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Heme Inhibition of δ-Aminolevulinic Acid Synthesis Is Enhanced by Glutathione in Cell-Free Extracts of *Chlorella*[1]

Jon D. Weinstein*, Robert W. Howell, Robert D. Leverette, Sonja Y. Grooms, Perry S. Brignola, Sandra M. Mayer, and Samuel I. Beale

Department of Biological Sciences, Clemson University, Clemson, South Carolina 29634-1903 (J.D.W., R.W.H., R.D.L., S.Y.G., P.S.B.); and Division of Biology and Medicine, Brown University, Providence, Rhode Island 02912 (S.M.M., S.I.B.)

In plants, algae, and many bacteria, the heme and chlorophyll precursor, δ-aminolevulinic acid (ALA), is synthesized from glutamate in a reaction involving a glutamyl-tRNA intermediate and requiring ATP and NADPH as cofactors. In particulate-free extracts of algae and chloroplasts, ALA synthesis is inhibited by heme. Inclusion of 1.0 mM glutathione (GSH) in an enzyme and tRNA extract, derived from the green alga *Chlorella vulgaris*, lowered the concentration of heme required for 50% inhibition approximately 10-fold. The effect of GSH could not be duplicated with other reduced sulfhydryl compounds, including mercaptoethanol, dithiothreitol, and cysteine, or with imidazole or bovine serum albumin, which bind to heme and dissociate heme dimers. Absorption spectroscopy indicated that heme was fully reduced in incubation medium containing dithiothreitol, and addition of GSH did not alter the heme reduction state. Oxidized GSH was as effective in enhancing heme inhibition as the reduced form. Co-protoporphyrin IX inhibited ALA synthesis nearly as effectively as heme, and 1.0 mM GSH lowered the concentration required for 50% inhibition approximately 10-fold. Because GSH did not influence the reduction state of heme in the incubation medium, and because GSH could not be replaced by other reduced sulfhydryl compounds or ascorbate, the effect of GSH cannot be explained by action as a sulfhydryl protectant or heme reductant. Preincubation of enzyme extract with GSH, followed by rapid gel filtration, could not substitute for inclusion of GSH with heme during the reaction. The results suggest that GSH must specifically interact with the enzyme extract in the presence of the inhibitor to enhance the inhibition.

ALA is a key, committed precursor to the biosynthesis of heme and Chl. In plants, algae, and many bacteria, ALA is synthesized from glutamate in a reaction involving a glutamyl-tRNA intermediate and requiring ATP and NADPH as cofactors (Kannangara et al., 1988; Beale, 1990; Beale and Weinstein, 1990). ALA synthesis occurs in the chloroplast stroma of eukaryotic cells. During chloroplast development, ALA formation is thought to be a rate-limiting step in tetrapyrrole biosynthesis, because administration of exogenous ALA causes massive accumulation of porphyrin and Mg-porphyrin intermediates. ALA biosynthesis appears to be highly regulated. In enzyme preparations from *Chlorella vulgaris* (Weinstein and Beale, 1985a), *Chlamydomonas reinhardtii* (Huang and Wang, 1986a), higher plants (Chereskin and Castelfranco, 1982), cyanobacteria (Rieble and Beale, 1991), and green sulfur bacteria (Rieble et al., 1989), this step is inhibited by heme when supplied in the micromolar concentration range. No other tetrapyrrole intermediate or end product is as effective an inhibitor as heme (Weinstein and Beale, 1985a). Genetic evidence suggests that heme may also regulate the production of the ALA biosynthetic enzymes (Huang and Wang, 1986a).

We recently developed a method to estimate the amount of heme in the chloroplasts that is not tightly associated with hemoproteins (Thomas and Weinstein, 1990, 1992). Using this method, we determined that the heme available for regulatory purposes is in the micromolar concentration range. However, the partition coefficient of heme between an artificial phospholipid membrane and an aqueous buffer is approximately 10^5, favoring the membrane (Rose et al., 1985). Therefore, the amount of heme available for regulation in an aqueous compartment would be extremely small. It has been proposed that heme that moves through an aqueous compartment travels via an aqueous carrier protein (Muller-Eberhard and Nikkilä, 1989; Smith, 1990). One proposed carrier is a member of the glutathione *S*-transferase family (Tipping et al., 1979; Vander Jagt et al., 1985; Takikawa et al., 1986; Caccuri et al., 1990). Some members of this protein family are known to bind heme, and, in some cases, GSH facilitates this binding (Caccuri et al., 1990). Therefore, we have investigated the effect of GSH on the heme inhibition of ALA synthesis in soluble extracts of the unicellular green alga, *C. vulgaris*.

MATERIALS AND METHODS

Materials

Tricine buffer and DTT were purchased from Research Organics, Inc. (Cleveland, OH). All porphyrins and metalloporphyrins were purchased from Porphyrin Products (Logan, UT). Gel filtration resin Spectra/Gel AcA 202 (1000 to 2500 mol wt cutoff) was from Medical Industries (Los Angeles, CA). All other biochemicals were of the highest purity avail-

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* Corresponding author; fax 1-803-656-0435.

Abbreviation: ALA, δ-aminolevulinic acid.

able from Sigma Chemical Co. (St. Louis, MO). All other salts and solvents were of analytical grade or better.

