Isolation and Functional Characterization of N-Methyltransferases That Catalyze Betaine Synthesis from Glycine in a Halotolerant Photosynthetic Organism * Aphanothece halophytica*

Received for publication, October 28, 2002, and in revised form, December 2, 2002

Rungaroon Waditee‡, Yoshito Tanaka‡, Kenji Aoki§, Takashi Hibino§, Hiroshi Jikuya¶, Jun Takanoto, Tetsuko Takabe, and Teruhiro Takabe**

*From the ‡Research Institute and §Graduate School of Environmental and Human Sciences, Meijo University, Nagoya 468-8502, Japan, ¶Shimadzu Company, Nakagyou-ku, Kyoto, 604-8511, Japan, and the †Graduate School of Agricultural Science, Nagoya University, Chikusa-ku, Nagoya 464-8601, Japan*

Glycine betaine (N,N,N-trimethylglycine) is an important osmoprotectant and is synthesized in response to abiotic stresses. Although almost all known biosynthetic pathways of betaine are two-step oxidation of choline, here we isolated two N-methyltransferase genes from a halotolerant cyanobacterium Aphanothece halophytica. One of gene products (ORF1) catalyzed the methylation reactions of glycine and sarcosine with S-adenosylmethionine acting as the methyl donor. The other one (ORF2) specifically catalyzed the methylations of dimethylglycine to betaine. Both enzymes are active as monomers. Betaine, a final product, did not show the feed back inhibition for the methyltransferases even in the presence of 2 M. A reaction product, S-adenosyl homocysteine, inhibited the methylation reactions with relatively low affinities. The co-expressing of two enzymes in Escherichia coli increased the betaine level and enhanced the growth rates. Immunoblot analysis revealed that the accumulation levels of both enzymes in A. halophytica cells increased with increasing the salinity. These results indicate that A. halophytica cells synthesize betaine from glycine by a three-step methylation. The changes of amino acids Arg-169 to Lys or Gln in ORF1 and Pro-171 to Gln and/or Met-172 to Arg in ORF2 significantly decreased V_{max} and increased K_{m} for methyl acceptors (glycine, sarcosine, and dimethylglycine) but modestly affected K_{m} for S-adenosylmethionine, indicating the importance of these amino acids for the binding of methyl acceptors. Physiological and functional properties of methyltransferases were discussed.

The most known biosynthetic pathways of betaine are the two-step oxidation of choline. Many bacteria, plants, and animals accumulate glycine betaine hereafter betaine under abiotic stress conditions (1–3). In these organisms, it was shown that betaine is synthesized by two steps, choline → betaine aldehyde → glycine betaine. The enzyme involved in the second step seems to be the same in plants, animals, and bacteria, namely NAD^+-dependent betaine-aldehyde dehydrogenase (4–6). By contrast, different enzymes are involved for the first step. In plants, it was catalyzed by a novel Rieske-type iron-sulfur enzyme choline monooxygenase (7, 8). In animals and many bacteria, the first step is catalyzed by membrane-bound choline dehydrogenase or soluble choline oxidase (9–11). In some bacteria, choline dehydrogenase and choline oxidase also catalyze the second oxidation step (9–11). It was suggested that betaine might be synthesized from glycine by a series of methylation reactions in archaebacterium Methanohalophilus portucaleensis (12) and anaerobic phototrophic sulfur bacterium Ectothiorhodospira halochloris (13). Betaine synthesis from simple carbon sources has also been suggested in aerobic heterotrophic eubacterium Actinopolyspora halophila (13) and halotolerant cyanobacterium of Aphanothece halophytica (14). Recently, the methyltransferase genes that are involved in betaine synthesis have been isolated from E. halochloris and A. halophila (15). Two methyltransferase genes were involved in E. halochloris. One of gene products catalyzed the methylation reactions of glycine and sarcosine to sarcosine and dimethylglycine, respectively (EcGSMT), whereas the other one catalyzed the methylations of sarcosine and dimethylglycine to dimethylglycine and betaine, respectively (EcSDMT) (15, 16). By contrast, one ORF was found in A. halophila of which the N- and C-terminal parts had homologous sequences to those of EcGSMT and EcSDMT, respectively (15). The functionality of A. halophila methyltransferase was not well shown due to the formation of cell pellet when expressed in Escherichia coli.

Glycine N-methyltransferase (GMT) catalyzing the methylation of glycine to sarcosine is known in mammalian cells although the enzymes catalyzing the further methylation steps do not occur (17, 18). The homology of amino acid sequences between mammalian GMT and EcGSMT was low. No homologous sequences to those of EcGSMT, EcSDMT, and A. halophila methyltransferase could be found. Therefore, it was interesting to examine whether betaine is synthesized from glycine by three-step methylation reactions in other organisms.

