Biochemical Evidence for Two Novel Enzymes in the Biosynthesis of 3-Dimethylsulfoniopropionate in *Spartina alterniflora*[1]

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3-Dimethylsulfoniopropionate (DMSP) is an osmoprotectant accumulated by the cordgrass *Spartina alterniflora* and other salt-tolerant plants. Previous in vivo isotope tracer and metabolic modeling studies demonstrated that *S. alterniflora* synthesizes DMSP via the route *S*-methyl-Met → 3-dimethylsulfoniopropylamine (DMSP-amine) → 3-dimethylsulfoniopropionaldehyde → DMSP and indicated that the first reaction requires a far higher substrate concentration than the second to attain one-half-maximal rate. As neither of these reactions is known from other organisms, two novel enzymes are predicted. Two corresponding activities were identified in *S. alterniflora* leaf extracts using specific radioassays. The first, *S*-methyl-Met decarboxylase (SDC), strongly prefers the L-enantiomer of *S*-methyl-Met, is pyridoxal 5′-phosphate-dependent, generates equimolar amounts of CO_2 and DMSP-amine, and has a high apparent K_m (approximately 18 mM) for its substrate. The second enzyme, DMSP-amine oxidase (DOX), requires O_2 for activity, shows an apparent K_m for DMSP-amine of 1.8 mM, and is not accompanied by DMSP-amine dehydrogenase or transaminase activity. Very little SDC or DOX activity was found in grasses lacking DMSP. These data indicate that SDC and DOX are the predicted novel enzymes of DMSP synthesis.

The tertiary sulfonium compound 3-dimethylsulfoniopropionate (DMSP) is accumulated by certain salt-tolerant angiosperms and many marine algae (Malin and Kirst, 1997; McNeil et al., 1999). DMSP is structurally analogous to a betaine and like betaines, functions as a cytoplasmic compatible solute or osmoprotectant and contributes to adaptation to osmotic and freezing stresses (Rhodes and Hanson, 1993; Karsten et al., 1996; Vianney et al., 1998). DMSP differs from betaines in that it contains sulfur instead of nitrogen and, in DMSP-accumulating plants, appears to act as a substitute for betaines when nitrogen is scarce (Colmer et al., 1996; Cooper and Hanson, 1998). Engineering accumulation of betaines or other osmoprotectants can improve osmotic or freezing stress resistance (Holmberg and Bülow, 1998; Nuccio et al., 1999). Since nitrogen often limits crop growth, and DMSP accumulation does not require nitrogen, DMSP synthesis is an attractive target for the engineering of stress resistance in low-nitrogen environments (McNeil et al., 1999).

DMSP biosynthesis is also environmentally important because DMSP is the main biogenic precursor of dimethylsulfide (DMS) released to the atmosphere from the oceans (Malin and Kirst, 1997) and is a likely precursor of DMS coming from land (Dacey et al., 1987; Paquet et al., 1994). Biogenic DMS plays a pivotal role in the global sulfur cycle, affects the pH of precipitation, and is believed to contribute to the regulation of global climate (Malin, 1996).

In vivo isotope labeling and modeling studies (Kocsis et al., 1998) demonstrated that DMSP biosynthesis in the saltmarsh cordgrass *Spartina alterniflora* proceeds from L-Met via *S*-methyl-Met (SMM), 3-dimethylsulfoniopropylamine (DMSP-amine) and 3-dimethylsulfoniopropionaldehyde (DMSP-ald; Fig. 1). The first and last steps in this pathway are the same as in the dicot *Wollastonia biflora*, but the central part is not. In *W. biflora*, SMM is converted directly to DMSP-ald without formation of DMSP-amine, most likely via a transamination/decarboxylation mechanism (Hanson et al., 1994; James et al., 1995; Rhodes et al., 1997). All angiosperms appear to produce SMM (Mudd and Datko, 1990; Bourgis et al., 1999), and to have enzymes that can catalyze the conversion of DMSP-ald to DMSP (Trossat et al., 1997; Vojtěchová et al., 1997). It is thus the conversion of SMM to DMSP-ald that is unique to DMSP synthesis, and *S. alterniflora* appears to have evolved specific enzymes that mediate this conversion. Besides predicting the existence of novel enzymes in *S. alterniflora* that convert SMM to DMSP-amine, and DMSP-amine to DMSP-ald, the in vivo tracer and modeling studies predicted that, of these enzymes, the first has a much higher K_m for its sulfonium substrate than the second (Kocsis et al., 1998).

We report here the identification, initial characterization, and assay procedures of two enzymes from *S. alterniflora* that catalyze the SMM → DMSP-amine and DMSP-amine → DMSP-ald steps. The first is SMM decarboxylase (SDC). The second is DMSP-amine oxidase (DOX). Comparative biochemical

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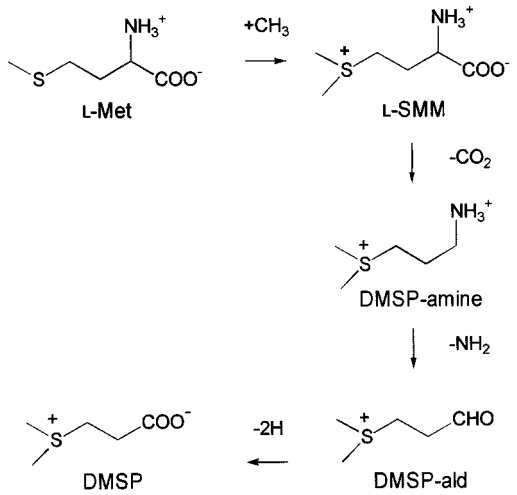

Figure 1. The DMSP biosynthesis pathway in *S. alterniflora*. Only the decarboxylation of SMM and conversion of DMSP-amine to DMSP-ald are unique to DMSP biosynthesis. Enzymes able to catalyze the other two steps are widespread in angiosperms.

data indicate that both are specific to the DMSP pathway.

