PROGRESS REPORT

Title: THE MAGNESIUM CHELATION STEP IN CHLOROPHYLL BIOSYNTHESIS
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The advances described in this report encompass work supported by DOE grant DE-FG09-89ER 13989 (\$140,000 in direct costs over three years) during the period 2/15/89 to the present, 6/20/91. The laboratory had no other source of support during this period. This report will be divided into two sections. The first will cover progress on the enzymology and regulation of the Mg-chelatase reaction in intact and broken chloroplast preparations. This work was the main objective of the original proposal and is the main focus of the renewal proposal. The second section will cover progress on the measurement of free heme content and heme efflux in isolated chloroplasts. This latter work was a natural outgrowth of the consideration of porphyrin flux through the magnesium and iron branches of the pathway. Each section will be organized by manuscripts in press or in preparation followed by preliminary results.

MG-CHELATASE

This work has been performed by a postdoctoral associate, Dr. Caroline Walker (since July 1989), with the assistance of a half-time technician, Ms. Laura Hupp (since April 1990). John Hartwell, a graduate student, joined the project in January of this year. Since July 1989 when full time work on this part of the project began, the work has resulted in two papers in press, one paper in preparation and several interesting leads for future work.

Walker, C.J. and Weinstein, J.D. (1991) Further characterization of the magnesium chelatase in isolated developing cucumber chloroplasts: Substrate specificity, regulation, intactness and ATP requirements. Plant Physiol. 95: 1189-1196. (Appendix A)

The published procedures of Castelfranco and co-workers (1, 2) were used as a starting point for the continued investigation of the magnesium chelatase reaction in intact chloroplasts from greening cucumber cotyledons. The first step in this investigation was to miniaturize and streamline the assay procedure. As described in Appendix A, the reaction volume was decreased from 1.0 ml to 0.25 ml, allowing rapid processing of samples in the microcentrifuge. The lengthy extraction of the reaction product into diethyl ether was also omitted. These minor changes allowed for an increase in the number of assays per chloroplast preparation and decreased by half the time required to process each sample. The incubation conditions were then optimized with respect to time and substrate and plastid concentrations. Under the optimized conditions a linear reaction rate was achieved for 50 min (Fig. 1, Appendix A). A standard assay time of 20 min was chosen to ensure that all assays measured rates rather than total accumulation of product.

The modified assay was used to probe the intactness and other reported requirements of the system. The intactness (as measured by the latency of 6-phosphogluconate dehydrogenase (3)) could be incrementally lowered by adjusting the concentration of osmoticum in the incubation. The activity loss was proportional to the intactness loss, confirming the intactness requirement for chloroplasts from greening cucumber cotyledons (Fig. 2, App. A). This intactness requirement has been the major obstacle to progress in characterizing this enzyme, and, to our knowledge, there have been no published reports on this activity since 1984. In the original proposal several strategies were outlined to determine the reason for the intactness requirement. The possibility was suggested that the ATP requirement might be related to the intactness requirement via the formation of an electrochemical gradient. This possibility was rejected by demonstrating that uncouplers and ionophores had no inhibitory effect in the presence of ATP (Table I).

Although the intactness requirement could not be overcome in cucumber chloroplasts, the Mg-chelatase activity in intact plastids could be further characterized. The ATP requirement could not be fulfilled by the non-hydrolyzable analogs of ATP, suggesting that it was required for something other than simple binding of Mg^{2+} or as an allosteric effector. A variety of Proto analogs were tested for their ability to serve as substrates (Fig. 3, Table III). Porphyrins having two hydrogens, two ethyls, or one ethyl and one vinyl (both isomers) at the 2 and 4 positions of the macrocycle

