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Reduction of *N*-Acetyl Methionine Sulfoxide in Plants[1]

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ABSTRACT

An enzymic activity which catalyzes the reduction of *N*-acetyl-methionine sulfoxide to L-*N*-acetyl-methionine has been observed in a wide variety of plant tissues. Its activity depended on the presence of dithiothreithol in the incubation medium. L-Methionine-sulfoxide was essentially inactive as a substrate. Of all the physiological reductants tested, only thioredoxin partially replaced dithiothreithol. When fractions obtained by gradient centrifugation of gently disrupted barley protoplasts were assayed for the reductase, the activity was largely associated with chloroplasts although approximately 15% was found in the cytosolic compartment. The enzyme, isolated from spinach chloroplasts, had a broad pH optima between 7.0 and 8.0, and its K_m for *N*-acetyl methionine sulfoxide is 0.4 millimolar. The possible participation of this ubiquitous enzyme in enzyme regulation is discussed.

Methionine[3] plays a key role in the biochemistry of a cell. As a precursor of *S*-adenosyl methionine, it is a direct participant in methylation reactions, and it serves as the precursor for the generation of ethylene in all plant tissues; as methionyl-tRNA, it initiates protein synthesis in cells; as an amino acid residue, it is found essentially in all proteins. Thus, any modification of the structure of methionine or the methionyl residue in proteins can perturb biochemical systems in the cell. Although it has been known for sometime that methionine is readily converted to its sulfoxide under mild oxidizing conditions, only recently has the importance of this reaction been realized (5).

The occurrence in *Escherichia coli* of an enzymic activity which reduced MetSO[4] to methionine was reported more than 30 years ago (13). This observation was later extended to yeast (1), plants (9), and animal tissues (10). The observations that Met residues in proteins can be oxidized under physiological conditions to MetSO residues, and that this oxidation is often associated with loss of biological activity and with pathological conditions (5), raises the possibility that this enzyme is involved in the reduction of protein MetSO residues with a concomitant restoration of enzyme activity. As a result of the work carried out with several organisms and tissues, it is now well established that animal tissues and microorganisms have a MetSO-peptide reductase which catalyzes the reduction of oxidized Met residues in proteins, restoring the biological activity of the protein (5). At least in the case of *E. coli*, two different enzymes catalyze the reduction of free MetSO and MetSO-peptide (3). However, only spinach leaf extract has been tested for MetSO reductase activity (3). In order to examine the properties and function of the enzyme, we report in this communication the distribution, localization, and properties of an enzyme in a number of plant tissues that reduces *N*-acetyl methionine sulfoxide to *N*-acetyl methionine.

MATERIALS AND METHODS

Materials. Spinach (*Spinacia oleracea*), potatoes (*Solanum tuberosum*), and leek (*Allium porrum*) leaves were from the local market. Safflower (*Carthamus tinctorius* var UC-1), pea (*Pisum sativum* var Alaska), castor bean (*Ricinus communis* var Baker 290), and barley (*Hordeum vulgaris* var CM 67) seeds were soaked for 24 h, and then transferred to vermiculite trays to continue the given time periods. Developing safflower and castor bean seeds were harvested from the field and stored at −20°C until used.

L-[^{35}S]Methionine (1488 Ci/mmol) and L-[^{14}C]methionine (13.8 Ci/mmol) were from New England Nuclear, and sodium-[^{14}C]bicarbonate (60 μCi/μmol) was obtained from Amersham Searle. Thioredoxin from spinach chloroplasts was a generous gift of Dr. B. B. Buchanan (University of California, Berkeley). All other reagents were from Sigma.