1 The abbreviations used are: EcGSMT, E. halochloris glycine sarcosine methyltransferase; EcSDMT, E. halochloris sarcosine dimethylglycine methyltransferase; GMT, glycine N-methyltransferase; IPTG, isopropyl-1-thio-β-D-galactopyranoside; AdoMet, S-adenosyl-l-methionine; AdoHcy, S-adenosyl-homocysteine; ApGDMT, A. halophytica dimethylglycine methyltransferase; ApGSMT, A. halophytica glycine sarcosine methyltransferase; EA, ethanolamine; Ptd, phosphatidyl; Ptd-ED, phosphatidylethanolamine.
A. halophytica is an oxygen-evolving halotolerant cyanobacterium that can grow in a wide range of salinity conditions from 0.25 to 3.0 M NaCl concomitant with the accumulation of betaine (14, 20). Previous studies have shown that A. halophytica has unique systems to survive under severe environmental conditions (20–24). Ribulose-1,5-bisphosphate carboxylase/oxygenase of A. halophytica dissociates easily into large and small subunits when betaine is absent (20). A. halophytica DnaK/Hsp70 has been shown to contain the longer C-terminal segment than other Dnak/Hsp70 family members (21) and exhibit extremely high protein folding activity at high salinity (22). It was also shown that A. halophytica Na+/H+ antiporter has novel ion specificity (23) and could confer the tolerance for salt stress of fresh water cyanobacterium capable of growing in sea water (24). Here, we show that in contrast to other oxygen-evolving photosynthetic organisms, A. halophytica synthesizes betaine from glycine by a three-step methylation. Two novel methyltransferase genes were isolated, and their functional properties were examined. From the mutagenesis approach, the information on the substrate binding sites in both enzymes was obtained.

**MATERIALS AND METHODS**

**Culture Conditions**—E. coli DH5α and E. coli BL21 (DE3) cells were grown at 37 °C in LB medium or M9 minimal medium supplemented with 0.2% glucose as a sole carbon source. Ampicillin was used at the final concentration of 50 μg/ml. A. halophytica cells were grown photoautotrophically in BG11 liquid medium plus 18 mM NaNO3 and Turk Island salt solution at 28 °C as previously described (19, 21). TSK BioAssist Q column was purchased from Tosoh Co. (Tokyo, Japan). Radiolabeled S-adenosyl-L-methionine was purchased from American Biosciences.

**Isolation of Methyltransferase Genes**—The genomic DNA of A. halophytica was partially sequenced by a shotgun method using a Shimadzu multidisciplinary DNA sequencing system (Shimadzu Co., Kyoto, Japan). Homology searches were made using the BLAST program as previously described (23). Two methyltransferase genes, ORF1 and ORF2, homologous to EcGSMT and EcSDMT were found and amplified by the PCR technique using the plasmid pORF1SK as a template. For the R169K mutant, the C-terminal part of ORF2 was amplified by the forward primer, ORF1-R/KF and ORF2-R, respectively, and contain the stop codon and the NcoI restriction site.

**Expression and Purification of Methyltransferase Genes in E. coli**—The plasmids, pORF1, pORF2, and pORF12, were transferred to E. coli BL21 (DE3). Cells expressing ORF1 and ORF2 were grown in LB medium containing 50 μg/ml ampicillin until A 620 nm reached 0.6–0.8. Then, 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added, and the cells were grown a further 3 h. The cells were harvested by centrifugation at 5,000 × g for 10 min and was twice with Buffer A (20 mM Tris-Cl, pH 8.0, containing 2 mM 2-mercaptoethanol). Total cell extracts were obtained by sonication with Kubota Insonator 201 M at 180 W for 15 min. Unbroken cells were removed by centrifugation at 10,000 × g for 10 min.

The ORF1 protein was precipitated by addition of ammonium sulfate (25% saturation). The precipitated fraction was dissolved in Buffer A dialyzed against the same buffer A. The dialyzed solubile fraction was applied to a TSK BioAssist Q column (4.6 mm × 5.0 cm). The ORF1 protein was eluted with a gradient of 0–1000 mM NaCl. Active fractions were pooled, dialyzed against buffer A, and applied to the same column. After three times of column chromatography, the purified ORF1 protein was obtained. For the purification of ORF2 protein, the cell extract was fractionated with ammonium sulfate (50–75% saturation). The precipitated fraction was dissolved in Buffer A, dialyzed against buffer A, and applied to the TSK BioAssist Q column. The ORF2 protein was eluted with a gradient of 0–1000 mM NaCl. Active fractions were pooled, dialyzed against Buffer A, and applied to the same column. After three times of column chromatography, the purified ORF2 protein was obtained.

