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Control of Free Methionine Production in Wild Type and Ethionine-resistant Mutants of *Chlorella sorokiniana*

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ABSTRACT

Mutants of *Chlorella sorokiniana* selected for resistance to the methionine analogue ethionine took up ethionine at the same rate as did the wild type strain. Cells of two ethionine-resistant mutants produced severalfold higher levels of free methionine and cysteine than did wild type cells.

Exogenous methionine had no apparent effect on free methionine production in a mutant that produces excessive levels of free methionine. Under the same conditions, production of free methionine was relatively inhibited in wild type cells and in a mutant that produces wild type levels of free methionine.

The results suggest that free methionine production in the wild type strain is subject to endproduct control, and that this control is lacking in one class of ethionine-resistant mutants.

In plants and microorganisms, methionine, lysine, threonine, and isoleucine are end products of a multibranched biosynthetic pathway that stems from aspartic acid, the common biosynthetic precursor. Much is known of the control mechanisms of this pathway in bacteria and fungi (15), but relatively little comparative work has been done with green plants. Dougall (5) reported several isotope competition experiments with cell suspensions of Paul's Scarlet Rose tissue in which suggestive, but not conclusive, evidence for end product control of the pathway was obtained. More recently, two of the enzymes common to the entire pathway, aspartokinase and homoserine dehydrogenase, were isolated from plants, and their feedback sensitivities to various end products of that pathway described (3, 4, 17, 18). In addition, nutritional studies with rice callus tissue (8), *Marchantia polymorpha* gemmalings (6), and *Lemna minor* plants (19) have provided some evidence that threonine and lysine cooperatively inhibit the aspartic pathway *in vivo*.

In this paper, we provide physiological evidence that production of free methionine in *Chlorella sorokiniana* is subject to end product control, and that one class of ethionine-resistant mutants lacks that control.

MATERIALS AND METHODS

Growth of Cells. *Chlorella sorokiniana* Shihira and Krauss, strain 7-11-05, was obtained from the University of Maryland Culture Collection. It was cultured on a minimal medium similar to that of Sorokin and Krauss (14), except that 1.75 g/l of KNO_3 was substituted for urea, and the pH of the medium was adjusted to 7.0, after autoclaving, by aseptically adding 10 ml/l of a sterile solution of 1 M potassium phosphate buffer, pH 7.5. Stock cultures were maintained at room temperature in continuous dim light on 2% agar slants of minimal medium containing 0.1% glucose.

The low sulfate medium used was the same as minimal medium, except that the concentration of $MgSO_4$ was 0.05 mM and $Mg(NO_3)_2$ was added to a final concentration of 2.0 mM. Cells used for inocula in this medium were also cultured on low sulfate medium.

During the experiments, cells were grown in liquid culture in nephelometer flasks or in test tubes held at a 30° angle in a lucite box mounted on a reciprocating shaker. The box was gassed with 5% CO_2 in air and maintained at 25 C under light of 300 ft-c intensity. The doubling time of the cells was 6 to 8 hr under these conditions. Only log phase cells were used as inocula.

Growth was measured as absorbance at 750 nm and converted to cells/ml by use of a standard curve obtained by counting cells in a Levy-Hausser type hemocytometer.

Mutant Selection. Most of the ethionine-resistant mutants were selected from wild type cells treated with a mutagen. Specifically, 1 ml (10^9 cells) of a stationary culture was centrifuged, and the cells were resuspended in 1 ml of minimal medium containing 100 μg of N-methyl-N'-nitro-N-nitrosoguanidine (filter-sterilized). After being shaken in the dark for 45 min at 25 C (0.1% survival), the cells were centrifuged, washed twice, and 0.1-volume aliquots were used to inoculate 10 ml of minimal glucose medium. The cells were then allowed to grow to stationary phase in the light. Aliquots (0.1 ml) containing about 10^8 cells were then streaked on ethionine gradient agar, placed in a humid atmosphere at 25 C in the light (150 ft-c) and allowed to grow 7 to 9 days. Discrete colonies were picked from the high ethionine portion of the gradient and tested and purified as described below.