were all almost as active as the natural substrate (with vinyl groups at positions 2 and 4). Of course, *bona fide* kinetic constants could not be obtained, because of the barrier presented by the plastid membranes. An important outcome of these experiments was the observation that porphyrins with vinyl groups were inhibitory at higher concentrations. Thus, when natural porphyrin endproducts (heme, protochlorophyllide and chlorophyllide) were tested as possible feedback inhibitors of the Mg-chelatase, they were found to be no more inhibitory than the substrate (Fig. 5). This finding reopened the question of how the pathway is regulated. It was previously thought that either chlorophyll or protochlorophyllide would accumulate causing a shut-down of Mg-chelatase, and direct the flux of porphyrins through the iron branch of the pathway (4). The resultant accumulation of heme would then shut-down ALA synthesis, turning off the entire pathway. This hypothesis is no longer tenable. The inhibitory effect of sulfhydryl-modifying reagents (PCMB, PCMBS, and NEM) was confirmed, and an inhibitory effect of metal ion chelators was noted. This effect was unexpected since these chelators stimulate the accumulation of Mg-porphyrins in intact tissue (5, 6). Finally a four-fold increase in Mg-chelatase activity during greening was observed (Fig. 4). Accompanying this rise in Mg-chelatase and chlorophyll, was an increase in the "free" heme content of the chloroplasts.

Walker, C.J. and Weinstein, J.D. (1991) *In vitro* assay of the chlorophyll biosynthetic enzyme, Mg-chelatase: Resolution of the activity into soluble and membrane-bound fractions. Proc. Nat. Acad. Sci. (USA) 88: In press. (Appendix B)

Obtaining an organelle-free preparation for biochemical characterization of the Mg-chelatase enzyme(s) has been the biggest obstacle to understanding the metabolic co-regulation of chlorophyll and heme formation in chloroplasts. It was also the primary goal of my original proposal. We have now attained this goal and have begun the detailed characterization of the enzymology, regulation and cell biology of this process.

The inability to stabilize Mg-chelatase in ruptured chloroplasts from cucumber cotyledons has hindered the most interesting biochemical investigations. Therefore, several plant tissues were screened for their ability to catalyze the Mg-chelatase reaction *in organello*. Chloroplasts isolated from peas, maize and barley are able to catalyze the reaction and have time courses which are linear for at least 40 min (data not shown). In all three cases, the plants were grown under a 12 h diurnal light/dark regime. The specific activities in barley and corn plastids were comparable to cucumber plastids. However, the specific activity in pea plastids was three to four-fold higher than in cucumber (Fig. 1, Appendix B). Consequently, the activity in pea chloroplasts was chosen for further investigation. Due to the lack of inhibition at higher porphyrin concentrations, deuteroporphyrin (hydrogens at positions 2 and 4) was used as the substrate in the following experiments.

Simple lysis of the plastids by two freeze/thaw cycles in hypotonic buffer resulted in a preparation still able to form Mg-Deutero with a specific activity 26% of intact plastids. Moreover, fractionation into soluble and membrane-associated components by centrifugation, resolved two fractions that were essential for reconstitution of activity (Table I, App. B). Boiling of either fraction resulted in inactivation, suggesting that both fractions contained one or more necessary protein components (Table II). In all the reconstitution experiments a sample of membrane-associated components, alone, was always tested for activity to ensure that the measured activity from reconstitution was not simply due to a small percentage of intact plastids which may have survived lysis. Omission of porphyrin substrate, ATP (and regenerating system) or extra Mg^{2+} also resulted in loss of activity. As expected, Proto was also a substrate for the reaction. Comparison of fluorescence emission and excitation spectra of the ether extracted reaction product with standards confirmed the presence of the magnesium rather than the zinc chelate of Proto. After an initial lag (5 min) the reaction rate was linear for 60 min. Routine fractionation of components by centrifugation at 12,000 x g may not have been sufficient to completely resolve soluble from membrane components. Therefore, soluble components were separated from membranes by a 1 h, 150,000 x g centrifugation, and the membranes given a buffer wash and

second centrifugation. Reconstitution of activity still required membrane and soluble components (Fig. 3). The reaction was still sensitive to sulfhydryl modifying reagents. Preparations of membrane-associated and soluble fractions from greening cucumber chloroplasts were not active upon reconstitution (Table IV). The soluble component from cucumber could reconstitute activity with the membrane fraction from peas, although membrane fraction from cucumber was not effective with the soluble fraction from peas. Thus, the inability of organelle-free preparations from cucumber chloroplasts to catalyze high rates of magnesium chelation is most likely due to the relative instability of the membrane fraction.

