PURIFICATION AND PROPERTIES OF *S*-ADENOSYL-L-METHIONINE: PHOSPHOMETHYLETHANOLAMINE *N*-METHYLTRANSFERASE FROM SPINACH

PURIFICATION AND PROPERTIES OF *S*-ADENOSYL-L-METHIONINE: PHOSPHOMETHYLETHANOLAMINE *N*-METHYLTRANSFERASE FROM SPINACH

by

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ABSTRACT

Under conditions of osmotic stress such as drought and salinity, many plants accumulate compatible organic solutes such as glycine betaine (Rhodes and Hanson, 1993). The primary metabolite choline is a precursor for glycine betaine synthesis in addition to being a component of phospholipids. In spinach leaves, choline synthesis involves three sequential *N*-methylations of phosphoethanolamine (PEA) in order to generate phosphocholine (PCho) via the pathway PEA → phosphomethylethanolamine (PMEA) → phosphodimethylethanolamine (PDEA) → PCho. The *S*-adenosyl-L-methionine (SAM) dependent *N*-methyltransferase phosphomethylethanolamine *N*-methyltransferase (PMEAMeT) can catalyze two of the three sequential steps: PMEA → PDEA → PCho (Dhadialla, 1999). This thesis describes a seven-step strategy for PMEAMeT purification from spinach leaves and provides evidence for the existence of two distinct enzymes with apparently overlapping capacities to use both PMEA and PCho as substrates.

A seven step purification strategy was used in this project which included the initial four-step strategy used by Dhadialla (1999) to partially purify PMEAMeT approximately 70-fold. The seven steps included precipitation of soluble leaf protein from spinach leaves by 1.8-2.6 M $(NH_4)_2SO_4$ fractionation followed by open column chromatography on DEAE Sepharose CL-6B, Phenyl Sepharose CL-4B, Macro-Prep® High Q, and Sephacryl S-100, then high performance chromatography on Mono Q HR 5/5 and Protein Pak SW-300. The highest fold purification achieved for PMEAMeT was 7,507-fold and yielded a specific activity of 3,243 nmol min^{-1} mg^{-1} protein. SDS-PAGE analysis of this sample and silver

staining of the polyacrylamide gel revealed that approximately 15 polypeptide bands are present in this sample and included two polypeptides with estimated molecular masses of 30 and 50 kDa.

Anion exchange chromatography on a Mono Q matrix followed by photoaffinity cross-linking with aliquots of individual fractions shown to have high PMEAMeT activity, shows that the 30 and 50 kDa photoaffinity cross-linked species can be partially resolved from each other by this matrix. This observation is best explained if the two polypeptides are not subunits of the same methyltransferase enzyme. Evidence that both the 30 and 50 kDa [^{3}H]SAM-binding polypeptides contribute to PMEAMeT activity was provided by showing that the inclusion of either PMEA or PDEA in the photoaffinity cross-linking assay prevented the binding of [^{3}H]SAM to either polypeptide; a result consistent with PMEA and PDEA serving as substrates for the enzyme(s) associated with both polypeptides. In addition, the presence of PEA in the photoaffinity cross-linking assay did not prevent the binding of [^{3}H]SAM to either polypeptide showing that in these species cross-linking is not prevented by phosphobases that are not suitable substrates for PMEAMeT.

This report describes for the first time the existence of two enzymes in spinach leaves that possess PMEAMeT activity. The role of these enzyme(s) in spinach might involve the maintenance of low PMEA and PDEA pool sizes. Low pools for these metabolites may be required to prevent the incorporation of PMEA and PDEA into the polar head groups of phospholipids in place of PCho. Whether the substitution of PMEA or PDEA for PCho is deleterious to phospholipid structure or function in plant membranes is unknown.

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LIST OF ABBREVIATIONS

AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BADH	Betaine aldehyde dehydrogenase
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CDP-Cho	Cytidine diphosphate choline
CDP-DEA	Cytidine diphosphate dimethylethanolamine
CDP-EA	Cytidine diphosphate ethanolamine
CDP-MEA	Cytidine diphosphate methylethanolamine
CMO	Choline monooxygenase
cpm	Counts per minute
Cho	Choline
DEA	Dimethylethanolamine
DEAE	Diethylaminoethyl
DMSP	3-Dimethylsulfoniopropionate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EA	Ethanolamine
HEPES	*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]
HPLC	High performance liquid chromatography

kDa	Kilodaltons
K-Pi	Potassium phosphate
MEA	Methylethanolamine
Mr	Molecular mass (relative)
MW	Molecular weight
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
OD	Optical density
P5CS	Δ1-Pyrroline-5-carboxylate synthetase
P-base	Phosphobase
PCho	Phosphocholine
PDEA	Phosphodimethylethanolamine
PDEAMeT	Phosphodimethylethanolamine *N*-methyltransferase
PEA	Phosphoethanolamine
PEAMeT	Phosphoethanolamine *N*-methyltransferase
Pi	Inorganic phosphate
PMEA	Phosphomethylethanolamine
PMEAMeT	Phosphomethylethanolamine *N*-methyltransferase
Ptd	prefix Phosphatidyl
PtdCho	Phosphatidylcholine
PtdDEA	Phosphatidyldimethylethanolamine
PtdEA	Phosphatidylethanolamine

PtdMEA	Phosphatidylmethylethanolamine
QAC	Quaternary ammonium compound
Rf	Relative mobility
Rubisco	Ribulose-1,5-bisphosphate carboxylase-oxygenase
SAH	*S*-Adenosyl-L-homocysteine
SAM	*S*-Adenosyl-L-methionine
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TLC	Thin layer chromatography
Tris	Tris(hydroxymethyl)aminomethane
UV	Ultraviolet

LITERATURE REVIEW

Plants have evolved various mechanisms which have allowed them to cope with conditions of osmotic stress, suboptimal temperature, or oxygen deficiency. Of these stresses, water deficiency is the major natural selective force in plant evolution and influences the natural distribution and commercial productivity of plants (Hanson and Hitz, 1982). Water deficiency stress can be the result of freezing, desiccation, or salinity and results in a change in the cellular water potential (Yancey et *al.*, 1982).

Water Stress

Plant water content is influenced by the quantity and quality of water in the surrounding environment and the metabolic responses to water deficit. Water movement occurs in response to gradients of water potential, or at least the result of osmotic and pressure potentials which are components which make up the total water potential of the plant (Kramer, 1988). The chemical potential of water or water potential (ψ_w) of a plant is the sum of the osmotic potential (ψ_s), turgor potential (ψ_p), and matric potential (ψ_m) (Hanson and Hitz, 1982). Osmotic potential (ψ_s) represents the concentration of solutes either inside or outside the cell and is given as a negative value, as solute concentration increases (more negative) plant water potential becomes negative and thus decreases. Turgor potential (ψ_p) is the positive pressure potential of the plant cell and is important in controlling cell growth and cell structural integrity (Hanson and Hitz, 1982). Cell expansion requires a positive turgor pressure although metabolic processes, the supply of solutes, and the availability of water are

also involved in this process. The matric potential (ψ_m) accounts for the decrease in water potential due to the interaction between water molecules and a solid surface thus reducing the tendency of water molecules to react chemically or to evaporate (Nilsen and Orcutt, 1996; Taiz and Zeiger, 1991). The overall contribution of matric potential to plant water potential is considered to be small, therefore water potential (ψ_w) can be represented as $\psi_s + \psi_p$ (Taiz and Zeiger, 1991). Severe water stress leads to inhibition of cell division, cell wall and protein synthesis, accumulation of solutes, closing of stomata, and inhibition of protein synthesis (Kramer, 1988). Thus the water potential (ψ_w) of a plant can provide an estimate of the extent of water stress a plant is experiencing.

Osmotic Adjustment

Osmotic adjustment occurs in response to a decline in water potential in organs such as roots, leaves, and hypocotyls and is the process by which plant ψ_w can decrease without a loss in turgor pressure (Morgan, 1984). The decline in ψ_w occurs via an increase in the concentration of solutes including those considered compatible solutes (Bohnert *et al.*, 1995). As soil dries, soil ψ_m and hence soil ψ_w become more negative relative to the ψ_w of the plant. Under these conditions, the gradient of ψ_w between the soil and the plant will not favor water uptake by the plant and plants may lose water by transpiration more rapidly than water will be replaced. This results in the passive concentration of solutes in the plant, loss of turgor and eventually the death of the plant. Osmotic adjustment, however, occurs via the accumulation of inorganic solutes (K^+, Ca^{2+}, Na^+), the synthesis of organic solutes (proline, mannitol and sorbitol, amino acids, and quaternary compounds such as glycine betaine), and changes in

cellular ion gradients (Bohnert *et al,*. 1995; Morgan, 1984).

Inorganic Solutes

During osmotic adjustment, the levels of three major inorganic constituents that increase include potassium (K^+), calcium (Ca^{2+}), and sodium (Na^+). Osmotic adjustment utilizing K^+, Ca^{2+} or Na^+ is only suitable within the vacuole because high concentrations of these ions in the cytoplasm would be toxic (Nilsen and Orcutt, 1996). As a result, the use of inorganic ions for osmotic adjustment is considered energetically inexpensive for the plant since no energy is utilized for the synthesis of carbon skeletons, energy is required to transport ions into the vacuole (Nilsen and Orcutt, 1996). In addition, high cytosolic concentrations of inorganic ions can perturb metabolism by inactivating enzymes (Greenway and Munns, 1980).

Plants growing in environments where there is a low water potential (halophytes and drought tolerant xerophytes) have higher intracellular osmolarity than glycophytes and mesophytes, and will often have higher proportions of inorganic anions (Cl^-, SO_4^{2-}) (Rains *et al.*, 1980). The major intracellular cation is usually K^+, although in many halophytes the Na^+ concentration is also high (Rains *et al.*, 1980). Salt ions in the growing medium are mainly responsible for causing osmotic stress and are therefore the most readily available solutes by which the plant can build up its own internal osmotic pressure (Rains *et al.*, 1980). However, high concentrations of inorganic ions are not the only mechanism that plants have evolved to establish an osmotic gradient during osmotic stress. The majority of the volume of a plant cell is taken up by the vacuole, thus the cytoplasm is located between two compartments which

contain high concentrations of salt: the cell wall spaces and the vacuole. Osmotic desiccation of the cytoplasm is avoided by the synthesis of organic solutes, which are considered metabolically expensive. However, the expense of organic solute accumulation in the cytoplasm is minimal when comparing the volume of the cytoplasm relative to the vacuole (Epstein, 1980).

Compatible Organic Solutes

As discussed above, plants accumulate compatible solutes (also referred to as "osmoprotectants") in response to dry or saline conditions (LeRudelier *et al.*, 1984; Wyn Jones and Storey, 1981). Osmoprotectants are small, non-toxic molecules that raise the osmotic potential of the cytoplasm without disrupting metabolism, and serve to stabilize protein and membrane structures (LeRudelier *et al.*, 1984). Solutes accumulated include primary metabolites such as proline, and secondary metabolites such as polyols and tertiary sulphonium and quaternary ammonium compounds.

Proline

Proline accumulates in many organisms including plants, eubacteria, marine invertebrates, protozoa, and algae experiencing conditions of drought, high salinity, freezing, or high temperatures (Nanjo *et al.*, 1999). In addition to acting as a compatible osmolyte, proline is involved in protecting enzymes from denaturation (Venekamp, 1989), scavenging free radicals (Smirnoff and Cumbes, 1989), and as a stress-related signal in *Arabidopsis* mutants (Werner and Finkelstein, 1995). Proline is also believed to protect plant tissues

against stress by acting as a nitrogen storage compound (McCue and Hanson, 1990). The role of proline in plant osmotolerance remains controversial. Many reports have shown a positive correlation between the accumulation of proline and osmotolerance in plants (Yoshiba *et al.*, 1997; Liu and Zhu, 1997; Peng *et al.*, 1996; Kishor *et al.*, 1995; and Handa *et al.*, 1986). However, for certain plant species no correlation has been shown between increased proline levels and an increase in salt tolerance (Moftah and Michel, 1987; Hanson and Hitz, 1982).

In plants proline is synthesized either from glutamate or ornithine (Liu and Zhu, 1997; Yoshiba *et al.*, 1997; Delauney and Verma, 1993). The pathway from glutamate is the primary route for the synthesis of proline under conditions of osmotic stress and nitrogen limitation while the pathway from ornithine predominates when nitrogen levels are high (Delauney and Verma, 1993). The biosynthetic pathway from L-glutamate to L-proline involves the intermediates glutamic semi-aldehyde (GSA), and pyrroline-5-carboxylate (P5C) (Yoshiba *et al.*, 1997). The genes encoding enzymes involved in proline biosynthesis have been cloned from higher plants (Yoshiba *et al.*, 1995; Delauney and Verma, 1990). These cloning efforts have shown that the first two steps beginning from glutamate are catalyzed by a single enzyme pyrroline-5-carboxylate synthetase (P5CS), a bifunctional rate-limiting enzyme with γ-glutamyl kinase (γ-GK) and Glutamate-5-semialdehyde dehydrogenase (GSA) activities (Yoshiba *et al.*, 1995; Hu *et al.*, 1992). P5CS activity is regulated by allosteric feedback inhibition by proline with activity decreasing as much as 50% relative to unstressed controls in the presence of 6 mM proline (Zhang *et al.*, 1995; Hu *et al.*, 1992). The concentration of proline in plant cells undergoing osmotic stress is reported to reach as high as 129 mM (Delauney and Verma, 1993). In *Brassica juncea* the free proline content of

whole seedlings as well as leaf tissue of plants at different stages of growth increased with increasing salt stress (Madan *et al.*, 1995). Proline content in seedlings increased from 5 to 7 mmol up to 195 mmol in *B. juncea* plants salt-stressed to 140 mM NaCl. Inhibition of P5CS activity by proline is thought to be overcome by a conformational change of the enzyme which would result in a loss of the effects of feedback regulation (Zhang *et al.*, 1995; Dandekar and Uratsu, 1988; Csonka *et al.*, 1988). Removal of feedback inhibition of P5CS in *Vigna aconitifolia* has been accomplished through site-directed mutagenesis resulting in a mutant enzyme (designated P5CSF129A) which retains similar kinetics to wild-type P5CS (Hong *et al.*, 2000). Transgenic tobacco (*Nicotiana tabacum*) plants expressing the mutant enzyme had two-fold higher levels of proline compared to transgenic tobacco plants expressing the wild-type P5CS enzyme (Hong *et al.*, 2000). In addition tobacco plants expressing the mutant enzyme were shown to be more salt tolerant when grown on a medium containing 200 mM NaCl (Hong *et al.*, 2000).

In the ornithine pathway of proline synthesis, ornithine can be converted to proline by two possible routes both involving the transamination of ornithine followed by cyclization and reduction (Stewart, 1981). In the first route the α-amino group on ornithine is transaminated by the enzyme O-α-aminotransferase (α-OAT) resulting in the formation of α-keto-δ-aminovaleric acid. This intermediate then cyclizes into pyrroline-2-carboxylic acid (P2C) which is then reduced to proline. In the second route the δ-amino group on ornithine is transaminated by the enzyme O-δ-aminotransferase (δ-OAT) resulting in the formation of glutamine-γ-semialdehyde which is cyclized into proline. It has been established that both the α-OAT and δ-OAT pathways occur in plants (Mestichelli *et al.*, 1979). The effects of a 200

mM NaCl stress on developing plants of *Arabidopsis thaliana* was studied by Roosens *et al.*, (1998). δ-OAT activity was measured in 12-d-old and 4-week-old plants incubated for 24 and 72 h in non-saline or 200 mM NaCl stress conditions. A δ-OAT activity of 28 μmol P5C mg^{-1} protein h^{-1} was measured in the control 12-d-old plants. In the salt-stressed plants the δ-OAT activity doubled after 24 h (42 μmol P5C mg^{-1} protein h^{-1}) and reached 82 μmol P5C mg^{-1} protein h^{-1} at 72 h. In addition to δ-OAT activity, the levels of δ-OAT mRNA and free-proline increased 20-fold in salt-stressed 12-d-old *A. thaliana* plants. No change in δ-OAT activity was measured in the 4-week-old control or salinized plants and no δ-OAT mRNA transcripts were detected. However, both free-proline levels and levels of P5CS mRNA increased 7-fold in salinized 4-week-old plants showing that proline is accumulated mainly by the glutamate pathway in salt-stressed mature *A. thaliana* plants (Roosens *et al.*, 1998).

Proline degradation has been shown to provide an alternate source of source of carbon and nitrogen in plants recovering from stress (Peng *et al.*, 1996). The oxidation of proline donates electrons to the respiratory electron transport chain and may provide energy that facilitates recovery of a plant from stress. Proline degradation also produces glutamate, which could act as a source of nitrogen for the synthesis of other amino acids. Proline levels are also regulated by levels of proline dehydrogenase (PDH) which is the enzyme responsible for the oxidation of proline into P5C (Kiyosue *et al.*, 1996). P5C is then further oxidized into glutamate by P5C dehydrogenase (Elthon and Stewart, 1981).

The relationship between free proline concentration and the levels of P5CS and proline dehydrogenase (PDH) mRNA in response to osmotic stress were studied in *A. thaliana* (Peng

et al., 1996). Under osmotic stress, P5CS mRNA levels increased over 6-fold after 12 h of dessication and 5.5-fold after 8 h of treatment with 200 mM NaCl, while levels of free proline increased 10- and 5.5-fold, respectively (Peng *et al.*, 1996). When the osmotic stress was released, P5CS mRNA levels declined and PDH mRNA levels increased 44- and 37-fold, for the dessication and 200 mM NaCl treatments, which resulted in a decline in level of free proline (Peng *et al.*, 1996). Elevated proline levels in tobacco plants containing the wild-type P5CS or transformed with P5CSF129A has been shown to reduce the levels of free radical production in response to osmotic stress (Hong *et al.*, 2000).

Polyols

The level of aliphatic polyols such as mannitol and sorbitol or cyclic polyols including *myo*-inositol and D-ononitol have been shown to increase in plants undergoing osmotic stress (Bohnert *et al.*, 1995). Polyols appear to function as both osmolytes and as osmoprotectants (Bohnert *et al.*, 1995). Polyols have also been thought to serve a role in osmoprotection by tightly associating with proteins, membranes, and enzymes through hydrogen bonding for protection against desiccation (Vernon and Bohnert, 1992; Yancey *et al.*, 1982). Biosynthesis of D-ononitol originates from glucose-6-phosphate which is first converted to *myo*-inositol-1-phosphate by *myo*-inositol-1-phosphate synthase and the *myo*-inositol-1-phosphate is further hydrolyzed to *myo*-inositol by *myo*-inositol-1-phosphate monophosphatase (Wanek and Richter, 1997). D-ononitol is then produced by methylation of *myo*-inositol by *myo*-inositol 6-*O*-methyltransferase (m6OMT), the enzyme that catalyzes the transfer of a methyl group from S-adenosyl-L-methionine (SAM) to the C-6 hydroxyl group of *myo*-inositol (Wanek and

Richter, 1995). In addition to its role as a precursor for D-ononitol, *myo*-inositol is also used by plants for the synthesis of vegetative storage carbohydrates such as stachyose and verbascose (Bohnert *et al.*, 1995).

D-ononitol can be epimerized by ononitol epimerase to give rise to D-pinitol in the ice plant *Mesembryanthemum crystallinum*. D-pinitol has been shown to accumulate in the cytosol of *M. crystallinum* cells to comprise over 70% of total soluble carbohydrate in salt-stressed plants (Paul and Cockburn, 1989). Studies on this plant have also shown Na^+ accumulation to be correlated with *myo*-inositol synthesis (Nelson *et al.*, 1999). The level of *myo*-inositol was found to increase in leaf mesophyll tissue and decrease in root tissue after a 100 mM NaCl treatment. *Myo*-inositol synthesis has been shown to be feedback-inhibited in yeast and in the roots of ice plant seedlings by the addition of exogenous myo-inositol (Nelson *et al.*, 1999; Ashburner and Lopes, 1995).

Tertiary Sulphonium and Quaternary Ammonium Compounds

Tertiary sulphonium compounds (TSCs) and quaternary ammonium compounds (QACs) found in plants feature a fully methylated sulfur or nitrogen atom giving these compounds a positive charge (Wyn Jones and Story, 1981). QAC's and TSC's are derived from amino acid precursors, and they have little or no effect on macromolecule-solvent interactions even when present at in high concentrations (Rhodes and Hanson, 1993). Both classes of compounds are zwitterionic, the property of being uncharged at neutral pH (Rhodes and Hanson, 1993). There are many different QACs which are found in plants, specifically choline-O-sulfate and the different betaines including glycine betaine, hydroxyproline betaine,

β-alanine betaine and proline betaine (Rhodes and Hanson, 1993). QAC's may have a negatively charged carboxyl group as is the case for betaines or a sulfate group as found in choline-*O*-sulfate (Wyn Jones and Story, 1981).

β-Dimethyl Sulfonic propionate

The TSC β-dimethyl sulfoniopropionate (DMSP) has been shown to act as a compatible osmolyte in many organisms, including algae (Gage *et al.*, 1997), bacteria (Lai *et al.*, 1991), and higher plants (Kocsis *et al.*, 1998; Trossat *et al.*, 1996; and Paquet *et al.*, 1994). DMSP is accumulated by various flowering plants and marine algae growing under conditions of osmotic stress, nitrogen deficiency and low temperatures (Trossat *et al.*, 1996; Dacey *et al.*, 1987). The accumulation of DMSP has been studied in the salt marsh grass *Spartina alterniflora* (Kocsis *et al.*, 1998; Colmer *et al.*, 1996; Dacey *et al.*, 1987), sugarcane (Paquet *et al.*, 1994), and in the coastal strand plant *Wollastonia biflora* (Trossat *et al.*, 1998; Trossat *et al.*, 1996; James *et al.*, 1995; and Hanson *et al.*, 1994). Concentrations of DMSP in *S. alterniflora* were found to be highest in leaves (80-300 μmol per g dry weight) and lowest in roots and rhizomes (20-60 μmol per g dry weight) (Dacey *et al.*, 1987). Ecologically, DMSP is important as the main biogenic precursor of atmospheric dimethylsulfide (DMS), which is important in the S-cycle, cloud formation and acid precipitation (Malin, 1996). In *S. alterniflora*, methionine (L-Met) is converted to DMSP via three intermediates: S-methyl-L-Met (SMM) → DMSP-amine → DMSP-aldehyde while in *W. biflora* only two intermediates: SMM → DMSP-aldehyde give rise to DMSP (Kocsis *et al.*, 1998). With respect to enzymes, *S*-adenosyl-L-Met *S*-methyltransferase (MMT) is

responsible for the methylation of Met to SMM and has been purified and characterized from *W. biflora* (James *et al.*, 1995). Localization of MMT to the cytosol in *W. biflora* has been shown by differential centrifugation of cell fractions (Trossat *et al.*, 1996). DMSP-aldehyde dehydrogenase (DDH) catalyses the oxidation of DMSP-aldehyde to DMSP in *W. biflora*. DDH activity was localized to the chloroplasts following fractionation of cell lysate by differential centrifugation and assaying for DDH activity in cell cytosolic and protoplast fractions (Trossat *et al.*, 1998; Trossat *et al.*, 1996).

The synthesis of DMSP has been studied in marine microalgae (Summers *et al.*, 1998) and was shown to be different from the pathway found in *W. bifloria* (Colmer *et al.*, 1996) and *S. alterniflora* (Kocsis *et al.*, 1998). In marine algae, L-Met is converted to DMSP via three intermediates: 4-methylthio-2-oxobutyrate (MTOB) → 4-methylthio-2-hydroxybutyrate (D-MTHB) → 4-dimethylsulfonio-2-hydroxybutyrate (D-DMSHB) (Kocsis *et al.*, 1998). The DMSP synthesis pathway in *S. alterniflora* resembles that of *W. biflora* in that DMSP is synthesized from L-met via the intermediates *S*-methyl-L-Met (SMM) and DMSP-aldehyde in both species (James *et al.*, 1995; Hanson *et al.*, 1994). However, the pathway of DMSP synthesis differs in *S. alterniflora* in that SMM is converted to DMSP-aldehyde via a pool of DMSP-amine which is an intermediate that has not been identified in DMSP production by *W. biflora* (Kocsis et *al.*, 1998).

Choline-*O*-Sulfate

The QAC choline-*O*-sulfate has been shown to accumulate in response to salt stress in the halophytic *Limonium spp.* and other members of Plumbaginaceae (Hanson and Gage,

1991). Choline-*O*-sulfate has also been shown to have an osmoprotective function in bacteria since exogenous application of this metabolite permits growth of *Escherichia coli* and *Salmonella typhimurium* cells in a liquid medium containing 0.6 M NaCl, a level normally inhibitory to the growth of these bacterial strains (Hanson and Gage, 1991). Hanson and Gage (1991) proposed a secondary role for choline-*O*-sulfate accumulation, namely in facilitating SO_4^{2-} detoxification for *Limonium spp.* since Limonium salt glands have a limited capacity for SO_4^{2-} secretion (Hanson and Gage, 1991). Increases of choline-*O*-sulfate in Limonium leaves would help alleviate long term SO_4^{2-} accumulation for plants growing in saline environments (Hanson and Gage, 1991).

