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Purification and Kinetic Properties of Betaine-Homocysteine Methyltransferase from *Aphanothece halophytica*

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Abstract. Betaine-homocysteine methyl transferase (BHMT) from *Aphanothece halophytica* was purified to homogeneity by hydroxyapatite, DEAE-Sepharose CL-6B and Sephadex G-200 column chromatography. A 24-fold purification and 11% overall yield were achieved with a specific activity of 595 nmol h^{-1} mg^{-1}. The subunit molecular weight was determined to be 45 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the native enzyme was found to have a molecular weight of 350 kDa, suggesting an octameric structure of the enzyme. The enzyme shows optimum activity at 37°C, pH 7.5. The apparent Km values for glycinebetaine and L-homocysteine were 4.3 mM and 1.3 mM, respectively. The enzyme was 70% inactivated by 5 mM dimethylglycine whereas the same concentration of sarcosine slightly inactivated the enzyme. Two analogs of glycinebetaine were also tested for enzyme inactivation and it was found that 5 mM choline inactivated 60% of the enzyme activity and 2.5 mM betaine aldehyde completely abolished the enzyme activity. NaCl at 200 mM or higher also completely inactivated the enzyme.

Aphanothece halophytica is an obligate halophilic unicellular cyanobacterium capable of growth in media containing NaCl concentrations ranging from 0.25 M to 3.0 M [25]. Cells of *A. halophytica* accumulate an organic osmoregulatory solute, glycinebetaine, as a means to alleviate damaging effects of cell dehydration brought on by osmotic stress [13, 20]. The osmoprotective effects of glycinebetaine have been well described for plants and bacteria. High concentration of glycinebetaine is not inhibitory to many enzymes and partial protection by glycinebetaine against NaCl or KCl inhibition has been reported for higher plant and cyanobacterial enzymes [12, 16]. The synthesis of glycinebetaine has been extensively studied in many bacteria and plants and is shown to involve a two-step oxidation of choline via betaine aldehyde [21]. Recently a three-step series of methylation reactions from glycine to glycinebetaine has been reported for two extreme halophiles [18]. In contrast, the degradation of glycinebetaine has rarely been studied in plants and bacteria. Glycinebetaine could be utilized as a growth substrate in *Rhizobium meliloti* by the action of betaine-homocysteine methyltransferase (BHMT, EC2.1.1.5) [6]. BHMT is an enzyme catalyzing a methyl transfer from glycinebetaine to homocysteine (Hcy) forming dimethylglycine and methionine, respectively. The enzyme has been mainly studied in mammals such as horse, pig, rat and human and is shown to be a hexamer of 45 kDa subunits [2, 4, 15, 22, 24]. The mammalian enzyme appears to be modulated by diet especially protein and methionine content [5, 19].

Previously we reported that BHMT activity of *A. halophytica* was increased upon salt downshock and starvation [10]. In the present work we further purified the enzyme to homogeneity and reported its kinetic properties.

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Materials and Methods

Organisms and growth conditions. *Aphanothece halophytica,* originally isolated from Solar Lake in Israel, was obtained in axenic culture from T. Takabe (Nagoya University). The organism was grown photoautotrophically in BG11 medium supplied with 18 mM $NaNO_3$ as previously described [12]. Cells were grown in 250-ml flasks containing 100 ml medium on a rotary shaker at 30°C without CO_2 supplementation. Continuous illumination was provided by cool-white fluorescent lamps at an irradiance of 60 μmol photon m^{-2} s^{-1}. To induce the formation of BHMT in the cells, the growth medium was added with 2 M NaCl and the cells were allowed to grow for 7 days. Cells

Table 1. Purification of BHMT from *A. halophytica*

Fraction	Protein (mg)	Activity (nmol/h)	Specific activity (nmol/h/mg)	Purification (fold)	Yield (%)
1. Crude	232.5	5727	25	1	100
2. Hydroxyapatite	28.8	2642	92	4	46
3. DEAE-Sepharose CL-6B	4.2	2093	498	20	37
4. Sephadex G-200	1.1	655	595	24	11

were harvested and suspended in the growth medium containing 0.5 M NaCl and growth was continued for 1 day before harvesting the cells. After washing the cell pellets with 50 mM Hepes-KOH pH 7.5, the cell pellets were kept frozen until used.