**Enzyme Activities**—Methyltransferase activities were measured according to the method of Raha et al. (25) with a slight modification. The reaction mixture consisted of 25 μl of 25 mM substrate (methyl acceptor), 25 μl of buffer B (125 mM Tris-Cl, pH 8.8, 2 mM 2-mercaptoethanol, 50 μM MgCl2, 160 μM EDTA), 25 μl of 4 mM S-adenosyl-L-methionine.
(AdoMet) (45 nCi of S-adenosyl-L-[14C]methionine), and 25 nM of methyltransferase. The reaction was started by the addition of enzyme. After incubation for 30 min at 37°C, the reaction was stopped by addition of 75 nM of charcoal suspension (133 g/liter in 0.1M acetic acid), which adsorbs the remained S-adenosyl-L-[14C]methionine, and incubated on ice for 10 min. After centrifugation for 10 min, 75 nM of supernatant was removed for assay, and the amount of 14C in methyl acceptors was measured with a liquid scintillation counter (model 3200C, Aloka Instruments Co., Tokyo, Japan). The enzyme activity was calculated as nanomoles of methyl groups transferred per min. The pH in the reaction mixture was adjusted by the following buffers, 125 mM of potassium phosphate (pH 6.0–7.0), 125 mM Hepes-KOH (pH 7.0–8.0), 125 mM Tris-HCl (pH 7.5–8.8), and 125 mM bicarbonate (pH 8.5–10.5). For the measurements of Km and Vmax, the concentrations of methyl acceptors (glycine, sarcosine, and dimethylglycine) were changed between 0 and 50 mM. Methyl donor, AdoMet, was changed between 0 and 10 mM. The reaction products of methyltransferases were identified by two methods, thin layer chromatography and time of flight mass spectroscopy (model KOMPACT MALDI IV tDE, Shimadzu/Kratos) (26). For thin layer chromatography, silica gel 60 (Merck) and phenol/water (80:20, v/v) solvent system were used. The radioactive spots were detected by autoradiography, and the authentic standards were revealed colorimetrically. The orRf values for glycine, sarcosine, dimethylglycine, and betaine were 0.23, 0.47, 0.60, and 0.67, respectively.

**Chemical Modifications of ORF1 and ORF2 with Phenylglyoxal**

ORF1 and ORF2 proteins (0.5 mg/ml) were incubated with 0, 2, 5, and 10 mM phenylglyoxal in 20 mM Tris-HCl buffer (pH 8.8) at 37°C. After 60 min, an aliquot (10 μl) of the reaction mixture was added to 2.0 ml of the assay mixture, and the residual enzyme activity was determined as above.

**Salt Upshock and Downshock of A. halophytica Cells**

A. halophytica cells were grown for 14 days in the growth medium described above, containing 0.5 or 2.5 M NaCl. The cells were transferred to the new medium containing 2.5 or 0.5 M NaCl, respectively. After appropriate times, cells were harvested and used for the Western blotting experiments.

**Other Methods**—SDS-PAGE and Western blotting analysis was carried out according to standard protocol (22). Antibodies raised against the ORF1 and ORF2 proteins were prepared by injection of purified ORF1 and ORF2 proteins into white New Zealand female rabbits, respectively (26). Protein concentrations were determined by Lowry's method. The molecular weights of native ORF1 and ORF2 proteins were estimated by a Superdex 200 column (60 × 2.6 cm) gel filtration column chromatography (Amersham Biosciences). Glycinebetaine content was analyzed by time of flight mass spectroscopy as described previously (26).
RESULTS

Cloning of Methyltransferase Genes from A. halophytica—During the genome sequencing of A. halophytica, two ORFs (ORF1 and ORF2) homologous to the EcGSMT and EcSDMT were found. The predicted gene products for ORF1 and ORF2 consist of 265 amino acids with a molecular mass of 31,211 Da and 277 amino acids with a molecular mass of 31,611 Da, respectively (Fig. 1A). The homology search revealed that a protein encoded by ORF1 is highly homologous to the EcGSMT (66% identity in amino acids) and N-terminal part of heterotrophic eubacterium A. halophila methyltransferase (61% identity in amino acids). The ORF1 protein showed some homology to the rat glycine N-methyltransferase (35% identity in amino acids) but essentially no homology to other methyltransferases including phosophoethanolamine N-methyltransferase (27). The protein encoded by ORF2 showed the highest homology to EcSDMT (52% identity in amino acids) and C-terminal part of A. halophila methyltransferase (49% identity in amino acids). No other homologous sequence was found. Among small molecule methyltransferases, consensus sequences for AdoMet-binding domains (motifs I, post I, II, and III) have been reported (28). As shown in Fig. 1A, the putative AdoMet-binding motifs were found in both ORF1 and ORF2 proteins although identification of post I, II, and III motifs were somewhat obscure.

Expression and Purification of the Cloned Genes in E. coli—The ORF1 and ORF2 were expressed in E. coli BL21 (DE3) under the control of the T7 promoter. It was found that both ORF1 and ORF2 proteins were expressed at high levels as soluble proteins (Fig. 2A). The SDS-PAGE suggested that molecular masses for ORF1 and ORF2 proteins were 33 and 31 kDa, respectively, which are in good agreement with the values calculated from the nucleotide sequences. Both proteins were purified to homogeneity by ammonium sulfate precipitation and ion-exchange column chromatography. Molecular masses of native enzymes for ORF1 and ORF2 were determined by gel filtration chromatography as 32 and 29 kDa, respectively (Fig. 2B). A part of the ORF1 protein was purified as a tetramer, but it was inactive (data not shown). These results indicate that both enzymes are monomer, which is the same to EcGSMT and EcSDMT (16) but different from the mammalian GMTs (18), which are tetramer.

