β-Alanine Betaine Synthesis in the Plumbaginaceae. Purification and Characterization of a Trifunctional, *S*-Adenosyl-L-Methionine-Dependent *N*-Methyltransferase from *Limonium latifolium* Leaves[1]

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β-Alanine (β–Ala) betaine is an osmoprotective compound accumulated by most members of the highly stress-tolerant family Plumbaginaceae. Its potential role in plant tolerance to salinity and hypoxia makes its synthetic pathway an interesting target for metabolic engineering. In the Plumbaginaceae, β-Ala betaine is synthesized by *S*-adenosyl-L-methionine-dependent *N*-methylation of β-Ala via *N*-methyl β-Ala and *N,N*-dimethyl β-Ala. It was not known how many *N*-methyltransferases (NMTases) participate in the three *N*-methylations of β-Ala. An NMTase was purified about 1,890-fold, from *Limonium latifolium* leaves, using a protocol consisting of polyethylene glycol precipitation, heat treatment, anion-exchange chromatography, gel filtration, native polyacrylamide gel electrophoresis, and two substrate affinity chromatography steps. The purified NMTase was trifunctional, methylating β-Ala, *N*-methyl β-Ala, and *N,N*-dimethyl β-Ala. Gel filtration and sodium dodecyl sulfate-polyacrylamide gel electrophoresis analyses indicated that the native NMTase is a dimer of 43-kD subunits. The NMTase had an apparent K_m of 45 μM *S*-adenosyl-l-methionine and substrate inhibition was observed above 200 μM. The apparent K_m values for the methyl acceptor substrates were 5.3, 5.7, and 5.9 mM for β-Ala, *N*-methyl β-Ala, and *N,N*-dimethyl β-Ala, respectively. The NMTase had an isoelectric point of 5.15 and was reversibly inhibited by the thiol reagent *p*-hydroxymercuribenzoic acid.

Many plants, bacteria, and marine algae accumulate quaternary ammonium compounds (QACs) in response to abiotic stresses such as drought and salinity (Gorham, 1995). QACs can accumulate to high concentrations to increase the osmotic pressure of the cytoplasm without perturbing metabolism (Yancey, 1994). They also stabilize enzymes and membranes (Yancey, 1994). The synthetic pathway to Gly betaine, the most common QAC, therefore has been the target of recent metabolic engineering efforts to improve plant stress tolerance (for review, see McNeil et al., 1999; Rathinasabapathi, 2000; Sakamoto and Murata, 2000). However, these efforts have met with only limited success due to metabolic constraints on the availability of the precursor choline (Hayashi et al., 1997; Nuccio et al., 1998; Huang et al., 2000).

Most members of the highly stress-tolerant plant family Plumbaginaceae accumulate β-Ala betaine instead of Gly betaine (Hanson et al., 1991, 1994). It was proposed that β-Ala betaine is a more suitable osmoprotectant than Gly betaine under saline hypoxic conditions because the first step in Gly betaine synthesis requires molecular oxygen (Hanson et al., 1991, 1994). Further, β-Ala betaine accumulation was proposed to be an evolutionary strategy to avoid metabolic limitations for choline (Hanson et al., 1994) because β-Ala betaine is synthesized from the ubiquitous primary metabolite β-Ala.

We have been investigating the synthesis and biological significance of β-Ala betaine in *Limonium latifolium,* Plumbaginaceae. Using radiotracer experiments we demonstrated that β-Ala betaine is synthesized by *S*-adenosyl-l-Met (Ado-Met)-dependent *N*-methylation of β-Ala via *N*-methyl β-Ala and *N,N*-dimethyl β-Ala (Rathinasabapathi et al., 2000; Fig. 1). Using a rapid and sensitive radiometric assay, Ado-Met-dependent *N*-methyltransferase (NMTase) activities were demonstrated in β-Ala betaine-accumulating members of the Plumbaginaceae (Rathinasabapathi et al., 2000). However, it was not known how many NMTases participate in the three sequential methylations. Therefore, we developed a seven-step protocol to purify NMTase activities from *L. latifolium* leaf tissue. Our analyses indicate that the purified native NMTase (pI 5.1), a dimer of 43-kD subunits, catalyzes all the three *N*-methylations in the synthesis of β-Ala betaine.