To our knowledge, this is the first report of high Mg-chelatase activity in broken chloroplast extracts. In the only other report of the Mg-chelatase in a broken plastid preparation, the activity of washed etiochloroplast membranes from cucumbers was only 2.5% of the activity from intact cucumber plastids (7) and was 1000-fold lower than typical activities with the reconstituted pea system. At present we cannot explain the difference in behavior of the pea and cucumber chloroplasts. The observation that Mg-chelatase activity requires at least two protein components was very surprising. Ferrochelatase, which catalyzes the analogous reaction of iron insertion, requires only a single membrane-bound polypeptide. Another striking difference between the two activities is the requirement for hydrolyzable ATP for magnesium but not iron insertion. These differences might suggest that the extra requirements are for some type of regulatory control. A few possibilities are briefly presented in the PNAS paper, and they will be discussed in the experimental section of this proposal.

Walker, C.J. and Weinstein, J.D. (1991) Localization of Mg-chelatase in pea chloroplasts. In preparation for submission to Plant Physiol. (Appendix C)

In our original report of organelle-free Mg-chelatase activity from pea chloroplasts (Appendix B), no attempt was made to distinguish the thylakoids from the envelopes in the membrane-requiring fraction. Under most fractionation conditions envelope membranes would be expected to stick non-specifically to thylakoid membranes (8). In the current work the intact chloroplasts were lysed by two cycles of freeze/thaw in a medium containing a low buffer concentration, 2 mM EDTA and no $MgCl_2$. Most of the thylakoids were removed by a 2 min. centrifugation at 12,000 x g, and the pellet was washed and resuspended in the same medium. The wash and first supernatant were combined and adjusted to 10 mM $MgCl_2$. This envelope-stroma mixture could then be used directly for routine experiments or further separated into stroma and crude envelope fractions by ultracentrifugation (30 min at 260,000 x g). This crude envelope pellet can then be washed and recentrifuged to remove stromal contamination.

When a crude envelope fraction is prepared as outlined above, it can reconstitute Mg-chelatase activity when combined with the stromal fraction (Table I, Appendix C). The envelope fraction is sixteen times more effective at reconstitution of activity than the thylakoid fraction on a membrane protein basis. The envelope fraction is contaminated by thylakoids only to a minor extent, 2% on a chlorophyll basis. A significantly cleaner envelope fraction can be obtained by ultracentrifugation of lysed plastids on a sucrose step gradient (9). Again the envelope fraction was more effective than the thylakoids in reconstituting Mg-chelatase activity, although a substantial amount of activity was lost. In this case there was only a 0.1% contamination by thylakoids. This loss of activity in the membranes could be explained by the data in Table II (Appendix C). It is apparent that washing the envelope membranes in buffer lacking $MgCl_2$ results in a substantial loss of activity, some of which can be recovered by adding back the wash proteins. In the separation of thylakoids from envelopes on the sucrose step gradient, only a low concentration of $MgCl_2$ was present to prevent the two types of membranes from sticking to each other.

The above data might suggest that the required membrane component is a peripheral protein which can be removed by washing. The following experiments argue against this interpretation. Purified intact plastids were treated with thermolysin, a calcium dependent protease. Control plastids were also treated thermolysin and calcium, but excess EGTA was included to sequester the calcium and inactivate the protease. After incubation, thermolysin-treated plastids were also

quenched with EGTA and intact chloroplasts were re-isolated by centrifugation through Percoll. The remaining intact plastids which were treated with active protease were only 37% as active as the control (Table III). These plastids were also lysed and assayed. Again, the control preparation was more active, but the difference was not as great as in intact plastids. Reasons for a larger effect of protease treatment when the plastids are assayed intact versus lysed are discussed in Appendix C. The result still stands that something accessible to a protease which cannot penetrate the outer envelope (10) is necessary for or contributes to Mg-chelatase activity.

