Final Technical Report for "The Mechanism of the Mg-chelatase
Step in Chlorophyll Biosynthesis"

Abstract

Mg-chelatase catalyzes the insertion of Mg²⁺ into protoporphyrin-IX (Proto) in the chlorophyll biosynthetic pathway. This is the first step unique to the chlorophyll pathway and is at the branchpoint between heme and chlorophyll biosynthesis. Previous work from our laboratory has shown that the enzyme from pea chloroplasts requires three distinct protein fractions (now known to contain the D, I and H subunits). The reaction requires ATP in two distinct steps: activation requiring two of the fractions (I and D) and metal ion insertion, requiring all three fractions. Work covered in this granting period includes the cloning and expression of the active form of one of the pea subunits and demonstration of the change in chromatographic behavior of the subunits upon activation with ATP

Work on chloroplast heme and chlorophyll biosynthesis has been carried out in my laboratory at Clemson University since 1988. For the most part the work has been funded by the Dept. of Energy (for which I remain most grateful). In December of 1998, I closed down my laboratory for personal reasons. During this period my colleagues and I were able to make many significant contributions to the field through our published work, contributions to national and international meetings and collaborations with colleagues. Our noteworthy work has been published in peer-reviewed journals (which are in the public domain), and there is no reason to restate the results in this report. Complete bibliographic reference to the work is given below:

- Luo, M., Weinstein, J.D. and Walker, C.J. (1999) Mg-chelatase subunit D from pea: Characterization of the cDNA, heterologous expression of an enzymatically active protein and immunoassay of the native protein. Plant Mol. Biol. 41:721-731.
- Guo, R., Luo, M. and Weinstein, J. D. (1998) Mg-chelatase from peas: Characterization of a soluble extract from chloroplasts and resolution into three required fractions. Plant Physiol. 116: 605-616.
- Walker, C.J. and Willows, R.D. (1997) Mechanism and regulation of Mg-chelatase. Biochem. J. 327, 321-333.
- Luo, M. and Weinstein, J. D. (1997) Cloning and Sequencing of a cDNA Encoding the Putative Magnesium-Chelatase Subunit D (Accession no. AF014399) from Pea (*Pisum sativum* L., cv. Spring) (PGR97-139) Plant Physiol. 115:315.
- Yu, G.-H. and Weinstein, J. D. (1997) Heme synthesis and breakdown in isolated developing pea chloroplasts. Plant Physiol. Biochem. 35: 223-234.
- Walker, C. J., Yu, G-H, and Weinstein, J. D. (1997) Comparative study of heme and Mg-protoporphyrin-IX (monomethyl-ester) biosynthesis in isolated pea chloroplasts; effects of ATP and metal ions. Plant Physiol. Biochem. 35: 213-222.
- Walker, C.J. and Weinstein, J.D (1995) Re-examination of the localization of Mg-chelatase within the chloroplast. Physiol. Plant. 94: 419-424.
- 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.

DOE Patent Clearance Granted

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- 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.
- Walker, C.J., Hupp, L.R., and Weinstein, J.D. (1992) Activation and stabilization of Mg-chelatase activity by ATP as revealed by a novel *in vitro* continuous assay. Plant Physiol. and Biochem. 30: 263-269.
- Thomas, J. and Weinstein, J.D. (1992) Free heme in isolated chloroplasts: An improved method of assay and its physiological importance. Plant Physiol. and Biochem. 30: 285-292.
- 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. Natl. Acad. Sci. (USA) 88: 5789-5793.
- 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.
- Thomas, J. and Weinstein, J.D. (1990) Measurement of heme efflux and heme content in isolated developing chloroplasts. Plant Physiol. 94: 1414-1423.

The paper at the top of the list was prepared during the last truncated funding period: February 1998 – December 1998 (with no cost extension until November 1999). While most of the experimental work for that paper was completed in the latter part of 1997 (covered under our previous DOE grant), some of the experimental tidying up and paper preparation was covered by the current grant. The work describes the expression of the D subunit of Mg-chelatase from pea in an enzymatically active form. The abstract from the paper concisely summarizes that work and is presented below:

Mg-chelatase catalyzes the insertion of Mg into protoporphyrin and lies at the branchpoint of heme and (bacterio)chlorophyll synthesis. In prokaryotes, three genes--BchI, D and H--encode subunits for Mg-chelatase. In higher plants, homologous cDNAs for the I, D and H subunits have been characterized. Since the N-terminal half of the D subunit is homologous to the I subunit, the C-terminal portion of the pea D was used for antigen production. The antibody recognized the chloroplast D subunit and was used to demonstrate that this subunit associated with the membranes in the presence of MgCl₂. The antibody immunoprecipitated the native protein and inhibited Mg-chelatase activity. Expression in *Escherichia coli* with a construct for the full-length protein (minus the putative transit peptide) resulted in induction of 24.5 kDa (major) and 89 kDa (minor) proteins which could only be solubilized in 6 M urea. However, when host cells were co-transformed with expression vectors for the full-length D subunit and for the 70 kDa HSP chaperonin protein, a substantial portion of the 89 kDa protein was expressed in a soluble form which was active in a Mg-chelatase reconstitution assay.

Unpublished work on the gene expression part of this work demonstrated that:

- An internal ribosome binding region accounted for the expression of the 24.5 kDa protein, and modification of this site with silent mutations eliminated the expression of the small protein.
- Expression of the protein was *E. coli* host strain specific.

- Modification of the D gene coding sequence (again by phenotypically silent mutation) to change codon usage to more closely mirror E.coli codon usage substantially increased the yield of the expressed protein.

In addition to the molecular work, we maintained a parallel effort on the biochemistry. Utilizing a completely soluble protein extract from pea chloroplasts we investigated the behavior of the individual subunits on a gel filtration column. Detection of the subunits relied on Western blots utilizing subunit-specific polyclonal antibodies. Our observations were as follows:

- We did not observe co-elution of all the subunits in a high molecular mass fraction indicative of a large complex containing all three subunits.
- The I subunit (42 kDa) for the most part migrated as a dimer.
- The D subunit (87 kDa) migrated in two fractions (one of very high molecular mass, the other as a monomer).
- The H subunit migrated as a monomer.
- Equilibration of the column with ATP did not substantially change these patterns.
- However preincubation of the extract with ATP did result in changes in the elution pattern. The amount of the D subunit in the high-molecular mass fraction decreased, a D peak of intermediate mass appeared, which coincided with a portion of the I subunit.

I must emphasize that these results were very preliminary and we did not have time to perform all the controls (using non-frozen extracts, etc.). The only conclusion that I would be confident of, at this time, is that preincubation of the chloroplast extract with ATP changes the mobility of some of the subunits on gel filtration. This would suggest the formation of some sort of complex.

At the conclusion of this work we made an effort to widely distribute our expression clone for the pea D subunit as well as antibodies to this subunit. In addition some almost-ready-to-publish work on the bacterial enzyme for formation of the fifth ring of bacteriochlorophyll was passed on to a colleague who was in a position to follow up on the work. We have now heard that the work has been repeated and confirmed and will be submitted for publication under the names of CJ Walker (co-PI on the present grant), D Bolivar and SI Beale with the tentative title, "Oxygen-independent Mg-protoporphyrin IX monomethyl ester cyclase activity from membranes of *Rhodobacter capsulatus*".