RESULTS

L. latifolium leaves are rich in phenolics, hampering our initial purification trials. To resolve this, several

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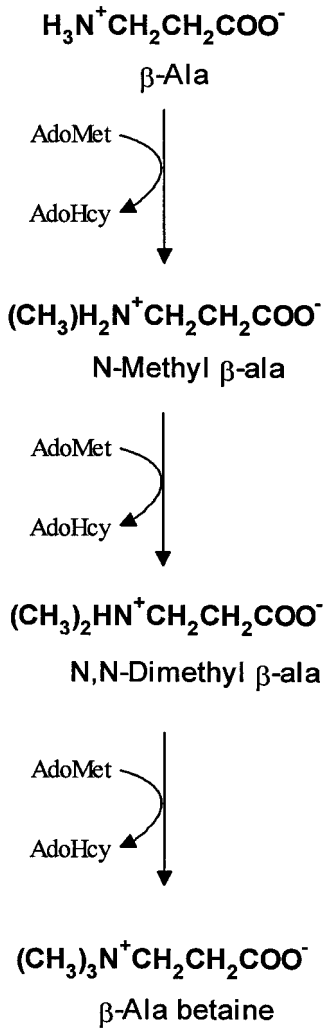

Figure 1. The synthetic pathway to β-Ala betaine. Each downward arrow represents an Ado-Met-dependent *N*-methylation step.

modifications were made to our previously described NMTase extraction method (Rathinasabapathi et al., 2000). These include increased measures against phenolics by the use of both nonionic polymeric adsorbent Amberlite XAD4 and polyvinyl polypyrrolidone (Loomis, 1974) and the inclusion of protease inhibitors in the extraction medium and the elution buffers used in early chromatography steps.

A series of steps were employed to purify the NMTase as detected by assays with β-Ala, *N*-methyl β-Ala, and *N,N*-dimethyl β-Ala (Table I). Each step was found to improve NMTase specific activities in smaller scale trials (data not shown). However, when scaled up, certain steps did not reproducibly improve purity (Table I; for example, heating and Sephacryl S-200 column chromatography).

Polyethylene glycol (PEG) precipitation step was employed primarily to concentrate the extracted protein in a stable form, achieving a 2-fold purification. In separate trials, heat treatment of the PEG fraction resulted in 2-fold improvement in specific activities (data not shown). DEAE-fractogel anion-exchange column chromatography improved specific activities to about 6-fold (Table I) as shown in Figure 2. NMTase activities eluted from DEAE-fractogel column between 125 and 200 mM KCl, ahead of the majority of proteins (Fig. 2).

Following anion-exchange chromatography, the protein fraction was purified by gel filtration chromatography on Sephacryl S-200. NMTase activity eluted as a single peak with an elution volume corresponding to a native molecular mass of 80 kD (data not shown). The use of protease inhibitors proved extremely valuable in this step. Without inhibitors, NMTase activity eluted in four peaks corresponding to 110, 80, 40, and 20 kD, the 80-kD NMTase being more than 50% of the total recovered activity and total activity recovered was substantially reduced (data not shown). Activity at 110 kD was probably due to protein aggregation.

N,N-Dimethyl β-Ala-epoxy-activated 1,6 diaminohexane (EAH) sepharose affinity matrix bound most proteins loaded (Fig. 3). β-Ala and *N,N*-Dimethyl β-Ala at 10 mM each were not sufficient to elute most NMTase activities from this column. Elution with 200 mM KCl was more effective (Fig. 3, inset), suggesting that the matrix also had anion-exchange characters besides affinity features.

Continuous elution gel electrophoresis using a Prep Cell improved specific activities about 34-fold

Table I. *Purification of an Ado-Met-dependent NMTase from 550 g fresh wt leaves of L. latifolium*
Fold-purification was calculated based on specific activities measured with β-Ala.

Step	Total Protein	Specific Activity			Fold Purification
		β-Ala	*N*-Methyl β-Ala	*N,N*-Dimethyl β-Ala	
	mg	*nmol h^{-1} mg^{-1} protein*			
Crude	2,533.3	8.3	8.1	12.8	1
10%–15% PEG	1,315.2	16.6	6.3	8.1	2
Heating	1,156.2	15.3	12.0	13.2	2
DEAE-fractogel	46.8	47.3	39.9	40.7	6
Sephacryl S-200	11.3	46.0	38.0	32.0	6
N,N-Dimethyl β-Ala:sepharose	5.1	104.0	71.0	70.0	13
Prep cell electrophoresis	0.65	285.3	185.4	174.8	34
Adenosine agarose	0.004	15,690.0	9,020.0	4,195.0	1,890