Growth of Algal Cells

Liquid cultures of *Chlorella vulgaris* Beijerinck, wild-type strain NG, were maintained in heterotrophic medium under continuous light at 25°C as described previously (Weinstein and Beale, 1985a). Cell densities were estimated by light scattering at 775 nm. Cell density *A* was determined with a Beckman DU-7 spectrophotometer on samples that were diluted with water to an apparent A_{775} of 0.1 to 0.4 before measurements were taken. Cell-doubling time was approximately 9.7 h. Cells for preparation of extracts were grown in 1-L flasks containing 500 mL of culture medium and were collected while in middle to late exponential growth phase (A_{775} approximately 6). In some experiments, where indicated, *Chlorella* strain C-10, which requires light for Chl formation, was used. These cells were grown in the dark and transferred to the light 2 h before collecting, as previously described (Avissar and Beale, 1989).

Preparation of Cell Extracts

Cell extracts were prepared as described before (Weinstein et al., 1986), and the proteins precipitating between 35 and 60% saturating $(NH_4)_2SO_4$ concentration were treated with NaCl to partially deplete the endogenous tRNA. A fraction containing a mixture of total cellular tRNAs was also prepared as described previously (Weinstein and Beale, 1985b).

Enzyme Assays

ALA formation from glutamate was assayed in 250 μL of reaction mixture containing 1 M glycerol, 50 mM Tricine, 15 mM $MgCl_2$, 5.0 mM ATP, 1.0 mM NADPH, 1.0 mM glutamate, 1.0 mM DTT, 20 μM pyridoxal phosphate, 0.7 to 1.2 mg of *Chlorella* protein extract, and 1.0 to 1.5 A_{260} units of *Chlorella* tRNA (final pH 7.85). Samples were incubated for 30 min at 30°C. ALA formation was quantitated spectrophotometrically by reaction with ethylacetoacetate to form ALA pyrrole, followed by reaction of the pyrrole with Ehrlich-Hg reagent (Weinstein et al., 1986). Any deviations from the standard procedure are noted in the text. A unit of enzyme activity is defined as that which catalyzes the formation of 1 nmol of ALA during the 30-min incubation. Protein was determined by the dye-binding method of Bradford (1976), using BSA as a standard.

Tetrapyrrole Solutions

Porphyrins and metalloporphyrins were dissolved in DMSO and stored as frozen concentrated stock solutions. The concentration of heme was determined from its *A* in ethanol:acetic acid:water (66.5:17:16.5, v/v/v), using an absorption coefficient of 1.44×10^5 M^{-1} cm^{-1} at 398 nm (Weinstein and Beale, 1983). The concentration of mesoheme was determined from the *A* of its pyridine hemochrome in 0.1 N NaOH, using an absorption coefficient of 1.40×10^5 M^{-1} cm^{-1} at 407 nm (Fuhrhop and Smith, 1975). The concentrations of protoporphyrin IX and Mg-protoporphyrin IX were determined from their absorption in diethyl ether, using absorption coefficients of 1.58×10^5 M^{-1} cm^{-1} at 404 nm and 3.08×10^5 M^{-1} cm^{-1}at 419 nm, respectively (Dawson et al., 1969). The concentration of Co-protoporphyrin IX was determined from its absorption in 0.1 N NH_4OH, using an absorption coefficient of 1.24×10^5 M^{-1} cm^{-1} at 421 nm (J. Yearyean, Porphyrin Products, Inc., personal communication). The concentration of Mn-protoporphyrin IX was determined from its absorption in 0.1 N NaOH, using an absorption coefficient of 2.5×10^4 M^{-1} cm^{-1} at 462 nm (Yonetani and Asakura, 1968). Before each experiment, tetrapyrrole stock solutions were thawed and diluted into working solutions of 10 mM KOH, which were used only for 1 d. In all experiments, the metalloporphyrin was added to the solution already containing the enzyme extract to facilitate binding to proteins rather than precipitation or binding to the reaction vessel. The volume of the KOH solution added to the incubations was kept <12 μL.

Analytical Spectrophotometry

All analytical spectrophotometric measurements were performed on a Cary 219 spectrophotometer (Varian Instruments, Sunnyvale, CA).

RESULTS

The ALA-forming activity of different enzyme preparations ranged between 3 and 8 units/mg of protein. The variation is attributed to differences in the quality of individual enzyme and tRNA preparations. Because of the variation, control incubations were performed for each experiment, and comparative activities are reported as percentages of the control, with the control activity stated for each experiment. Addition of 2.0 μM heme to the standard assay mixture decreased ALA formation to 64% of the control level (Fig. 1). Addition of GSH up to 4.0 mM had very little effect on ALA formation

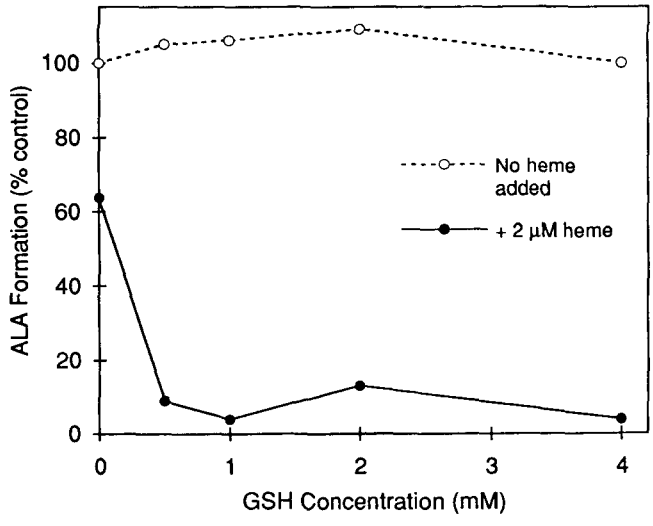

Figure 1. Effect of GSH on ALA synthesis in the presence and absence of 2 μM heme. The activity in the control incubation without added GSH or heme was 3.1 units/mg of protein.