Choline sulfotransferase (CST) is responsible for the formation of choline-*O*-sulfate from choline using 3-phosphoadenosine-5-phosphosulfate as the sulfate donor. The activity of this enzyme has been identified and characterized in *Limonium spp.* (Rivoal and Hanson, 1994). *Limonium perezii* plants salinized gradually to 40% (v/v) artificial sea water showed a increase in CST specific activity of 4-and 6-fold in extracts prepared from leaves and roots, respectively (Rivoal and Hanson, 1994). Osmotic shock of *Limonium perezii* cell cultures with 20% (v/v) artificial sea water or 19% (w/v) polyethylene glycol resulted in 2-to-4 fold increases in CST activity (Rivoal and Hanson, 1994).

Glycine Betaine

Glycine betaine biosynthesis proceeds by the pathway of choline → betaine aldehyde → glycine betaine. The two-step synthesis of glycine betaine has been shown to occur in chloroplasts of spinach (Weigel et al., 1988; Hanson et al., 1985). The first step (oxidation

of choline to betaine aldehyde) is catalyzed by choline monooxygenase (CMO), a ferredoxin-dependent stromal enzyme that contains a Rieske-type [2Fe-2S] center and a mononuclear Fe-binding site (Nuccio *et al.*, 1998; Russell *et al.*, 1998; Rathinasabapathi *et al.*, 1997). CMO enzyme activity and mRNA levels were shown to increase 3- and 5-fold, respectively in sugar beet (*Beta vulgaris*) leaves subject to drought treatment (Russell *et al.*, 1998). After 3 days of re-watering the plants following the drought treatment, CMO enzyme activity and mRNA levels were then shown to return to levels equal to those measured prior to drought (Russell *et al.*, 1998). Western blot hybridization analysis of CMO protein in sugar beet leaves removed from plants before and after drought treatment showed that changes in CMO protein levels correlated with CMO enzyme activity, suggesting CMO regulation is not post-translational but rather post-transcriptional (Nuccio *et al.*, 1998; Russell *et al.*, 1998).

The product of CMO activity, betaine aldehyde, is further oxidized to glycine betaine by the stromal enzyme betaine aldehyde dehydrogenase (BADH) with NAD^+ or $NADP^+$ serving as cofactors (Rhodes and Hanson, 1993). BADH has been purified from the leaves of spinach plants and activity localized to the chloroplast stroma (Weretilnyk and Hanson, 1989). Osmotic stress-induced increases of glycine betaine levels in spinach have been associated with increases in both CMO and BADH activity (Brouquisse *et al.*, 1989; Weretilnyk and Hanson, 1989; Weigel *et al.*, 1986). Studies done with transgenic tobacco constitutively expressing a spinach cDNA encoding CMO did not accumulate glycine betaine to levels high enough to provide increased osmotic stress tolerance (Nuccio *et al.*, 1998). The authors proposed that in tobacco the supply of choline is the major limiting factor in glycine betaine synthesis.

Choline Biosynthesis

In plants studied to date, choline has been shown to be synthesized from free-ethanolamine (EA). The source of EA is believed to be either through the decarboxylation of free serine (Mudd and Datko, 1989a,b) or the serine moiety of phosphatidylserine (Moore, 1982). Free-EA is phosphorylated to phosphoethanolamine (PEA) via EA kinase through the reaction: EA+ATP → PEA+ADP (Fig. 1). Choline synthesis is thought to proceed via sequential *N*-methylations of: EA (free-base route) or intermediates ultimately produced from, PEA (phosphobase route), CDP-EA (nucleotide route), or phosphatidylEA (phosphatidyl route) (Fig. 1). For example, in *Lemna paucicostata* PtdCho synthesis occurs through the phosphobase route by the sequential *N*-methylation of PEA (Mudd and Datko, 1986; Mudd and Datko, 1989c). However, for other plants a combination of these routes may prevail. The first methylation step of PtdCho synthesis in soybean and carrot occurs through an *S*-adenosyl-L-methionine (SAM)-dependent methylation of P-EA to P-MEA but subsequent *N*-methylations leading to PtdCho may occur solely at the phosphatidyl level as found in soybean, or both the phosphobase and phosphatidyl routes as found in carrot (Datko and Mudd, 1988). Regardless of the predominant pathways, the synthesis of PMEA from PEA has been shown to be physiologically irreversible and has been proposed to be the committing step in the methylation of PEA to PtdCho in higher plants (Datko and Mudd, 1988; Hitz *et al.*, 1981). An exception has been reported for castor bean endosperm where PtdCho synthesis has been shown to involve the direct *N*-methylation of EA → MEA rather than PEA → PMEA (Prud'homme and Moore, 1992a). The subsequent *N*-methylations occur at both the phosphobase and phosphatidylbase levels forming PCho and PtdCho, respectively

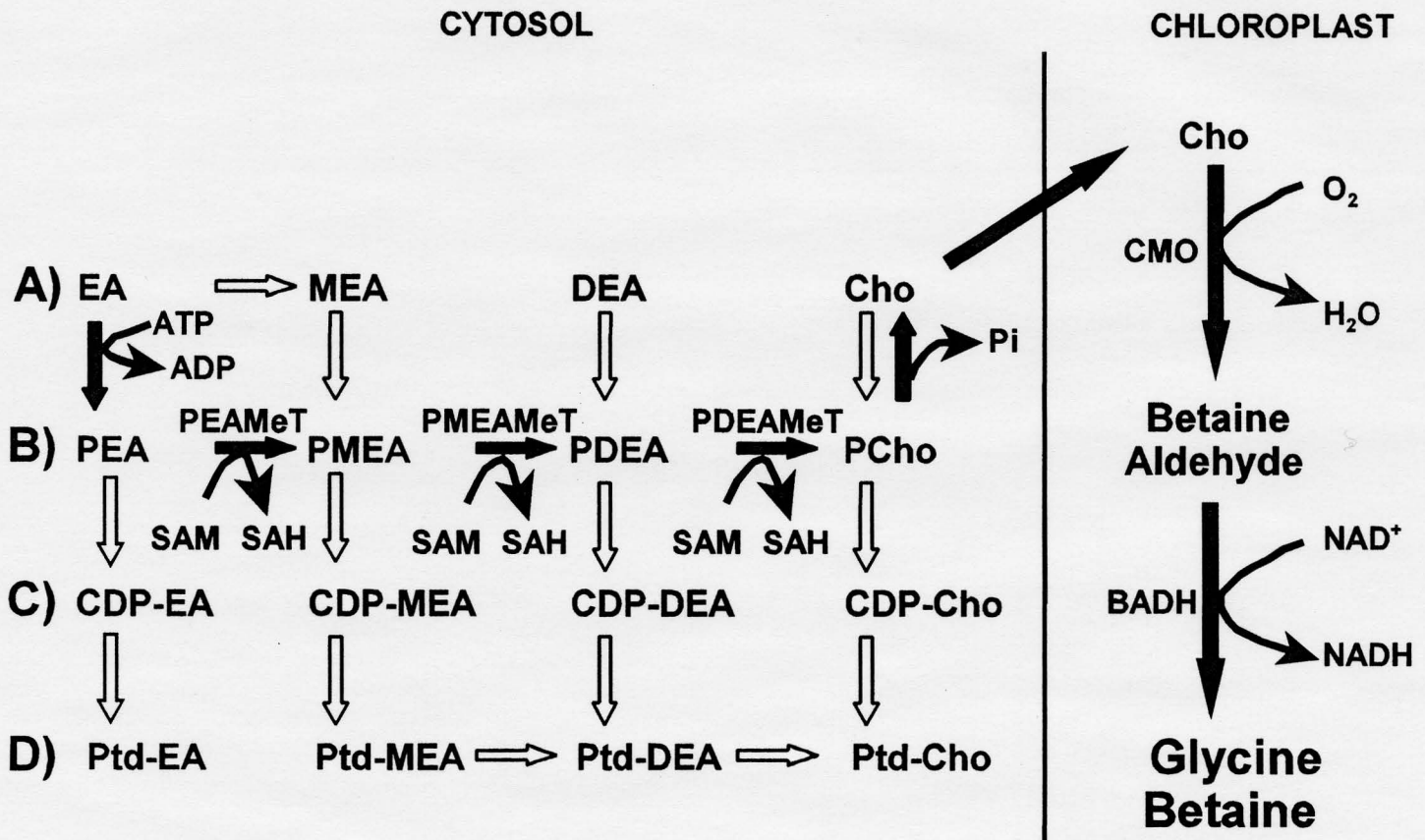
(Prud'homme and Moore, 1992b).

In spinach, choline synthesis is the result of three *N*-methylations of PEA via SAM-dependent methyltransferases designated PEAMeT, PMEAMeT and PDEAMeT, respectively (Summers and Weretilnyk, 1993). Differential centrifugation of spinach leaf extracts showed that the enzymes catalyzing the three *N*-methylations from PEA to PCho are cytosolic in spinach leaf cells (Weretilnyk *et al.*, 1995). These reactions are shown by the solid arrows in Figure 1. The first methyltransferase, PEAMeT, was purified over 5,000-fold and reported to have an estimated Mr of 54 kDa (Smith *et al.*, 2000). PEAMeT activity is reported to be absent in spinach roots (Weretilnyk and Summers, 1992), and is greatly diminished in spinach leaves kept in the dark for prolonged periods (Weretilnyk *et al.*, 1995), or in spinach leaves purchased at the supermarket (Smith *et al.*, 2000; Dhadialla , 1999). In contrast, PMEA and PDEA *N* -methylating activities were found in all of these sources (Weretilnyk *et al.*, 1995; Weretilnyk and Summers, 1992). Thus in spinach, PMEAMeT activity and PDEAMeT activities do not decline upon exposure to prolonged periods of darkness (Weretilnyk *et al.*, 1995).

The presence of three *N*-methylating activities in spinach leaves (PEA → PMEA → PDEA → PCho) and only two *N*–methylating activities (PMEA → PDEA → PCho) in root tissues provides evidence that there are at least two enzymes which can *N*-methylate PMEA. Following a 200 mM NaCl-shock treatment to spinach plants, all three *in vitro N*-methylation activities increased 2-fold (Weretilnyk *et al.*, 1995). In *Lemna paucicostata,* plants grown in the presence of 3 μM choline were used to assay all three *N*-methylating activities and the *in vivo* and *in vitro* rates of PEAMeT activity were shown to decrease by 80% of the control

FIGURE 1: Metabolic grid showing the possible routes of choline and glycine betaine synthesis from ethanolamine

At least in theory, choline synthesis can occur through any combination of four possible routes. Each of the four routes is delineated by the chemical mature of the intermediates involved: A) free base route B) phosphobase route C) nucleotide route D) phosphatidyl route. Solid arrows represent the pathway of choline and glycine betaine synthesis pathway found in spinach for which there is both *in vivo* radiotracer and *in vitro* enzyme activity evidence (Summers and Weretilnyk, 1993; Coughlan and Wyn Jones, 1982). The open arrows represent other possible pathways of choline synthesis for which there is *in vivo* or *in vitro* radiotracer evidence (Prud'homme and Moore, 1992a; Prud'homme and Moore, 1992b; Weretilnyk *et al.*, 1989; Datko and Mudd, 1988; Mudd and Datko, 1986; Hitz *et al.*, 1981). Arrows are shown as being unidirectional for simplicity and are not meant to preclude reversible reactions. Ethanolamine (EA) is phosphorylated to phosphoethanolamine (PEA) by ethanolamine kinase (EAK). The enzymes responsible for the phosphobase route and glycine betaine synthesis in spinach are designated (in order): PEA *N*-methyltransferase (PEAMeT), PMEA *N*-methyltransferase (PMEAMeT), PDEA *N*-methyltransferase (PDEAMeT), choline monooxygenase (CMO) and betaine aldehyde dehydrogenase (BADH). PCho is hydrolyzed to release choline by PCho phosphatase.


CYTOSOL
CHLOROPLAST
A) EA
MEA
DEA
Cho
ATP
ADP
Pi
B) PEA
PEAMeT
PMEA
PMEAMeT
PDEA
PDEAMeT
PCho
SAM SAH
SAM SAH
SAM SAH
C) CDP-EA
CDP-MEA
CDP-DEA
CDP-Cho
D) Ptd-EA
Ptd-MEA
Ptd-DEA
Ptd-Cho
Cho
O_2
CMO
H_2O
Betaine Aldehyde
NAD^+
BADH
NADH
Glycine Betaine

level whereas PMEAMeT and PDEAMeT activities were only reduced by 30 and 40% (Mudd and Datko, 1989a). In cell suspensions of carrot and soybean grown in 50 μM choline, PtdCho synthesis was inhibited 98% and 77%, respectively (Mudd and Datko, 1989b). Since PEAMeT and at least one other enzyme are able to utilize both PMEA and PDEA as substrates, it is unclear whether any change in the flux rates through the phosphobase route as a result of salt stress or growth in the presence of choline reflects an altered regulation of one or possibly both enzymes.

Photolabeling of *N*-methyltransferases with *S*-adenosyl-L-methionine

Methylation reactions occur in all organisms and involve a variety of different substrates such as amino acids, nucleic acids, proteins, and lipids (Park *et al.*, 1993). Thus methyltransferases are responsible for important biological roles such as lipid synthesis or nucleic acid and protein modifications (Ahmad and Rao, 1994; Subbaramaiah and Simms, 1992; Takata and Fujioka, 1992; Som and Friedman, 1990). The main methyl donor involved in transmethylation reactions is *S*-adenosyl-L-methionine (SAM). SAM, radiolabeled with ^{3}H in the methyl group, has been shown to specifically and covalently bind to the active site of various methyltransferases with exposure to short wave UV light. Enzymes modified in this manner include phosphatidylethanolamine methyltransferase (PtdEA MeT) (Pajares *et al.*, 1984), *Eco*RII methyltransferase (*Eco*RII MeT) (Som and Friedman, 1990), cytosine-5-methyltransferase (CheR MeT) (Subbaramaiah and Simms, 1992), guanidinoacetate methyltransferase (GA MeT) (Takata and Fujioka, 1992), and phosphoethanolamine *N*-methyltransferase (PEAMeT) (Smith *et al.*, 2000). Since methyltransferases use a common

substrate, namely SAM, the sequence homology of the SAM binding site is, not surprisingly, highly conserved (Takata and Fujioka, 1992; Subbaramaiah and Simms, 1991,1992; Som and Friedman, 1991). When both *Eco*RII MeT and CheR MeT were successfully cross-linked with [^{3}H]SAM, both enzymes were found to be stable over a wide pH range, and showed no loss of radioactivity after treatments with 10% trichloroacetic acid, 2.5% SDS, or 10 mM dithiothreitol (Subbaramaiah and Sims, 1992; Som and Friedman, 1990). Labeling efficiency has been shown to be a function of the concentration of enzyme, concentration of [^{3}H]SAM, intensity of the UV light, and the duration of irradiation (Finta *et al.*, 1995; Ahmad and Rao, 1994; Subbaramaiah and Simms, 1992; Takata and Fujioka, 1992; Som and Friedman, 1990). Following UV irradiation of a reaction mixture containing EcoRII MeT in the presence of [^{3}H]SAM, the mixture was resolved by electrophoresis through an SDS-polyacrylamide gel and the position of the radioactive band identified by exposing the gel to an X-ray film (Som and Friedman, 1990). However, no radioactivity was found associated with the polypeptide corresponding to EcoRII MeT if the reaction mixture was not exposed to UV light (Som and Friedman, 1990). Furthermore, EcoRII MeT, that was heat-inactivated or exposed to UV light prior to the addition of [^{3}H]SAM did not incorporate radioactivity (Som and Friedman, 1990).

Many different amino acids including cysteine, lysine, and arginine have been shown to covalently cross-link with nucleotides upon exposure to UV light (Kierdaszuk and Eriksson, 1988; Smith and Meun, 1968). Photolabeling of CheR MeT was inhibited in the presence of sulfhydryl reagents (2-nitrobenzoic acid, *N*-ethylmaleimide) indicating cysteine is present at the [^{3}H]SAM binding site and oligonucleotide-mediated mutagenesis of the CheR

MeT SAM-binding site showed that cys^{31} is involved in the photolabeling reaction (Subbaramaiah and Simms, 1992). Photolabeling of *Eco*RII MeT revealed that cys^{186} at the SAM-binding site is converted to *S*-methylcysteine (Som and Friedman, 1991; Subbaramaiah *et al.*, 1991). Competitive inhibitors of methyltransferases, sinefungin and SAH, have been shown to inhibit photolabeling of *Eco*RII MeT, GA MeT, CheR MeT, *Eco*P15 DNA MeT, and *Kpn*I DNA MeT when such inhibitors are present in 2-15 fold higher concentrations than SAM in the photolabeling assay (Finta *et al.*, 1995; Ahmad and Rao, 1994; Subbaramaiah and Simms, 1992; Takata and Fujioka, 1992; Som and Friedman, 1990).

The presence of substrates to be methylated can also affect the extent of photolabeling of SAM-dependent methyltransferases by [^{3}H]SAM. The enzyme protein-*O*-carboxylmethyltransferase transfers a methyl group from SAM to an aspartic or glutamate residue on the substrate calmodulin (Hurst *et al.*, 1984). The binding of [^{3}H]SAM to protein-*O*-carboxylmethyltransferase under UV irradiation was inhibited in the presence of 1.0 μg of calmodulin (Hurst *et al.*, 1984). Purified phenylethanolamine *N*–methyltransferase irradiated under UV light in the presence of the substrate phenylethanolamine was also not labeled by [^{3}H]SAM (Hurst *et al.*, 1984). Photolabeling can be used to deduce the abundance of various MeT's by the inclusion of the substrate in the photolabeling assay to help identify specific MeT's. Wanek and Richter (1995) used this approach to study the synthesis of the compatible solute ononitol (see Polyols). In this study they used photolabeling to identify *myo*-inositol 6-O-methyltransferase from *Vigna umbellata* by the inclusion of *myo*-inositol or SAH to the incubation mixture. A single polypeptide was identified on a fluorograph of an SDS-PAGE gel which disappeared when the photolabeling reaction included either *myo*-inositol or SAH.

MATERIALS AND METHODS

CHEMICALS, RADIOISOTOPES, AND SUBSTRATES

Chemicals and enzymes used were purchased from Sigma Chemical Co. (St. Louis, MO) unless noted otherwise. H_2O used to prepare reagents was purified by a Barstead NANOpure II water purification system.

S-adenosyl-L-methionine (SAM) (Boehringer Mannheim Canada) was dissolved in 0.01 N H_2SO_4:ethanol 9:1 (v/v). The concentration of SAM was measured spectrophotometrically at 257 nm using a UVIKON 930 spectrophotometer and calculated from the absorbance using the molar extinction coefficient of 15 M^{-1} cm^{-1} (Eloranta *et al.*, 1976). Once the concentration of the stock solution of SAM was determined, the solution was diluted to 12 mM with 0.01 N H_2SO_4:ethanol 9:1 (v/v), dispensed into 25-µL aliquots in 0.5 mL microfuge tubes, and then stored at -20 °C. *S*-[methyl-^{3}H]adenosyl-L-methionine ([^{3}H]SAM) was purchased from New England Nuclear (NET 155H) as a 10 mM sulfuric acid solution: ethanol (9:1 v/v) with a specific activity of 85 Ci/mmol and concentration of 0.55 mCi mL^{-1}. The [^{3}H]SAM was received frozen in dry ice, thawed and dispensed into 10–µL aliquots in 0.5 mL microfuge tubes. These aliquots were frozen rapidly in the remaining dry ice and stored at -20 °C. *S*-[methyl-^{14}C]adenosyl-L-methionine ([^{14}C]SAM) was purchased from New England Nuclear (NEC 363) as a 10 mM sulfuric acid solution: ethanol (9:1 v/v) with a specific activity of 59 mCi/mmol and concentration of 0.02 mCi mL^{-1}. The [^{14}C]SAM was received frozen in dry ice, thawed and dispensed into 10–µL aliquots in 0.5 mL microfuge tubes. These aliquots were frozen rapidly in the remaining dry ice and stored at -20 °C.

PEA (Sigma, A7007) substrate was dissolved in 0.1 N HCl to a final concentration of 7.5 mM and stored at -20 °C. Substrates PMEA and PDEA were synthesized by the method of Datko and Mudd (1988) using 12 mg of dipalmityl-phosphomethylethanolamine (PtdMEA, Avanti Polar Lipids, 850851) or dipalmityl-phosphodimethylethanolamine (PtdDEA, Avanti Polar Lipids, 850854). The Ptdbases were dissolved in 1 mL of 5% v/v Triton X-100 by mixing end-over-end overnight in 15 mL conical tubes. 160 U of phospholipase C (Boehringer Mannheim, 691950) grade XI was mixed with 140 μL of dimethylgutarate buffer pH 7.5 NaOH and the mixture was then desalted by centrification through Sephadex G-25 (previously equilibrated with dimethylgutarate buffer-NaOH, pH 7.5). Phosphobases were released from the Ptdbases by hydrolysis with 360 U of phospholipase C for 40 min at 37 °C. The reaction was stopped by the addition of 3.2 mL methanol and 1.6 mL chloroform to each tube. For phase separation, 2.06 mL of H_2O and 4.8 mL chloroform were also added, the contents of the tubes were vortexed to mix and then the tube was centrifuged at 700 rpm in a table top centrifuge for 5 min. The top phase, containing the P-base, was transferred to a 13 x 100 mm test tube and N-evaporated at 40 °C (Meyer N-EVAP Organomation Model No. 111 Berlin, MA. USA). The dried residue was dissolved in 150 µL of 0.5 N HCl and stored at -20 °C. Concentrations of PMEA and PDEA were determined by hydrolyzing the phosphate groups from the P-base and measuring the phosphate (Pi) released by the method of Martin and Tolbert (1983). The Pi assay was performed in duplicates with the control containing H_2O and tubes with substrates PMEA or PDEA. Initially, 4 µL of PMEA or PDEA stock solution was diluted 10 times with H_2O. Alkaline phosphatase buffer was made by mixing 1.58 mL of phosphate incubation buffer (50

mM $NaHCO_3$ (pH 10.4 22 °C), 1 mM $MgCl_2$, 0.1 mM $ZnSO_4$) with 28 U of alkaline phosphatase (Boehringer Mannheim, 713023) and stored on ice (no longer than 1 h) before being used for the Pi assay. Into each assay tube 226 µL of the alkaline phosphatase buffer, 19 µL of H_2O and 5 µL of H_2O or 10-fold diluted P-base were added, the contents were vortexed to mix and the tubes incubated in a water bath at 30 °C for 18 h. After incubation the reaction was stopped with 250 µL 10% (w/v) TCA and vortexed to mix. The concentration of Pi was determined by incubating 250 µL of the stopped alkaline phosphatase assay with 250 µL of ammonium molybdate for 90 min at 37 °C. The absorbance of the reaction mixture was read at 820 nm and the optical density plotted was compared to a phosphate standard curve in order to determine the concentration of Pi released by the P-base substrates. Stock PMEA and PDEA solutions were diluted to 7.5 mM with 0.1 N HCl and stored at -20 °C.

Dowex 50W(H^+) X8-200 resin (J.T. Baker 1905-05) was prepared by resuspending 300 gm of resin in approximately 600 mL of H_2O. The mixture was gently stirred, the resin beads were allowed to settle, and the H_2O was decanted and discarded. After several such "washes" the resin was protonated by successive treatment with 1 N HCl. Approximately 800 mL of 1 N HCl was added to the washed resin and the mixture was gently stirred. After the resin beads had settled, the 1 N HCl was decanted and discarded. This was repeated at least 30 times. To remove excess acid the beads were "washed" with H_2O as described above until the pH of the H_2O decanted from the beads was between 6 and 7. The regenerated resin was stored at 4 °C until used. For use, disposable Evergreen columns (Diamed #208-3384-060) were rinsed with H_2O, filled with 1 mL of the regenerated Dowex resin, and the matrix

washed with at least 5 column volumes of H_2O before being used for phosphobase enzyme assays.

PHOSPHOBASE *N*-METHYLTRANSFERASE ASSAYS

Methyltransferase activities attributed to PEAMeT, PMEAMeT, and PDEAMeT were assayed using a procedure modified from one described by Datko and Mudd (1988). Each methyltransferase activity was assayed using the relevant phosphobase substrate namely PEA, PMEA, or PDEA. Each assay consisted of 100 μL of 150 mM Hepes-KOH buffer (pH 7.8, 22 °C)/1 mM Na_2-EDTA, 5 µL of 7.5 mM phosphobase (PEA, PMEA, or PDEA), 2.5 µL of 12 mM SAM, 1 μL of [^{3}H]SAM (0.55 μCi; 1.22x10^6 dpm), 21.5 μL H_2O, and 25 µL of sample extract to a final volume of 150 µL. Control assays used 25 µL of H_2O in place of sample extract. In order to carry out assays more efficiently, a mixture of the above components was prepared to which the sample extract was later added to start the assay (i.e. 15 μL of a 10-fold concentrated mixture was aliquoted into each of 10 assay tubes). Upon addition of the sample extract the mixture was gently vortexed to mix and the assay tube was incubated in a water bath at 30 °C for 30 min. The reaction was stopped by the addition of 1 mL ice cold H_2O, the diluted mixture was vortexed to mix and the tubes were placed on ice until the subsequent steps were performed. In order to distinguish ^{3}H present in [^{3}H]SAM from ^{3}H present in the [^{3}H]P-base products, a 1-mL aliquot of the diluted reaction mixture was applied to the prepared column containing 1-mL Dowex 50W (H^+) X8-200 resin. The contents of the column was washed three times with 0.5 mL H_2O each time and then the column was transferred to a 16 x 150 mm disposable test tube. P-bases bound to the Dowex

resin were eluted by washing the resin with 10 mL of 0.1N HCl. The eluate was vortexed and a 1-mL volume was transferred to a vial containing 5 mL Ready Safe® fluor (Beckman). The contents of the scintillation vial were vortexed to mix and the amount of radioactivity present quantified with a Beckman LS 1801 liquid scintillation counter. Counting efficiency for ^3H in 1 mL of 0.1 N HCl using 10 mL of Ready Safe fluor was determined previously to be 41.6% (unpublished). When counts per min (cpm) approached 3000 (equivalent to 2.75 nM SAH), sample extracts were diluted several-fold using 50 mM Hepes-KOH (pH 7.8, 22 °C), 1 mM Na_2-EDTA, 5 mM DTT in order to ensure linearity of the assay. SAH at a concentration of 10 nM was shown to inhibit PEAMeT activity by 53% and PMEAMeT by 66% (Smith *et al.*, 2000; Dhadialla, 1999).

