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Induction of δ-Aminolevulinic Acid Synthase Activity and Inhibition of Heme Synthesis in *Euglena gracilis* by *N*-Methyl Mesoporphyrin IX[1]

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ABSTRACT

N-Methyl mesoporphyrin IX, an inhibitor of heme synthesis, increases extractable δ-aminolevulinic acid (ALA) synthase activity when administered to growing cultures of *Euglena gracilis* Klebs strain Z Pringsheim in micromolar concentrations. Wild-type light-grown green cells and white aplastidic cells exhibited 2.8-fold and 1.8-fold increases, respectively, in ALA synthase activity within five to six hours after incubation with 4 × 10^{-6} molar N-methyl mesoporphyrin IX. Protoheme levels were decreased and ^{59}Fe incorporation into heme was inhibited by N-methyl mesoporphyrin IX, indicating that, as in animal cells, N-methyl mesoporphyrin IX acts specifically to block iron insertion into protoporphyrin IX. Chlorophyll synthesis in wild-type cells was not affected within the first 6 hours after administration of N-methyl mesoporphyrin IX.

In animals, fungi, and bacteria, the first identified step of the tetrapyrrole biosynthetic pathway leading to hemes and Chl is catalyzed by ALA[3] synthase (succinyl-CoA: glycine *C*-succinyl transferase [decarboxylating], EC 2.3.1.37). Plants and algae synthesize ALA by a different route, from the intact carbon skeleton of glutamate or a closely related compound. The plant pathway, which is as yet incompletely characterized, has been shown to operate in bluegreen and green algae, including *Euglena*, as well as in greening leaves. Until quite recently, ALA synthase had not been detected in O_2-evolving photosynthetic organisms (for review, see 2, 14).

It was recently reported that *Euglena gracilis* contains ALA synthase (6). High levels of ALA synthase activity were measured in extracts of aplastidic mutant cells and dark-grown wild-type cells, and lower levels were found in light-grown wild-type cells. A study of the regulatory properties of *Euglena* ALA synthase is being undertaken with the goal of determining the physiological role of the enzyme in these cells (4, 5).

N-Methyl mesoporphyrin IX is a powerful inhibitor of ferrochelatase (protoheme ferrolyase, EC 4.99.1.1), the enzyme catalyzing iron insertion into protoporphyrin IX, which is the last step in the protoheme biosynthetic pathway (9). This compound was reported to cause accumulation of porphyrins and induction of hepatic ALA synthase within 4 h after injection into mice. We now report that *N*-methyl mesoporphyrin IX increases extractable ALA synthase activity, inhibits incorporation of exogenous ^{59}Fe into heme *in vivo*, and diminishes heme levels in *Euglena*, but has no effect on Chl formation within the first 6 h after administration.

MATERIALS AND METHODS

Cultures of *Euglena gracilis* Klebs strain Z Pringsheim and an aplastidic mutant derived from this strain, $W_{14}ZNa1L$ (23), were kindly provided by H. Lyman (State University of New York, Stony Brook, NY). Cells were grown at 23°C and 32 μE $m^{-2}s^{-1}$ of cool white and red fluorescent light in a glucose-based heterotrophic medium as previously described (6). Cell population densities were determined with a Coulter Counter (Model ZBI, Coulter Electronics). Chl was extracted into methanol and quantitated by spectrophotometry, using the absorption coefficients of MacKinney (19). ALA synthase was extracted and assayed by the methods previously reported (6), except that cells were disrupted by sonication for four periods using a Sonifier (Model W 185, Heat Systems—Ultrasonics), and ALA was measured by condensation with ethyl acetoacetate at pH 6.8 (18) followed by color development with Ehrlich Hg reagent (26). Spectrophotometry was performed on a Cary Model 219 instrument (Varian).

