

Measurement of Heme Efflux and Heme Content in Isolated Developing Chloroplasts¹

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ABSTRACT

Hemes destined for cytosolic hemoproteins must originate in one of the cellular compartments which have the capacity for heme synthesis, namely the chloroplast or the mitochondria. Since developing chloroplasts from greening cucumber (*Cucumis sativus*, cv. Sumter) cotyledons are known to contain complete heme and chlorophyll biosynthetic pathways, they were tested for their capacity export hemes. Picomole quantities of heme were measured by reconstitution of the heme with apo-peroxidase and subsequent determination of peroxidase activity. The assay method was sensitive (as little as 0.7 picomole of heme could be detected in a volume of 100 microliters) and was linear with heme concentration. When intact plastids were incubated with apo-peroxidase, a steady-state rate of efflux between 0.12 and 0.45 picomole heme/minute/milligram plastid protein was measured. The efflux rate was not due to plastid breakage and could be enhanced by incubating with the heme precursor, δ -aminolevulinic acid. Cold acetone extraction removed 47 ± 17 picomoles heme/milligram plastid protein from the total b-type heme pool in the chloroplasts (166 ± 9 picomoles heme/milligram protein, by acid-acetone extraction). The reconstitution technique provided a similar estimate of readily exchangeable heme in the plastid, 37 ± 8 picomoles heme/milligram protein (or 6 micromolar in the plastids). These values may be indicative of a 'free heme pool' which exists in the chloroplast.

Hemoproteins are widely distributed in all eukaryotic cells. In addition to the electron transport Cyt of chloroplasts and mitochondria, the cytosol of plant cells contains a number of relatively abundant hemoproteins which include: catalase, Cyt P-450, and peroxidase. The mechanism by which the cytosolic apo-proteins receive their hemes from the site of heme synthesis is not well understood in either plant or animal cells.

In animal cells, the enzymes required for heme biosynthesis are distributed between the cytosol and the mitochondria (9). The final step, insertion of iron into protoporphyrin-IX (catalyzed by ferrochelatase), occurs on the matrix side of the inner mitochondrial membrane (11). Thus, the heme must traverse two lipid bilayers, and then be transported through the cytosol to its site of utilization. Although transport proteins have been implicated in intracellular transport of heme from the mitochondrial surface (6, 23), the mechanism of

heme movement across the membrane(s) is unknown. Using heme-loaded liposomes as a model system, it has been proposed that heme passively diffuses through the membrane to become associated with a heme binding protein at the surface (4). It has also been suggested that heme efflux varies inversely with the degree of energy coupling in the mitochondria and is dependent on the concentration and nature of the heme binding protein outside the mitochondria (14, 15).

In plants the situation is likely to be more complicated. Both the mitochondria and the chloroplast have the capacity to catalyze the final steps in heme biosynthesis (16, 18), and it is possible that either organelle provides the heme for cytosolic hemoproteins (see model in Fig. 1). It is not known if plant mitochondria are similar to animal mitochondria in having the early steps in heme biosynthesis distributed between cytoplasm and the organelle (24). However, it is clear that chloroplasts are capable of catalyzing all the steps of heme and Chl biosynthesis within the intact organelle (2, 18).

Since chloroplasts have a complete and functional heme biosynthetic pathway, and much is already known about chloroplast tetrapyrrole physiology, we have chosen to examine heme efflux from intact developing cucumber (*Cucumis sativus*, cv Sumter) chloroplasts. As a first step in this examination, an extremely sensitive assay has been developed for 'free'² or loosely bound heme. The assay is based on the ability of horseradish peroxidase apoenzyme to reconstitute with heme to form an active enzyme. This assay was used to demonstrate efflux of heme from intact plastids and to estimate the amount of free heme in the plastids.

MATERIALS AND METHODS

Chemicals and Biochemicals

Horseradish peroxidase apoenzyme, BSA, guaiacol, heme, ALA³, ATP, sorbitol, Percoll, NADP, and 6-phosphogluconic acid were purchased from Sigma Chemical Co. Tricine buffer and DTT were purchased from Research Organics, Inc. All other salts and solvents were purchased from either Sigma or Fisher.

² The terms 'free heme' and 'free heme pool' are used, operationally, to refer to those hemes which can combine with apo-peroxidase. Given the low solubility of heme in aqueous solution, these hemes are most likely dissolved in the membranes or adhering, nonspecifically, to proteins. The concept of a 'free heme pool' will be considered in more detail in the "Discussion."

³ Abbreviation: ALA, δ -aminolevulinic acid.

¹ This research was supported, in part, by a Department of Energy grant, DE-FG09–89ER13989 (J. D. W.), and by a Clemson University Department of Agronomy assistantship (J. T.).

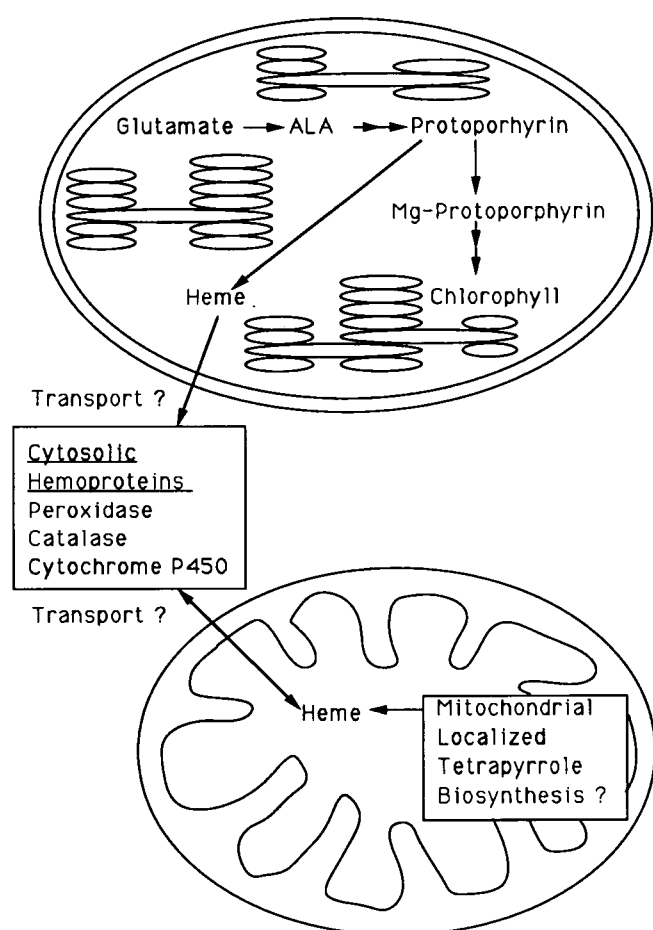


Figure 1. The model summarizes what is known of the relative abilities of plant chloroplasts and mitochondria to synthesize hemes and illustrates the requirement for heme efflux or transport to supply the needs of cytosolic hemoproteins.

