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REVERSIBLE INACTIVATION OF EXTRACELLULAR THIAMINASE I IN *BACILLUS THIAMINOLYTICUS*

I. INACTIVATION BY THE PRIMARY SUBSTRATE AND REACTIVATION BY THE SECONDARY SUBSTRATE

KIROKU SUZUKI AND JUN-ICHI OOBA

Department of Agricultural Chemistry, University of Shimane, Matsue, Prefecture of Shimane (Japan)

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SUMMARY

Extracellular thiaminase I (thiamine:base 2-methyl-4-aminopyrimidine-5-methenyltransferase, EC 2.5.1.2) in *Bacillus thiaminolyticus* was inactivated by incubation with any of its primary substrates. The activity lost could be restored by incubation at 37 °C for 60 min after dialysis. Any secondary substrate tested was found to inhibit the inactivation and to restore the lost activity. Each substrate at high concentration overcame the effect of the other substrate. Neither inactivation nor reactivation proceeded in the enzymic assay mixture, since the mixture contained $1.5 \cdot 10^{-3}$ M thiamine and $2.5 \cdot 10^{-2}$ M pyridine. Consequently, it is possible to assay active and inactive thiaminase I activity separately in the mixed solution.

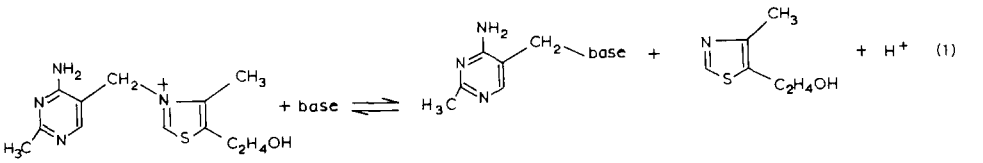
Inactivation was inhibited only 50% by *p*-chloromercuribenzoate (PCMB) at $2 \cdot 10^{-4}$ M which inhibited thiaminase I reaction completely. PCMB at the same concentration inhibited completely the reactivation by pyridine, while it did not prevent the inhibitory action of pyridine on the inactivation. On the basis of these results, the mechanism of the inactivation was discussed.

INTRODUCTION

Thiaminase I (thiamine:base 2-methyl-4-aminopyrimidine-5-methenyltransferase, EC 2.5.1.2) requires two kinds of substrates, a (2-methyl-4-amino-5-pyrimidinyl)methyl (PM) compound and an acceptor base, for the enzymic reaction[1,2]. The reaction with thiamine as a PM-compound is represented in Eqn 1.

We found that extracellular thiaminase I (ref. 3) in *Bacillus thiaminolyticus*

Abbreviations: PM-, (2-methyl-4-amino-5-pyrimidinyl)methyl; PCMB, *p*-chloromercuribenzoate.



was transformed reversibly into its inactive (or less active) form by incubation with any of its primary substrates (PM-compounds).

This paper describes the effects of primary and secondary substrates (acceptor bases) on the decrease and restoration of extracellular thiaminase I activity, and the changes of activity during enzymic assay.

MATERIALS AND METHODS

Organism

B. thiaminolyticus, strain YUSM 1001, was kindly supplied by Prof. R. Hayashi, University of Yamaguchi.

Preparation of enzyme

Crude extracellular thiaminase I was prepared by the procedure of Douthit and Airth[3]. This preparation had an enzymic activity of about 7000 munits/ml and a specific activity of 550 munits/mg of protein. This preparation was diluted 270-fold with distilled water and incubated for 60 min at 37 °C before use in the study, because activity increased by 50% during the first 45 min (presumably due to the activation of the contaminating inactive enzyme) and subsequently it remained constant.

Inactivation and reactivation of enzyme

To 1.5 ml of a preincubated (or inactivated) enzyme solution, 0.15 ml of 0.15 M citrate–phosphate buffer (pH 6.5) and 0.15 ml of the appropriate reagent solution were added. The mixture was incubated for 60 min at 37 °C. The concentration of each reagent is presented in the legend to each figure. If needed, the incubated mixture was dialyzed against 1 l of 0.01 M citrate–phosphate buffer (pH 6.5) before enzymic assay (at 4 °C, for 5 h).

Enzymic assay

Thiaminase I activity was assayed by a modification[4] of the heteropyrithiamine (1-PM-pyridine) method[5]. If needed, additional components were added to the mixture, as indicated in the legend for each figure. One unit was defined as the amount of enzyme which produced 1 μmole of heteropyrithiamine per min at 37 °C.

