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Enzymatic Synthesis of the Methyl Group of Methionine*

VI. The Catalytic Role of S-adenosylmethionine in the Enzyme System of *Escherichia coli*

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The enzymatic components involved in the transfer of the methyl group of 5-methyltetrahydrofolate to homocysteine to yield tetrahydrofolate and methionine have been studied. An enzyme containing a derivative of vitamin B_{12} as a prosthetic group catalyzes this reaction in the bacterial system isolated from cells of a mutant (113-3) of *Escherichia coli* grown on vitamin B_{12}. The cofactors, reduced flavin adenine dinucleotide and S-adenosylmethionine, are required for this reaction and may be generated from appropriate precursors by enzymatic systems, FAD reductase and S-adenosylmethionine synthetase, present in crude preparations of the B_{12}-enzyme. A method has been described for the further purification of B_{12}-enzyme and its separation from these other enzymes.

S-adenosylmethionine does not act as an intermediate of this reaction but participates in a catalytic manner. S-adenosylhomocysteine does not replace homocysteine as a methyl acceptor in this reaction.

The biosynthesis of the methyl group of methionine from 5-methyltetrahydrofolate (Reaction 1),

5-methyltetrahydrofolate + homocysteine → tetrahydrofolate + methionine (1)

by an enzyme system isolated from *E. coli* strain 113-3 grown on vitamin B_{12} has been shown in Paper IV[1] of this series to require reduced pyridine dinucleotide, ATP, Mg^{++}, FAD, and an enzyme fraction that contains a derivative of vitamin B_{12} as a prosthetic group (B_{12}-enzyme).

The purification of the B_{12}-enzyme and the separation of a protein fraction (D fraction), which contains at least two enzymatic components, have been reported in prelimary communications. One of the components was shown to be an FAD reductase[2]. Further experiments suggested that the D fraction also contained a cofactor that is bound to protein and an enzyme that forms the cofactor from ATP[3]. Mangum and Scrimgeour[4] have studied Reaction 1 with

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the pig liver enzyme and have shown that S-adenosylmethionine may replace ATP in this system.

We have now shown that the cofactor synthesized from ATP by fraction D of the bacterial system is probably identical with S-adenosylmethionine. This paper reports experiments concerned with the role of S-adenosylmethionine in methionine synthesis in the bacterial system and presents evidence that the two enzymatic components referred to collectively as D fraction are in fact an FAD reductase and S-adenosylmethionine synthetase. A procedure for separation of these two enzyme activities from B_{12}enzyme is presented.

EXPERIMENTAL

Materials

Coenzymes, ATP, and S-adenosylmethionine were obtained from California Corporation for Biochemical Research and Pabst Laboratories, Inc. L-methionine labeled in the methyl group with ^{14}C was obtained from New England Nuclear Corporation and S-adenolsylmethionine-methyl-^{14}C was obtained from Tracerlab, Inc. S-adenolsylhomocysteine was kindly provided by Dr. W. Sakami and partially purified pig heart reductase by Dr. Thanos Evangelopoulos.

D,L-5-methyltetrahydrofolate was synthesized by the procedure of Keresztesy and Donaldson[5]. Only one of the stereoisomers is utilized by the enzymatic system. $FADH_2$ was prepared chemically by catalytic hydrogenation with 10 % palladium on charcoal.

Enzyme assays

FAD reductase. The reaction mixture contained in 4 ml: enzyme, FAD, 200 mμmoles, DPNH, 1 μmole, and phosphate buffer, 200 μmoles. The incubation was carried out at 25°C. The decrease in absorbancy at 450 mμ under anaerobic conditions was determined at 30 sec intervals. A unit of FAD reductase activity is defined as that quantity of enzyme that carries out the reduction of 100 mμmoles of FAD per min under the conditions of experiments described above.