The gradient plates were prepared by placing a solution of ethionine in a glass cylinder (10 mm × 10 mm) set in the center of a 9-cm Petri dish containing 10 ml of minimal glucose 2% agar medium. The cylinder was sealed to the solidified agar by dipping the end of the cylinder in melted agar before placing it on the solidified agar. One milliliter of minimal glucose medium containing 25 mg of DL-ethionine (0.15 M) was pipetted into the cylinder, and the plate was allowed to "dry" until most of the solution had passed into the agar (1–2 days). Cell suspensions were then streaked across the gradient from the cylinder to the outer rim of the Petri dish. Further evaporation was minimized by placing the plates in a transparent polyethylene bag.

Presumptive mutants were tested and purified by streaking on minimal glucose agar containing 10 mM DL-ethionine. Dis-

crete colonies, presumably arising from single cells, were transferred to tube slants of minimal agar medium for stock maintenance. One ethionine-resistant mutant, E-27, was obtained from a spontaneous mutation observed when untreated wild type cells were streaked across ethionine agar.

Seven mutant strains were chosen for more intensive study. These mutant strains grew as well in the presence of 10 mM DL-ethionine as did the wild type in its absence.

Assimilation of Labeled Compounds. L-Methionine-carboxy-^{14}C, L-ethionine-ethyl-1-^{3}H, or $^{35}SO_4^{2-}$ was added to the *Chlorella* cultures, in test tubes or flasks, and incubated in the light as described above. At designated intervals, 1-ml aliquots were removed from the cultures, filtered through membrane filters and washed with 10 ml of water. The filters were dried at 60 C overnight and counted in 10 ml of toluene-POPOP scintillation fluid.

Analysis of Sulfur-35 Amino Acids in Cells and Medium. Cells grown in low sulfate medium with added $^{35}SO_4^{2-}$ were harvested by centrifugation and washed twice with water. One μmole each of carrier L-methionine, D,L-homocysteine, D,L-cystathionine, D,L-cysteine, and GSH were added to the cells, and the free amino acids were extracted by boiling the cells in water for 2 min. The extracts were passed through columns (0.8 cm × 3.75 cm) of Dowex 50 H^+ form resin. After washing the columns with 30 ml of water, the amino acids were eluted with 10 ml of 3 M NH_4OH.

The NH_3 and H_2O were evaporated and the residue was dissolved in 88% formic acid. Aliquots were spotted on paper (22 × 58 cm) and oxidized by overlaying with performic acid and sodium molybdate catalyst (9) for 1 hr. The above procedure oxidizes methionine, homocysteine, cystathionine, cysteine, and GSH to methionine sulfone, homocysteic acid, cystathionine sulfone,[1] cysteic acid, and GSH sulfonic acid, respectively.

The chromatograms were developed overnight in descending fashion with methanol-pyridine-1.25 N HCl (37:4:8), and the amino acids were visualized with ninhydrin (0.3% ninhydrin in 95% ethanol-collidine, 9:1). Each chromatogram was cut into 26 sections (2 × 3 cm) and counted in 10 ml of toluene-POPOP scintillation fluid. The radioactive spots were identified by co-chromatography with authentic amino acid standards. A representative chromatogram is shown in Figure 1 to indicate the resolution obtained. No peaks of ^{35}S-homocysteic acid or ^{35}S-cystathionine sulfone were clearly identifiable on chromatograms of any of the samples.

To confirm the identity and to determine the degree of purity of the radioactive peaks observed on the chromatograms, representative samples of cell and medium extracts were subjected to paper electrophoresis in 4.9 M formic acid, pH 1.6, at 2000 v for 2 hr. Radioactivity found in cysteic acid and GSH sulfonic acid peaks agreed closely with the amounts obtained by paper chromatography. Homocysteic acid was clearly resolved in the system, but no corresponding peak of radioactivity was observed in the samples. Methionine sulfone and cystathionine sulfone were not resolved by electrophoresis, but since the latter had been shown to be negligible by paper chromatography all of the radioactivity in that peak could be assigned to methionine sulfone. Radioactivity in the methionine sulfone peaks averaged 34% lower than those on the chromatograms, indicating some contamination of the methionine sulfone peaks on the chromatograms with other ^{35}S compounds. However, since the differences in methionine levels of interest among the strains were an order of magnitude greater than these analytical differences, paper chromatography was used to quantitate all of the ^{35}S amino acids.