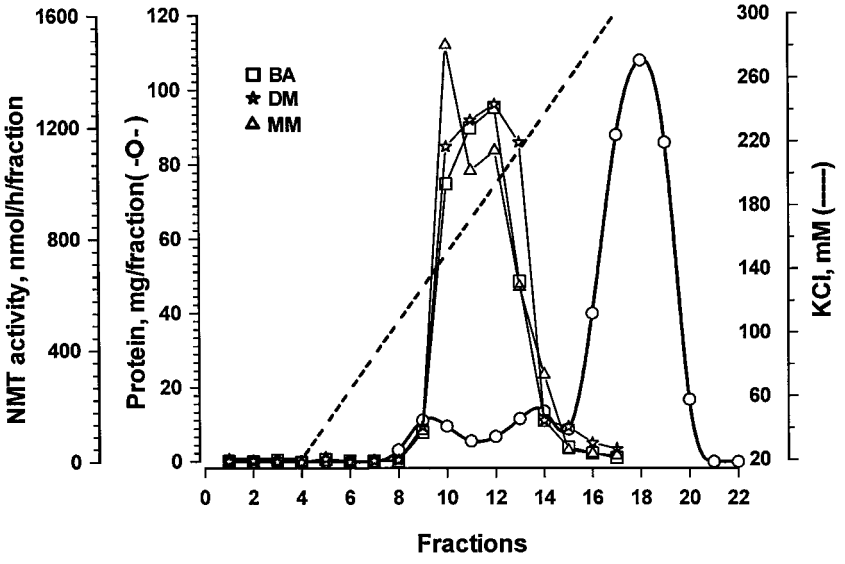

Figure 2. Anion-exchange chromatography using DEAE-fractogel. The procedure is described in "Materials and Methods." NMTase activities (nmol h^{-1}/fraction) against β-Ala (BA), *N*-methyl β-Ala (MM), and *N,N*-dimethyl β-Ala (DM) are indicated by squares, triangles, and stars, respectively. The predicted KCl gradient (20–300 mM) is shown in a dotted line. Protein content (mg/fraction), estimated by the modified Lowry's method (Peterson, 1977), is shown in white circles.

(Table I). From this step onward, however, the enzyme was labile and the steps needed to be performed without interruption. In the buffer system employed, the NMTase activities eluted 6 to 9 mL after the dye front's elution. Adenosine agarose effected about a 1,890-fold increase in specific activities (Fig. 4). The purified fraction methylated β-Ala, *N*-methyl β-Ala, and *N,N*-dimethyl β-Ala (Table I). However, the specific activities with *N*-methyl β-Ala and *N,N*-dimethyl β-Ala were less than those with β-Ala (Table I). The enzyme was labile in this fraction, especially for the activity against *N,N*-dimethyl β-Ala, with about 50% loss over 12 h on ice. SDS-PAGE analysis indicated that the purified protein fraction had one major protein at about 43 kD (Fig. 5, lane B). There were minor contaminants at around 66 kD, appearing as a faint doublet in a silver-stained gel (Fig. 5, lane B). A protein band was generated at around 25 kD upon storage of the purified protein at −80°C and the more it generated, the longer the storage period (data not shown).

When a partially purified protein fraction was subjected to photoaffinity labeling with *S*-adenosyl-L-[*methyl*-^{3}H]Met, the 43-kD protein was labeled (Fig. 5, lane C). When *S*-adenosyl-L-homo-Cys at 217 μM was added prior to crosslinking, the photoaffinity labeling was completely inhibited (Fig. 5, lane D). Experiments showed that the 43-kD affinity-labeled sub-

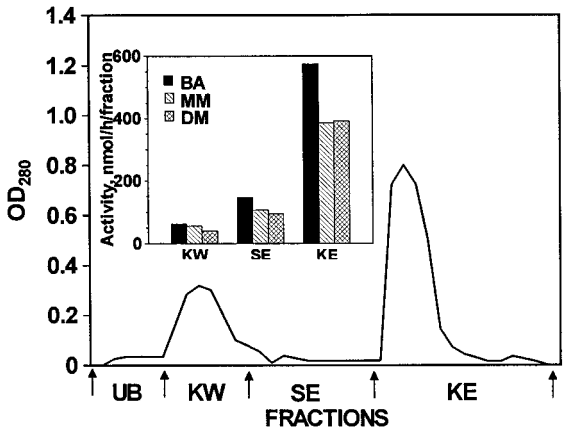

Figure 3. *N,N*-Dimethyl β-Ala substrate affinity column chromatography. Protein elution profile by OD_{280} (optical density at 280 nm) is shown for the unbound fraction (UB) and elutions (KW = 50 mM KCl wash, SE = substrate elution, and KE = 200 mM KCl elution). NMTase activities (nmol h^{-1}/fraction) with β-Ala (BA), *N*-methyl β-Ala (MM), and *N,N*-dimethyl β-Ala (DM) measured in the wash and the elutions are shown in the inset.

