The Magnesium Chelation Step in Chlorophyll Biosynthesis

Progress Report

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PROGRESS REPORT

The progress described in this report encompasses work supported by DOE grant DE-FG09-89ER13989 for the period 2/15/92 to the present 6/14/94. The goals of the project were to continue investigating the enzymology of Mg-chelatase and to investigate the co-regulation of heme and chlorophyll formation in intact plastids. During this period the laboratory had additional support (two years) from USDA to investigate heme metabolism in chloroplasts. This report is arranged so that the progress is described by reference to manuscripts which are published, under review or in preparation. A brief introduction to our work from previous DOE support will set the stage for the current progress report.

In 1989 our basic approach was to start with the system in cucumber chloroplasts described by Castelfranco and co-workers and further characterize the intact chloroplast system [50]. However, the inability to stabilize Mg-chelatase in ruptured chloroplasts from cucumber cotyledons hindered the most interesting biochemical investigations. Using pea chloroplasts as a starting point, we were able to obtain the first organelle-free preparation with high Mg-chelatase activity [1]. The breakthrough came with the realization that Mg-chelatase consisted of multiple components and required both soluble and membrane-associated proteins.

The ability to assay and fractionate Mg-chelatase activity in vitro has allowed investigation of the protein components and reaction mechanism of this enzyme. As a first step we developed a continuous fluorescent assay which allows a closer examination of the reaction kinetics [51]. The material used for this assay, designated LM/S, was a subplastidic fraction from which most of the thylakoid membranes could be removed, leaving the lighter chloroplast membranes (probably envelopes) plus soluble chloroplast proteins. This fraction was highly active and could be separated by ultracentrifugation into a completely soluble fraction and a membrane fraction, both of which were required for reconstitution of activity. The most intriguing result obtained from this new continuous assay was that there was an initial lag period before the onset of Mg-deuteroporphyrin (Mg-Deutero) accumulation and that preincubation with ATP could overcome the initial lag in the reaction kinetics. This observation suggested that ATP might be functioning as an activator. In addition the protein concentration dependence curve for this enzyme system was concave up rather than linear. This work set the stage for the following investigation of the enzyme mechanism.

Enzymology

Walker, C.J. and Weinstein, J.D (1994) The magnesium insertion step of chlorophyll biosynthesis is a two-stage reaction. Biochem. J. 299: 277-284. (Appendix A)

The results from the previous paper suggested that ATP might be involved in an activation process and that this process might involve protein-protein interactions (non-linear protein concentration dependence). A separate activation step implies that the actual magnesium insertion step must be a distinct process. Thus we set out to prove that the Mg-chelatase reaction was a two-step process, with each step having qualitatively and quantitatively different requirements. By manipulating the preincubation conditions, while holding the incubation conditions constant (and vice versa), we could determine requirements for the activation and magnesium insertion steps independently. In several respects they proved to be quite different. Activation was optimal at higher proteins concentrations and required ATP (Figs 1 & 2, Appendix A). Preincubation in the absence of ATP resulted in a loss of activity (Fig 1). Although the preincubation effect (activation) requires the presence of both LM and S fractions (Table 1), it was not clear if, once activated, a single fraction could catalyze the magnesium insertion step (Table 2). When an ATP trap (glucose plus hexokinase) was added to the assay during the magnesium insertion stage, the reaction was instantly stopped (Fig 4), thus demonstrating that ATP is required for both stages. However, the activation and magnesium insertion steps could be differentiated on the basis of their respective ATP requirements. Activation requires higher ATP concentrations than does the magnesium insertion step (Fig 5). In addition the activation step could utilize the slowly hydrolyzable ATP

analog, adenosine 5'-O-(3-thiotriphosphate) (ATP_γS), but the magnesium insertion step could not utilize this analog (Fig 6). Finally, the activation step could be reversibly inactivated by chilling (Fig 3 and Table 3); ATP_γS could not prevent this cryo-inactivation.