Purification of BHMT. About 5 g of cell pellets were suspended in 10 ml of grinding buffer consisting of 50 mM Hepes-KOH pH 7.5, 10 mM EDTA, 5 mM DTT and 10% glycerol. Lysozyme was added to the suspension at 5 mg/ml and incubated at 37°C for 1 h. The supernatant obtained after centrifugation (14,000 g, 10 min) was loaded on to a hydroxyapatite column (1.8 × 7.0 cm) pre-equilibrated with buffer A (25 mM potassium phosphate buffer pH 7.5 containing 1 mM EDTA and 10 mM β-mercaptoethanol). The proteins were eluted with a linear gradient of 0-500 mM potassium phosphate buffer pH 7.5 containing 1 mM EDTA and 10 mM β-mercaptoethanol. The fractions (2 ml) containing enzyme activity were pooled and loaded on to a DEAE-Sepharose CL-6B column (1.7 × 20 cm) pre-equilibrated with buffer A. The proteins were eluted with a linear gradient of 0-1.0 M KCl prepared in buffer A. Active fractions were pooled, concentrated and desalted before loading on to a Sephadex G-200 column (1.1 × 60.5 cm) pre-equilibrated with buffer A. The enzyme was eluted with the same buffer at a flow rate of 0.2 ml/min. The column was calibrated with the following protein standards from Bio-Rad Laboratories, thyroglobulin (670 kDa), immunoglobulin G (158 kDa), ovalbumin (44 kDa) and myoglobin (17 kDa). The void volume and the total bed volume were determined by the use of blue dextran 2000 and potassium ferricyanide, respectively.

BHMT assay. BHMT activity was assayed as previously described [10]. Fresh homocysteine was prepared from L-homocysteine thiolactone immediately before enzyme assay. The reaction in a final volume of 1.5 ml contained 40 mM potassium phosphate buffer pH 7.5, 7 mM L-homocysteine freshly prepared from L-homocysteine thiolactone, 7 mM [methyl-^{14}C] glycinebetaine (20,000 dpm), which was prepared from [methyl-^{14}C] choline (55 mCi/mmol) from Amersham by using choline oxidase from *Alcaligenes* sp. (Sigma Chemical Co.) [8]. The reaction was started by adding the enzyme sample to the reaction mixture. Following a 60 min incubation at 37°C, the reaction product, [methyl-^{14}C] methionine, was isolated by Dowex 1 × 4 (hydroxyl form) column and quantitated by liquid scintillation counting.

Other methods. The molecular weight of BHMT in the denatured state was determined by SDS-PAGE according to the method of Laemmli [14] on a 10% slab gel. The standard proteins used were bovine serum albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa) and soybean trypsin inhibitor (20.1 kDa). Protein was determined by a sensitive dye-binding method according to Bradford [1] using bovine serum albumin as standard.

Results

Purification of BHMT. A typical purification of BHMT from *A. halophytica* is summarized in Table 1. The enzyme was purified 24-fold after 3 steps of column chromatography, giving a preparation with a specific activity of 595 nmol h^{-1} mg^{-1}. The step of DEAE-Sepharose CL-6B effectively removed the bulk of undesired proteins. Slight improvement of the purity of the enzyme was obtained after the final step by Sephadex G-200 column. As shown in Fig. 1 there were at least two other protein peaks still present after Sephadex G-200 column chromatography. The peak fractions containing BHMT activity were pooled, dialyzed and subjected to SDS-PAGE which showed a single band of 45 kDa (Fig. 2A). The molecular weight of the enzyme as estimated from Sephadex G-200 column was 350 kDa (Fig. 2B). These overall results suggest that *A. halophytica* BHMT is most likely an octamer of 45 kDa subunits.