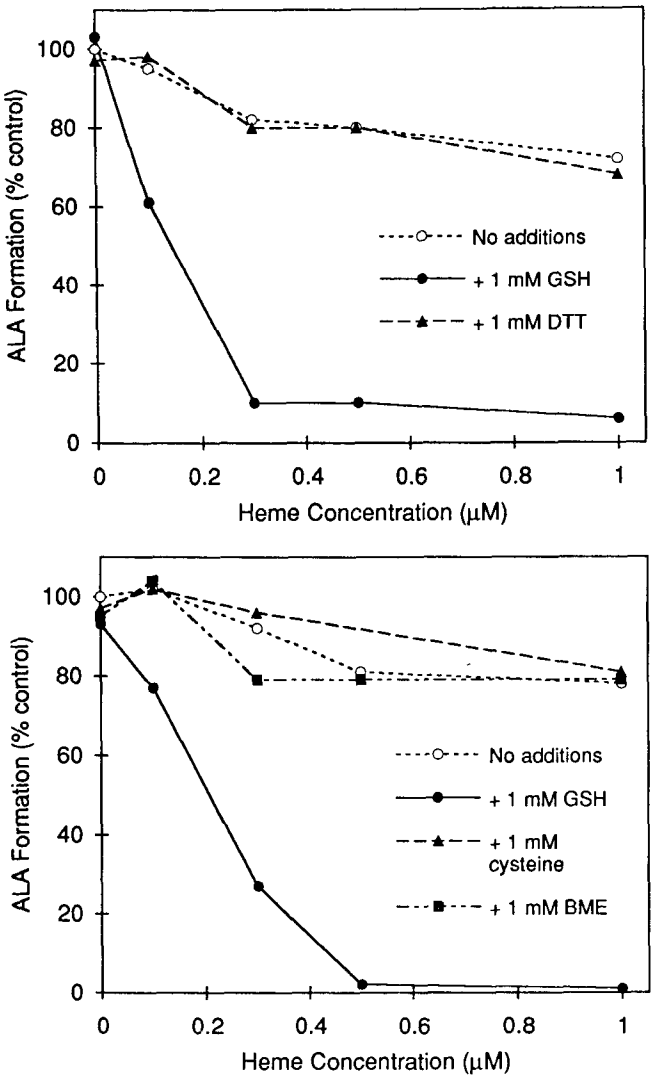

Figure 2. Effects of reduced sulfhydryl-containing compounds on heme inhibition of ALA synthesis. The activity in the control incubations for the DTT experiment (top) was 5.2 units/mg of protein, and for the other experiment (bottom) it was 5.0 units/mg of protein. BME, β-Mercaptoethanol.

in the absence of heme. However, when 2.0 μM heme was added to the assay mixture together with 0.5 mM GSH, the activity decreased to 10% of the control level. GSH concentrations <0.5 mM were not as effective (data not shown), and GSH was not significantly more effective at concentrations up to 4.0 mM (Fig. 1).

The effectiveness of GSH in enhancing heme inhibition was determined by measuring enzyme activity at various heme concentrations in the presence and absence of 1.0 mM GSH. With this enzyme preparation, in the presence of 1.0 mM GSH, 50% inhibition was achieved with <0.3 μM heme, and nearly complete inhibition occurred with 1 μM heme (Fig. 2). In the absence of added GSH, <20% inhibition was achieved with 0.3 μM heme and <40% inhibition was reached at 1.0 μM heme. An inhibition of 50% occurred at 2.3 μM heme (data not shown). The heme concentration required for the same degree of inhibition was approximately 8-fold lower in the presence of GSH than in its absence.

It was originally hypothesized that the unexpected effect of GSH on enhancing heme inhibition was caused by the extra reduced thiol added to the incubation mixture. Therefore, several reduced sulfhydryl-containing compounds were tested for their ability to replace GSH as an enhancing agent for heme inhibition. In each case, heme concentrations ranging from 0.1 to 1.0 μM were tested in the presence and absence of 1.0 mM GSH. These activities served as reference points for comparing the effects of the test compounds relative to GSH. Although 1.0 mM DTT was present in the original cell extracts, it was possible that the sulfhydryl groups became oxidized during storage. Therefore, fresh DTT was prepared and added just before the incubation. Thus, for the incubations containing additional DTT, the total concentration in the incubation mixture (reduced plus oxidized) was 2.0 mM. When the Ehrlich-Hg reagent was added to the ALA-pyrrole from these incubations, the samples formed a cloudy precipitate (possibly due to formation of Hg-DTT). Therefore, immediately before the *A* was read, all samples containing extra DTT were centrifuged for 2 min at 13,000*g* to remove the turbidity. In addition to GSH and DTT, the other reduced sulfhydryl-containing compounds tested were Cys and β-mercaptoethanol. None of the added compounds, other than GSH, were effective in enhancing heme inhibition (Fig. 2).

