BBA 61130

Feedback inhibition of methylene–tetrahydrofolate reductase in rat liver by S-adenosylmethionine

A feedback-type regulation of the enzyme methylene–tetrahydrofolate reductase (5-methyltetrahydrofolate:NAD oxidoreductase, EC 1.1.1.68) (Eqn. 1) by methionine or *S*-adenosylmethionine (SAM) has been proposed[1–4] to account for the well-known effects of methionine on folic acid-mediated metabolism.

$$5,10\text{-}CH_2\text{-}H_4\text{folate} + NADH + H^+ \rightarrow 5\text{-}CH_3\text{-}H_4\text{folate} + NAD^+ \quad (1)$$

Previous experiments[5] failed to show an influence of dietary methionine on the level of this enzyme in rat liver homogenates. We therefore tested the effect of methionine, choline, betaine, creatinine and SAM on the activity of this enzyme *in vitro* since the tissue levels of these compounds should depend on methionine intake. Of these, only SAM showed an inhibitory effect at physiological concentration.

The standard assay was performed in the back direction of Eqn. 1 with 5-$^{14}CH_3$-H_4PteGlu as substrate and menadione as artificial electron acceptor[6]. The 5,10-CH_2-H_4PteGlu formed dissociates easily to yield labeled formaldehyde which can be isolated as the dimedone adduct[7,8]. The incubation mixture (vol. 0.6 ml) contained: 320 mμmoles of (±)-5-$^{14}CH_3$-H_4PteGlu (110 disint./min per mμmole), 5 mμmoles FAD, 2 μmoles menadione (not completely dissolved), 5 μmoles ascorbate, 100 μmoles phosphate buffer (pH 6.3), 1 μmole ethylenediaminetetraacetic acid and

TABLE I

INHIBITION OF METHYLENE–TETRAHYDROFOLATE REDUCTASE BY SAM IN THE STANDARD ASSAY

Addition	*Conditions*	*Specific activity (mμmoles $min^{-1} \cdot mg^{-1}$)*	*% Inhibition*
—	undialyzed, 30°	0.13	0
0.16 mM SAM			64
0.8 mM SAM			93
—	undialyzed, 35°	0.18	0
0.8 mM SAM			75
—	dialyzed 12 h at 0°, 30°	0.23	0
0.16 mM SAM			25
0.8 mM SAM			40
0.8 mM SAH	undialyzed, 30°		0
0.16 mM SAM 0.16 mM SAH			40
0.16 mM SAM 0.8 mM SAH			22
0.8 mM SAM 0.16 mM SAH			83
0.8 mM SAM 0.8 mM SAH			68

Abbreviations: SAM, *S*-adenosylmethionine; PteGlu, pteroylglutamic acid; SAH, *S*-adenosylhomocysteine.

an amount of enzyme that produced maximally 30 mμmoles formaldehyde. The incubation period was 1 h at 30°; 0.3 ml of a solution of 3 mg dimedone per ml 1 M acetate buffer (pH 4.5) was then added, the mixture heated for 5 min at 95° and cooled in ice. The formaldehyde–dimedone adduct was extracted into 3 ml of toluene by vigorous shaking with a Vortex mixer for 15 sec. The phases were then separated by centrifugation and 1 ml of the toluene phase counted in a Packard scintillation counter with internal standardization. The blank was the value obtained when the enzyme was added after the incubation. Liver homogenates were prepared from fresh rat liver with 3 vol. of 0.05 M phosphate buffer, pH 7.2, in a Potter–Elvehjem homogenizer and then centrifuged at 30 000 × *g* for 30 min. SAM iodine was purchased from Calbiochem.

The extent of inhibition by SAM (Table I) was very much dependent on the incubation temperature and the treatment of the liver homogenate. Under standard incubation conditions 0.16 mM SAM and 0.8 mM SAM caused 64% and 93% inhibition. At 35° the activity of the enzyme was 1.4-fold higher than at 30°, but inhibition by 0.8 mM SAM, dropped to 75%. Dialysis (and "aging" at 0°) increased the activity similarly and led to an even higher loss in sensitivity to the inhibitor. Furthermore, all attempts to purify the enzyme caused desensitization against inhibition. Similar effects have been observed on other regulatory enzymes[9]. The enzyme from pig liver was inhibited by SAM in the same concentration range and also desensitized upon storage at 0°. The rat liver enzyme may also be inhibited by *S*-adenosylethionine in view of the inhibition produced by a combination of ethionine and ATP, each compound alone having little or no effect. Of several compounds tested, only *S*-adenosylhomocysteine (SAH) (a gift from Professor Dr. L. JAENICKE, Köln) reversed

