ELSEVIER

Methods of Nutritional Biochemistry

An assay for betaine-homocysteine methyltransferase activity based on the microbiological detection of methionine

Jacob D. Mulligan,* Timothy J. Laurie,* and Timothy A. Garrow*†

**Department of Food Science and Human Nutrition, and †Division of Nutritional Sciences, University of Illinois, Urbana, IL USA*

Betaine-homocysteine methyltransferase (BHMT: EC 2.1.1.5) catalyzes a methyl transfer from betaine to homocysteine (Hcy) forming dimethylglycine and methionine (Met), respectively. We have developed an assay for BHMT activity based on the microbiological detection of one of its products, Met. The microbiological assay uses Escherichia coli *J5-3, a Met auxotroph. When cultured in minimal media, J5-3 growth increases linearly with Met supplementation up to 50 μM (2.24 μg/mL). Although not sensitive enough for kinetic analyses, this assay is adequate for measuring activity levels in crude or purified enzyme preparations using saturating substrate concentrations. When BHMT activity was measured in crude liver extracts, using both the radioactive and microbiological assays, the values obtained show a high degree of correlation ($r^2 > 0.93$) over an eight-fold range of activities. We have used the microbiological assay to identify stachydrine as a naturally occurring methyl donor substrate for BHMT. The sensitivity of detecting BHMT activity by the microbiological assay for Met can be increased at least an order of magnitude by using dimethylacetothetin, a sulfonium analog of betaine, as the methyl donor substrate.* (J. Nutr. Biochem. 9:351–354, 1998) © *Elsevier Science Inc. 1998*

Keywords: betaine-homocysteine methyltransferase; betaine; methionine; homocysteine; dimethylacetothetin; dimethylpropiothetin; stachydrine

Introduction

Betaine-homocysteine methyltransferase (BHMT: EC 2.1.1.5) is required for the oxidation of choline, a process that is largely restricted to the liver and kidney of mammals.[1,2] BHMT is an abundant protein in liver,[3-5] and recent nutrition studies indicate that BHMT gene expression is dramatically influenced by the level of methionine (Met) and choline or betaine in the diet.[6,7] Because BHMT is one of only three metabolic enzymes that utilize homocysteine (Hcy), and elevated levels of blood homocyst(e)ine increase the risk for the premature development of vascular disease,[8] there is renewed interest in the enzyme's potential role in modulating plasma homocyst(e)ine in normal and pathophysiologic states.

The most commonly employed assay for BHMT activity uses [^{14}C-*methyl*]-betaine. Following incubation of the reaction mixture for 1 to 2 hours, labeled betaine is separated from labeled dimethylglycine and Met by chromatography on Dowex 1, and the conversion is measured by scintillation counting. However, [^{14}C-*methyl*]-betaine is not commercially available and most laboratories produce this substrate by converting [^{14}C-*methyl*]-choline to [^{14}C-*methyl*]-betaine using a bacterial choline oxidase system. This report describes a new procedure to assay BHMT activity that does

Address correspondence and reprint requests to Timothy A. Garrow, 463 Bevier Hall, 905 South Goodwin Avenue, University of Illinois, Urbana, IL 61801 USA.
Received January 8, 1998; accepted March 24, 1998.
This research was supported by grants from the University of Illinois' Agricultural Experiment Station (60-0305), the American Heart Association (Illinois Affiliate, GB-10 gar), and the National Institute of Diabetes and Digestive and Kidney Diseases (52501-01) to T.A. Garrow.
A preliminary report of this work was presented at Federation of American Societies for Experimental Biology, April 1996, Washington, D.C. [Laurie, T.J. and Garrow, T.A. (1996). A microbiological assay for betaine-homocysteine methltransferase activity using microtiter plates. *FASEB J.* **10(3),** A472].