Heme is known to exist in neutral aqueous solutions primarily in a dimeric form (Brown et al., 1970). GSH might act to break heme dimers and thereby increase the effective concentration of free heme. However, the binding of GSH to the Fe atom of heme is similar to that of Cys (Bayer et al., 1974), which does not enhance heme inhibition of ALA synthesis. To examine further a possible role for GSH in increasing the effective heme concentration by breaking heme dimers, two agents that have been reported to break heme dimers, imidazole (O'Carra, 1975) and BSA (Brown et al., 1974), were tested for the ability to influence heme inhibition of ALA synthesis. Imidazole (1 mM) had no effect on heme inhibition, nor did it affect ALA-synthesizing activ-

Table I. *Effects of GSH, imidazole, and BSA on heme inhibition*

The activity in the control incubation without added heme, GSH, imidazole, or BSA, was 4.1 units/mg of protein. The final concentrations were as follows: heme, 1.0 μM; GSH, 1.0 mM; imidazole, 1.0 mM; BSA, 20 μM. Extract of *Chlorella* strain C-10 cells was used in this experiment.

Components Added	ALA Formation
	% control
None (control)	100
GSH	103
Imidazole	99
BSA	118
Heme	69
Heme + GSH	18
Heme + imidazole	66
Heme + BSA	84

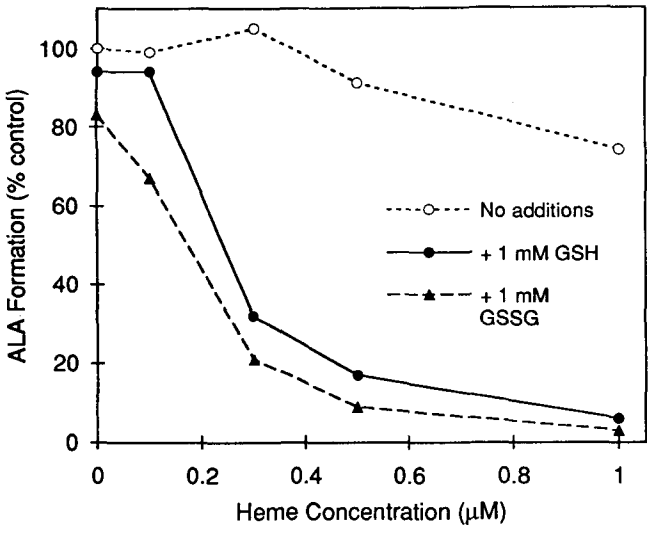

Figure 3. Effect of GSSG on heme inhibition of ALA synthesis. The activity in the control incubation was 7.8 units/mg of protein.

ity in the absence of heme (Table I). BSA (20 μM) decreased the inhibitory effect of 1 μM heme, presumably by binding to heme and lowering the free heme concentration (Table I). BSA also measurably increased ALA formation by the enzyme extract in the absence of added heme, presumably by lowering the level of endogenous free heme in the enzyme preparation.

The ineffectiveness of reduced sulfhydryl-containing compounds other than GSH in enhancing heme inhibition led to the testing of GSH derivatives. GSSG was as effective as GSH (Fig. 3). It is possible that GSSG was converted to GSH in situ by DTT or via an endogenous GSH reductase. The finding that GSSG was effective suggested the possibility that, in the presence of GSH reductase, which is likely to be present in the cell extract (Foyer and Halliwell, 1976), GSSG might indirectly inhibit ALA formation by causing NADPH to become depleted. Activity of the control incubation containing 1.0 mM GSSG but no heme was slightly lower than the control incubation without GSSG (Fig. 3). Additional incubations were done in which the NADPH concentration was increased from 1.0 to 2.0 mM. Under these conditions, GSSG alone did not inhibit ALA formation in the absence of added heme, but GSSG was still effective in enhancing heme inhibition (Table II).

Table II. *Effects of oxidized GSH and S-methyl-GSH on heme inhibition*

The final concentrations of the above components were as follows: heme, 1.0 μM; GSSG, 0.5 mM; S-methyl-GSH, 2.0 mM. In this experiment, NADPH was 2.0 mM instead of 1.0 mM, and the mixture was incubated for 5 min at room temperature before substrates were added to initiate the reaction.

Components Added	ALA Formation	
	units/mg of protein	*% control*
None (control)	7.1 ± 0.1	100
GSSG	7.4 ± 0.2	104
S-Methyl-GSH	6.7 ± 0.1	94
Heme	5.5 ± 0.1	77
Heme + GSSG	1.2 ± 0.1	17
Heme + S-methyl-GSH	3.8 ± 0.0	54
Heme + GSSH + S-methyl-GSH	1.4 ± 0.1	20

Table III. *Effects of protoporphyrin IX, Mg-protoporphyrin IX, and mesoheme*

In experiment 1, the concentration of porphyrins and metalloporphyrins was 2.0 μM. In experiment 2, heme and mesoheme were supplied at 0.5 μM. The GSH concentration in both experiments was 1.0 mM.