The finding that an active component of the Mg-chelatase system is present in the chloroplast envelope and is accessible to the outside is consistent with the results of Castelfranco and co-workers who first proposed this idea because they found that the membrane impermeable mercurial reagent, PCMBs, inhibited Mg-chelatase activity in intact cucumber cotyledons (2). It is also consistent with the recent observation that protochlorophyllide reductase in green tissue is localized on the cytosolic side of the outer envelope (10).

Since the membrane component(s) required for reconstitution of activity with stroma are associated with the envelope(s), the preparation could be partially purified by removing the thylakoids. The envelope-stroma mix was very active (line 3, Table III), and activity was linearly proportional to protein concentration after an initial lag (Fig. 2). The envelope-stroma mix was stable enough to be stored at -80°C for at least a week without loss of activity (Table IV). In contrast if the stroma and envelopes are stored separately, considerable activity is lost in one week. It is likely that it is the envelope component which loses activity, since a stromal fraction concentrated by ammonium sulfate precipitation has retained its activity for at least a month at -80°C . The envelope-stroma mix was further characterized with respect to optimum substrate concentrations (Fig. 1A and B). Interestingly, the water soluble porphyrin, deuteroporphyrin disulfonic acid was also a substrate for the reaction. It was not active in the intact plastid system (our unpublished results). This porphyrin is a substrate for the bacterial but not the mammalian ferrochelatase (11).

Other preliminary results; (Appendix D)

Using the highly active intact chloroplast system from peas, we have begun to develop a continuous spectrofluorometric assay for Mg-chelatase activity. In this assay the intact plastids are placed directly in a cuvette with buffer, MgATP and Dp. The sample chamber is temperature controlled, the reaction initiated by the addition of Dp, and MgDp formation is followed by the increase in fluorescence at 584 nm with time. A sample trace is presented in Fig. 1 (Appendix D). As would be expected, the slopes of the linear part of these traces increase directly with increasing amounts of plastids (Fig. 2). The beauty of a continuous assay is that it allows one to modify the experiment while it is in progress, and it is especially useful when testing new compounds (inhibitors or other effectors) and/or conditions. Also, more information can be deduced from a single assay since time-dependent changes are observed directly. In a stopped assay this information is lost or requires several separate time points. A significant problem with the assay of Mg-chelatase in the intact chloroplast is quenching of the fluorescence by endogenous chlorophyll. Since the envelope-stroma mix is highly active and has only a minor contamination with chlorophyll, the quenching problem can now be surmounted.

A continuous assay in intact chloroplasts will still be useful to monitor intermediate flux before and after the branch-point in the pathway. In a preliminary experiment, the porphyrin precursor, ALA, was supplied to intact plastids and Proto accumulation was followed by the increase in fluorescence at 634 nm. When ALA and MgATP were supplied simultaneously, only MgProto increased (fluorescence emission at 595 nm); there was little or no accumulation of Proto (Fig. 3). Thus, it appears that, in the presence of ATP, the system is tightly coupled such that, Proto is immediately (and quantitatively?), converted to MgProto. Although we could not follow heme formation in the same system, it may be possible to do so in the future. Camadro and Labbe (12) have shown that ferrochelatase in yeast has a zinc chelatase activity which can be followed by fluorescence. In our system we have also observed a zinc chelatase activity in the presence of zinc and absence of ATP. However since the emission maxima of the zinc and Mg chelates only differ

by about 4 nm, it will be very difficult to quantitate small amounts of one in the presence of large amounts of the other.

FREE HEME AND HEME EFFLUX

Since heme levels can regulate porphyrin synthesis at the level of ALA formation, and can serve as an alternate end-product for the substrate of the Mg-chelatase reaction, heme metabolism within the chloroplast cannot be ignored. We have devised a method to measure very small amounts of "free" heme in biological tissue, and have used this assay to measure "free" heme levels in the plastids as well as to demonstrate that intact chloroplasts are capable of heme efflux. This project has now significantly diverged from the original goal of understanding the branchpoint regulation of heme and chlorophyll biosynthesis, and separate funds to support the heme work have now been awarded. The progress on the heme work (one paper published and one in preparation) will be summarized in less detail since the proposed research on Mg-chelatase is not as heavily reliant on these results. A graduate student, Jim Thomas, was the sole benchworker on this project.

