KINETIC EVIDENCE FOR AN ENZYME-BOUND INTERMEDIATE IN THE BIOSYNTHESIS OF METHIONINE

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1. Introduction

Methionine formation follows the equation

homocysteine + 5-CH_3-FH_4 → methionine + FH_4 *

(see refs. [1, 2]). In *Escherichia coli* [2], as well as in animals [3], this reaction is catalyzed by the cobalamin enzyme methionine synthetase which in addition requires SAM [4] as an activator. Stavrianopoulos and Jaenicke [5] and Weissbach and Taylor [6] isolated and identified labelled methylcobalamin from an incubation mixture containing methionine synthetase, 5-$^{14}CH_3$-FH_4 and SAM, but no homocysteine. From these experiments it seems probable that in the course of the above reaction enzyme-bond methylcobalamin is formed as an intermediate. Yet we did not regard these results unequivocal since the methylcobalamin might have been formed by the 'unnatural' reaction conditions, i.e. the absence of homocysteine in the reaction mixture.

According to W. W. Cleland [7], two-substrate reaction mechanisms with an ordered sequence of steps ('ping-pong' mechanisms) can be differentiated from 'random' processes by kinetic studies, in which the concentration of only one of the substrates is changed, keeping all other parameters constant. From such measurements evidence is presented to support our assumption.

Abbreviations: FH_4, 5,6,7,8-tetrahydrofolic acid; 5-CH_3-FH_4, 5-methyl-5,6,7,8-tetrahydrofolic acid; SAM, S-adenosylmethionine.

2. Materials and Methods

The enzyme was purified by ammonium sulfate fractionation of an *E. coli* extract as described in [5] and stabilized by storage in 50% glycerol at pH 8 and −20°. Even at 0° the enzyme looses activity rapidly. Protein was assayed according to Folin-Ciocalteu [8]. DL-homocysteine and DL-5-CH_3-FH_4 were prepared according to [5]. 48.6% of the DL-5-CH_3-FH_4 was converted to FH_4, if the enzymatic reaction was run to completion. DL-5-CH_3-FH_4 was measured by its O.D. (ϵ_M^{290} 25 000). Initial DL-homocysteine concentrations were determined according to [9].

SAM-chloride was a product of B.D.H. Ltd., Poole; its concentration was assayed spectrophotometrically (ϵ_M^{260} 15 400 [10]). FAD and NADH were purchased from Sigma, St. Louis, and Boehringer, Mannheim, respectively. The concentration of FH_4 produced during the reaction was assayed according to ref. [11] modified to a micromethod (Eppendorf-Photometer, Netheler and Hinz, Hamburg; ϵ_M^{365} 22 600). Preceding experiments had confirmed the formation of equimolar amounts of both FH_4 and methionine (determined microbiologically).

3. Results and Discussion

A typical experiment is shown in fig. 1. It demonstrates that the kinetics of the reaction support the assumption of a 'ping-pong' mechanism with a methylcobalamin-containing enzyme intermediate: on incubation of methionine synthetase with its substrates under standard conditions, but varying con-

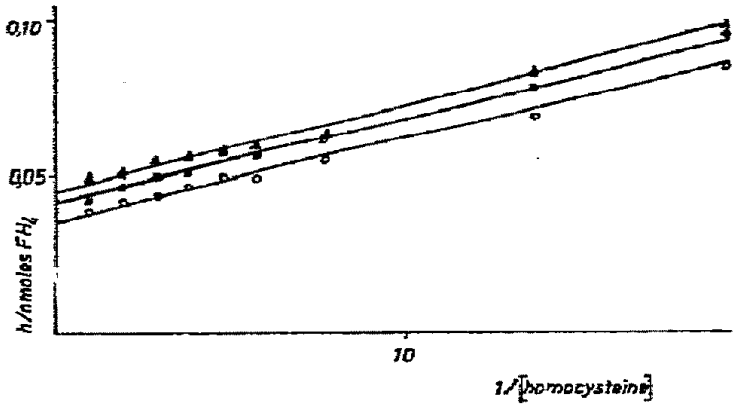
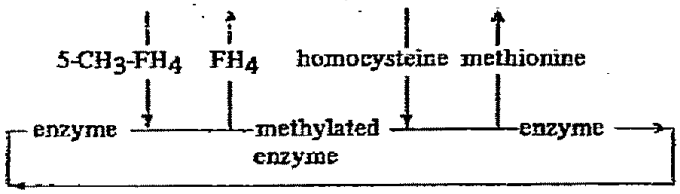


Fig. 1. Dependence of reciprocal reaction velocities on reciprocal substrate concentrations. Each incubation mixture contained 5-CH_3-FH_4 and homocysteine as indicated, 72 μg enzyme, 25 nmoles SAM, 25 nmoles FAD, 0.7 μmoles NADH and 1.0 mg NADH-FAD-oxidoreductase from *E. coli* [5] in 0.25 ml 0.1 M sodium phosphate buffer pH 7.2 and was incubated for 30 min at 31° in an atmosphere of nitrogen in the dark. Each value represents the average of triplicate analyses. Their deviations do not exceed 6%. ○ 1.0 mM, ● 0.412 mM, ▲ 0.200 mM 5-CH_3-FH_4.

centrations of either 5-CH_3-FH_4 or homocysteine, a series of parallel straight lines is obtained in the double reciprocal plot. Due to the lability of even the stabilized enzyme, only those values are plotted which were obtained in the same experimental run.

At 5-CH_3-FH_4 concentrations higher than 2 mM, substrate inhibition occurs. Whether this effect is due to the substrate itself or to an unknown contaminant of 5-CH_3-FH_4 was not investigated. Deviations from linearity at low substrate concentrations are caused by the rapid decomposition of the labile substrates prior to incubation. These experiments give additional evidence for the following reaction sequence (scheme 1), characterized by an oscillation of the enzyme between a methylated and a demethylated form.



Scheme 1.

The steps preceding the first reaction, i.e. the role of SAM in the labilization and transfer of the methyl group of 5-CH_3-FH_4 to the enzyme-bound cofactor, still remain to be investigated. Possibly the latter step is aided by the reduction of cobalamin which renders the central atom more nucleophilic.

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