Protoheme was extracted by slight modification of the method of Stillman and Gassman (25). All operations were carried out at ice temperature. Cells were extracted with three 5-ml portions of 90% acetone containing 10^{-2} M NH_4OH. These extracts were discarded. The cells were then extracted with 2 ml acetone containing 2% HCl. A second 1-ml acetone-HCl extraction supernatant was added to the first, and then 2 ml peroxide-free diethyl ether were added to the combined acetone extracts. After mixing, 6 ml H_2O were added, the solution was thoroughly mixed, and briefly centrifuged to separate the phases. The upper ether phase was removed and the lower phase was extracted with 1 ml ether. The combined heme-containing ether phases were backwashed once with 1 ml H_2O. After evaporation of the ether, protoheme was determined by the reduced minus oxidized pyridine hemochromogen method (11).

^{59}Fe uptake and incorporation into heme were measured by adding 10 μCi $^{59}FeCl_3$ to cells growing in 4 ml normal medium. After 6 h incubation at the standard growth conditions with orbital shaking in the light, cells were harvested by centrifugation. Heme was isolated as described above, and ^{59}Fe was counted in a Packard Gamma Counter Model 5210. Samples of the cell suspension and the medium after removal of cells by centrifugation were also counted to measure uptake of ^{59}Fe into cells.

N-Methyl mesoporphyrin IX was prepared from the dimethyl ester by hydrolysis in 6 N aqueous HCl for 18 h. Its concentration was determined from its Soret band light absorption peak at 406

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[3] Abbreviation: ALA, δ-aminolevulinic acid.

Table I. *Green wild-type Z Cells and Aplastidic Strain W_{14}ZNa1L Cells were Grown in the Light for 6 Hours in the Presence of 10 μCi of $^{59}FeCl_3$, with and without Added 2.5 × 10^{-6} M N-Methyl Mesoporphyrin IX*

Heme was isolated as described under "Materials and Methods." Values of duplicates are shown with experimental ranges indicated.

Cell Type	Treatment	^{59}Fe Supplied	Uptake of ^{59}Fe			^{59}Fe Appearing in Heme Fraction		
		cpm	*cpm*	*% of supplied label*	*% of controls*	*cpm*	*% of supplied label*	*% of control*
W_{14}ZNa1L	Control	4.0 ± 0.1 × 10^6	4.05 ± 0.51 × 10^5	10.0 ± 1.2		3500 ± 300	0.087 ± 0.003	
W_{14}ZNa1L	Treated	4.0 ± 0.1 × 10^6	2.25 ± 0.80 × 10^5	5.6 ± 2.0	56	1710 ± 30	0.042 ± 0.001	48
Z	Control	4.0 ± 0.1 × 10^6	9.29 ± 1.32 × 10^5	23.4 ± 3.4		7970 ± 770	0.203 ± 0.007	
Z	Treated	4.0 ± 0.1 × 10^6	5.36 ± 0.50 × 10^5	13.1 ± 1.0	56	2480 ± 40	0.061 ± 0.000	30
Z	Zero time control	4.0 ± 0.1 × 10^6	0	0		0	0	
Z	Carryover control	4.0 ± 0.1 × 10^6	3.90 × 10^6	100		40	0.00011	

Table II. *Growing Cultures of Aplastidic Euglena gracilis Strain W_{14}ZNa1L were Incubated in the Light for 5 Hours in the Presence of the Indicated Concentrations of N-Methyl Mesoporphyrin IX*

ALA synthase values were determined in duplicate.

		ALA Synthase Activity	
	cells ml culture^{-1}	*nmol ALA 30 min^{-1} ml culture^{-1}*	*nmol ALA 30 min^{-1} 10^7 cells^{-1}*
Initial value	1.50 × 10^6	7.17 ± 0.06	47.75 ± 0.15
Control	1.88 × 10^6	9.56 ± 0.04	50.83 ± 0.19
4 × 10^{-7} M *N*-methyl mesoporphyrin IX	1.84 × 10^6	10.05 ± 0.13	54.62 ± 0.71
4 × 10^{-6} M *N*-methyl mesoporphyrin IX	1.74 × 10^6	15.59 ± 0.29	89.60 ± 1.67

nm in 0.1 N HCl, using the molar absorption coefficient of 1.93 × 10^5 at 389 nm given for mesoporphyrin monocation (24). Concentrations determined in this manner are only approximate, because the true absorption coefficient of *N*-methyl mesoporphyrin IX has not been reported.