Plant Material and Chloroplast Isolation

Greening cucumber (*Cucumis sativus*, cv Sumter) cotyledons were used as a source of developing chloroplasts. Seeds (purchased from Asgrow Seed Co.) were germinated on absorbant paper, placed in flats containing moist vermiculite, and grown in the dark at room temperature (22–25°C) for 6 or 7 d. Seedlings were greened at room temperature for 14 to 16 h under white fluorescent room lights, supplemented by a 75 W incandescent ‘Grow’ (General Electric) light (approximately $75 \mu \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ PAR) before use.

Chloroplasts were isolated from 40 to 50 g of cotyledons by gentle grinding in a mortar and pestle with 200 mL ice-cold grinding buffer (0.5 M sorbitol, 50 mM Tricine, 1.0 mM DTT, 1.0 mM EDTA, 1.0 mM MgCl_2 , 0.1% (w/v) BSA [pH 7.85]). A crude plastid pellet was obtained by differential centrifugation (21). The plastids were further separated from other cellular debris by a gentle centrifugation through a Percoll cushion by a procedure modified from Fuesler *et al.* (8). The crude plastids were gently resuspended in 5 mL grinding buffer, layered onto two tubes (15 × 100 mm), each containing an 11.5 mL cushion of grinding buffer supplemented with

45% (v/v) Percoll, and centrifuged at 500g for 15 min in a Sorvall HB-4 rotor. The plastid pellet was resuspended in 3 to 5 mL incubation buffer (grinding buffer lacking BSA and DTT), and recentrifuged (1000g for 1.5 min) to wash out the Percoll, BSA, and DTT, which interfere with the assay procedures. The final pellet of intact plastids was resuspended in incubation buffer and immediately used in heme efflux experiments.

Chloroplast Incubations

Chloroplasts were routinely incubated in flat bottomed glass vials (1.5 cm diameter, 4.4 cm high), containing 250 to 1200 μL incubation buffer and 0.5 to 2.0 mg of plastid protein. The incubations were supplemented with 360 nM horseradish peroxidase apoenzyme and 50 μM freshly prepared FeSO_4 , and then incubated for various periods of time at 28°C. During the incubation period the samples were subjected to intermittent gentle mixing. The inclusion of apo-peroxidase provided a heme sink as well as a method for quantifying heme. The FeSO_4 was included to provide iron for heme synthesis. The incubations were partially protected from the room lighting by incubation in a black heating block. At fixed points during the timed plastid incubation, small aliquots were removed and centrifuged for 7 s at 13,500g in a Brinkmann Eppendorf microcentrifuge. The clear supernatant was removed and incubated for 1 h at 30°C. This second incubation allowed full reconstitution of the heme with the apo-peroxidase and will be referred to as the ‘reconstitution incubation’ to distinguish it from the first chloroplast incubation. In some cases (for the total heme measurements in broken plastids), 10 mM levulinic acid, a competitive inhibitor of ALA dehydratase, was included in the reconstitution incubation to prevent *de novo* synthesis of heme. Samples were stored on ice (usually overnight) before assaying for peroxidase activity.

Measurement of Heme Recovery

Peroxidase activity, resulting from reconstitution of the apoenzyme with chloroplast-derived heme, was used to quantify the heme. For each experiment, a separate standard curve of reconstituted peroxidase activity *versus* heme concentration was prepared. Standard samples contained 360 nM apo-peroxidase, 5.0 to 200 nM heme, and sufficient incubation buffer to bring the final volume of each sample to 100 μL . Samples for the standard curve were also given a reconstitution incubation (30°C for 1 h).

Peroxidase activities were assayed using guaiacol and hydrogen peroxide as substrates (26). Assay volumes were 1.0 mL and contained final concentrations of 15 mM guaiacol, 1.76 mM hydrogen peroxide, and 0.025% (w/v) Triton X-100. Substrates were dissolved in water and were prepared fresh each day. The reactions were initiated by addition of 5 to 170 μL of sample or standard to a cuvette containing the substrates and, at least, 620 μL , of 50 mM potassium phosphate buffer (pH 7.0) preequilibrated to 30°C. Peroxidase activity was measured by the increase in absorbance at 470 nm with time. In the guaiacol assay, 4 mol of oxidized substrate are required to form 1 mol of chromophore (26); therefore, the increase in absorbance with time is not linear

until a sufficient concentration of oxidized intermediates accumulate. Thus, the peroxidase activity was not measured until an absolute absorbance of 0.1 unit was reached. The volume of reconstituted peroxidase (plastid supernatant or heme standard) added to each assay was empirically adjusted to yield rates between 0.05 and 0.8 A_{470} units per min. If the rates were slower, there was a risk of peroxide-dependent inactivation of the reconstituted peroxidase, probably through Compound III formation (26). Background peroxidase activity from samples which were incubated without apo-peroxidase was subtracted from the activities of the experimental samples.

Other Procedures

Apo-peroxidase stock solutions were prepared at a concentration of $9.0 \mu\text{M}$ in 50 mM potassium phosphate buffer (pH 7) and stored at -80°C prior to use. Concentrations were determined using a millimolar extinction coefficient of 20 at 278 nm (25). Heme stock solutions were prepared in DMSO, and working solutions were freshly prepared in 10 mM KOH. Concentrations were determined using a mM extinction coefficient of 144 at 398 nm in a solvent consisting of 66.5% ethanol, 17% acetic acid, and 16.5% water (v/v) (29).

