Assay, Purification and Characterization of Cob(I)alamin Adenosyltransferase of *Protaminobacter ruber*

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Summary A simple spectrophotometric method for assay of ATP: cob(I)alamin Co- β -adenosyltransferase was established. By using this method, specific activity of the enzyme in *Protaminobacter ruber* was found to be the highest in the logarithmic growth phase. The enzyme was highly purified from the cells in that phase and characterized. The molecular weight of the enzyme was estimated as 44,000 by gel filtration. The optimal pH and temperature were about 8.0 and 50°C, respectively. Among divalent metal ions tested, only Mg^{2+} was effective for the enzyme activity. K_m values for ATP and hydroxocobalamin were 250 μM and 26.3 μM , respectively. Substrate analogs such as GTP and CTP had only small activity in the enzymatic reaction.

Key Words *Protaminobacter ruber*, adenosylcobalamin, ATP: B_{12S} adenosyl transferase, adenosylation, hydroxocobalamin

Cob(I)alamin (B_{12S}) adenosyltransferase [EC 2.5.2.17], which catalyzes the conversion of cobalamin compounds to adenosylcobalamin (adenosyl-B₁₂) according to Eq. 1, has been found in microorganisms (1-3) and mammals (1, 4).

The adenosylating enzyme has been partially purified from only a few sources and characterized (1, 2).

A facultative methylotroph, *Protaminobacter ruber*, also possesses the enzyme (5). The development of any simple method for assaying it will be useful, since reported methods including the use of labeled compounds are very complicated (1). Therefore, we first developed a convenient spectrophotometric assay method.

This paper describes this assay method and the partial purification and

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characterization of the adenosylating enzyme from *P. ruber*.

MATERIALS AND METHODS

Cultivation conditions. *P. ruber* NR-1 was grown at 30°C aerobically by the conventional method or two-step cultivation method as described previously (5, 6). In the latter method, cobaltous ion was used instead of the [⁵⁷Co] cyanocobalamin used in the former (5).

Purification of P. ruber adenosylating enzyme.

1) Cell-free extracts. The cell-free extracts of *P. ruber* were prepared as described previously (7) except that the volume of the buffer added per 1 g of the cells was 10 ml instead of *ca.* 5 ml. In the following purification steps, all operations were carried out at 0–5°C.

2) Ammonium sulfate fractionation. The cell-free extracts were fractionated with ammonium sulfate. Solid ammonium sulfate was added to the extracts to 40% saturation. The precipitate which contained almost all of the adenosylating enzyme activity was dissolved in 0.05 M potassium phosphate buffer.

3) DEAE-Sephacel column chromatography. The solution was dialyzed against 2 liters of 0.01 M potassium phosphate buffer (pH 7.5) for 3 h. The dialyzate was applied to a DEAE-Sephacel column (1.8 × 7 cm) which had been equilibrated with 0.01 M potassium phosphate buffer (pH 7.5). After washing the column with 72 ml of the same buffer, the adenosylating enzyme was eluted with 252 ml linear gradient of KCl (0–0.5 M) in 0.01 M potassium phosphate buffer (pH 7.5). Each 18 ml fraction was collected. Almost all of the adenosylating enzyme activity was obtained in fraction No. 10, which eluted in *ca.* 0.2 M KCl. The active fraction of this step was used for the investigation of enzyme properties unless otherwise stated.

4) Sephadex G-100 column chromatography. The concentrated protein solution (0.3 ml) from the preceding step was loaded on a Sephadex G-100 column (1.2 × 45 cm) equilibrated with 0.05 M potassium phosphate buffer containing 0.3 M KCl and 10% glycerol. Elution was done with the same buffer and 1.6 ml fractions were collected. Maximum activities of adenosylating enzyme were found in fractions Nos. 22 and 23.

Assays. The reaction mixture for assay of adenosylating enzyme contained hydroxocobalamin, 0.04 μmol; ATP (dissolved in 1 M Tris-HCl buffer, pH 7.5), 1.2 μmol; NADH, 0.2 μmol; DTT, 2.0 μmol; FMNH₂, 0.08 μmol; KCl, 40 μmol; MgCl₂, 40 μmol; Tris-HCl buffer (pH 7.5), 40 μmol; and an appropriate amount of the enzyme in a final volume of 0.7 ml. The reaction mixtures (0.65 ml), lacking the presence of FMNH₂ were put into test tubes (1.2 × 12 cm) with silicone stoppers and kept at 0°C in the dark. All the following operations were carried out in the dark. An atmosphere of argon was created by passing the gas into each tube *via* a syringe needle inserted through a tiny gap between the tube and the stopper. Then, FMNH (0.05 ml) freshly prepared by the method of Taylor and Weissbach (8) was injected into each tube *via* a syringe. Test tubes were stoppered tightly and incubated at 37°C

for 1 h or the indicated time.

