METHYLMALONYL-CoA MUTASE IN A METHANOL-UTILIZING BACTERIUM, *PROTAMINOBACTER RUBER*

Kazuyoshi SATO, Shunsaku UEDA and Shoichi SHIMIZU
Department of Food Science and Technology, Nagoya University, Furo-Cho, Chikusa-Ku, Nagoya 464, Japan

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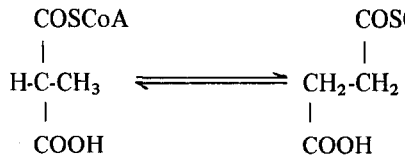
1. Introduction

Many of the methanol-utilizing bacteria produce vitamin B_{12} [1–3], which is presumed as playing an important role in the metabolism of the cells. However, little is known on the physiological roles of vitamin B_{12} in these microorganisms.

Recently we isolated a pink-pigmented methanol-utilizing bacterium, classified as *Protaminobacter ruber*. It was found to synthesize a considerable amount of vitamin B_{12}, mainly in a form of adenosyl-B_{12} and a little in a form of methyl-B_{12}.

Since we could demonstrate the existence of the B_{12}-dependent methionine synthetase in the bacterium [4], methyl-B_{12} would be considered to function as the active site of this transmethylase. However, in what reaction the adenosyl-B_{12} formed predominantly in the cells of *P. ruber* participates has not been elucidated yet. Here we present an evidence that adenosyl-B_{12}-dependent methylmalonyl-CoA mutase catalyzing the following reaction exists in the cells of *P. ruber*.



2. Materials and methods

Protaminobacter ruber isolated by us was grown aerobically at 30°C on a medium containing $NH_4H_2PO_4$ 4.0 g, KH_2PO_4 2.0 g, $Na_2HPO_4 \cdot 12\ H_2O$ 3.0 g, $MgSO_4 \cdot 7\ H_2O$ 0.2 g, $CaCl_2 \cdot 2\ H_2O$ 10 mg, $FeSO_4 \cdot 7\ H_2O$ 5 mg, $MnSO_4 \cdot n\ H_2O$ 5 mg, $CoSO_4 \cdot 7\ H_2O$ 1 mg, $ZnSO_4 \cdot 7\ H_2O$ 0.18 mg, $Na_2MoO_4 \cdot 2\ H_2O$ 0.23 mg, H_3BO_3 0.28 mg, $CuSO_4 \cdot 5\ H_2O$ 0.016 mg and 10 ml of methanol in 1 litre of distilled water. The pH was adjusted to 7.2 with 40% KOH. Growth was measured turbidimetrically at 660 nm with Akiyama D. S. 374 Fuji spectrophotometer (ADS value of 100 corresponds to about 1 of OD). The cells were harvested after about 30 h cultivation, in which ADS value reached nearly 300.

In order to prepare the cell-free extracts 1.7 g (wet weight) of *P. ruber* was ground with 5.0 g of alumina by using a mortar and pestle on ice. When cells were disrupted well, 10 ml of 0.05 M potassium phosphate buffer (pH 7.0) was added to it and grinding was continued for further several minutes. A clear supernatant was obtained by the centrifugation at 15 000 $\times g$ for 30 min and dialyzed overnight twice against 1 litre of 0.01 M potassium phosphate buffer (pH 7.0). This dialysate was used for the assay of enzyme activity.

Methylmalonyl-CoA mutase was assayed according to a slightly modified procedure of Stadtman et al. [5]. The incubation mixtures and the incubation conditions are described in the legend of table 1. After the incubation, the mixtures were heated at 100°C for 3 min and the residual methylmalonyl-CoA stable to this heat treatment in contrast to unstable succinyl-CoA was determined by the hydroxamic acid method [6]. The chief modification of the procedure was the centrifugation at 15 000 $\times g$ for 30 min before and after the reaction with hydroxamic acid in order to obtain a clear solution by removing the precipitates

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such as inactivated enzyme. The prompt measurement of optical density at 540 nm was very important, because the color would fade gradually.

Methylmalonyl-CoA was prepared from methylmalonic acid through its mixed anhydride with ethylchlorocarbonic acid [7]. All other chemicals were obtained from commercial source.

