10 to 100% depending on how the acetone precipitation was done In contrast to the enzyme inactivated by acetone precipitation, the activity of native enzyme was not affected by the incubation with iron and dithiothreitol.

The time course of reactivation of acetone-treated enzyme by incubation with iron and dithiothreitol was examined at various incubation temperatures (Fig. 1) and at various pH values (Fig. 2). The initial rate of reactivation of the enzyme was increasingly greater but the stability of the enzyme was lower as the incubation temperature was raised, as shown in Fig 1. Incubation at 24°C resulted in a progressive increase of the enzyme activity up to 30 min, and thereafter a very slow decrease of the enzyme activity. The pH for maximal rate of restoration of the enzymatic activity was 7.5 or higher, although the enzyme appeared to be more unstable at higher pH values as shown in Fig. 2. Thus, the reconstitution process of the enzyme by incubation with iron and dithiothreitol was a time-, temperature- and pH-dependent process.

Effect of various concentrations of iron on reactivation of tyrosine 3-monooxygenase inactivated by acetone precipitation is shown in Fig. 3. A maximal restoration of the enzyme activity was observed at iron concentrations of 50—100 μM. When iron was omitted from the incubation mixture, no significant reactivation of the enzyme was observed. Table II demonstrates the specificity of the requirement for iron for restoration of the enzyme activity. No cations tested other than ferrous or ferric ion were effective in restoring activity to the inactivated enzyme. The restoring activity observed with ferric

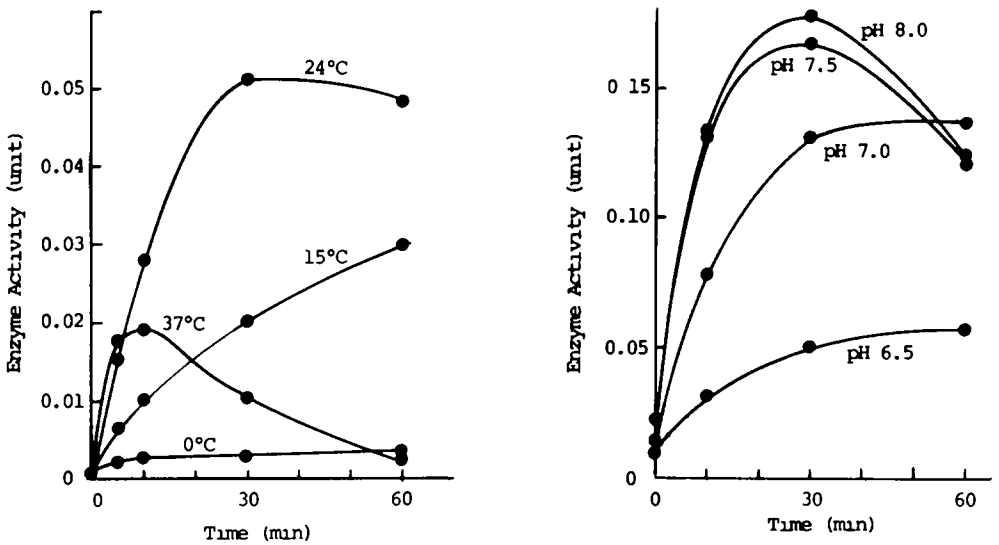

Fig 1 Effect of temperature on restoration of tyrosine 3-monooxygenase activity Reactions were carried out as described, except that the temperature and period of incubation for reactivation were varied as indicated.

Fig 2 Effect of pH on restoration of tyrosine 3-monooxygenase activity Reactions were carried out as described, except that the pH of incubation mixture and the period of incubation for reactivation were varied as indicated

ion was probably due to rapid reduction to ferrous ion by dithiothreitol which was present during incubation, since the result of iron analysis suggested the rapid conversion of ferric ion to ferrous ion in the incubation mixture even under aerobic conditions Thus, the restoration of the enzyme activity absolutely and specifically required the presence of iron, presumably the presence of ferrous ion.

Fig. 4 shows the effect of different concentrations of dithiothreitol on the

TABLE II

SPECIFICITY OF IRON FOR RESTORATION OF TYROSINE 3-MONOOXYGENASE ACTIVITY

Reactions were carried out as described, except that $FeSO_4(NH_4)_2SO_4$ was omitted from the incubation mixture for restoration and indicated cations were added at a concentration of 100 μM

Additions (100 μM)	Enzyme activity		Additions (100 μM)	Enzyme activity	
	units	%		units	%
None	0	0	$ZnCl_2$	0	0
$FeSO_4(NH_4)_2SO_4$	0 056	100	$MnCl_2$	0 004	7
$FeCl_2$	0 052	93	$CdCl_2$	0	0
$FeCl_3$	0 040	71	$MgCl_2$	0 001	2
$CoCl_2$	0 002	4	$CaCl_2$	0 002	4
$NiCl_2$	0 001	2	$BaCl_2$	0	0
$CuCl_2$	0 001	2			

