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RECYCLING OF METHIONINE SULFUR IN A HIGHER PLANT BY TWO PATHWAYS CHARACTERIZED BY EITHER LOSS OR RETENTION OF THE 4-CARBON MOIETY

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SUMMARY

When duckweed was grown in the presence of [^{35}S, U-^{14}C]-methionine the ratio of [^{14}C]-4-carbon moiety relative to ^{35}S in protein methionine was less than in the administered methionine. This decrease demonstrates a preferential recycling of the sulfur moiety relative to the 4-carbon moiety of methionine. This pathway probably plays a hitherto unsuspected general role in plants, such as in polyamine synthesis. The ratio of [^{14}C]-methyl relative to ^{35}S of protein methionine was also less than in the administered methionine. This decrease is tentatively attributed to a rapid utilization of the methyl moiety of methionine for transmethylation, accompanied by recycling of homocysteine to methionine. Transmethylation was estimated to be the dominant route for methionine metabolism.

INTRODUCTION

During studies of methionine biosynthesis in higher plants, we examined the metabolism of [^{35}S, U-^{14}C]-methionine by growing duckweed, *Lemna paucicostata*. The results, reported here, indicate that methionine sulfur is recycled by two pathways in *Lemna*. In one, the sulfur is reincorporated without the 4-carbon moiety into methionine; in the other, the sulfur and the 4-carbon moiety are reincorporated together.

MATERIALS AND METHODS

Materials. L-[^{35}S]- and L-[U-^{14}C]-methionine (Amersham) were purified by paper chromatography in 2-propanol:88% formic acid:water (7:1:2) containing 10 mM 2-mercaptoethanol. Carrier L-methionine was then added to obtain the desired specific activity, and any methionine sulfoxide present reduced to methionine by incubation for 1 hr at 100° in 0.7 M 2-mercaptoethanol -

Abbreviations: SEM, standard error of mean; PC, phosphatidylcholine; MTA, 5'-methylthioadenosine; AdoMet, (S)-*S*-adenosyl-L-methionine; AdoHcy, *S*-adenosyl-L-homocysteine; ACC, 1-aminocyclopropane-1-carboxylic acid; MTR, 5-methylthioribose.

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10 mM potassium phosphate pH 7.5. The two isotopic preparations were then combined. The mixture was filter sterilized, and aliquots added to medium 4 (1) to final concentrations of 2 or 0.67 μM [^{35}S, ^{14}C]-methionine.

Growth of *Lemna* with [^{35}S,^{14}C]-methionine. *Lemna* was pregrown for 3.5-3.8 doublings in the presence of the appropriate concentration of methionine under standard conditions (1) such that virtually all tissue carbon derives ultimately from sucrose. Colonies (4 to 6) were then transferred to medium containing [^{35}S, ^{14}C]-methionine, and allowed to grow under the same conditions for 3.8 to 4 generations. The mean doubling time for triplicate cultures grown in 0.67 μM methionine was 41.3 hrs (SEM , 0.4 hrs); in 2 μM methionine, 46.8 hrs (SEM, 0.3 hrs). Plants grown concurrently in the absence of external methionine had a doubling time of 37.4 hrs (SEM, 0.9 hrs).

Isolation, purification, and degradation of protein methionine. Plants were homogenized in 10% TCA and the pellets obtained after centrifugation were reduced with DTT, reprecipitated and washed with TCA (2), and hydrolyzed with 3 N mercaptoethanesulfonic acid (Pierce Chemical) (3). Radioactive methionine was purified by paper chromatography, first in its unmodified form, then successively as the sulfoxide (4) and carboxymethylsulfonium salt (5). Radioactive methionine added to the medium was subjected to the same procedures. Methionine carboxymethylsulfonium salt was degraded (5) to homoserine (4-carbon moiety) and methylthioacetic acid which were separated on Dowex 50 H^+. Radioactivity in the sulfur moiety was determined from the amount of radioactivity remaining after oxidation of methylthioacetic acid with HNO_3-HCl (6). Radioactivity in the methyl moiety of methionine was determined from the difference between the radioactivity present in methylthioacetic acid and the sulfur moiety. Details of the above methods will be published separately (Giovanelli *et al.*, manuscript in preparation).

Estimates of preferential methionine sulfur recycling and transmethylation.