Preparation of the Radiolabeled Precursor. The radiolabeled precursor, AcMet^{35}SO, was prepared from L-[^{35}S]methionine by a modified procedure of Brot *et al.* (4) involving first acetylation and then oxidation of the acetylated intermediate. Two μl 0.1 M L-methionine were mixed with 140 μCi (15 μl) L-[^{35}S]methionine and 100 μl water, and the solution was incubated with 10 μl of 4 N NaOH and 1 μl acetic anhydride for 10 min at room temperature. Additionally, 2 μl acetic anhydride and 4 μl 4 N NaOH were added, and the reaction mixture was allowed to stand for 10 min more at room temperature. A pH of approximately 9.0 was maintained during the acetylation reaction. To the completed acetylation mixture was added 1 μl 30% H_2O_2 and 20 μl 5 N HCl (final pH approximately 2.0) and the reaction was allowed to continue for 10 min at room temperature. At the end of the oxidation, 2.5 ml water were added, and the mixture was lyophilized. The dry product was dissolved in a minimal volume of water and applied onto a Silica Gel G plate for purification. Plates were developed with butanol:acetic acid:water (60:15:25), and radioactive areas were located with a Packard Radiochromatogram Scanner (model 7201). Under these conditions, the intermediate product, Ac^{35}SMet, showed a R_F of 0.7, and the final product, AcMet^{35}SO, 0.3. Bands corresponding to both products were scraped and eluted with water. The pH of the substrate solutions were adjusted to neutrality with diluted NaOH. [^{14}C]MetSO was prepared from 20 μCi [^{14}C]Met by reaction with H_2O_2 as described above.

Preparation of Plant Extracts. To test AcMSO reductase in different tissues, 2 to 3 g fresh tissue were ground with a mortar and pestle in 3 volumes grinding buffer consisting of 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM KCl, 1 mM β-mercap-

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[3] In all references to methionine, the L-isomer was employed.

[4] Abbreviations: MetSO, methionine sulfoxide; AcMetS, *N*-acetyl-methionine; AcMetSO, *N*-acetyl methionine sulfoxide.

toethanol, and 10% (v/v) glycerol (3). The homogenate was filtered through one layer of Miracloth and centrifuged at 30,000*g* for 20 min. The pellet was discarded and the supernatant used immediately as the enzyme extract. Chloroplasts were isolated from spinach leaves by grinding 10 to 20 g tissue in 4 volumes of the same buffer described above in which glycerol was replaced by 0.4 M sucrose. The homogenate was filtered and then centrifuged at 3000*g* for 1 min. To disrupt the chloroplasts, the chloroplast pellet was resuspended in grinding buffer (with glycerol, without sucrose), and the suspension was stirred for 10 min and then used directly or separated into the stroma phase and the grana phase by centrifugation at 20,000*g* for 20 min.

To purify the chloroplast AcMetSO reductase, the method described by Wolosiuk *et al.* (18) was followed, starting with 900 g of leaves and ending at the acetone precipitation step. Although the procedure was originally designed to isolate chloroplast thioredoxins, the acetone fraction had a very high and stable AcMetSO reductase activity and, therefore, it was used as a source of the enzyme in several experiments.

Analysis of Methionine Sulfoxide in Seed Protein. Analysis for protein MetSO in pea seeds was carried out essentially as described by Neumann (16). Dry pea seeds from which the seed coat had been removed were homogenized in an Omni mixer with 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 5% mercaptoethanol for three 1-min bursts. The brei was filtered through two layers of Miracloth and the soluble protein precipitated with 10% TCA. The precipitated protein was collected by centrifugation (1500*g*, 5 min) and the pellet was washed twice with acetone. The pellet was finally resuspended in 15% NaOH. Duplicate 0.5-ml aliquots containing 5 to 10 mg protein were sealed under vacuum in pyrex tubes (100 mm × 12 min). Hydrolysis was carried out overnight at 100°C at the end of which time the tubes were opened, neutralized with HCl, and the amino acids analyzed on a Durrum Amino Acid Analyzer D-500 by Alan Smith of this campus. The results reported represent the average of four determinations.

Subcellular Distribution of the AcMetSO Reductase. Barley protoplasts were prepared according to Day *et al.* (8). After lysis, the protoplast suspension was layered onto a 30 to 60% (w/w) linear sucrose gradient containing 10 mM Tris-HCl (pH 7.5), and centrifuged at 20,000 rpm for 30 min in a Beckman SW27 swing-out rotor. Fractions of 1 ml were collected from the gradient, and the AcMetSO reductase activity was determined in each fraction as described below. Fumarase (12), catalase (14), ribulose BP carboxylase (17), and P-enolpyruvate carboxylase, determined as in Wishnick and Lane (17) but using P-enolpyruvate instead of ribulose bisphosphate as the substrate, were used as enzyme markers. Chl was determined according to Bruinsma (7), and protein by the Bradford method (2) with BSA as the standard.