It is obvious from these data that the Mg-chelatase reaction can be divided into two different stages, activation and magnesium insertion. However, the mechanism for each step is still a matter of speculation. The fact that an ATP-dependent process is necessary for activation suggests that the activation may play a regulatory role for chlorophyll synthesis. Ideas concerning the mechanisms and implications for each step will be discussed more fully in the experimental section of this proposal.

Localization

Walker, C.J. and Weinstein, J.D (1994) Re-examination of the localization of Mg-chelatase within the chloroplast. submitted to Physiol. Plant. (Appendix B)

In 1984 Fuesler, et al. [23] reported that in intact plastids Mg-chelatase is more susceptible to the membrane impermeant sulfhydryl inhibitor, *p*-chloromercuribenzenesulfonate (PCMBs), than is a subsequent enzyme in the biosynthetic pathway, Mg-Proto monomethyl (oxidative) cyclase. Both enzymes were equally susceptible to the more membrane permeable analog of the inhibitor, *p*-chloromercuribenzoate (PCMB). On this basis they postulated that the cyclase is protected by a membrane barrier while the Mg-chelatase is on the outside of this barrier. The obvious candidate for the barrier is the chloroplast inner envelope. Since that time two other enzymes of the chlorophyll biosynthetic pathway have been localized to the chloroplast envelope, protoporphyrinogen oxidase [52], and protochlorophyllide reductase [53]. In addition our group published a Mg-chelatase assay using a subplastidic fraction depleted in thylakoids [51]; the implication being that a component of the chelatase is localized to the plastid envelope. This information plus information from protein import studies has been used to suggest a grand theory that the chloroplast envelope is the staging ground for thylakoid assembly [54, 55]. Fuesler's [23] paper played a key role in the formulation of this theory.

Since we had an in vitro assay for Mg-chelatase, we set out to confirm the results of the Fuesler paper. PCMB and PCMBs are equally effective inhibitors of Mg-chelatase in the subplastidic fraction, LM/S (Fig 1, Appendix B). However, in intact plastids PCMBs is a less effective inhibitor than the more permeable PCMB (Fig 2). The four-fold difference in the IC₅₀s suggests that the chelatase is protected by a membrane barrier. The same phenomenon is observed in pea and cucumber chloroplasts. Another way to get at the localization question is to investigate the site of ATP utilization. In chloroplasts from cucumbers the Mg-chelatase reaction can be driven by photosynthetically generated ATP (Table 1, and ref. [24]). An external ATP trap (hexokinase plus glucose) has no effect on Mg-chelatase activity when it is driven by photosynthetically generated ATP. If Mg-chelatase were localized in the interenvelope space, we would expect to see a decrease in activity, because ATP in this space would be in free equilibrium with the outside medium via the large pores in the outer envelope. Protease treatment of intact plastids had no effect on the activity of the LM component subsequently isolated from the treated plastids (Table 2). We, as well as others have assumed that Mg-chelatase (or a component thereof) is a membrane associated protein. This assumption was based on analogy to ferrochelatase and was supported by our own fractionation data [51]. However, when we purified thylakoids and envelopes, the reconstituted Mg-chelatase activity was too low to make firm conclusions about which membrane system contained the activity. In addition we found that the "membrane associated component" could fractionate with the thylakoids or the envelopes, depending on the MgCl₂ concentration used in the chloroplast lysis buffer. In our routine preparations of LM/S, magnesium is added after removal of the thylakoids so that the activity associates with the LM. In fact, the "membrane associated component" could be solubilized by washing the membranes in buffer containing low MgCl₂ concentrations (1 mM). Thus a completely soluble and active enzyme system could be prepared by chloroplast lysis in a large volume of low salt buffer (Table 3). This characteristic

explains why activity could not be recovered in the clean membrane fractions: the membrane component was washed away. It should be noted that the putative genes for the Mg-chelatase subunits encode proteins which are soluble and should be localized to the chloroplast stroma [45, 49].