RESULTS

Extraction and Assay of SDC Activity

Amino acid decarboxylases can be conveniently assayed by measuring the release of $^{14}CO_2$ from ^{14}C-carboxyl-labeled substrate. However, such assays must first be validated by confirming that CO_2 and amine production are in a 1:1 molar ratio because other reactions in plant extracts may lead to loss of the carboxyl group as CO_2 (Birecka et al., 1985). To test this, L-[U-^{14}C]SMM was used as substrate, and [^{14}C]DMSP-amine and $^{14}CO_2$ were quantified. [^{14}C]DMSP-amine formation was readily detected (Fig. 2A), and ^{14}C quantification indicated a CO_2:DMSP-amine molar ratio of 0.97 ± 0.01 (mean ± SE; n = 3). SDC was therefore assayed in subsequent work by measuring $^{14}CO_2$ release from L-[1-^{14}C]SMM. SDC activity showed a strong preference for the L enantiomer of SMM. D-[1-^{14}C]SMM gave <4% of the activity observed with L-[1-^{14}C]SMM (Fig. 2B).

Most amino acid decarboxylases have a pyridoxal 5′-phosphate (PLP) coenzyme, but a few use a covalently-bound pyruvate as prosthetic group instead (Recsei and Snell, 1984; John, 1995). We therefore tested the effect of including PLP (0.1 or 1 mM) in the buffers used to extract and assay SDC. Adding PLP to the assay buffer doubled activity and adding it to both extraction and assay buffers increased activity 18-fold. These data indicate that SDC requires PLP for stability as well as activity. PLP (0.1 mM) was therefore added to all SDC buffers. Progress curves showed that the rate of $^{14}CO_2$ production from L-[1-^{14}C]SMM declined slowly with time (Fig. 2C). This was not due to substrate depletion or product inhibition (see below) and presumably not to abortive transamination (and hence, inactivation) of the coenzyme because PLP was present to replace the inactivated species (John, 1995). We therefore attribute the activity loss to inactivation of the enzyme itself. A 75-min incubation time was adopted for subsequent work. Using this incubation time, $^{14}CO_2$ formation was approximately linearly related to enzyme level (not shown).

Characteristics of SDC Activity

SDC showed a broad pH optimum around 7, retaining ≥75% of the maximum activity between pH 6.5 and 8. SDC activity was one-half-maximal at about 18 mM SMM, and its V_{max} was estimated as 0.28 nmol min^{-1} mg^{-1} protein (crude extract). Unlike some other decarboxylases (Grossfeld et al., 1984), SDC is not highly sensitive to inhibition by the amine product. A 2-fold molar excess of DMSP-amine over SMM had no effect on activity. SDC activity appears

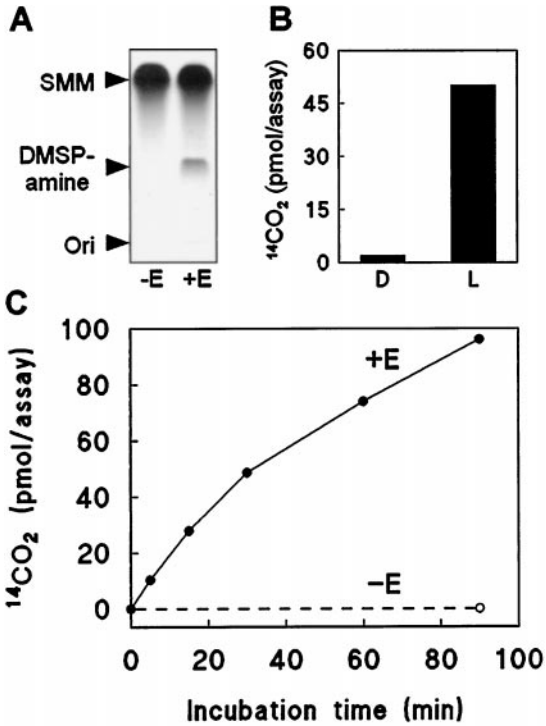

Figure 2. Characterization of SDC activity in *S. alterniflora* leaf extracts. Buffers contained 0.1 mM PLP. A, Formation of [^{14}C]DMSP-amine from L-[U-^{14}C]SMM. L-[U-^{14}C]SMM (1.12 nmol, 10.7 kBq) was incubated with (+E) or without (−E) extract (860 μg of protein) for 75 min. The assay mixtures were separated using TLC system 1, and ^{14}C was detected by autoradiography. The positions of standards and the origin (ori) are marked. B, SDC activities assayed using D- or L-[1-^{14}C]SMM as substrate. Assays contained 520 μg of protein. Data are means of triplicates. SE values were ≤4% of the means. C, Progress of $^{14}CO_2$ release from L-[1-^{14}C]SMM, plus or minus extract (330 μg of protein). Data points are means of triplicates. SE values were ≤4% of the means.

not to be a side reaction mediated by one of the decarboxylases found in all plants. This was shown by assaying SDC activity in the presence of a 20-fold molar excess of five L-amino acids for which specific decarboxylases are known (Stevenson et al., 1990; Kumar et al., 1997), all except one (Glu) being structural analogs of SMM (Table I). None of these compounds caused the drastic inhibition that would be expected were SMM a poor alternative substrate for their respective decarboxylases. Met, *S*-adenosyl-L-Met (Ado-Met), Arg, and Glu gave ≤18% inhibition. Orn inhibited activity by 60%.