Incubations. Unless otherwise stated, the incubation mixture contained in a final volume of 50 μl, 30 μl enzyme extract, 1 μM AcMet^{35}SO, 10 mM DTT, 20 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, 34 mM KCl, and 7% glycerol (not present in the experiments carried out with the partially purified preparation from spinach chloroplasts). Normally incubations were carried out at 32°C for 1 h.

Analysis of the Products. At the end of the incubation time, 0.5 ml 0.5 N HCl and 1.5 ml ethyl acetate were added to the reaction mixture (3). After vigorous stirring and centrifugation for 2 min in a clinical table-top centrifuge, radioactivity was determined by liquid scintillation with a 1-ml aliquot of the organic phase. Under these conditions, less than 1% of the substrate was in the organic phase whereas 50% of *N*-acetyl Met^{35}S was in the organic phase. Values in all the data presented were corrected to 100% recovery. Blank experiments were carried out routinely using both AcMet^{35}S and AcMet^{35}SO. The nature of the ^{35}S product extracted with ethyl acetate was confirmed by TLC under the conditions described above.

In experiments involving the presence of [^{14}C]MetSO, after stopping the reaction with 20 μl isopropanol, products were separated by TLC as described above.

RESULTS

Nomenclature. The term acetyl-methionine-sulfoxide reductase is employed in this paper since *N*-acetyl-methionine-sulfoxide was routinely used in the assay substrate. This substrate was selected on the basis that it was easy to prepare and assay. Moreover, it has been reported to be a substitute for methionyl sulfoxide residues in enzyme proteins (3–5).

Table I. *AcMetSO Reductase Activity in Several Species and Tissues*

The incubation mixture contained the enzymic extract prepared as described under "Material and Methods" (50–200 μg protein), 1 μM AcMet^{35}SO, and 10 mM DTT, in a volume of 40 μl.

Species	Organ	Specific Activity
		pmol/mg prot·h
Pea	Leaves	15
Pea	Germinating seeds[a]	69
Barley	Leaves	66
Barley	Germinating seeds[a]	29
Castor bean	Leaves	34
Castor bean	Germinating seeds[a]	30
Castor bean	Mature seeds	16
Safflower	Germinating seeds[a]	10
Leek	Leaves	28
Potato	Tuber	18
Spinach	Chloroplasts	85

[a] Seeds were germinated in the dark at RT (pea, barley, safflower) or at 30°C (castor bean) during 3d.

Table II. *Distribution of the AcMetSO Reductase in Pea Seedlings*

Part	Specific Activity
	pmol/mg prot·h
Shoots	165
Cotyledons	7
Roots	11

Table III. *Distribution of AcMetSO Reductase and Enzyme Markers among the Subcellular Compartments of Barley Protoplasts*

	Distribution[a]		
	Soluble Cytosolic Proteins	Chloroplasts	Others
		%	
AcMetSO reductase	24	63	13
Ribulose BP carboxylase	15	75	11
P-enolpyruvate carboxylase	93	1	6
Fumarase	73	0	27
Catalase	73	10	17
Chl	2	73	26
Protein	25	66	9

[a] Figures expressed percentages of the total activity or content determined along the sucrose gradient, 25 fraction of 1 ml each. The cytosolic (soluble) compartment includes fractions 1 to 5; intact chloroplasts comprise fractions number 11 to 19. 'Others' refers to those organelles collected in fractions 6 to 10, including mitochondria and broken chloroplasts. Negligible amounts of each markers were detected in the bottom of the gradient, fractions 20 to 25.

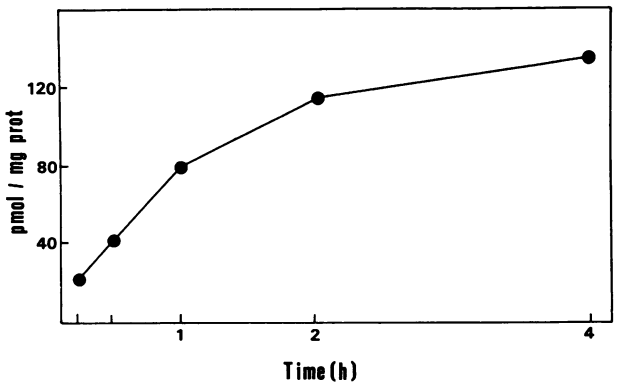

FIG. 1. Time course of the reduction of AcMet^{35}SO by disrupted spinach chloroplasts.

