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THE MAGNESIUM CHELATION STEP IN CHLOROPHYLL BIOSYNTHESIS

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TECHNICAL REPORT FOR 1990

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PROGRESS REPORT AND EXPERIMENTAL PLAN FOR THE FOLLOWING YEAR

The insertion of magnesium ion into the central position of protoporphyrin IX (Proto) is the first enzymatic step unique to chlorophyll biosynthesis. This step is dependent on Proto, MgATP, and appears to require an intact chloroplast for catalysis. Insertion of iron into Proto to form heme also occurs in the chloroplast. Thus, the metal ion chelation steps are at the branch point of the tetrapyrrole pathway in the chloroplast. A complete understanding of the regulation of MgProto formation will also require an understanding of heme metabolism. The progress report will be divided into two sections, one on MgProto formation and one on heme metabolism.

MgProto formation. We have been working with greening cucumber cotyledons as a source of chloroplasts for our investigations. Our results from the cucumber chloroplast system have recently been submitted to Plant Physiology for publication, and the manuscript is attached to this report (appendix A). Dr. Caroline Walker, the post doc on this project, was invited to present this work to the Gordon Conference on the Chemistry and Biology of Pyrroles this last summer. It was also presented in poster format to the American Soc. of Plant Phys. meetings last summer. Using an assay system which measures rates rather than total accumulation of product, we have made several significant advances in the areas of: substrate specificity, nature of the ATP requirement, rise in activity during greening, and the role of potential feedback inhibitors. Progress on Mg-chelatase has continued on three fronts: 1) Assay of activity in different plants leading to more active preparations and the possibility of identifying mutants of this enzyme(s). 2) Development of a continuous assay. 3) Characterization of the activity in broken or organelle-free preparations.

1) We are now able to measure reliable activities in chloroplasts isolated from peas, corn and barley. In all three cases, the plants were grown under a diurnal light-dark schedule. Although the conditions have not been optimized for the corn and barley plastids, time-course experiments suggest that the activities (on a mg plastid protein basis) are comparable to the activity in cucumber plastids. These plants are particularly important (for our studies) in that putative genetic lesions in Mg-chelatase activities have been identified. These mutants have been identified on the basis of Proto accumulation in intact plants. Unfortunately, there are several points in the tetrapyrrole biosynthetic pathway where an enzymatic lesion may result in Proto accumulation. Potential collaborators on characterizing a mutant include: Dr. Mary Polacco at the Univ. of Missouri (for corn) and the Carlsberg group (Dr. Dieter von Wettstein) in Copenhagen (for barley). The Mg-chelatase activity in isolated pea plastids is seven to ten times more active than in the cucumber chloroplasts. The activity is still dependent upon added MgATP and a porphyrin substrate. We now routinely use deuteroporphyrin (Dp) as a substrate. This porphyrin is as active as Proto in both cucumber and pea chloroplasts when supplied at saturating concentrations (as determined by Vmax). In addition Dp is more water soluble and less inhibitory at higher concentrations compared to Proto. The fluorescence emission maximum of MgDp is shifted 12 nm to the blue compared to MgProto, which moves the fluorescence peak of the synthesized product further away from the chlorophyll.

2) Using the highly active pea chloroplast system, 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. 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 trying out new compound 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. Perhaps, most useful will be the ability to monitor flux through several intermediates 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 no accumulation of Proto. 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 (1982, , BBA, 707: 280-288) 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.

Currently, there are several problems which need to be solved before this assay will be of routine use. Quenching due to the endogenous chlorophyll is a major problem which limits the assay to fairly low protein concentrations. The multiplicity of environments in which the product may localize (stroma vs membrane) makes calibration of the relative fluorescence with product very complicated, because Mg-porphyrins have very different fluorescence yields depending upon the polarity of the solvent. Light scattering is also a problem in this system. A new Aminco/SLM spectrofluorimeter was recently acquired by one of our colleagues. This fluorimeter can store and manipulate data, do rapid scans etc.) Therefore, by judicious choice of assay conditions and use of the new fluorimeter we should be able to solve or minimize these problems.