Noncovalently bound hemes were estimated by acid-acetone extraction (22, 29). Free hemes and other pigments were removed from 1 mL of concentrated plastids by washing four times with 5 mL portions of cold 80% (v/v) acetone containing 10 mM NH_4OH . The noncovalently bound hemes were extracted with four washes of 5 mL of 80% (v/v) acetone containing 5% (v/v) concentrated HCl. The extracted hemes were transferred to chloroform:butanol (2:1, v/v), concentrated and washed on a DEAE-Sepharose column, and finally purified and quantitated by reverse phase HPLC as described (22, 29). A Hitachi model L-6200 pump and gradient controller equipped with a Hibar LiChrosorb ODS column (4×250 mm) with $5 \mu\text{m}$ particles, and a Perkin-Elmer model LC-85B spectrophotometric detector was used for the chromatography. The hemes were eluted with an isocratic solvent system consisting of 66.5% ethanol, 17% acetic acid, and 16.5% water (by volume) at a flow rate of 1 mL/min. Absorbance was monitored at 398 nm and hemes were quantified by comparison to authentic standards.

Chloroplast intactness was estimated by the latency of 6-phosphogluconate dehydrogenase activity (19). For each experiment the intactness at the beginning and end of the chloroplast incubation was determined from a sample incubated under the same conditions as the experimental samples. In addition, in experiments where chloroplasts were intentionally lysed, loss of intactness was confirmed by this assay. If necessary, samples could be stored on ice for an hour before assaying intactness, since the intactness of samples stored on ice did not appreciably change for periods up to 100 min.

Packed chloroplast volumes were estimated by centrifuging a concentrated suspension of intact chloroplasts in Hopkins tubes for 2 min at top speed in a table-top clinical centrifuge. The average value for two separate preparations was $6.5 \pm 1.1 \mu\text{L}/\text{mg}$ plastid protein.

Plastid protein was determined by the Bradford dye binding

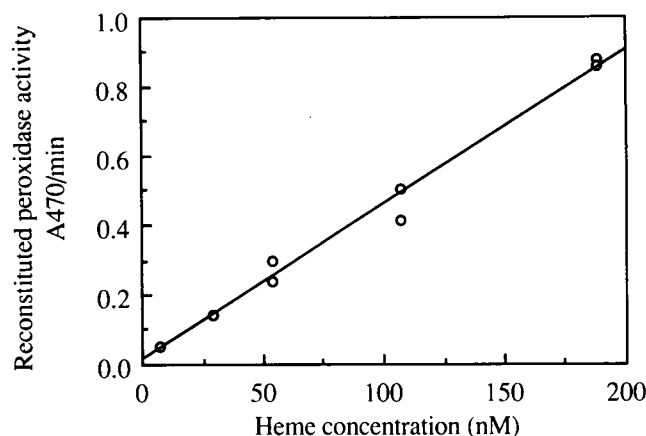


Figure 2. Reconstituted peroxidase activity is linear with heme concentration. The reconstitution was carried out in a total volume of 100 μL of incubation buffer in the presence of 360 nM apo-peroxidase. After a 1-h reconstitution incubation at 30°C , peroxidase activity was measured as described in the text. Duplicates were prepared for each heme concentration.

method (3), using BSA as a standard. All absorbance measurements were made on a Cary 219 spectrophotometer.

RESULTS

Reconstitution of Peroxidase Activity is a Sensitive Assay of Heme Concentration

Horseradish peroxidase apo-enzyme spontaneously reconstitutes with heme to give the active holo-enzyme (25). This property was used to develop a sensitive assay for free heme (*i.e.* heme which is not tightly bound to proteins, either covalently or noncovalently). A standard curve of reconstituted peroxidase activity *versus* heme concentration is shown in Figure 2. Heme concentration on the abscissa was the amount of heme present during the reconstitution incubation, and activities were normalized to a 20 μL sample addition for the 1 mL assay. Activity was linear with the range of heme concentrations tested. Amounts of heme as low as 0.7 pmol could be accurately measured. For the lowest standard concentration, 80 μL of the standard incubation volume (100 μL) was added to the peroxidase assay. In this particular sample the measured slope (average of duplicates) was $0.208 \pm 0.003 A_{470}/\text{min}$, which, when normalized to a 20 μL addition, yielded a value of $0.052 A_{470}/\text{min}$ that was plotted on the standard curve. For standards or biological samples, the deviation from the average of duplicate samples was rarely greater than 5%. In all the following figures except Figure 3, both duplicate values are plotted.

Heme Efflux from Intact Developing Chloroplasts

When intact chloroplasts were incubated in the presence of apo-peroxidase, the reconstituted peroxidase activity in the plastid-free supernatants increased with time (Fig. 3, dashed line). In this experiment, the standard chloroplast incubation procedure was modified by preincubating the plastids at 28°C for 7 min in the presence or absence or exogenously added

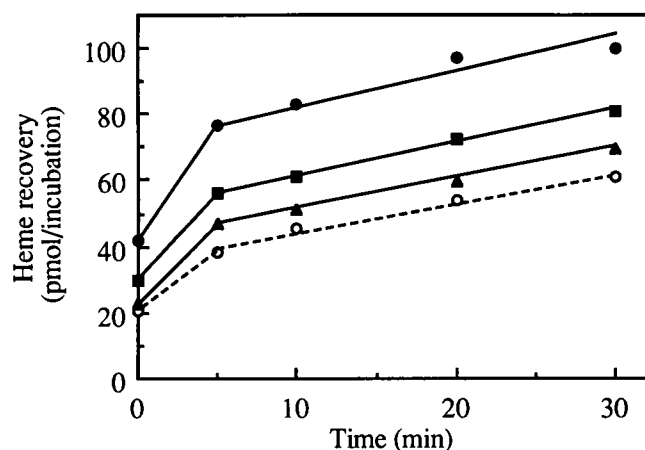


Figure 3. Heme efflux from intact chloroplasts. Intact chloroplasts (2.6 mg protein) were preincubated without apo-peroxidase in a volume of 1.2 mL in the presence or absence of exogenous heme (exogenous heme: ○, none; ▲, 12 pmol; ■, 30 pmol; ●, 60 pmol). After the 7 min preincubation, the samples were chilled on ice, apo-peroxidase added, and the plastids were incubated as described in "Materials and Methods." Aliquots were sampled at various times, the chloroplasts removed by centrifugation, and heme recovery measured after reconstitution by the peroxidase assay as in Figure 2.