Substrate Specificities and Kinetic Properties for ORF1 and ORF2 Proteins—We tested whether the ORF1 and ORF2 enzymes could synthesize betaine from glycine. AdoMet acts as methyl donor in both enzymes. The reaction products of ORF1 and ORF2 enzymes were analyzed by thin layer chromatography and time of flight mass spectroscopy as described under “Materials and Methods.” It was found that the ORF1 protein catalyzed the methyl transfer to glycine and sarcosine but not to dimethylglycine (Fig. 3A). By contrast, the ORF2 protein catalyzed the methyl transfer to dimethylglycine but not to glycine and sarcosine. Neither ethanolamine or ethanolamine derivatives were N-methylated by both ORF1 and ORF2. None of tested amino acids, except glycine, were N-methylated. Acids such as isovaleric-, n-butyric-, propionic-, and t-butylacetic-acid were also inactive as acceptors of methyl group. These results indicate that the ORF1 specifically catalyzed the methylation reactions of glycine and sarcosine, whereas the ORF2 catalyzed the methylation of dimethylglycine, and therefore, betaine synthesis from glycine is possible if two enzymes work together. The ORF1 and ORF2 were designated as ApGSMT and ApDMT, respectively.

Kinetic parameters for the methyl transfer reactions by ORF1 and ORF2 were examined. The apparent kinetic parameters for substrates were determined with other substrates present in excess. Both enzymes displayed Michaelis-Menten kinetics for their substrates (data not shown). Fig. 3B shows that the apparent V_max value of ORF1 was significantly smaller than those of EcGSMT (18) and human GMT (6.3 mM) but higher than that of rat GMT (0.44 mM). The apparent K_m values of ORF1 for sarcosine (0.8 mM) and of ORF2 for dimethylglycine (0.8 mM) were also smaller than those of EcGSMT (2.3 mM) and EcSDMT (6.1 mM) for sarcosine and of EcSDMT (4.9 mM) for dimethylglycine. By contrast, the apparent K_m values of ORF1 and ORF2 for AdoMet were similar or slightly larger than those of EcGSMT, EcSDMT, and mammalian GMTs. The V_max values of ORF1 and ORF2 were
Fig. 3. Substrate specificity and kinetic parameters for ORF1 and ORF2 proteins. A, substrate specificity. Activities were measured at pH 8.8 and represented by relative values. The concentration of AdoMet was 1 mM. The concentration of methyl group acceptors was 25 mM. B, kinetic parameters. The apparent kinetic parameters for substrates were determined with other substrates present in excess. The concentrations of fixed substrates were 1 mM for AdoMet and 25 mM for methyl group acceptors. Each value shows the average of three independent measurements (S.E. was within 15%).

**FIG. 3**. Substrate specificity and kinetic parameters for ORF1 and ORF2 proteins. A, substrate specificity. Activities were measured at pH 8.8 and represented by relative values. The concentration of AdoMet was 1 mM. The concentration of methyl group acceptors was 25 mM. B, kinetic parameters. The apparent kinetic parameters for substrates were determined with other substrates present in excess. The concentrations of fixed substrates were 1 mM for AdoMet and 25 mM for methyl group acceptors. Each value shows the average of three independent measurements (S.E. was within 15%).

**B) Kinetics parameters of ORF1 and ORF2**

| Enzyme   | Apparent Km (mM) | Vmax (µmol/min/mg) |
|----------|------------------|--------------------|
|          | Glycine          | Sarcosine          | Dimethylglycine | AdoMet |          |
| ORF1     | 1.5              | 0.8                | 0.70           | 0.50   | 0.29    |
| ORF2     | 0.50             | 0.18               | 0.21           | 1.2    | 0.13    |
| EcGSMT   | 18               | 0.42               | 0.28           | 0.14   |
| EcSDMT   | 6.1              | 4.9                | 0.21           | 1.2    |
| ratGMT   | 0.44             | 0.036              | 0.16           | 0.83   |
| humanGMt | 6.3              | 0.27               |                |        |

a) Ref. 16  b) Ref. 18  c) average obtained for two substrates  d) [AdoMet]_{0.5}: concentration which gives a half-maximal activity.

similar or lower than those of EcGSMT and EcSDMT, respectively. As a whole, the results indicate that the affinities for the methyl acceptors were higher in *A. halophytica* than those in *E. halochloris*, whereas the affinities for the methyl donor and *V* max were similar between them.

Inhibitors for ApGSMT and ApDMTs—Inhibitors for ApGSMT (ORF1) and ApDMT (ORF2) were examined. As shown in Fig. 4, acetate inhibited the methylation reactions of glycine and sarcosine by ApGSMT about 78 and 62%, respectively. Dimethylglycine also inhibited these reactions about 67 and 39%, respectively. Other amino acids and ethanolamine derivatives did not inhibit or only slightly inhibited the activity of ApGSMT. The methyl transfer reaction of dimethylglycine by ApDMT was moderately inhibited by n-butyric acid. However, amino acids including glycine and acids such as t-butylic-, isovaleric-, and propionic-acid essentially did not inhibit its reaction. These results indicate that acetate and dimethylglycine were inhibitors for ApGSMT and n-butyric acid was an inhibitor for ApDMT.