SDC Activity in Grasses That Do Not Accumulate DMSP

Comparative biochemistry provided further evidence that the SDC activity is due to an enzyme specific to DMSP synthesis. SDC activity in *S. alterniflora* was compared with its activity in three other grasses, one of which is another *Spartina* species, that contain very little or no DMSP (Paquet et al., 1994; Kocsis et al., 1998). The housekeeping enzymes, catalase and malate dehydrogenase (MDH), were also assayed as controls for the quality of the extracts. Their activities were fairly similar in all species (Table II). In contrast only *S. alterniflora* had high SDC activity. *Spartina patens* showed 30- to 40-fold less activity, and neither maize (*Zea Mays*) nor wheat (*Triticum aestivum*) had detectable activity (Table II).

Assay for the Conversion of DMSP-Amine to DMSP-Ald

Because DMSP-amine could, a priori, be converted to DMSP-ald by a transaminase, dehydrogenase, or oxidase, we developed a radioassay able to measure any of these activities. The principles of the assay are schematized in Figure 3 and can be briefly stated as follows: (a) [^{35}S]DMSP-amine is used as substrate. When [^{35}S]DMSP-ald is formed, it decomposes rapidly and spontaneously to give [^{35}S]DMS (Trossat et al., 1996); (b) the [^{35}S]DMS is trapped in 30% H_2O_2, which oxidizes it to non-volatile [^{35}S]dimethyl sulfoxide (DMSO); (c) as some of the [^{35}S]DMSP-ald formed may be chemically or enzymatically oxidized to [^{35}S]DMSP, [^{35}S]DMSP formation is measured at the end of the assay by fractionating the reaction mixture or by decomposing the [^{35}S]DMSP to [^{35}S]DMS by injecting cold NaOH; and (d) catalase is added to the assay to destroy H_2O_2 generated by DOX activity, or diffusing from the DMS trap.

The assay was tested first with purified hog kidney diamine oxidase, for which DMSP-amine is a substrate (Bardsley et al., 1971). After a 75-min incubation, not followed by NaOH treatment, 19.6% of the

Table I. *Effect of L-amino acids on decarboxylation of L-1-[-^{14}C]SMM by S. alterniflora extract*

Assays contained 50 μM L-[1-^{14}C]SMM, 1 mM unlabeled L-amino acid, and desalted leaf extract corresponding to 520 μg of protein. The L-amino acids were neutralized with KOH or HCl. Data are the means of duplicates. SE values were ≤1% of the means.

Amino Acid Added	Inhibition
	%
None	0[a]
Met	0
S-adenosyl-L-Met	18
Arg	9
Orn	60
Glu	7

[a] The production of $^{14}CO_2$ in the no-addition control was 48.9 pmol per assay.

Table II. *SDC activity in leaf extracts of various grasses*

SDC activity was measured in desalted leaf extracts of *S. alterniflora* and three grasses that do not accumulate DMSP, *S. patens,* maize, and wheat. SDC was assayed using an L-[1-^{14}C]SMM concentration of 36 μM and a 75-min incubation time. Catalase and MDH were assayed spectrophotometrically. The experiment was repeated, with similar results.

Species	SDC Activity[a]	Housekeeping Enzyme Activity	
		Catalase	MDH
	pmol h^{-1} mg^{-1} protein	*μmol min^{-1} mg^{-1} protein*	
S. alterniflora	23.9	55.4	10.5
S. patens	0.8	49.5	16.1
Maize	<0.3	40.5	10.2
Wheat	<0.2	188	7.4

[a] Assay data were converted to units of pmol h^{-1} mg^{-1} protein by assuming a constant $^{14}CO_2$ production rate. Because the rate declines slowly with time (Fig. 2C), this slightly underestimates activity.

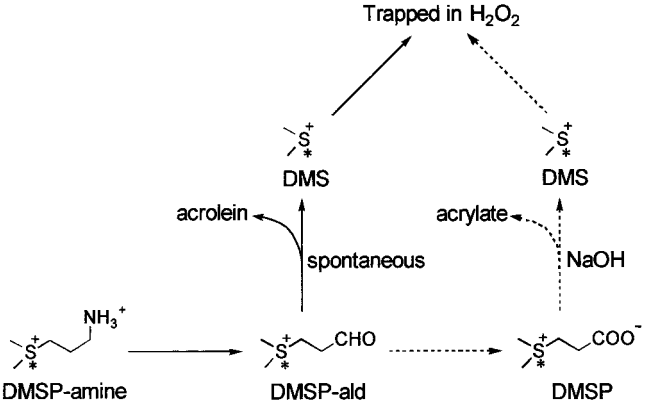

Figure 3. Assay for the enzyme-catalyzed conversion of [^{35}S]DMSP-amine to [^{35}S]DMSP-ald. The assay was carried out in potassium-phosphate buffer, pH 8, in which [^{35}S]DMSP-ald decomposes spontaneously to acrolein and [^{35}S]DMS with a half-life of <4 min (Trossat et al., 1996). The [^{35}S]DMS released partitions into the gas phase and is trapped on a filter disc soaked in 30% H_2O_2. The H_2O_2 oxidizes [^{35}S]DMS to [^{35}S]DMSO, which is non-volatile. In certain conditions, some [^{35}S]DMSP-ald may be oxidized to [^{35}S]DMSP before it breaks down. At the end of the assay, [^{35}S]DMSP can be decomposed to acrylate and [^{35}S]DMS by treatment with NaOH. *, ^{35}S radiolabel.