heme. This preincubation was used to allow the exogenous heme to become incorporated into the chloroplast membranes prior to the addition of apo-peroxidase. After the preincubation period, the plastids were chilled, apo-peroxidase added, and the plastids incubated under standard chloroplast incubation conditions. At zero time and several time points during the chloroplast incubation, an aliquot of plastids was removed, centrifuged, and the heme determined by the reconstitution of peroxidase activity as described in "Materials and Methods." In the sample without exogenous heme (dashed line), there was a small but significant amount of activity in the zero time sample (see below and "Discussion"). When the samples were transferred to 28°C, there was a continuous increase in the amount of heme available for reconstitution. In this and other experiments, the increase during the first 5 min was faster than the overall slow increase observed for the latter part of the time-course. The slow rate was assumed to be the steady-state rate of heme efflux (*i.e.* heme available for reconstitution at the surface of the chloroplast). In this and all the following experiments, the rate of heme efflux has been calculated from the period between the first 5 and 30 min of the chloroplast incubation. The other data in Figure 3 show time courses for heme recovery from samples in which the chloroplasts were supplemented with exogenous heme during the preincubation period. These lines have the same shape as the sample without exogenous heme. In fact, the slopes for the slow increase in heme recovery were similar whether or not exogenous heme was added, thus reinforcing the concept of a steady-state rate of heme efflux from chloroplasts. However, the 0 and 5 min time points are considerably higher than the unsupplemented sample, suggesting that exogenous heme was recovered in the first 5 min of the incubation. The total recovery of exogenous heme was 83, 60, and 65% for samples spiked with 12, 30, and 60 pmol of heme, respectively.

Plastid Breakage Cannot Account for Heme Efflux

The microcentrifugation between the plastid incubation and the reconstitution incubation did not cause the contents of the chloroplast to leak out. The supernatants were clear and contained less than 1.0% of the total recoverable 6-phosphogluconate dehydrogenase activity present in 100% intact plastids. However, the possibility that breakage during the plastid incubation could account for heme efflux was tested by measuring both heme efflux and the loss of plastid intactness concurrently with heme recovery from broken plastids. In this experiment (Fig. 4), heme efflux from intact plastids was measured as in "Materials and Methods." In addition, an aliquot of chloroplasts from the same preparation was lysed by dilution with four volumes of incubation buffer, lacking the sorbitol osmoticum, and two cycles of rapid freeze-thaw. The broken plastids were then given a standard chloroplast incubation side-by-side with the intact plastids (diluted with isoosmotic buffer to the same plastid concentration). When the broken plastids were sampled during the chloroplast incubation, they were centrifuged for 2 min at 4°C instead of 7 s. The longer centrifugation was required to pellet the majority of the membrane fragments, which still contained heme that would be accessible to the apo-peroxidase during the reconstitution incubation. The reconstituted peroxidase activity in the supernatants of these samples was taken as a measure of the plastid heme reconstituted in 100% broken plastids at each time point. If each value was multiplied by the cumulative loss of intactness at each time point, a theoretical set of points could be generated which would estimate the heme efflux due to plastid breakage during the incubation. The results of this experiment are shown in Figure 4, panel A. The top line shows the time course for heme recovery from 100% broken plastids. The middle line is heme efflux from intact plastids, and the dashed bottom line is the theoretical contribution of plastid breakage to heme efflux. In this experiment plastid intactness steadily decreased from 100 to 93% during the 30 min incubation. The theoretical efflux rate due to breakage (slope = 0.059 pmol heme/mg protein/min) was only 22% of the efflux rate from intact plastids (0.27 pmol heme/mg plastid protein/min).

In the experiments described above, the values measured at zero time were often higher than expected for a zero-time control, and the rates of efflux during the first 5 min were sometimes higher than the rates during the last 25 min. This behavior might be due to continued reconstitution of apo-peroxidase with heme derived from nonpelletable material still present in the supernatants (despite the longer centrifugation) which were collected during the timed plastid incubation. This effect would artificially increase the time that apo-peroxidase and heme containing plastid fragments are in contact with each other (timed plastid incubation plus reconstitution time) and, thus, would increase the amounts of heme recovered. The magnitude of this effect was estimated by incubating the plastids (intact or broken) without apo-peroxidase for the indicated period of time, centrifuging, and then adding the apo-peroxidase to the supernatants for the standard reconstitution incubation. The recovery of heme from nonpelletable material is shown in panel B of Figure 4. The heme recovery in the nonpelletable material from the 5-min sample