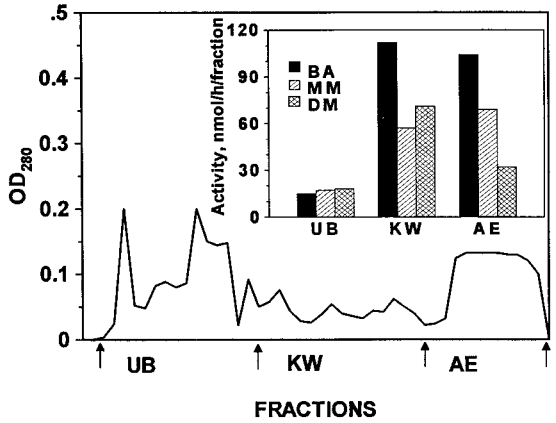

Figure 4. Adenosine agarose affinity chromatography. See "Materials and Methods" for details. Protein elution profile by OD_{280} (optical density at 280 nm) of fractions is shown for unbound (UB), 200 mM KCl wash (KW), and substrate elution with 5 mM Ado-Met (AE). Note that absorbance in the Ado-Met elution is largely due to the Ado-Met and not protein. NMTase activities (nmol h^{-1}/fraction) with β-Ala (BA), *N*-methyl β-Ala (MM), and *N,N*-dimethyl β-Ala (DM) measured in the unbound fraction; 200 mM KCl wash and Ado-Met elution are shown in the inset.

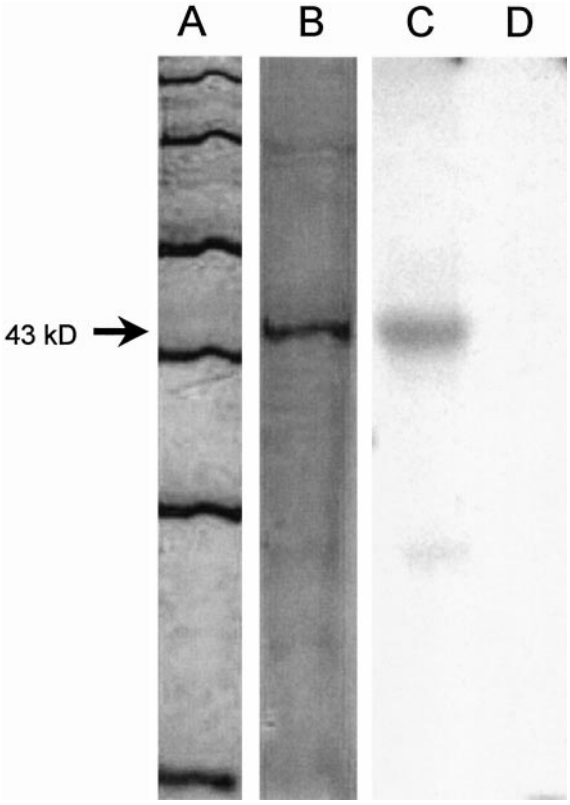

Figure 5. SDS-PAGE analysis of the purified *L. latifolium* NMTase and Photoaffinity labeling. Lane A, Precision SDS-protein markers (Bio-Rad 161-0362). Lane B, SDS-denatured protein (20 ng) from the adenosine agarose step (Table I), separated in a 12% (w/v) acrylamide gel, and stained with silver stain. Lane C, Partially purified (100-fold) NMTase fraction following photoaffinity labeling with *S*-Adenosyl-L-[*methyl*-^{3}H]Met, SDS-PAGE, and autoradiography. Lane D, Partially purified (100-fold) NMTase fraction following photoaffinity labeling with *S*-Adenosyl-L-[*methyl*-^{3}H]Met in the presence of *S*-adenosyl-L-homo-Cys (AdoHCy), SDS-PAGE, and autoradiography.

unit was degrading during storage producing a labeled band about 25 kD in size (data not shown).

The reactions catalyzed by the NMTase exhibited Michaelis-Menten kinetics with respect to its substrate saturation response. The response for varying Ado-Met and β-Ala are shown in Figure 6. Similar plots for *N*-methyl β-Ala and *N,N*-dimethyl β-Ala were employed (data not shown) to derive the kinetic parameters listed in Table II. At 10 mM β-Ala, Ado-Met exhibited substrate inhibition above 200 μM (Fig. 6A). Apparent K_m for Ado-Met was 45 μM. Apparent K_m for the methyl acceptor substrates determined at 100 μM Ado-Met was around 5 mM (Table II). The catalytic efficiency V_{max}/K_m values were comparable for the three methyl acceptors (Table II). AdoHCy was highly inhibitory to the NMTase: 50% inhibition was achieved at 40 μM AdoHCy at 10 mM β-ala and 100 μM Ado-Met.

Isoelectric focusing (IEF) experiments indicated a single peak of activity at a pI of 5.15. The sulfhydral reagent *p*-hydroxymercuribenzoic acid highly inhibited the NMTase (Table III). This inhibition was partially reversible by DTT, suggesting that cysteines are involved in the active site of the NMTase.