In the first experiments reported, an unidentified peak of radioactivity (unknown A of Table III) was observed between cysteic acid and GSH sulfonic acid on chromatograms of cell extracts. It did not co-chromatograph with S-adenosylmethionine, which, in any case, is degraded by the alkaline desalting conditions. It was not observed in later experiments.

^{35}S amino acids excreted into the medium were analyzed in the manner described above.

Labeled Compounds. L-Ethionine-ethyl-1-^{3}H (42 mc/mmole) and carrier-free $H_2{}^{35}SO_4$ were purchased from New England Nuclear and L-methionine-carboxy-^{14}C (11 mc/mmole) from Schwarz BioResearch.

Scintillation Counting. Radioactivity was measured with a Packard Tri-Carb liquid scintillation spectrometer Model 3003. Aqueous samples were counted with 10 ml of Aquasol (New England Nuclear) or with 15 ml of Bray's solution (2). Samples on paper or membrane filters were counted with 10 ml of toluene scintillation fluid containing 5 g/l PPO and 0.3 g/l dimethyl POPOP.

RESULTS AND DISCUSSION

Growth Studies. Wild type strain 7-11-05 and two of the ethionine-resistant mutants were cultured in minimal medium with or without 0.5 mM L-ethionine. The cell doubling time for each of the cultures is presented in Table I. Ethionine greatly inhibited the growth of the wild type cells, causing their doubling time to increase nearly 6-fold, but had no effect on mutants E-1 or E-12.

Uptake of Label from Methionine-^{14}C and Ethionine-^{3}H. In order to determine whether ethionine resistance might be due to impaired uptake, the kinetics of methionine-^{14}C and ethionine-^{3}H incorporation by the wild type strain and E-1 and E-12 mutants were determined. The results in Table II demonstrate that the capability of the mutants to take up ethionine or methionine is comparable to or greater than that of the wild type cells. This rules out the possibility that ethionine resistance in these mutants is due to a reduced capability for taking up ethionine.

Production of Free Sulfur Amino Acids. One common

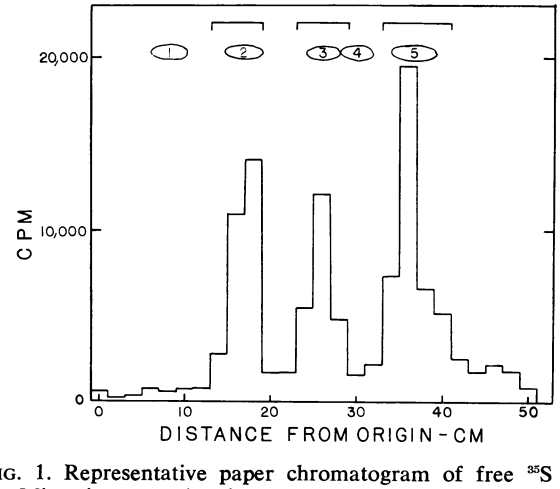

FIG. 1. Representative paper chromatogram of free ^{35}S amino acids. Migration of authentic cystathionine sulfone (1), cysteic acid (2), GSH sulfone (3), homocysteic acid (4), and methionine sulfone (5) was visualized by reaction with ninhydrin. The total radioactivity enclosed by the brackets was used for quantitating the respective ^{35}S amino acids.

[1] The exact oxidation state of cystathionine is apparently unknown but is here designated as cystathionine sulfone for convenience.

Table I. *Doubling Times for Wild Type Chlorella sorokiniana and Two Mutants Cultured in the Presence or Absence of Ethionine*

Values are averages of two replicate cultures.

Strain	Doubling Time	
	0.5 mM L-ethionine	No ethionine
	hr	
Wild type	42.5	7.5
E-1	7.8	7.5
E-12	7.5	7.0

Table II. *Net Uptake of Ethionine-^{3}H or Methionine-^{14}C by Wild Type Strain and Two Ethionine-resistant Mutants*

Cells were grown in minimal medium supplemented with 0.05 mM ethionine-^{3}H or 0.05 mM methionine-^{14}C. The growth conditions and experimental procedure are given in the text. Each value is the average of two replicate cultures.