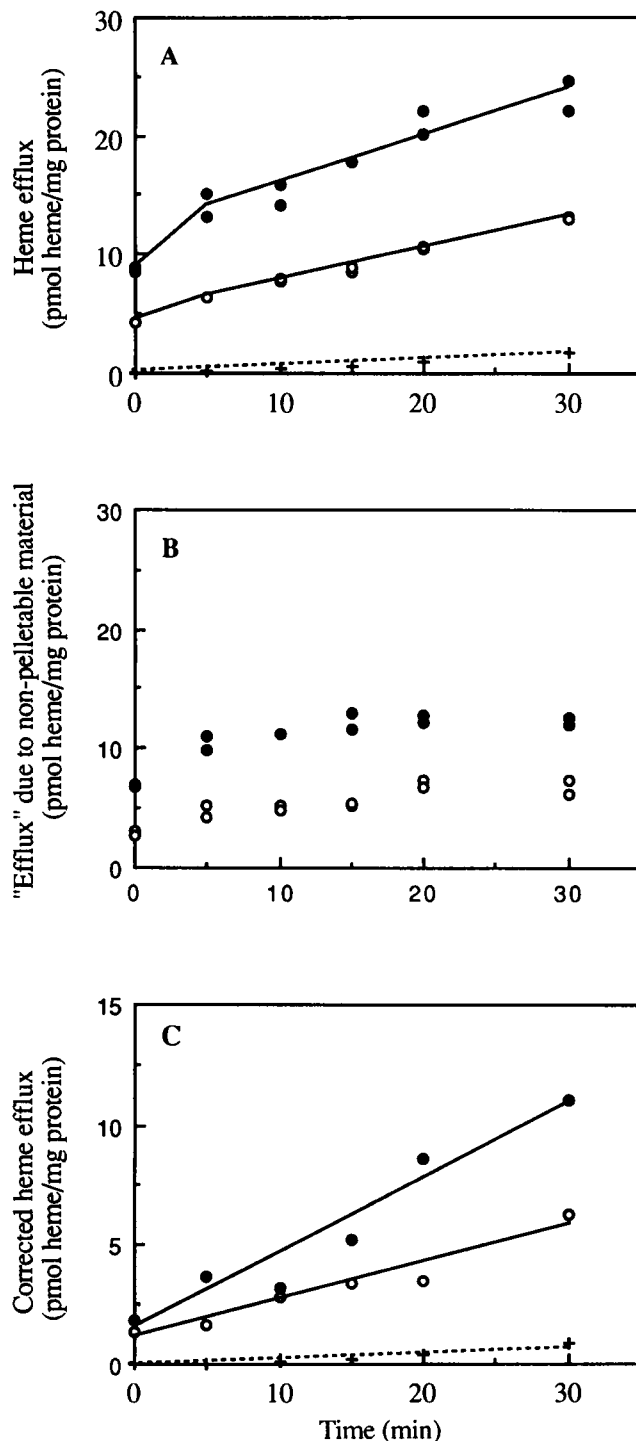


Figure 4. Heme efflux is not due to plastid leakage. Heme recovery from intact (○) or freeze-thawed (●) plastids was measured as described in "Materials and Methods." Panel A, the uncorrected efflux data from intact plastids (○) and recovery data from broken plastids (●) are shown by the solid lines. The dashed line is an estimate (described in the text) of the possible contribution of plastid breakage to efflux. Panel B, heme and/or heme-containing fragments remaining in the supernatants after centrifugation of intact or broken plastids. The plastids were incubated in the absence of apo-peroxidase. After centrifugation, the heme remaining in the supernatant was determined by adding apo-peroxidase to the supernatant for a reconstitution

accounts for 74% of the heme recovered in the same time from intact plastids (panel A). The contribution of the non-pelletable material to the total decreases as the time course progresses. At 30 min this contribution is 52%. The effect of heme recovery from non-pelletable material (during the reconstitution incubation) can be removed by subtracting the values in panel B from the corresponding values in panel A. The corrected efflux rate (0.16 pmol heme/mg plastid protein/min) is linear with time as shown in panel C. The contribution of plastid breakage to this rate is 0.028 pmol heme/mg protein/min, or only 18%.

Effect of Plastid Concentration on Efflux

Apparent heme efflux was a function of chloroplast concentration in the incubation mixture. At concentrations above a threshold of 0.06 mg protein per incubation, heme efflux from intact plastids, in 30 min, was linear with plastid protein concentration (Fig. 5).

Effect of Heme Precursors

Addition of an exogenous heme precursor, ALA, stimulated heme efflux (Fig. 6). When 50 μ M ALA was added, the efflux rate increased by 31%. This effect was not significantly enhanced by higher concentrations of ALA (data not shown).

Measurement of the Free Heme Pool

An estimate of the magnitude of the free heme pool was obtained by direct extraction of plastids and quantitation of the hemes by HPLC. Noncovalently bound heme prosthetic groups can be removed from their apo-proteins by acid-acetone extraction. However, if the tissue is preextracted with cold acetone, loosely bound hemes can be removed without denaturing Cyt and other b-type hemoproteins. These properties were exploited to estimate the concentration of free hemes in the plastids. Two identical sets of freshly prepared plastids were subjected to acid-acetone extraction and the hemes purified and quantified by HPLC as described in "Materials and Methods." One set was acid-acetone extracted directly, and the other was preextracted with cold and slightly basic acetone before the acid-acetone extraction. The difference between these two values should be equal to the amount of free heme. As seen in Table I, experiment 1, the free heme concentration obtained in this manner is 47 ± 17 pmol heme/mg plastid protein. A value of 34 ± 4 was obtained for a separate preparation of plastids (experiment 2).

A similar estimate of the magnitude of the free heme pool could be obtained by a variation of the experiment described above for heme recovery from broken plastids. Two aliquots of plastids were removed from the standard chloroplast incubation at each time point. In the first, heme efflux was

incubation. Panel C, heme efflux (○) and recovery (●) was corrected for continuous extraction of heme from membranes or proteins during the reconstitution incubation by subtracting the hemes in the non-pelletable fractions (panel B) from the raw data of panel A. The contribution of plastid breakage to efflux (dashed line) was calculated as described in the text. The average values are plotted.