After terminating the reaction with 0.1 ml of 1 N HCl, the denatured protein was removed by centrifugation at $15,000 \times g$ for 10 min at 4°C. The supernatant was loaded onto an Amberlite XAD-2 (48–100 mesh) column (0.6×3 cm). After washing the column with 3 ml of distilled water, cobalamin compounds were eluted with 25% ethanol and 2.9 ml of eluate was collected. After the addition of 0.1 ml of 1 N HCl to the eluate, the optical density of the solution was measured at 350 nm and the solution was then exposed to the light of a 200 W tungsten lamp (Toshiba photorelector lamp) for 5 min at a distance of 10 cm. In order to completely convert cobalamin compounds into hydroxocobalamin, the test tubes were vigorously shaken once or twice during the exposure to light. The optical density of the light-exposed solution was also measured at 350 nm. The amount of adenosylcobalamin was calculated according to Eq. 2.

$$\text{Adenosyl-B}_{12} (\mu\text{g}) = \frac{A_{350}(+h\nu) - A_{350}}{0.008} \times V \quad (2)$$

Where $A_{350}(+h\nu)$ = optical density at 350 nm of light-exposed eluate, A_{350} = optical density at 350 nm of eluate before exposure to light and V = volume (ml) of eluate from the Amberlite XAD-2 column. The difference of optical density at 250 nm by the analogous treatment of acidic adenosylcobalamin solution (1 $\mu\text{g}/\text{ml}$) was determined as 0.008 by separate experiments using authentic adenosylcobalamin. Optical density was measured with a Hitachi model 624 digital spectrophotometer. Protein was determined by the method of Lowry *et al.* (9).

Molecular weight determination. The concentrated adenosylating enzyme preparation from *P. ruber* was applied to a column (1.2×45 cm) of Sephadex G-100 equilibrated with 0.05 M potassium phosphate buffer (pH 7.5) containing 0.3 M KCl and 10% glycerol. Molecular weight standards used for gel filtration were bovine serum albumin (M.W. 68,000), human serum albumin (M.W. 45,000), chymotrypsinogen (M.W. 25,000) and cytochrome *c* (M.W. 12,500).

RESULTS

Growth of P. ruber and the specific activity of cob(I)alamin adenosyltransferase

P. ruber was grown by the conventional (Fig. 1 (A)) and two-step (Fig. 1 (B)) cultivation methods. The specific activity of adenosylating enzyme was higher in the early logarithmic phase of growth. The latter two-step cultivation method was more suitable for obtaining a large amount of enzyme having high specific activity. Therefore, *P. ruber* was cultivated by the two-step cultivation method.

Purification of adenosylating enzyme

Table 1 summarizes the results of purification of *P. ruber* adenosylating enzyme, which was purified more than 300-fold in nearly 20% yield. The increase of the specific activity in the step of ammonium sulfate fractionation is probably due to

Fig. 1. Time courses of growth and specific activities of adenosylating enzyme from *P. ruber* cultured in (A) conventional and (B) two-step cultivation methods.

Table 1. Purification of adenosylating enzyme from *P. ruber*.

Step	Volume (ml)	Total protein (mg)	Total activity (nmol/h)	Specific activity (nmol/mg/h)	Purification (fold)
Crude extract	59	696	3,410	4.9	1
Ammonium sulfate 0-40%	16	261	4,390	16.8	3.5
DEAE-Sephacel fraction 10	18	16	4,100	256	52
Sephadex G-100 fraction 22 ^a		0.23	340	1,480	300
Sephadex G-100 fraction 23 ^a		0.17	310	1,820	370

^a Calculated from the units recovered from an aliquot.

removal of inhibitor(s). Most of the adenosylating enzyme was recovered in the 0-40% ammonium sulfate fraction. Most impurity was eluted after the elution of adenosylating enzyme in the case of DEAE-Sephacel column and before the elution of the enzyme in the case of Sephadex G-100 column. Therefore, the enzyme was highly purified and a very nearly single major band was obtained by 7% polyacrylamide disc-gel electrophoresis.

Molecular weight

The apparent molecular weight of *P. ruber* adenosylating enzyme was estimated as ca. 44,000 by gel filtration on Sephadex G-100 (Fig. 2).

Optimal pH and temperature

Figure 3 shows the effect of pH on the adenosylating enzyme activity. The pH optimum was about 8. As shown in Fig. 4, the adenosylating enzyme was most active around 50°C, whereas the enzyme activity rapidly decreased at temperatures

Fig. 2. Estimation of the molecular weight of *P. ruber* adenosylating enzyme by gel filtration of Sephadex G-100.

Fig. 3. Effect of pH on the activity of *P. ruber* adenosylating enzyme. The enzyme activity was determined under the standard assay conditions except that the pH of the buffer was varied as indicated. The buffers used were sodium barbital-HCl buffer (—●—) and Tris-HCl buffer (- -○- -).

Fig. 4. Effect of temperature on the activity of *P. ruber* adenosylating enzyme. The enzyme activity was determined under the standard assay conditions except that the temperature was varied as indicated.

higher than 50°C because of inactivation by heat denaturation.