CHLOROPHYLL AND PROTEIN CONCENTRATION

Protein concentration of the sample extracts was determined by a colorimetric method developed by Bradford (1976). Each protein assay consisted of 700 µL H_2O, 200 µL Bio-Rad Bradford's Reagent (Bio-Rad), and 100 µL protein extract. The mixture was gently vortexed to mix and the absorbance read at 595 nm after 15 min. A control assay with 800 µL H_2O and 200 µL Bradford's Reagent was used to zero the absorbance of the spectrophotometer. Protein concentrations were calculated by comparison to a standard curve generated using bovine serum albumin (BSA). When required, protein extracts were diluted with H_2O prior to performing the Bradford's protein assay.

Chlorophyll concentration was determined for each batch of leaf tissue ground. Routinely 25 µL of crude extract was combined with 3 mL of 80% (v/v) acetone in a 13 X

100 mm test tube that was covered with foil to exclude light. The mixture was vortexed and then centrifuged at 22 °C for 3-5 min at speed 7 in an IEC clinical tabletop centrifuge. The absorbance of the supernatant was then read at 700, 663, and 645 nm. Chlorophyll concentration was calculated in $\mu g^{-1}mL^{-1}$ using the absorption coefficients of Arnon (1949) and the formula: $20.2(A_{645}-A_{700}) + 8.02\ (A_{663}-A_{700})$.

ENZYME EXTRACTION PURIFICATION PROCEDURE

Enzyme Extraction

Spinach (*Spinacea oleracea*) leaves were purchased from a local supermarket and stored (1-2 h) at 4 °C until used. All procedures were done on ice or at 4 °C. A batch consisting of 1.5 to 2.5 kg spinach leaves were de-veined, coarsely chopped with a razor blade, weighed, and then homogenized in a Waring blender with two volumes of grind buffer (100 mM Tris-HCl (pH 7.8, 4 °C), 2 mM Na_2-EDTA, and 5 mM dithiothreitol (DTT, BioShop Canada)). The brei was filtered through four layers of cheesecloth and two layers of miracloth (Calbiochem) and the filtrate was collected into a cold 2 L beaker. A 25 µL aliquot was taken for a chlorophyll determination and the remaining filtrate was centrifuged at 4 °C for 11 min at 12,000*g* in 250-mL centrifuge bottles using a Beckman JLA 16.25 rotor. The pellets were discarded and the supernatants pooled in a chilled 4 L beaker. A 1-mL aliquot of the crude sample supernatant was removed, desalted and then used for methyltransferase assays and protein concentration determination. Desalting was performed by centrifuging 180 µL of sample through Sephadex G-25 medium (Pharmacia) equilibrated with Hepes buffer containing 100 mM Hepes-KOH (pH 7.8, 4 °C), 1 mM Na_2-EDTA, and

5 mM DTT (Datko and Mudd, 1988).

The first purification step involved $(NH_4)_2SO_4$ precipitation. The filtrate collected above was slowly stirred at 4°C and $(NH_4)_2SO_4$ (267 gm L^{-1}) was added stepwise to a final concentration of 1.8 M (Wood, 1976). After stirring for 40 min, the sample was centrifuged at 4 °C for 11 min at 12,000*g*. The pellet was resuspended in grind buffer and a 1-mL aliquot was removed, desalted and used for a MeT assay and protein determination. This pellet had no PMEAMeT activity and so was discarded. The supernatant was stirred at 4 °C with gradual addition of $(NH_4)_2SO_4$ (139 gm L^{-1}) to a final concentration of 2.6 M, stirred for 40 min, and then centrifuged at 4 °C for 11 min at 12,000*g*. The 2.6 M $(NH_4)_2SO_4$ supernatant was discarded since no P-base MeT activity was found in this fraction. The 1.8 - 2.6 M $(NH_4)_2SO_4$ pellet was dissolved in a minimal volume (50-100 mL) of Buffer A (100 mM Tris-HCl pH 7.8, 4 °C, 1 mM Na_2-EDTA, 10% (v/v) glycerol, and 5 mM DTT). A 180 µL aliquot was removed, desalted by centrifugation through Sephadex G25 equilibrated in Hepes buffer (100 mM Hepes-KOH (pH 7.8, 4 °C), 1 mM Na_2-EDTA, and 5 mM DTT) and used for P-base MeT activity assays and protein concentration. The remainder of the 1.8 - 2.6 M $(NH_4)_2SO_4$ fraction was dialysed to remove residual $(NH_4)_2SO_4$ using dialysis tubing with a 12,000 to 14,000 MW cut-off suspended in 2 L of Buffer A at 4 °C. Dialysis was performed overnight and the following day and required four changes with 2 L of Buffer A each time. A 180 µL aliquot of the dialysed 1.8 - 2.6 M $(NH_4)_2SO_4$ fraction was removed, desalted by centrifugation through Sephadex G25 as described above and used for P-base MeT activity assays and protein concentration measurements. The remainder of the fraction was flash frozen in liquid N_2 in 50-mL conical polypropylene tubes and stored at -80 °C until further

use. This entire grinding and fractionation procedure was repeated five times with successive batches of spinach leaves.

Column Chromatography

For all low pressure column chromatography steps a Gilson Model 740 Protech system controller with software running on a IBM/PC was used to control the operation of two Gilson Minipulse 3 peristaltic pumps via a Gilson 506B system interface. A Gilson 112 UV detector was hooked up to a Kipp and Zoen BD112 chart recorder for continuously measuring the absorbance of the column effluent at 280 nm. For all high pressure chromatography a Waters 625 LC system controller, Waters model 625 high performance liquid chromatography (HPLC), Waters 486 tunable absorbance spectrophotometer, and a Gilson FC 204 fraction collector were interfaced with a IBM/PC running Baseline 810 software. All equipment except the computers were kept in a Coldstream refrigeration unit set to 4 °C. Buffers used for column chromatography steps were filtered through 0.45 μm membrane filter (Gelman Sciences FP-450) prior to use.

Following column chromatography procedures, pooled fractions were concentrated and dialysed at 4 °C by ultrafiltration using an Amicon 8050 stirred cell concentrator with a YM-30 membrane (Millipore) To prevent losses incurred through the binding of proteins to the ultrafiltration cell, the entire cell and stirrer were passivated with 6% (v/v) polyethylene glycol (PEG) at 22 °C overnight (Blatt *et al.*, 1968). The ultrafiltration apparatus was then equilibrated by passing two volumes of dialysis buffer through the membrane. P-base MeT assays and protein determinations were performed on the ultrafiltrate flow-through in addition

to the pre- and post- concentrated samples.

Each of the five bulk preparations was processed independently for each purification step until the volume of purified sample extract decreased to where samples could be combined and managed more easily. Between each purification step, samples were frequently concentrated and dialysed, flash frozen in liquid N_2 and then stored at -80 °C in passivated conical tubes. Samples were thawed for use in subsequent steps and no losses of enzyme activity were detected following repeated freeze-thaw cycles carried out in this manner.

Anion Exchange Chromatography on DEAE Sepharose CL-6B

DEAE Sepharose CL-6B (Sigma DCL-6B-100) anion exchange matrix was poured into a Bio-Rad column (5 cm X 20 cm, 325 mL bed volume). The column was washed and regenerated between each sample application using a series of solvents in the following order: 1 L 1 N NaOH, 2 L H_2O, 1 L 1 M Na-acetate pH 3.0, and finally 2 L of H_2O. For use, the DEAE matrix was equilibrated with 1 L Buffer A at a flow rate of 1.0 mL min^{-1} before the sample was loaded. The sample extract was thawed, centrifuged at 10,000*g* for 10 min to remove any particulates, and after removal of a 180-µL aliquot, the extract was diluted 2-fold with Buffer A and then loaded onto the column. Column flow-through was collected following both the sample loading and subsequent column wash and aliquots from each were assayed for P-base MeT activity and protein concentration. When A_{280} of the effluent stabilized near zero, protein bound to the column was eluted with a linear 1 L 0 - 500 mM NaCl gradient. Eluate from the column was collected with a fraction collector set to collect 5-mL fractions in 13 X 100 mm disposable test tubes. A 100-μL aliquot was removed from

every fifth fraction and used to assay for P-base MeT activity and protein concentration in order to prepare an elution profile for this purification step. Fractions with the highest PMEAMeT activity were pooled, a 180-μL aliquot was removed for enzyme activity and protein assays, and the remainder was dispensed into 50-mL conical tubes, flash frozen with liquid N_2 and then the tubes were stored at -80 °C. The results obtained from assaying P-base MeT activity and protein concentration in the sample load and subsequently pooled eluate were used to calculate the % recovery of PEAMeT, PMEAMeT, and PDEAMeT activities and protein for samples representing all stages of the DEAE column fractionation procedure.

Hydrophobic Interaction Chromatography on Phenyl Sepharose CL-4B

Phenyl Sepharose CL-4B (Sigma P-7892) hydrophobic interaction media was poured into a Bio-Rad column (2.5 cm X 20 cm, 100 mL bed volume). The column matrix was washed and regenerated in a 500 mL beaker with the following solvents in order: 1 L H_2O, 500 mL 25% (v/v) ethanol, 500 mL 50% (v/v) ethanol, 500 mL 99% (v/v) ethanol, 1 L n-butanol, 500 mL 99% ethanol, and finally with 1 L of H_2O. The column was equilibrated with 500 mL of Buffer A containing 25% (w/v)$(NH_4)_2SO_4$ at 1.0 mL min^{-1}. The pooled sample prepared by chromatography on DEAE Sepharose (above) was thawed, inverted slowly to mix, and $(NH_4)_2SO_4$ (140 gm L^{-1}) was added to the sample until a final concentration of 1.0 M $(NH_4)_2SO_4$ was reached. A 150-μL aliquot of the sample was removed for P-base MeT activity measurement and protein determination; the remainder was loaded onto the column at a flow rate of 0.5 mL min^{-1}. The non-adsorbed protein was washed from the column with Buffer A containing 25% $(NH_4)_2SO_4$ at 1 mL min^{-1} until the A_{280} of the effluent approached

zero. The effluent from the column was collected, its volume determined, and an aliquot was assayed for PMEAMeT activity and protein concentration. The adsorbed proteins were eluted from the column at a flow rate of 1.0 mL min^{-1} with a 1 L linear gradient of 25%-0% $(NH_4)_2SO_4$/0%-50% ethylene glycol using 25% $(NH_4)_2SO_4$ (w/v) in Buffer A and 50% ethylene glycol (v/v) in Buffer A in the two solvent reservoirs for mixing. The column eluate was collected in 6-mL fractions into 13 X 100 mm disposable test tubes. Gradient elution was followed by a 0.2 L wash with 50% ethylene glycol (v/v) in Buffer A. An aliquot from every fifth fraction was assayed for PMEAMeT activity and protein concentration in order to plot an elution profile for this purification step. Fractions with the highest PMEAMeT activity were pooled, $(NH_4)_2SO_4$ was added to a final concentration of 2.6 M (0.413 gm mL^{-1}), and the solution was stirred for 40 min before centrifugation at 15,000*g* for 11 min. The supernatant was discarded and the pellet was resuspended in Buffer A, dialyzed and then concentrated to approximately 15 mL using a passivated Amicon ultrafiltration cell. Concentrated and dialyzed samples were used to assay for P-base MeT activity and protein concentration, then flash frozen in liquid N_2, and stored at -80 °C. P-base MeT activities and protein concentrations of the sample loaded onto the column and the pooled sample obtained following the column were compared to estimate the % recoveries of PEAMeT, PMEAMeT, and PDEAMeT activities and protein following chromatography on this matrix.

Anion Exchange Chromatography on Macro-Prep® High Q

High Q anion exchange media (Bio Rad 156-0040) was poured into a Bio-Rad column (2.5 cm X 20 cm, 50 mL bed volume). The column matrix was washed and regenerated with

the following solvents in order: 1 L 0.1 M NaOH, 100 mL 1.0 M NaCl, and finally with 1 L of H_2O at a flow rate of 1.0 mL min^{-1} before equilibration with 0.5 L Buffer A. Samples prepared by chromatography on Phenyl Sepharose were thawed, mixed, a 150-μL aliquot removed, and the remainder diluted with Buffer A to <10 mg protein mL^{-1}. The aliquot was assayed for P-base MeT activity and protein concentration and the diluted sample was loaded onto the column matrix at a flow rate of 1.0 mL min^{-1}. After loading, the column was washed with Buffer A to remove non-adsorbed proteins until the A_{280} of the effluent approached zero. Effluent from the column was collected, its volume measured, and an aliquot removed for PMEAMeT activity and protein concentration determinations. Adsorbed proteins were eluted from the matrix at 1.0 mL min^{-1} with a 0.5 L linear gradient of 0 - 0.3 M NaCl in Buffer A followed by a 100 mL wash of Buffer A containing 0.3 M NaCl. The column eluate was collected in 5-mL fraction volumes in 13 X 100 mm disposable test tubes. An aliquot was taken from every fifth fraction and assayed for PMEAMeT activity and protein concentration in order to plot an elution profile for this purification step. Fractions with high PMEAMeT activity were pooled, dialyzed, and concentrated to approximately 10 mL using a passivated Amicon Ultrafiltration cell. Concentrated and dialyzed samples were assayed for P-base MeT activity and protein concentration, then flash frozen in liquid N_2, and stored at -80 °C. P-base MeT activities and protein concentrations of the sample loaded along with those of the pooled sample were used to estimate the % recoveries of PEAMeT, PMEAMeT, and PDEAMeT activities and protein following chromatography on this matrix.

Gel Filtration Chromatography on HiPrep® Sephacryl S-100

A 320-mL Sephacryl S-100 High Resolution gel filtration column (Pharmacia Biotech 26/60 #17-1194-01) was initially washed with 400 mL of H_2O at 2.1 mL min^{-1} and then with 1 L of 0.2 M NaOH at 0.5 mL min^{-1}. The column was then washed with 400 mL of H_2O and equilibrated with 640 mL of Buffer B (20 mM Tris-HCl (pH 7.8, 4 °C), 150 mM NaCl, 5 mM DTT, 5% (v/v) glycerol). In the first trial on Sephacryl S-100 (denoted later as S-100#1) the sample loaded onto the column was prepared by pooling fractions from two separate High Q chromatography runs from spinach leaves which were ground on Nov 18/98 and Dec 2/98. The two extracts prepared by chromatography on High Q were thawed, pooled and then dialyzed and concentrated to a final volume of approx. 10 mL using a passivated Amicon ultrafiltration cell. In the second trial on S-100 (later denoted as S-100#2) samples prepared by several runs on High Q (arising from bulk spinach leaf preparations Feb 22/99, Jan 11/99, and Mar 2/99) were thawed, pooled, and then concentrated as described above. An 150-μL aliquot of the pooled sample to be loaded was taken and assayed for P-Base MeT activity and protein concentration before the remainder (15 mL) was loaded onto the column at 0.25 mL min^{-1}. In S-100#1, elution of adsorbed proteins from the column was carried out at 1.0 mL min^{-1} with 250 mL of Buffer B, while for S-100#2 elution of adsorbed proteins from the column was carried out at 0.25 mL min^{-1} with 250 mL of Buffer B. In both S-100 trials, 2-mL fractions were collected in 13 X 100 mm disposable test tubes and aliquots from every fifth fraction collected were assayed for PMEAMeT activity and protein concentration in order to plot an elution profile for this purification step. Samples with high PMEAMeT activity were pooled, a 150 μL aliquot was removed and then the remaining sample was

dispensed into passivated 15-mL conical tubes, flash frozen in liquid N_2, and stored at -80 °C. Comparison of P-base MeT activities and protein content between the sample load and the final pooled sample prepared from this column were used to calculate the % recovery activity of PMEAMeT and protein following gel filtration chromatography on Sephacryl S-100.

Anion Exchange Chromatography on Mono Q® HR 5/5

A 1-mL Mono Q HR 5/5 anion exchange column (Pharmacia 17-0546-01) was washed with 300 μL 5% (v/v) Triton X-100, 300 μL 2 M NaCl, 300 μL 2 M NaOH, 400 μL of 45% (v/v) acetic acid and then with 500 mL H_2O at a flow rate of 0.5 mL min^{-1}. In order ensure the column matrix was free of impurities, a mock 0 - 1 M NaCl gradient prepared in Buffer C (20 mM Tris-HCl (pH 7.8, 4 °C), 5 mM DTT, 5% (v/v) glycerol) was run before equilibration with 600 mL of Buffer C. Fractions numbered 55 to 59 that were collected during chromatography on Sephacryl S-100 (#2) were pooled, dialyzed and concentrated with Buffer C to a volume of 6.4 mL using a Amicon Centricon 30. A 100 μL aliquot of the pooled and concentrated sample extract was removed for P-Base MeT activity and protein concentration determination before the remainder was loaded onto the column at 0.5 mL min^{-1}. In the next Mono Q chromatography, the Sephacryl S-100 (#1) sample fractions numbered 19 to 21 were thawed, pooled, and concentrated as described above. A 100 μL aliquot of the pooled and concentrated sample extract was removed for P-Base MeT activity and protein concentration determination before the remainder was loaded onto the column at 0.5 mL min^{-1}. The non-adsorbed protein was washed from the column with Buffer C at 0.5 mL min^{-1} until

the A_{280} of the effluent approached zero. Flow-through from the column was collected, the volume determined, and an aliquot was assayed for PMEAMeT activity and protein concentration to calculate the amount of PMEAMeT activity present in the effluent. Adsorbed proteins were eluted from the column matrix by a linear 30 mL 0 - 1 M NaCl gradient in Buffer C at 0.5 mL min^{-1}. The column eluate was collected in 1-mL fractions. A 100 μL aliquot was taken from every fraction and used to assay for PMEAMeT activity and protein concentration in order to plot an elution profile for this purification step. Samples with high PMEAMeT activity were pooled, a 150 μL aliquot was removed, and the remainder was flash frozen in liquid N_2 and stored at -80 °C. Comparison of P-base MeT activities and protein content in the load and pooled sample extracts were used to calculate the % recovery activity of PMEAMeT activity and protein following chromatography on the Mono Q anion exchange matrix.

Gel Filtration Chromatography on Protein Pak® SW-300

A Beckman 421 HPLC controller, Beckman 112 solvent delivery HPLC pump, Waters 421 tunable absorbance detector, and a Gilson FC 204 fraction collector were assembled and operated at 22 °C. A 13.3 mL Protein Pak 300 SW HPLC gel filtration matrix (Waters 011757) was first washed using 300 mL of 0.2 μm filtered H_20 at a flow rate of 0.5 mL min^{-1} and then equilibrated with 50 mL of Buffer D (50 mM Tris-HCl (pH 7.4, 22 °C), 100 mM KCl, 1 mM DTT and 5% (v/v) glycerol) at a flow rate of 0.5 mL min^{-1}. Samples prepared by chromatography on Mono Q (50 μL volume loaded per run) were thawed and loaded onto a 100 μL sample loop using a 1 cc syringe (B-D J09602). Proteins were carried

through the column with 5 mL of Buffer D at a flow rate of 0.5 mL min^{-1}. The initial 2-mL flow-through was collected in a 15-mL conical polypropylene tube and then stored temporarily at 4 °C. Next, 100-μL fractions were collected in siliconized 1.5 mL microfuge tubes (Fisherbrand, 05-541-13). Collection tubes were kept cool by sitting the microfuge tubes in 13 X 100 mm test tubes filled with water chilled to 4 °C. Proteins eluting from the column matrix were continuously monitored by measuring the A_{280} and recording the output using a Kipp and Zonen BD112 chart recorder. Fractions associated with absorbance peaks were assayed for PMEAMeT activity as were aliquots taken from the initial flow-through and sample loaded. Fractions found to have PMEAMeT activity were subjected to SDS-PAGE analysis (see below) in order to determine which polypeptides corresponded to PMEAMeT protein.

SDS-POLYACRYLAMIDE GRADIENT GEL ELECTROPHORESIS

Sample extracts were separated electrophoretically on a 7.5-15% SDS-polyacrylamide gradient gel using the buffer system of Nelville (1971). Extracts were diluted with an equal volume of SDS-solubilizing buffer (60 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 1% (w/v) SDS, 1% (w/v) DTT and 0.002% (w/v) bromophenol blue), vortexed lightly and incubated at 90 °C for 3 min immediately before loading onto the gel (Merrick, 1983). For Mr determination, polypeptides of known Mr (Bio-Rad SDS-PAGE low range molecular weight kit, cat# 161-0304) were diluted in SDS-solubilizing buffer (at a ratio of 1/20 or 1/100 for visualizing polypeptides by Coomassie dye or silver staining, respectively) and then incubated at 90 °C for 3 min before loading onto the gel. Electrophoresis was performed at a constant

current of 15 mamp/1.5 mm gel thickness for 5 hours at 15 °C until the SDS-solubilizing buffer dye was approx 0.2 cm from the bottom of the gel.

Gels were stained with either silver reagent or a Coomassie protein stain after electrophoresis was completed. For silver staining, gels were first soaked in 50% (v/v) reagent grade methanol overnight before staining following the method of Wray *et al.*, (1981). Gels to be stained with the Coomassie protein stain were soaked overnight in Coomassie solution (0.1% (w/v) Coomassie Brilliant Blue R250, 25% (v/v) isopropanol, 10% (v/v) glacial acetic acid and 0.1% (w/v) cupric acetate). Destaining to remove excess dye was done by gentle agitation in 40% (v/v) methanol and 7% (v/v) glacial acetic acid using foam plugs to speed up the process. Both silver and Coomassie dye stained gels were then soaked in gel drying solution (40% (v/v) methanol, 7% (v/v) glacial acetic acid, and 3% (v/v) glycerol) for 2 h. Coomassie dye stained gels to be used for fluorography were soaked in Amplify (Amersham NAMP 100) fluorographic reagent for 30 to 45 min with gentle agitation. Gels were dried between two sheets of hydrated cellophane (Bio-Rad 1650963) and air dried (Wallevik and Jensenius, 1982). The position of polypeptide standards and the top and bottom of the gel were marked using ink spiked with [^{14}C]choline. Dried gels were exposed to X-ray film (Kodak X-OMAT-AR) at -80°C for periods up to 3 months before the X-ray films were developed using a Kodak M35A X-OMAT Diagnostic Imaging Processor.

PHOTOAFFINITY CROSS-LINKING OF [^{3}H]SAM TO PMEAMeT

For photoaffinity cross-linking, 60 μg of partially purified sample extract was dispensed into individual wells of an Falcon 3911 Microtest III assay plate (Becton

Dickinson) kept on ice. All plasticware used was dimethyldichlorosilane (BDH 331644V) treated in order to prevent protein binding to the surface of the wells. [^{3}H]SAM was dispensed into each well to a final concentration of 200μM and the contents of the well were mixed. PEA, PMEA, PDEA, PCho, or K-Pi, if used, were added to a final concentration of 1 mM and were dispensed into wells containing protein samples before exposure to UV light. A Mineralight UVS-54 lamp (short wave 254 nm, Ultraviolet Products Inc.) was set directly on top of the microtitre plate and the methyltransferase/[^{3}H]SAM/P-base mixture was exposed to UV light for 30 min. The reaction was stopped by the addition of an equal volume of SDS-solubilizing buffer to each well. The contents of each well were transferred to individual 0.5 mL microfuge tubes, heated at 90 °C for 3 min, and then loaded onto a 7.5% to 15% SDS-PAGE gradient gel for electrophoresis. Polypeptides were revealed by staining the gels with a Coomassie dye stain solution followed by destaining, and any radiolabeled polypeptides were identified by fluorography as described above.