[^{35}S]DMSP-amine was converted to [^{35}S]DMS (in the trap), 2.1% to [^{35}S]DMSP, and 0.06% to [^{35}S]DMSO (in the reaction mixture). These data show that [^{35}S]DMSP formation can be significant, and that little oxidation of [^{35}S]DMS to [^{35}S]DMSO occurs in the reaction mixture. This is important because DMSO is not volatile and would not be transferred to the trap.

Evidence for a DOX in *S. alterniflora* Extracts

Desalted *S. alterniflora* extracts gave high rates of DMSP-amine → DMSP-ald conversion in the above assay. The activity was not stimulated by α-keto acids or PLP (Table III), indicating that it is not due to a transaminase. Nor was activity increased by adding flavins (Table III) or pyridine nucleotides (Fig. 4), making a dehydrogenase unlikely. Although NAD and NADP did not increase activity, they caused a switch in the major reaction product from [^{35}S]DMS to [^{35}S]DMSP (Fig. 4). This is anticipated because *S. alterniflora* is expected to possess NAD(P)-linked DMSP-ald dehydrogenase activity (Kocsis et al., 1998). Adding NAD(P) as well as DMSP-amine to *S. alterniflora* extracts therefore reconstitutes the last two steps in the DMSP pathway (Fig. 1). The above results suggest, by elimination, that the enzyme mediating the DMSP-amine → DMSP-ald conversion is an oxidase. Direct evidence for this was obtained by removing O_2 from the assay using Glc oxidase plus Glc (Table IV). The Glc oxidase/Glc system reduced activity by 96% when used alone, and by 99% when combined with a nitrogen atmosphere.

Characteristics of DOX Activity

Fractionation of assay mixtures showed that [^{35}S]DMSP became a major product as the amount of extract increased, but that [^{35}S]DMSO formation was never very important (Fig. 5A). The relationship between [^{35}S]DMSP formation and extract concentration is presumably due mainly to DMSP-ald dehydrogenase activity, supported by traces of NAD(P) left after desalting (compare with Fig. 4) and by NAD(P)H oxidase activity in the extract. Total [^{35}S]DMSP-ald formation, whether estimated from the sum of labeled products after fractionation or from total [^{35}S]DMS production when assays were treated with base, was almost the same and was

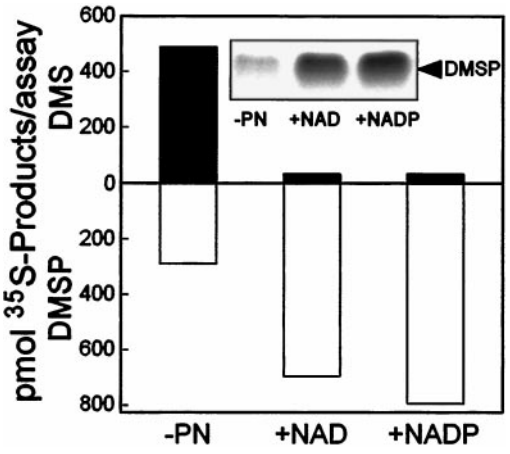

Figure 4. Effect of pyridine nucleotides on conversion of [^{35}S]DMSP-amine to [^{35}S]DMS and [^{35}S]DMSP by *S. alterniflora* leaf extract. After desalting twice, extract (470–720 μg of protein per assay) was incubated for 75 min with 25 nmol (3.7 kBq) of [^{35}S]DMSP-amine, minus (−PN) or plus 1 mM NAD or NADP. L-Ascorbate (5 mM) was included in the assay buffer. Data are means of two or three replicates, normalized to 500 μg of protein per assay. SE values were ≤12% of the means. The identity of the [^{35}S]DMSP formed was confirmed by TLC and autoradiography (inset).

Table III. *Effects of α-keto acids, PLP, or flavins on the conversion of [^{35}S]DMSP-amine to [^{35}S]DMSP-ald by S. alterniflora leaf extract*

Assays contained 1 mM [^{35}S]DMSP-amine and desalted extract (78 μg of protein). The concentration of α-keto acids, FMN and FAD, was 1 mM. The PLP concentration was 0.1 mM. Incubation was for 75 min. The conversion of [^{35}S]DMSP-amine to [^{35}S]DMSP-ald was estimated from total [^{35}S]DMS production when base was added after incubation. Values are the means of duplicate assays. SE values were ≤2% of the means.

Addition	Activity
	pmol/assay
None	924 (100)[a]
α-Ketoglutarate	940 (101)
Oxaloacetate	796 (86)
Pyruvate	897 (97)
Glyoxylate	887 (99)
β-Hydroxypyruvate	849 (95)
PLP	891 (97)
Flavin mononucleotide	813 (88)
Flavin adenine dinucleotide	870 (94)

[a] Values in parentheses are activities as percent of the no-addition control.

Table IV. *O_2-Dependence of the conversion of [^{35}S]DMSP-amine to [^{35}S]DMSP-ald by S. alterniflora leaf extract*

Assays contained 1 mM [^{35}S]DMSP-amine and desalted extract (75 μg of protein) and were carried out in air except where indicated. The final concentration of Glc was 100 mM. Glucose oxidase (GOX) was added at 500 units per assay. Catalase was omitted so that H_2O_2 generated by GOX would not be reconverted to O_2. The conversion of [^{35}S]DMSP-amine to [^{35}S]DMSP-ald was estimated from total [^{35}S]DMS production when base was added after incubation. Subsequent fractionation of reaction mixtures confirmed that no [^{35}S]DMSO was formed. Values are the means of duplicate assays. SE values were ≤3% of the means.