It is known that AdoMet-dependent methyltransferases are strongly inhibited by the reaction product S-adenosyl-L-homocysteine (AdoHcy) (29). Fig. 5A shows that the activities of ApGSMT and ApDMT were also inhibited by AdoHcy. However, the *K* values for ApGSMT and ApDMT were in the range of 0.3–0.6 mM, which were significantly larger than those of mammalian GMTs, 0.035–0.08 mM (17), and other methyltransferases (e.g. 0.4 µM for tRNA methyltransferase) (29). As shown in Fig. 5B, a final product, betaine, did not inhibit the methyl transfer reactions of ApGSMT and ApDMT at all. These results indicate that ApGSMT and ApDMT were weakly inhibited by the reaction products, AdoHcy and betaine, which might be suitable for the accumulation of high concentrations.

Effects of pH Activities Profiles and Salts on the Activities of ApGSMT and ApDMTs—The pH activity profiles of ApGSMT and ApDMT are shown in Fig. 6A. The maximal activities for both enzymes were around pH 8.8. The activities were remained high at more alkaline pH but decreased sharply at the acidic side of the optimal pH.

**Effects of pH Activities Profiles and Salts on the Activities of ApGSMT and ApDMTs**—The pH activity profiles of ApGSMT and ApDMT are shown in Fig. 6A. The maximal activities for both enzymes were around pH 8.8. The activities were remained high at more alkaline pH but decreased sharply at the acidic side of the optimal pH.

NaCl and KCl were two important salts for *A. halophytica* (14, 20). NaCl and KCl inhibited the methylation reactions of ApGSMT and ApDMT with different patterns (Fig. 6, B and C). At 1.0 M NaCl, methyl transfers to glycine by ApGSMT and to
dimethylglycine by ApDMT were inhibited only about 17%, whereas the methyl transfer to sarcosine by ApGSMT was inhibited about 70% (Fig. 6B). By contrast, KCl inhibited the methyl transfer reactions by ApGSMT and ApDMT to the similar extends, about 50% at 1.0 M KCl (Fig. 6C). These results indicate that the activities of ApGSMT and ApDMT were affected moderately by NaCl and KCl although the methylation of sarcosine by ApGSMT was inhibited considerably.

Salt-induced Increase of Methyl Transfer Activities and Enzyme Levels of ApGSMT and ApDMT in A. halophytica Cells—Although the above results strongly suggest that A. halophytica cells synthesize betaine from glycine by a three step methylation, we investigated whether the glycine-methylation pathway operates in A. halophytica cells. The results shown in Fig. 7A clearly indicate that A. halophytica cells have methyltransferase activities of glycine, sarcosine, and dimethylglycine using AdoMet as a methyl donor. All methyltransferase activities increased about 1.6–2.5-fold upon the increase of NaCl from 0.5 M to 2.5 M. By contrast, the methyltransferase activities of glycine, sarcosine, and dimethylglycine almost could not be detected in Synechococcus sp. PCC 7942 cells.

Western blotting experiments showed that the accumulation levels of ApGSMT and ApDMT increased upon the increase of NaCl in growth medium from 0.5 M to 2.5 M and decreased upon the decrease of NaCl from 2.5 M to 0.5 M (Fig. 7B). These results support the viewpoint that A. halophytica cells synthesize betaine from glycine by a three-step methylation and that betaine levels are regulated by salts in concomitant with the levels of ApGSMT and ApDMT.

Co-expression of ApGSMT and ApDMT in E. coli Cells—To investigate whether ApGSMT and ApDMT could synthesize betaine in E. coli, the ApGSMT and ApDMT were co-expressed in BL21 cells. The control cells could synthesize betaine by the E. coli betaine synthesis genes (choline dehydrogenase and betaine-aldehyde dehydrogenase). As shown in Fig. 8, it was found that the E. coli (BL21) cells co-expressing ApGSMT and ApDMT grew faster than the control cells. Upon the increase of salinity, the growth rates of both cells decreased, but the growth rates of co-expressing cells were always higher than the control cells. Accumulation levels of betaine in the co-expressing cells were 3- to 5.5-fold higher than those of control cells. These results suggest that the co-expression of ApGSMT and ApDMT could synthesize betaine about 2- to 4.5-fold more than the E. coli betaine synthesis genes, which caused the enhanced growth rate of transformed E. coli cells.