THIN LAYER CHROMATOGRAPHY

A sample fraction prepared by gel filtration on SW300 was assayed for PEAMeT, PMEAMeT, and PDEAMeT activity with [^{14}C]-labeled SAM and samples were processed as for the [^{3}H]SAM based assays. However, a 20 μL volume of substrates PEA, PMEA, PDEA, and PCho each at final concentration of 300 mM was added to the 10 mL of 0.1 N HCl eluted from the Dowex 50W(H^+) columns. The [^{14}C]-labeled and non-labeled phosphobases were flash-frozen in liquid N_2 and concentrated by freeze drying using a Labconco Freezone Plus 6 freeze dryer. The freeze-dried sample was dissolved with 200 μL

0.1 N HCl and then the container was washed with two additional 200 μL washes of 0.1 N HCl. The 600 μL collected was concentrated by N-evaporation at 40 °C. The dried samples were then dissolved in 10 μL of 0.1 N HCl and stored at -80 °C before further processing. Aliquots of each sample were then analyzed by thin layer chromatography (TLC). A Silica G plate (Macherey-Nagel, 20 X 20 cm) was oven dried for 24 h at 50 °C and then allowed to cool to room temperature. A pencil line was drawn onto the plate 1 cm from the bottom with points marked at 1 cm intervals. In order to drive off any contaminants, the plate was equilibrated overnight in a tank with 110 mL of 50-50-10 (v/v/v) n-butanol, methanol, HCl. In the morning, the plate was allowed to dry and then 1 μL or 4 μL of each sample dissolved in 0.1N HCl were applied in 0.5 μL aliquots. A fan was used to aid evaporation of the sample solvent between each application. Plates were then placed into the TLC tank and developed by allowing the solvent to ascend for 6 h. The plate was then air-dried and radiolabeled P-bases identified following a 1 week exposure of the plate to X-ray film (Kodak X-OMAT-AR) at -80 °C. The X-ray film was developed using a Kodak M35A X-OMAT Diagnostic Imaging Processor. In order to visualize the relative positions of the non-radiolabeled phosphobases the plate was then evenly sprayed with a Pi-specific acid molybdenum blue reagent that consisted of: 85.6 mM $(NH_4)_6MO_7O_{24} \cdot 4H_2O$, 0.0076% (v/v) concentrated HCl, 0.05% (v/v) perchloric acid made up to a 50 mL volume with acetone. After air drying, the plates were exposed to UV light Mineralight UVS-54 lamp (short-wave 254 nm, Ultraviolet Products Inc.) for approximately 30 min at a distance of 5 cm. All of the phosphorous-containing compounds stained blue and their relative mobility (R_f) was determined by measuring the distance from the origin to the midpoint of the respective blue spot.

BSA PROTEIN STANDARD CURVE

In order to accurately quantify the level of protein in fractions eluting from a SW-300 gel filtration column, a standard curve was prepared against known concentrations of BSA. Seven serial dilutions of BSA at 0.000781, 0.0156, 0.0312, 0.0625, 0.125, 0.25, and 0.5 mg mL^{-1} concentrations were prepared using a 1 mg mL^{-1} BSA stock concentration. Dilutions were made by initially transferring 0.5 mL of the BSA stock solution to a 13 X 100 mm test tube containing 0.5 mL of H_2O, thus resulting in a final concentration of 0.5 mg mL^{-1} BSA. The contents of the tube were lightly vortexed and a 0.5 mL volume was transferred into another 13 X 100 mm test tube containing 0.5 mL of H_2O giving a final concentration of 0.25 mg mL^{-1} BSA. This procedure was repeated five more times in order to dilute BSA to 0.000781 mg mL^{-1}. Samples were then transferred into a 1 mL quartz cuvette and the absorbance at 280 nm was read against a H_2O blank. The A_{280} absorbance values were then plotted against the protein concentration of the samples giving the equation of the standard curve as:

$y= 1.6496(x) + 4.671X10^{-4}$

Protein concentration for SW-300 eluted sample fractions could then be calculated using the absorbance at 280 nm measured for each eluted sample fraction. Figure 8 shows BSA concentration in mg mL^{-1} plotted against the absorbance at 280 nm for each concentration.

RESULTS

Partial Purification of PMEAMeT

In order to obtain sufficient plant material from which to isolate PMEAMeT protein, five bulk extractions of spinach leaves were performed (Nov. 18/98, Dec. 2/98, Jan. 11/99, Feb. 22/99, and Mar. 2/99). The material chosen was spinach available in local markets because this source of leaves is greatly diminished in PEAMeT activity (Dhadialla, 1999). PEAMeT activity in spinach plants exposed to prolonged (48 h) exposure to continuous dark Weretilnyk *et al.*, (1995) was reduced to 25% of the activity level present in control plants growing under normal diurnal conditions (8h light / 16h dark). In contrast, PMEAMeT and PDEAMeT activities in leaves of these dark-treated plants remained almost the same at 86 and 108%, respectively, of control levels (Weretilnyk *et al.*, 1995). The absence of any apparent regulation of PMEAMeT activity by light allowed for the use of spinach leaves which were purchased from a local market and, in fact, made this a preferred source of leaf material because the PMEA *N*-methylating activity contributed by PEAMeT was also greatly diminished.

PMEAMeT was partially purified from soluble proteins extracted from spinach leaves using, as a first stage, four steps of a purification strategy devised by Sharon Dhadialla (Dhadialla, 1999) and then augmenting this strategy with chromatographic separations on other column matrices. Specifically, the initial four steps involved extraction of soluble leaf proteins, preparation of a 1.8 - 2.6 M $(NH_4)_2SO_4$ protein fraction from the crude leaf extract and then successive separations of the 1.8 - 2.6 M $(NH_4)_2SO_4$ fraction by column

TABLE I: Schematic key to steps used in the partial purification of PMEAMeT

SPINACH HARVEST DATE	Nov 18/98	Dec 2/98	Jan 11/99	Feb 22/99	March 2/99
Purification Steps[a]	1.8 to 2.6 M $(NH_4)_2SO_4$	1.8 to 2.6 M $(NH_4)_2SO_4$	1.8 to 2.6 M $(NH_4)_2SO_4$	1.8 to 2.6 M $(NH_4)_2SO_4$	1.8 to 2.6 M $(NH_4)_2SO_4$
	DEAE-Sepharose	DEAE-Sepharose	DEAE-Sepharose	DEAE-Sepharose	DEAE-Sepharose
	Phenyl-Sepharose	Phenyl-Sepharose	Phenyl-Sepharose	Phenyl-Sepharose	Phenyl-Sepharose
	High Q	High Q	High Q	High Q	High Q
	S-100		S-100		
	Mono Q		Mono Q		
	SW-300		SW-300		

[a] 1.8 to 2.6M $(NH_4)_2SO_4$ = dialysed 1.8 to 2.6 M $(NH_4)_2SO_4$ fraction preparations, DEAE = DEAE-Sepharose CL 6B, Phenyl-Sepharose = Phenyl-Sepharose Cl-4B, High Q = FPLC High Q, S-100 = Sephacryl S-100, Mono Q = HPLC Mono Q, SW-300 = HPLC Protein Pak SW-300

chromatography on DEAE CL-6B anion exchange matrix, Phenyl-Sepharose CL-4B hydrophobic interaction, and High Q anion exchange. In this study, samples prepared through to the chromatography step on High Q anion exchange were then subjected to additional separation steps involving gel filtration through Sephacryl S-100, HPLC Protein Pak SW-300, and anion exchange chromatography on a Mono Q anion exchange matrix. A summary of the purification steps performed on the five crude spinach preparations is given in Table I. PMEA-methylating (PMEAMeT) activity and protein concentration determinations were performed at all stages of purification including samples loaded onto columns, the column effluent, selected fractions collected following chromatography and any samples pooled, dialyzed and concentrated following chromatography. PEAMeT and PDEAMeT activities were frequently measured as well. Enzyme activity and protein determinations allowed for specific activity determinations as well as estimates of the recoveries for total enzyme activity and total protein following each purification step.

In order to facilitate handling of large sample volumes, the five bulk crude extractions described above were processed through to the chromatography step on the High Q matrix as individual, independent preparations. The results obtained at each step of the five preparations are shown in Tables II through VI and are identified individually by the date of the initial crude extraction from leaves and will be discussed in greater detail below. Following the High Q anion exchange step, however, fractions containing PMEAMeT activity from separate preparations were frequently pooled in order to provide sufficient PMEAMeT activity to detect during subsequent purification steps (Table I). For example, the crude preparations prepared on Nov 18/98 and Dec 2/98 were processed separately through to High

Q anion exchange whereupon fractions containing high PMEAMeT activity from the two separations were pooled and processed together on a Sephacryl S-100 column (Table VII). Likewise, crude preparations from Jan 11/99, Feb 22/99, and Mar 2/99 were pooled at a similar stage of purification and then processed as a pooled sample on a Sephacryl S-100 matrix (Table VIII).

In addition to the chromatography steps described above, other strategies for the purification of PMEAMeT were also explored. Affinity chromatography using an SAH-Sepharose column has been used successfully for the purification of *S*-adenosyl-L-methionine:nicotinic acid *N*-methyltransferase from cell cultures of *Glycine max* (Upmeier *et al.*, 1988) and furanocoumarin *O*-methyltransferase from *Ruta graveolens* (Sharma and Brown, 1978). Three trials were used to test the ability of SAH-Sepharose and each trial involved the application of 241 nmol min^{-1} of PMEAMeT activity from a pooled Phenyl Sepharose sample (Feb 22/99) onto a 2-mL SAH-Sepharose column. Several strategies were attempted to try to selectively elute PMEAMeT activity from the bulk of the protein adsorbed onto this column and they included eluting adsorbed proteins with 2 X 4 mL of 20 mM Bis-Tris-Propane (pH 8.5, 4 °C), 1 mM Na_2-EDTA, 5 mM DTT, 10% (v/v) glycerol containing either buffer alone, 100 μM SAH, 200 μM SAH, 50 mM NaCl/200 μM SAH, or 200 mM NaCl. However, all of the PMEAMeT activity and protein adsorbed to the SAH-Sepharose matrix was only eluted by the buffer containing 200 mM NaCl and no purification of PMEAMeT was achieved. In another trial, a pH gradient (pH 6.5-8.0) with the same buffer (see above) containing 50 mM NaCl was used to elute adsorbed proteins from the SAH-Sepharose matrix. Again all of the PMEAMeT activity and the proteins adsorbed on the

SAH-Sepharose matrix were eluted only after the addition of 200 mM NaCl to the buffer. In the final trial to selectively elute PMEAMeT activity from the adsorbed proteins on the SAH-Sepharose matrix, the buffer was modified to include 50 mM NaCl with 100 μM PCho and 200 μM SAH, or 50 mM NaCl with 100 μM PMEA and 200 μM SAH. In this final trial, PMEAMeT activity and adsorbed proteins eluted from the SAH-Sepharose matrix only after 200 mM NaCl was added to the buffer.

Failure of the partially purified spinach PMEAMeT protein to be selectively eluted from the SAH-Sepharose matrix lead to testing the potential use of an adenosine-agarose matrix. Adenosine-agarose affinity chromatography has been used to purify *S*-adenosyl-L-methionine: methyl chloride transferase from *Batis maritima* (Ni and Hager, 1998), *S*-adenosyl-L-methionine: halide/bisulfide from *Brassica oleracea* (Attieh *et al.*, 1995), and tobacco *O*-methyltransferase from *Nicotiana tabacum* (Dumas *et al.*, 1988). A volume equivalent to 241 $nmol^{-1}min^{-1}$ of PMEAMeT activity from a desalted pooled Phenyl Sepharose sample (Feb 22/99) was applied to a 0.5-mL adenosine-agarose matrix. A buffer containing 20 mM Bis-Tris-Propane (pH 8.5, 4 °C), 1 mM Na_2-EDTA, 5 mM DTT, 10% (v/v) glycerol was used to elute non-adsorbed proteins from the adenosine-agarose matrix and this was followed by additions of buffer containing either 25 mM NaCl, 50 mM NaCl, 100 mM NaCl, 100 mM NaCl/1 mM SAM, 200 mM NaCl, or 200 mM NaCl/1mM SAM. PMEAMeT and protein concentration assays of the column fractions showed that all of the PMEAMeT and the bulk of the proteins were present in the first sample fraction eluted from the column matrix (results not shown). A second trial was performed using the same approach but using a sample purified by High Q chromatography (Nov 18/98). In this trial all fractions had almost

equal PMEAMeT activity and protein concentration suggesting that no purification of PMEAMeT was achieved by using this matrix.

Extraction and Fractionation by $(NH_4)_2SO_4$ Precipitation

As reported by Dhadialla (1999), PEA-methylating activity was substantially reduced relative to PMEA and PDEA methylating activities in the crude homogenate prepared from market spinach (Tables II through VI). In general, PEAMeT activity was only 2 to 13% of PMEAMeT activity levels in these extracts. This contrasts with levels of PEAMeT in leaf extracts of plants grown under controlled environment conditions which is generally around 69% of the level of total PMEAMeT activity (Smith *et al.*, 2000).

Recoveries of all three P-base MeT activities upon $(NH_4)_2SO_4$ fractionation of soluble protein crude leaf extracts were variable for the five different trials. The recoveries for enzyme activities found in the 1.8 to 2.6 M $(NH_4)_2SO_4$ fraction were, in general, higher than 50% of those total activities estimated to be present in crude extracts. The average recovery of total protein at this purification step was 26% for the five crude preparations (Tables II to VI). Considering total enzyme activities with estimates of total protein, estimated average fold-purifications of 2.8, 3 and 3 for PEAMeT, PMEAMeT and PDEAMeT, respectively, were obtained (Tables II to VI). No enzyme activity was detected for any of these substrates in the <1.8 M or >2.6 M $(NH_4)_2SO_4$ fractions.

DEAE Sepharose CL-6B Anion Exchange Chromatography

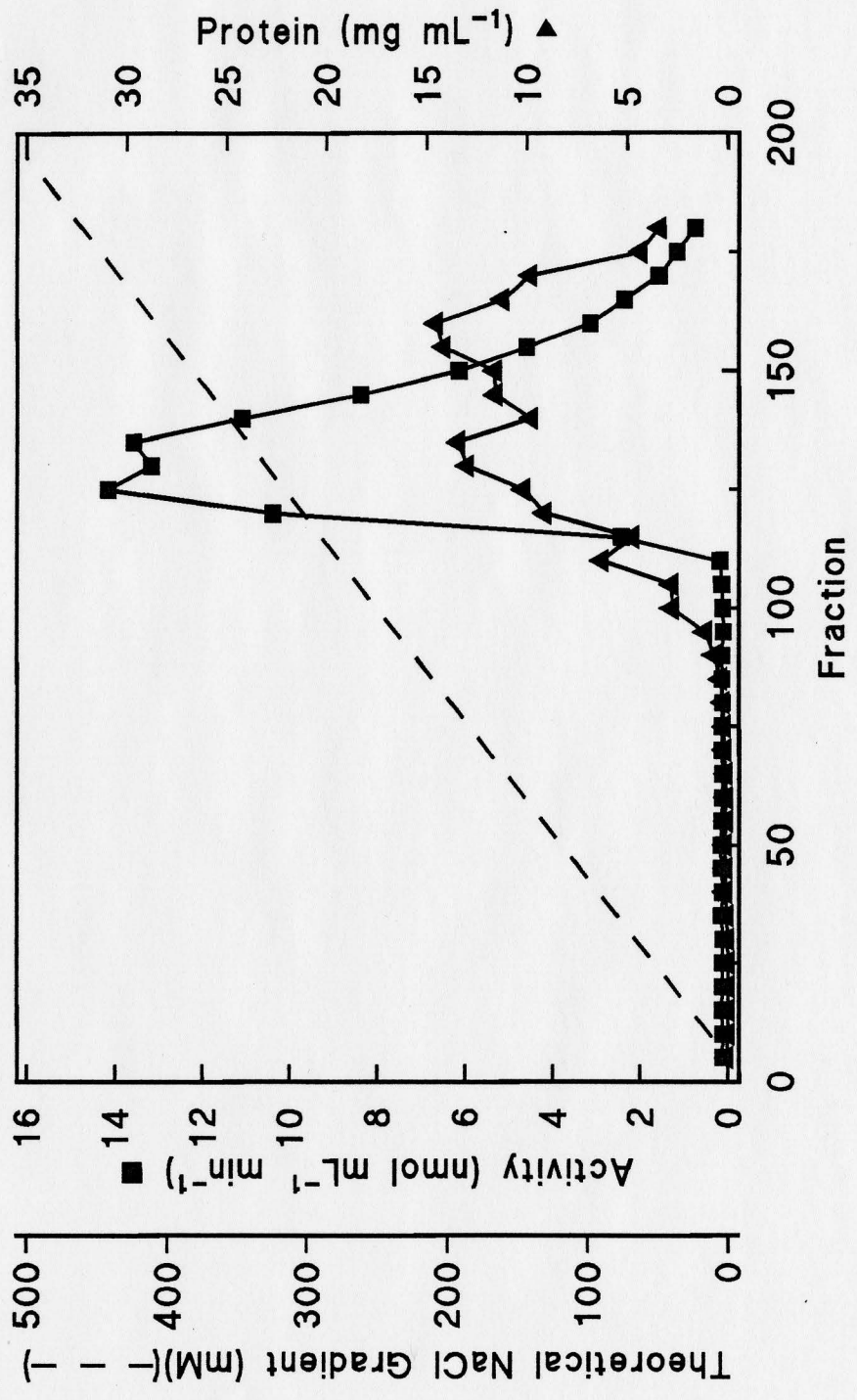
As described in the Materials and Methods section, the 1.8 - 2.6 M $(NH_4)_2SO_4$

fraction was dialysed and then flash-frozen for storage until the anion exchange step could be carried out. For application onto the DEAE Sepharose matrix, the dialysed 1.8 - 2.6 M $(NH_4)_2SO_4$ fraction was diluted 2-fold with Buffer A in order to ensure that the protein concentration of the sample loaded onto the matrix did not exceed 100 mg mL^{-1}. Of the protein loaded onto the DEAE Sepharose, an average of 68% adsorbed to the matrix and the remainder was found in the effluent containing non-adsorbed protein. No PMEAMeT activity was detected in the effluent. Figure 2 shows a typical elution profile for the DEAE Sepharose matrix when a linear 0 to 500 mM NaCl gradient is used to elute proteins adsorbed to this matrix. Fractions containing PMEAMeT activity were found to elute from the column in fractions that coincided with the first of two peaks of protein eluting from the column. The fractions associated with high PMEAMeT activity were then pooled, concentrated, and dialysed following this chromatography step.

Recoveries of PMEAMeT activity in these pooled and concentrated samples ranged from a low of 13% (Table II) to a high of 163% (Table IV) of the total activity detected in crude extracts, while recoveries for protein were below 10% of that found in the crude preparations. In general, the samples obtained following this column fractionation showed a 9- or 10-fold improvement in PMEAMeT specific activity over the crude extract, however an improvement of 21-fold was achieved using the Jan 11/99 sample (Table IV). PEAMeT and PDEAMeT activities were also detected in pooled and concentrated samples prepared by DEAE Sepharose. Recoveries of PEA- and PDEA-methylating activities ranged from 1% to 13% (Table II) to a high of 121% (Table V) and 213% (Table IV) for these two reactions, respectively. The fold-increase in PEAMeT specific activity was variable between the trials

FIGURE 2: Anion exchange chromatography on DEAE Sepharose (CL-6B) media

Representative elution profile of PMEAMeT activity (■, PMEA substrate), and adsorbed proteins (▲) from a DEAE Sepharose matrix (325 mL bed volume). Adsorbed proteins were eluted by a 1 L linear 0 to 0.5 M NaCl gradient (- - -) at 1.0 mL min^{-1} and the recoveries for PMEAMeT activity and protein are reported in Table V. The eluate was collected in 5 mL fractions and sample fractions containing the highest PMEAMeT specific activity were pooled before purification by Phenyl Sepharose (CL-4B) hydrophobic interaction chromatography.


Protein (mg mL^{-1}) ▲
35
30
25
20
15
10
5
0
200
150
100
50
0
Fraction
Activity (nmol mL^{-1} min^{-1}) ■
16
14
12
10
8
6
4
2
0
Theoretical NaCl Gradient (mM)(– – –)
500
400
300
200
100
0

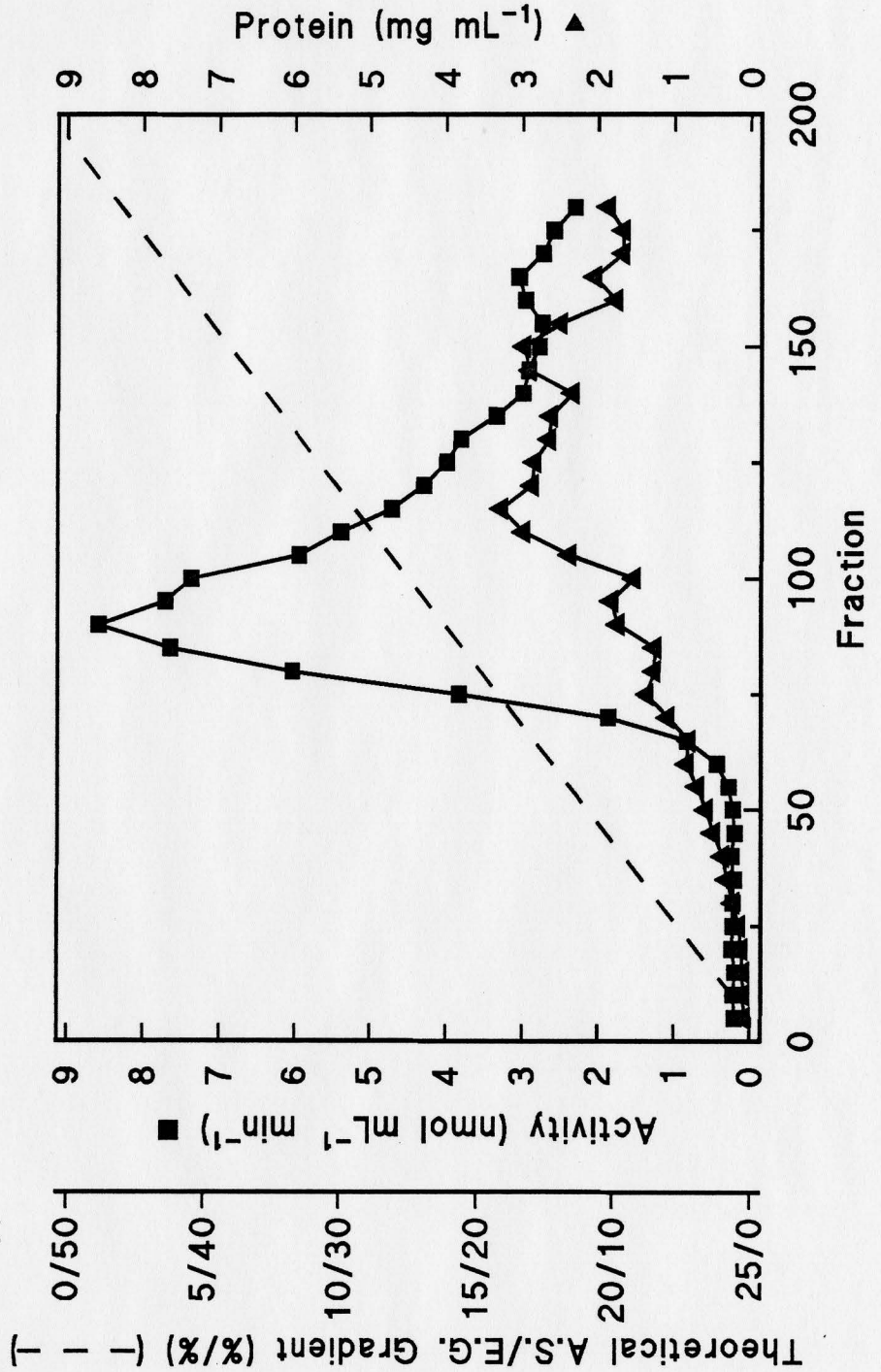
ranging from a low of 0.24-fold (Table III) to a high of 21-fold (Table V) and did not seem to correspond well to the more similar fold changes seen for PMEAMeT and PDEAMeT for any given trial (see Tables II and VI). In contrast, the fold improvement for PDEA-methylating specific activity following this chromatography step was comparable to that found for PMEAMeT activity for any given trial. Thus the highest improvement in specific activity was 21-fold for PMEAMeT and 27-fold for PDEAMeT in the Jan 11/99 preparation (Table IV).

Phenyl Sepharose Hydrophobic Interaction Chromatography

The pooled sample prepared by chromatography on the DEAE column was flash-frozen and stored at -80°C until chromatography on the Phenyl Sepharose hydrophobic matrix. Once the Phenyl Sepharose matrix was cleaned and equilibrated (see Materials and Methods), the sample to be loaded was thawed, an aliquot was removed for verifying the P-base *N*-methylating activities and protein concentration, and then $(NH_4)_2SO_4$ was added to the remainder of the sample to a final concentration of 1.0 M $(NH_4)_2SO_4$ (see Materials and Methods). Of the total protein loaded onto the Phenyl Sepharose matrix, 80% was adsorbed and the remainder was collected in the effluent. No PMEAMeT activity was found in the effluent containing non-adsorbed proteins. Figure 3 shows a typical elution profile for Phenyl Sepharose hydrophobic interaction chromatography where adsorbed proteins are eluted from the matrix by a 25-0%/0-50% $(NH_4)_2SO_4$/ethylene glycol gradient in Buffer A. PMEAMeT activity elutes as a unimodal peak with PMEAMeT activity trailing into subsequent column fractions (Fig. 3). The profile for protein concentration in column fractions is much broader

FIGURE 3: Hydrophobic interaction chromatography on Phenyl Sepharose (CL-4B) media

Representative elution profile of PMEAMeT activity (■, PMEA substrate) and adsorbed proteins (▲) from a Phenyl Sepharose matrix (100 mL bed volume). The concentration of $(NH_4)_2SO_4$ was made to 1.0 M in the pooled sample recovered from DEAE Sepharose anion exchange chromatography before loading onto the Phenyl Sepharose matrix. The elution profile shown is for the sample reported in Table V. Adsorbed proteins were eluted by a linear 1 L 25-0% / 0-50% $(NH_4)_2SO_4$ (A.S.)/ethylene glycol (E.G.) gradient prepared in Buffer A (- - -) at a flow rate of 1.0 mL min^{-1}. The eluate was collected in fractions of 6 mL each. Sample fractions having a high PMEAMeT specific activity were pooled before further purification by High Q anion exchange chromatography.