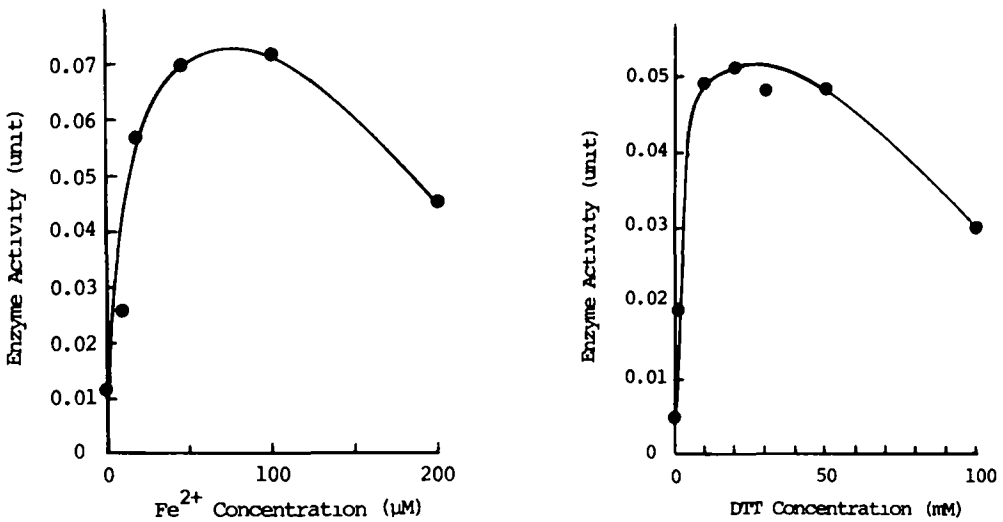

Fig 3 Effect of iron concentration (Fe^{2+}) on restoration of tyrosine 3-monooxygenase activity Reactions were carried out as described, except that the concentration of $FeSO_4(NH_4)_2SO_4$ in the incubation mixture for restoration was varied as indicated

Fig 4 Effect of dithiothreitol (DTT) concentration on restoration of tyrosine 3-monooxygenase activity Reactions were carried out as described, except that the concentration of dithiothreitol in the incubation mixture for restoration was varied as indicated

restoration of inactivated tyrosine 3-monooxygenase in the presence of 50 μM ferrous ion. Restoration of the enzyme activity was maximal at concentrations of 10—50 mM and declined with higher concentrations of dithiothreitol. The specificity of the requirement for dithiothreitol for restoration of the enzyme activity was also examined (Table III). The experiment was carried out under an argon atmosphere in order to avoid autooxidation of sulfhydryl agents and reducing agents in the incubation mixture. Under the standard experimental

TABLE III

EFFECT OF SULFHYDRYL AGENTS AND REDUCING AGENTS ON RESTORATION OF TYROSINE 3-MONOOXYGENASE ACTIVITY

Reactions were carried out as described, except that 10 mM sulfhydryl agents or reducing agents were added to incubation mixture for restoration in place of dithiothreitol as indicated and the reaction was carried out under an argon atmosphere

Additions (10 mM)	Enzyme activity	
	units	%
None	0 001	2
Dithiothreitol	0 064	100
Glutathione	0 018	28
2-Mercaptoethanol	0 017	27
Cysteine	0 013	20
Ascorbic acid	0 002	3
Dithionite	0 008	13
Dithionite + Dithiothreitol	0 017	27

conditions, aerobic or anaerobic conditions did not affect the restoration of the enzyme activity Dithiothreitol could be replaced by other sulfhydryl agents such as glutathione, 2-mercaptoethanol or cysteine, although dithiothreitol was the most effective.

Discussion

During our attempts to purify tyrosine 3-monooxygenase from rat adrenal medulla, it was observed that the presence of glycerol, Tween 80 and EDTA in an enzyme solution markedly stabilized the enzyme during the course of purification (Okuno, S. and Fujisawa, H., unpublished data), and fractionation procedure by acetone precipitation resulted in complete loss of enzyme activity even in the presence of glycerol, Tween 80 and EDTA. Since there is some circumstantial evidence that tyrosine 3-monooxygenase may be an iron protein [1], the loss of enzyme activity by acetone precipitation was presumed to be due to the loss of iron in the enzyme. The results that the restoration of the enzyme activity absolutely and selectively required the presence of iron in the incubation mixture are in accord with this contention.

Petrack et al. [5] have reported that partially purified tyrosine 3-monooxygenase from bovine adrenal medulla which had lost 80—90% of the activity upon standing overnight at room temperature restored the activity following incubation with ferrous ion and mercaptoethanol. They have reported that mercaptoethanol could not be replaced by cysteine, glutathione or dithiothreitol. In contrast, the results of our own experiments demonstrated that various sulfhydryl agents including glutathione, mercaptoethanol and cysteine could replace dithiothreitol in restoring activity, although dithiothreitol was the most effective. Thus, although there are some differences in the specificity of the requirement for sulfhydryl agents for restoration of the enzyme activity, both the studies appear to exhibit basic similarities in the requirement for both iron and a sulfhydryl agent for restoration of enzyme activity.

The presence of glycerol, Tween 80 and EDTA appeared to stabilize the enzyme inactivated by acetone precipitation as well as native enzyme, since the inactivated enzyme could be stored in the presence of glycerol, Tween 80 and EDTA at 4°C at least for several days without significant loss of its ability to restore the activity. Instability of tyrosine 3-monooxygenase was likely to be due to two major factors, a conformational change of the enzyme protein and the loss of iron in the active site. The former appeared to be prevented by the presence of glycerol, Tween 80 and EDTA and the latter appeared to be restored by incubation with iron and dithiothreitol.

The results presented in this report provide not only strong circumstantial evidence that iron may be present in the active site of tyrosine 3-monooxygenase and may participate in the enzyme reaction but also a useful tool for investigation of the role of the iron in the enzyme reaction, although the ultimate answer to the question of whether the enzyme is really an iron protein cannot be provided until pure enzyme becomes available for use in studying the molecular properties and the reaction mechanism of the enzyme.

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