Site-directed Mutagenesis of Arg-169 in ApGSMT—Among the many AdoMet-dependent methyltransferases, the consensus sequences on AdoMet-binding domains have been proposed (28, 29). But, the structural information on methyl acceptors are largely unknown. Previously, no information was available on the amino acids of methyltransferases involved in betaine synthesis. Here, we focused on the methyl acceptor binding domains. Fortunately, Arg-176 in rat GMT has been proposed as a possible glycine binding site (17). It was found that several
amino acids around Arg-176 in rat GMT are conserved among four methyltransferases (Fig. 1). To test the role of Arg for substrate binding in ApGSMT, a chemical modification study of Arg was carried out by using phenylglyoxal. When ApGSMT was incubated with phenylglyoxal as described under "Materials and Methods," the glycine methyltransferase activity decreased. At 10 mM phenylglyoxal, the activity decreased to 23% of the control activity (data not shown). By contrast, the inhibition of dimethylglycine methyltransferase activities of ApDMT by phenylglyoxal was negligible. These data suggest the importance of Arg for methyltransferase in ApGSMT but not in ApDMT. To test more directly, the Arg-169 in ApGSMT was...
modified to Lys and Glu by the site-directed mutagenesis (Fig. 9). The mutants, R169K and R169E, were expressed in *E. coli* at similar levels to wild type and purified as soluble proteins. It was found that the R169E mutant was completely inactive (Fig. 9). By contrast, the *V* \(_{\text{max}}\) values of R169K were about 10–20% of the wild-type enzyme when glycine and sarcosine were used as methyl group acceptors. The mutant R169K could not catalyze the methylation of dimethylglycine. The apparent *K* \(_{m}\) values of R169K for glycine and sarcosine increased about 50- and 63-fold, respectively, whereas the apparent *K* \(_{m}\) values for AdoMet changed only within 2-fold. These results suggest that the Arg-169 in ApGSMT was involved in binding of glycine and sarcosine but not of AdoMet.

**Site-directed Mutagenesis of Pro-171 and Met-172 in ApDMT**—Substrate specificity of ApDMT was unique, and we have no homologous enzyme that could be compared with it.
based on structural information. However, it was found that five amino acids after motif III, FTDPM, were conserved among three methyltransferases as shown in Figs. 1 and 10. Therefore, we tested the role of Pro-171 and Met-172 as a possible methyl group binding site in ApDMT. Three mutants, P171Q, M172R, and P171Q/M172R, were constructed, expressed in E. coli, and purified as soluble proteins. As shown in Fig. 10, the mutants, M172R and P171Q/M172R, were almost inactive. By contrast, the $V_{\text{max}}$ value of P172Q was about 27% of the wild-type enzyme when dimethylglycine was used as a methyl group acceptor. Glycine and sarcosine were ineffective as methyl group acceptors. The apparent $K_m$ value for dimethylglycine increased about 15-fold, but the apparent $K_m$ value for AdoMet changed only within 2-fold. These results suggest that the Pro-171 and Met-172 in ApDMT were involved in binding of dimethylglycine.

**DISCUSSION**

Present results clearly indicate that in contrast to other oxygen-evolving photosynthetic organisms, betaine is synthesized from glycine by a three-step methylation in a halotolerant cyanobacterium *A. halophytica*. This conclusion was supported by several experimental evidences. Isolated genes exhibited homology to recently discovered glycine methyltransferase genes (Fig. 1) (15, 16). The purified enzymes catalyzed the methyl transfer reactions to glycine, sarcosine, and dimethylglycine (Fig. 3). The methyl transfer activities of glycine, sarcosine, and dimethylglycine were detected in *A. halophytica*.
but not in *Synechococcus* sp. PCC 7942, a betaine non-accumulating cyanobacterium (Fig. 7A). The accumulation levels of ApGSMT and ApDMT in *A. halophylica* cells increased upon the increase of salinity (Fig. 7B). Co-expression of ApGSMT and ApDMT in *E. coli* cells increased the accumulation levels of betaine (Fig. 8).

Two methyltransferase genes, encoding ApGSMT and ApDMT, were isolated from *A. halophylica* (Fig. 1). ApGSMT catalyzed the methylation reactions of glycine and sarcosine, whereas ApDMT specifically catalyzed the methylation reaction of dimethylglycine (Fig. 3). Homologous sequences to ApGSMT and ApDMT were only found in *E. halochloris* and *A. halophila*. *A. halophylica* methyltransferases were different from that of *A. halophila* in which one gene was found (15). ApGSMT and ApDMT of *A. halophylica* were similar to EcGSMT and EcSDMT of *E. halochloris*, respectively, but substrate specificity of ApDMT was different from EcSDMT (Fig. 3).