S-adenosylmethionine synthetase. Synthesis of S-adenosylmethionine was determined by measurement of the incorporation of ^{14}C into S-adenosylmethionine from methionine-methyl-^{14}C when this substrate was incubated with ATP, 20 μmoles, $MgSO_4$, 100 μmoles, and KCN, 20 μmoles, in a volume of 0.2 ml. The S-adenosylmethionine was isolated by paper chromatography[6]. The spots on the paper corresponding to S-adenosylmethionine were cut out and after elution with 0.1 % acetic acid the radioactivity of the eluate was measured with a Nuclear-Chicago Internal Gas Flow Counter. In control experiments 71 to 73 % of ^{14}C labeled S-adenosylmethionine was recovered by this procedure. The amount of S-adenosylmethionine formed was linear with respect to time and enzyme concentration. A unit of S-adenosylmethionine synthetase activity is defined as that quantity of enzyme that catalyzes at 37°C the synthesis of 1 μμmole of S-adenosylmethionine per h under the conditions described above.

B_{12}-enzyme. The amount of B_{12}-enzyme was estimated by measurement of the radioactivity in the protein fractions isolated from extracts of *E. coli* 113-3 grown on ^{60}Co-labeled vitamin B_{12}. The arbitrary units of B_{12}-enzyme activity are proportional to the radioactivity and are expressed in terms of cpm per ml of enzyme solution. This enzymatic activity was also determined by measurement of the amount of methionine synthesized after a three h incubation with an excess of the necessary components of the system. Methionine synthesis was measured with a microbiological assay as described previously[7]. Details of incubation conditions are given in the legend of Table 2.

RESULTS

Separation of the enzymatic components required for Reaction 1. Purification of the B_{12}-enzyme was carried out to the calcium phosphate gel eluate stage[7]. Separation of the FAD reductase and S-adenosylmethionine synthetase from the B_{12}-enzyme was obtained by chromatography on two successive hydroxylapatite

columns. The protein solution eluted (320 ml, 600 mg protein) from the gel was equilibrated with 0.01 M phosphate buffer at pH 7.5 and applied to the first hydroxylapatite column (4.4 × 13 cm). To elute the protein from the column the phosphate buffer concentration was increased from 0.01 M to 0.02 and 0.03 M in a stepwise manner. A partial separation of the three enzymatic activities was obtained with this column as shown in Fig. 1. Protein fraction I, which elutes with 0.01 M buffer, contains small quantities of FAD reductase activity and S-adenosylmethionine synthetase activity. The first protein fraction that elutes with 0.03 M phosphate buffer (fraction II) contains most of the S-adenosylmethionine synthetase and small amounts of B_{12}-enzyme. The B_{12}-enzyme (cobalt labeled) elutes shortly afterwards as a narrow radioactive band and is contaminated with a small quantity of S-adenosylmethionine synthetase. Peak IV is a yellow fraction, which contains the FAD reductase activity. Each fraction was assayed in two ways, first for its individual activity, which is shown in Fig. 1, and secondly as a component of the methionine synthesizing system as described by Reaction I. Two different procedures were used for this latter assay system. The first contained FAD and DPNH and required FAD reductase for the demonstration of methionine synthesis from 5-methyltetrahydrofolate and homocysteine. In the second assay system $FADH_2$ replaced FAD reductase, FAD and DPNH. Since the FAD reductase activity could be replaced by either pig heart reductase or chemically prepared $FADH_2$, further studies were not made on the reductase fractions. Inspection of Table I shows that the B_{12}-enzyme fraction has no reductase activity, but that it contains some S-adenosylmethionine synthetase. A maximal rate of conversion of substrate to methionine was obtained when aliquots of the fraction corresponding to peak II in Fig. 1 were added to the assay system containing ATP, Mg^{++}, $FADH_2$ and B_{12}-enzyme (Table 1).

Table 1. Requirement for enzyme fractions in Reaction 1. Each vessel contained in 1 ml: phosphate buffer, pH 7.4, 50 μmoles; homocysteine, 5 μmoles; ATP, 0.4 μmoles; $MgSO_4$, 0.8 μmoles; D, L-5-methyltetrahydrofolate, 0.228 μmoles in experiment 1 and 0.456 μmoles in experiment 2; and, where indicated: FAD reductase, 25 μg (peak IV); S-adenolsylmethionine synthetase, 20 μg (peak II); and B_{12}-enzyme, 13 μg. Assay A contained FAD, 0.08 μmoles; DPNH, 1.1 μmoles and was carried out in an atmosphere of N_2. Assay B contained $FADH_2$, 0.2 μmoles, and was performed in an atmosphere of H_2.