N-Methyl mesoporphyrin IX dimethyl ester was a generous gift from F. De Matteis (Medical Research Council, Surrey, England). $^{59}FeCl_3$ was purchased from New England Nuclear. Other chemicals were obtained from Sigma and Fisher.

RESULTS

Heme Synthesis. *N*-Methyl mesoporphyrin IX at 2.5 × 10^{-6} M inhibited incorporation of exogenous ^{59}Fe into heme in both aplastidic and wild-type cells growing in the light (Table I). The uninhibited rate of ^{59}Fe incorporation into heme was 2.3-fold greater in wild-type control cells than in aplastidic control cells. This is consistent with the generally higher cellular levels of protoheme present in the wild-type cells (Beale, unpublished). Compared to their respective controls, ^{59}Fe incorporation into heme was reduced by 70% in wild-type cells and 52% in aplastidic cells in the presence of 2.5 × 10^{-6} M *N*-methyl mesoporphyrin IX. Although it was noted that uptake of ^{59}Fe appeared to be inhibited by *N*-methyl mesoporphyrin IX (Table I), the amount of label taken up by all cells was far in excess of the amount that was incorporated into heme.

A zero time control incorporated no measurable ^{59}Fe into the heme fraction. To assess the degree of nonspecific carryover of ^{59}Fe into the final heme-containing fraction, an additional zero time control experiment was performed in which ^{59}Fe was added to cells after the culture medium was removed but before the first acetone extraction was performed. This control approximates the condition where all of the supplied label is taken up but none is incorporated into heme, and serves to measure the maximum possible degree of nonspecific carryover of ^{59}Fe into the final heme fraction. Only trace quantities of label were detected in this fraction (Table I).

Attempts at measuring *Euglena* ferrochelatase activity *in vitro* were unsuccessful (data not shown).

ALA Synthase Induction. Growing cultures of aplastidic *Euglena* cells were incubated for 5 h with two concentrations of *N*-methyl mesoporphyrin IX, 4 × 10^{-7} M and 4 × 10^{-6} M. The lower concentration affected growth and ALA synthase levels only slightly; the higher concentration inhibited growth slightly and caused an almost two fold increase in extractable ALA synthase activity (Table II).

Wild-type cells growing in the light were incubated for 6.5 h in the presence or absence of 4 × 10^{-6} M *N*-methyl mesoporphyrin IX. At this concentration, the compound inhibited growth slightly, caused extractable ALA synthase activity to increase almost three fold, and had little if any effect on Chl synthesis (Table III).

Heme Content. After 6 h of growth in the light and the presence of 4 × 10^{-6} M *N*-methyl mesoporphyrin IX, cellular protoheme content of wild-type cells was reduced to 74% of the control value, and after 24 h, treated cells had only 59% of the control level of protoheme (Table IV).

DISCUSSION

Although *Euglena* cells possess ALA synthase (4–6), they also are capable of synthesizing ALA from five-carbon precursors by the still incompletely characterized "plant" pathway (22, Beale, unpublished). It was proposed (6) that in this organism, ALA synthase is involved in nonplastid, *e.g.* mitochondrial, tetrapyrrole biosynthesis. If this is the case, then it would be expected that ALA synthase levels would not necessarily co-vary with Chl formation rates, but would instead respond to the cellular demand for heme. The hypothesis can be tested by measuring the response of the cells to the presence of *N*-methyl mesoporphyrin IX, because this compound inhibits heme formation by interfering with the last step, *i.e.* iron insertion into protoporphyrin IX (9), and thus it does not directly block any step in the Chl pathway.

The effectiveness of *N*-methyl mesoporphyrin IX *in vivo* was assessed by measuring the rate of exogenous ^{59}Fe incorporation into heme in growing cells. At 2.5 × 10^{-6} M, *N*-methyl mesoporphyrin IX was effective in lowering the rate of exogenous ^{59}Fe incorporation into heme by more than 50% in both wild-type and aplastidic cells. Uptake of ^{59}Fe also appeared to be inhibited by *N*-methyl mesoporphyrin IX. The total amount of label taken up, even by inhibited cells, was 100 times greater than the amount appearing in heme. Whereas it is unlikely that uptake was the limiting factor in the label incorporation into heme, it is possible that Fe uptake may be indirectly affected by the rate of heme synthesis, in response to the cellular demand for iron as a heme precursor.