Reagents

Heteropyrithiamine chloride hydrochloride was prepared by the method of Matsukawa and Yurugi[6], PM-OH chloride and 5-(2-hydroxyethyl)-4-methylthiazole by that of Watanabe[7], *S*-PM-cysteine by that of Murata *et al.*[8], and *N*-PM-aniline hydrochloride by that of Matsukawa and Yurugi[9]. Dimethialium, 4,5-dimethylthiazole, thiamine tetrahydrofurfuryl disulfide and thiamine propyl disulfide were kindly supplied by Dr J. Suzuoki, Takeda Industries, Ltd, 3-PM-5-(2-chloroethyl)-4-methylthiazole and 5-(2-chloroethyl)-4-methylthiazole by Dr T. Yusa, Sankyo Co.

TABLE I

EFFECTS OF THIAMINE-RELATED COMPOUNDS ON THE INACTIVATION OF THIAMINASE *I*

Reagent ($6 \cdot 10^{-6}$ M)	*Relative activity (%)*	*Decomposition by thiaminase I in B. thiaminolyticus***
Before incubation	100	
None	99	
Thiamine, 0 °C	97	
Thiamine	17	+*
Heteropyrithiamine	15	+*
PM-OH	93	−*
S-PM-cysteine	100	−**
Thiamine tetrahydrofurfuryl disulfide	90	−*
Thiamine propyl disulfide	91	−*
Amprolium	97	−**
3-PM-5-(2-chloroethyl)-4-methylthiazole	13	+*
Dimethialium	15	+**
1-PM-4-(2-hydroxyethyl)-5-methylimidazole	104	−**
N-PM-aniline	18	+*
Thiamine pyrophosphate	20	+*
5-(2-hydroxyethyl)-4-methylthiazole	102	

* See refs 13–18.

** Each substance (10^{-3} M) was incubated with 50 munits/ml of the enzyme and 10^{-3} M 5-(2-hydroxyethyl)-4-methylthiazole at 37 °C for 3 h. After incubation, thiamine formed in the mixture was determined by the thiochrome method or the method of Prebluda and McCollum[19].

*** +, decomposed; −, not decomposed.

Ltd, amprolium by the Dainippon Pharmaceutical Co. Ltd, and 1-PM-4-(2-hydroxyethyl)-5-methylimidazole by Dr T. Ichikawa, Osaka University. All other reagents were of analytical grade.

Thiamine content in the mixture was determined by the thiochrome procedure[10].

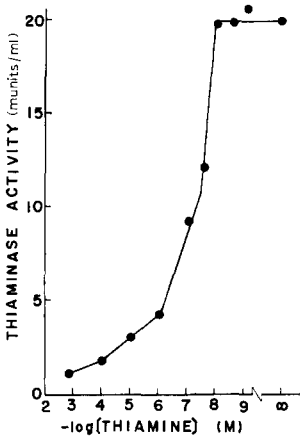
RESULTS

Inactivation of extracellular thiaminase I by its primary substrate

Table I shows the change of extracellular thiaminase I activity on incubation with $6 \cdot 10^{-6}$ M thiamine-related compound. All substances which were primary substrates for thiaminase I inactivated the enzyme, an effect not shown by other substances.

Fig. 1 shows the effect of thiamine concentration on inactivation. At a concentration of 10^{-8} M, inactivation was not observed. At $3 \cdot 10^{-8}$ M and above, inactivation was intensified with increase in thiamine concentration. However, 6% of the activity before incubation remained even after incubation with $1.5 \cdot 10^{-3}$ M thiamine. Though further incubation with $1.5 \cdot 10^{-3}$ M thiamine slowly gave rise to inactivation, there was still residual activity after 3 h incubation.

Fig. 2 shows the effect of dialysis on the lost activity. Dialysis alone did not restore the lost activity, but incubation at 37 °C for 1 h after dialysis restored it to the same level as the activity in the control.



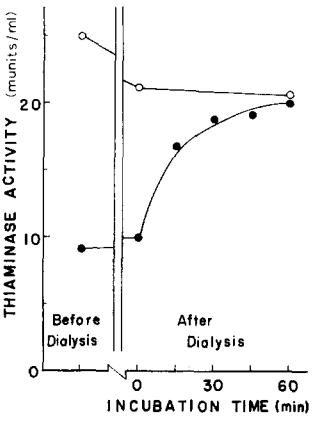
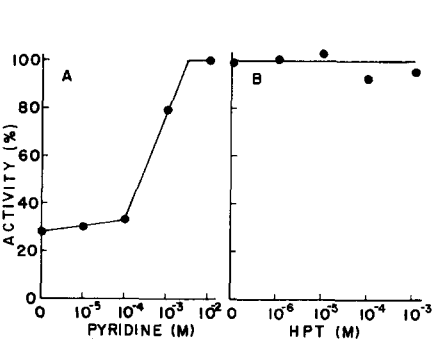


Fig. 1. Effect of thiamine concentration on the inactivation of thiaminase I. Incubation was carried out as described in Materials and Methods.