Components Added	ALA Formation	
	units/mg of protein	*% control*
Experiment 1		
None (control)	7.1 ± 0.2	100
Heme	5.8 ± 0.1	82
Heme + GSH	0.68 ± 0.05	10
Protoporphyrin IX	6.7 ± 0.1	94
Protoporphyrin IX + GSH	7.7 ± 0.4	108
Mg-protoporphyrin IX	6.2 ± 0.2	87
Mg-protoporphyrin IX + GSH	6.9 ± 0.3	97
Experiment 2		
None (control)	5.6 ± 0.1	100
Heme	5.4 ± 0.1	96
Heme + GSH	1.2 ± 0.1	21
Mesoheme	5.4 ± 0.1	96
Mesoheme + GSH	2.0 ± 0.1	36

S-methyl-GSH, which is not a reductant and not a substrate for GSH reductase, enhanced heme inhibition, although not to the same extent as GSH or GSSG (Table II). This enhancing effect was concentration dependent (data not shown). S-methyl-GSH did not interfere with the effect of GSSG on enhancing heme inhibition.

In previous reports, heme was the only tested tetrapyrrole that inhibited ALA synthesis in *Chlorella* extracts (Weinstein and Beale, 1985a). Because GSH has such a dramatic effect on heme inhibition, it was thought possible that other potentially inhibitory tetrapyrroles might also measurably inhibit ALA synthesis in the presence of GSH. Protoporphyrin IX and Mg-protoporphyrin IX were both slightly inhibitory in the absence of GSH (Table III, experiment 1). However, the effect of GSH was to decrease the inhibitory effects of these compounds, in contrast to the enhancing effect of GSH on heme inhibition. Mesoheme, a heme derivative in which the vinyl groups at positions 2 and 4 of the macrocycle are replaced by ethyl groups, acted like heme: there was slight inhibition in the absence of GSH, and the inhibition was much greater in the presence of GSH (Table III, experiment 2).

A possible role for GSH is to reduce the iron in heme to the ferrous oxidation state. Ascorbate has been used to chemically reduce hemes (O'Carra, 1975). When ascorbate, from a freshly prepared solution, was added to the incubation mixture at a final concentration of 2.0 mM, ALA formation was marginally lower at lower heme concentrations than with no added reductant, but the effect of ascorbate was not nearly as pronounced as with GSH (Table IV).

The inability of other possible heme reductants to replace

Table IV. *Effect of ascorbate on heme inhibition*

The activity in the control incubation without added heme, ascorbate, or GSH, was 5.40 units/mg of protein. The concentrations of ascorbate and GSH in the incubations were 2.0 and 1.0 mM, respectively.

Heme Concentration	ALA Formation		
	No additions	Ascorbate	GSH
μM	% control		
0.0	100	99	98
0.1	101	79	60
0.3	87	77	13
0.5	90	78	12
1.0	71	74	7

GSH in enhancing heme inhibition led to an examination of the heme oxidation state in the incubation medium. The absorption spectrum of heme was recorded in incubation buffer from which DTT had been omitted. The spectrum was then rerecorded after the serial addition of, first, 1.0 mM DTT, then 1.0 mM GSH, and finally, solid sodium dithionite (2.5–3 mg/mL; the approximate final concentration was 15 mM). The addition of DTT changed the spectrum from that of oxidized heme to reduced heme (Dawson et al., 1969), and the further additions of GSH and sodium dithionite did not further alter the spectrum significantly (Fig. 4). GSH, when added to buffer containing heme but lacking DTT, did not reduce the heme (data not shown).

Another approach to determining whether GSH acts by reducing heme is to use a physiologically nonreducible heme analog. Co-protoporphyrin IX is a heme analog that is inactive as a substrate for enzyme reactions that require reduced heme as a substrate (Maines et al., 1977; King and Brown, 1978; Yoshida and Kikuchi, 1978). Co-protoporphyrin IX inhibited ALA formation in a concentration-dependent manner, and the inhibition was greatly enhanced by 1.0 mM GSH (Fig. 5). The approximate Co-protoporphyrin IX concentrations required for 50% inhibition of ALA synthesis in the absence and presence of 1.0 mM GSH were 5.0 and 0.4 μM, respectively. Although Co-protoporphyrin IX is considered to be a physiologically nonreducible heme analog, absorption spectroscopy indicated that it was partially reduced in incubation buffer containing 1.0 mM DTT (Fig. 6). However, the degree of reduction was not changed when 1.0 mM GSH was added to the medium. Mn-protoporphyrin IX, another physiologically nonreducible heme analog, inhibited ALA formation by <35% at any concentration up to 12.5 μM, and the inhibition was not significantly affected by GSH (data not shown).

A possible role for GSH is to react with a protein in the incubation mixture to form a disulfide adduct or other stable association that confers increased sensitivity to heme. Alternatively, the GSH or GSSG might catalyze some other stable change in the structure of a protein involved in ALA synthesis, rendering it more sensitive to heme inhibition. After the enzyme extract has been exposed to GSH, it might retain its enhanced sensitivity to heme in the absence of free GSH during the assay. To test this possibility, the enzyme extract