Let S = entry of sulfate sulfur into methionine (nmoles/colony)

U = uptake of administered methionine (nmoles/colony)

$$R = \frac{[^{14}C]\text{-4-carbon}/^{35}S \text{ in protein methionine}}{[^{14}C]\text{-4-carbon}/^{35}S \text{ in medium methionine}}$$

C = entry of sucrose carbon into the 4-carbon moiety of methionine (nmoles/colony)

S was determined (7, Giovanelli *et al.*, manuscript in preparation) essentially from the accumulation of ^{35}S from sulfate in total tissue methionine and its products. U was determined by dividing the amount of methionine taken up (dpm taken up/specific activity of administered methionine) by the final number of colonies. R was calculated from the ratios listed in Table I. C was calculated from the following equation which was derived (Giovanelli *et al.*, manuscript in preparation) using the specific activities of the sulfur moiety of protein methionine ([U/U + S] x specific activity of ^{35}S-methionine in medium) and the 4-carbon moiety of protein methionine ([U/U + C] x specific activity of [^{14}C-4-carbon]-methionine in medium):

$$C = \frac{U(1 - R) + S}{R}$$

The derivation assumes free mixing of methionine taken up from the medium with that synthesized by the plant. In plants growing in $^{35}SO_4^{2-}$ and 2 μM methionine, no significant difference between the specific activity of soluble and protein methionine was detected (Giovanelli *et al.*, unpublished), indicating that extensive compartmentation of soluble methionine does not occur.

Preferential methionine sulfur recycling = C - S.

By analogy with the above, entry of sucrose carbon into the methyl moiety of methionine is given by the expression:

$$\frac{U\,(1 - R') + S}{R'}$$

where $R' = \dfrac{[^{14}C]\text{-methyl}/^{35}S \text{ in protein methionine}}{[^{14}C]\text{-methyl}/^{35}S \text{ in medium methionine}}$

Determination of ethylene. Duplicate *Lemna* cultures were grown for 4.6 and 4.9 doublings in 2 μM [U-^{14}C]-methionine and the air streams leaving the cultures dispersed into solutions of mercuric perchlorate (8). [^{14}C]-Ethylene evolution was determined from the amount of radioactivity in this solution.

Polyamine determination. Plants grown in the presence of non-radioactive methionine were homogenized in 10% TCA containing tracer amounts of [^{3}H]-spermidine (New England Nuclear). The homogenate was clarified by centrifugation and polyamines in the supernatant solutions determined by ion exchange chromatography (9). Quantitative recovery of spermidine was established from the amount of ^{3}H in the supernatant solutions.

Estimation of PC. Plants grown in the presence of non-radioactive methionine were harvested, mixed with tracer amounts of [N-methyl-^{14}C]-L-α-PC, dipalmitoyl (Amersham), and the phospholipids extracted according to Folch *et al.* (10). PC was purified by thin layer chromatography with chloroform:n-propanol:propionic acid: water (2:3:2:1) on silica gel (Anasil H, 250μ, from Analabs), and determined as inorganic phosphate after digestion with perchloric acid (11). The amount of ^{14}C recovered allowed correction for losses of PC during the isolation and purification.

RESULTS AND DISCUSSION

Lemna was grown for several generations in the presence of exogenous [^{35}S, U-^{14}C]-methionine. Protein methionine was then isolated and degraded. Ratios of [^{14}C]-4-carbon/^{35}S and of [^{14}C]-methyl/^{35}S were determined and compared to the same ratios for the administered methionine (Table I). Each of these ratios in protein methionine had decreased relative to the corresponding ratio in administered methionine. Furthermore, the ratios for protein methionine observed in plants growing in the presence of 0.67 μM methionine

Table I. Ratios of [^{14}C]-4-carbon/^{35}S and [^{14}C]-methyl/^{35}S of methionine in growth medium and in protein isolated from *Lemna* growing in 2 μM and 0.67 μM methionine.

