Thiaminokinase in Parent and Pyrithiamine Mutant *Staphylococcus aureus*

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1. As a result of the mutation of *Staphylococcus aureus* by pyrithiamine, deletion of the enzyme thiaminokinase in the system occurs. Some properties of thiaminokinase including the effects of pH, pyrophosphate donor nucleotides and metal ions on the enzyme in the parent *S. aureus* have been studied. Cell-free extract from mutant strain has been studied under similar conditions and thiaminokinase activity was found to be absent. Addition of thiamine (10 $\mu\text{g./ml.}$) to the medium containing pyrithiamine (required for the growth of the mutant strain) did not give rise to thiaminokinase activity in the mutant bacteria. 2. The parent and the mutant strains of *S. aureus* have been studied for the fermentative production of acetylmethylcarbinol (3-hydroxybutan-2-one) and the mutant strain did not produce acetylmethylcarbinol under the conditions used.

In previous publications (Das & Chatterjee, 1962*a,b*, 1963) it was reported that there were changes in the morphological and biochemical characters in a pyrithiamine mutant of *Staphylococcus aureus* (*S. aureus* A) when compared with the parent pyrithiamine-sensitive strain (*S. aureus*). Unlike *S. aureus*, which requires thiamine for its growth (Knight, 1935, 1937; Kögl & Van Wagtenodonk, 1938), *S. aureus* A grows in the absence of thiamine but requires pyrithiamine, a potent inhibitor of *S. aureus*.

Thiamine is required for metabolic processes in the living system in the form of thiamine pyrophosphate and the conversion of thiamine into thiamine pyrophosphate is catalysed by the enzyme thiaminokinase (ATP-thiamine pyrophosphotransferase, EC 2.7.6.2) in the presence of a suitable pyrophosphate donor and Mg^{2+} . Since *S. aureus* A does not require thiamine for its metabolism, thiaminokinase activity in the mutant was compared with that of the parent *S. aureus*. Also, because acetylmethylcarbinol (3-hydroxybutan-2-one) is formed from pyruvate in the presence of thiamine pyrophosphate by the bacteria, quantitative estimation of the compound was made in both the strains. The results of these investigations, including some studies on the nature of thiaminokinase in these strains, are presented.

MATERIALS AND METHODS

Organisms. A laboratory strain of *S. aureus* and a mutant of this organism were used. The method of preparation of the pyrithiamine mutant of *S. aureus* A was described by Das & Chatterjee (1962*a*).

Preparation of cell-free extract. Cells were grown in solid agar media containing glucose (3.0g.), Difco vitamin-free casein hydrolysate (4.0g.), cysteine (20mg.) and nicotinic acid (20mg.) in 11. of water with 2% agar. The medium was supplemented with thiamine (1 $\mu\text{g./ml.}$) for *S. aureus* and with pyrithiamine (1 $\mu\text{g./ml.}$) for *S. aureus* A. After 18 hr. incubation at 37°, the cells were harvested with 0.85% NaCl soln. (0°). The cells were disintegrated in 50mm-tris buffer, pH 7.0, with Ballotini beads (grade 12) in a Mickle disintegrator. Viscous supernatant was obtained by centrifugation of the disintegrated mass at 13000*g* for 30 min. at 0°. This cell-free extract was used as the enzyme source. The protein content was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951).

Assay of thiaminokinase. The incubation mixture contained 2.5 μmoles of thiamine, 2.5 μmoles of ATP, 3.5 μmoles of MgSO_4 , 35 μmoles of tris buffer, pH 7.0, and cell-free extract equivalent to 4mg. of protein in a final volume of 2.5 ml. The system was incubated for 60 min. at 37°. The reaction was stopped by heating for 2 min. in a boiling-water bath. Portions were taken and the formation of thiamine pyrophosphate was determined manometrically; the evolution of CO_2 from pyruvate in the presence of thiamine pyrophosphate was measured at different intervals by using alkali-washed yeast at 30° as described by Westenbrink (1955). The assay mixture contained 200 μmoles of potassium pyruvate, 300 μmoles of phosphate buffer, pH 6.2, 40 μmoles of MgCl_2 and 0.2 ml. of alkali-washed yeast (equivalent to 4mg. dry wt. of the organism) in a total volume of 3.2 ml. The evolution of CO_2 was measured at 10 min. intervals for 1 hr.

Identification of thiamine derivatives. The reaction products of thiamine derivatives were identified by paper chromatography by the method of Siliprandi & Siliprandi (1954). The solvent was propan-1-ol-water-0.5*M*-acetate buffer (pH 5.4) (7:2:1, by vol.). Thiamine derivatives were identified by comparison with authentic samples and the spots located with u.v. light.