Protein (mg mL^{-1}) ▲
Activity (nmol mL^{-1} min^{-1}) ■
Fraction
Theoretical A.S./E.G. Gradient (%/%) (– – –)
0/50
5/40
10/30
15/20
20/10
25/0

than that for fractions of peak PMEAMeT activity such that fractions containing higher protein levels are associated with low levels of PMEAMeT activity (Fig. 3).

Recovery of PMEAMeT activity in the pooled and dialysed samples ranged from a low of 12% (Table II) to a high of 68% (Table IV) of the total activity detected in the crude extracts. An overall average of only 3% protein of that detected in the crude extracts was recovered in the fractions containing high PMEAMeT activity. PMEAMeT specific activity improved, on average, 13.4-fold with a low of 6-fold for the Nov 18/98 preparation (Table II) to a high of 22-fold for the Feb 22/99 preparation (Table V). PEAMeT activity was only detected in the Dec 2/98 preparation and absent in all other preparations. For the Dec 2/98 preparation, 78% PEAMeT activity relative to the levels found in the crude extract was recovered which represents a 19-fold improvement in specific activity over the crude sample extract although the total activity is admittedly very low. Recovery for PDEAMeT activity ranged from a low of 15% (Table II) to a high of 93% (Table IV) of the total activity detected in the crude extract. PDEAMeT specific activity improved an average of 16.4-fold with a low of 8-fold (Table II) for the Nov 18/98 preparation to a high of 26-fold for the Feb 22/99 preparation (Table V). Again the fold-increase in PMEAMeT and PDEAMeT specific activities rose to a comparable degree following chromatography on Phenyl Sepharose. The highest fold-improvement in specific activity of PMEAMeT and PDEAMeT following chromatography on Phenyl Sepharose were 22 and 26-fold, respectively, for the Feb 22/99 preparation.

High Q Anion Exchange Chromatography

Dialysed and concentrated samples from Phenyl Sepharose chromatography were flash-frozen and stored at -80°C until chromatography on High Q anion exchange. After the High Q matrix was cleaned and equilibrated with Buffer A (see Materials and Methods), the sample prepared by chromatography on Phenyl Sepharose was thawed, and then diluted to a maximum of 10 mg protein mL^{-1} using Buffer A prior to being loaded onto the High Q matrix (see Materials and Methods). On average, of the total protein loaded onto the High Q anion matrix only 48% was adsorbed and the remainder was collected in the effluent. PMEAMeT activity assays of the effluent fraction showed no activity associated with the non-adsorbed proteins. Figure 4 shows a typical elution profile for the High Q anion exchange chromatography matrix with adsorbed proteins being eluted by a linear 0-300 mM NaCl gradient in Buffer A. PMEAMeT activity was found in fractions associated with a unimodal peak and showed little trailing of PMEAMeT activity in later fractions (Fig. 4). In addition, the peak of protein in the eluted fractions was separated from the PMEAMeT activity peak (Fig. 4). Fractions with the highest PMEAMeT activity were pooled and concentrated following this chromatography step.

Recovery of PMEAMeT activity in the pooled and concentrated samples ranged from a low of 0.36% (Table V) to a high of 49% (Table III) relative to that found in the crude extract. On average, only 0.29% protein of that present in the crude extract was recovered. PMEAMeT specific activity improved, on average, 70-fold with a low of 21-fold for the Feb 22/99 preparation (Table V) to a high of 180-fold for the Jan 11/99 preparation (Table IV). PEAMeT activity was only detected at a very low level (15 nmol min^{-1}) in the Dec 2/98

FIGURE 4: Anion exchange chromatography on Macro-Prep® High Q media

Representative elution profile of PMEAMeT activity (■, PMEA substrate) and adsorbed proteins (▲) from a High Q anion exchange matrix (50 mL bed volume). Samples having high PMEAMeT activity recovered from Phenyl Sepharose (CL-4B) were dialysed, concentrated, and then diluted to 1.0 mg mL^{-1} with Buffer A (see Materials and Methods) before being loaded onto the column. The elution profile shown is for the sample reported in Table IV. Adsorbed proteins were eluted by a linear 0.5 L 0-300 mM NaCl gradient prepared in Buffer A (- - -) at a flow rate of 1.0 mL min^{-1}. The eluate was collected in fractions of 5 mL each. Sample fractions having a high PMEAMeT specific activity were pooled, concentrated and dialysed before further purification by Sephacryl S-100 gel filtration chromatography.

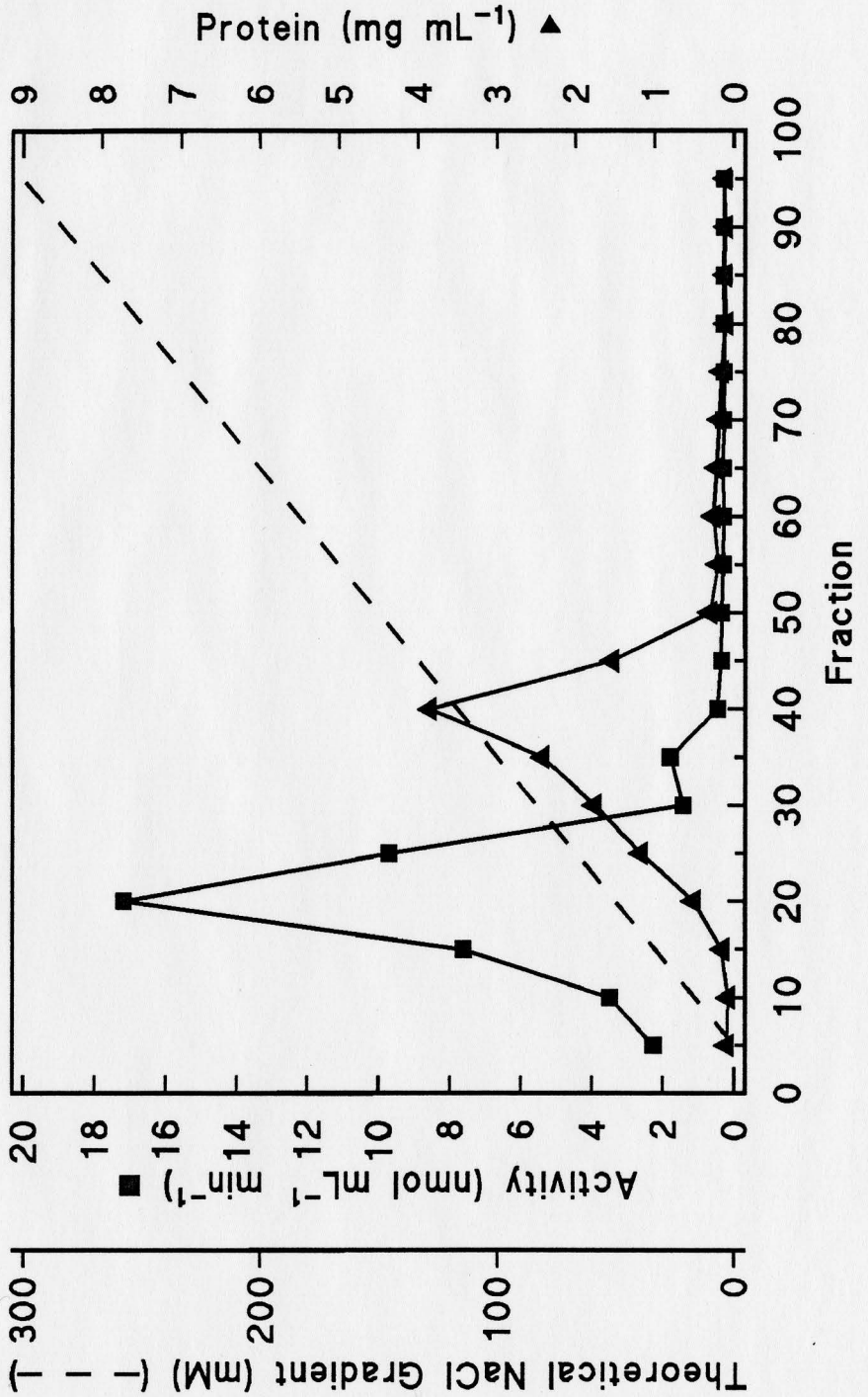

Protein (mg mL^{-1}) ▲
Activity (nmol mL^{-1} min^{-1}) ■
Theoretical NaCl Gradient (mM) (– – –)
Fraction
0
1
2
3
4
5
6
7
8
9
0
2
4
6
8
10
12
14
16
18
20
0
100
200
300
0
10
20
30
40
50
60
70
80
90
100

TABLE II: Recoveries of phosphobase *N*-methylating activities during the partial purification of PMEAMeT from spinach leaves harvested on November 18/98

PURIFICATION STEP (elution condition)	TOTAL PROTEIN[a] mg	TOTAL ACTIVITY[a] nmol min^{-1}			SPECIFIC ACTIVITY nmol min^{-1} mg^{-1}			FOLD-PURIFICATION		
		PEA	PMEA	PDEA	PEA	PMEA	PDEA	PEA	PMEA	PDEA
Crude	16693 (100)	1019 (100)	7708 (100)	7228 (100)	0.061	0.462	0.433	1	1	1
1.8 TO 2.6 M $(NH_4)_2SO_4$ Dialysed	4945 (30)	379 (37)	6400 (83)	5364 (74)	0.077	1.294	1.085	1	3	3
DEAE Sepharose (0 to 0.5 M NaCl)	606 (4)	9 (1)	1015 (13)	982 (13)	0.014	1.675	1.621	0.24	2	1
Phenyl-Sepharose CL-4B (25-0% AS/ 0-50% EG)[b]	326 (2)	N.D.	918 (12)	1102 (15)	N.D.	2.811	3.373	N.D.	6	8
High-Q (0 to 0.3 M NaCl)	26 (0.15)	N.D.	384 (5)	477 (6.5)	0.044	14.832	18.388	N.D.	32	42

[a]Values given in parentheses represent the % recoveries relative to the Crude extract.

[b]AS and EG represent $(NH_4)_2SO_4$ and ethylene glycol, respectively.

N.D.= none detected

TABLE III: Recoveries of phosphobase *N*-methylating activities during the partial purification of PMEAMeT from spinach leaves harvested on December 2/98

PURIFICATION STEP (elution condition)	TOTAL PROTEIN[a] mg	TOTAL ACTIVITY[a] nmol min^{-1}			SPECIFIC ACTIVITY nmol min^{-1} mg^{-1}			FOLD-PURIFICATION		
		PEA	PMEA	PDEA	PEA	PMEA	PDEA	PEA	PMEA	PDEA
Crude	19840 (100)	376 (100)	8000 (100)	6360 (100)	0.019	0.403	0.321	1	1	1
1.8 TO 2.6M $(NH_4)_2SO_4$ Dialysed	3634 (18)	246 (65)	5246 (66)	3583 (56)	0.068	1.444	0.986	4	4	3
DEAE Sepharose (0 to 0.5 M NaCl)	1980 (10)	107 (28)	6995 (87)	6570 (103)	0.054	3.533	3.318	3	9	10
Phenyl-Sepharose CL-4B (25-0% AS/ 0-50% EG)[b]	818 (4)	295 (78)	4698 (59)	4121 (65)	0.036	5.741	5.035	19	14	15
High-Q (0 to 0.3 M NaCl)	140 (1)	15 (4)	3900 (49)	3578 (56)	0.111	27.845	25.542	6	69	80

[a]Values given in parentheses represent the % recoveries relative to the Crude extract.

[b]AS and EG represent $(NH_4)_2SO_4$ and ethylene glycol, respectively.

TABLE IV: Recoveries of phosphobase *N*-methylating activities during the partial purification of PMEAMeT from spinach leaves harvested on January 11/99

PURIFICATION STEP (elution condition)	TOTAL PROTEIN[a] mg	TOTAL ACTIVITY[a] nmol min^{-1}			SPECIFIC ACTIVITY nmol min^{-1} mg^{-1}			FOLD-PURIFICATION		
		PEA	PMEA	PDEA	PEA	PMEA	PDEA	PEA	PMEA	PDEA
Crude	16566 (100)	908 (100)	8498 (100)	5461 (100)	0.055	0.513	0.329	1	1	1
1.8 TO 2.6 M $(NH_4)_2SO_4$ Dialysed	3670 (22)	746 (82)	5392 (63)	3589 (66)	0.203	1.469	0.978	4	3	3
DEAE Sepharose (0 to 0.5 M NaCl)	1281 (8)	651 (72)	13860 (163)	11617 (213)	0.508	10.823	9.071	9	21	27
Phenyl-Sepharose CL-4B (25-0% AS/ 0-50% EG)[b]	661 (4)	N.D.	5774 (68)	5085 (93)	N.D.	8.734	7.693	N.D.	17	23
High-Q (0 to 0.3 M NaCl)	20 (0.12)	N.D.	1869 (22)	1737 (32)	N.D.	92.098	85.586	N.D.	180	260

[a]Values given in parentheses represent the % recoveries relative to the Crude extract.

[b]AS and EG represent $(NH_4)_2SO_4$ and ethylene glycol, respectively.

N.D.= none detected

TABLE V: Recoveries of phosphobase *N*-methylating activities during the partial purification of PMEAMeT from spinach leaves harvested on February 22/99

PURIFICATION STEP (elution condition)	TOTAL PROTEIN[a] mg	TOTAL ACTIVITY[a] nmol min^{-1}			SPECIFIC ACTIVITY nmol min^{-1} mg^{-1}			FOLD-PURIFICATION		
		PEA	PMEA	PDEA	PEA	PMEA	PDEA	PEA	PMEA	PDEA
Crude	20041 (100)	270 (100)	12426 (100)	9509 (100)	0.014	0.621	0.474	1	1	1
1.8 TO 2.6 M $(NH_4)_2SO_4$ Dialysed	3669 (18)	172 (63)	7287 (58)	5295 (56)	0.047	1.986	1.443	3	3	3
DEAE Sepharose (0 to 0.5 M NaCl)	1179 (6)	328 (121)	8343 (67)	8502 (89)	0.278	7.076	7.211	21	11	15
Phenyl-Sepharose CL-4B (25-0% AS/ 0-50% EG)[b]	329 (2)	N.D.	4534 (36)	4012 (42)	N.D.	13.759	12.174	N.D.	22	26
High-Q (0 to 0.3 M NaCl)	3 (0.15)	N.D.	45 (0.36)	36 (0.37)	N.D.	12.842	10.071	N.D.	21	21

[a]Values given in parentheses represent the % recoveries relative to the Crude extract.

[b]AS and EG represent $(NH_4)_2SO_4$ and ethylene glycol, respectively.

N.D.= none detected

TABLE VI: Recoveries of phosphobase *N*-methylating activities during the partial purification of PMEAMeT from spinach leaves harvested on March 2/99

PURIFICATION STEP (elution condition)	TOTAL PROTEIN[a]	TOTAL ACTIVITY[a] nmol min^{-1}			SPECIFIC ACTIVITY nmol min^{-1} mg^{-1}			FOLD-PURIFICATION		
		PEA	PMEA	PDEA	PEA	PMEA	PDEA	PEA	PMEA	PDEA
Crude	19512 (100)	557 (100)	10318 (100)	7361 (100)	0.029	0.529	0.378	1	1	1
1.8 TO 2.6 M $(NH_4)_2SO_4$ Dialysed	8526 (44)	381 (68)	8281 (80)	6115 (83)	0.045	0.971	0.717	2	2	2
DEAE Sepharose (0 to 0.5 M NaCl)	1530 (8)	117 (21)	7440 (72)	6303 (86)	0.076	4.861	4.118	3	9	11
Phenyl-Sepharose CL-4B (25-0% AS/ 0-50% EG)[b]	739 (4)	N.D.	3136 (30)	2717 (37)	N.D.	4.245	3.678	N.D.	8	10
High-Q (0 to 0.3 M NaCl)	77 (0.039)	N.D.	1901 (18)	1812 (25)	N.D.	24.836	23.667	N.D.	47	62

[a]Values given in parentheses represent the % recoveries relative to the Crude extract.

[b]AS and EG represent $(NH_4)_2SO_4$ and ethylene glycol, respectively.

N.D.= none detected

preparation (Table III) and was absent from all other preparations. Levels of PDEAMeT activity were generally comparable to levels of PMEAMeT activity in all five of the preparations (compare Tables II-VI). Recovery of PDEAMeT ranged from a low of 0.37% (Table V) to a high of 56% (Table IV) of the total activity estimated in the crude extract. PDEAMeT specific activity improved an average of 93-fold with a low of 21-fold for the Feb 22/99 preparation (Table V) to a high of 260-fold for the Jan 11/99 preparation (Table IV). The low recoveries of PMEAMeT activity (0.36%) and PDEAMeT activity (0.37%) in the Feb 22/99 preparation were the result of omitting glycerol in Buffer A. The highest fold improvement in specific activity of PMEAMeT and PDEAMeT were 180- and 260-fold, respectively, for the Jan 11/99 preparation.

Sephacryl S-100 Gel Filtration Chromatography

In order to prepare a sample with sufficient PMEAMeT activity to carry out gel filtration chromatography on the Sephacryl S-100 media, High Q-prepared samples from Nov. 18/98 and Dec. 2/98 crude preparations were pooled and concentrated to a volume of 15 mL using a passivated Amicon centricon 30 concentrator (see Materials and Methods). In addition, a second sample was prepared by combining samples carried through to the High Q chromatography step from crude preparations completed on Jan. 11/99, Feb. 22/99, and Mar. 2/99. This latter sample was pooled and concentrated (as above) to a volume of 8.5 mL (see Materials and Methods). Sephacryl S-100 chromatography performed on the pooled samples of Nov. 18/98 and Dec. 2/98 is designated S-100#1 while Sephacryl S-100 chromatography performed on the pooled samples from Jan. 11/99, Feb. 22/99, and Mar.

FIGURE 5: Gel filtration chromatography on HiPrep® Sephacryl S-100

Representative elution profile of PMEAMeT activity (■, PMEA substrate) and proteins (▲) from a Sephacryl S-100 gel filtration matrix (320 mL bed volume). Samples recovered from High Q anion exchange chromatography having high PMEAMeT activity were concentrated to approximately 5 mL using a passivated Amicon ultrafiltration cell (see Materials and Methods) before being loaded onto the column. The elution profile shown is for the sample reported in Table VII. Proteins were eluted from the column by 250 mL of 150 mM NaCl in Buffer B at a flow rate of 0.25 mL min^{-1}. The eluate was collected in fractions of 2-mL volume each (V=eluted volume Vo=Void volume). Sample fractions having a high PMEAMeT specific activity were pooled, concentrated and dialysed before further purification by Mono Q anion exchange chromatography.

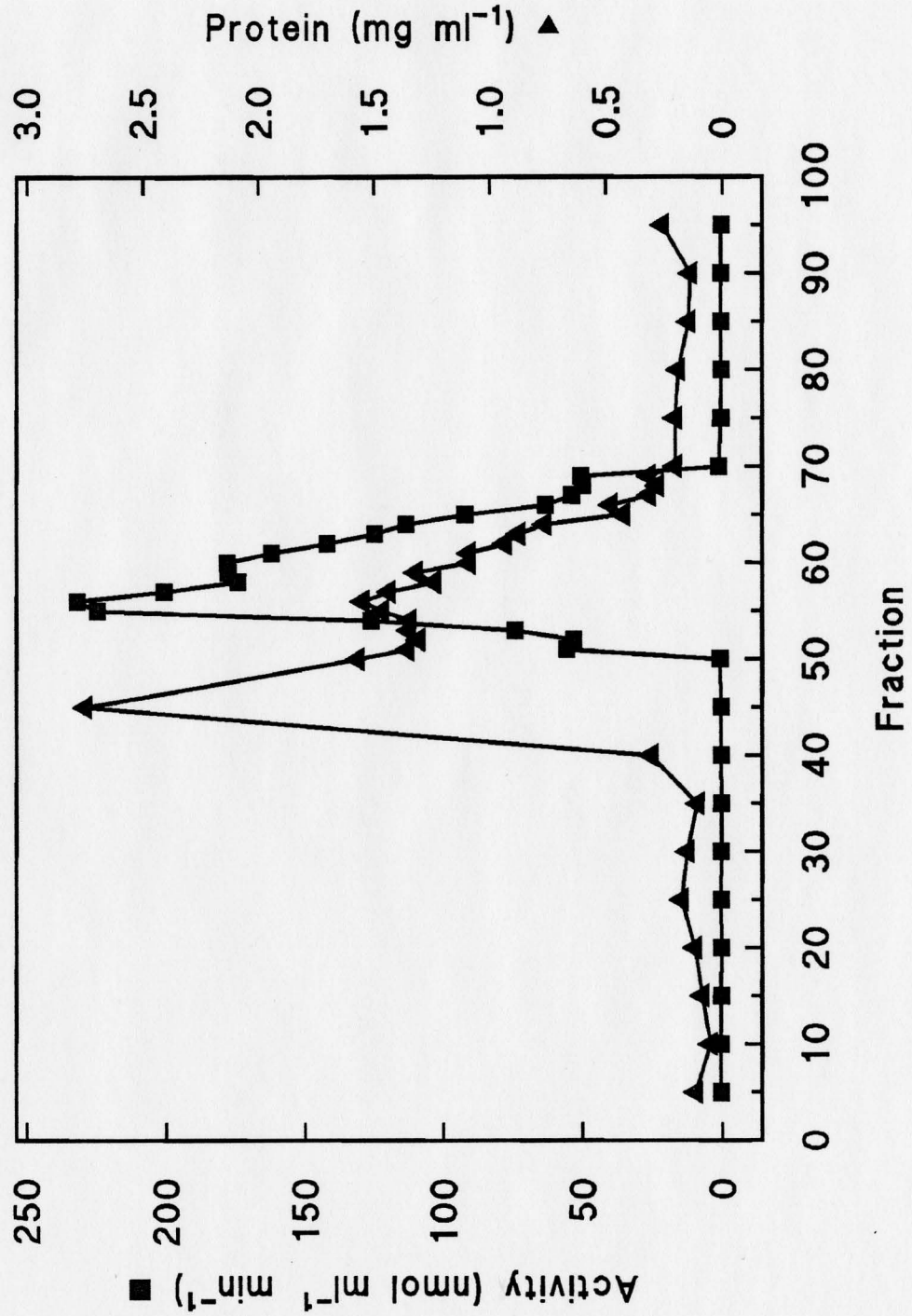

Protein (mg ml^{-1}) ▲
3.0
2.5
2.0
1.5
1.0
0.5
0
Activity (nmol ml^{-1} min^{-1}) ■
250
200
150
100
50
0
0 10 20 30 40 50 60 70 80 90 100
Fraction

TABLE VII: PMEAMeT activity recovered in fractions collected after gel filtration on Sephacryl S-100#1[a].

SAMPLE	TOTAL PMEAMeT ACTIVITY[b] nmol min^{-1}	TOTAL PROTEIN[b] mg	SPECIFIC ACTIVITY nmol min^{-1} mg^{-1}	FOLD-PURIFICATION
Column Load	5082 (100)	59.313 (100)	34.553	80
Fraction 19	1354.145 (27)	12.380 (21)	109.382	253
Fraction 20	1774.260 (35)	8.260 (14)	214.801	497
Fraction 21	1515.935 (30)	6.065 (10)	249.948	578

[a]Samples from extractions dated Nov 18/98 and Dec 2/98 were subject to chromatography on High Q anion exchange matrix and then pooled for application to the Sephacryl S-100 matrix (see Materials and Methods for details).
[b]Values in parentheses represent the % recoveries relative to the sample loaded on the column.

TABLE VIII: PMEAMeT activity recovered in fractions collected after gel filtration on Sephacryl S-100#2[a].

SAMPLE	TOTAL PMEAMeT ACTIVITY[b] nmol min^{-1}	TOTAL PROTEIN[b] mg	SPECIFIC ACTIVITY nmol min^{-1} mg^{-1}	FOLD-PURIFICATION[c]
Column Load	3228.725 (100)	95.260 (100)	33.894	61
Fraction 55	449.568 (14)	2.928 (3)	153.541	277
Fraction 56	463.890 (14)	3.102 (3)	149.545	270
Fraction 57	401.530 (14)	2.872 (3)	139.808	252
Fraction 58	348.234 (12)	2.484 (3)	140.191	253
Fraction 59	355.486 (11)	2.642 (2)	134.552	243
Fraction 60	356.210 (11)	2.184 (2)	163.100	294
Fraction 61	324.306 (10)	2.184 (2)	148.492	268
Fraction 62	284.244 (9)	1.872 (2)	151.840	274
Fraction 63	249.800 (8)	1.758 (2)	142.093	257

[a]Samples from extractions dated Jan 11/99, Feb 22/99 and Mar 2/99 were subject to chromatography on a High Q anion exchange matrix and then pooled for application to the Sephacryl S-100 matrix (see Materials and Methods for details).
[b]Values in parentheses represent the % recoveries relative to the sample loaded on the column.
[c]Fold-purification relative to the average of the crude leaf extracts from Jan 11/99, Feb 22/99, and Mar 2/99.