[^{14}C]-4-carbon/^{35}S			[^{14}C]-methyl/^{35}S		
Medium Methionine	Protein methionine		Medium Methionine	Protein methionine	
	2 μM methionine	0.67 μM methionine		2 μM methionine	0.67 μM methionine
1.125 (0.004)	1.008 (0.004)	0.961 (0.006)	0.307 (0.005)	0.129 (0.002)	0.0841 (0.0039)
1.132 (0.008)	1.032 (0.009)	0.946 (0.006)	0.282 (0.004)	0.135 (0.005)	0.0815 (0.0020)
1.119 (0.009)	1.038 (0.008)	0.961 (0.006)	0.283 (0.004)	0.140 (0.005)	0.0859 (0.0041)
Means (SEM) of individual values:					
	[a]	[a,b]		[a]	[a,c]
1.125 (0.004)	1.026 (0.009)	0.956 (0.005)	0.291 (0.008)	0.135 (0.003)	0.0838 (0.0013)

Each individual value for medium methionine is one of triplicate assays of the one preparation of [^{35}S,U-^{14}C]-methionine used to prepare all media. Each individual value for protein methionine represents assay of one of the triplicate cultures. The SEM (shown in parentheses) for each of these individual values reflects variation in replicate determinations of radioactivity in a given sample.

P values were derived from the t test.

[a] Different from the corresponding ratio for medium methionine at the level of $P < 0.001$.

[b] Different from the corresponding ratio for protein methionine from plants grown in 2 μM methionine at the level of $P < 0.01$.

[c] Different from the corresponding ratio for protein methionine from plants grown in 2 μM methionine at the level of $P < 0.001$.

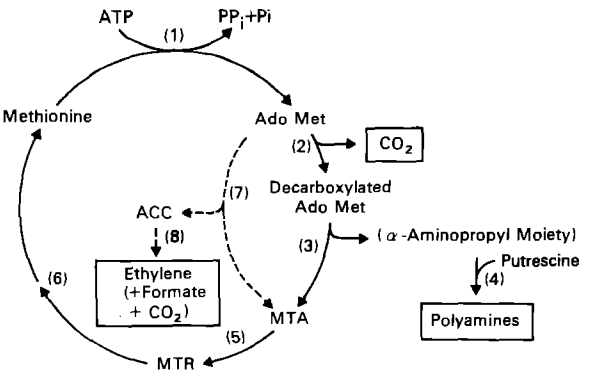

Fig. 1. Proposed pathway for preferential methionine sulfur recycling in higher plants. The sulfur and methyl moieties of methionine are recycled *via* MTA, while the 4-carbon moiety is converted to the products shown in boxes. Reactions (1)-(4) have been recently reviewed (7). Reactions (7) and (8), shown in broken lines, are unique to ethylene biosynthesis, and have been reviewed by Kende *et al.* (25). The stoichiometry of polyamine and MTA synthesis shown would not apply if spermidine synthesis proceeded by an alternate pathway: aspartic semialdehyde + putrescine → carboxyspermidine → spermidine. In *Lathyrus sativus* seedlings this pathway has been reported (26) to coexist with the classical pathway shown in the Figure, but the extent to which the alternate pathway may occur in other plants is not known. Reaction (5) is catalyzed by MTA nucleosidase (EC 3.2.2.9) (16, 19, 27, 28). While it is clear (16) that the methyl and sulfur moieties of MTA are metabolized to methionine (Reaction [6]), the origin of the 4-carbon moiety of regenerated methionine in plants is not known. Animals (29) and *Candida utilis* (30, 31) convert the thiomethyl moiety and ribose carbons of MTA to the thiomethyl and 4-carbon moieties, respectively, of methionine. This pathway operating in *Lemna* under our conditions would provide for the observed conservation of radioactivity in the sulfur moiety and entry of unlabeled carbon (derived ultimately from sucrose) into the 4-carbon moiety of recycled methionine.

were less than for those growing in the presence of 2 μM methionine. All differences were highly significant as evidenced by the agreement between replicates and by the low P values (Table I).

The decreases in the ratios of $[^{14}C]$-4-carbon/^{35}S demonstrate that the plants had metabolically separated the sulfur and 4-carbon moieties of methionine and preferentially reincorporated the sulfur back into methionine.[1] Such preferential recycling of the sulfur relative to the 4-carbon moiety of

[1] No single enzyme is known that could account for such decreases by exchange of the 4-carbon or methyl moiety relative to the sulfur moiety. In fact, consideration of the mechanism of cystathionine γ-synthase (EC 4.2.99.9) (12) and methionine γ-lyase (EC 4.4.1.11) (12, 13) leads us to conclude that any exchange catalyzed by either of these enzymes would result in increases, rather than the observed decreases, in the ratio of $[^{14}C]$-4-carbon/^{35}S, and in no change in $[^{14}C]$-methyl/^{35}S.