Kinetic properties of BHMT. The pH profile of BHMT showed an optimum at pH 7.5 with a slight decrease in activity at either pH 7.0 or 8.0. The optimum temperature for BHMT activity occurred at 37°C. At higher temperature of 45°C, the enzyme still retained its activity with about one-fourth of that observed at 37°C. Apparent Km values as determined by Lineweaver-Burk plot for substrates glycinebetaine and L-homocysteine were found to be 4.3 mM and 1.3 mM, respectively. Vmax value of BHMT when glycinebetaine was a variable substrate was 645 nmol h^{-1} mg^{-1}.

Inhibition by substrate analogs and salt. Four compounds bearing resemblance to glycinebetaine substrate were tested for inhibitory effect on BHMT activity. Betaine aldehyde was the most potent inhibitor resulting in the complete loss of enzyme activity at 2.5 mM (Table 2). Choline at 5 mM inhibited 60% of the enzyme activity. Slight inhibitory effect was observed for sarcosine. Marked inhibition occurred with dimethylglycine yielding about one-third of the original activity.

The presence of NaCl also inhibited the activity of BHMT (Fig. 3). Half of the original activity was retained

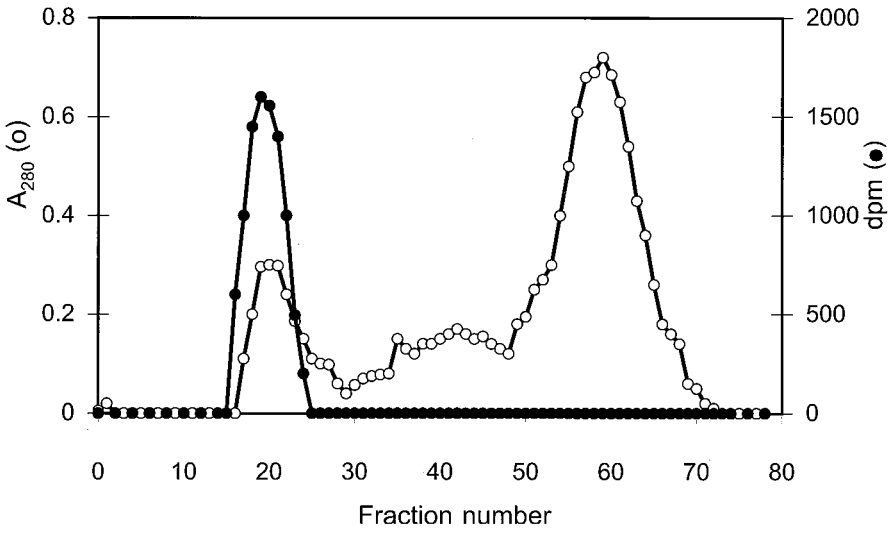

Fig. 1. Sephadex G-200 chromatography of *A. halophytica* BHMT. Experimental details are described under Materials and Methods. Fractions of 1 ml were collected and BHMT activity is indicated as dpm of [methyl-^{14}C] methionine formed.

Table 2. Effect of substrate analogs on BHMT

Compound added	Activity (%)
None	100
2 mM Dimethylglycine	36
5 mM Dimethylglycine	30
2 mM Sarcosine	96
5 mM Sarcosine	90
5 mM Choline	40
2.5 mM Betaine aldehyde	0

at 100 mM NaCl. The concentration of NaCl at 200 mM or higher resulted in a total loss of enzyme activity.

Discussion

BHMT has been reported to exist primarily in liver and kidney of mammals [4, 15, 22, 24]. The importance of BHMT in mammals is due to its action for lowering the level of plasma Hcy. Therefore the role of mammalian BHMT is concerned mainly with the metabolism of sulfur-containing compounds. In contrast, very few reports were available on BHMT in organisms other than mammals. The bacterium, *Rhizobium meliloti*, exhibited BHMT activity when cells were grown under low osmolarity [23]. Very recently the cyanobacterium, *Aphanothece halophytica*, was shown to degrade glycinebetaine by the action of BHMT under salt downshock and starvation [10]. It appears that BHMTs in mammals and bacteria play different roles, i.e., metabolism of sulfur-containing compounds in the former and osmoregulation in the latter.