TABLE II

INHIBITION OF METHYLENE–TETRAHYDROFOLATE REDUCTASE BY SAM, ASSAYED IN REDUCING DIRECTION

Incubations contained: 1 μmole (±)-5,10-CH_2-H_4PteGlu, 0.1 μmole FAD, 7.5 μmoles NADH, 100 μmoles phosphate buffer, pH 6.8, 10 mg ascorbic acid, SAM, and enzyme in a total of 1 ml. Incubation was for 2 h at 30° under helium.

SAM (mM)	*5-CH_3-H_4PteGlu formed (mμmoles)*	*% Inhibition*
—	116	0
0.05	85	27
0.1	74	46
0.5	0	100
1.0	0	100

the inhibition by SAM but did not activate the enzyme when added alone (Table I).

The inhibition was also demonstrated in the physiological forward direction of the enzyme reaction (Table II). 5-Methyl-H_4PteGlu formed from 5,10-CH_2-H_4PteGlu and NADH was estimated as the difference of *Lactobacillus casei* and *Streptococcus faecalis* activities before and after the reaction[6].

A kinetic study was done in the direction of 5-methyl-H_4PteGlu oxidation at 4 concentrations of either substrate and inhibitor. $1/v$ *vs.* $1/[S]$ (Lineweaver–Burk) plots were linear and indicated a mixed competitive and non-competitive type of inhibition. To compare the effect of the inhibitor under different conditions, the inhibitor concentration causing 50% inhibition (I_{50}) was estimated from the data for a substrate concentration of $2 \cdot 10^{-4}$ M which is twice the K_m of 5-CH_3-H_4PteGlu. Values are: $6 \cdot 10^{-5}$–$8 \cdot 10^{-5}$ M for the enzyme in a fresh extract, $3 \cdot 10^{-4}$ and $8 \cdot 10^{-4}$ M for two preparations of desensitized enzyme, and $3 \cdot 10^{-4}$ M in the presence of 0.8 mM SAH. In the latter two cases inhibition apparently did not approach 100% at high inhibitor concentrations. Thus, the difference between fresh and dialyzed enzyme preparations is much bigger than could be accounted for by removal of endogenous tissue SAM. The effects of SAM and SAH on the enzyme activity may be explained most simply by assuming competitive binding of these compounds to an allosteric regulatory site on the enzyme different from the catalytic, substrate-binding site. This is strongly supported by the fact that the enzyme can be desensitized against inhibition[9].

Additional support for this concept is given by the data in Table III on the protective effects of substrate and inhibitor against heat inactivation of the enzyme.

TABLE III

EFFECTS OF SUBSTRATE AND INHIBITOR ON INACTIVATION AND DESENSITIZATION OF METHYLENE-TETRAHYDROFOLATE REDUCTASE BY HEATING

The samples were incubated for 3 min at 50°. After cooling the added compounds were removed by adsorption on Norite. Activity and inhibition were determined in the standard assay system.

Addition during heating	*% Original activity*	*% Inhibition by 0.8 mM SAM*
None	32	29
2 mM SAM	100	88
2 mM SAH	51	60
0.5 mM CH_3-H_4PteGlu	94	27

SAM and SAH protected both the catalytic and the inhibitory site, but the substrate protected only the former. Preincubation of partly desensitized enzyme with 1 mM SAM also restored sensitivity to SAM.

We propose that the inhibition of methylene–tetrahydrofolate reductase activity by SAM functions as a feedback-type metabolic regulation *in vivo* and might explain in part the effects of methionine on folic acid metabolism. A connection between methionine intake and the level of SAM in the tissues has recently been demonstrated by BALDESSARINI[10] who found that intraperitoneal injection of 130 μmoles DL-methionine increased rat liver SAM from an average normal range of 65 mμmoles to 290 mμmoles per g wet weight. Such SAM concentrations cause appreciable inhibition of the enzyme in the *in vitro* assay. Studies on the correlation of

methionine content in the diet to SAM levels in tissues over a longer period are, however, still missing.

This work was supported by U.S. Public Health Service Grant No. AM 08171-03.

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Received January 2nd, 1967

Biochim. Biophys. Acta, 139 (1967) 217–220