N-Methyl mesoporphyrin IX caused apparent induction of ALA synthase in both light-grown green wild-type cells and white aplastidic cells. The relatively greater percentage increase in ALA synthase induced in wild-type cells, compared to the effect in

Table III. *Light-Grown Cultures of Euglena gracilis Strain Z Were Incubated in the Light for 6.5 Hours in the Presence or Absence of 4 × 10^{-6} M N-Methyl Mesoporphyrin IX*

ALA synthase values were determined in duplicate.

		ALA Synthase Activity		Chlorophyll	
	cells ml culture^{-1}	*nmol ALA 30 min*$^{-1}$ *ml culture*$^{-1}$	*nmol ALA 30 min*$^{-1}$ 10^7 *cells*$^{-1}$	*nmol ml culture*$^{-1}$	*nmol* 10^7 *cells*$^{-1}$
Initial value	3.51×10^6	3.01 ± 0.02	17.10 ± 0.11	22.2	63.3
Control	5.25×10^6	4.84 ± 0.08	18.40 ± 0.30	31.7	60.3
N-Methyl mesoporphyrin IX	4.73×10^6	12.25 ± 0.09	51.67 ± 0.36	31.7	67.0

Table IV. *Light-Grown Cultures of Euglena gracilis Strain Z were Incubated in the Light for 6 and 24 Hours in the Presence or Absence of 4 × 10^{-6}M N-Methyl Mesoporphyrin IX*

Protoheme was extracted and assayed as described in "Materials and Methods." Zero and 6 h values were determined in duplicate. Figures in parentheses indicate percent of control values.

	Protoheme		
	0 h	6 h	24 h
	pmol 10^7 *cells*$^{-1}$		
Control	324 ± 28	252 ± 9	270
N-Methyl mesoporphyrin IX		186 ± 2 (74)	160 (59)

aplastidic cells, is probably related to the lower basal level of the enzyme in the green cells. The observed 3-fold lower activity in extracts obtained from untreated green cells, compared to the levels in untreated white cells, was reported previously (6).

ALA formation is generally considered to be the rate-limiting step for Chl synthesis during greening. This has been inferred in higher plants (20) and greening dark-grown *Euglena* (16) from the observed stimulation of Chl or Pchl formation in response to administration of exogenous ALA. Moreover, in many plant tissues and algae (1, 3), including *Euglena* (21), Chl formation decreases in stoichiometric ratio to ALA accumulation when ALA conversion to other Chl precursors is blocked by administration of levulinic acid, a competitive inhibitor of ALA dehydratase. However, under other conditions, when ALA is available, other factors undoubtedly become rate-limiting for Chl synthesis. *N*-Methyl mesoporphyrin IX did not affect the rate of Chl formation in greening wild-type *Euglena* cells during the first 6 h after administration, even though extractable ALA synthase activity was increased and heme levels were depressed by the compound. These results are consistent with the hypothesis that the ALA which is formed via ALA synthase is not a precursor to Chl.

Heme functions as an allosteric inhibitor and repressor of ALA synthase in photosynthetic bacteria (7, 15, 17), and it appears to act as a central regulator of bacteriochlorophyll synthesis in *Rhodopseudomonas spheroides* (17) by controlling the part of the pathway that is common to both heme and Chl formation. Because certain iron chelators have been found to induce accumulation of porphyrin and Mg-porphyrin Chl precursors while blocking heme synthesis in etiolated plant tissues (10, 12, 13), it has been proposed that heme may act to repress ALA formation in plants, thus regulating Chl precursor synthesis in a manner similar to the case in photosynthetic bacteria (8, 10). The ineffectiveness of *N*-methyl mesoporphyrin IX in influencing the rate of Chl synthesis in *Euglena*, even though it causes a decrease in the cellular heme content, argues against a direct role for heme in controlling the synthesis of Chl in this organism. To the extent that regulation of Chl synthesis in *Euglena* is similar to that in higher plants, these results suggest that iron chelators may affect Chl precursor accumulation in plants by a mechanism other than through causing a decrease in heme level.

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