Effect of divalent metal ions

Various divalent cations were compared as regards the ability to stimulate the enzyme activity at a concentration of 57 μM . As summarized in Table 2, maximal activity was obtained in the presence of Mg^{2+} . No other divalent cations showed any positive effects.

Effect of substrate analogs

Several ATP analogs were tested for activity as substrate of the adenosylating enzyme; the results are shown in Table 3. ATP was a better substrate than any other nucleoside triphosphates. Both the amount of enzyme and the incubation time were augmented for the substrate analogs. All substrate analogs tested were active in the adenosylating enzyme reaction, although their activities were very low.

Kinetic properties of P. ruber adenosylating enzyme

The enzymatic reaction was linear with an incubation time of up to 2 to 2.5 h. The K_m values determined by Lineweaver-Burk plots were 250 μM and 26.3 μM for ATP and hydroxocobalamin, respectively.

DISCUSSION

Cob(I)alamin adenosyltransferase plays an important role in the supply of adenosylcobalamin which participates as coenzyme in various cobalamin-

Table 2. Effect of metal ions on *P. ruber* adenosylating enzyme activity.

Metal ion	Adenosyl-B ₁₂ formed (μ g)
None	8.6
Mg ²⁺	13.9
Mn ²⁺	5.8
Co ²⁺	4.8
Zn ²⁺	6.7
Ca ²⁺	2.4
Cd ²⁺	3.8

Table 3. Substrate specificity of *P. ruber* adenosylating enzyme for nucleoside triphosphate.

The enzyme activity was determined as described in MATERIALS AND METHODS except for the following modification. Each nucleoside triphosphate (1.2 μ mol) substituted for ATP was added as a substrate to the reaction mixture. The combined fractions (No. 8–12) in Sephacel column chromatography were used as the enzyme source. Incubation was carried out for 9 h except the case of the reaction with ATP, where incubation was carried out for 1 h and one-tenth of the enzyme solution was used.

Substrates	Cobalamin derivatives formed (μ g) ^a	Relative activity (%)
ATP	1,557	100
GTP	20	1.3
ITP	6	0.4
CTP	28	1.8
UTP	23	1.5

^a Each value was obtained by subtracting the control value (2 μ g) for the reaction in the mixture containing no substrate.

dependent enzymatic reactions. Although several assay methods for the adenosylating enzyme have been described, very few simple and reliable assay methods are available. A spectrophotometric assay method has been developed (2, 10), but it was necessary to use the purified enzyme, since the absorbance at 525 nm as used for the assay was not very strong and was affected by the components of the mixture. Our spectrophotometric assay method overcame those problems by using Amberlite XAD-2 in the assay and by measuring absorbance at 350 nm, where the main peak of hydroxocobalamin exists. Pure argon gas was used in this assay method to maintain an anaerobic atmosphere, but nitrogen or hydrogen gas may be used in a similar way for cobalamin-dependent methionine synthase assay (5, 8, 11). Since cobalamin compounds in the enzymatic assay could be quantitatively and partially purified with Amberlite XAD-2, the assay was reliable and reproducible. This method seems to be more convenient than that with labeled ATP (5) and was

successfully applied to the assay of *P. ruber* adenosylating enzyme.

The molecular weight of the adenosylating enzyme was about 44,000 as determined by the gel filtration method (Fig. 2) and is the smallest among the various cobalamin-dependent enzymes.

P. ruber adenosylating enzyme has several properties not shared by other microorganisms. The metal requirement of *P. ruber* enzyme was unique since its activity was stimulated only by Mg^{2+} , in contrast with stimulation by Mn^{2+} and partial stimulation by Co^{2+} , Mg^{2+} and Zn^{2+} for the adenosylating enzymes of *Clostridium tetanomorphum* (1,2) and *Propionibacterium shermanii* (1,12). The enzyme from cobalamin-requiring *Lactobacillus leichmannii*, whose substantial adenosylating activity is associated with ribosomes, is stimulated by Mn^{2+} and Mg^{2+} (3). Ca^{2+} , Cd^{2+} and Zn^{2+} have an inhibitory effect and Mn^{2+} is also inhibitory at a concentration of 10 mM. Adenosylating enzyme requires K^+ as monovalent cation (1,3), which is partially replaced by NH_4^+ and Na^+ . In this experiment, hydroxocobalamin was used instead of B_{12s} . K_m values for ATP and hydroxocobalamin were 250 μM and 26.3 μM , respectively.

It has been reported that nucleoside triphosphates besides ATP can act as substrate for the adenosylating enzyme from *C. tetanomorphum* (1,2), and *L. leichmannii* (3), while not in the case of *P. shermanii* (1,12). The enzyme from *P. ruber* had very little but significant activity with the nucleoside triphosphates tested other than ATP (Table 3). Considerable nonspecificity with respect to the nucleoside triphosphate in *C. tetanomorphum* enzyme might indicate the formation of various cobalamin derivatives in the cells (2), but this would probably not be applicable in the case of *P. ruber*.

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