From this work we can not determine the localization of Mg-chelatase in the chloroplast. We can say that the original conclusion drawn from the Fuesler et al. paper (that the chelatase is localized externally to the inner envelope) is probably incorrect. Thus, one of the key pieces of evidence supporting the theory of the envelope as a site for assembly of thylakoid components is no longer valid. If this intriguing theory is to stand, other evidence must be found to support it. Finally, this work has provided useful information for obtaining a completely soluble enzyme system for further manipulation (described in more detail in the experimental section).

Enzymology and developmental regulation of cucumber Mg-chelatase

Hartwell, J. G. and Weinstein, J. D. (1994) Magnesium chelatase from chloroplasts of developing cucumber cotyledons: properties of the subplastidic system and light regulation of two components. In preparation for submission to Plant Physiol. (Figures and tables are in Appendix C)

After the publication of our work on the pea chloroplast Mg-chelatase, Lee, et al. [56] published a paper on a subplastidic preparation from cucumber etiochloroplasts which suggested that the properties of the pea and cucumber systems were vastly different from each other. There are two important differences between the two systems: 1) The cucumber system is contained entirely in the membranes and is not stimulated by addition of soluble proteins. 2) Recovery of activity from the lysed cucumber chloroplasts requires lysis in the presence of ATP (20 mM) and ultracentrifugation in the presence of Proto (300 μ M). It seemed unlikely that the two plant systems could be so different. Therefore we characterized the Mg-chelatase system from cucumber chloroplasts in light of our experience with the pea system. LM/S prepared from 12 h greened cucumber chloroplasts was highly active and not stimulated by addition of the thylakoids (Table I, Appendix C). In fact LM/S was inhibited by addition of thylakoids in a concentration dependent manner (Fig 1). Inclusion of ATP in the lysis buffer was not necessary and did not stimulate the activity in the resultant LM/S fraction (data not shown). The substrate requirements for the LM/S were optimized (Table II). A high $MgCl_2$ concentration (30 mM) was required for optimum activity (Fig 2). This optimum is similar in the pea LM/S system. It is also similar for the cucumber subplastidic enzyme system catalyzing the oxidative cyclase reaction [57]. The optimum ATP concentration (between 1 and 1.5 mM) is about 10-fold lower than that of the membranous system of Lee, et al. [56]. This difference is readily explained by the four-fold longer incubation period and absence of an ATP regenerating system in the assay of the membranous Mg-chelatase. As with pea LM/S, Mg-chelatase activity versus cucumber LM/S protein concentration is concave up rather than linear (Fig 3), and cucumber LM/S can be fractionated into LM and S by ultracentrifugation (Table III). Thus far the pea and cucumber LM/S systems have identical properties with the exception that the pea system is more active. The higher activity of the pea LM/S is due to the more active LM component as can be seen in the mix and match experiment with pea and cucumber LM and S fractions (Table IV).

What explanation can be given for the differences in behavior between the two cucumber systems? We tried to duplicate the lysis and fractionation procedure of Lee et al using chloroplasts from 12 hour greened cotyledons instead of etiochloroplasts. We were unable to measure activity in the membranous pellet alone or the pellet plus soluble proteins, when we used the same amounts of protein as did Lee, et al. [56]. However, we were able to measure activity when the membrane fraction was recombined with a much larger amount of soluble proteins (closer to the protein concentrations used for our own LM/S assays). Thus, when lysed and fractionated by the method of Lee et al. [56], chloroplasts obtained from 12 h greened cucumber cotyledons also have Mg-chelatase activity which requires recombination of two fractions.