Thomas, J. and Weinstein, J.D. (1990) Measurement of heme efflux and heme content in isolated developing chloroplasts. Plant Physiol. 94: 1414-1423. (Appendix E)

Heme which is not tightly bound to a hemoprotein can spontaneously combine with the apoenzyme of horseradish peroxidase (commercially available) to reconstitute the active holoenzyme. Under conditions of apo-peroxidase excess, reconstituted peroxidase activity is directly proportional to heme concentration (Fig. 2, app. E). This assay was first used to document the phenomenon of heme efflux from intact chloroplasts (Figs. 3 and 4). Only a small portion of the efflux rate could be attributed to plastid breakage, and rate could be enhanced by administration of the heme precursor, ALA (Fig. 6). Incubation of lysed plastids with the apo-peroxidase allowed access to heme embedded in internal membranes, and yielded a rough estimate of the internal "free" heme concentration within the plastids.

In plants, both the mitochondria and the chloroplasts have the capacity for the final steps in heme biosynthesis (4). The chloroplast has, in addition, the ability to form heme from general uncommitted metabolites (13). This ability has not been documented in plant mitochondria, and whether they are actively involved in heme synthesis is unknown. However, one of these organelles must be able to supply the hemes for cytosolic hemoproteins. We have now documented that *in vitro*, chloroplasts are able to export hemes. Whether this process happens *in situ* is a matter for further study. In animals and yeast heme is known to regulate a wide array of metabolic functions including transcription and translation (14, 15). In plants and algae it is known that heme can regulate ALA synthesis and it has recently been shown to regulate the transcription of genes for the apo-proteins of phycobiliproteins (16, 17). Although both of these effects have been shown *in vitro*, there was no information on the amount of heme available for regulation *in situ* until this report.

Thomas, J. and Weinstein, J. D. (1991) Free heme in isolated chloroplasts: An improved method of assay and physiological importance. In preparation for submission to Plant Physiol. or Plant Physiol. Biochem. (Appendix F, selected tables and figures from Jim Thomas's Ph.D. dissertation which will be used in preparing the manuscript)

Our original measurements of "free" heme levels in chloroplasts were complicated by the possibility of the apo-peroxidase being able to "steal" heme from legitimate hemoproteins. This possibility was suggested by the ability of apo-peroxidase to remove hemes from some types of hemoproteins (Fig. 1, Appendix F). However, incubation of lysed chloroplasts with apo-peroxidase at 10°C (rather than 30°C), and more thorough removal of chloroplast membranes before assaying reconstituted peroxidase activity, resulted in a kinetics of reconstitution which levels off after six to seven hours (Fig. 2). Reconstitution at 30°C continually rises until all the

available b-type heme is sequestered. The fact that a small portion of the heme is susceptible to removal by apo-peroxidase would suggest that this heme is not tightly bound. Exogenously added heme (by definition free) is recovered in three hours (Fig. 3). Thus, free heme is recovered more rapidly than heme bound as a part of stable hemoproteins. The measured concentration of plastid free heme responded in a rational manner to treatments which should logically alter the heme levels (Table I). Pretreatment of the plastids with a heme precursor (ALA) or inhibitor of heme oxygenase (PCMB) (18) increased free heme levels. Although free heme and total non-covalent heme increased during greening, the free heme reached a maximum considerably earlier (Fig. 4). This difference in kinetics is suggestive of a precursor-product relationship. It could also be demonstrated that the endogenous free heme in cucumber chloroplasts does have a relevant physiological effect. If isolated intact plastids are pre-treated with apo-peroxidase to serve as a sink for endogenous free heme, the subsequent rate of *in organello* ALA synthesis is raised by 30% (Table II). Thus, endogenous levels of heme are, in fact, regulating ALA synthesis.

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