Experiment	B_{12}-enzyme*	S-adenosyl-methionine synthetase	FAD reductase	Methionine formed	
				Assay A	Assay B
				mμmoles	
1	+			10	43
		+		4	10
			+	7	9
	+	+		24	100
	+		+	18	56
	+	+	+	107	106
2	+				10
		+			10
	+	+			184

* B_{12}-enzyme in experiment 1 was the effluent of the first chromatography on hydroxylapatite; in experiment 2, the effluent of the second chromatography on hydroxylapatite.

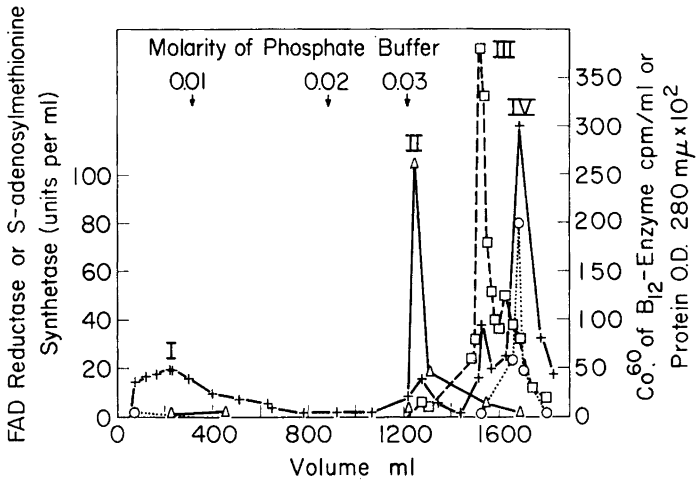

Fig. 1. First hydroxylapatite column (4.4 × 13 cm). 320 ml of protein solution were applied to the column and eluted successively with 600 ml of 0.01 M, 300 ml of 0.02 M, and 600 ml of 0.03 M phosphate buffer, pH 7.5. B_{12}-enzyme □, FAD reductase O, S-adenosylmethionine synthetase △, protein +.

In preliminary studies bacterial extracts were prepared by grinding the cells with alumina and by removal of nucleic acid with protamine sulfate according to Takeyama and Buchanan[8]. Under these conditions a single protein peak containing most of the S-adenosylmethionine synthetase and FAD reductase eluted from the first hydroxylapatite column with 0.01 M phosphate buffer. For this reason preliminary reports refer to D fraction rather than to the individual activities.

B_{12}-enzyme free of S-adenosylmethionine synthetase activity was obtained by rechromatography of fraction III (Fig. 1) on hydroxylapatite under conditions described in Fig. 2. It can be seen that the B_{12}-enzyme eluted from this column has a relatively constant ratio of radioactivity to absorbancy at 280 mμ. This material was free of S-adenosylmethionine synthetase activity.

Function of ATP in methionine biosynthesis. The demonstration that the D fraction contained two separate enzyme activities made it appear likely that one was concerned with ATP utilization. Attempts were made to find the products formed from ATP during methionine biosynthesis.

The crude B_{12}-enzyme (calcium phosphate gel eluate) contained sufficient S-adenosylmethionine synthetase and traces of methionine to catalyze the formation of limited amounts of S-adenosylmethionine now known to be required for the synthesis of methionine from 5-methyltetrahydrofolate and homocysteine.