3) We have just recently been able to demonstrate Mg-chelatase activity in broken plastids isolated from peas. In these experiments intact plastids were suspended in hypo-osmotic buffer (containing MgATP and an ATP regenerating system) and subjected to two cycles of freeze-thaw to break the membranes. A crude fraction of thylakoid (T) was obtained by centrifuging (13,000 g) for three min. The pellet was washed with more hypotonic buffer and re-centrifuged. The original supernatant was centrifuged (13,000 g) for 20 min to obtain a crude envelope pellet (E) and a crude stromal supernatant (S). When assayed alone, none of the fractions were very active (Fig. 3). When S plus T were assayed together, the resultant activity was 55% more than the total of the two activities added together. When two portions of S was combined with T, the resultant activity was 230% more than the additive activities. The envelope fraction had no effect on the reconstitution. Boiling either the S fraction or the T fraction eliminates the reconstituted activity. This experiment has now been repeated several times and we are convinced that the organelle-free activity is not an artifact. This result is not as simple as one might hope. If the plastids were broken and assayed with an ATP regenerating system consisting of phosphocreatine and creatine kinase (Fig. 4) instead of the usual (and more expensive) PEP and pyruvate kinase, the stromal fraction alone was very active. Although optimum conditions have not yet been thoroughly investigated, this stromal activity (700 pmol/mg protein/20 min) was as high as that obtained in intact cucumber chloroplasts, and more than 10% of the activity of intact pea chloroplasts. When S plus T, were assayed together, the reconstituted activity was only 25% more active than the additive activities. In intact plastids, both ATP regenerating systems were equally effective. While we cannot explain these observations at the present time, they may provide an important clue as to the nature of the ATP requirement. Another important and useful result from these experiments is that the fractions retain a significant amount of activity when frozen for up to five days at -80°C (Fig. 5). This characteristic will permit us to run several experiments with a single chloroplast preparation. The organelle-free Mg-chelatase preparation is clearly the most important and exciting part of this project and we will be concentrating our efforts on characterizing this activity.

Heme metabolism. A project which relates porphyrin metabolism in the chloroplast to the rest of the cell has been ongoing in my laboratory. In the chloroplast, if Proto is not utilized for chlorophyll formation, it is used for heme formation. Since heme levels can regulate porphyrin synthesis, at the level of ALA formation, and can serve as an alternate end-product for Proto, heme metabolism within the chloroplast cannot be ignored. We have devised a method to measure very

small amounts of "free" heme in biological tissue. The basis for this assay is the ability of free heme to reconstitute with the apoenzyme of horseradish peroxidase to yield an active peroxidase. Under conditions of apo-peroxidase excess, reconstituted peroxidase activity is directly proportional to heme concentration. We 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, and thus, have the ability to supply heme for cytosolic hemoproteins. This work has been accepted for publication in Plant Physiology (Nov. issue), and the manuscript is included as appendix B. This work was also presented at the Gordon Conference on the Chemistry and Biology of Pyrroles and the American Soc. of Plant Phys. meetings last summer. Work on heme metabolism is proceeding along two lines: 1) Further characterization of "free" heme and heme efflux. 2) Characterization of ferrochelatase activity in chloroplasts.

1) 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. Therefore we had to define "free" heme operationally. We are currently working to refine these measurements by incubating the apo-peroxidase and lysed chloroplasts at low temperature. Under these conditions, one can clearly observe two kinetic components for reconstitution (Fig. 6). The kinetic break is magnified when the chloroplasts are intentionally spiked with excess free heme. Tentatively, we suggest the slow rise may be due to heme stealing and/or reconstitution with heme that is more inaccessible, for whatever reason. The fast rise is likely due to free heme; in spiked and unspiked samples the fast component lasts for the same amount of time. When the reconstitution is carried out at 30°C, the two kinetic components are not clearly resolvable. If the validity of this approach can be confirmed, we will go one to investigate physiological parameters which may be expected to alter the free heme levels (herbicide and inhibitor treatment, greening, etc.). Future efflux experiments will concentrate on comparing chloroplasts and mitochondria, possible energy requirements and a search for cytosolic heme binding proteins.

2) Since one expects the branch point of metabolic pathways to be regulated, it will be important to compare the properties of both enzymes leading from the branch point. Although ferrochelatase has been characterized in spinach chloroplast membranes, we feel that it is important to study both enzymes from the same tissue under as similar conditions as possible. To this end, we are beginning a characterization of the ferrochelatase in pea chloroplasts. We will use conventional techniques for this assay (pyridine hemochromogen) until such time that we can prove that the easier fluorescent assay of Zn-chelatase activity does, in fact, reflect "real" ferrochelatase activity. We are currently concerned that ferrochelatase activity *in situ* may be a function of reducing conditions and/or iron supply. These studies are just beginning.

The number of questions that we can ask (and probably answer) are quickly exceeding the number of hands required to do the experiments. Currently the heme project is the thesis project of a grad student who should be finishing up in the Spring of '91. A new graduate student has joined the lab and may take over this project. I will be seeking additional support (from NSF) for the heme project.