Similar to betaine (trimethylglycine), choline is a trimethyl compound of ethanolamine (EA), and synthesized from EA via free-base, phospho-base, and phosphatidyl (Ptd)-base, depending on the species (1, 27). Several N-methyltransferases are involved in choline biogenesis. For instance, three-step methylation reactions of phosphatidylethanolamine (Ptd-EA), Ptd-monomethyl-EA, and Ptd-dimethyl-EA have been catalyzed by a single Ptd-EA N-methyltransferase in the cases of *Rhodobacter sphaeroides* and liver (30, 31). However, in yeast, these reactions are catalyzed by two enzymes, one mediating the first methylation of Ptd-EA and another mediating the last two (32). In the phospho-base pathway, presence of three separate N-methyltransferases has been reported in nerve tissues (33), whereas in plants, three-step methylation reactions were catalyzed by a single enzyme comprising two similar tandem methyltransferase domains (27). Thus, the biogenesis of N-trimethyl compounds occurs via diversity methyltransferases. Despite of diversity methyltransferases, betaine synthesis from glycine was only observed in halotolerant organisms (15). This suggests that the methyltransferase genes, encoding ApGSMT and ApDMT, in *A. halophylica* might evolve independently from those of methyltransferases in choline biogenesis and mammalian glycine N-methyltransferase.

Fig. 4 shows that acetate inhibited the methyl transfers to glycine and sarcosine. The inhibition of glycine methylation by acetate in mammalian GMTs has been reported (17, 18). These results suggest some similarity of methyl acceptor domain between ApGSMT and mammalian GMT although mammalian GMTs could not catalyze the methyl transfer to sarcosine. Mammalian GMTs are tetrameric enzyme (17, 18), whereas ApGSMT was monomeric. It has also been reported that rat GMA plays another function such as a major folate-binding protein (34). Despite these functional differences between ApGSMT and mammalian GMTs, it was found that the amino acid sequence after AdoMet binding motif III was relatively conserved between them (Fig. 9). Especially Arg-176 in rat GMA, the putative glycine binding site in rat GMA, was conserved. The chemical modification of Arg by phenylglyoxal and site-directed mutagenesis experiments (Fig. 9) showed that the Arg-169 in ApGSMT plays an important role for binding of glycine and sarcosine but not of AdoMet.

ApDMT is a unique enzyme. Its substrate specificity was different from that of EcSDMT (Fig. 3). The amino acid sequence after AdoMet binding motif III in ApDMT did not show the homology to that of mammalian GMTs. Site-directed mutagenesis of Pro-170 and Met-171 showed that these amino acids play important role for binding of dimethylglycine, but not of glycine or sarcosine. The results of Figs. 9 and 10 suggest that the amino acid sequence just after AdoMet binding motif III might play important role for binding of methyl acceptor group in small molecule N-methyltransferases, which remains to be tested experimentally.

AdoHcy is a potent inhibitor in almost all AdoMet-dependent methyltransferases. For the continuous synthesis of betaine, AdoHcy must be removed by AdoHcy-hydrolase, which catalyzes both synthesis and hydrolysis reactions. The results of Fig. 4 show that in contrast to most other AdoMet-dependent methyltransferases, the activities of ApGSMT and ApDMT were inhibited by AdoHcy with relatively low affinities. The Kᵢ values of ApGSMT and ApDMT were about 10-fold higher than that of rat GMA and 1000-fold higher than that of tRNA methyltransferase (Fig. 5A). Allowance of relatively high concentrations of AdoHcy during the betaine synthesis could shift the equilibrium to the hydrolysis direction that would be suitable for the synthesis of large amount of betaine.

Fig. 5F shows that one of final product, betaine, did not inhibit the methyl transfer activities of ApGSMT and ApDMT. This property is also suitable for the synthesis of high concentrations of betaine. The results of Fig. 4 indicate that acetate and dimethylglycine are inhibitors for ApGSMT whereas n-butylic acid was an inhibitor for ApDMT. Any examined amino acids except glycine, ethanolamines derivatives, and several acids did not affect the methyl transfer activities. These facts suggest that betaine synthesis could occur relatively without interference of metabolites.

Hitherto, extensive studies have been carried out for genetic engineering of betaine accumulation (35, 36). All of them used the genes encoding choline-oxidizing enzymes such as choline monoxygenase, choline dehydrogenase, choline oxidase, and betaine-aldehyde dehydrogenase. However, the produced betaine levels were generally low. Several factors limiting the betaine levels are considered. Supply of choline, transport of choline to the place choline oxidizing enzyme localized, and increase of choline-oxidizing enzyme levels might be important to improve the abiotic tolerance of plants. The data of Fig. 8 show that ApGSMT and ApDMT could produce large amount of betaine in *E. coli*. Therefore, overexpression of ApGSMT and ApDMT in photosynthetic organisms are interesting subjects to be tested, which is in progress in this laboratory.

Acknowledgments—We thank Eiko Tsuzekawa for expert technical assistance.