The purification of BHMT from *A. halophytica* reported here shows the activity enrichment of 24-fold with a final yield of 11%. The preparation was judged to be homogeneous by SDS-PAGE analysis yielding a single band of Mr 45 kDa (Fig. 2A). The low specific activity of *A. halophytica* BHMT is similar to that reported for human and rat liver BHMTs [3, 17]. Although the subunit Mr of 45 kDa reported here for *A. halophytica* BHMT is consistent with those for rat, human and pig liver BHMTs [15, 22, 24], its molecular structure appeared to be an octamer as opposed to a hexamer found in mammalian BHMTs [15, 22].

The apparent Km values for glycinebetaine and L-Hcy of *A. halophytica* BHMT are much higher than those reported for rat and human enzymes [4, 15, 22]. The estimated high Km for L-Hcy would arise due to the oxidation of Hcy during the kinetic analysis of the enzyme. This was likely as dithiothreitol was not included in the enzyme assay. Another possible factor might be a result of incomplete hydroxide-dependent conversion of Hcy thiolactone to Hcy [3], thus the concentration of Hcy used in the enzyme assay would represent the higher value than its actual concentration. Km for glycinebetaine of *A. halophytica* BHMT was relatively high (4.3 mM). The intracellular concentration of glycinebetaine of *A. halophytica* has been reported to be in the range of several hundred millimolar especially when cells were subjected to high osmolarity [13, 20]. This intracellular level of glycinebetaine is much higher than the Km (glycinebetaine). It is obvious that *A. halophytica* BHMT is at all times saturated with glycinebetaine. The fact that Vmax of *A. halophytica* BHMT is low suggests inefficient binding of glycinebetaine and/or Hcy to the enzyme. This is consistent with the observed high Km for both glycinebetaine and Hcy. At this stage the cause of low specific activity of BHMT from *A. halophytica* is

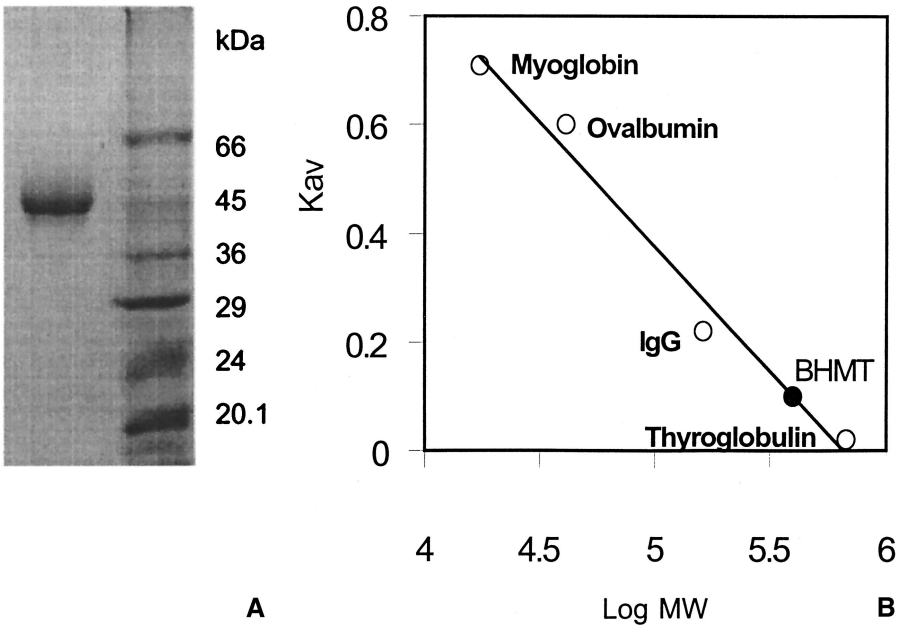

Fig. 2. (**A**) Sodium dodecyl sulfate gel electrophoresis of *A. halophytica* BHMT after chromatography on Sephadex G-200. Proteins were visualized by a Coomassie staining procedure. Left lane shows purified *A. halophytica* BHMT (5 μg) and right lane shows molecular weight markers standard in kDa. (**B**) Estimation of the molecular mass of *A. halophytica* BHMT by gel filtration chromatography.