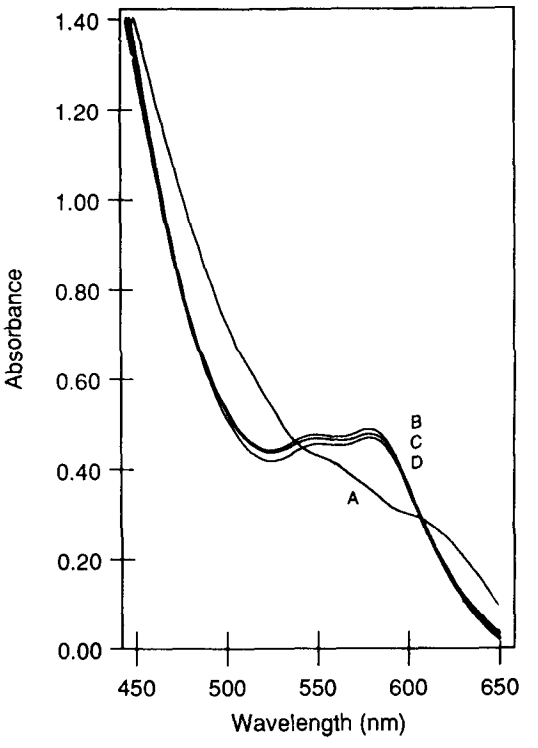

Figure 4. Absorption spectrum of heme (125 μM) in incubation buffer from which DTT was omitted. The buffer composition was 1 M glycerol, 50 mM Tricine, 15 mM $MgCl_2$, 20 μM pyridoxal phosphate, pH 7.9. Spectra were recorded before any additions (A) and after the serial addition of, first, 1.0 mM DTT (B), then, 1.0 mM GSH (C), and finally, 15 mM sodium dithionite (D).

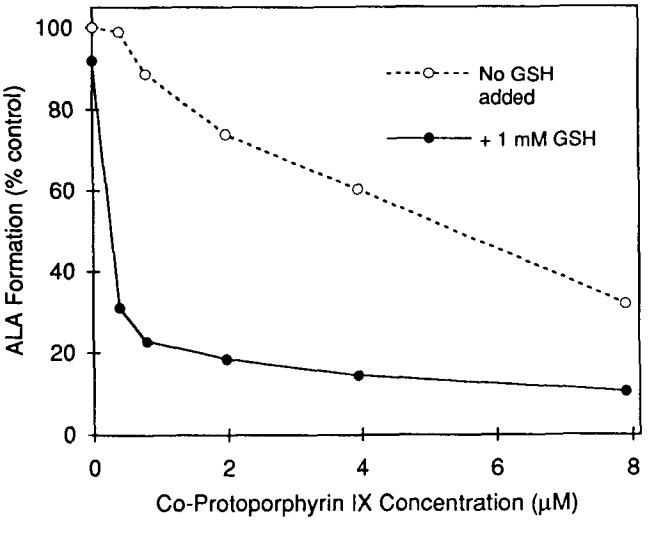

Figure 5. Effect of Co-protoporphyrin IX on ALA synthesis in the presence and absence of 1.0 mM GSH. The activity in the control incubation was 6.91 units/mg of protein. Extract of *Chlorella* strain C-10 cells was used in this experiment.

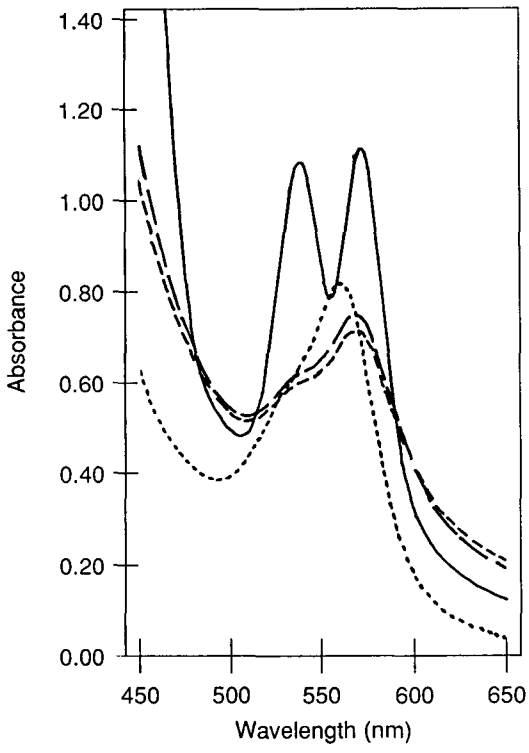

Figure 6. Absorption spectrum of Co-protoporphyrin IX (80 μM) in incubation buffer from which DTT was omitted. Spectra were recorded before any additions (——) and after the serial addition of, first, 1.0 mM DTT (— — —), then, 1.0 mM GSH (- - - - -), and finally, 15 mM sodium dithionite (. . . .).

was preincubated with GSH, and then the GSH was removed before the activity of the extract was assayed. Because previous work had shown that ALA-forming activity is greatly reduced during a preincubation without ATP (Weinstein and Beale, 1985a), both ATP (3.0 mM) and GSH (0.5 mM) were included with the enzyme extract in a 20-min preincubation at 30°C. At the end of the preincubation, the mixture was chilled and rapidly passed through a small gel filtration column to remove the ATP and GSH. Heme inhibition was then tested in the presence and absence of 1.0 mM GSH. As with nonpreincubated extracts, GSH in the incubation mixture greatly enhanced the heme inhibition of the GSH-pretreated enzyme extract (Fig. 7).

It is possible that the GSH effect is mediated by a heme-binding protein whose affinity for heme is increased by GSH. GSH *S*-transferase has been proposed as a heme carrier protein, and its affinity for heme is increased by GSH (Caccuri et al., 1990). The addition of commercial bovine liver GSH *S*-transferase (Sigma) had no effect on the heme inhibition of ALA synthesis or its enhancement by GSH in *Chlorella* extracts (data not shown).