Distribution and Localization of the AcMetSO Reductase Activity. The distribution of AcMetSO reductase in a number of plants is summarized in Table I. Activity was observed in all the seven species of plants tested, which include both mono- and dicotyledoneous species as well as different tissues. Peptide methionine sulfoxide reductase activity had been previously reported in yeast, bacteria, and animal tissues (5). The results presented here show that AcMetSO reductase is, indeed, widespread in the plant kingdom, which indicates that all kinds of cells have the ability of reducing methionyl sulfoxide residues.

Furthermore, AcMetSO reductase was tested in the 30,000*g* supernatants from roots, cotyledons, and shoots of 10-d-old pea seedlings. Results in Table II indicate that the aerial components of the seedling contain the highest level of enzyme. Studies on the subcellular distribution of the enzyme were carried out with barley protoplasts, and the results showed that the activity was mostly in the chloroplast, although a not negligible part of the total activity seems to occur in extrachloroplast compartments (Table III). On the basis of enzyme markers, about 85% of the activity is located in the chloroplasts of barley protoplasts, whereas the remaining 15% seems to be cytosolic (Table III). Finally, when chloroplasts isolated from spinach leaves were disrupted by osmotic shock and fractionated by centrifugation, the activity was mainly associated with the stroma phase (results not shown.

Properties of the Enzyme. Since AcMetSO reductase activity is largely confined to the chloroplast stroma, this fraction was used to investigate the component requirements and properties of the enzyme.

The time course of the reduction of AcMet^{35}SO by a suspension of broken spinach chloroplasts is shown in Figure 1. The reaction is linear for 1 h. The activity of this crude preparation decreased rapidly when stored at 0°C, and after 24 h only half of the original activity remained in the extract. The more stable and partially purified preparation (see "Materials and Methods") was therefore used in further studies (Fig. 2).

The reduction of AcMet^{35}SO to AcMet^{35}S increased linearly with the amount of enzyme preparation, at least up to 20 μl (94 μg), the highest concentration tested (Fig. 2A). As indicated in Figure 2B, in the presence of 5 μl enzyme extract, there was a linear response to increased substrate concentrations up to approximately 4 μM AcMet^{35}SO. Since our substrate was prepared from L-methionine under conditions at which a mixture of 50% of each isomers, L-AcMet-d-SO and L-AcMet-l-SO was formed (11), it was of interest to determine whether or not both isomers were equally effective as substrates. As shown in Figure 2C, the kinetics of the reduction of AcMet^{35}SO strongly suggests that the enzyme does not have isomer specificity, since after 6 h of incubation the percentage of reduction was almost 90%, and the curve approaches asymptotically the level of complete reduction of the substrate.

Table IV. *Reductants for AcMetSO Reductase from Spinach Chloroplasts*

Experiments were carried out by using the partially purified spinach AcMetSO reductase prepared as described in "Materials and Methods" and 2 μM AcMet^{35}SO. The control experiment contained 10 mM DTT; 15 μg spinach thioredoxin were added where expressed; the Fd system consisted of 10 mM NADPH, 6 μg spinach Fd, and 0.15 IU spinach Fd reductase.

Treatment	Relative Activity
	%
Control[a]	100
− DTT + 5 mM NADPH + thioredoxin	28
− DTT + 10 mM glutathione	4
− DTT + 10 mM ascorbate	1
− DTT + 50 mM NAD(P)H	0
− DTT + Fd system	0
− AcMetSO reductase + DTT	0
− AcMetSO reductase + DTT + thioredoxin	13

[a] The specific activity obtained in the control experiment was 3.9 nmol/mg protein.

On the other hand, free MetSO, tested as [^{14}C]MetSO, was essentially ineffective when tested with the 30,000*g* supernatants prepared from germinating pea seeds and spinach chloroplasts, and very little reduction was found after incubation with the partially purified preparation from spinach chloroplasts (data not shown). These results indicate that the plant enzyme is specific for MetSO with the amino group blocked, in this case with an acetyl function.

