METHIONINE SYNTHESIS: DEMONSTRATION OF THE REVERSIBILITY OF THE REACTION

H.RÜDIGER and L.JAENICKE
Institut für Biochemie der Universität, Köln, Germany

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1. Introduction

The synthesis of methionine by methionine synthetase ($CH_3.FH_4$ – homocysteine – methyltransferase *) from *E. coli* proceeds as follows:

$$CH_3.FH_4 + \text{homocysteine} \rightarrow FH_4 + \text{methionine}.$$

The reaction seems to run unidirectionally to the right and it can be used to determine $CH_3.FH_4$ [1]. Stavrianopoulos and Jaenicke [2] have, however, presented evidence that the reaction can in certain conditions be forced backwards, at least partially. They enzymically transferred the *S*-methyl group from SAM to FH_4, using a highly purified enzyme which was activated by SAM. This result was indirectly confirmed by Taylor and Weissbach [3,4] who detected an exchange of methyl groups between methylated enzyme and $CH_3.FH_4$ contaminated with FH_4. They also established that the exchange reaction was accelerated by additional FH_4. Since a preparation of $CH_3.FH_4$ uncontaminated by FH_4 was not available to these authors, they could not demonstrate the expected lack of exchange with $CH_3.FH_4$ alone.

To study the back reaction it is necessary to determine either the homocysteine or the $CH_3.FH_4$ formed, because the decreases in methionine or FH_4 during the reaction are too small to be measured. Homocysteine is very susceptible to oxidation by air and is not easily determined. $CH_3.FH_4$ may, however, be assayed very specifically by enzymic demethylation to FH_4 [1] but, to avoid high blank values, it is indispensible to free the $CH_3.FH_4$ of FH_4 prior to the determination of the former. Although several methods exist for separating folates of different oxidation states or numbers of glutamate residues or one-carbon residues [e.g. 5–10], no efficient method has been described for separating $CH_3.FH_4$ and FH_4. In the course of our investigations of methionine synthesis, we have developed methods of purifying the substrates of this reaction. In this paper we describe the separation of $CH_3.FH_4$ from FH_4 and the application of this method to demonstrate the back reaction of methionine synthesis.

* Abbreviations used: FH_4, 5,6,7,8-tetrahydrofolic acid; $CH_3.FH_4$, 5-methyl-5,6,7,8-tetrahydrofolic acid; SAM, *S*-adenosylmethionine; DTT, dithiothreitol.

2. Materials and methods

L-Methionine was purchased from Schuchardt, München, D-methionine (less than 0.04% L-methionine) from EGA-Chemie, Steinheim. Homocysteine-thiolactone hydrochloride (Fluka AG, Buchs) was converted to free homocysteine according to ref. [2]. Dithiothreitol was a product of Calbiochem, Los Angeles; FMN was from E.Merck, Darmstadt; TEAE-cellulose from Serva Entwicklungslabor, Heidelberg. SAM was prepared according to [11]; FH_4 according to [12]. Aquocobalamin was a generous gift from Dr. L.Mervyn, Glaxo Laboratories, Greenford.

2.1. *Estimation of $CH_3.FH_4$*

The incubation mixture (250 to 450 μl) contained, in addition to the sample to be analysed (e.g. the eluate from the TEAE-cellulose column), 12.5 μmoles Na-phosphate pH 7.2, 2.5 μmoles homocyste-

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ine 0.21 mg methionine synthetase from *E. coli*, specific activity 4.4 μmoles h^{-1} mg^{-1} [11], 0.25 μmoles SAM, 2.5 nmoles aquocobalamin, 25 nmoles FMN, and 2.5 μmoles DTT. The mixture was incubated at 31° in the dark under nitrogen for 2 h. FH_4, which had been formed from $CH_3.FH_4$, was determined by formylation according to [13]. Blanks were kept at 0° during incubations.

2.2. *Back reaction*

The incubation mixture (9.4 ml) contained 202 μmoles L-methionine, 159 μmoles FH_4, 9 mg methionine synthetase, specific activity 19 μmoles h^{-1} mg^{-1} [11], 10 μmoles SAM, 1 μmole FMN, 0.1 μmole aquocobalamin, and 50 μmoles DTT. Prior to incubation (2 h at 31° under nitrogen in the dark), the solutions were brought to pH 7.2. In control experiments, either enzyme or L-methionine were omitted or D-methionine was substituted for L-methionine. After incubation the mixtures were adsorbed to a TEAE-cellulose column and eluted with 0.15 M-NH_4HCO_3, 0.05 M mercaptoethanol. Fractions containing $CH_3.FH_4$, or having its retention volume, were pooled and rechromatographed on the same column to achieve quantitative separation from FH_4. After the second run, $CH_3.FH_4$ was identified by its retention volume and assayed by enzymic demethylation.