DISCUSSION

The effect of GSH on heme inhibition of ALA synthesis in *Chlorella* extracts is dramatic: 1.0 mM GSH lowers by a factor of 10 the heme concentration required for 50% inhibition. This effect cannot be caused solely by the presence of a free sulfhydryl group on GSH, because other compounds with free sulfhydryl groups (Cys, DTT, and β-mercaptoethanol) are not effective. Also, the effect cannot be caused by a specific association of GSH with heme, because other compounds that bind heme (Cys, imidazole, BSA) do not enhance heme inhibition of ALA synthesis. The increased heme sensitivity caused by GSH is not attributable to oxidation-reduction properties of GSH, because the heme reductant, ascorbate, has no effect and GSSG is as effective as GSH. Although it is possible that GSSG is reduced to GSH during the incubation in the presence of NADPH, via an NADPH-linked GSH reductase, additional evidence arguing against a mechanism based on the reducing power of GSH is the fact that *S*-methyl-GSH is somewhat effective in enhancing heme inhibition. Absorption spectroscopy indicated that heme was fully reduced in standard incubation medium, which contains DTT, and, therefore, the inhibition-enhancing effect of GSH cannot be attributed to an influence on the oxidation state of heme.

GSH does not confer enzyme sensitivity to protoporphyrin IX or Mg-protoporphyrin IX, which were previously shown not to have strong inhibitory effects on ALA synthesis (Weinstein and Beale, 1985a). However, GSH does enhance the inhibitory action of two nonphysiological metalloporphyrins, mesoheme and Co-protoporphyrin IX. The Co-protoporphyrin IX inhibition and its enhancement by GSH support the conclusion that GSH does not exert its effect on heme inhibition by reducing the heme iron from the ferric to the ferrous state. Co-protoporphyrin IX apparently cannot be reduced under physiological conditions, and it is not converted to biliverdin by NADPH-linked microsomal heme oxygenase, an enzyme that requires reduced heme as a sub-

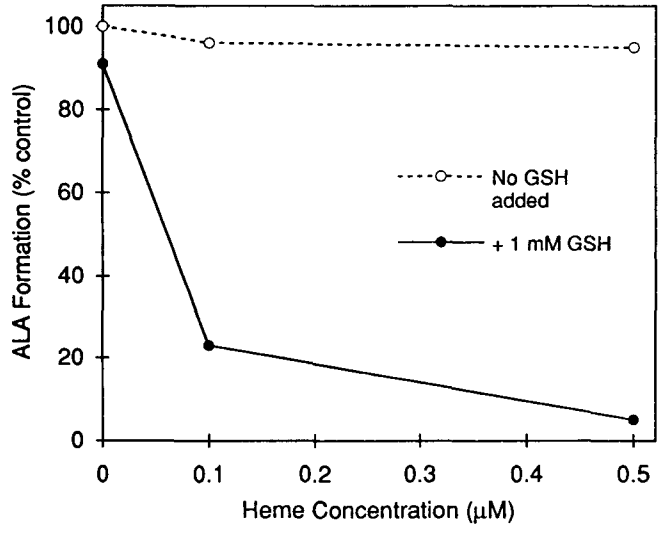

Figure 7. Effect of GSH preincubation on heme inhibition. The enzyme extract was preincubated with 0.5 mM GSH and 3.0 mM ATP as described in the text. After the preincubation, GSH and ATP were removed by gel filtration, and the extract was tested for heme inhibition in the presence and absence of 1.0 mM GSH. The activity of the control incubation was 5.7 units/mg of protein.

strate (Maines et al., 1977; King and Brown, 1978; Yoshida and Kikuchi, 1978). Rather, it is an inhibitor of this enzyme (Drummond and Kappas, 1982; Yoshinaga et al., 1982; Abraham et al., 1986). Moreover, Co-protoporphyrin IX is an inhibitor, but not a substrate, of Fd-linked algal heme oxygenase (G. Rhie and S.I. Beale, unpublished work).

On the other hand, Co-protoporphyrin IX can be converted to biliverdin nonenzymically after it has been chemically reduced with the strong reductant, dithionite (Vernon and Brown, 1984). The reduction potential of Co-protoporphyrin IX is considerably lower than that of heme ($E'_o = -0.265$ versus +0.015 V for the respective pyridine complexes [Lemberg and Legge, 1949]). Despite the low reduction potential, absorption spectroscopy indicated that Co-protoporphyrin IX is partially reduced in incubation medium containing DTT. However, the degree of reduction was not influenced by the presence of GSH. Therefore, the enhancement by GSH of the sensitivity of ALA synthesis to inhibition by Co-protoporphyrin IX, as for heme, cannot be attributed to reduction of the metalloporphyrin by GSH.

The reduction potential of Mn-protoporphyrin IX is even lower than that of Co-protoporphyrin IX ($E'_o = -0.387$ V for the pyridine complex [Lemberg and Legge, 1949]). However, because this metalloporphyrin had relatively little effect on ALA synthesis, either in the presence or absence of GSH, it was not useful as a probe for determining whether reduction of the chelated metal affects inhibition.