Strain	5 Hr		12 Hr		24 Hr	
	Ethionine	Methionine	Ethionine	Methionine	Ethionine	Methionine
	nmoles/10^{10} cells					
Wild type	100	203	80	177	65	169
E-1	130	362	88	211	61	123
E-12	107	178	78	149	52	120

mechanism of antimetabolite resistance in mutants of microorganisms is the excessive production of the corresponding metabolite. Consequently, the wild type strain and six of the ethionine-resistant mutants were examined for levels of free sulfur-containing amino acids. To accomplish this the cells were grown for six generations in a medium containing $^{35}SO_4^{2-}$, and the amount of radioactivity found in each of the S amino acids extracted from the cells or excreted into the medium was used as a quantitative measure of that compound.

Two of the ethionine-resistant mutants, E-1 and E-7, produced excessive amounts of both free methionine and cysteine when compared to the wild type strain (Tables III and IV). The intracellular level of free methionine in E-1 was three times that of the wild type, while cysteine was about twice that of the wild type. The corresponding comparison for methionine in E-7 cells was 4-fold and for cysteine 6-fold higher than that of wild type cells. Since all of the strains excreted about one-third of their total supply of free S amino acids into the medium, the proportionate increase in production of free methionine and cysteine by E-1 and E-7, on a whole-culture basis, was about the same as noted above for intracellular levels of these amino acids. The data suggest that ethionine resistance in mutants E-1 and E-7 is associated with the overproduction of free methionine.

Kvitko and Golubtsova (12) recently reported increased cysteine and methionine levels in several slow growing ethionine-resistant mutants of *C. vulgaris*. Of the six mutants studied here, only E-7 grew somewhat more slowly (9.5 *versus* 8.0 hr doubling time) than did the wild type strain.

Effects of Exogenous Methionine on Methionine Production. The observation that several ethionine-resistant mutants produced higher levels of free methionine and cysteine than did the wild type strain could be interpreted to mean that the mutants lacked control over the synthesis of those amino acids. If that interpretation is correct, then exogenously supplied methionine, the end product or end product precursor, should have no effect on the endogenous synthesis of methionine by mutants E-1 and E-7 but should suppress free methionine production by wild type cells. Two mutants, along with the wild type, were selected to test this hypothesis. E-1 was selected as a mutant that overproduces free methionine and E-12 as an ethionine-resistant mutant that does not produce significantly greater levels of free methionine than does the wild type (Tables III and IV).

To obtain valid data from this experimental approach, it was necessary to demonstrate that exogenously supplied methionine actually accumulated to comparable intracellular levels in all three test strains. Thus the three strains were grown in low sulfate medium containing 1 mM methionine-^{14}C for 13.5 hr, and the cells were then extracted and analyzed for free methionine-^{14}C content. Higher concentrations of exogenous methionine (5 mM) were somewhat inhibitory to the growth of all three strains. The rate of methionine-^{14}C uptake was similar for each of the strains. After 13.5 hr the intracellular levels of

Table III. *Content of Free Sulfur-containing Amino Acids in Cells of Wild Type and Ethionine-resistant Mutants*

Cells were grown for 46 hr in low sulfate medium (5-ml cultures) containing approximately 8.2×10^6 cpm of $^{35}SO_4^{2-}$. Each value is the average of two replicate cultures.

Experiment	Strain	Cysteine	Unknown A	Glutathione	Methionine
		nmoles/10^{10} cells			
1	Wild type	62.9	63.3	54.1	54.4
	E-1	113.1*	117.1	76.0	151.8*
	E-12	50.4	72.3	66.4	92.8
	SE	7.2	20.8	17.4	8.8
2	Wild type	31.7	9.6	22.0	26.6
	E-7	177.2*	46.9*	94.7*	118.5*
	E-27	25.9	9.5	21.9	22.1
	E-31	68.5	16.1	26.8	63.0
	E-32	118.9	22.0	54.0	70.2
	SE	25.9	6.8	53.2	17.9

* Significantly different from wild type at the 95% level as determined by Dunnett's procedure for comparing several treatments simultaneously with a control or standard treatment (7). In this and succeeding tables the SE is a pooled estimate from the several strains in each experiment.