655 Avenue of the Americas, New York, NY 10010
0955-2863/98/$19.00
PII S0955-2863(98)00025-4

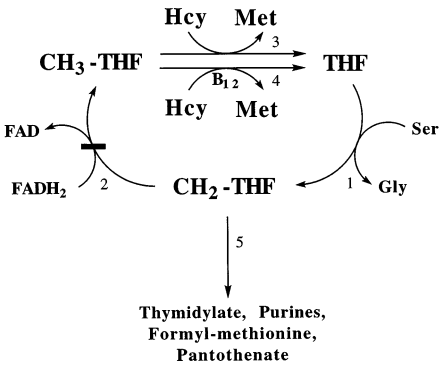

Figure 1 Folate-dependent methionine biosynthesis in *Escherichia coli*. THF, tetrahydrofolate; CH_2-THF, 5,10-methylene THF; CH_3-THF, 5-methyl THF; Hcy, homocysteine; Met, methionine; Ser, serine; Gly, glycine; FAD, flavin adenine dinucleotide (oxidized form); $FADH_2$, flavin adenine dinucleotide (reduced form); B_{12}, cobalamin. Reactions: 1, serine hydroxymethyltransferase; 2, 5,10-methylene THF reductase; 3, methionine synthase (cobalamin-independent); 4, methionine synthase (cobalamin-dependent); 5, miscellaneous reactions that utilize one carbon units from the folate pool. The black bar represents the metabolic block in *Escherichia coli* J5-3.

not require the use of radiolabeled betaine or the separation of products from substrates by ion exchange chromatography. The method is based on the microbiological determination of Met. The assay for Met uses *Escherichia coli* J5-3, which lacks 5,10-methylene tetrahydrofolate (THF) reductase (EC 1.1.1.68) activity, resulting in an absolute Met auxotrophy. *Figure 1* depicts the metabolic relationships exploited to develop this assay.

Methods and materials

Materials

Ninety-six well microtiter plates (Falcon #3072) were purchased from Fisher Scientific (Pittsburgh, PA USA). *E. coli* strain J5-3, which lacks 5,10-methylene THF reductase activity, was obtained from the *E. coli* Genetic Stock Center (Yale University: CGSC #6850; New Haven, CT USA). Betaine hydrochloride, L-Met, and D,L-Hcy thiolactone hydrochloride were purchased from Sigma Chemical Company (St. Louis, MO USA). Dimethylacetothetin was synthesized as previously described.[5] Dimethylpropiothetin and stachydrine were purchased from TCI America (Portland, OR, USA) and Indofine Chemical Company, Inc. (Somerville, NJ, USA), respectively. The chemicals used for the L-amino acid-defined diets were purchased from Dyets (Bethlehem, PA, USA). All other reagents were of the highest purity commercially available.

Diets and animal protocol

The amino acid-defined diets used in this study were similar to those previously described.[7] The diets varied only in the levels of Met (1, 2, or 3 g/kg diet) and choline bitartrate (0, 0.625, 1.25, 2.5, and 6.25 g/kg diet) or betaine (3.8 g/kg). Diets also contained 1% succinylsulfathiozole to prevent the microbial metabolism of choline in the gastrointestinal tract.[9] Due to antimicrobial use, 50 mg menadione sodium bisulfite was added per kilogram diet.

Three-week-old Sprague-Dawley rats (Harlan, Indianapolis, IN USA) were housed as previously described[7] and were initially fed a control amino acid-defined diet for 3 days, which contained adequate Met (3 g/kg) and choline bitartrate (2.5 g/kg) and was devoid of betaine. Following the adaptation period, rats were divided into 17 groups such that mean body weights among groups ($n = 5$) were not significantly different. Sixteen dietary treatments were used and the effect these treatments had on hepatic BHMT activity and gene expression will be reported elsewhere. The liver extracts prepared from these rats were used to validate the procedure of measuring BHMT activity by the microbiological determination of Met as described in this report.

This study was approved by the University of Illinois' Laboratory Animal Care Advisory Committee.

Measurement of BHMT activity

BHMT activity was measured in crude liver extracts, or purified fractions of pig liver BHMT,[5] using the standard radioassay as previously described,[5] or by our new assay using the microbiological determination of Met as follows.

E. coli J5-3 (*pro-22, metF63*) were grown in Vogel-Bonner (VB) minimal media (50 mL) containing 0.2% glucose, 50 μg/mL L-proline, 50 μg/mL Met, and 0.001% thiamin. When the cells reached an OD_{600} of approximately 1, the culture was centrifuged at 5000 g for 10 minutes to pellet the cells. The Met containing supernatant was discarded and the cells were resuspended in 50 mL of VB. The cells were again centrifuged, the supernatant discarded, and the cells resuspended in 50 mL of VB. A final wash was performed as above except the cells were resuspended in 5 mL VB containing 20% (v/v) glycerol. The cells were then stored frozen at −80°C until used for the microbiological determination of Met.