Addition	Activity
	pmol/assay
None	730 (100)[a]
Glc	725 (100)
GOX	675 (93)
Glc/GOX	30 (4)
Glc/GOX (N_2)[b]	5 (1)

[a] Values in parentheses are activities as percent of the no-addition control. [b] The head space of the reaction was flushed with N_2 for 1 min before starting the assay.

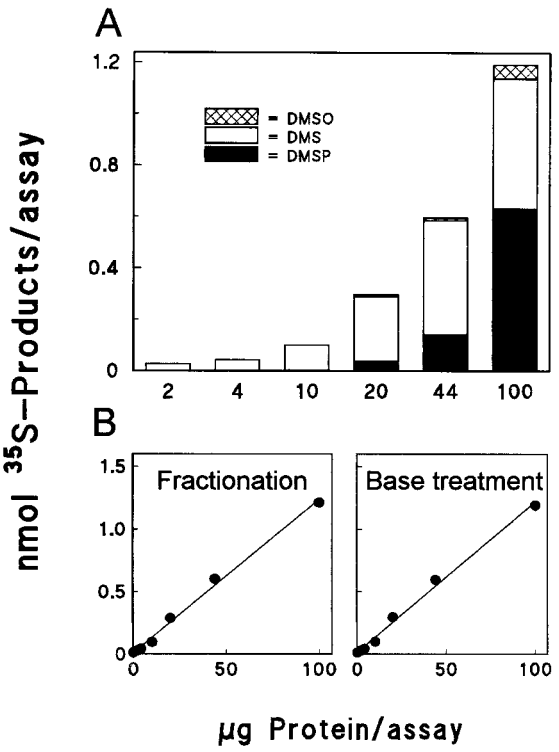

Figure 5. Effect of *S. alterniflora* extract concentration on the nature and amounts of the products formed in the DOX assay. Assays containing 1 mM [^{35}S]DMSP-amine and extract equivalent to 2 to 100 μg of protein were incubated for 75 min, and then either fractionated as described in "Materials and Methods" or treated with 25 μL of 17% NaOH to decompose [^{35}S]DMSP to [^{35}S]DMS. A, Proportions of [^{35}S]DMS, [^{35}S]DMSP, and [^{35}S]DMSO formed as a function of the amount of protein per assay. B, Relationship between the amount of protein per assay and either the summed ^{35}S-products obtained by fractionation ([^{35}S]DMS + [^{35}S]DMSP + [^{35}S]DMSO) (left) or the total amount of [^{35}S]DMS formed when assays were treated with base (right). Data are means of two to five replicates. The sum of the labeled reaction products, plus remaining [^{35}S]DMSP-amine, equaled the amount of [^{35}S]DMSP-amine substrate added to the assay.

linearly related to the amount of protein (Fig. 5B). The base-treatment procedure, which is simpler, was therefore adopted for routine use. [^{35}S]DMSP-ald formation was linear with time for 2 h (not shown). DOX activity was maximal at pH 7.5 to 8, and showed a V_{max} of 0.37 nmol min^{-1} mg^{-1} protein (crude extract), and an apparent K_m for DMSP-amine of 1.8 mM. L-Ascorbate (5 mM) inhibited activity, but improved enzyme extraction. It was therefore routinely added to the DOX extraction buffer and removed by desalting.

Because DMSP-amine is structurally similar to diamines and polyamines, the selectivity of DOX was investigated by adding a 5- or 20-fold molar excess of unlabeled di- and polyamines to DOX assays (Fig. 6). The polyamines, spermidine and spermine, had little effect, but the diamines, 1,3-diaminopropane, putrescine, and cadaverine reduced DOX activity by

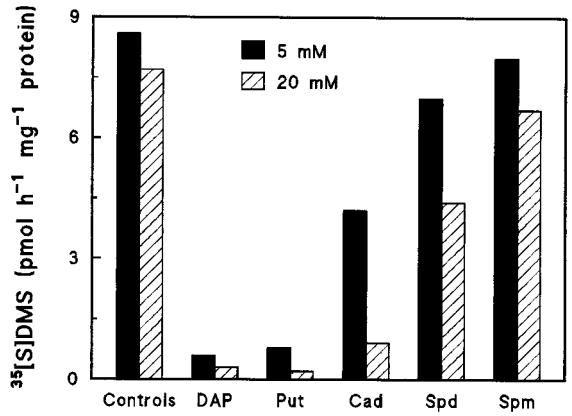

Figure 6. Effect of diamines and polyamines on DOX activity in *S. alterniflora* leaf extract. Assays contained 1 mM [^{35}S]DMSP-amine, and either 5 or 20 mM unlabeled amine or an equivalent quantity of KCl (controls). The reaction mixtures were treated with NaOH to convert [^{35}S]DMSP to [^{35}S]DMS. Data are means of duplicate assays. SE values were ≤2% of the means. DAP, 1,3-Diaminopropane; Put, putrescine; Cad, cadaverine; Spd, spermidine; Spm, spermine.

90% to 98% when present in 20-fold excess. This shows that DOX is not highly selective for DMSP-amine and suggests that it may be related to diamine oxidases. Because these are copper-containing enzymes (Smith, 1985), we attempted to remove enzyme-bound copper using 10 mM diethyldithiocarbamate or 200 mM EDTA. These treatments gave preparations that were stimulated 20% to 30% by 1 mM $CuSO_4$, which was not the case for untreated controls. As copper is hard to strip out of some amine oxidases (Hysmith and Boor, 1988), this result is not inconsistent with a copper requirement.