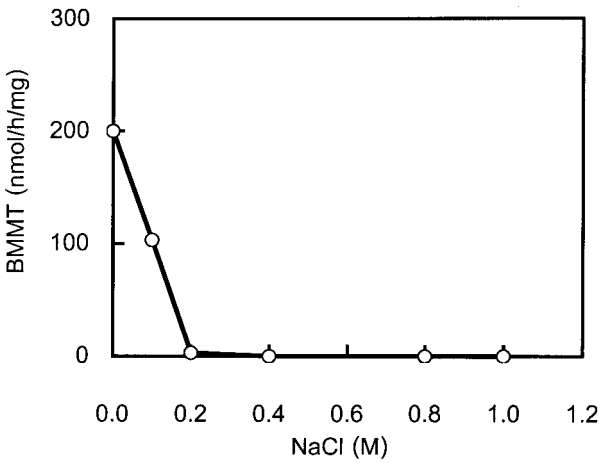

Fig. 3. Inhibition of BHMT by NaCl. Enzyme assays were performed as described under Materials and Methods in the presence of various concentrations of NaCl.

unknown. It is worth mentioning that in the case of human BHMT, the enzyme appeared to polymerize to integral aggregates in the absence of thiol reagents [22].

The intracellular content of Hcy in cyanobacteria has never been reported. However, under normal growth condition the level of Hcy is expected to be relatively low since high level of Hcy can lead to hyperhomocysteinemia which is especially harmful in mammals [19]. Previously we showed that BHMT activity of *A. halophytica* is increased when the osmolarity of the growth medium is decreased [10]. Also we have shown that increased osmolarity resulted in an increased oxidation of choline to glycinebetaine via betaine aldehyde in *A. halophytica* [11]. It is therefore likely that the changes in osmolarity may also affect the metabolism of sulfur amino acid whereby BHMT plays a regulatory role in modulating the level of glycinebetaine and Hcy in particular. This avenue of research using *A. halophytica* is now under study in our laboratory.

The product of BHMT-catalyzed reaction, dimethylglycine, has been reported to be a potent inhibitor of pig, rat, human and bacterial BHMTs [4, 7, 17, 22]. The present study also shows that dimethylglycine is a strong inhibitor of *A. halophytica* BHMT (Table 2). However, the most potent inhibitor is betaine aldehyde which is an analog of the glycinebetaine substrate. Sarcosine displays very little inhibition on the enzyme activity. The finding that betaine aldehyde, an intermediate of choline-glycinebetaine pathway, completely abolishes BHMT activity suggests that the synthesis and the degradation of glycinebetaine do not occur simultaneously. This is substantiated by the evidence that no BHMT activity was observed when *A. halophytica* cells were subjected to high osmolarity [10], the condition which caused a high rate of glycinebetaine synthesis and accumulation [11]. BHMT activity of *A. halophytica* could be detected when high osmolarity condition was shifted to low osmolarity condition [10]. However, the above interpretation should be taken with some precautions. The concentration of betaine aldehyde (2.5 mM) used in the present study would likely be much higher than the actual concentration existing intracellularly. Normally betaine aldehyde levels in most organisms are very low due to its toxicity.

Furthermore, salt-stressed *A. halophytica* appeared to have a high turnover of betaine aldehyde because of its rapid conversion to glycinebetaine [11].

High level of NaCl could effectively inhibit BHMT activity of *A. halophytica* (Fig. 3). Reed, *et al.* [20] found that intracellular Na^+ of *A. halophytica* increased from 80 to 180 mM when the strength of the external medium was raised from 50% sea water (ca. 0.3 M NaCl) to 400% sea water (ca. 2.4 M NaCl). On the other hand, intracellular Cl^- of *A. halophytica* was increased from 35 mM to 150 mM when NaCl in the growth medium was raised from 0.5 M to 2.0 M [9]. It can then be envisaged that high osmolarity causes an increase in intracellular Na^+ and Cl^- of *A. halophytica* which in turn prevents the degradation of glycinebetaine. This is supportive of the evidence that glycinebetaine can be maintained at high level under high external osmolarity.

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