The BHMT assay was performed as previously described for the radioassay[5] except 2-mercaptoethanol and tracer [^{14}*C-methyl*]-betaine were not added. Following incubation at 37°C, the reaction tubes were placed in ice water and 500 μL of ice-cold water was added. Marbles were placed on top of the tubes, which were then transferred to a boiling water bath for 5 minutes. Each reaction mixture was then transferred into a polypropylene microcentrifuge tube and centrifuged at 12,000 g for 15 minutes. Supernatants were assayed for Met as described below.

Two hundred microliters of minimal media containing 5×10^6 colony forming units of *E. coli* J5-3 was added to each microtiter well. The culture media contained VB salts, 0.2% glucose, 50 μg/mL L-proline, and 0.001% thiamin (w/v). BHMT assay supernatants (10–100 μL) and water were added so that the final volume of each microtiter well was 300 μL. A reaction mixture containing crude extract but lacking betaine was used as a blank for each test sample. A standard curve was prepared with the same reaction mixture except enzyme was omitted and known amounts of L-Met were added. A well lacking Met was used as a blank for the standard curve. All samples were assayed in duplicate. Once the additions were completed, the plates (with lids) were placed in a 37°C incubator for 30 hours. Following incubation, the cells were gently resuspended and the optical density (450 nm) of each well was measured using an ELx800 automated microtiter plate reader (Bio-Tek Instruments, Winooski, VT, USA). The absorbance of the appropriate blank was subtracted and the amount of Met formed by each BHMT-containing extract could then be calculated from the standard curve. A unit of BHMT activity is defined as a nmole of Met formed per hour.

Statistics

The comparison of the microbiological and radio assays for BHMT activity were compared by a simple regression analysis. The slopes and intercepts of the standard curves for Met as measured by the microbiological assay were analyzed by an analysis of variance.

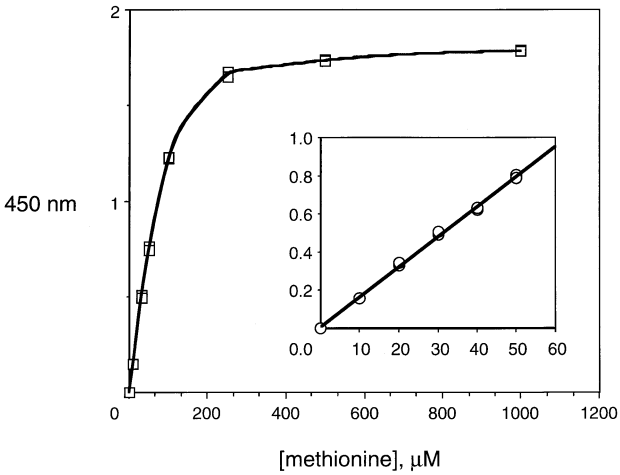

Figure 2 Influence of methionine concentration on growth of *Escherichia coli* strain J5-3. Details are described in Materials and Methods. Two cultures were grown at each methionine concentration and the final optical density of each culture is plotted. The inset shows a regression ($r^2 = 0.99$) of bacterial turbidity versus methionine concentrations up to 50 μM (2.24 μg/mL).

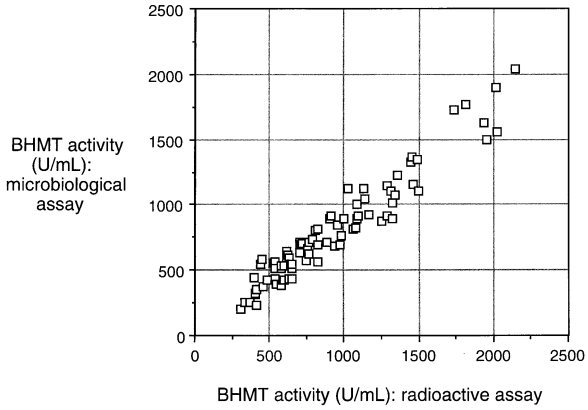

Figure 3 Correlation of assays to quantitate betaine-homocysteine methyltransferase (BHMT) activity. Extracts were prepared by homogenizing rat liver in 5 volumes of potassium phosphate buffer (30 mM, pH 7.6) containing 2 mM EDTA and 5 mM 2-mercaptoethanol. Following centrifugation, supernatants were assayed by the standard radioactive assay (abscissa) or by the microbiological detection of methionine (ordinate). The average protein (× ± SD) concentration in the crude liver extracts were 14.7 ± 1.8 mg/mL. A unit of activity is defined as 1 nmol Met formed per hour.