The observation by Mangum and Scrimgeour[4] that S-adenosylmethionine acts catalytically in the pig liver enzymatic system, was confirmed in the *E. coli* system. The data in Table 2 show that S-adenosylmethionine can replace S-adenosylmethionine synthetase, ATP and Mg^{++}. An absolute requirement for methionine in the reaction was demonstrated by use of material eluted on the

Table 2. Requirement for S-adenosylmethionine in Reaction 1. All vessels contain in 1 ml: B_{12}-enzyme, 1.5 μg; homocysteine, 5 μmoles; D,L-5-methyltetrahydrofolate, 480 mμmoles; $FADH_2$, 0.4 μmoles; phosphate buffer, pH 7.4, 50 μmoles; and, where indicated: S-adenosylmethionine, 20 mμmoles; ATP, 100 mμmoles; Mg^{+2}, 1 μmole, methionine, 20 mμmoles. The vessels were incubated for 3 h under H_2.

Vessel	Additions	Methionine mμmoles
1	None	8
2	S-adenosylmethionine	231
3	ATP + Mg^{+2} + S-adenosylmethionine synthetase	8
4	ATP + Mg^{+2} + S-adenosylmethionine synthetase + methionine	169
5	ATP + Mg^{+2} + methionine	14
6	Methionine + S-adenosylmethionine synthetase	25
7	ATP + Mg^{+2} + S-adenosylmethionine synthetase + methionine	215

extreme right side of peak I of Fig. 1, an area of the chromatogram that contains some S-adenosylmethionine synthetase but is free of methionine.

Role of S-adenosylmethionine in the methyl transfer reaction. Although catalytic amounts of S-adenosylmethionine are required in the methyl transfer reaction, there is no significant conversion of S-adenosylmethionine to methionine either directly or by transfer of the methyl group to homocysteine. The inability of S-adenosylmethionine to serve as a precursor of methionine is best demonstrated in vessel 6 of Table 3 where the substrate, 5-methyltetrahydrofolate, is omitted from the incubation system. On chromatography of incubation mixtures that contained both 5-methyltetrahydrofolate and labeled S-adenosylmethionine (methyl ^{14}C) the primary source of methionine methyl was 5-methyltetrahydrofolate regardless of the ratio of the concentrations of the two substances. These experiments serve to demonstrate that S-adenosylmethionine is not an intermediate of the reactions of methionine biosynthesis.

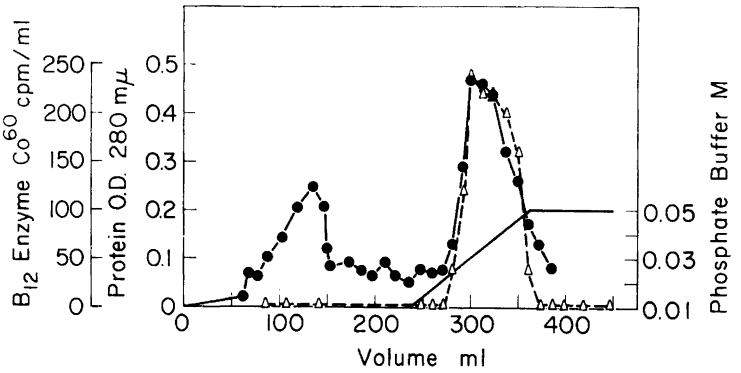

Fig. 2. Second hydroxylapatite column (1.0 × 10 cm). 106 ml of B_{12}-enzyme solution from peak III of Fig. 1, equilibrated with 0.01 M phosphate buffer, pH 7.5, were applied to the column and elution was carried out with 140 ml of 0.01 M phosphate buffer and then more concentrated phosphate buffer was applied as a linear concentration gradient varying from 0.01 M to 0.05 M (70 ml of each). B_{12}-enzyme △, protein •.

Table 3. Non participation of S-adenosylhomocysteine in methionine biosynthesis. All vessels contain in 1 ml: B_{12}-enzyme, 4.6 μg; phosphate buffer, pH 7.4, 50 μmoles; 2-mercaptoethanol, 50 μmoles; and $FADH_2$, 0.2 μmoles. The vessels were incubated for 3 h under H_2.