Table IV. *Sulfur Amino Acids Excreted by Cells of Wild Type and Ethionine-resistant Mutants*

Cells were grown for 46 hr in low sulfate medium (5-ml cultures) containing approximately 8.2×10^6 cpm of $^{35}SO_4^{2-}$. Each value is an average of two replicate cultures.

Experiment	Strain	Cysteine	Glutathione	Methionine
		nmoles/10^{10} cells		
1	Wild type	15.1	10.8	20.0
	E-1	72.6*	18.8	47.1
	E-12	18.7	16.4	50.8
	SE	5.2	5.4	8.6
2	Wild type	13.8	18.8	12.8
	E-7	95.6*	36.4	69.9*
	E-27	22.5	28.7	21.2
	E-31	74.8*	36.4	46.9
	E-32	62.6*	20.2	31.9
	SE	10.4	8.0	11.0

* Significantly different from wild type at the 95% level (7).

free methionine-^{14}C were similar for E-1 and the wild type strain but somewhat higher for E-12 (Table V). From these data we concluded that any end product control exerted by exogenous methionine would be at least as great in E-1 and E-12 as in the wild type cells.

To examine the effect of exogenous methionine on endogenous free methionine production, we grew the three strains in low-sulfate medium containing nonradioactive 1 mM methionine for a length of time (10 hr) deemed sufficient to permit any end product control to be fully expressed. Radioactive SO_4^{2-} was then added to the medium, and 4 hr later the cells were harvested and the distribution of ^{35}S in the free S amino acids was determined. The uptake of $^{35}SO_4^{2-}$ by each of the strains was monitored to permit correction of the resulting data for any uptake differences. Exogenous methionine inhibited $^{35}SO_4^{2-}$ uptake from 15 to 30% (Table VI), an effect also reported to occur with *C. pyrenoidosa* (16).

The distribution of ^{35}S in free amino acids at the end of the 4-hr $^{35}SO_4^{2-}$ period is shown in Table VI. The experiment was performed twice, and results were similar from both experiments. The data in Table VI disclose one dramatic difference in ^{35}S distribution attributable to exogenous methionine. Mutant E-1 accumulated over two and one-half times more endogenous free methionine-^{35}S in the presence of nonradioactive exogenous methionine than in its absence. This accumulation of endogenous methionine in the presence of a high level of exogenous methionine was not observed with the wild type and E-12 strains. These results are consistent with the postulate that exogenous methionine expanded an intracellular pool (10) of free methionine which diluted, and thus trapped, the methionine-^{35}S synthesized in or transported to that pool. Continued synthesis of methionine-^{35}S by mutant E-1 caused a buildup of endogenous methionine-^{35}S in this pool, while partial suppression of methionine-^{35}S synthesis in the wild type and E-12 strains by exogenous methionine accounted for their lack of methionine-^{35}S buildup.

This explanation appears to account for only about half of the methionine-^{35}S buildup (79 nmoles/10^{10} cells, calculated from data in Table VI) by mutant E-1 in the presence of exogenous methionine, since the data in Table V indicate that exogenous methionine can expand the pool of free methionine in E-1 by only 35 nmoles per 10^{10} cells. The latter value represents the maximum amount of methionine-^{35}S buildup possible from saturation of this expanded pool with labeled methionine. The explanation for the additional buildup of 44 nmoles of methionine-^{35}S per 10^{10} cells is unclear. However, the discrepancy may simply reflect a difference in size of the endogenous-free methionine pools, and consequently their potential for expansion, in this experiment and in the one reported in Table V. Differences in endogenous pool sizes were observed in the two successive experiments reported in Tables III and IV.

An alternative explanation considered for the buildup of endogenous methionine-^{35}S in mutant E-1 in the presence of a high level of exogenous methionine was that E-1 differed from the wild type by having a lowered inducible capacity to degrade or utilize methionine. Such an explanation is not consistent with the data in Table V which indicate that the high level of exogenous methionine-^{14}C supplied to E-1 cells was degraded or utilized as rapidly in these cells as in wild type or E-12 cells. A diminished rate of degradation or utilization in that experiment would have resulted in higher levels of free methionine-^{14}C in E-1, relative to wild type and E-12, because all three strains took up similar amounts of exogenous methionine-^{14}C (Table V). Thus, a lowered capacity for degrading or utilizing methionine does not appear to be a reasonable explanation for the buildup of methionine-^{35}S in mutant E-1 in the presence of high exogenous methionine. Rather, the offered explanation invoking a lack of end product control of methionine synthesis in E-1 is favored.