The greening cucumber cotyledon system has been used as a model for chloroplast development and there is already a substantial body of information available on the physiology of

pigment biosynthesis in this system. Since we have already observed an increase in the Mg-chelatase activity of intact plastids during greening [50], and since we now have the ability to separate at least two fractions required for activity, we decided to see if the two fractions might be differentially regulated during greening. The strategy was as follows: A large stock of standard LM/S from 12 h greened cotyledons was stored in separate aliquots at -80°C . For each greening time point seedlings were grown in the dark for up to 6.5 d. Trays of seedlings were exposed to white light for various periods of time (up to 18 h). At the end of the light period chloroplasts and LM/S were prepared. Standard LM/S and variable LM/S were ultracentrifuged to prepare four fractions, standard LM and S, and variable LM and S. The activities of the variable fractions were determined by assaying increasing amounts of the variable fraction with a constant excess amount of the complementary standard fraction. In each case the relationship between activity and the amount of variable fraction was linear and intersected the ordinate at zero; the slope was considered to be the specific activity of the variable fraction. For each of two complete greening curves the results are presented as % specific activity versus chlorophyll accumulation. Cotyledon chlorophyll accumulation is used instead of greening time so that this work can be more directly compared to other studies which may have slightly different greening conditions. A three hour time point (corresponding to approximately 33 nmol Chl/gfw) is the earliest greening time measured, because the yield of intact chloroplasts was too low from completely etiolated tissue. As observed before, the Mg-chelatase activity in intact chloroplasts increases four-fold until the cotyledon chlorophyll content reaches 430 nmol chl/gfw (9 h) after which the activity slowly declines (Fig 4). Similar greening kinetics are observed for the specific activities of the LM/S (slopes of the linear portion of activity vs LM/S concentration graphs) and S fractions (Figs 5 and 6, respectively). In contrast for the LM fraction, the rise to the maximum is somewhat sharper (between 6 and 9 h) and there is no comparable decline in activity of this fraction during the course of the 18 h greening experiment. These results suggest that two of the Mg-chelatase components are differentially regulated and support the idea that one of the components may play a regulatory role (activation?) which may be distinct from, but necessary for catalysis.

Co-ordination of Mg-Proto and heme formation in intact plastids

Walker, C. J., Yu, G-H, and Weinstein, J. D. (1994) Distribution of precursors between the iron and magnesium branches of the tetrapyrrole pathway in isolated pea chloroplasts; effect of ATP and metal ions. In preparation for Plant Physiol. (Figures and tables are in Appendix D).

Chloroplasts have the capacity to synthesize both heme and chlorophyll, which are needed in precise but different amounts in the mature plastid. The method by which chloroplasts achieve a balance of these two end products is not known. We have attempted to advance our understanding of this process by measuring heme synthesis and Mg-Proto synthesis simultaneously, under identical conditions in the same tissue. Mg-Proto is the first committed intermediate in chlorophyll formation and accumulation of this intermediate is indicative of the capacity of the magnesium branch of the pathway. Although this is an obvious approach, no direct measurements of this type have been previously reported. There have only been indirect measurements made in whole plant tissue [5].

Heme and chlorophyll are thought to share a common biosynthetic pathway up to the level of Proto, with ALA as the first intermediate. Therefore our approach was to incubate intact chloroplasts with ALA for 30 min in darkness, then quench the incubation and measure heme, Proto and Mg-Proto. Heme was quantitated by its specific radioactivity after extraction and HPLC (^{14}C -ALA was used as a substrate). Proto and Mg-Proto were extracted with acetone and quantitated fluorometrically. In Figure 1 (Appendix D), the accumulation of Mg-Proto and heme are compared under identical conditions (4 mM MgATP, 1 mM FeSO_4 , 5 mM DTT) at a range of ALA concentrations. Between 10 and 100 μM ALA, Mg-Proto synthesis exceeded heme synthesis by about 10 fold (this is also true up to 1 mM ALA). Proto was not detectable in these samples indicating that the two chelatases (Mg and Fe) are present at high enough activities to utilize all of the flux through the pathway. Since ATP is needed for Mg-chelatase activity, but not

for heme synthesis, we examined the effect of ATP on heme synthesis (Table 1). In the absence of ATP, we only observed the accumulation of Proto and heme. The accumulation of Proto suggests that ferrochelatase may be limiting under these conditions. In the presence of ATP, no Proto accumulated and heme synthesis decreased dramatically, although the deficit in heme accumulation is insufficient to account for Mg-Proto formation. Thus, there was a second effect of ATP: the total porphyrins (Proto + Mg-Proto + heme) doubled in the presence of ATP, indicating that ATP gives an overall stimulation of the pathway. Since no (or very little) Proto accumulates under these conditions, the ability to form Mg-Proto appears to facilitate porphyrin formation from ALA.