Vessels	S-adenosyl methionine	Homocysteine	D, L-5-methyl tetrahydrofolate	S-adenosyl homocysteine	Methionine synthesis
	μmoles	μmoles	μmoles	μmoles	mμmoles
1	0.02	5.0	0.73	–	287
2	0.02	5.0	0.73	2.0	264
3	0.02	–	0.73	2.0	0
4	–	5.0	0.73	2.0	2
5	–	5.0	0.73	0.02	6
6	0.50	5.0	–	–	5

Role of S-adenosylhomocysteine. In view of the interest in S-adenosylhomocysteine as a possible acceptor of methyl groups, we have carried out experiments to determine whether it participates in the reactions of our enzymatic system. As shown in Table 3, S-adenosylhomocysteine could not replace homocysteine as an acceptor of methyl groups from 5-methyltetrahydrofolate nor could it substitute for S-adenosylmethionine.

DISCUSSION

The data presented in the foregoing sections have demonstrated that the last step in methionine synthesis (Reaction I) in the bacterial system involves the participation of S-adenosylmethionine and a reducing agent such as $FADH_2$. One of these two essential components of the reaction may be generated from DPNH and FAD in the presence of a flavin reductase and the other from ATP and methionine in the presence of Mg^{++} and S-adenosylmethionine synthetase. Both of these enzymes are present in a protein fraction (D fraction) and may be separated from B_{12}-enzyme by chromatography on hydroxylapatite. We have been able to confirm in the bacterial system the earlier observation of Mangum and Scrimgeour[4] that S-adenosylmethionine is an essential component of the pig liver enzyme system.

In both instances S-adenosylmethionine participates in catalytic quantities. This compound is not an intermediate of the reaction or reactions concerned with the transfer of the methyl group of 5-methyltetrahydrofolate to homocysteine. Furthermore, when S-adenosylmethionine is added to the reaction system in substrate quantities it is not significantly converted to methionine, at least at a rate commensurate with the overall reaction. These experiments thus leave in question the role that S-adenosylmethionine is playing in the reaction. Since this essential agent is required only in catalytic quantities it is possible that it may be functioning by activation of B_{12}-enzyme in some as yet unknown manner. Activation by transfer of a methyl or adenosyl group to the B_{12} prosthetic group of the enzyme are two possibilities currently under investigation in this and other laboratories[9–11].

As a corollary to these experiments with S-adenosylmethionine, we have also found that S-adenosylhomocysteine does not function as a reactant of Reaction 1. Neither does it replace homocysteine as a methyl acceptor nor does it substitute

for S-adenosylmethionine as an "activator" of the enzymatic system. These data thus do not support the scheme of reactions proposed for methionine synthesis by Jaenicke[12] and his collaborators.

Recently a number of papers have appeared describing transfer reactions in microbial systems, involving S-adenosylhomocysteine and S-adenosylmethionine[13–15]. Evidence has been presented for the occurrence of the following reactions:

$$\text{S-adenosylmethionine} + \text{homocysteine} \xrightarrow{\text{methyl transfer}} \text{methionine} + \text{S-adenosylhomocysteine} \qquad (2)$$

$$\text{S-adenosylmethionine} + \text{homocysteine} \xrightarrow{\text{adenosyl transfer}} \text{methionine} + \text{S-adenosylhomocysteine} \qquad (3)$$

Reactions 2 and 3 appear in the same form but the metabolic origin of methionine carbon is different in the two processes. The suggestion has been made that S-adenosylhomocysteine (or a metabolic product) formed in the above reactions acts as a methyl acceptor for a C_1 unit, presumably the methyl group of 5-methyltetrahydrofolate (Reaction 4).

$$\text{S-adenosylhomocysteine} \xrightarrow{C_1 \text{ unit}} \text{S-adenosylmethionine} \qquad (4)$$

Although evidence for the existence of Reactions 2 and 3 is well documented, the connection of these reactions with methyl synthesis *de novo,* has not been established (Reaction 4). In view of the evidence to the contrary there seems to be little ground for the postulation of the participation of S-adenosylmethionine and S-adenosylhomocysteine as intermediates of methionine synthesis *de novo.*

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