With increased concentrations of the reductant, enzyme activity increased markedly to 5 mM DTT and then essentially showed little further increase up to 40 mM DTT (Fig. 2D). Under the conditions of enzymic reduction, even at higher levels of DTT (50 mM), no nonenzymic conversion of AcMetSO to AcMetS occurred (Table IV). Neither NADH nor NADPH at several concentrations replaced DTT as a reductant. Several other physiological reductants were examined. Table IV summarizes the results obtained by testing different reductant systems. Among the six reductants used, only purified thioredoxin plus NADPH could partially replace DTT (10 mM). Addition of thioredoxin and NADPH to a 30,000*g* supernatant isolated from germinating pea and barley seeds and etiolated barley leaves, produced a remarkable stimulation of the AcMetSO reductase (Table V), although in these cases, incubations were carried out in the presence of DTT to make sure that thioredoxin was in its reduced form (18). On the other hand, intact spinach chloroplast failed to reduce AcMet^{35}SO in the light and in the absence of DTT, under conditions of significant incorporation of [^{14}C]acetate into fatty acids was observed. These results suggest, therefore, that thioredoxin may be the physiological electron carrier involved in the reduction reaction, in agreement with previous results obtained from *E. coli* (4). Finally, the enzyme showed optimal activity at pH ranging from 7.0 to 8.0 (data not shown).

Physiological Factors on AcMetSO Reductase Activity. The variation of AcMetSO reductase activity was followed in pea, safflower, castor bean, and barley seedlings grown in the dark and in the light (pea and barley only). Except in castor bean, where a peak of activity was found after 5 d of germination, the general trend seems to be toward decreasing MetSO reductase activity (Table VI). Interpretation of these data is difficult since there could be intense activity in a specific tissue zone in the total plant, with little or no activity in the rest of the plant. Finally, AcMetSO reductase was measured in four sections of