DOX Activity in Grasses That Do Not Accumulate DMSP

The activities of DOX, catalase, and MDH were measured in *S. alterniflora* and in the non-DMSP-accumulating species *S. patens*, maize, and wheat

Table V. *DOX activity in leaf extracts of various grasses*

DOX activity was measured in extracts of *S. alterniflora* and three grasses that do not accumulate DMSP. DOX was assayed with a [^{35}S]DMSP-amine concentration of 1 mM and a 75-min incubation time using total [^{35}S]DMS production when base was added after incubation. Catalase and MDH were assayed spectrophotometrically. Data are means of duplicates. SE values were ≤12% of the means.

Species	DOX Activity	Housekeeping Enzyme Activity	
		Catalase	MDH
	pmol h^{-1} mg^{-1} protein	*μmol min^{-1} mg^{-1} protein*	
S. alterniflora	10,800	180	16.8
S. patens	145	85	16.7
Maize	44	68	12.2
Wheat	157	235	10.0

(Table V). DOX activity in *S. alterniflora* was about 100-fold higher than in the other plants, whereas catalase and MDH activities were quite similar in all species. These data indicate that the DOX activity in *S. alterniflora* is due principally to an enzyme specific to DMSP synthesis and not to an oxidase of widespread occurrence.

DISCUSSION

We have identified novel enzyme activities in *S. alterniflora* that catalyze the two steps unique to DMSP synthesis in this species, namely the decarboxylation of SMM and the oxidation of DMSP-amine to DMSP-ald. These enzymes are SDC and DOX. We also devised convenient radioassays for SDC and DOX and improved the procedure for synthesizing the [^{35}S]DMSP-amine substrate for the DOX assay. SDC and DOX activities were shown to be robust inasmuch as they withstand freeze-thaw treatment, desalting, and concentration. Together, these advances open the way for future work to purify and characterize SDC and DOX, and to clone their cDNAs.

The in vivo flux through the pathway SMM → DMSP-amine → DMSP-ald → DMSP was estimated from computer modeling of radiotracer data to be 1.6 ± 0.7 nmol min^{-1} g^{-1} fresh weight (Kocsis et al., 1998). The V_{max} values for SDC and DOX activities in *S. alterniflora* extracts (0.28 and 0.37 min^{-1} mg^{-1} protein, respectively) are adequate to account for this flux because leaf protein content in *S. alterniflora* is about 10 mg g^{-1} fresh weight. Modeling of the in vivo labeling data also predicted that the K_m for SMM decarboxylation would be an order of magnitude higher than that for DMSP-amine oxidation (310 versus 5.8 nmol g^{-1} fresh weight, each value being subject to an error of about ± 50%; Kocsis et al., 1998). This prediction agrees well with the 10-fold difference between the apparent K_m values that we estimated for substrates of SDC (about 18 mM) and DOX (1.8 mM).

The absence or very low level of SDC activity in grasses that do not accumulate DMSP indicates that SDC is an enzyme specific to the DMSP pathway. The relative insensitivity of SDC to inhibition by L-amino acids reinforces this inference by showing that SDC activity is unlikely to be a side-reaction mediated by the ubiquitous decarboxylases whose physiological substrates are Met, Ado-Met, Arg, Orn, or Glu. The lack of Ado-Met inhibition also suggests that SDC is not closely related to Ado-Met decarboxylase, even though SMM is very similar in structure to Ado-Met. Additional evidence that SDC and Ado-Met decarboxylase are unrelated is that SDC requires PLP, whereas Ado-Met decarboxylase belongs to the small group of enzymes that use a catalytic pyruvoyl residue, not PLP (Xiong et al., 1997). On the other hand, the modest inhibition of SDC by L-Orn suggests a possible relationship to Orn decarboxylase. Inasmuch as eukaryotic Orn decarboxylases and other basic amino acid decarboxylases share amino acid sequence homology (Sandmeier et al., 1994), such a relationship could offer an indirect approach to the cDNA cloning of SDC.

The very low DOX activities in grasses that lack DMSP imply that DOX is an enzyme associated specifically with DMSP synthesis. The trace of activity found in these species may be because plant diamine oxidases have some activity toward DMSP-amine, as has been demonstrated for the porcine kidney enzyme (Bardsley et al., 1971). Whatever the nature of the slight DOX activity in species that do not accumulate DMSP, this activity is consistent with the finding that such species have a low capacity to oxidize exogenously supplied DMSP-amine (Kocsis et al., 1998). Assuming DOX to be a specific enzyme, it might a priori be related to diamine oxidases or to polyamine oxidases because both types of enzymes occur in grasses (Smith, 1985; Suzuki and Hagiwara, 1993), and DMSP-amine can be considered to be a diamine or polyamine analog. Diamine oxidases and polyamine oxidases are quite different, the former being copper enzymes with a covalently bound topa quinone cofactor and the latter being flavin-containing enzymes (Smith, 1985; Klinman, 1996). That DOX is far more sensitive to inhibition by diamines than polyamines, and modestly stimulated by copper after chelation treatment, suggests that it is more likely to be a member of the copper amine oxidase family. The substantial sequence identity among the members of this family (Padiglia et al., 1998) may, as for SDC, permit the cDNA cloning of DOX by a homology-based approach.