REFERENCES

1. Rhodes, D., and Hanson, A. D. (1993) *Annu. Rev. Plant Physiol. Mol. Biol.* 44, 357–384.
2. Kempf, B., and Bremer, E. (1998) *Arch. Microbiol.* 170, 319–330.
3. Takabe, T., Nakamura, T., Nomura, M., Hayashi, Y., Ishitani, M., Muramoto, Y., Tanaka, A., and Takabe, T. (1997) in *Stress Responses of Photosynthetic Organisms* (Satoh, K., and Murata, N., eds) pp. 115–132, Elsevier Science, Amsterdam.
4. Weretilnyk, E. A., and Hanson, A. D. (1989) *Proc. Natl. Acad. Sci. U. S. A.* 87, 2745–2749.
5. Falkenberg, P., and Strom, A. R. (1990) *Biochim. Biophys. Acta* 1034, 253–259.
6. Chern, M.-K., and Pietruszko, T. (1995) *Biochem. Biophys. Res. Commun.* 213, 561–568.
7. Burket, M., Lafontaine, P. J., and Hanson, A. D. (1995) *Plant Physiol.* 108, 581–588.
8. Rathinasabapathi, B., Burnet, M., Russell, B. L., Gage, D. A., Liao, P.-C., Nye, G. J., Scott, P., Golbeck, J. H., and Hanson, A. D. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 3454–3458.
9. Lamark, T., Kaasen, I., Eshoo, M. W., McDougall, J., and Strom, A. R. (1991) *Microb. Ecol.* 2044, 39, 1049–1064.
10. Bohl, J., Kemp, B., Schmidt, R., and Bremer, E. (1996) *J. Biol. Chem.* 178, 5121–5129.
11. Yamada, H., Mori, N., and Tani, Y. (1979) *Agric. Biol. Chem.* 43, 2173–2177.
12. Kai, M.-C., Yang, D.-R., and Chuang, M.-J. (1999) *Appl. Environ. Microbiol.* 65, 828–833.
13. Galinski, E. A., and Truper H. G. (1994) *FEBS Microbiol. Rev.* 15, 95–108.
14. Reed, R. H., Borowicka, L. J., Mackey, M. A., Chudek, J. A., Foster, R., Warr, S. R. C., Moore, D. J., and Stewart, W. D. P. (1996) *FEBS Microbiol. Rev.* 39, 51–56.
15. Nyyssola, A., Kerovuo, J., Kaukinen, P., von Weymarn, N., and Reinikainen, T. (2000) *J. Biol. Chem.* 275, 22196–22201.
16. Nyyssola, A., Reinikainen, T., and Leisola, M. (2001) *Appl. Environ. Microbiol.* 67, 2044–2050.
Betaine Synthesis in Halotolerant Cyanobacterium

17. Huang, Y., Komoto, J., Konishi, K., Takata, Y., Ogawa, H., Gomi, T., Fujioka, M., and Takusagawa, F. (2000) J. Mol. Biol. 298, 149–162
18. Ogawa, H., Gomi, T., Takusagawa F, and Fujioka M. (1998) Int. J. Biochem. Cell Biol. 30, 1044–1049
19. Ishitani, M., Takabe, T., Kojima, K., and Takabe, T. (1993) Aust. J. Plant Physiol. 20, 693–703
20. Incharoensakdi, A., Takabe, T, and Akazawa, T. (1986) Plant Physiol. 81, 693–703
21. Lee, B. H., Hibino, T., Jo, J., Viale, A. M., and Takabe, T. (1997) Plant Mol. Biol. 35, 763–775
22. Hibino, T., Kaku, N., Yoshikawa, H., Takabe, T., and Takabe, T. (1999) Plant Mol. Biol. 40, 409–418
23. Waditee, R., Hibino, T., Tanaka, Y., Nakamura, T., Incharoensakdi, A., and Takabe, T. (2001) J. Biol. Chem. 276, 36931–36938
24. Waditee, R., Hibino, T., Nakamura, T., Incharoensakdi, A., and Takabe, T. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 4109–4114
25. Raha, A., Wagner, C., MacDonald, R. G., and Bresnick, E. (1994) J. Biol. Chem. 269, 5750–5756
26. Hibino, T., Waditee, R., Araki, E., Ishikawa, H., Aoki, K., Tanaka, Y., Takabe, T. (2002) J. Biol. Chem. 277, 41352–41360
27. Nuccio, M. L., Ziemak, M. J., Henry, S. A., Weretilnyk, E. A., Hanson, A. D. (2000) J. Biol. Chem. 275, 14095–14101
28. Kagan, R. M., and Clarke, S. (1994) Arch. Biochem. Biophys. 310, 417–427
29. Schluckebier, G., Kozak, M., Bleimling, N., Weinhold, E., and Saenger, W. (1997) J. Mol. Biol. 265, 56–67
30. Vance, D. E., Wallkey, C. J., and Cui, Z. (1997) Biochim. Biophys. Acta 1348, 142–150
31. Arondel, V., Benning, C., and Somerville, C. R. (1993) J. Biol. Chem. 268, 16005–16008
32. Kanipes, M. I., and Henry, S. A. (1997) Biochim. Biophys. Acta 1348, 134–141
33. Mukherjee, S., Freysz, L., and Kanfer, J. N. (1995) Neurochem. Res. 20, 1233–1237
34. Cook, R. J., and Wagner, C. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 3631–3634
35. Rontein, D., Basset, G., and Hanson, A. D. (2002) Metab. Eng. 4, 49–56
36. Chen, T. H., and Murata, N. (2002) Curr. Opin. Plant Biol. 5, 250–257