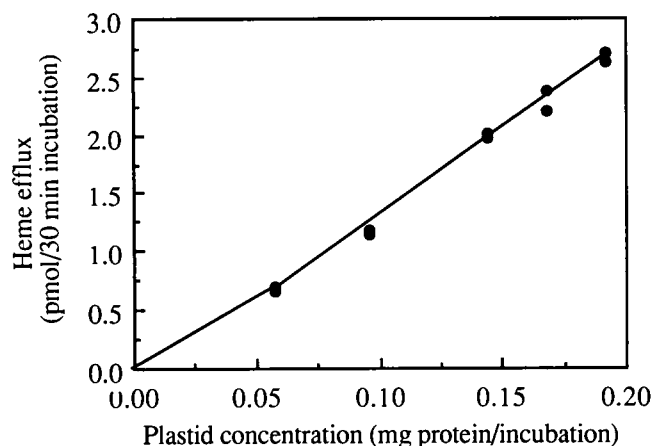


Figure 5. Heme efflux is dependent upon plastid concentration. Increasing amounts of intact chloroplasts were incubated in 1.2 mL suspension buffer containing 360 nM apo-peroxidase. The incubations were sampled at zero time and 30 min. Heme efflux was measured as described in "Materials and Methods." Efflux over 30 min was obtained by subtracting the zero time values from the 30 min values.

measured as usual. The second was subjected to two cycles of freeze-thaw in liquid N_2 and the broken plastids were given a prolonged reconstitution incubation of 1.5 h. Plastid membrane fragments were then removed by a 2 min centrifugation at 13,000g. The aim of the prolonged reconstitution was to allow complete reconstitution of all free hemes, including those embedded in the internal membranes. Levulinic acid, a competitive inhibitor of ALA dehydratase, was included in the reconstitution incubations to prevent synthesis of new hemes during this period. The heme efflux from intact plastids was 0.121 pmol heme/mg plastid protein/min (slope of the solid line, Fig. 7). However, the heme concentration measured in broken plastids was high at zero time, 44.4 pmol heme/mg protein (intercept of the linear regression line), and decreased at a rate of 0.085 pmol heme/mg protein/min (slope of the dotted line, Fig. 7). At least five separate plastid preparations have been assayed for total free heme by this method. Of 12 measurements on these five preparations, the average value (\pm SD) was 36.9 ± 8 pmol heme/mg plastid protein.

The results of an experiment, in which the free heme in a chloroplast suspension was measured as a function of chloroplast protein concentration, are shown in Table II. The plastid preparation used in this experiment is the same one used for the direct extraction measurement (experiment 1, Table I). The measured free heme concentration (pmol/mg plastid protein) did not increase with increasing plastid protein concentration in the incubation.

DISCUSSION

Enzymes for the final steps of heme biosynthesis are localized within mitochondria and chloroplasts of higher plants (16–18). Thus, one or both of these compartments must be capable of heme export to provide hemes for cytosolic hemoproteins. In the chloroplast, heme may be involved in regulating the activity of heme and Chl precursor biosynthesis

(1). In addition, heme has recently been shown to regulate the transcription of genes for phycobilin apo-proteins in *Cyanidium caldarium* (28). In view of their important and long-recognized role as prosthetic groups for photosynthetic Cyt and their newly discovered role in regulation, we have begun a study of heme metabolism in intact developing chloroplasts.

Peroxidase activity, derived from reconstitution of apo-peroxidase with unbound heme, has been used as a sensitive and reproducible measure of minute amounts of free heme. Using a slightly modified standard peroxidase assay, as little as 0.7 pmol of heme could be measured. The assay has not been pushed to its limits, and more sensitivity could probably be gained with different peroxidase substrates. In addition to sensitivity, the assay has an advantage over chemical detection systems which rely on the peroxidative properties of free heme (7) in that the apo-enzyme serves as a sink to stabilize the heme and prevent (or delay) its biological breakdown or adsorption to glass or plastic surfaces.

Incubation of intact, developing plastids with apo-peroxidase has been used to detect heme and measure its efflux. In each experiment there was a significant amount of heme measured at zero incubation time. This amount was variable from preparation to preparation and most likely resulted from heme embedded in membrane fragments which did not pellet during the 7 s centrifugation. After the zero time point, efflux appeared to be biphasic with a rapid phase lasting for 5 to 10 min and a slower steady-state phase lasting for at least 25 min. When apo-peroxidase was present in excess, the overall rate, fast plus slow, was independent of apo-peroxidase to plastid ratio, and dependent solely on the amount of plastids (Fig. 5). The rapid phase most likely arises from reconstitution of heme localized in the outer plastid envelope, or from heme which is not compartmentalized within the plastids (and, which does not pellet during the centrifugation after the chloroplast incubation). The slower, steady-state phase probably results from reconstitution of hemes originally localized in more interior portions of the chloroplast. This interpreta-

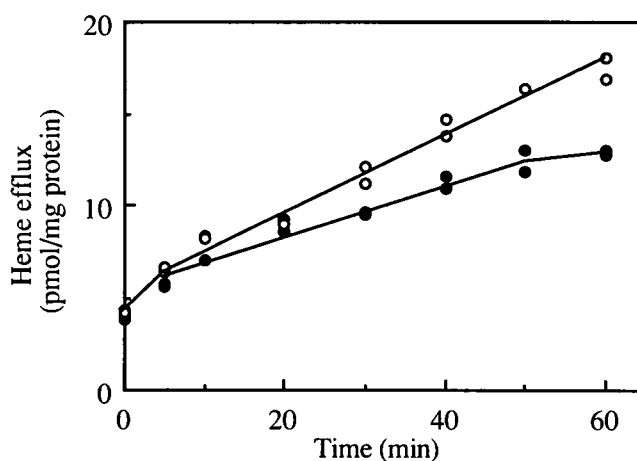


Figure 6. Heme efflux is stimulated by supplying a heme precursor. Intact plastids were supplied with 50 μ M ALA (O) at the beginning of the incubation. No exogenous ALA added (●). Heme efflux was measured as in "Materials and Methods."

Table I. Free Heme Concentration as Measured by Extraction and HPLC

Freshly isolated chloroplasts were either preextracted with cold acetone, followed by acid-acetone, or extracted directly with acid-acetone. Heme in the acid-acetone phase was partially purified by solvent partition and chromatography on DEAE-Sephrose. The recovered heme was purified by reverse-phase HPLC and quantified by comparison of absorption (398 nm) peak heights with standards.