2/99 is designated S-100#2 (Tables VII and VIII). Figure 5 shows a typical elution profile of PMEAMeT activity and protein concentration in fractions collected from the eluate of the Sephacryl S-100 matrix. PMEAMeT activity eluted from the column in fractions that yielded a unimodal peak that was separate from the peak associated with protein. Following this PMEAMeT activity peak, PMEAMeT activity was found to trail in subsequent fractions (Fig. 5).

Table VII summarizes the results of Sephacryl S-100 gel filtration for the S-100#1 sample. The flow rate for this trial was set to 1.0 mL min^{-1}. For fractions containing high levels of PMEAMeT activity, recovery of total PMEAMeT activity in individual fractions ranged from a low of 27% to a high of 35% of the total PMEAMeT activity present in the S-100#1 sample loaded onto the column (Table VII). Of the total protein loaded onto the column, 45% was present in the fractions that had the highest PMEAMeT activity (Table VII). The PMEAMeT specific activity of selected fractions are compared to the averaged PMEAMeT specific activity (i.e. average of Nov 18/98 and Dec 2/98) of crude samples. Values ranged from a low of 253-fold in Fraction 19 to a high of 578-fold in Fraction 21 (Table VII). The latter fraction contained only 10 % of the protein present in the sample loaded onto the column (Table VII). Fractions having the highest PMEAMeT specific activity were pooled, concentrated, and dialyzed with Buffer B before flash-freezing and storage at -80°C.

Table VIII summarizes the results of Sephacryl S-100 gel filtration on the S-100#2 sample. In this trial the flow rate was set to 0.25 mL min^{-1}. For fractions containing high levels of PMEAMeT activity, the estimated recovery of PMEAMeT in individual fractions

ranged from a low of 4% to a high of 14% of that present in the sample loaded onto the column (Table VIII). Total protein recovered in fractions containing high PMEAMeT activity ranged from a low of 1% to a high of 3% of that present in the S-100#2 sample (Table VIII). Therefore, PMEAMeT specific activity improved, on average, 265-fold for a single fraction, Fraction 65, showing a 389-fold improvement in specific activity relative to the averaged specific activity of the crude extracts (i.e. average of Jan 11/99, Feb 22/99, and Mar 2/99).

Mono Q HR 5/5 Anion Exchange Chromatography

Three independent HPLC Mono Q anion exchange chromatography trials (designated Mono Q #1, #2, and #3) were performed. In the first Mono Q chromatography trial, sample fractions 55 to 59 prepared by chromatography on Sephacryl S-100 (S-100 Trial no.2) were pooled, dialysed with Buffer C, and concentrated to a volume of 6.4 mL prior to chromatography on the Mono Q anion exchange matrix. Of the total protein loaded onto the Mono Q anion exchange matrix, 73% was adsorbed and the remainder was collected in the effluent. No PMEAMeT activity was found in the effluent containing the non-adsorbed proteins.

For the first Mono Q trial, adsorbed proteins were eluted from the matrix by a linear 0 to 1.0 M NaCl gradient prepared in Buffer C. For fractions containing high levels of PMEAMeT activity, recovery of total PMEAMeT activity ranged from a low of 14% (Fraction 9) to a high of 23% (Fraction 6) of the total PMEAMeT activity present in the sample loaded onto the column (Table IX). Recovery of protein in fractions with the highest PMEAMeT activity ranged from a low of 6% (Fraction 7) to a high of 39% (Fraction 9) of

FIGURE 6: Anion exchange chromatography on Mono Q® HR 5/5

Elution profile of PMEAMeT activity (■, PMEA substrate) and adsorbed proteins (▲) from a Mono Q anion exchange matrix (1 mL bed volume). Samples having high PMEAMeT activity recovered from Sephacryl S-100 were concentrated and dialyzed with Buffer B to a volume of approximately 10 mL using a passivated Amicon Centricon 30 (see Materials and Methods) before being loaded onto the column. Panel A shows an elution profile of a 30 mL, 0-1M NaCl gradient in Buffer B. The results of this elution is reported as Mono Q Trial no.1 in Table IX. Panels B and C show elution profiles for a 30 mL, 0-3 M NaCl gradient in Buffer B for Mono Q purification trials 2 and 3, respectively. These trials are reported in Table IX. The eluate was collected in 1 mL fractions. Sample fractions having a high PMEAMeT specific activity were further purified by SW-300 gel filtration chromatography.

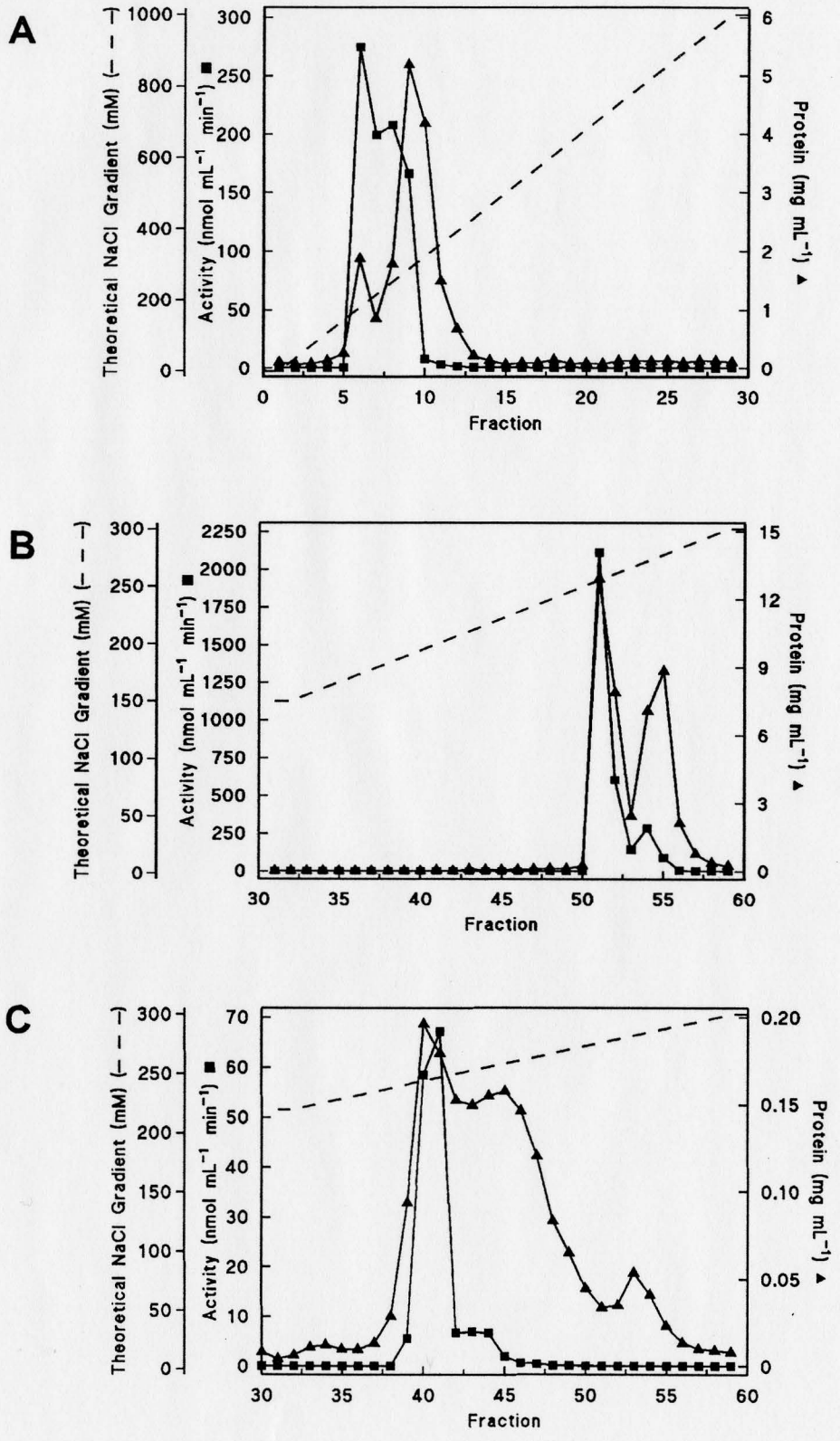

A
Theoretical NaCl Gradient (mM) (– – –)
Activity (nmol mL⁻¹ min⁻¹) ■
Protein (mg mL⁻¹) ▲
Fraction
B
Theoretical NaCl Gradient (mM) (– – –)
Activity (nmol mL⁻¹ min⁻¹) ■
Protein (mg mL⁻¹) ▲
Fraction
C
Theoretical NaCl Gradient (mM) (– – –)
Activity (nmol mL⁻¹ min⁻¹) ■
Protein (mg mL⁻¹) ▲
Fraction

TABLE IX: Different elution conditions from Mono Q HR 5/5 led to varying fold-purifications of PMEAMeT

TRIAL No.	ELUTION CONDITION	SAMPLE (Source)	TOTAL PMEAMeT ACTIVITY[a] nmol min^{-1}	TOTAL PROTEIN[a] mg	SPECIFIC ACTIVITY nmol min^{-1} mg^{-1}	FOLD-PURIFICATION[b,c]
1.	0-1.0 M NaCl	Column Load (S-100 #2 Fractions 55-59)	1199.622 (100)	13.453 (100)	89.173	161[b]
		Fraction 6	274.446 (23)	1.873 (14)	146.527	265[b]
		Fraction 7	199.393 (17)	0.848 (6)	235.133	425[b]
		Fraction 8	207.886 (17)	1.782 (14)	116.648	211[b]
		Fraction 9	166.413 (14)	5.188 (39)	32.077	58[b]
2.	0-3.0 M NaCl	Column Load (S-100 #1 Fractions 19-21)	3617.992 (100)	33.350 (100)	108.486	251[c]
		Fraction 51	2111.435 (58)	12.926 (39)	163.348	378[c]
		Fraction 52	606.193 (17)	7.885 (24)	76.879	178[c]
		Fraction 53	145.385 (4)	2.439 (7)	59.608	138[c]
3.	0-3.0M NaCl	Column Load (S-100 #2 Fraction 54)	164.020 (100)	1.754 (100)	93.528	169[b]
		Fraction 40	29.200 (18)	0.098 (6)	297.954	538[b]
		Fraction 41	33.580 (21)	0.090 (6)	374.950	677[b]

[a]Values in parentheses represent the % recoveries relative to their respective sample loaded onto the column.

[b]Fold-purification relative to the average of the crude leaf extracts from Jan 11/99, Feb 22/99, and Mar 2/99.

[c]Fold-purification relative to the average of the crude leaf extracts from Nov 18/98 and Dec 2/98.

the total protein loaded onto the column (Table IX). The specific activity varied from a low of 58-fold (Fraction 9) to a high of 425-fold (Fraction 7) relative to the average specific activity of the crude sample extracts. Panel A of Figure 6 shows the elution profile of PMEAMeT activity and protein concentration in column fractions of the first Mono Q trial. PMEAMeT activity elutes as a unimodal peak with a shoulder and very little trailing activity in subsequent column fractions, while the profile for protein concentration shows a small peak (1.87 mg mL^{-1}) coinciding with the PMEAMeT activity peak fraction (Fraction 6) followed by another peak following the shoulder of PMEAMeT activity.

In the second Mono Q trial, sample Fractions 19 to 21 prepared from chromatography on Sephacryl S-100 (S-100 Trial no.1) were pooled, concentrated to a volume of 10 mL and dialyzed with Buffer C prior to chromatography on the Mono Q anion exchange matrix. Up to 70% of the total protein loaded onto the Mono Q matrix was adsorbed and the remainder was collected in the effluent. No PMEAMeT activity was found in the effluent containing non-adsorbed proteins. Adsorbed proteins were eluted from the matrix by a linear 0 to 300 mM NaCl gradient prepared in Buffer C. For fractions containing high levels of PMEAMeT activity, recovery of total PMEAMeT activity ranged from a low of 4% (Fraction 53) to a high of 58% (Fraction 51) of the total PMEAMeT activity loaded on the column (Table IX). Recovery of protein in fractions with the highest PMEAMeT activity ranged from a low of 7% (Fraction 53) to a high of 39% (Fraction 51) of the total protein present in the sample loaded onto the column (Table IX). Therefore, specific activity of PMEAMeT increased from a low of 138-fold (Fraction 53) to a high of 378-fold (Fraction 51) relative to the average specific activity of the crude sample extracts. Panel B of Figure 6 shows the elution profile

of PMEAMeT activity and protein concentration in column fractions of the second Mono Q trial. PMEAMeT activity elutes as a single prominent unimodal peak followed by a minor peak with very little PMEAMeT activity trailing in subsequent column fractions. The elution profile for protein concentration shows two peaks, the first protein concentration peak (12.92 mg mL^{-1}) coincides with the peak of PMEAMeT activity and consists of 52% of the total protein eluted from the column while the second peak (9.00 mg mL^{-1}) is associated with comparatively little PMEAMeT activity.

For the third Mono Q trial, a single fraction (Fraction 54) from the second S-100 trial was diluted 3-fold with Buffer C and then loaded onto the Mono Q anion exchange matrix. Adsorbed proteins were eluted from the anion matrix by a linear 0 to 300 mM NaCl gradient prepared in Buffer C. Only 12% of the total protein loaded onto the Mono Q matrix was recovered in fractions that contained high levels of PMEAMeT activity (Table IX). No PMEAMeT activity was found in the effluent fraction containing non-adsorbed proteins. Of all of the fractions of column eluate collected, two fractions (Fractions 40 and 41) contained 40% of the total PMEAMeT activity loaded onto the column (Table IX). The specific activity of Fraction 40 increased 538-fold relative to the average specific activity of the crude sample extracts. The highest fold-improvement in specific activity from Mono Q anion exchange chromatography was found in Fraction 41 which showed a 677-fold increase in specific activity relative to the average specific activity of the crude sample extracts. Panel C of Figure 6 shows the elution profile of PMEAMeT activity and protein concentration in column fractions of the third Mono Q trial. A single PMEAMeT activity peak was found with no trailing activity in subsequent column fractions while the profile for protein concentration

shows a broad peak that includes column fractions that contain no PMEAMeT activity.

SW-300 Gel Filtration Chromatography

For the final step in PMEAMeT purification, a sample fraction collected from a Mono Q anion exchange chromatography trial was used. An aliquot of the sample was removed in order to determine the P-base MeT activities and protein concentration before applying the remainder to a SW-300 gel filtration column. Sample Fraction 51 that was collected during Mono Q chromatography Trial no.2 (see above), contained the highest total PMEAMeT activity (2111.435 nmol min^{-1}) and was selected for both trials on the SW-300 gel filtration matrix. A total of 50 μL of this Mono Q fraction was injected onto the SW-300 column and elution was carried out at flow rates of 0.5 mL min^{-1} or 1.0 mL min^{-1} for Trials 1 and 2, respectively. Figure 7 shows an elution profile of PMEAMeT activity and protein detected in selected fractions collected during elution from the SW-300 column. In this trial, Buffer D (see Materials and Methods) was used at a flow rate of 1.0 mL min^{-1}. Protein concentrations were estimated using the absorbance at 280 nm for each fraction and estimating the protein concentration by comparison of the A_{280} for the sample against a A_{280} standard curve generated using BSA as a standard (Fig. 8). PMEAMeT activity eluted in fractions that yielded a single, unimodal peak in the profile (sample Fractions numbered 63-71) with almost no enzyme activity detected trailing in the subsequent fractions. Proteins eluted from this column to yield several prominent peaks (e.g.: Fractions numbered 31-39, 55-75, and 115-120) (Fig. 7).

Table X summarizes the results of the two trials on the SW-300 gel filtration matrix.

FIGURE 7: Gel filtration chromatography on Protein Pak SW-300

Representative elution profile of PMEAMeT activity (·····) and proteins (—-) from a Protein Pak SW-300 gel filtration matrix (13.3 mL bed volume). 50 μL of Fraction 51 recovered from Mono Q Trial no.2 having 2111.435 nmol mL^{-1} min^{-1} PMEAMeT activity was loaded onto the SW-300 matrix. The elution profile shown is for SW-300 Trial no.1 reported in Table X. Proteins were eluted from the column by 5 mL of Buffer C at a flow rate of 0.5 mL min^{-1} (see Materials and Methods). The eluate was collected in 0.1 mL fractions. The A_{280} was used to calculate protein concentration by calibration against a BSA standard curve (Fig. 8).

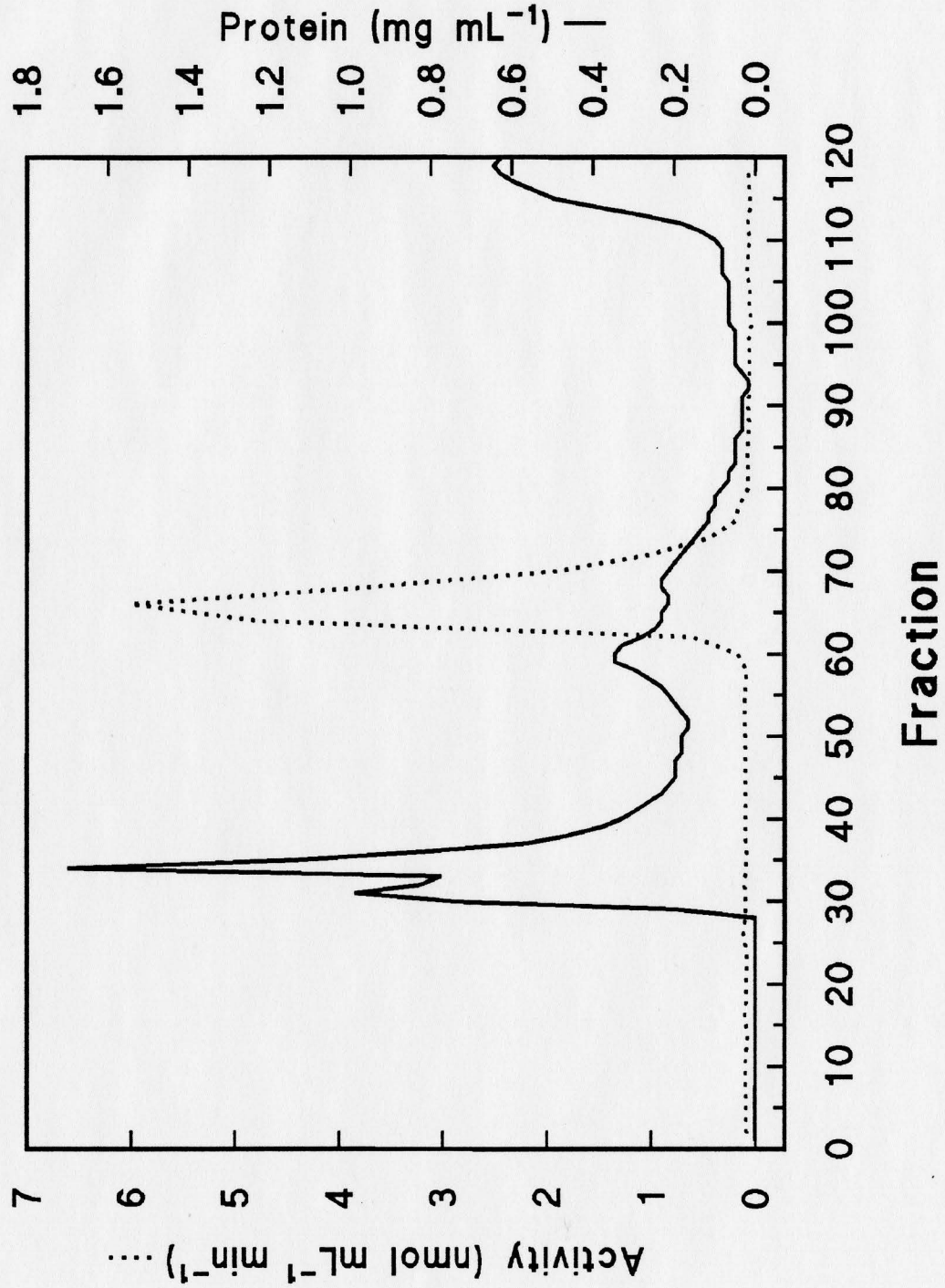

Protein (mg mL^{-1}) —
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Activity (nmol mL^{-1} min^{-1}) ····
7
6
5
4
3
2
1
0
0 10 20 30 40 50 60 70 80 90 100 110 120
Fraction

FIGURE 8: Protein standard curve of BSA concentration and absorbance at 280 nm

A 1 mg mL^{-1} BSA stock solution was serially diluted with H_2O to prepare 0.000781, 0.0156, 0.0312, 0.0625, 0.125, 0.25, and 0.5 mg mL^{-1} concentrations (see Materials and Methods). The absorbance at 280 nm of each BSA concentration was measured using a 1 mL quartz cuvette zeroed using H_2O. The equation of the line is given by: $y= 1.6496(x) + 4.671x10^{-4}$. The protein concentration for fractions collected following elution from the SW-300 gel filtration column was determined by comparing the OD_{280} for each fraction to the corresponding μg protein value on the standard curve.

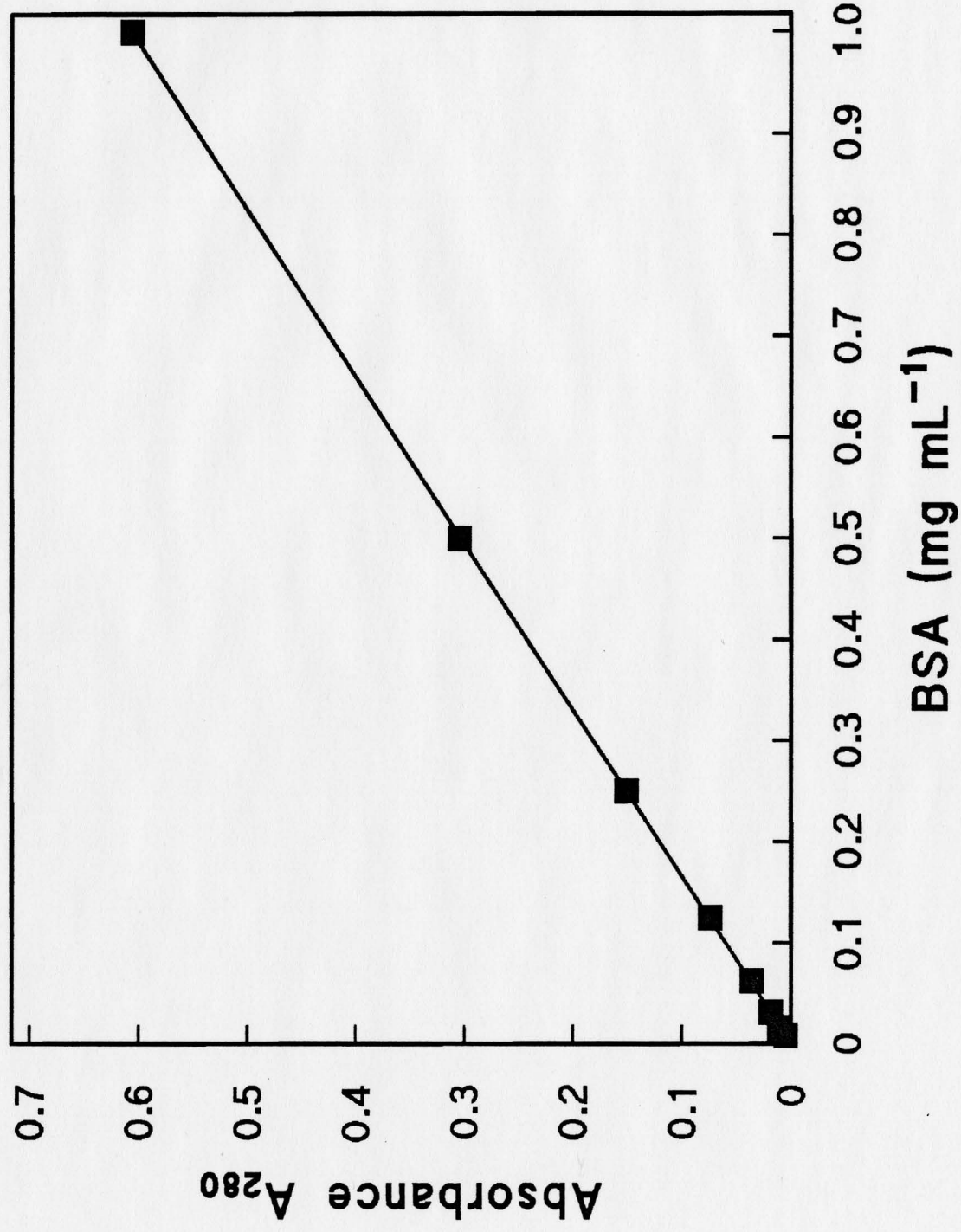

Absorbance A_{280}
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
BSA (mg mL^{-1})

TABLE X: Different elution conditions from SW-300 gel filtration chromatography led to varying fold-purifications of PMEAMeT

TRIAL No.	ELUTION CONDITION	SAMPLE (Source)	TOTAL PMEAMeT ACTIVITY[a] nmol min^{-1}	TOTAL PROTEIN[a] mg	SPECIFIC ACTIVITY nmol min^{-1} mg^{-1}	FOLD-PURIFICATION[b]
1.	0.5 mL min^{-1}	Column Load (Mono Q #2 Fraction 51)	105.571 (100)	0.646 (100)	163.421	378
		Fraction 67	30.101 (28)	0.0515 (8)	584.485	1353
		Fraction 68	29.766 (28)	0.0306 (5)	972.745	2252
		Fraction 69	23.511 (28)	0.0193 (3)	1218.186	2820
		Fraction 70	8.9001 (8)	0.0116 (2)	767.251	1776
2.	1.0 mL min^{-1}	Column Load (Mono Q #2 Fraction 51)	105.571 (100)	0.646 (100)	163.421	378
		Fraction 65	21.898 (21)	0.0396 (6)	552.979	1280
		Fraction 66	30.663 (29)	0.0231 (3)	1327.402	3073
		Fraction 67	29.612 (28)	0.0124 (2)	2388.064	5527
		Fraction 68	20.385 (19)	0.00706 (1)	2887.393	6684
		Fraction 69	13.029 (12)	0.00417 (0.6)	3124.461	7232
		Fraction 70	6.843 (6)	0.00211 (0.03)	3243.127	7507

[a]Values in parentheses represent the % recoveries relative to the column load sample.