methionine has been previously reported among plants only in ripening apples, which are unusual in synthesizing massive amounts of ethylene (see 14 for review). In this tissue, methionine is metabolized predominantly to ethylene (15), and the sulfur moiety is recycled *via* MTA (16) (Fig. 1). In *Lemna*, ethylene is not a major product of methionine metabolism, since no [^{14}C]-ethylene evolution was detected during several doublings of *Lemna* in 2 μM [U-^{14}C]-methionine. An upper limit, based on the sensitivity of detection of any radioactive ethylene evolved, was two orders of magnitude below the radioactivity accumulated in protein methionine. Based on studies with eukaryotes (17), among the alternative sources of MTA in *Lemna* and the many other vegetative tissues that normally evolve little or no ethylene (18) are AdoMet hydrolyase (EC 3.3.1.2) and polyamine synthesis. Limited studies (19, 20) suggest that the latter is by far the more important. Accordingly, the polyamine contents of *Lemna* were measured (Table II). For plants growing in 2 and 0.67 μM methionine, spermidine accounted for 37 and 51%, respectively, of preferential methionine sulfur recycling. These percentages would be higher to the extent that polyamine turnover occurs. Such turnover has been demonstrated in cultured carrot cells (21). Based on these observations, we suggest that preferential methionine sulfur recycling plays a general role in plants, conserving methionine sulfur and preventing the buildup of inhibitory concentrations of MTA (17) during polyamine synthesis.

The decreases in the ratios of [^{14}C]-methyl/^{35}S in protein methionine relative to those in medium methionine[1] (Table I) may reasonably be ascribed to the established reactions for recycling of the homocysteine moiety of methionine during transmethylation: methionine → AdoMet → AdoHcy → homocysteine → methionine (7, 22). These data provide, for the first time, a quantitative estimate of the extent to which transmethylation occurs in a higher plant. As shown in Table II, plants growing in each concentration of methionine transferred 17-18 nmoles of methyl moieties during an interval in which they accumulated 4.4 nmoles of protein methionine and recycled 1.2-1.4 nmoles

Table II. Tentative estimates of preferential methionine sulfur recycling and transmethylation compared to contents of spermidine and phosphatidylcholine (PC), respectively

Methionine in medium	Preferential methionine sulfur recycling	Spermidine content	Transmethylation	Methyl groups in PC
μM	nmoles/colony	nmoles/colony	nmoles/colony	nmoles/colony
2	1.4[a] (1.1-1.8)[b]	0.52 (0.04)[c]	18[a] (15-22)[b]	6.9 (0.27)[c]
0.67	1.2[a] (1.0-1.4)[b]	0.61 (0.06)[c]	17[a] (14-20)[b]	6.9 (0.15)[c]

Mean protein methionine/colony was 4.4 nmoles (SEM, 0.2; range of 3.9-5.1) for plants growing in 2 μM methionine. This value is essentially the same as that for plants growing in the absence of added methionine (mean of 4.4 nmoles/colony; SEM, 0.3; range of 3.6-5.6). Protein methionine in plants growing in 0.67 μM methionine was therefore presumed to be also 4.4 nmoles/colony. No spermine was detected.

[a] Calculated as described in Materials and Methods using the following mean values. For *Lemna* growing in 2 μM methionine, $U = 13.2$ and $S = 1.1$. For *Lemna* growing in 0.67 μM methionine, $U = 5.7$ and $S = 0.9$. Values of R and R' were derived from the mean values listed in Table I.

[b] Calculated as in (a), except that experimental values of U, S, R and R' were selected to yield the lowest and highest possible (extreme) values shown.

[c] Mean of determinations on two cultures, with SEM shown in parentheses.

of methionine sulfur without retention of the original 4-carbon moiety. Transmethylation thus appears to be the dominant pathway for methionine metabolism. As is also shown in Table II, PC methyl groups account for approximately 40% of the transmethylation, and would account for more to the extent that turnover of PC occurs. Turnover times as short as 6 hrs for the methyl groups of PC have been reported (23) in organelle membranes of castor bean. PC is therefore probably a major product of transmethylation in *Lemna*. This suggestion may extend to the plant kingdom in general, since PC is a dominant phospholipid in plants (24).

The findings described were obtained with plants growing in the presence of exogenous methionine, and it is not known to what extent the two pathways for recycling of methionine sulfur were affected by these growth conditions. This question is currently being examined.

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