Acetylmethylcarbinol production. This was estimated by the method of Westerfeld (1945). For this purpose the strains *S. aureus* and *S. aureus* A were grown in liquid media (pH 7.0) containing glucose (3.0g.), vitamin-free casein hydrolysate (4.0g.), K_2HPO_4 (5.0g.), nicotinic acid (20mg.) and cysteine (20mg.) in distilled water (11.). Erlenmeyer flasks (250 ml.) containing this medium (50 ml.) were inoculated with approximately the same number of viable cells (about 10^6) and incubated at 37° in a rotary shaker. The medium for *S. aureus* was supplemented with thiamine (1 μ g./ml.) and that for pyrithiamine mutant was supplemented either with pyrithiamine (1 μ g./ml.) or with thiamine (10 μ g./ml.) and pyrithiamine (1 μ g./ml.). Growth of the cells was measured turbidimetrically in a Klett-Summerson photoelectric colorimeter (filter no. 54). Neopyrithiamine (pyrithiamine) and oxythiamine were purchased from Calbiochem, Los Angeles, Calif., U.S.A. Thiamine monophosphate and thiamine pyrophosphate were gifts from Sigma Chemical Co., St Louis, Mo., U.S.A.

RESULTS

Estimation of thiaminokinase activity in *S. aureus* and *S. aureus* A. When the cell-free extracts of the parent and the mutant strains were used as the enzyme source for thiaminokinase-activity assays, no evolution of carbon dioxide occurred with extracts of the pyrithiamine mutant strain, indicating the absence of the enzyme thiaminokinase, whereas extracts of the parent strain showed thiaminokinase activity (Fig. 1). On increasing the amount of cell-free extract from the parent strain in the assay system, carbon dioxide evolution was increased, indicating increased thiamine pyrophosphate formation, but this did not occur with extract from the mutant strain (Fig. 2).

Effects of pyrithiamine and oxythiamine on the thiaminokinase activity in *S. aureus*. As pyrithiamine and oxythiamine both inhibit the growth of *S. aureus* and the former is a much more effective inhibitor, the effect of these thiamine analogues on thiaminokinase was investigated. Pyrithiamine had a very strong inhibitory action on staphylococcal thiaminokinase, whereas oxythiamine was much less effective (Table 1).

Effect of pH on thiaminokinase activity of *S. aureus*. The effect of pH on the activity of thiaminokinase in both *S. aureus* and *S. aureus* A was investigated. The thiaminokinase activity of *S. aureus* is quite sensitive to changes of pH, activity being strongly inhibited at acid pH values. No thiaminokinase activity was noted in extracts of *S. aureus* A in the pH range 5–8. The optimum pH for thiaminokinase activity was found to be 7.4–7.5. The enzyme is strongly inhibited at pH 5.0 (Fig. 3).

Effect of pyrophosphate donor nucleotides on the formation of thiamine pyrophosphate by staphylococcal thiaminokinase. The nucleotides GTP, ITP, CTP and ATP were tested to determine their

Fig. 1. Evolution of CO₂ by decarboxylation of potassium pyruvate by using alkali-washed yeast, in the presence of thiamine pyrophosphate formed by cell-free extract from *S. aureus* (○), and evolution of CO₂ with cell-free extracts of *S. aureus* A (●). Details of the incubation systems are given in the Materials and Methods section.

Fig. 2. Effect of increasing the amount of cell-free extract on the formation of thiamine pyrophosphate as indicated by CO₂ evolution in the thiaminokinase assay system (○, cell-free extract of *S. aureus*; ●, cell-free extract of *S. aureus* A). Details of the incubation system are given in the Materials and Methods section.

efficiency as pyrophosphate donors to thiamine by the action of staphylococcal thiaminokinase with cell-free extracts of *S. aureus* and *S. aureus* A. With extracts from the parent strain, ATP was the most effective, and CTP the least effective, as a pyrophosphate donor. Addition of ATP, GTP, ITP or CTP to the assay system did not give thiaminokinase activity in cell-free extracts of *S. aureus* A (Table 2). Nor did replacement of Mg^{2+} by Mn^{2+} in the assay system give thiaminokinase activity.

Acetylmethylcarbinol production by *S. aureus* and *S. aureus* A. Estimation of acetylmethylcarbinol in the liquid media of the parent and the mutant

Table 1. *Effect of thiamine analogues on staphylococcal thiaminokinase*

The reaction mixture contained 2.5 μ moles of ATP, 3.5 μ moles of $MgSO_4$, 35 μ moles of tris buffer, pH 7.0, and cell-free extract of *S. aureus* equivalent to 4 mg. of protein and was incubated at 37° for 60 min. Samples were taken and the formation of thiamine pyrophosphate was measured manometrically by the evolution of CO_2 from pyruvate by using alkali-washed yeast as described in the Materials and Methods section.