DISCUSSION

We have previously used radiotracer experiments to demonstrate that *N*-methylation of β-Ala in *L. latifolium* has three steps via *N*-methyl β-Ala and *N,N*-dimethyl β-Ala (Rathinasabapathi et al., 2000; Fig. 1). Methyltransferase activities were shown in leaf extracts of *L. latifolium* and other β-Ala betaine-accumulating members of the Plumbaginaceae (Rathinasabapathi et al., 2000), but it was not known how many methyltransferases were involved. Sequential methylations in comparative biochemistry are known to involve one or many methyltransferases (Kodaki and Yamashita, 1987; Ridgway and Vance, 1988; Weretilnyk et al., 1995). A bifunctional NMTase participating in both $^{\alpha}$N-methylation of the small subunit of Rubisco and $^{\epsilon}$N methylation of the large subunit of Rubisco has been reported in tobacco

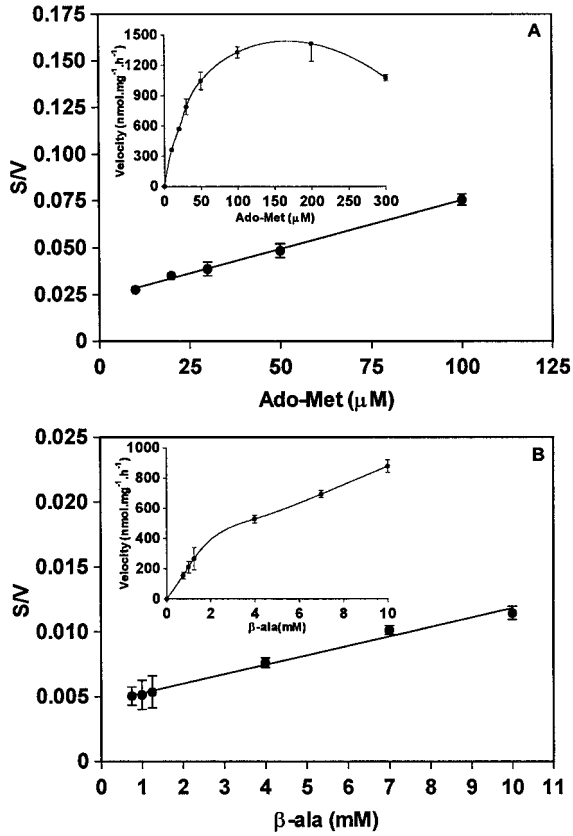

Figure 6. Kinetic analysis of *L. latifolium* NMTase protein. A, Effect of varying Ado-Met concentration on the reaction velocity shown in a plot of s/v versus s, where s is the substrate concentration and v is the velocity. Ado-Met concentration was varied from 0 to 300 μM and β-Ala concentration was kept at 10 mM. Inset shows the direct plot. B, Effect of varying β-Ala on the reaction velocity shown in a plot of s/v versus s. β-Ala levels were varied between 0 and 10 mM. Ado-Met concentration was kept at 100 μM.

Table II. *Kinetic parameters of NMTase from L. latifolium leaves*

Replots of data from substrate response experiments were used to determine the value of the kinetic parameters.

Substrate	Apparent K_m	V_{max}	V_{max}/K_m Catalytic Efficiency
	mM	nmol mg^{-1} h^{-1}	
β-Ala	5.28	1,216	230
N-Methyl β-Ala	5.68	1,290	227
N,N-Dimethyl β-Ala	5.87	1,697	289
Ado-Met	0.045	1,922	43,094

(*Nicotiana rustica*) and pea (*Pisum sativum*; Ying et al., 1999). In phosphocholine synthesis in spinach (*Spinacia oleracea*), an NMTase catalyzes three sequential methylations of phosphoethanolamine (Nuccio et al., 2000). A monomeric NMTase catalyzes two sequential methylations in caffeine synthesis in tea leaves (Kato et al., 1999).

Our initial attempts to purify the NMTase from *L. latifolium* indicated enzyme polymorphism by gel filtration analysis but this proved to be due to endogenous proteolysis of the enzyme and was eliminated by the use of protease inhibitors. Such generation of enzyme polymorphisms due to protease activity has been documented in other systems (for example, see Serrano, 1986). It appears that proteolytic (or other) degradation products of *L. latifolium* NMTase still retain activity, perhaps at a reduced level. Such degradations can affect one substrate-binding site more than others. In our experiments, the activity against *N, N*-dimethyl β-Ala was more labile than the other two NMTase activities (last step, Table I). The simplest explanation for the reduced specific activities with *N,N*-dimethyl β-Ala and to a limited extent with *N*-methyl β-Ala in the purified enzyme (Table I) is that their binding sites are more prone to degradation than that of β-Ala. Differential response in the restoration of activity following inhibition by *p*-hydroxy mercuribenzoic acid (Table III) also suggests that the dimethyl substrate may interact with the enzyme differently from that of the other two methyl acceptors.