3. Results and discussion

The conversion of methylmalonyl-CoA to succinyl-CoA by cell-free extracts from *P. ruber* was confirmed as shown in table 1. The enzymatic reaction was dependent on adenosyl-B_{12}. A little activity without adenosyl-B_{12} was probably due to endogeneous one. Cyano-B_{12} could not replace with adenosyl-B_{12}, but rather inhibited the reaction. This fact strongly supports that the methylmalonyl-CoA mutase reaction is strictly dependent on adenosyl-B_{12}. The boiled enzyme did not catalyze the reaction at all, giving the same value as the enzyme was omitted from the reaction mixture. In this experiment, the incubation was carried out for one hour, a considerably long time, in order to put the reaction forward well enough for the clear demonstration of the mutase reaction. Since the methylmalonyl-CoA is a mixture of two diastereoisomers, the enzymatically active isomer will correspond to 375 nmol. Accordingly, the amount of the methylmalonyl-CoA disappeared in the complete system of table 1 seems to represent almost complete conversion into succinyl-CoA and a relatively higher activity than expected one would probably be caused by the isomerization of methylmalonyl-CoA into the active isomer during the incubation.

Table 1
Enzymatic activity of methylmalonyl-CoA mutase

Conditions	Methylmalonyl-CoA disappeared (nmoles)
Complete system	445
– Adenosyl-B_{12}	60
– Adenosyl-B_{12} + Cyano-B_{12}	24
– Enzyme + Boiled enzyme	0

The complete system contained maleate buffer (pH 6.5) 100 μmol, methylmalonyl-CoA 750 nmol, adenosyl-B_{12} (or cyano-B_{12}) 2 nmol, and crude extract (protein 4.75 mg) in a total volume of 0.85 ml. After 1 h incubation at 37°C in the dark, the samples were heated at 100°C for 3 min and the residual methylmalonyl-CoA was determined by the hydroxamic acid method.

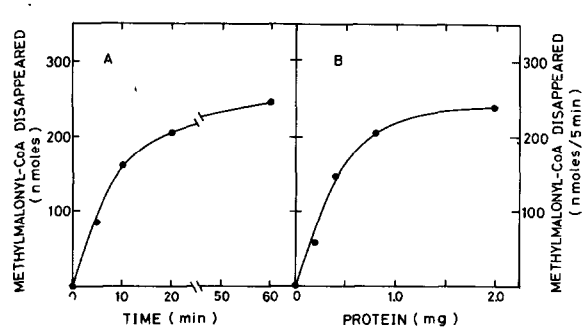

Fig.1. Dependence of methylmalonyl-CoA mutase reaction on the length of incubation period and the protein concentration. The reaction mixtures contained protein 0.25 mg (A) or at the level indicated (B), maleate buffer (pH 6.5) 100 μmol, methylmalonyl-CoA 750 nmol and adenosyl-B_{12} 2 nmol in a total volume of 0.8 ml. The reactions were run in the dark at 30°C for the time indicated (A) or for 5 min (B).

Figure 1 shows the effect of the incubation time and protein concentration on the enzymatic activity. With 0.25 mg of extract protein, the activity was nearly maximum after 20 min, although there was some increase in the activity through 1 h of incubation. The rate of disappearance of methylmalonyl-CoA was linear with the protein level nearly up to 0.4 mg per assay and reached a maximum in about 2 mg of protein used. The specific activity of the mutase from *P. ruber* is calculated as 75 nmol/min/mg of protein from the data of fig.1. This specific activity is comparable to that of *Propionibacterium shermanii* [8,9], which is known as one of the best producers of methylmalonyl-CoA mutase and the extracts of which has the specific activity much higher than in the extract from animal tissues [8,10]. Methylmalonyl-CoA mutase takes an important part in both the conversion of pyruvate to propionate in *Propionibacteria* and propionate to succinate in animal tissues [11,12]. This methylmalonyl-CoA mutase seems very important also in *P. ruber* judging from the occurrence of its very high specific activity.

The formation of succinyl-CoA as the product in this mutase reaction was confirmed by the paper chromatography of the labeled compound derived from [^{14}C]methylmalonyl-CoA.

As the methylmalonyl-CoA mutase was detected in the crude extract obtained from the cells grown on propanediol, fructose or succinate as a sole carbon and energy sources and their levels of the activity were almost similar to the one from methanol-grown cells, so this mutase is not considered as the enzyme inducible only when the bacterium was grown on methanol. But the physiological importance of this enzyme system would be also supposed from the fact that *P. ruber* does not grow on methanol medium under the strictly cobalt-deficient condition i.e. vitamin B_{12}-deficient condition, while the supplementation of succinate to the medium can restore the growth of the bacterium considerably [13]. These results including the identification of the product in this mutase reaction will be reported elsewhere.

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