Since Fe^{2+} is required for ferrochelatase activity, it was present also in the Mg-Proto accumulation assays so that the reaction conditions were identical to those for heme accumulation. Unexpectedly, Fe^{2+} caused a 60% increase in the accumulation of Mg-Proto from ALA (Table 2) as did several other divalent metal ions (Table 3), the most potent of which was Mn^{2+} . This result was unexpected because Fe^{2+} inhibits Mg-chelatase when assayed in intact chloroplasts with either Proto or Deutero (an artificial porphyrin substrate) (Table 4). These results are not contradictory: Mg-chelatase activity is present in sufficiently high activities that even when it is inhibited 63% by Fe^{2+} , there is still sufficient enzyme to utilize all of the Proto produced from an ALA substrate in our Mg-Proto accumulation assays (Table 4). We must conclude that with respect to utilization of exogenous ALA, Mg-chelatase is not a rate limiting enzyme in the pathway. The mechanism by which divalent metal ions achieve this stimulation was investigated further. Fe^{2+} has no stimulatory effect on the conversion of ALA to Proto (Table 5), indicating that the presence of ATP is required for the metal ion effect. Also the effect is not mediated by heme formation, because it can still be observed (Table 2) in the presence of N-methyl mesoporphyrin (NMMP) a potent inhibitor of ferrochelatase [58]. In broken chloroplasts (assayed at a high protein concentration to minimize dilution of the plastid contents), Fe^{2+} and Mn^{2+} gave no stimulation of Mg-Proto accumulation (Table 6). It was interesting to note that in these lysed chloroplasts, Proto accumulation can be seen in the presence of ATP indicating that the close linkage of magnesium chelation to Proto formation is lost. There was one enzyme in the porphyrin synthesis pathway which was a potential candidate for a divalent metal ion stimulation; namely ALA dehydratase, which catalyses the conversion of ALA to porphobilinogen (PBG) and requires magnesium for activity. Therefore we looked for a metal ion stimulation in the conversion of PBG to Mg-Proto in which the ALA dehydratase step would be circumvented. In fact, the metal ion stimulation of Mg-Proto accumulation was even stronger using PBG as a substrate than using ALA (Table 7). Thus, it seems unlikely that ALA dehydratase plays a direct role in the stimulation by metal ions. It is possible that some enzyme(s) between ALA dehydratase and Mg-chelatase are stimulated by divalent metal ions, but if so, this stimulation has not been reported.

In summary, the only circumstances in which metal ion stimulation is observed are intact chloroplasts in the presence of ATP. We tested to see if this stimulation was constant over a long time period and found that at 1 mM ALA this is the case (Fig 2, panel A). However, at 0.1 mM ALA we observed that the first 10 minutes of Mg-Proto accumulation was very rapid compared to the linear rate from 10 to 60 min (Fig 2, panel A). In Figure 2, panel B the timescale has been expanded to focus on this initial rapid phase, which seems to be complete in just 6 minutes, after which the rate of Mg-Proto accumulation is almost identical to the rate seen in the absence of Fe^{2+} . The metal ion effect is transitory, thus it seems unlikely that it is due to metal ion enhanced precursor uptake. Unfortunately we cannot yet define the mechanism of the metal ion enhanced formation of Mg-Proto from ALA.

This work has contributed to our understanding of the coordination of heme and chlorophyll synthesis in intact plastids. Under conditions where both can be synthesized, Mg-Proto formation is ten-times more rapid than heme synthesis. ATP inhibits heme synthesis, either by stimulating Mg-chelatase and a preferential utilization of porphyrin precursors for Mg-Proto, or by a more direct inhibitory effect on ferrochelatase which has been reported [59], but never confirmed. ATP and divalent metal ions stimulate ALA utilization by an, as yet, unknown mechanism(s).