FIGURE LEGENDS

Fig. 1 Continuous assay of MgDp formation in intact pea chloroplasts. ATP and an ATP regenerating system were added to a mixture of intact pea plastids at zero time. Dp was added at the time indicated. The sample chamber was maintained at 30°C and the cuvette contents were not stirred.

Fig. 2 Activity as a function of protein concentration as measured in a continuous assay. The relative activities were determined by measuring the slopes of the linear part of the traces such as the one shown in Fig. 1. Note that activity is directly proportional to the amount of protein only within a limited protein concentration range. This limitation is probably due to quenching of the product fluorescence by the increasing amounts of chlorophyll present in the chloroplast membranes.

Fig. 3 Reconstitution of Mg-chelatase activity with fractions of lysed pea chloroplasts. S, stroma; T, thylakoids; E, envelope.

Fig. 4 Reconstitution of Mg-chelatase activity with fractions of lysed pea chloroplasts. In this case the chloroplasts were lysed with an ATP regenerating system comprised of phosphocreatine and creatine kinase. Note that the S fraction, alone, is very active. S, stroma; T, thylakoids; E, envelope.

Fig. 5 Stromal Mg-chelatase activity is stable enough to store at -80°C for five days. This fraction was also prepared from chloroplasts lysed with the phosphocreatine ATP regenerating system. The concave-up shape of the activity vs protein concentration curve suggests that the stromal fraction contains more than one component.

Fig. 6 Reconstitution of apo-peroxidase with the heme in lysed cucumber chloroplasts on ice. In the upper line the sample was spiked with exogenous heme. Note the slow and fast phases of reconstitution in both samples.

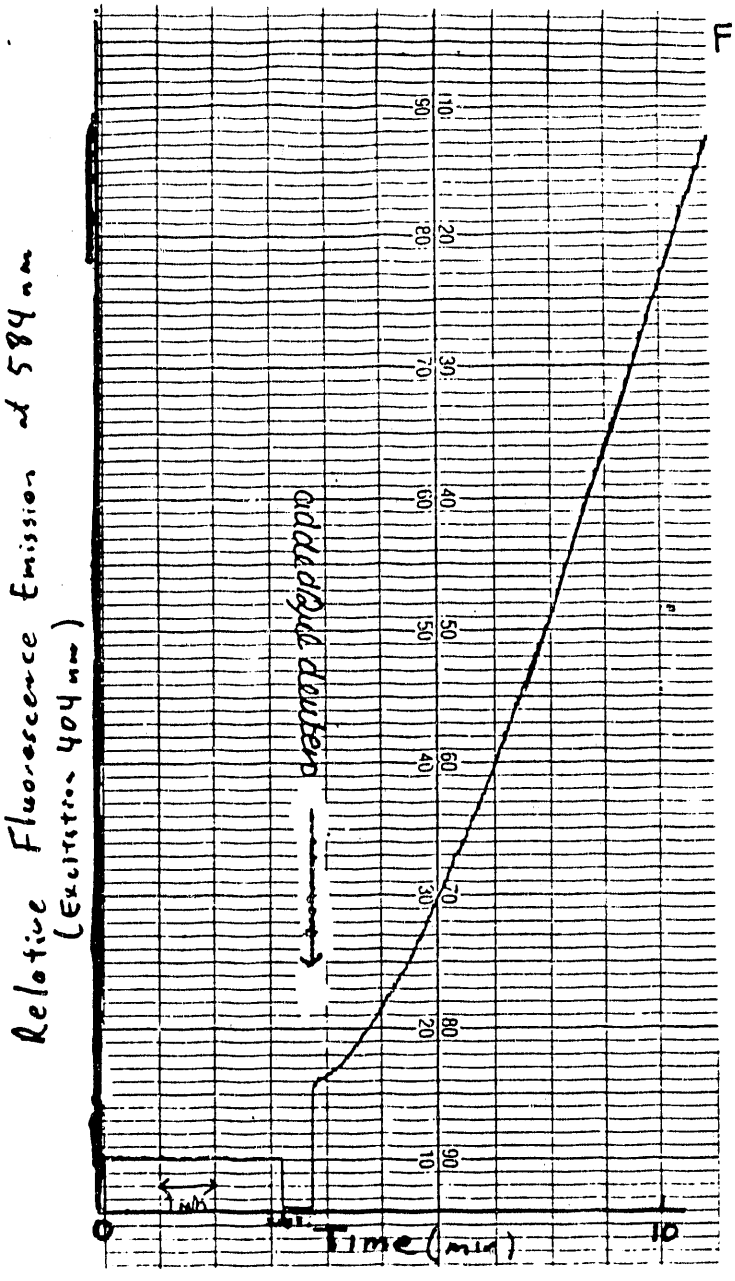
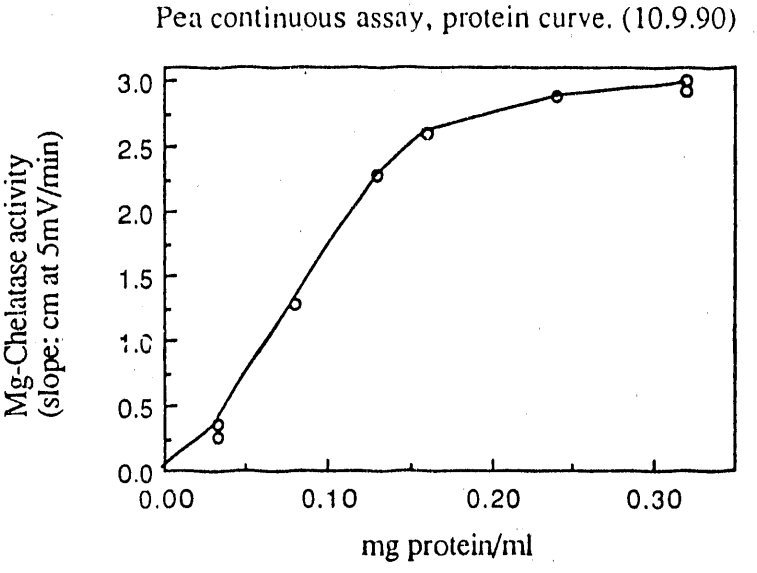