Although our results have ruled out a number of explanations for the effect of GSH on enhancing heme inhibition of ALA synthesis, other possible explanations remain. One interesting possibility is that GSH may facilitate the binding of heme to a specific binding or carrier protein. The heme-protein complex may then mediate the inhibition of ALA synthesis. One protein that has been suggested as a heme carrier is a member of the GSH *S*-transferase family of enzymes (Tipping et al., 1979; Vander Jagt et al., 1985; Takikawa et al., 1986; Caccuri et al., 1990). It has been demonstrated that GSH causes a severalfold increase in the heme-binding affinity of one form of this enzyme (Caccuri et al., 1990). However, the putative role of GSH *S*-transferase as a heme carrier has been questioned (Kirschner-Zilber et al., 1989). In any case, the addition of bovine liver GSH *S*-transferase to the incubation mixture had no effect on heme inhibition of ALA synthesis or its enhancement by GSH in *Chlorella* extracts.

Yet another possibility is a spontaneous or enzyme-catalyzed process whereby GSH or GSSG reacts with specific sulfhydryl groups on the ALA-forming enzymes or heme-binding protein to form a disulfide adduct. Alternatively, the GSH may form a stable noncovalent association with a protein or stably modify a protein in some other way. The adduct-bearing or modified protein would then be the form that enhances sensitivity to heme or binds heme more effectively. However, if the effect of GSH is on the ALA-forming enzymes or a heme-binding protein, then our results show that this effect is transitory, because pretreatment of the enzyme extract with GSH was not sufficient to confer enhanced heme sensitivity after free GSH was removed by gel filtration.

After a preliminary report of this work appeared (Weinstein et al., 1989), the effect of GSH on the inhibition of ALA synthesis in isolated developing cucumber chloroplasts was reported (Huang and Castelfranco, 1990). In that work, addition of exogenous GSH to isolated intact chloroplasts caused inhibition of ALA synthesis in a concentration-dependent manner. Subsequently, it was reported that broken thylakoid membranes inhibit ALA synthesis when added to intact chloroplasts (Castelfranco and Zeng, 1991). This inhibition was enhanced by the addition of GSH. The authors proposed that the inhibitory agent in the thylakoid membranes might be heme, a possibility that is consistent with our demonstration of the existence of a substantial pool of uncommitted heme in cucumber chloroplasts (Thomas and Weinstein, 1990, 1992).

It is of interest that GSH enhancement of heme inhibition is not universally observed. GSH had no effect on heme inhibition of purified glutamyl-tRNA reductase from *Synechocystis* sp. PCC 6803 (Rieble and Beale, 1991). Furthermore, GSH also had no effect on the heme sensitivity of ALA synthesis in crude extracts of *Synechocystis* sp. PCC 6803 or *Euglena gracilis* (S.M. Mayer, S. Rieble, and S.I. Beale, unpublished results). On the basis of the pattern of results reported and observed thus far, GSH enhancement of heme inhibition appears to be a property of ALA synthesis in plants and chlorophyte algae.

The GSH concentration in mature chloroplasts is in the millimolar range (Foyer and Halliwell, 1976). Therefore, ALA synthesis may be more sensitive to heme inhibition in vivo than would have been apparent from previous in vitro measurements of heme inhibition in cell extracts that were not supplemented with GSH. Although the chloroplast GSH concentration is known to vary, especially in response to stress (Alscher, 1989), GSH concentrations in developing plastids apparently have not been reported (R.G. Alscher, personal communication). If the plastid GSH concentration increases appreciably during development, then ALA synthesis would become increasingly sensitive to heme inhibition and would be maximally sensitive in mature chloroplasts. Increasing plastid GSH concentration could be an important contributing factor, together with changes in plastid heme concentration (Walker and Weinstein, 1991; Thomas and Weinstein, 1992), in reducing ALA synthesis from the high rate required for rapid pigment biosynthesis during development to a lower rate that is sufficient for maintenance activity as Chls and plastid hemes reach their final concentrations in maturing chloroplasts.

GSH enhancement of heme inhibition may also be physiologically significant for recovery from chilling stress in chilling-sensitive plants. In the leaves of cucumber, a chilling-sensitive species, it has been reported that during prolonged exposure to high light (1000 μmol m^{-2} s^{-1}) at 5°C, the levels of both Chl and GSH decrease substantially (Wise and Naylor, 1987). The low GSH level in the stressed leaves may facilitate Chl resynthesis during recovery from the chilling stress.

It is now well established that ALA synthesis in plants and algae occurs via a glutamyl-tRNA intermediate, and it has been shown that heme inhibits the NADPH-dependent reduction of this intermediate to glutamate-1-semialdehyde (or a cyclized derivative) (Huang and Wang, 1986b). However,

it has also been shown that, under some conditions, heme can also inhibit the preceding step, catalyzed by ATP-dependent glutamyl-tRNA synthetase (Chang et al., 1990). Although the physiological relevance of the heme inhibition of this step was questioned by the authors, the possibility that GSH might also increase the sensitivity of glutamyl-tRNA synthetase to heme inhibition would have far-reaching implications. In all cases that have been examined, there is only one chloroplast-localized glutamyl-tRNA synthetase (Chang et al., 1990), and the single pool of chloroplast glutamyl-tRNA is used for the synthesis of both proteins and ALA (Schneegurt et al., 1988). Therefore, it is possible that GSH may also increase the sensitivity of plastid protein synthesis to inhibition by heme and, thus, play a role in coordinating the rates of synthesis of pigments and proteins in developing plastids.

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