In the protocol presented here, heating and Sephacryl S-200 chromatography do not improve NMTase specific activities (Table I) and hence can be omitted. However, those steps were included here because small-scale experiments indicated that heating step can achieve up to 5-fold purification and Sephacryl S-200 chromatography can reveal enzyme polymorphism resulting from degradation. Adenosine agarose affinity chromatography was the most useful step in achieving purity (Fig. 4), similar to the experiences of other investigators with other methyltransferases (Attieh et al., 1995; James et al., 1995). By purifying a trifunctional NMTase to 1,890-fold (based on specific activity with β-Ala), we demonstrate for the first time that a single enzyme participates in β-Ala betaine synthesis in *L. latifolium*. Photoaffinity labeling of the 43-kD subunit (Fig. 5) is consistent with this result. Also, when fractions from the Prep Cell electrophoresis step were analyzed by SDS-PAGE, the intensity of the 43-kD band correlated to NMTase specific activities (data not shown).

Our results have an important implication for metabolic engineering experiments: The β-Ala betaine synthetic pathway potentially can be installed in any plant by expressing a single NMTase transgene. The precursor to β-Ala betaine, β-Ala, also participates in the synthesis of pantothenate, a vital metabolite in all plants. Analyses indicate that the pool size of free β-Ala in plants including *Limonium* spp. is about 100 nmol g^{-1} fresh weight (B. Rathinasabapathi, unpublished data; Bouchereau et al., 1999) and is modulated by stress (Mayer et al., 1990). Pantothenate content in plants has been reported to be between 100 and 46,000 nmol g^{-1} fresh weight in various plants (Mozafar, 1994), the wide variation suggesting a high metabolic flexibility in pantothenate synthesis and utilization.

The NMTase purified from *L. latifolium* shares many characteristics typical of other NMTases. The enzyme exhibited substrate inhibition for the cosubstrate Ado-Met (Fig. 6). The apparent K_m for Ado-

Table III. *Inhibition of L. latifolium NMTase by p-hydroxymercuribenzoate*

Activities are expressed as percent total relative to control assays containing 5 mM dithiothreitol (DTT). They were assayed, 30 min total time in each case, against β-Ala (BA), *N*-methyl β-Ala (MM), and *N,N*-dimethyl β-Ala (DM) as described in "Materials and Methods." Values are means and SEs from three determinations. *p*HMB, *p*-Hydroxymercuribenzoate.

Treatment	% Activity BA	% Activity MM	% Activity DM
Control, 5 mM DTT in the assays	100 ± 4.6	100 ± 6.2	100 ± 11.4
Minus DTT	59.2 ± 1.1	58.7 ± 1.3	87.3 ± 16.0
Minus DTT plus 0.2 mM *p*HMB	1.3 ± 0.7	2.4 ± 1.4	0.4 ± 0.2
0.2 mM *p*HMB 10 min + 5 mM DTT for 30 min	22.6 ± 2.9	23.0 ± 2.9	55.2 ± 7.6

Met was 45 μM at 10 mM β-Ala, the value comparable with many other NMTases reported in the literature (for examples, see Upmeier et al., 1988; Kato et al., 1999). Based on apparent K_m values determined in the presence of 100 μM Ado-Met and catalytic efficiencies estimated by V_{max}/K_m values (Table II), the NMTase has comparable affinities toward β-Ala, *N*-methyl β-Ala, and *N, N*-dimethyl β-Ala. The apparent K_m values for β-Ala and its derivatives observed here are an order of magnitude higher than that reported for other plant NMTases participating in diverse metabolic pathways (Upmeier et al., 1988; Houtz et al., 1991; Kato et al., 1999). Further kinetic characterization of this enzyme therefore is warranted using an assay that will avoid the substrate inhibition by Ado-Met (e.g. by removing the AdoHCy from the assay as it is formed, for example). More stable but pure enzyme preparations for such characterization may become possible by producing the protein in a recombinant expression system in the future.

The native NMTase is a dimer of 43-kD subunits with a pI of 5.15. The pH optimum was 8.0 and the NMTase was highly inhibited by AdoHCy. An analysis of amino acid sequences of other NMTases of plant origin—phosphoethanolamine NMTase, Rubisco NMTase, and putrescine NMTase—indicated that their theoretical pIs fall between 4.69 and 6.20 and their molecular mass between 37 and 56 kD. Purification and characterization of the NMTase involved in β-Ala betaine synthesis opened up opportunities for cDNA cloning, biochemical characterization of this NMTase, and understanding the functional significance of this pathway.