Results

Figure 2 shows a typical growth response curve of J5-3 in minimal media with varying concentrations of Met. The inset shows that the optical density (450 nm) of the cultures versus initial Met concentration is linear from 0 to 50 μM (2.24 μg/mL); we used this region as the standard curve to quantitate the amount of Met produced in a BHMT catalyzed reaction. Regression analysis of the standard curve showed that the correlation of optical density versus Met concentration was excellent ($r^2 = 0.99$). The slopes and intercepts of the standard curves from 5 different days did not statistically differ when analyzed by an analysis of variance. Hence, the standard curve is very reproducible. Furthermore, the addition of any BHMT reaction components, namely, bovine serum albumin, Hcy, betaine, Tris, potassium phosphate, 2-mercaptoethanol, crude extract, and dimethylglycine, did not affect the slope or intercept of the standard curve when tested at the maximum concentrations that could be present in a microtiter well. For example, assuming the maximum level of BHMT assay supernatant assayed would be 100 μL, substrates were tested at concentrations assuming no BHMT activity was present in the test sample, whereas dimethylglycine was tested at a level based on the assumption that the maximum amount of betaine that can be converted to dimethylglycine while maintaining the near linearity of the BHMT assay is 10% of the initial betaine content.

Figure 3 shows a regression of hepatic BHMT activity (U/mL) as measured by both the radioactive and microbiological assays. Duplicate assays varied less than 5% for both methods and thus were averaged for clarity. The liver samples were from rats fed varying levels of Met, choline, and betaine using combinations predicted to result in significant variations in hepatic BHMT activity. Up to eightfold changes in BHMT activity were observed.

Discussion

This report describes an assay for BHMT activity based on the microbiological detection of one of its products, Met. The assay uses saturating levels of both substrates. The microbiological assay requires approximately the same amount of hands-on time to perform as the standard radioactive assay, but due to the required sterile technique, more technical expertise is required. The method described here used microtiter plates but this procedure works equally well with culture tubes by scaling up all volumes 10-fold. However, the use of microtiter plates facilitates assaying a large number of samples simultaneously.

The metabolic relationships that make this assay possible can be seen in *Figure 1*. When grown in minimal media, *E. coli* obtains all of the one carbon units to support folate-dependent reactions,[10] including the biosynthesis of Met from Hcy, from serine (60%) and glycine (40%) as follows. The 3-carbon of serine can be transferred to THF, forming 5,10-methylene THF and glycine by a reaction catalyzed by serine hydroxymethyltransferase (EC 2.1.2.1, reaction 1). The 2-carbon of glycine can ultimately be transferred to THF forming 5,10-methylene THF, by the reactions catalyzed by the glycine cleavage system (not shown). 5,10-Methylene THF lies at a branch point in folate metabolism, it can be used directly for thymidylate biosynthesis or oxidized to 10-formyl THF and used in purine, formylmethionine, or pantothenate biosynthesis. All of these possibilities are depicted in *Figure 1* by reaction 5. Alternatively, 5,10-methylene THF can be reduced to 5-methyl THF by 5,10-methylene THF reductase. The only reactions that utilize 5-methyl THF in *E. coli* are the cobalamin-independent and cobalamin-dependent Met synthases (EC 2.1.1.14 and EC 2.1.1.13, reactions 3 and 4, respectively). Because *E. coli* strain J5-3 lacks 5,10-methylene THF reductase activity (reaction 2), the resulting absence of 5-methyl THF precludes the methylation of Hcy, thus the

bacterium has an absolute requirement for Met. Further, *E. coli* does not transport folates[11] and therefore cannot utilize any folate present in crude extracts.

Figure 2 shows that the turbidity of *E. coli* J5-3 cultures is linear when Met concentrations are varied up to 50 μM (2.24 μg/mL). This region of linearity was used to quantitate the portion of the Met produced by BHMT catalyzed reactions. *Figure 3* shows that the microbiological method of measuring BHMT activity in crude liver extracts agrees well with those values obtained from the standard radioactive assay. It should be noted that BHMT reaction mixtures contain D,L-Hcy and therefore some D-Met is produced. However, we have previously reported that BHMT displays a strong preference for the L-isomer.[5] Further, *E. coli* utilizes D-Met equally effectively as L-Met for growth (not shown), and therefore the predictably small amount of D-Met produced is inconsequential. As can be seen in *Figure 3*, the amount of Met produced as measured by either assay is significantly correlated ($r^2 = 0.93$).