Related work on heme regulation of the tetrapyrrole formation

Weinstein, J.D., Howell, R.W., Leverette, R.D., Grooms, S.Y., Brignola, P.S., Mayer, S.M., and Beale, S.I. (1993) Heme inhibition of δ -aminolevulinic acid synthesis is enhanced by glutathione in cell-free extracts from *Chlorella*. Plant Physiol. 101: 657-665.

This work addresses regulation of the supply of precursors to the porphyrin biosynthetic pathway, but does not directly impinge upon the magnesium chelation step of chlorophyll biosynthesis. As such, it will be discussed only briefly. ALA is the first committed precursor to chlorophyll and heme biosynthesis. It has been known for a long time that heme inhibits ALA synthesis from glutamate in vitro [3]. However, the reported degree of inhibition from a given concentration of heme appeared to vary quite widely with different ALA synthesizing preparations from the same species and from different species. It is also known that the heme binding affinity of glutathione-transferase (which can bind heme and was suspected of being a heme carrier protein [60]) is enhanced by glutathione [61]. Therefore we determined the effect of glutathione on the inhibition of ALA synthesis by heme in extracts from the green alga *Chlorella vulgaris*. One mM glutathione reduced the concentration of heme required for 50 % inhibition by a factor of 10 (Fig 2, Appendix E). The effect of glutathione could not be duplicated by other sulfhydryl-containing compounds and was not due to the redox state of the heme iron. Preincubation of the enzyme extract with glutathione was not sufficient to confer enhanced heme sensitivity (Fig 7). The effect of glutathione on the inhibition of ALA synthesis by Co-Proto was the same as for heme (Fig 5). The effect of glutathione is also observed on thylakoid inhibition of ALA synthesis in intact cucumber chloroplasts [62].

Other progress on the enzymology and regulation of Mg-chelatase will be discussed in the appropriate section of the proposed experimental work.

PROPOSED RESEARCH

Hypothetical model for the structure, function and regulation of Mg-chelatase.

On the basis of our results, and by analogy to other enzyme systems, we can begin to envisage a working model for Mg-chelatase. Since enzyme activity requires ATP and two or more protein components, it is obvious that ferrochelatase can no longer be used as a model for understanding the over-all Mg-chelatase reaction. In contrast, the recently published paper on cobalt-chelatase from *Pseudomonas denitrificans* suggests many parallels with Mg-chelatase [63]. This enzyme system consists of two soluble components each of which are inactive alone, but together they catalyze cobalt insertion into the corrin ring (hydrogenobrynic acid α, ϵ -diamide) during coenzyme B₁₂ synthesis. One component consists of a single polypeptide of M_r, 140,000 and is encoded by the *cobN* gene. The other component, M_r, 450,000 is heteromeric, consisting of multiple copies of two polypeptides, M_r, 38,000 and M_r, 80,000, which are encoded by the *cobS* and *cobT* genes, respectively. Co-chelatase activity also has an absolute requirement for hydrolyzable ATP (K_m = 0.22 mM). Unfortunately, nothing else is known about the nature of the ATP requirement or whether the reaction takes place in multiple steps. Thus, the limited information available for Mg-chelatase suggests that it is more closely related to Co-chelatase.

Our results demonstrating that the Mg-chelatase reaction is a two-stage process, activation followed by Mg insertion, allows us to further elaborate on a hypothetical model. Activation requires protein-protein interactions and the presence of hydrolyzable ATP (Appendix A). These results suggest the possibility of a protein kinase, although there are other well-known examples of ATP mediated protein-protein interactions that result in an activation: Rubisco activase, glutamine synthetase (adenylation results in inactivation), assembly of Rubisco by chaperonins. Another characteristic of the Mg-chelatase system is cryoinactivation (Appendix A). This phenomenon is typical of enzymes that are active as oligomers [64]; no monomeric or dimeric enzyme has been reported to be subject to cryoinactivation [65]. Many multimeric enzymes also have a lag period in