FIG 1



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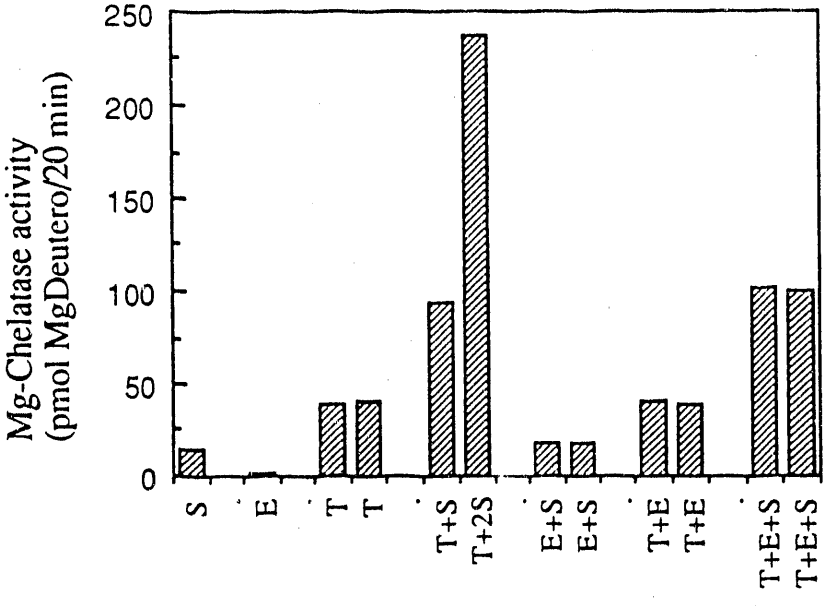