3. Results and discussion

The complete separation of $CH_3.FH_4$ from FH_4 on a column of TEAE-cellulose is shown in fig. 1. TEAE-cellulose is reported to be similar to DEAE-cellulose in respect of its degree of substitution at the amino groups [14]. Nevertheless, the separation properties of both ion exchangers are quite different since almost no separation of $CH_3.FH_4$ from FH_4 can be achieved on DEAE-cellulose [9].

In table 1 are shown the results of an experiment in which the back reaction, starting from FH_4 and methionine, was measured together with three controls run simultaneously. The reverse reaction proceeds in the same conditions as are optimal for methionine synthesis. In the control experiments the yields of $CH_3.FH_4$ were one order of magnitude lower than with the complete system; thus it may be concluded that the back reaction is dependent on the presence of both the enzyme and L-methionine. When enzyme was omitted there was no peak at the retention volume of $CH_3.FH_4$, the value given in parentheses represents a slightly but unspecifically raised background. In the control without L-methionine there was a very small peak, possibly some $CH_3.FH_4$ had been formed from SAM either via its hydrolysis to methionine or by direct transfer of its methyl group. Stavrianopoulos and Jaenicke [2] have indicated that such a transfer can indeed occur enzymically. These authors, however, used an enzyme which was dependent on premethylation by SAM. In the

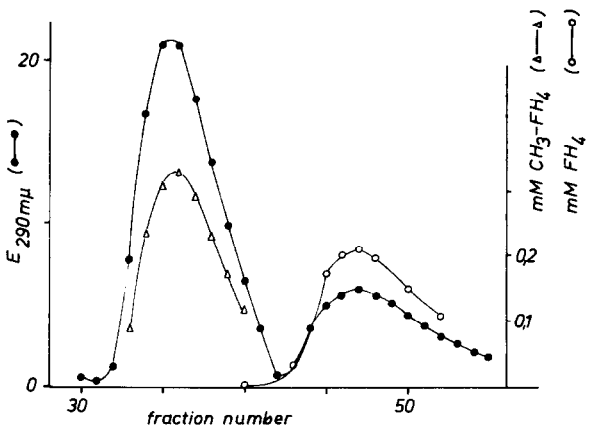


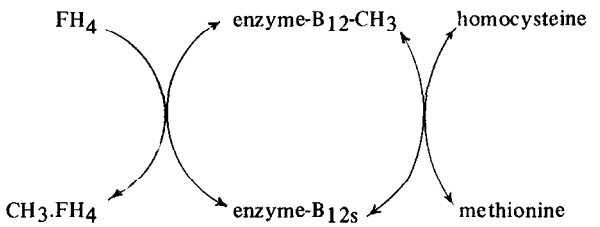
Fig. 1. Chromatographic separation of $CH_3.FH_4$ and FH_4. Separation was performed on a TEAE-cellulose column (1 × 33 cm) with 0.15 M NH_4HCO_3, 0.05 M mercaptoethanol. Fractions of 4 ml/30 min were collected, FH_4 was determined by chemical formylation (13), $CH_3.FH_4$ by enzymatic demethylation (11), followed by formylation.

Table 1
Enzymatic formation of $CH_3.FH_4$ from methionine and FH_4. For details see experimental section.

	nmoles $CH_3.FH_4$ formed
Full system	474
Enzyme omitted	(26) *
L-methionine omitted	15
D-methionine substituted for L-methionine	49

* No $CH_3.FH_4$ peak could be observed after column chromatography.

present work the enzyme used was active in the absence of SAM, presumably because the method used isolated the methylated form of the enzyme which therefore needed to premethylation to catalyse the following cycle and does not exchange methyl groups with SAM [11]:



In the last control experiment, where D-methionine was substituted for L-methionine, there was a slight synthesis of $CH_3.FH_4$, although clearly L-methionine is the preferred substrate.

The enzyme activity used in this experiment would have been enough to convert 700 times the amount of $CH_3.FH_4$ formed back to FH_4 during the incubation. Thus it may be assumed that thermodynamic equilibrium had been established between the reactants. From the data given it is possible to calculate an equilibrium constant of 7×10^{-6} and a reaction-free enthalpy of +7.1 kcal $mole^{-1}$ for the formation of $CH_3.FH_4$ and homocysteine.

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