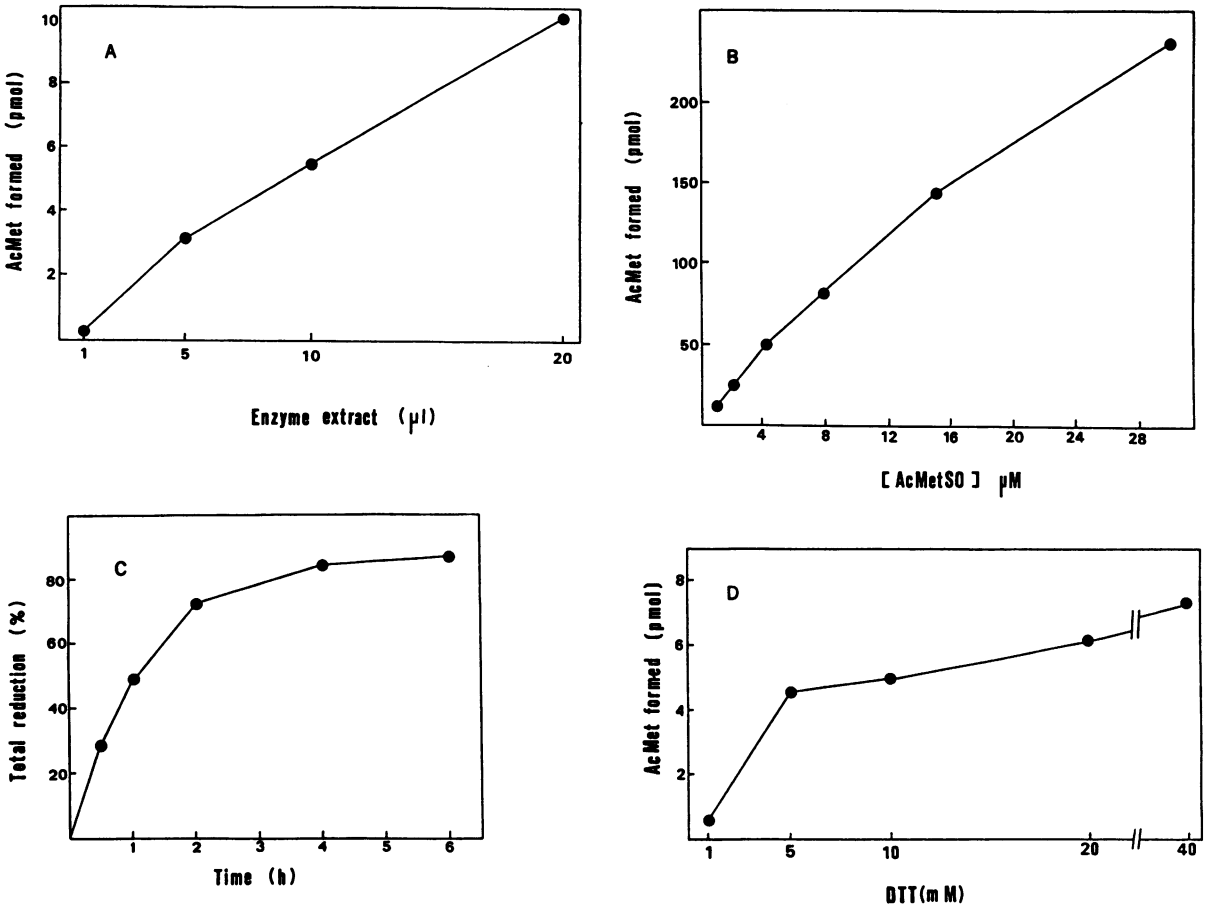

FIG. 2. Properties of the chloroplastic AcMetSO reductase. The partially purified preparation from spinach chloroplasts (see "Materials and Methods"), containing 4.7 mg protein/ml, was used. A, Effect of the concentration of enzyme. B, Effect of the concentration of substrate; 5 µl of enzyme extract (24 µg) were used. C, Time course of the reduction of AcMet^{35}SO; the incubation medium contained 7 µM AcMet^{35}SO, 10 mM DTT, and enzyme extract (1.3 mg/ml). D, Effect of the concentration of DTT.

Table V. *Effect of the Addition of Spinach Thioredoxin on the AcMetSO Reductase Present in the 30,000g Supernatant Prepared from Different Tissues*

Tissue	Total Reduction		Stimulation
	−Thiore doxin	+Thiore doxin	
	pmol		*-fold*
Germinating pea seeds[a]	17	26	1.5
Germinating barley seeds[a]	2	10	5.0
Etiolated barley leaves[b]	3	10	3.3

[a] Seeds germinated during 24 h.
[b] Three-d-old leaves.

green barley leaves, and the results showed no marked difference in the various growth regions of the blade, averaging about 60 pmol/mg protein conversion of substrate to product.

Methionine Sulfoxide Content in Seed Protein. It was found that the methionine content of dormant pea seed was 42 nmol/mg protein and MetSO content was 9.3 nmol/mg protein; *i.e.* 18% of the total methionine in pea proteins was as the sulfoxide residue. It would be of interest to use these proteins in the future as substrates for the *N*-acetyl-methionyl-sulfoxide reductase.

DISCUSSION

Because of the key role that methionine plays in plant systems, it became important to determine the presence, distribution, and properties of the AcMetSO reductase. The data presented here clearly demonstrate the widespread occurrence of this enzyme in the plant cell, in particular in the chloroplast. As yet, however, a direct test has not been made to determine if this enzyme is active with MetSO peptides or proteins. Presumably, the generation of MetSO proteins would occur when high levels of H_2O_2

Table VI. *Effect of Time of Germination on AcMetSO Reductase Content*

Seeds were germinated in the dark (d) or in the light (l). The 30,000*g* supernatant was prepared as described under "Materials and Methods" and incubated with 1 µM AcMet^{35}SO and 10 mM DTT at 32°C for 1 h.

Tissue	Castor Bean (d)	Safflower (d)	Pea (d)	Pea (l)	Barley (d)	Barley (l)
d			*pmol/mg prot·h*			
1	ND[a]	ND	ND	92	ND	117
2	ND	ND	95	95	121	81
3	30	10	91[b]	47	48[b]	81
4	64	9	72[b]	42	59[b]	75
5	94	13	63[b]	23	88[b]	40
6	32	4	35[b]	34	72[b]	45
7	0	0	27	ND	ND	ND
14	0	ND	0	ND	ND	ND

[a] ND, not determined.
[b] Mean of two independent experiments.

are permitted to accumulate in the plant cell. It is also possible that the hydroperoxyl derivatives of fatty acids could catalyze the conversion of Met residues to MetSO residues in key proteins.

It is also tempting to speculate that this enzyme might be involved in thioredoxin-linked systems in chloroplasts. In two desaturation systems (6, 15), high levels of catalase increase the activity of the desaturation reaction, presumably by removing H_2O_2 which is being generated by the flow of electrons from NADPH via a linking carrier system to molecular O_2. It is equally tempting to suggest that the AcMetSO reductase may function to convert MetSO residues to methionyl residues in key proteins involved in the desaturation reaction.

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