Sample	Heme Recovered	
	Experiment 1	Experiment 2
	(pmol/mg plastid protein)	
A. Direct acid-acetone extraction of plastids	166 ± 9	149 ± 2
B. Acetone wash followed by acid-acetone extraction	119 ± 14	115 ± 4
Free heme (A minus B)	47 ± 17	34 ± 4

tion is supported by two different results. First, exogenously added heme, associated with the chloroplast, is recovered during the first 5 min of incubation, after which the rate of efflux was independent of the amount of exogenous heme supplied (Fig. 3). We expected that, as the exogenous heme was preincubated with the intact plastids for only 7 min at 28°C, it would have been preferentially associated with the outer membrane. Since the steady-state rate of efflux was not affected by exogenous heme, the heme exported during the steady-state phase probably originated from an interior compartment of the plastid, not readily accessible to the outside. Second, when the heme precursor, ALA, was administered to the plastids, the increase in the efflux rate was not manifested until after the rapid phase (Fig. 6). This evidence reinforces the interpretation that heme recovered during the steady-state phase results from efflux or transport rather than simple

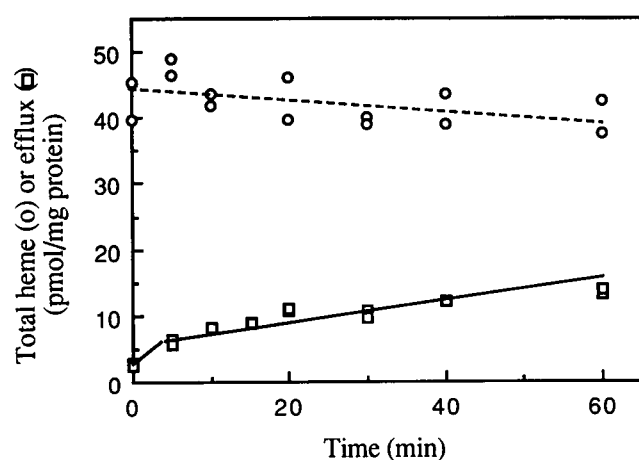


Figure 7. Heme breakdown may compete with heme efflux. Heme efflux from intact plastids (□) was measured as in "Materials and Methods." Total heme (○) measurements were obtained by incubating intact chloroplasts with apo-peroxidase for the indicated times. At the end of the incubation the plastids were broken (freeze-thaw in hypo-osmotic buffer), and the free hemes in the total plastid fragments and soluble components were allowed to reconstitute with the apo-peroxidase for an additional 1.5 h. At the end of the reconstitution incubation, the plastid fragments were removed by centrifugation, and reconstituted heme was measured by reconstituted peroxidase activity.

recovery of heme associated with the outer membrane and/or fragments.

Questions of whether or not this process is energy-requiring or carrier-mediated have not yet been addressed. Mitochondria isolated from rat liver demonstrated an energy requirement for heme retention rather than efflux (15). However, studies with model systems indicate no energy or carrier requirements (see below).

When heme binding proteins were reconstituted with heme from heme loaded liposomes, the transfer rate was biphasic and independent of the concentration of the heme binding proteins (4). The authors suggested that the two rates reflect two populations of heme, each embedded in the lipid bilayer, but having a different orientation with respect to the outside. Thus, the slower rate results from the hemes having their propionic acid tails oriented toward the interior of the liposome and extra time is required to reorient so that the polar portion of the heme becomes accessible to the binding proteins. Of course, the situation in chloroplasts is more complex, as there is a more intricate membrane system and there may be ongoing processes of synthesis and breakdown.

In these experiments each plastid preparation was monitored for intactness by measuring the latency of 6-phosphogluconate dehydrogenase activity (19). Although the intactness loss was small during a 30 min incubation (10%), the contribution of plastid breakage to heme efflux was estimated to be less than 23% of the steady-state rate from intact plastids (Fig. 4). The estimate was made by multiplying the cumulative

Table II. Free Heme Concentration as Measured by Reconstitution with Apo-peroxidase

The total free heme concentration of freshly isolated plastids was measured by breaking the plastids (freeze-thaw in hypo-osmotic buffer) in the presence of apo-peroxidase and incubating for 1.5 h. Plastid fragments were removed by centrifugation, and heme content was estimated by the reconstitution assay.

Plastid Concentration during Reconstitution	Heme Concentration
mg protein/mL	pmol heme/mg plastid protein
0.49	32.7 ± 0.9
0.97	45.0 ± 10.8
1.75	36.6 ± 0.5

intactness loss by the heme recovery from 100% broken plastids for each time point. This estimate is an upper limit, because the heme recovery from 100% broken plastids was magnified by the inability to completely sediment membrane fragments in a 2-min centrifugation. Any membrane fragments present in the reconstitution incubation are an additional source of heme, which would then (erroneously) be included with the total heme recovered from the plastid incubation. Although the supernatants from the centrifugation of intact plastids were clear, they contained significant amounts of heme, possibly embedded in small membrane fragments or adhering to soluble proteins. This extra heme was quantified by adding the apo-peroxidase to the postcentrifugation supernatants (Fig. 4B). The original efflux and heme recovery values were corrected by subtracting the heme which remained in the supernatants (Fig. 4C). Although the new efflux rate was lower, the contribution of breakage to the corrected rate was similar, only 18%. Thus, only a minor fraction of the measured heme efflux rate could be attributed to plastid breakage.

Isolated chloroplasts have the capacity to synthesize hemes and Chl from common precursors. During greening, the supply of ALA is the rate limiting step of Chl formation (1) and, possibly, heme formation as well. When ALA is administered to greening plastids, protoporphyrin and various Mg-porphyrins will accumulate. The amount and identity of the accumulated Mg-porphyrins will depend upon the cofactors and/or light regime provided during the incubation (1). As yet, no net accumulation of heme has been noted in previous studies where plastids were incubated with porphyrin precursors such as ALA, possibly due to the inability to measure small amounts of free heme. Thus, the iron branch of the pathway is often ignored in many discussions of the regulation of Chl biosynthesis. Using the sensitive reconstitution assay, a net increase in the rate of heme efflux was observed when the plastids were administered exogenous ALA at 50 μM (Fig. 6). By inference, the increase in the rate of heme efflux was most readily explainable as a result of the net synthesis of new heme. The observation that the increase in efflux was not enhanced by higher concentrations of ALA could be due to several reasons. Above a certain threshold, efflux may not be limited by the supply of heme. Heme synthesis may be saturated at low levels of ALA, or the experimental conditions may not have favored heme synthesis. Finally, when isolated plastids are incubated with ALA, substantial quantities of protoporphyrin accumulate (5). This resultant increase in protoporphyrin concentration may have interfered with either efflux or the reconstitution assay.