[b]Fold-purification relative to the average of the crude leaf extracts from Nov 18/98 and Dec 2/98.

In SW-300 Trial no. 1, recovery of total PMEAMeT activity relative to that present in the sample loaded onto the column ranged from a low of 8% in Fraction 70 to an average of 28% in Fractions 67, 68, and 69. These four fractions taken together account for up to 92% of the total PMEAMeT activity loaded onto the SW-300 matrix. Recovery of total protein relative to that present in the sample loaded ranged from a low of 2% in Fraction 70 to a high of 8% in Fraction 67. Overall only 18% of the total protein loaded onto the SW-300 matrix was present in the fractions containing PMEAMeT activity. The highest PMEAMeT fold-purification achieved was 2,820-fold over the specific activity determined for the crude spinach extract preparation. For the second trial on SW-300, the flow rate of Buffer D was increased to 1.0 mL min^{-1} in order to minimize diffusion effects on the sample moving through the SW-300 matrix. Recovery of total PMEAMeT activity relative to that present in the sample loaded ranged from a low of 6% in Fraction 70 to a high of 29% in Fraction 66. All of the PMEAMeT activity loaded onto the SW-300 matrix can be accounted for by addition of the total activity in the individual sample fractions collected from this column (Table X). Again, a single unimodal peak of total PMEAMeT activity was found with total activity per fraction comparable to the first trial. Recovery of total protein relative to that present in the sample loaded ranged from a low of 0.03% in Fraction 70 to a high of 6% in Fraction 65. Overall only 13% of the total protein loaded onto the SW-300 matrix was present in the fractions containing PMEAMeT activity. The highest PMEAMeT fold-purification achieved for the seven-step purification scheme was 7,507-fold relative to the crude spinach extract preparation (Table X). This sample (Fraction 70) had only 6% of the total PMEAMeT activity and 0.03% of the total protein loaded onto the SW-300 matrix. In addition, sample fractions

numbered 67 and 68 contained 28 and 19% of the total PMEAMeT activity loaded and showed 5,527 and 6,684 fold-purifications, respectively, relative to the crude spinach leaf extract. All fractions were flash frozen in liquid N_2 and stored at -80 °C for subsequent analysis.

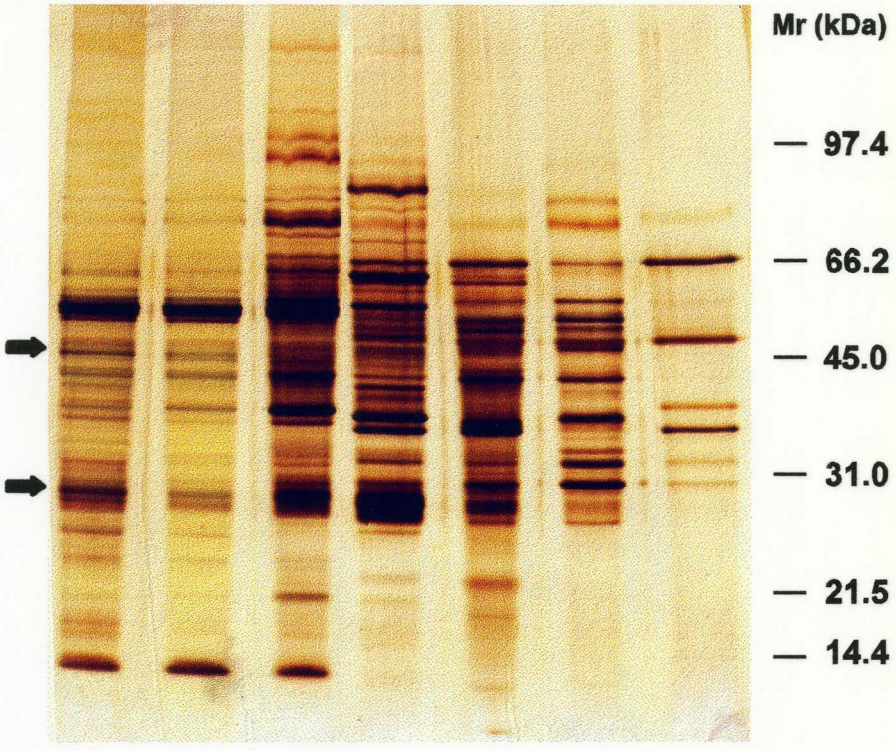
SDS-Polyacrylamide Gradient Gel Electrophoresis Analysis

Polypeptide Profile of Representative Samples Obtained at Various Steps of PMEAMeT Purification

Figure 9 shows the polypeptide banding patterns in representative samples which were taken from each step in the purification of PMEAMeT. In the crude extract and $(NH_4)_2SO_4$ fraction shown in lanes 1 and 2, respectively, a prominent polypeptide having a Mr around 52-55 kDa is present. This polypeptide corresponds to the large subunit of ribulose 1,5 bisphosphate carboxylase/oxygenase or Rubisco which has a Mr of 55 kDa and makes up in excess of 50% of the soluble leaf proteins in plants (Raven *et al.*, 1999). Rubisco protein is still present after anion exchange on DEAE Sepharose (lane 3) and is almost completely removed from the samples after samples have been subjected to anion exchange on a High Q matrix (lane 4). The overall contribution of Rubisco protein to the total protein in each sample decreases with each successive purification step and with its removal, other polypeptides originally present in the sample at a lower abundance become more pronounced on the SDS-polyacrylamide gel (compare lanes 1 to 7). Two arrows to the left of the gel denote Rf positions corresponding to 30 and 50 kDa. The significance of these Mr estimates is shown in Figure 11, Panel A (discussed below). In comparing the complexity of polypeptide

FIGURE 9: Silver-stained SDS-PAGE gradient gel showing polypeptide composition of samples prepared during steps of PMEAMeT purification.

Lanes 1 to 6 were loaded with 2 μg each while Lane 7 was loaded with 0.25 μg protein. Sample preparation and electrophoresis conditions were as reported in Materials and Methods. Polypeptides were separated by a 7.5-15% SDS-Polyacrylamide gel, and the gel was then silver stained. *Lane 1,* Crude extract (Mar. 2/99); *Lane 2,* $(NH_4)_2SO_4$ fraction (Mar. 2/99); *Lane 3,* DEAE Sepharose (Mar. 2/99); *Lane 4,* High Q Anion (Mar 2/99); *Lane 5,* S-100 gel filtration (Fraction #65 S-100 #2); *Lane 6,* Mono Q Anion (Fraction #6 Mono Q #1); *Lane 7,* SW-300 gel filtration (Fraction #54 SW-300 #2). The amount of PEAMeT and PMEAMeT activity (nmol mL^{-1} min^{-1}) and PMEAMeT specific activity (nmol min^{-1} mg^{-1}) of each sample loaded is given below the gel. Arrows along the left of the gel denote the approximate positions of the 30 and 50 kDa polypeptides which have been shown to photoaffinity cross-link with [^{3}H]SAM.



	1	2	3	4	5	6	7
PEAMeT Activity (nmol mL^{-1} min^{-1})	0.18	1.29	3.76	0.58	None detected	None detected	None detected
PMEAMeT Activity (nmol mL^{-1} min^{-1})	3.35	28.07	240.01	144.05	92.27	274.44	4.87
PMEAMeT Specific Activity (nmol min^{-1} mg^{-1})	0.03	0.05	0.08	0.10	214.58	146.60	287.25

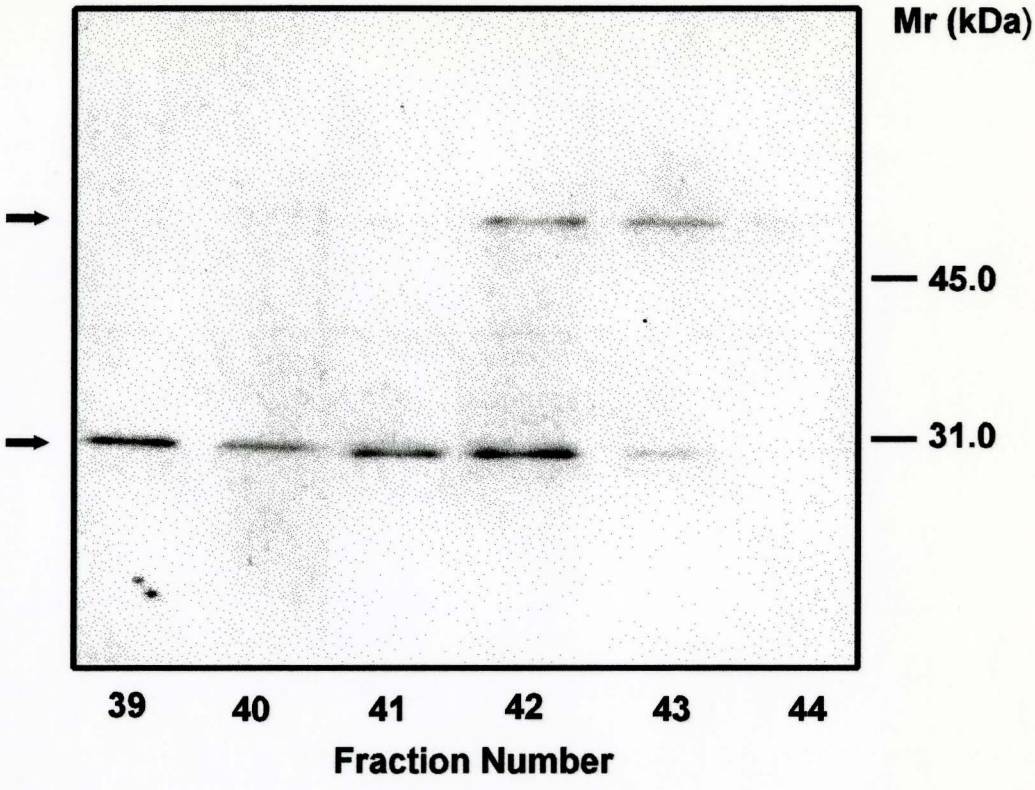
banding and specific activity measurements between samples from the crude extract (lane 1) through to gel filtration chromatography on SW-300, lane 7, the highest improvement in PMEAMeT specific activity occurred after gel filtration on Sephacryl S-100. For this latter sample (lane 7) only 7 prominent polypeptides were revealed by silver-staining the gel.

Separation of the 30 and 50 kDa Polypeptides by Mono Q Anion Exchange Chromatography

Photolabeling of SAM-dependant methyltransferases with [^{3}H]SAM by irradiation under UV light is a method that has been used for the identification of various methyltransferases (Smith *et al.*, 2000; Takata and Fujioka, 1992; Som and Friedman, 1990; Pajares *et al.*, 1984). The whole SAM molecule is considered to bind covalently, specifically and irreversibly to the SAM-binding site through its methyl group (Subbaramaiah and Simms, 1992; Som and Friedman, 1990). This method was employed in order to identify which polypeptides present in the gel shown in Figure 9 represent PMEAMeT protein. We chose to apply photoaffinity cross-linking to sample fractions with high PMEAMeT activity prepared by Mono Q chromatography (Fig. 10). Figure 10 shows the fluorograph of photoaffinity cross-linked samples of successive fractions across the peak of PMEAMeT activity following chromatography on Mono Q. Arrows to the left of the figure designate the positions of the 30 and 50 kDa polypeptides that are photoaffinity cross-linked with [^{3}H]SAM. In the lanes loaded with samples from fractions numbered 39-41, the 30 kDa [^{3}H]SAM-labeled polypeptide is prominent while the 50 kDa [^{3}H]SAM-labeled polypeptide is faint or absent from these lanes (Fig. 10). In Fraction 42 both polypeptides are fairly equally

FIGURE 10: Anion exchange chromatography on Mono Q can separate the [^{3}H]-labeled 30 kDa and [^{3}H]-labeled 50 kDa polypeptides

30 μL aliquots of sample Fractions 39 to 44 collected following Mono Q anion exchange chromatography Trial no.3 were mixed with 3.0 μL of [^{3}H]SAM and irradiated with 254 nm UV light for 30 min on ice. Sample preparation and electrophoresis conditions were as reported in Materials and Methods. The samples were then loaded onto a 7.5-15% SDS-Polyacrylamide gel and the polypeptides were separated electrophoretically. The gel was stained with Coomassie dye and exposed to Amplify fluorographic reagent for 45 min before drying (see Materials and Methods). A X-ray film was exposed to the dried gel for 3 months. The total PMEAMeT activity loaded (nmol min^{-1}) and specific activity (nmol min^{-1} mg^{-1}) of each sample is given below the gel. Arrows along the left side indicate positions of the 30 and 50 kDa polypeptides that photoaffinity cross-link with [^{3}H]SAM.



Total PMEAMeT Activity (nmol min^{-1})	0.055	0.27	0.089	0.24	0.26	0.080
Specific Activity (nmol mg^{-1} min^{-1})	53.23	218.34	64.90	181.81	252.56	102.71

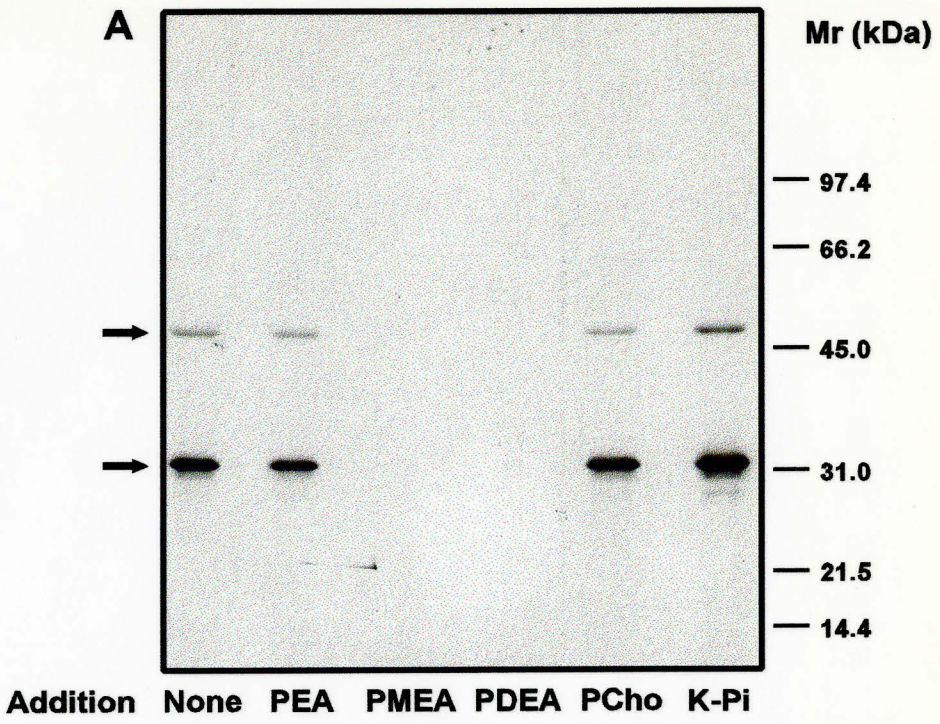
represented but in Fraction 43 the 50 kDa species is more prominent. PMEAMeT activity elutes from this matrix as two separate peaks, the first peak occurs in fraction number 40 (0.27 nmol min^{-1}) where the 30 kDa [^{3}H]SAM-labeled polypeptide is present and the second peak occurs in fraction number 43 (0.26 nmol min^{-1}) in which the 50 kDa [^{3}H]SAM-labeled polypeptide is prominent. It is important to note that all of the fractions which were photoaffinity cross-linked (Fig. 10) contained PMEAMeT activity and, as such, both the 30 and 50 kDa polypeptides are associated with PMEAMeT.

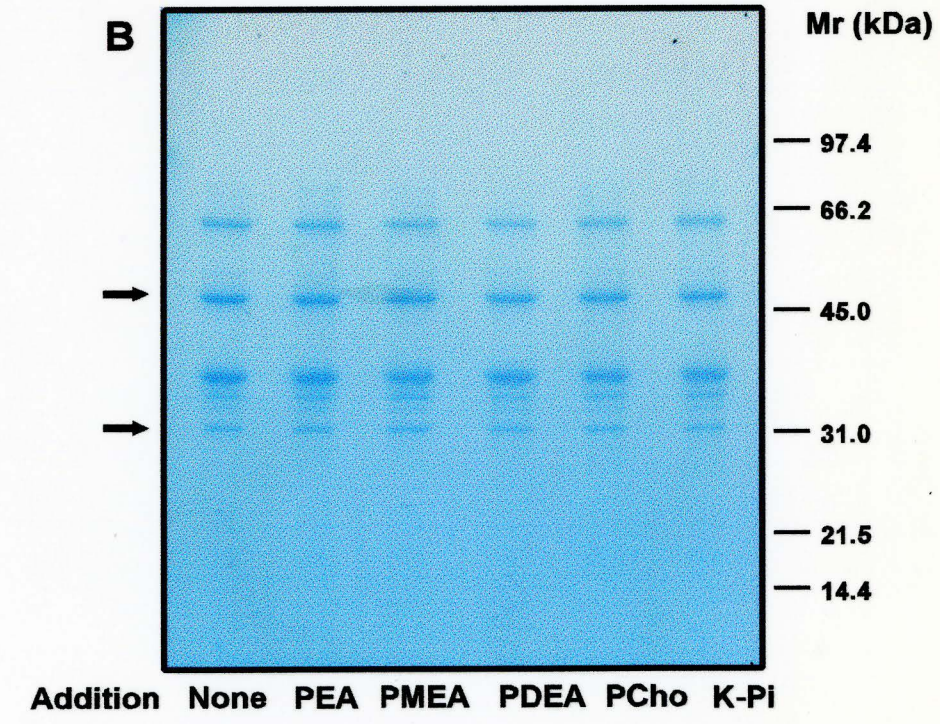
Photoaffinity Cross-linking of [^{3}H]SAM to PMEAMeT and Analysis by SDS-PAGE and Fluorography

In order to determine if both 30 and 50 kDa polypeptides are associated with PMEAMeT activity or some other methyltransferase present in the sample extract, a sample purified 1,353-fold for PMEAMeT activity was photoaffinity cross-linked to [^{3}H]SAM in the presence and absence of either PEA, PMEA, PDEA, PCho or K-Pi (see Materials and Methods). A fluorograph of this gel shows two [^{3}H]SAM-labeled polypeptides present in the lane loaded with the photoaffinity assay sample to which no additions were made (Fig. 11, Panel A). The estimated molecular mass for these polypeptides were calculated to be 30 and 50 kDa (by comparing their relative mobility (R_f) on the gel and their molecular weights against a standard curve generated using a set of protein Mr standards). The addition of P-base substrates PMEA or PDEA to the photoaffinity cross-linking assay completely prevented [^{3}H]SAM from binding to either polypeptide (Fig. 11, Panel A) as indicated by the absence of radioactivity associated with the 30 and 50 kDa polypeptides on the fluorograph. Both the

FIGURE 11: Photoaffinity cross-linking of [^{3}H]SAM to PMEAMeT in the presence and absence of: PEA, PMEA, PDEA, PCho, or K-Pi

1.65 μg (10 μL) of sample (Fraction 67 from SW-300 #1) was mixed with 2.0 μL of [^{3}H]SAM (0.55 μCi μL^{-1}). Either PEA, PMEA, PDEA, PCho or K-Pi were added to a final concentration of 1 mM. The mixture was then irradiated with 254 nm UV light on ice for 30 min. Sample preparation and electrophoresis conditions were as reported in Materials and Methods. The entire sample volume was then loaded onto a 7.5-15% SDS-polyacrylamide gel and polypeptides were separated electrophoretically. The gel was stained with Coomassie dye and exposed to Amplify fluorographic reagent for 45 min before drying. Panel A shows a fluorograph of the gel following a 1.5 month exposure to X-ray film. Panel B is the Coomassie-stained gel and shows that equal quantities of protein were loaded onto each lane of the gel. Additions to the photoaffinity cross-linking assay are given along the bottom of the gel. Arrows along the left side show positions of 30 and 50 kDa polypeptides that photoaffinity cross-link with [^{3}H]SAM.


A
Mr (kDa)
97.4
66.2
45.0
31.0
21.5
14.4
Addition None PEA PMEA PDEA PCho K-Pi


B
Mr (kDa)
97.4
66.2
45.0
31.0
21.5
14.4
Addition None PEA PMEA PDEA PCho K-Pi

30 and the 50 kDa polypeptides were present on the fluorograph when PEA, PCho, or K-Pi were included in the photoaffinity cross-linking assay indicating that none of these additions prevented cross-linking of these polypeptides to [^{3}H]SAM. The differences in relative intensity of bands between treatments and the control sample may relate to the amount of protein loaded. However Figure 11 Panel B shows the Coomassie-stained gel which was exposed to the X-ray film, and this gel shows that equal amounts of protein (1.65 μg) were loaded for each sample.

SDS-Polyacrylamide Gradient Gel Electrophoresis of the Sample Purified 7,500-fold

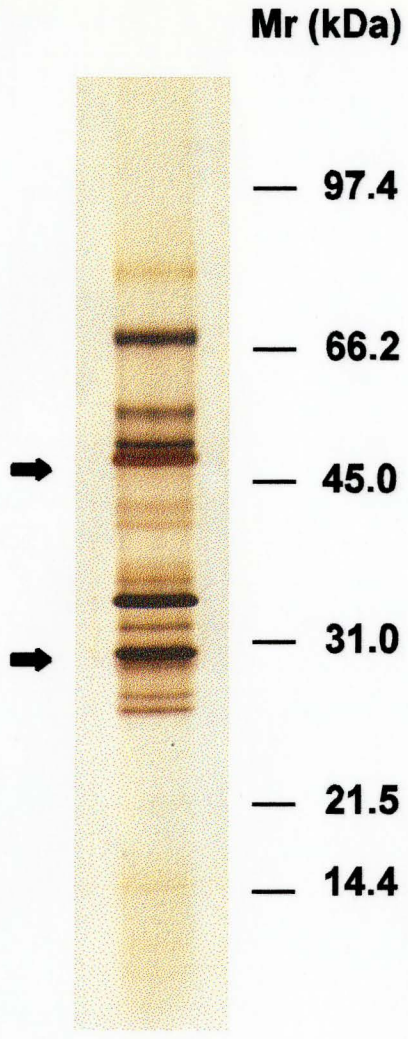
SDS-PAGE analysis was used to determine the number of polypeptides present in the 7,500-fold purified sample. A total of 0.10 μg of protein was loaded onto the gel and silver-staining revealed approximately 15 polypeptides still present in this sample (Fig. 12). Presumably these polypeptides are associated with proteins that share similar physical properties to PMEAMeT and so could not be separated from PMEAMeT by the seven-step purification strategy used.

Thin Layer Chromatography of Phosphobases Produced from the PMEAMeT Assay

The level of [^{3}H]phosphobases in the 0.1 N HCl eluted from the Dowex 50W (H^+) cation exchange columns of phosphobase *N*-methyltransferase assays provides information about the rate of transfer of methyl groups from [^{3}H]SAM to the substrates PEA, PMEA, or PDEA (Mudd and Datko, 1989a,b,c). Furthermore, the calculated PMEAMeT activity is based upon the assumption that PMEAMeT transfers a [^{3}H]-methyl group from SAM to

FIGURE 12: SDS-PAGE analysis of the sample purified over 7,500-fold

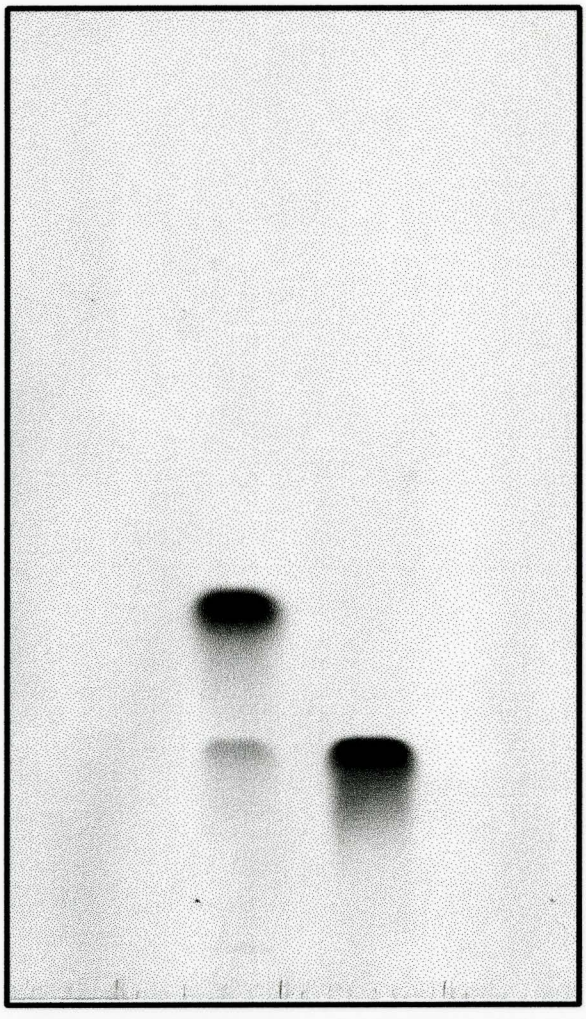
A 5 μL (0.105 μg) aliquot of sample fraction number 70 from SW-300 Trial no.2 was added to 5 μL of SDS-solubilizing buffer, incubated at 90 °C for 3 min to denature proteins, and loaded onto a 7.5-15% SDS-polyacrylamide gel. Proteins were then separated by electrophoresis at a constant current of 15 mamps until the SDS-solubilizing buffer reached the bottom of the gel. The gel was then silver stained and dried (see Materials and Methods). The amount of PMEAMeT activity (nmol mL^{-1} min^{-1}) and specific activity (nmol min^{-1} mg^{-1}) of the sample loaded is given below the gel. Arrows along the left of the gel denotes the positions of the 30 and 50 kDa polypeptides which were previously shown to photoaffinity cross-link with [^{3}H]SAM.