MATERIALS AND METHODS

Chemicals

If not otherwise indicated, chemicals used were from Sigma Chemical Co (St. Louis) and were of the highest purity available. Amberlite XAD-4 resin beads (Aldrich, Milwaukee, WI) were washed in 20-column volumes each of methanol and water and stored in water at 4°C until use. *S*-Adenosyl-L-[*methyl*-^{3}H]Met was purchased from NEN Life Science Products (Boston) at a specific activity of 82 Ci mmol^{-1} (3 TBq mmol^{-1}) and used without further purification. Ado-Met chloride salt was purified using Whatman CM 52 ion-exchange chromatography according to Chirpich (1968). *N*-Methyl β-Ala and *N,N*-dimethyl β-Ala were synthesized as described previously (Rathinasabapathi et al., 2000). *N,N*-Dimethyl β-Ala sepharose 4B affinity resin was prepared by coupling the amino group of 1,6 diaminohexane in EAH-sepharose (Amersham-Pharmacia Biotech, Piscataway, NJ) to the carboxyl group of *N,N*-dimethyl β-Ala, using a carbodiimide procedure (Hoare and Datta, 1990). Adenosine agarose affinity resin was prepared from 5′-AMP-agarose by the method of James et al. (1995).

Plant Material

Seeds of *Limonium latifolium* (Sm.) O. Kuntze were from Park Seed Co. (Greenwood, SC). Plants were grown in Metro-Mix 200 (Scotts-Sierra, Marysville, OH) in wooden boxes (2 feet × 2 feet × 8 inches deep) in a greenhouse in Gainesville between August 1999 and August 2000. The plants were fertilized once a week using a 0.02% solution of a fertilizer (N:P:K, 20:20:20).

Enzyme Extraction

Fully expanded leaves were harvested, briefly washed in a mild soap solution, and rinsed in de-ionized water prior to extraction. Leaves were sliced into about 1-cm-wide strips, frozen in liquid nitrogen, and ground to a powder in a mortar. The powder was transferred to a blender containing freshly prepared extraction medium, 400 mL per 100 g fresh weight leaves. The extraction medium contained the following in 0.1 M Tris-HCl (pH 8): 0.2 M sodium tetraborate, 2 mM DTT, 5 mM EDTA, 10% (v/v) glycerol, 4% (w/v) insoluble polyvinyl polypyrrolidone, 6% (w/v) Amberlite XAD-4, 10 μM leupeptin, 0.2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 1 μM pepstatin A, 1 μM Bestatin, 1 μM E-64, and 1 mM 1,10-phenanthroline. The tissue was blended in the extraction buffer for 3 min at maximum speed, filtered through four layers of autoclaved cheesecloth, and centrifuged at 20,000*g* for 30 min in a refrigerated centrifuge (model J2-HS, Beckman Instruments, Fullerton, CA). The supernatant (crude extract) was saved for further purification (see below). An aliquot of the crude extract was desalted by passage through Sephadex G-25 columns (PD10, Amersham Pharmacia) prior to assays for total protein and NMTase activities.

Enzyme Assay

The NMTase activities with β-Ala, *N*-methyl β-Ala, and *N,N*-dimethyl β-Ala were assayed using a radiometric method (Rathinasabapathi et al., 2000), with modifications as stated below. The assay mixture contained 54 μL of enzyme preparation in a total volume of 100 μL containing 0.1 M Tris-HCl buffer (pH 8.0), 2 mM DTT, 10 mM methyl acceptor, 100 μM Ado-Met, and 0.027 μM *S*-Adenosyl-L-[*methyl*-^{3}H]Met (200 nCi of radioactivity). Following incubation at 30°C for 30 min, the reactions were stopped by the addition of 10 μL of 10% (w/v) trichloroacetic acid containing 1 mM of methylated products as unlabeled carrier. Activated charcoal (38 mg mL^{-1}) in 0.1 N acetic acid, 250 μL per assay, was added and centrifuged for 5 min. The radioactive product in the supernatant was quantified in 75% (v/v) Ready Gel using a liquid-scintillation counter (Beckman Instruments). The counting efficiency was 30%.

Enzyme Purification

All protein purification steps were done at 4°C. For column chromatography steps, a low-pressure column chromatography system (Bio-Rad, Hercules, CA) consisting of a peristaltic pump, UV monitor, a fraction collector,

and a chart recorder was used. All columns were equilibrated in buffer A (20 mM Tris-HCl [pH 8.0], 10% [v/v] glycerol, and 2 mM DTT), prior to use. If required, protein preparations between purification steps were concentrated using a 10-kD cutoff Centriprep (Millipore, Bedford, MA) centrifugal filter device.