Addition	CO_2 evolution (μ l.)
Thiamine (2.5 μ moles)	40
Thiamine (2.5 μ moles) + pyrithiamine (1 μ mole)	20
Thiamine (2.5 μ moles) + pyrithiamine (2 μ moles)	5.2
Thiamine (2.5 μ moles) + oxythiamine (1 μ mole)	36
Thiamine (2.5 μ moles) + oxythiamine (5 μ moles)	20
Thiamine (2.5 μ moles) + oxythiamine (8 μ moles)	12

Fig. 3. Effect of variation of pH on the thiamine pyrophosphate formation by cell-free extract of *S. aureus* as the source of thiaminokinase as indicated by the evolution of CO_2 in the thiaminokinase assay system. Buffers used were: 10mm-acetate buffer in the pH range 5.0-5.6; 10mm-phosphate buffer (KH_2PO_4 - Na_2HPO_4) in the pH range 5.8-6.8; 10mm-tris-HCl buffer in the pH range 7.0-8.0. Other details of the incubation system are given in the Materials and Methods section.

strains was made after different periods of incubation at 37°. There was a steady accumulation of acetylmethylcarbinol in the medium with *S. aureus* but none with *S. aureus* A (Table 3). In another experiment, where thiamine (10 μ g./ml.) as well as pyrithiamine (1 μ g./ml.) was added to the medium with *S. aureus* A, no acetylmethylcarbinol formation was found.

Table 2. *Effect of different nucleotides on the formation of thiamine pyrophosphate by staphylococcal thiaminokinase*

Reactions were carried out as described in the Materials and Methods section except for the addition of ATP, GTP, CTP or ITP as indicated. Evolution of CO_2 was measured after 60 min. No enzyme activity was noted under the conditions described above with enzyme source from *S. aureus* A.

Addition	Evolution of CO_2 with the enzyme from <i>S. aureus</i> (μ l.)
Thiamine (2.5 μ moles)	0
Thiamine (2.5 μ moles) + ATP (2.5 μ moles)	40
Thiamine (2.5 μ moles) + GTP (2.5 μ moles)	36
Thiamine (2.5 μ moles) + CTP (2.5 μ moles)	30
Thiamine (2.5 μ moles) + ITP (2.5 μ moles)	34

Table 3. *Acetylmethylcarbinol production by S. aureus and S. aureus A*

As a sample of acetylmethylcarbinol was not available, the concentration of the same is expressed in terms of E_{530} in a Bausch and Lomb colorimeter. Turbidity of the bacterial growth was measured in a Klett-Summerson photoelectric colorimeter (filter no. 54), as described in the Materials and Methods section.

Incubation period (hr.)	Organism	Growth of the bacteria (Klett units)	Amount of acetyl- methylcarbinol/ 100 ml. of the broth (E_{530})
12	<i>S. aureus</i>	60	0
	<i>S. aureus</i> A	48	0
18	<i>S. aureus</i>	110	0.40
	<i>S. aureus</i> A	115	0.00
24	<i>S. aureus</i>	150	0.76
	<i>S. aureus</i> A	160	0.00
36	<i>S. aureus</i>	157	1.2
	<i>S. aureus</i> A	162	0.00
48	<i>S. aureus</i>	155	2.80
	<i>S. aureus</i> A	161	0.00

DISCUSSION

Thiaminokinase in cell-free extracts of staphylococci is strongly inhibited by pyrithiamine (Table 1) as was found with the thiaminokinase of baker's yeast (Kaziro, 1959) and chicken (Woolley, 1951). Inhibition of the thiaminokinase leads to a deficiency of thiamine pyrophosphate and, since this substance plays a key role in carbohydrate metabolism, *S. aureus* cannot grow in the presence of pyrithiamine.

The mutant organism cannot synthesize thiamine and its metabolic pattern is completely altered. The absence of thiaminokinase from *S. aureus* A

presumably means that thiamine is not present in the mutant bacteria and thus reactions requiring thiamine pyrophosphate cannot occur. Thiaminokinase activity was not observed with cell-free extracts of *S. aureus* A in the presence of various pyrophosphate donor nucleotides or metal ions, and changing the pH of the system had no effect.

The absence of acetylmethylcarbinol production by the mutated strain is further evidence that thiaminokinase is lost as a result of the mutation of *S. aureus* in the presence of pyrithiamine. Even when the mutated strain was grown in a medium containing thiamine as well as pyrithiamine, neither thiaminokinase activity nor acetylmethylcarbinol production could be demonstrated.

Therefore, as a result of the mutation of *S. aureus* in the presence of pyrithiamine, the detrimental effect of pyrithiamine is by-passed in the micro-organism with subsequent deletion of thiaminokinase, which is the primary site of action of this antimetabolite, and the metabolic characteristics of the bacteria are thereby altered. The exact role of this antimetabolite in the altered metabolism of the mutant strain cannot be definitely proposed, though it has been found (A. K. Sinha & G. C. Chatterjee, unpublished work) that pyrithiamine is

deaminated to oxypyrithiamine by the culture filtrate and no pyrithiamine pyrophosphate could be detected in the bacterial system.

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