MATERIALS AND METHODS

Plants

Spartina alterniflora Loisel. and *Spartina patens* (Ait.) Muhl. were collected from Crescent Beach, Florida and grown in un-drained pots in a naturally-lit greenhouse at 15°C to 35°C. *S. alterniflora* was grown in a 10:1 (w/w) mixture of sand:potting soil. *S. patens* (Fafard mix 3-B, Conrad Fafard, Agawam, MA) was grown in soil from the collection site. Maize (*Zea mays* L. cv NK 508) was grown in potting soil in the same greenhouse conditions. Wheat (*Triticum aestivum* L. cv Florida 310 or cv Bob White) was grown in potting soil in a chamber with a 12-h photoperiod (200–300 μE m^{-2} s^{-1}; 25°C day/22°C night). Plants were watered as required and fertilized weekly with Peters soluble fertilizer (20-20-20, NPK, Scotts-Sierra Horticultural Products, Marysville, OH).

Chemicals and Radiochemicals

L-[^{35}S]Met (43.5 MBq nmol^{-1}), L-[U-^{14}C]Met (9.56 kBq nmol^{-1}), and [^{14}C]formate (1.79 kBq nmol^{-1}) were obtained from NEN Life Science Products (Boston), D-[1-

^{14}C]Met (2.07 kBq nmol^{-1}) was from Moravek Biochemicals (Brea, CA), and L-[1-^{14}C]Met (2.03 kBq nmol^{-1}) was from American Radiolabeled Chemicals (St. Louis). Specific radioactivities were adjusted with unlabeled compounds. 3-Methylthiopropylamine (MTP-amine) from Chem Service (West Chester, PA) was used to prepare DMSP-amine as described (Kocsis et al., 1998). DMSP was from Research Plus (Bayonne, NJ). L-SMM iodide was converted to the hydrochloride form by ion-exchange (Kocsis et al., 1998). Ion-exchange resins were purchased from Bio-Rad Laboratories (Hercules, CA).

Radiochemical Syntheses

D- and L-[1-^{14}C]SMM (2.07 and 2.03 kBq nmol^{-1}, respectively) were synthesized from D- and L-[1-^{14}C]Met using methanol as the methylating agent (Gage et al., 1997). L-[^{35}S]SMM (370 kBq nmol^{-1}) was made from L-[^{35}S]Met in the same way. L-[U-^{14}C]SMM (9.56 kBq nmol^{-1}) was synthesized from L-[U-^{14}C]Met and Ado-Met using Met *S*-methyltransferase (Trossat et al., 1996). After synthesis, labeled SMM was isolated (>98% radiochemical purity) using thin layer electrophoresis (TLE) system 2 (James et al., 1995). [^{35}S]DMSP (52–277 Bq nmol^{-1}) was isolated from *S. alterniflora* leaf sections fed with L-[^{35}S]Met or L-[^{35}S]SMM (Kocsis et al., 1998). [^{35}S]DMSO was obtained by treating [^{35}S]DMSP with 17% (w/v) NaOH for 2 h to liberate [^{35}S]DMS (White, 1982; Reed, 1983), which was trapped and converted to [^{35}S]DMSO on a 1-cm number 3 paper disc (Whatman, Clifton, NJ) containing 20 μL 30% (w/w) H_2O_2 (Kiene and Linn, 2000). The [^{35}S]DMSO was then eluted with water. To prepare [^{35}S]DMSP-amine (370–740 kBq nmol^{-1}), L-[^{35}S]Met (25 nmol) was decarboxylated by incubating (2 h, 37°C, under N_2) in 0.1 mL of 0.2 M succinate-NaOH buffer, pH 5.0, containing 1 mM PLP and 11 mg of autumn fern acetone powder (Kocsis et al., 1998). The reaction mixture was applied to 1-mL AG-1 (OH^-) and BioRex-70 (H^+) columns arranged in series. After washing the columns with water, [^{35}S]MTP-amine and its sulfoxide were eluted from the BioRex-70 column with 5 mL of 1 N HCl, and lyophilized. The dry sample was then dissolved in 0.1 mL of water plus 4 μL of 70% (w/w) thioglycolic acid and heated at 95°C for 3 h to reduce the sulfoxide to [^{35}S]MTP-amine. Excess thioglycolate was removed by lyophilization. Methylation of [^{35}S]MTP-amine gave [^{35}S]DMSP-amine, which was isolated by thin-layer chromotography (TLC; Kocsis et al., 1998). The radiochemical yield of [^{35}S]DMSP-amine was 40%. Radiochemical purity was >98% as determined by TLC and TLE. The inclusion of the thioglycolate reduction step almost doubled the [^{35}S]DMSP-amine yield compared to the procedure described previously (Kocsis et al., 1998).

Enzyme Extraction

Tissue was pulverized in liquid N_2 and thawed in extraction buffer (2 mL g^{-1} fresh weight). Unless otherwise stated, the extraction buffer for SDC (final pH of 7.2) was 50 mM potassium-phosphate, 5 mM dithiothreitol, 1 mM Na_2EDTA, 0.1 mM PLP, and 5 mM L-ascorbic acid. The extraction buffer for DOX was the same except that the pH was 8.0 and PLP was omitted. Subsequent steps were at 4°C. The homogenate was centrifuged (10,000*g* for 10 min) and the supernatant was desalted using PD-10 columns (Amersham-Pharmacia Biotech, Uppsala) that were equilibrated and eluted with extraction buffer for SDC or with extraction buffer minus ascorbate (desalting buffer) for DOX. The desalted extracts were clarified by centrifugation (16,000*g* for 5 min) and concentrated about 10-fold with Centricon-30 units (Amicon, Beverly, MA). In some cases samples were then frozen in liquid N_2 and stored at −80°C, which did not affect enzyme activity. Protein was estimated by the Bradford (1976) method using bovine serum albumin as standard.