Protein precipitating between 10% (w/v) and 15% (w/v) PEG 8000 (Fisher Biotech, Fair Lawn, NJ) was dissolved in buffer A. The NMTase activities were stable in this fraction for at least 2 months when stored at −80°C. For heat treatment, 25 mL of the PEG-precipitated protein dissolved in buffer A was exposed to 50°C in a water bath for 15 min. The preparation then was centrifuged at 20,000*g* for 20 min and the supernatant was collected. For anion-exchange chromatography, protein (about 40–50 mg) from the heat treatment step was loaded onto a column (13.5 cm × 3 cm) containing 50 mL DEAE-fractogel ion exchanger (EM Separations Technology, Gibbstown, NJ). The column was washed with 50 mL buffer A and then with 90 mL buffer A containing 20 mM KCl. The bound proteins were then eluted from the column with 104-mL linear 20- to 300-mM KCl gradient in buffer A containing 0.1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride. Fractions (7.5 mL) were collected and assayed for NMTase activities and protein. Fractions with specific activities equal to and above that of the load were pooled and concentrated to 1 to 2 mL prior to gel filtration. Gel filtration was performed on a 70- × 1.7-cm Sephacryl S-200 HR column (Amersham Pharmacia). Fractions (3 mL each) were assayed for NMTase activities and protein, and those with specific activities equal to or above that of the load were pooled. The pooled fractions from the gel filtration step were loaded onto a *N,N*-dimethyl β-Ala-EAH Sepharose 4B affinity column (5- × 0.8-cm i.d., 2 mL). The column was washed with buffer A, and with 50 mM KCl. The bound proteins were eluted using buffer A containing 10 mM each of β-Ala and *N,N*-dimethyl β-Ala and using buffer A containing 200 mM KCl. Substrate elution and the 200 mM KCl elution were pooled and concentrated to 1.3 mL before being loaded on to a continuous electrophoresis prep cell (model 491, Bio-Rad). The prep cell used a native-gel column made up of 40 mL of 6% (w/v) acrylamide in 24 mM Tris-3-[cyclohexylamino]-1-propane sulfonic acid buffer, pH 9.3 (McLellan, 1982). Electrophoresis was at 300 V for 2 h with 24 mM Tris-3-[cyclohexylamino]-1-propane sulfonic acid buffer, pH 9.3, and the proteins were eluted with buffer A. Fractions (3 mL each) were assayed for NMTase activities and protein. Fractions with specific activities equal to and above that of the load were pooled, concentrated, and loaded onto an adenosine agarose affinity gel (3-mL column). Nonspecific proteins were washed off the column with buffer A containing 0.2 M KCl and the bound proteins were eluted with 5 mM Ado-Met and 0.2 M KCl in buffer A. The eluate was concentrated prior to NMTase and protein assays.

Estimation of Native Molecular Mass

Gel filtration was performed using Sephacryl S-200 column chromatography as described above. The column was calibrated with marker proteins alcohol dehydrogenase (150 kD), bovine serum albumin (66 kD), ovalbumin (45 kD), and cytochrome C (12.4 kD).

Estimation of Protein

Protein was estimated after precipitating it from appropriate volumes of fractions using Lowry's method as modified by Peterson (1977). Bovine serum albumin was used as the standard.

SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli (1970) in 12% (w/v) separation gel and 5% (w/v) stacking gel. Proteins were visualized with Coomassie Brilliant Blue or silver stain.

Estimation of pI

A protein fraction purified about 10-fold was subjected to IEF in an IsoGel agarose IEF plate pH 3 to 10 system (FMC Bioproducts, Rockland, ME) at 1,000 V for 40 min. The anolyte was 0.5 M acetic acid (pH 2.6) and the catholyte was 1 M NaOH, pH 13. Two lanes in the IEF plate were stained with Coomassie Blue to visualize the proteins and the rest of the agarose gel was sliced into 2-mm strips and assayed for NMTase activities. Maximum activities against all the three methyl acceptors corresponded to pH 5.15 in a standard curve of pIs for known standard proteins focused in the same IEF plate.

Photoaffinity Labeling

To identify the protein subunit(s) binding to Ado-Met, photoaffinity labeling (Som and Friedman, 1990) was done on protein samples at various stages of purification from the ion-exchange chromatography stage onward using the method as described by Smith et al. (2000).

Kinetic Characterization

A partially purified enzyme preparation after the anion-exchange column chromatography step (Table I) was used. The activity was stable in this fraction when stored at −80°C for up to 2 months. The assay procedure and conditions were similar to that described above except that the duration of the assay was reduced to 20 min and the substrate concentrations were varied as indicated. The enzyme concentration employed (15 μg of protein per assay) gave a linear reaction velocity during the incubation period (data not shown). Kinetic constants were derived from the x and y intercepts of a linear plot of s/v versus s drawn from triplicate assay results (Henderson, 1993), where s is the substrate concentration and v is the velocity. The experiment was repeated twice with similar results.

Effect of a Thiol Reagent

Protein purified using PEG precipitation was assayed with or without added DTT in the presence and absence of the thiol reagent *p*-hydroxymercuribenzoic acid.

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