One advantage of the microbiological system for determining BHMT activity is that compounds can be tested for methyl donor activity without having to produce the compounds in radiolabeled form. However, the compounds or their demethylated product cannot be toxic to *E. coli*, or catabolized to Met by a Hcy-independent pathway. We have used the microbiological assay to compare the relative substrate effectiveness of other known methyl donors for BHMT using purified pig liver enzyme. Compared with betaine (2 mM; relative value of 1), dimethylacetothetin (5 mM) and dimethylpropiothetin (10 mM) were 37- and 8-fold more effective as BHMT substrates than betaine, respectively. These data are consistent with previous reports.[5,12,13] In other studies using the purified pig liver enzyme we tested whether 5-methyl THF (2 mM) or stachydrine (20 mM) could function as methyl donors. The latter compound, like dimethylpropiothetin and folate, occurs naturally in some plants. The results indicated that pig liver BHMT cannot catalyze a methyl transfer from 5-methyl THF, although the betaine analog stachydrine, also known as N,N-dimethylproline or proline betaine, could serve as methyl donor (data not shown). However, stachydrine was only 20% as effective as betaine.

References

1 Sunden, L.F.S., Renduchintala, M.S., Park, E.I., Miklasz, S.D., and Garrow, T.A. (1997). Betaine-homocysteine methyltransferase expression in porcine and human tissues and chromosomal localization of the human gene. *Arch. Biochem. Biophys.* **345,** 171–174

2 Finkelstein, J.D., Kyle, W.E., and Harris, B.J. (1971). Methionine metabolism in mammals. Regulation of homocysteine methyltransferases in rat tissue. *Arch. Biochem. Biophys.* **146,** 84–92

3 Skiba, W.E., Taylor, M.P., Wells, M.S., Mangum, J.H., and Awad, W.M. (1982). Human hepatic methionine biosynthesis. *J. Biol. Chem.* **257,** 14944–14948

4 Lee, K-H., Cava, M., Amiri, P., Ottoboni, T., and Lindquist, R.N. (1992). Betaine-homocysteine methyltransferase from rat liver: Purification and inhibition by a boronic acid substrate analog. *Arch. Biochem. Biophys.* **292,** 77–86

5 Garrow, T.A. (1996). Purification, kinetic properties, and cDNA cloning of mammalian betaine-homocysteine methyltransferase. *J. Biol. Chem.* **271,** 22831–22838

6 Emmert, J.L., Garrow, T.A., and Baker, D.H. (1996). Hepatic betaine-homocysteine methyltransferase activity in the chicken is influenced by dietary intake of sulfur amino acids, choline, and betaine. *J. Nutr.* **126,** 2050–2058

7 Park, E.I., Renduchintala, M.S., and Garrow, T.A. (1997). Diet-induced changes in hepatic betaine-homocysteine methyltransferase activity are mediated by changes in steady-state level of its mRNA. *J. Nutr. Biochem.* **8,** 541–545

8 Motulsky, A.G. (1996). Nutritional ecogenetics: Homocysteine-related arteriosclerotic vascular disease, neural tube defects, and folic acid. *Am. J. Hum. Gen.* **58,** 17–20

9 Zeisel, S.H., daCosta, K.A., Youssef, M., and Hensey, S. (1989). Conversion of dietary choline to trimethylamine and dimethylamine in rats: Dose-response relationship. *J. Nutr.* **119,** 800–804

10 Dev, I.K., and Harvey, R.J. (1982). Sources of one-carbon units in the folate pathway of *Escherichia coli. J. Biol. Chem.* **257,** 1980–1986

11 Green, J.M., Nichols, B.P., and Matthews, R.G. (1996). Folate biosynthesis, reduction, and polyglutamylation. In *Escherichia coli and Salmonella* (F.C. Neidhardt, ed.), pp. 665–673, ASM Press, Washington, D.C, USA

12 Dubnoff, J.W. and Borsook, H. (1948). Dimethylthetin and dimethylpropiothetin in methionine synthesis. *J. Biol. Chem.* **176,** 789–796

13 Maw, G.A. (1958). Thetin-homocysteine transmethylase. *Biochem. J.* **70,** 168–173