Fig. 2. Effect of dialysis on the activity lost by thiamine treatment. Enzyme was inactivated by $6 \cdot 10^{-6}$ M thiamine (37 °C, 60 min) and twice dialyzed for 5 h in a cold room (4 °C), followed by incubation at 37 °C. It was assayed for activity after each treatment. ●, thiamine-treated enzyme; ○, enzyme without thiamine treatment.

Changes in activity during enzymic assay

In the heteropyrithiamine method, the plot of the amounts of heteropyrithiamine formed against the reaction time has been found to be linear[4,5] although the assay mixture contained 1.5 mM thiamine. The assay mixture also contained $2.5 \cdot 10^{-2}$ M pyridine, a secondary substrate. Fig. 3 shows the effect of pyridine on the



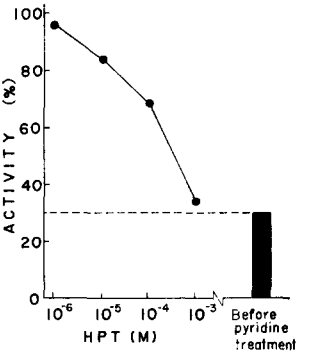


Fig. 3. Effect of pyridine on the inactivation of thiaminase I by heteropyrithiamine (HPT). (A) Effect of pyridine at various concentrations on the inactivation by $6 \cdot 10^{-6}$ M heteropyrithiamine. (B) Effect of $2.5 \cdot 10^{-2}$ M pyridine on the inactivation by heteropyrithiamine at various concentrations. Activities are expressed as % of those in the controls (no heteropyrithiamine and no pyridine in A, $2.5 \cdot 10^{-2}$ M pyridine in B).

Fig. 4. Effect of $2.5 \cdot 10^{-2}$ M pyridine on inactive thiaminase I. An enzyme solution which had been preincubated with 10^{-6} M heteropyrithiamine (HPT) (37 °C, 60 min) was divided into five parts. Four were incubated with $2.5 \cdot 10^{-2}$ M pyridine and heteropyrithiamine at various concentrations (37 °C, 60 min); the other part was kept at 0 °C for 60 min. After incubation, the five mixtures were dialyzed and the dialyzates were assayed for activity. Activities are expressed as % of the activity before preincubation.

inactivation by heteropyrithiamine. Pyridine at $2.5 \cdot 10^{-2}$ M inhibited completely the inactivation by heteropyrithiamine. These findings indicate that pyridine in the assay mixture is responsible for the prevention of inactivation by thiamine.

Fig. 4 shows the effect of $2.5 \cdot 10^{-2}$ M pyridine on the activity lost by a heteropyrithiamine treatment. Incubation with pyridine was capable of restoring the lost activity, in spite of the presence of heteropyrithiamine in the mixture, as long as its concentration was low. Heteropyrithiamine at 10^{-3} M inhibited almost completely the restoration of activity by pyridine. These findings suggest that a primary substrate at higher concentrations prevents the reactivation by pyridine, and hence that the reactivation of inactive thiaminase I does not proceed in an assay mixture which contains $1.5 \cdot 10^{-3}$ M thiamine besides pyridine. This possibility was confirmed by the time course of heteropyrithiamine formation in the assay mixture by thiamine-treated enzyme (37 °C, 60 min; with $6 \cdot 10^{-6}$ M). The plot of the amounts of heteropyrithiamine formed in the mixture against the reaction time was found to be linear till 80 min after the beginning of incubation (37 °C).

TABLE II

EFFECTS OF SECONDARY SUBSTRATES ON THE INACTIVATION AND REACTIVATION OF THIAMINASE *I*

Incubated mixtures were dialyzed before assay. The concentrations of primary substrates were $6 \cdot 10^{-6}$ M and those of secondary substrates, except aniline, were 10^{-2} M.

Treatment		*Relative activity (%)*
First incubation 37 °C, 60 min	*Second incubation 37 °C, 60 min*	
None	—	100
N-PM-Aniline	—	20
	+ aniline ($2 \cdot 10^{-3}$ M)	85
+ aniline ($2 \cdot 10^{-3}$ M)	—	73
Dimethialium	—	19
	+ 4,5-dimethylthiazole	95
+ 4,5-dimethylthiazole	—	98
3-PM-5-(2-chloroethyl)-4-methylthiazole	—	16
	+ 5-(2-chloroethyl)-4-methylthiazole	86
+ 5-(2-chloroethyl)-4-methylthiazole	—	70
Thiamine	—	17
	+ 5-(2-hydroxyethyl)-4-methylthiazole	62
+ 5-(2-hydroxyethyl)-4-methylthiazole	—	72

Effects of secondary substrates on inactivation and reactivation

Table II shows the effects of four acceptor nucleophiles on the inactivation by their corresponding primary substrates and on the reactivation. All showed the same effects on both reactions as observed with pyridine.