Table V. *Free Methionine-^{14}C in Cells Supplied with Exogenous Methionine-^{14}C*

Each 30-ml culture was grown for 13.5 hr in low sulfate medium supplemented with 1 mM L-methionine-^{14}C (4.0×10^3 cpm/nmole). The cells were then extracted and analyzed for free methionine-^{14}C. Each value is an average of two replicate cultures.

Strain	Free Methionine-^{14}C in Cells	Total ^{14}C in Cells
	nmoles/10^{10} cells	
Wild type	31.5	278
E-1	35.0	378
E-12	71.0	363

Table VI. *Effect of Exogenous Methionine on the Distribution of ^{35}S in Intracellular Free Amino Acids*

Cells were grown in low sulfate medium (35-ml cultures) with or without 1 mM L-methionine for 10 hr. Carrier-free $H_2{}^{35}SO_4$ was then added to give a specific activity of approximately 9.5×10^4 cpm/nmole, and the cells were harvested 4 hr later. To calculate the specific activity, the slight depletion of nonradioactive SO_4^{2-} during the initial 10 hr was estimated from the cell increase during that period and the rate of ^{35}S accumulation (per cell increase) during the subsequent 4 hr. Amino acid values were normalized for $^{35}SO_4^{2-}$ uptake differences using the wild type control uptake value as the norm. Each value is the average of two replicate cultures.

Strain	Treatment	^{35}S in Cells	Cysteine	GSH	Methionine
		cpm $\times 10^{-4}$/10^7 cells	*nmoles/10^{10} cells*		
Wild type	Control	8.2	74	46	38
	1 mM L-methionine	7.0	33*	46	32
	SE	1.0	7.6	5.6	2.9
E-1	Control	7.3	77	38	50
	1 mM L-methionine	5.1*	56	49	129**
	SE	0.4	11.0	5.2	10.6
E-12	Control	7.9	75	51	64
	1 mM L-methionine	6.1	65	51	53
	SE	1.5	12.7	7.5	4.9

* Significantly different from control at the 95% level as determined by the *t* test.

** Significantly different from control at the 98% level as determined by the *t* test.

The effects of exogenous methionine on the sulfur amino acid metabolism of the strains studied here were not limited to the production of free methionine. A decrease in the level of free cysteine-^{35}S, a proposed S-donor for methionine (9), accompanied the apparent inhibition of endogenous free methionine-^{35}S production by exogenous methionine in wild type cells, relative to mutant E-1 (Table VI). These data suggest that methionine may regulate the reduction of SO_4^{2-} as in the case of *Neurospora* (11).

The above experiments show that the nature of ethionine resistance is quite different in mutants E-1 and E-12. E-1 produces and excretes more free methionine than the wild type, and its production is relatively insensitive to control by exogenously supplied methionine. In contrast, E-12 produces levels of methionine not significantly different from those of the

ethionine-sensitive wild type strain and also exhibits an apparent end product control of methionine production similar to that of the wild type.

Ethionine toxicity to green plants is not fully understood. Ethionine is known to inhibit Chl synthesis in cultured soybean cells (13), presumably by antagonizing the methylation of Mg protoporphyrin by S-adenosylmethionine. However, in other organisms ethionine is known to act in one or more of several different ways (1), and resistant mutants are known to use various counteracting mechanisms, of which overproduction of methionine is but one (11).

CONCLUSION

This study provides physiological evidence of end product control of methionine production in *Chlorella*. This conclusion is based partially on the observation that one class of ethionine-resistant mutants produces three to four times as much free methionine as does the wild type strain. This conclusion was also supported by the further observation that in the presence of a high level of exogenous methionine mutant E-1 exhibits no apparent inhibition of free methionine production, whereas in wild type cells its production is relatively inhibited.

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