Enzyme Assays

Assays were carried out at 23°C to 25°C. Radiochemical assays were agitated gently on a rotary shaker. No spontaneous breakdown of labeled substrates occurred during the assays. Unless otherwise noted, SDC was assayed in extraction buffer (pH 7.2) with 36 to 50 μM L-[1-^{14}C]SMM using 25-μL reactions and a 75-min incubation time. Assays were carried out in 12- × 75-mm glass tubes closed by a rubber serum stopper to which a CO_2 trap (a 1-cm Whatman number 3 paper disc containing 20 μL of 2 N KOH) was attached with a pin. Reactions were stopped by injecting 100 μL of 10% (w/v) trichloroacetic acid, and incubated for 1 h to maximize transfer of $^{14}CO_2$ to the trap. The trapped $^{14}CO_2$ was quantified by scintillation counting after letting chemiluminescence subside. The trapping efficiency was determined to be 98% using [^{14}C]formate and formate dehydrogenase (Sigma F-5632). SDC activity data were corrected accordingly. SDC activity in potassium-phosphate buffer was twice that in bis(2-hydroxyethyl) iminotris(hydroxymethyl) methane-HCl (Bis-Tris-HCl), *N*-(2-hydroxyethyl) piperazine-*N*′-(2-ethanesulfonic) acid-KOH (HEPES-KOH), or 3-(*N*-morpholino) propanesulfonic acid (MOPS-KOH).

Except where noted, DOX was assayed in desalting buffer (pH 8.0) containing 1 mM [^{35}S]DMSP-amine (2–6 kBq) and 1 to 2 × 10^3 units of bovine liver catalase (Sigma C-40) per assay. The reaction volume was 25 μL in a system like that used for SDC assays except that the stopper was Teflon-lined and the paper disc contained 20 μL of 30% H_2O_2 to trap [^{35}S]DMS. Routine assays were run for 75 min, stopped by injecting 25 μL of 17% (w/v) NaOH, and incubated for 1 h to maximize breakdown of reaction products to [^{35}S]DMS and transfer of [^{35}S]DMS to the trap. ^{35}S was quantified by scintillation counting. Trap efficiency was determined to be 97% using base-mediated decomposition of [^{35}S]DMSP as the [^{35}S]DMS source (White, 1982; Reed, 1983). DOX activity data were corrected accordingly. To analyze the labeled products of the assay prior to their decomposition by NaOH, reaction mixtures were fractionated as described below. To remove O_2, 100 mM β-D-(+)-Glc plus Glc oxidase (Sigma G-9010, 500 units per assay) were added and catalase was omitted. DOX activity was at

least as high in potassium-phosphate buffer as in other buffers tested (the ranking was potassium-phosphate = HEPES-KOH > Bis-Tris-propane-HCl > Bis-Tris-HCl).

Catalase assays (final volume of 1 mL) contained 65 mM potassium-phosphate, pH 7.0, 0.036% (w/v) H_2O_2, and 1 μL of extract. The reaction was monitored by the decrease in A_{240}. MDH assays (0.8 mL) contained 0.1 M Tris-acetate, pH 8.0, 0.2 mM NADH, 2.5 mM oxaloacetate, and 1 μL of extract. Oxaloacetate-dependent NADH oxidation was measured by the fall in A_{340}.

DMSP-Amine:CO_2 Stoichiometry in the SDC Assay

To measure both DMSP-amine and CO_2 formation, L-[U-^{14}C]SMM was used as substrate, $^{14}CO_2$ was trapped as above, and [^{14}C]DMSP-amine was isolated and quantified as follows. After adding unlabeled DMSP-amine and SMM carriers (0.1 μmol each), reaction mixtures were fractionated using 1-mL AG-1 (OH^-) and BioRex-70 (H^+) columns arranged in series. Both columns were washed with water. DMSP-amine and SMM were then eluted from the BioRex-70 column with 5 mL of 1 N HCl and the eluate was lyophilized. DMSP-amine was separated from SMM using TLC system 1 (James et al., 1995), detected by autoradiography, and quantified by scintillation counting. The recovery of DMSP-amine was determined to be 61% by spiking unlabeled reaction mixtures with [^{35}S]DMSP-amine. This value was used to correct [^{14}C]DMSP-amine data.

Analysis of Labeled Products in the DOX Assay

To analyze DMSO, DMSP, and DMSP-amine, DOX reaction mixtures (not treated with NaOH) were mixed with DMSP, DMSO, and DMSP-amine carriers (0.2–1 μmol) and fractionated on 1-mL columns of AG-1 (OH^-), BioRex-70 (H^+), and AG-50 (H^+) arranged in series. Each column series was washed with 10 mL of water. The effluent contained DMSO. DMSP-amine was eluted from BioRex-70 with 5 mL of 1 N HCl and DMSP from AG-50 with 5 mL of 2.5 N HCl. Samples of the effluent and eluates were counted. When only [^{35}S]DMSP and [^{35}S]DMS were analyzed, a 1-mL mixed resin (AG-1 [OH^-]:BioRex-70 [H^+], 2:1, v/v) column replaced the corresponding separate columns. Recoveries were determined by spiking unlabeled reactions with [^{35}S]DMSP, [^{35}S]DMSO, or [^{35}S]DMSP-amine, and experimental data were corrected accordingly. The identity of [^{35}S]DMSP was authenticated using TLC system 1 and TLE system 1 of James et al. (1995).

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