Effects of p-chloromercuribenzoate on inactivation and reactivation

Table III shows the effects of *p*-chloromercuribenzoate, an SH-blocking reagent, on four reactions related to thiaminase I. In contrast to a more than 99% inhibition of thiaminase I reaction, *p*-chloromercuribenzoate at a concentration of $2 \cdot 10^{-4}$ M inhibited the inactivation by only 48% and did not at all prevent the inhibition by pyridine of the inactivation. Moreover, *p*-chloromercuribenzoate at the same concentration inhibited completely the reactivation by pyridine. The effects of two SH-

TABLE III

EFFECTS OF PCMB ON THE INACTIVATION, REACTIVATION AND INHIBITION BY PYRIDINE OF THE INACTIVATION

The concentrations of pyridine and PCMB in the incubation mixtures were $2.5 \cdot 10^{-2}$ and $2 \cdot 10^{-4}$ M, respectively. Before enzymic assay, mercaptoethanol was added to each mixture to give a concentration of 10^{-2} M. Each incubation was carried out at 37 °C for 60 min.

Treatment		*Relative activity (%)*		
First incubation	*Second incubation*	*1*	*2*	*3*
None	—	100	100	100
+ PCMB	—	102		
Thiamine $6 \cdot 10^{-6}$ M	—	14		
+ PCMB	—	55		
Thiamine $1.5 \cdot 10^{-3}$ M	—		9	
+ pyridine, + PCMB*	—		107	
Heteropyrithiamine $6 \cdot 10^{-6}$ M	—			40
	+ pyridine			96
	+ pyridine, + PCMB			35

* Thiaminase I reaction was inhibited by more than 99% during incubation.

protective reagents, mercaptoethanol and dithiothreitol, on the inactivation and reactivation were also examined. At concentrations of 10^{-2} M, both were found to have similar effects on the two reactions as observed with pyridine. By estimations of thiamine in the mixtures after incubation (37 °C, 1 h), their effects were probably due to their action as secondary substrates. No residual thiamine was detected in either mixture, while $5.2 \cdot 10^{-6}$ M thiamine (87% of the amount added) remained in the control (where only thiamine had been added) after the incubation.

DISCUSSION

The findings in the present study indicate that the reversible inactivation of thiaminase I is initiated by the interaction between the enzyme and its primary substrate. From the kinetic study of bacterial thiaminase I, Lienhard[11] suggested that the enzyme reaction proceeded in two steps, that is, the formation of PM-enzyme with release of a nitrogenous base, followed by reaction of the PM-enzyme with an acceptor nucleophile. Consequently, there are two possibilities for the inactivation process; of which only one would involve the PM-enzyme. The effects of *p*-chloromercuribenzoate found here exclude neither of the two possibilities. Thiaminase I in *B. thiaminolyticus* was found to be an SH-enzyme[12]. The effect of *p*-chloromercuribenzoate on the inactivation suggests that an SH group at the active site is not directly involved in inactivation. If the inactivation process involved the PM-enzyme, it would follow that an SH group at the active site of the enzyme was involved in the reaction of the PM-enzyme with a secondary substrate. As shown in Table III, pyridine exhibits its effect on the inactivation independently of its nucleophilic attack. It seems likely that the interaction between a secondary substrate and the enzyme inhibits the conformational change of the enzyme initiated by the addition of a primary substrate alone. *p*-Chloromercuribenzoate inhibited completely the reactivation by pyridine. A possible explanation is that the nucleophilic attack by a

secondary substrate is involved in the reactivation, that is, thiaminase I changes into its less active form. This possibility is strengthened by the finding that there was residual activity in every inhibition experiment in the present study. However, it is difficult to determine Michaelis constants for thiamine and pyridine in the mixture of active and inactive thiaminase I, because a decrease of either of the two substrates may produce the change in activity during assay. In order to decide whether the enzyme is transformed into its inactive form or its less active one, it is necessary to separate the two forms of the enzyme.

Mercaptoethanol and dithiothreitol are likely to be secondary substrates; some thiol compounds have been found to be secondary substrates[11,13]. Hereafter, the use of these two compounds as SH-protective reagents should be avoided for any study on the inactivation of thiaminase I in *B. thiaminolyticus*.

The enzymic assay mixture used here prevents both inactivation and reactivation. Consequently, it is possible to estimate two forms of the enzyme separately in the mixed solution by assaying for activity before and after the activation of the inactive form. There are two possible procedures for activating inactive enzyme: one is the incubation of an enzyme solution after the removal of primary substrate by dialysis; the other is incubation with a secondary substrate at high concentration without the removal of primary substrate.

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