PMEAMeT Activity (nmol mL^{-1} min^{-1})	68.43
Specific Activity (nmol min^{-1} mg^{-1})	3243.13

FIGURE 13: Autoradiogram of a thin layer chromatography plate used to separate and identify phosphobase products of an assay performed with an 2,820-fold purified PMEAMeT sample

A sample fraction from SW-300 gel filtration chromatography purified 2,820-fold for PMEAMeT activity was assayed for PEAMeT, PMEAMeT, and PDEAMeT activity with [^{14}C]SAM for 30 min. Unlabeled carrier PEA, PMEA, PDEA, and PCho were added to a final concentration of 300 mM to the 9 mL of 0.1 N HCl eluted from the Dowex 50W(H^+) columns of the P-base MeT assay. The [^{14}C]-labeled P-base products with unlabeled carriers were then flash frozen, concentrated by freeze drying, and the dried samples were each dissolved in a total of 600 μL of 0.1 N HCl (see Materials and Methods). The 600 μL volume was concentrated by N-evaporation at 40 °C and the dried sample was redissolved in 10 μL of 0.1 N HCl. Aliquots of each sample were analyzed by thin layer chromatography (TLC) on Silica G plates. 4μL of each 0.1 N HCl dissolved sample were applied to the plate, air dried, and then the plates were placed into a TLC tank containing 50-50-10 (v/v/v) n-butanol, methanol, and conc HCl, respectively. The plates were developed by allowing the solvent to ascend for 6 h. The positions of the non-radiolabeled P-bases on the stained TLC plate are shown along the right side of the figure. Radiolabeled P-bases were identified after exposure of the dried TLC plate to X-ray film for 1 week. The P-base substrate used in each P-base MeT assay is given along the bottom of the fluorograph.



Addition	PEA	PMEA	PDEA	H_2O
PMEAMeT Activity ($nmol\ mL^{-1}\ min^{-1}$)	None detected	36.21	34.30	

PMEA, thus producing [^{3}H]PDEA and SAH as sole reaction products. The validity of this assumption can be determined by TLC analysis of the radiolabeled products eluted from the Dowex matrix. That is, TLC can be used to identify and quantify the radiolabeled P-base products (Fig. 13). Consequently, a preparation purified 2,820-fold for PMEAMeT activity was selected to identify and quantify the P-base MeT reaction products. For the TLC procedure, [^{14}C]SAM was used as the radiolabeled substrate instead of [^{3}H]SAM which allowed for the radiolabeled phosphobases to be identified by exposing the TLC plate directly to X-ray film without the use of a fluorographic reagent.

When the phosphobase PEA was included in the PMEAMeT assay no [^{14}C]-labeled phosphobase products were identified on the fluorograph (Fig. 13). This was also evident in the calculation of the PEA *N*-methylating activity of the 2,820-fold purified SW-300 sample which showed no activity associated with the P-base PEA. The inclusion of P-base PMEA in the 30 min PMEAMeT assay resulted in the formation of [^{14}C]PDEA and some [^{14}C]PCho which can be clearly seen on the fluorograph (Fig. 13). This represents a PMEA *N*-methylating activity of 36.210 nmol mL^{-1} min^{-1}. When PMEAMeT was assayed with the P-base PDEA only [^{14}C]PCho was identified (Fig. 13). The PDEAMeT *N*-methylating activity of the SW-300 sample was 34.302 nmol mL^{-1} min^{-1}. The last lane of the TLC is a control lane where H_2O was added to the PMEAMeT assay in lieu P-base substrate and, as expected, shows no [^{14}C]-labeled products on the fluorograph.

DISCUSSION

Phosphobase *N*-methyltransferase Activities

There are at least two *N*-methyltransferase enzymes in spinach leaves that are believed to synthesize PCho from PEA (Fig.1). The first enzyme designated PEAMeT can convert PEA → PMEA → PDEA → PCho (Smith *et al.*, 2000). Thus while PEAMeT is named in recognition of its capacity to convert PEA → PMEA, it also contributes towards PMEAMeT activity (PMEA → PDEA), and PDEAMeT activity (PDEA → PCho). To further complicate nomenclature, the second enzyme designated PMEAMeT can convert PMEA → PDEA → PCho and therefore has PMEAMeT and PDEAMeT activities (Fig. 1) (Dhadialla, 1999 and this thesis). To date, no enzyme with the sole capacity to convert PDEA → PCho has been identified for any plant source.

PEAMeT was purified from spinach leaves over 5,400-fold and found to have an estimated molecular mass of 54 kDa using SDS-PAGE and photoaffinity cross-linking with [^{3}H]SAM (Smith *et al.*, 2000). PEAMeT has also been shown to be inhibited to varying extents by the metabolites SAH, PCho, K-Pi and by the cations $Mn^{2+}$and Co^{2+} but not Mg^{2+} (Smith *et al.*, 2000). Comparing spinach leaf extracts from unsalinized plants to those prepared from plants exposed to 200 mM NaCl showed that in the salinized plants, PEAMeT activity was up-regulated approximately 2.9-fold while PMEAMeT and PDEAMeT activities were only up-regulated approximately 1.7 and 1.5-fold, respectively (Weretilnyk and Summers, 1992) Weretilnyk *et al.*, (1995) showed that only PEAMeT activity was diurnally regulated in spinach plants, being highest at the end of an 8 h light period and lowest at the

end of a 16 h dark period while the PMEAMeT and PDEAMeT activities were apparently unaffected during the photoperiod. Furthermore, in spinach plants that were exposed to a dark period exceeding 40 h, almost no PEAMeT activity was detected while PMEAMeT and PDEAMeT activities were found to be unchanged (Weretilnyk *et al.*, 1995). This latter observation made spinach plants purchased from a local market an ideal source of enzyme for PMEAMeT purification since the level of PEAMeT activity would already be greatly diminished and hence its contribution towards PMEA methylation activity would be similarly reduced.

PMEAMeT from spinach leaves was previously partially purified 43-fold using a four-step purification strategy involving $(NH_4)_2SO_4$ fractionation, anion exchange chromatography on DEAE Sepharose, hydrophobic interaction chromatography on Phenyl Sepharose, and anion exchange chromatography on a High Q anion exchange matrix (Dhadialla, 1999). Studies done on this partially purified PMEAMeT showed that PMEAMeT activity has a slightly alkaline pH optimum between 8.5-9.0 compared to PEAMeT which has a pH optimum closer to that of the cytosol at 7.8. The native molecular masses of both PMEAMeT and PEAMeT have been estimated to be 76.4 kDa and 77 kDa, respectively, by HPLC gel filtration chromatography on a Protein Pak SW-300 matrix (Smith et al., 2000; Dhadialla, 1999). However there is a discrepancy of 23 kDa in the molecular mass estimate for PEAMeT under denaturing conditions on SDS-PAGE where a 54 kDa polypeptide was shown to photoaffinity cross-link with [^{3}H]SAM (Smith *et al.*, 2000). The nature of this 23 kDa difference in the molecular mass estimate is presently not understood (Weretilnyk, personal communication). PMEAMeT has also been shown to be inhibited by the metabolites SAH and

PCho and unlike PEAMeT, PMEAMeT activity is inhibited by the cation Mg^{2+} (Dhadialla, 1999; Smith *et al.*, 2000).

Starting with crude spinach leaf extracts, PMEAMeT was purified by selecting fractions obtained at each step that displayed high levels of PMEA *N*-methylating activity (Tables II to X). To evaluate the efficiency of each step, recoveries for PEA, PMEA and PDEA *N*-methylating activities were estimated. Although the spinach plants that were used for the PMEAMeT purification were purchased from a local supermarket, PEAMeT activity was detected in all five of the crude spinach leaf preparations (Tables II to VI). However, the overall total PEAMeT activity in the crude spinach leaf extracts was only 8% of the total PMEAMeT and PDEAMeT activities (compare Tables II to IV). After precipitation with $(NH_4)_2SO_4$ the average total PEAMeT activity was only 63% of that present in the crude extracts (compare Tables II to IV) and declines to only 49% after the sample preparations were subjected to anion exchange chromatography on the DEAE Sepharose CL-6B matrix (compare Tables II to IV). After hydrophobic interaction chromatography on Phenyl Sepharose CL-4B, PEAMeT activity in the resulting sample was below the detection limits of the assay in four of the five sample preparations (Tables II, IV, V, VI). In the Dec 2/98 preparation the total PEAMeT activity measured increases from 107 nmol min^{-1} after chromatography on the DEAE matrix, to 295 nmol min^{-1} after chromatography on the Phenyl Sepharose matrix (Table III). This apparent increase in enzyme activity is not likely a result of an overall increase in the amount of PEAMeT protein in the preparation, but rather results from the removal of proteins and metabolites in the sample which are inhibitory to PEAMeT activity. Similar "gains" in PEAMeT activity were observed during the purification of

PEAMeT and PMEAMeT (Smith, 1995; Dhadialla, 1999). No PEAMeT activity was measured in any of the preparations after gel filtration chromatography on Sephacryl S-100.

PMEAMeT Purification Strategy

The presence of PMEAMeT at all of the stages of purification was determined by assaying PMEA → PDEA activity. Although PEAMeT is able to catalyze both PMEA → PDEA activity and PDEA → PCho activity, the contribution of PEAMeT activity to that of PMEAMeT was found to be low or absent from the most pure preparations (see discussion above). Smith (1995) reported that rates of conversion of PEA to PMEA and PMEA to PDEA by PEAMeT were roughly equivalent. In this case, one would expect that PMEAMeT activity could never exceed the rate of PEAMeT activity if both reactions were catalyzed by a single enzyme. In this study, this is clearly not the case since PMEAMeT activity is enriched in the absence of PEA → PMEA *N*-methylating activity following chromatography on Sephacryl S-100 (Fig. 9).

The seven-step PMEAMeT purification strategy used to partially purify PMEAMeT exploited properties of the protein such as its capacity to precipitate with $(NH_4)_2SO_4$, interaction with different anionic and hydrophobic matrices, and mobility through two gel filtration matrices with different resolving powers (Tables II to X). Of the various matrices, gel filtration on Sephacryl S-100 and on Protein Pak SW-300 provided the highest overall fold-improvement of specific activity between what was loaded on the column and the sample recovered following chromatography (Tables II to X). Overall, PMEAMeT was purified in excess of 7,500-fold after gel filtration chromatography on SW-300 (Table X, Fig. 13). This

sample (Fraction 70, SW-300#2) contained 6.843 nmol min^{-1} total PMEAMeT activity with only 0.00211 mg of total protein yielding a specific activity of 3,243.127 nmol min^{-1} mg^{-1} (Fig. 13). The percent recovery of PMEAMeT activity in this sample was only 0.043% of the total PMEAMeT activity present in the crude extract.

Sample Fraction 70 from SW-300 Trial no.2 was analyzed by SDS-PAGE and at least 15 bands can be seen following silver staining of the gel (Fig. 13). The lack of a homogeneous PMEAMeT preparation even after being purified over 7,500-fold and being subjected to six different column matrices shows that there are still other polypeptides that must share sufficiently similar properties to PMEAMeT that are difficult if not impossible to remove. Taking into account the large quantity of spinach leaves that were used in the preparation of crude extracts (10 kg), and that the level of protein in the sample that was purified 7,500-fold contained only 0.00005% of the total soluble protein in the crude extract, it is apparent that PMEAMeT is not an abundant protein in spinach leaves. PEAMeT protein in the 5,400-fold purified sample is reported to also be low (0.0003%) relative to total soluble protein in the crude extract (Smith *et al.*, 2000). The low estimated abundance for PEAMeT and PMEAMeT is not extraordinary since the total protein levels of purified enzymes choline monooxygenase and betaine aldehyde dehydrogenase account for 0.0007% and 0.006% of the total soluble proteins in the crude leaf extracts, respectively (Burnet *et al.*, 1995; Weretilnyk and Hanson, 1989).

Affinity chromatography using an SAH-Sepharose column has been shown to bind other methyltransferase enzymes allowing for a selective means of enzyme purification (Upmeier *et al.*, 1988; Sharma and Brown, 1978). In addition to the seven purification steps

used for the PMEAMeT purification, affinity chromatography using SAH-Sepharose and adenosine-agarose matrices was attempted in this study. In the trials performed using an SAH-Sepharose column, several strategies were used to try to selectively elute PMEAMeT protein from the remaining proteins in a sample purified 22-fold by chromatography on a Phenyl Sepharose matrix (see Materials and Methods). Some of the elution conditions used included elution by 100 or 200 μM SAH in the presence or absence of NaCl, but no combination of adjuvants to the buffer led to any improvement in the purification of PMEAMeT. The non-selective behavior of the SAH-Sepharose matrix towards PMEAMeT protein led to affinity chromatography trials using an adenosine-agarose matrix. Adenosine-agarose chromatography has been successfully used for the purification of several SAM-methyltransferases (Ni and Hager, 1998; Attieh *et al.*, 1995). An adenosine-agarose matrix was loaded with 241 nmol min^{-1} of the same sample used in SAH-Sepharose trials and all of the PMEAMeT activity loaded was found in the column effluent. A similar approach and comparable outcome for affinity chromatography on adenosine-agarose for a less pure preparation was reported by Dhadialla (1999).

In order to limit losses incurred while performing the different purification steps, several precautions were taken to maintain enzyme stability. These measures included initially grinding the spinach leaves in grind buffer adjusted to pH 7.8, a pH that is similar to that of the cytoplasm of spinach cells (pH 7.5) (Gout *et al.*, 1992). A 1 mM concentration of Na_2-EDTA was included in most of the buffers to chelate any divalent metal cations that could be inhibitory to the enzyme activity and 5 mM DTT was used to protect the enzyme from inactivation in the presence of oxygen (Scopes, 1982; Stryer, 1981). DTT is essential to

maintain PEAMeT activity (Summers, personal communication). Glycerol at a final concentration of 10 % (v/v) was included in most of the buffers used during chromatography as it has been shown to maintain enzyme stability (Eisenthal and Danson, 1992). Enzyme stability is also facilitated by keeping the enzyme proteins concentrated at all times (Eisenthal and Danson, 1992). Passivation of all plasticware with either 6% (w/v) PEG or dimethyldichlorosilane to minimize the binding of proteins was also found necessary for the purification of PMEAMeT as is true for PEAMeT. Throughout the purification procedure, samples were routinely concentrated by ultrafiltration using a stirred cell concentrator or Amicon Centricon 30 prior to freezing the sample in liquid N_2 and storage at -80 °C.

Photoaffinity Cross-linking of [^{3}H]SAM to PMEAMeT Shows Polypeptides at 30 kDa and 50 kDa Correlate with PMEAMeT Activity

Photoaffinity cross-linking of *S*-adenosyl-L-methionine-dependant methyltransferase enzymes with [^{3}H]SAM has been used for the identification and characterization of several methyltransferase enzymes including phosphoethanolamine *N*-methyltransferase (PEAMeT), guanidinoacetate methyltransferase (GA MeT), cytosine-5-methyltransferase (CheR MeT), and phosphatidylethanolamine-methyltransferase (PtdEA MeT) (Smith *et al.*, 2000; Takata and Fujioka, 1992; Suddaramaiah and Simms, 1992; Pajares *et al.*, 1984). UV irradiation with short wave (254 nm) light results in the covalent and irreversible binding of [^{3}H]SAM to the SAM-binding site of active enzymes (Som and Friedman, 1990). In order to identify which polypeptides in a crude extract or partially purified sample correspond to PMEAMeT, sample fractions representative of the PMEAMeT activity peak collected after anion exchange

chromatography on Mono Q were photoaffinity cross-linked with [^{3}H]SAM and analyzed by SDS-PAGE and fluorography. In Figure 10, two polypeptides of approximately 30 and 50 kDa are revealed on X-ray film after a 3 month exposure of the film to the dried SDS-PAGE gel. Figure 10 also shows that the 30 and 50 kDa [^{3}H]SAM-labeled polypeptides elute differentially from the Mono Q anion matrix: the 30 kDa polypeptide elutes earlier from the column (Fraction 39) than the 50 kDa polypeptide (Fraction 42). When PMEAMeT activity present in sequentially collected fractions are compared, two peaks of PMEAMeT activity are detected: the first peak is associated with the appearance of the 30 kDa [^{3}H]SAM-labeled polypeptide (Fraction 40, 0.27 nmol min^{-1}) with the second peak associated with the appearance of the 50 kDa [^{3}H]SAM-labeled polypeptide (Fraction 43, 0.26 nmol min^{-1}). PMEAMeT activity was contributed by both species in Fraction 42 (0.24 nmol min^{-1}) where both the 30 kDa and 50 kDa polypeptides are shown to co-elute from the Mono Q anion exchange matrix. The enrichment of the two polypeptides in different fractions collected following Mono Q is evidence that there two separate polypeptides associated with PMEAMeT activity and raises an interesting question regarding the structure of native PMEAMeT given its estimated 76 kDa Mr (Dhadialla, 1999). That is, while the sum of the two polypeptides approximates the anticipated Mr for the native PMEAMeT (30 and 50 kDa), the capacity of Mono Q to resolve these species is not consistent with these two polypeptides being subunits of a single, heterodimeric enzyme.

Photoaffinity cross-linking experiments were used by Dhadialla (1999) to identify two radio-labeled polypeptides of approximate Mr at 28.6 and 26 kDa in a sample purified 29-fold following chromatography on a High Q anion exchange resin. The apparent absence of the

50 kDa polypeptide in this partially purified sample could reflect its absence from the sample loaded onto the High Q matrix or a greatly diminished abundance of this species in the sample fractions collected and pooled following chromatography on High Q. In photoaffinity cross-linking experiments performed using sample purified 180-fold by High Q anion exchange (Jan 11/99 preparation), [^{3}H]SAM-labeled polypeptides of 30 and 50 kDa in size were identified after fluorography (results not shown). Since the smaller 26 kDa polypeptide identified by Dhadialla (1999) was not seen following any of the photoaffinity cross-linking experiments performed for this thesis, this SAM-binding polypeptide must have been removed or substantially depleted from the samples tested. It is worth noting that aside from being present in fractions with PMEAMeT activity, no specific test was performed to show that the 26 kDa product identified by Dhadialla (1999) is related to PMEAMeT.

When photoaffinity cross-linking assays are carried out in the presence of a suitable substrate for methylation, binding of [^{3}H]SAM to the methyltransferase under UV light is prevented. In this way, one can use photolabeling assays with [^{3}H]SAM to test a variety of possible substrate(s) for any particular methyltransferase. SDS-PAGE analysis of the crosslinked polypeptides can help identify the proteins that methylate specific substrates. This technique was used in the identification of SAM-binding methyltransferases such as *myo*-inositol 6-*O*-methyltransferase and protein-*O*-carboxylmethyltransferase (Wanek and Richter, 1995; Hurst et al., 1984). In order to determine whether the 30 and the 50 kDa polypeptides are associated with PMEAMeT activity or not, photoaffinity cross-linking was performed on a 1,353-fold purified sample purified by chromatography on SW-300. PEAMeT, unlike PMEAMeT, is able to *N*-methylate PEA. The inclusion of the PEAMeT substrate, PEA, did

not prevent the binding of [^{3}H]SAM to either of the two polypeptides. PCho, which is the final product of both PMEAMeT and PEAMeT *N*-methylation and an inhibitor for both enzymes (Smith et al, 2000; Dhadialla, 1999), also had no effect on the binding of [^{3}H]SAM to either polypeptides. K-Pi has been shown to act as both an inhibitor of PEAMeT (Smith *et al.*, 2000) and to a lesser extent, of PMEAMeT (Dhadialla, 1999). The inclusion of K-Pi in the photoaffinity cross-linking assay did not prevent the binding of [^{3}H]SAM to either polypeptide. However, the inclusion of phosphobase substrates PMEA or PDEA in the photoaffinity cross-linking assay prevents [^{3}H]SAM from being covalently bound to both the 30 and 50 kDa polypeptides, a finding consistent with both P-bases being suitable substrates for these proteins. The specificity shown in the binding of [^{3}H]SAM to the 30 and 50 kDa polypeptides in the presence and absence of PEA, PMEA, PDEA, PCho, or K-Pi shows that the both the 30 and the 50 kDa polypeptides are associated with PMEAMeT activity. Moreover, since both polypeptides use PMEA and PDEA as substrates, both are associated with a PMEAMeT enzyme that can catalyze the sequential reactions of PMEA→ PDEA→ PCho.

Future Molecular Experiments on PMEAMeT

Since PEAMeT and PMEAMeT are both able to *N*-methylate PMEA and PDEA, it is anticipated that these two methytransferase enzymes share regions of similar if not identical amino acid sequence. Recently, the cDNA encoding PEAMeT was isolated by functional complementation of a *Schizosaccharomyces pombe cho2-* mutant. The cDNA encodes a protein with PEAMeT activity and shows no ethanolamine- or phosphatidylethanolamine *N*-

methyltransferase activity (Nuccio *et al.*, 2000). In a comparable experimental approach using a *Saccharomayces cerevisiae opi3* mutant, an *Arabidopsis thaliana* cDNA sequence encoding PEAMeT (*AtNMT1*) has also been isolated (Bolognese and McGraw, in press).

The cDNA sequence for *AtNMT1* was used as a template to identify genes related to the one encoding PEAMeT by performing a BLAST search against the *Arabidopsis thaliana* genome posted on the National Center for Biotechnology Information (NCBI) web site. Three genes in *A. thaliana* were found to share homology with PEAMeT and are identified as *F25P22.1* and *T1N15.23,* which lie on chromosome 1, and the third *MEB5.19* lies on chromosome 3. Of the three genes the last one on chromosome 3 encodes *AtNMT1* given the high degree of identity found. A potential avenue for future research would be to obtain full -length cDNAs or Expressed Sequence Tags corresponding to *F25P22.1* and *T1N15.23* in order to determine if these two genes encode PMEAMeT enzymes. This determination could be performed by over-expression of their full-length cDNAs in *E. coli* strains engineered to express cDNA inserts at high levels and then biochemically verifying the presence of PMEAMeT activity in cell-free extracts of the bacteria. This approach is possible because PMEAMeT activity is not present in cell-free extracts of *E. coli* (data not shown).

Access to clone(s) encoding PMEAMeT from either Arabidopsis or spinach would permit one to address many questions regarding the properties and role(s) of these enzymes *in vitro* and *in planta.* For example, why do two enzymes with apparently identical substrate usage exist? Are both enzymes equally efficient with respect to Km's for PMEA and PDEA or could distinctions in this regard underlie the necessity for two enzymes to operate in

plants? Do these enzymes operate in different compartments in the cell? Does the presence of two PMEAMeT enzymes capable of converting PMEA to PCho reflect the need by the plant to ensure that neither PMEA nor PDEA accumulate under conditions where PEAMeT activity is reduced? What might the consequences be if either or both enzymes were inactivated or otherwise down-regulated (using antisense suppression or gene knock-out approaches with transgenic plants)? It is worth noting that the pool sizes for PMEA and PDEA are small for spinach, including salt-stressed spinach where the overall flux rate of choline synthesis is increased (Summers and Weretilnyk, 1993; Coughlan and Wyn Jones, 1982). The maintenance of low pool sizes could be beneficial for the plant, including for stressed plants. A possible role for these enzyme(s) might be to prevent the incorporation of PMEA and PDEA into phospholipids in lieu of or in addition to PCho. Why or if the incorporation of PMEA or PDEA into the polar head group of a membrane phospholipid is a problem is unknown at present. Finally, access to clones encoding these gene products would enable us to determine if these genes are differentially regulated in plants. That is, when plants are salt-stressed or placed in the dark, are the genes for both PMEAMeT enzymes constitutively expressed and are their products equally stable? One could envision a scenario where the differential regulation of these genes might result in the apparently constitutive enzyme activity reported for PMEAMeT in spinach leaves (Weretilnyk *et al.*, 1995).

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