If intact chloroplasts can support heme efflux, it becomes important to know how much heme is available for this process. Presumably, these hemes are part of a free heme pool, in which the heme is available for a myriad of metabolic fates, including: efflux, regulation, breakdown, and formation of new hemoproteins. Since heme is an amphipathic molecule, it is not likely to exist free in aqueous solution within the chloroplast. In rat hepatocytes, the calculated distribution of hemes between phospholipid bilayers and heme carrier proteins suggests that 98% of the heme not associated with stable hemoproteins would be incorporated into membranes (27). Yet, proteins with high affinity heme binding sites can

readily remove hemes from phospholipid bilayers (4). In addition, it has been shown that hemes in stable hemoproteins may be transferred to other apo-hemoproteins, depending upon their relative heme dissociation constants and concentrations (10, 20). Thus, the free heme pool will consist of little to no heme free in aqueous solution, heme dissolved in membranes, and heme associated with a variety of hemoproteins, each with its own dissociation constant for heme. We have estimated the magnitude of the free heme pool using two different techniques; direct extraction with organic solvents and reconstitution of broken plastids with apo-peroxidase.

When broken plastids were incubated with the apo-peroxidase, hemes, associated with the membranes, were slowly transferred to the apo-peroxidase (solid circles, Fig. 4A). This characteristic was used to estimate the total amount and concentration of free heme in the chloroplasts. Repeated measurement of this heme pool resulted in an estimate of 37 ± 8 pmol heme per mg plastid protein. When the free heme pool was estimated by direct extraction and quantitation by HPLC, values in close agreement with those of the reconstitution technique were obtained ($\sim 47 \pm 17$ pmol heme/mg protein). Both techniques have their inherent problems, but the agreement between the two estimates reinforces their validity.

Values obtained by the reconstitution technique are dependent on the relative affinities of the endogenous hemoproteins and apo-peroxidase for heme under the specified incubation conditions. In our hands, only 5% of the heme in horse heart myoglobin was transferred to an equimolar amount of apo-peroxidase in a 3-h incubation (as measured by recovery of peroxidase activity). Similarly, when bovine liver catalase was incubated with equimolar (on a heme basis) apo-peroxidase, there was no loss of catalase activity over 3 h. Under similar incubation conditions, bovine hemoglobin gave up half of its heme to apo-peroxidase. Thus, we cannot say with complete assurance that the free heme measured by the reconstitution technique does not also contain heme transferred from Cyt or other hemoproteins. However, depending upon their relative heme dissociation constants, the hemes of plastid Cyt and hemoproteins may be able to enter the free heme pool of the plastids. It should be noted that the possibility of heme transfer from Cyt and other hemoproteins to apo-peroxidase does not complicate the measurements of heme efflux since the plastid envelope would protect the internal contents of the plastid from the apo-peroxidase.

There is a physiological component to the uncertainty in the free heme measurements due to an ongoing process of heme degradation which would compete with reconstitution. Evidence for this degradative process is provided by recovery of only 60 to 83% of the exogenously added heme in the experiment shown in Figure 3, and the negative slope of the line obtained for total heme *versus* incubation time of intact plastids (dashed line, Fig. 7). Another source of experimental variation in the heme measurements is the developmental state of the chloroplast. Preliminary experiments suggest that free heme increases during greening (J Thomas, CJ Walker, unpublished observations).

A crude estimate of total plastid volume was obtained by measuring the packed plastid volume in a Hopkins tube.

Although methods exist for measuring the volume of the aqueous compartment of the chloroplast, the solubility properties of free heme suggest that it would be dissolved in the membranes or associated with soluble or membrane-bound heme binding proteins. Thus, the crude volume measurement provides a better estimate of the free heme volume than the aqueous volume alone. This estimate (6.5 $\mu\text{L}/\text{mg}$ plastid protein) was used to express free heme in terms of heme concentration within the plastids. A surprisingly high concentration of 5.7 μM heme in the plastids could be estimated for plastids from plants which had been greened for 14 to 16 h. Even considering the physiological sources of error in the heme estimation (variation with greening and possible breakdown) together with the crudeness of the volume measurement, these data still indicate micromolar heme concentrations within the plastid. As far as we are aware, this value has not been estimated before, even though the expression 'physiologically relevant concentration range' is not uncommon in the literature.

The relative heme concentration in the chloroplast has been implicated in the regulation of ALA synthesis at the level of enzyme activity (1). In intact plastids from greening cucumbers, exogenous heme concentrations as low as 0.4 μM inhibited ALA synthesis by 50% (13). Similar concentrations of heme have been found to inhibit organelle- and membrane-free preparations of ALA synthesizing enzymes from algae (12, 30). Heme has also been implicated in the regulation of transcription of the chloroplast encoded genes for allophycocyanin and phycocyanin in the red alga, *Cyanidium caldarium* (28). Thus, a knowledge of the range of possible internal plastid heme concentrations is essential to ascertain whether or not the regulation by heme observed *in vitro* or in feeding experiments is relevant to the *in situ* situation.

In summary, a new sensitive method has been developed to quantitate small amounts of free or easily exchangeable heme in biological systems. Using this method, chloroplasts were shown to have the capacity for heme efflux and, by inference, the capacity to supply other cellular compartments with heme for assembly of hemoproteins and for possible regulatory roles. The question as to whether chloroplasts, mitochondria, or both, are the source for cytosolic hemes cannot be answered at this point. Studies similar to those described above are planned for mitochondria isolated from similar or identical tissue. Finally, the heme concentration of intact developing chloroplasts has been estimated. This estimate can now be used for comparison of the heme concentration required for regulation in *in vitro* experiments. Studies are in progress to determine how the plastid heme concentration varies with development and the possible role of breakdown and synthesis in maintaining the plastid heme concentration.

ACKNOWLEDGMENT

We would like to thank Dr. C. J. Walker for useful discussions and her helpful criticism of the manuscript.

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