FIG 3

FIG 4

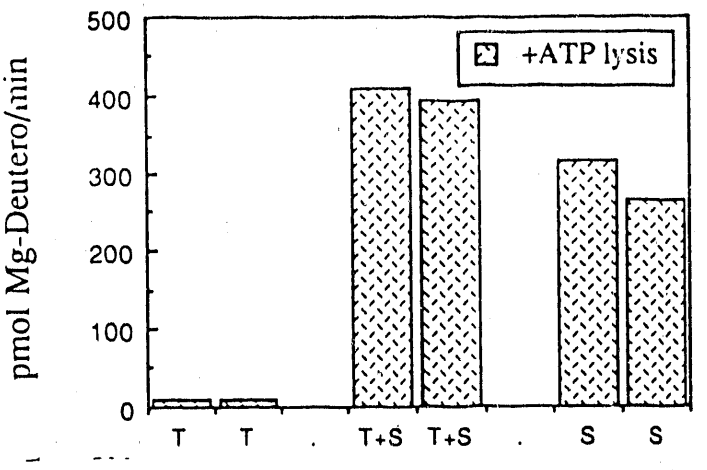


FIG 5

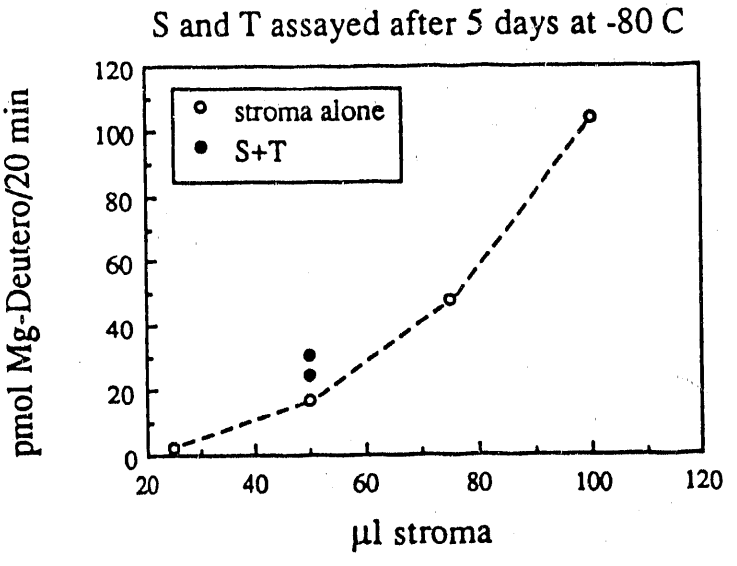
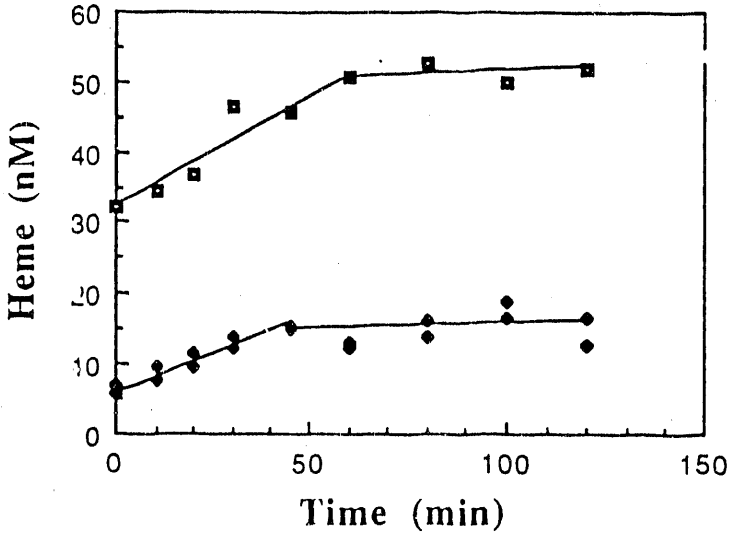


FIG 6



PROJECT SUMMARY FOR THE ANNUAL REPORT

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The Magnesium Chelation Step in Chlorophyll Biosynthesis

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In photosynthetic organisms, the biogenesis of energy transducing membranes requires the coordinate synthesis of prosthetic groups, proteins, and various lipids. Two of the major prosthetic groups, chlorophyll and heme, share a common biosynthetic pathway that diverges at the point of metal insertion into protoporphyrin IX (Proto). Insertion of iron leads to the formation of hemes, while insertion of magnesium is the first step unique to chlorophyll formation. This project is directed toward identifying the enzyme(s) responsible for magnesium chelation and elucidating the mechanism which regulates the flux of precursors through the branch point enzymes in isolated chloroplasts. Using intact chloroplasts from greening cucumber cotyledons, we have confirmed the ATP requirement for Mg-Proto formation. Use of non-hydrolyzable ATP analogs, uncouplers and ionophores has lead to the conclusions that ATP hydrolysis is necessary, but that this hydrolysis is not linked to the requirement for membrane intactness by transmembrane ion gradients or electrical potentials. The enzyme(s) are flexible with respect to the porphyrin substrate specificity, accepting porphyrins with -vinyl, -ethyl or -H substituents at the 2 and 4 positions. The activity increases approximately four-fold during greening. Possible physiological feedback inhibitors such as heme, protochlorophyllide, and chlorophyllide had no specific effect on the activity. The activity has now been assayed in barley, corn and peas, with the system from peas almost ten-fold more active than the cucumber system. Work is continuing in pea chloroplasts with the development of a continuous assay and investigation of the feasibility of characterizing an active, organelle-free preparation.

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