

# NITROGEN CONTROL OF CHLOROPLAST DEVELOPMENT AND DIFFERENTIATION

## Annual Progress Report

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## Project Summary

### Nitrogen Control of Chloroplast Development and Differentiation

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The growth and development of plants and photosynthetic microorganisms is commonly limited by the availability of nitrogen. When the synthesis of amino acids, nucleic acids and pigments is restricted by nitrogen deprivation, these organisms appear to respond by altering the activities of photosynthesis and other metabolic pathways. Hence, the classical symptom for nitrogen deficiency is chlorosis. Our work concerns understanding the mechanisms by which plants and algae that are subjected to nitrogen deprivation alter the composition of photosynthetic membranes and enzymes involved in photosynthetic carbon metabolism. Toward these ends, we study biosynthetic and gene expression processes in the unicellular green alga *Chlamydomonas reinhardtii* which is grown in an ammonium-limited continuous culture system. We have found that the expression of nuclear genes, including those encoding for light-harvesting proteins, are severely repressed in nitrogen-limited cells whereas, in general, chloroplast protein synthesis is attenuated primarily at the level of mRNA translation. Conversely, nitrogen deprivation appears to lead to enhanced synthesis of enzymes that are involved in starch and storage lipid deposition. In addition, as a possible means by which photosynthetic electron transport activities and ATP synthesis is sustained during chronic periods of nitrogen deprivation, thylakoid membranes become enriched with components for chlororespiration. Characterization of the chlororespiratory electron transport constituents, including cytochrome complexes and NAD(P)H dehydrogenase is a major current effort. Also, we are striving to isolate the genes encoding chlororespiration proteins toward determining how they and others that are strongly responsive to nutrient availability are regulated.

### 1990-1991 Progress Report and Continuation Request

During the past two years we have strived to resolve three major aspects of the effects of nitrogen availability on chloroplast biogenesis and physiology: 1) Isolation and characterization of cloned nuclear genes that are differentially expressed as a function of nitrogen availability; 2) Biochemical and physiological characterization of the components of the chlororespiratory electron transport pathway that is prominent in nitrogen-limited chloroplasts; 3) Resolution of the consequences of impaired synthesis of chlorophyll and/or carotenoids, a classical symptom of nitrogen-deficiency, on the expression of nuclear and chloroplast genes encoding pigment-binding proteins. Two publications, one of which is in review, have been generated and are appended.

The identification of nuclear genes that are differentially expressed as a function of nitrogen levels provided to continuous cultures of *Chlamydomonas* entails a rather novel protocol. As outlined in the original proposal, the small nuclear genome size of *Chlamydomonas* enables direct screening of genomic lambda phage libraries via plaque hybridization with radiolabeled cDNAs generated by reverse transcription of mRNA preparations from control vs. N-limited cultures. Although a multitude of plaques that exhibit positive or negative control by nitrogen have been identified and confirmed to be differentially expressed, we discovered that it was necessary to modify the procedure for plaque-purification and subcloning of the 13-22 kDa inserts. This was dictated by the finding that many of the phage harbor genes that are constitutively expressed in addition to contiguous loci that are nitrogen-regulated. Hence, differential hybridization signals become diminished as plaque purification proceeds due to saturation with radiolabeled, constitutive cDNA probes. In these

instances, we have resorted to amplification of phage from unpurified plaques and utilization of excised inserts for Southern blot analyses with the N-limited and control cDNAs. Upon identification of the gene fragments that are differentially expressed, preparative agarose gel electrophoresis is employed for isolation of the DNA of interest and its subcloning into plasmids. These, together with larger clones that have been successfully obtained through plaque-purification, are then used to reaffirm gene copy number in Southern blots, to characterize the transcription products detected in northern blots, and finally to assess the nature the gene product and its possible regulatory features by sequencing. The last aspect of this project has just been initiated and will be a major effort during the forthcoming funding period.

As established in the accompanying reprint, a somewhat surprising characteristic of nitrogen-limited cells is the high activity of chlororespiration and the coincidental high abundance of NAD(P)H dehydrogenase and novel cytochromes (h1 and h2) associated with thylakoid membranes. We have proposed that the development of this activity enables the maintenance of the photosynthetic electron transport chain under conditions when CO<sub>2</sub> reduction might be (or need to be) attenuated. Thus, nitrogen-limited chloroplasts become somewhat distended with starch and triglyceride deposits when cell growth and division is restricted by limitations of amino acids and nucleotide. There are several outstanding problems about chlororespiration that include: 1) Is the pathway requisite for prevention of sundry photoinhibitory reactions and, hence, cell survival? 2) What are the biochemical features of the chlororespiratory electron transport chain with regard to the dehydrogenase, putative cytochrome b<sub>c</sub> complex and cytochrome oxidase? 3) Where are the subunits of chlororespiratory electron transport encoded?

For problem 1, it will be necessary to generate mutants with impaired chlororespiratory activity and test for their ability to survive nitrogen deficiency. Alternatively, specific inhibitors of chlororespiration might be employed but the best of these, antimycin and myxothiazol are also inhibitors of mitochondrial respiration. Moreover, since we control the extent of nitrogen deficiency through the use of a continuous culture system, the inhibitor approach would be quite costly. Hence, the mutant approach is more appropriate if useful strains can be selected. We have obtained one mutant candidate accidentally. Mutant 6.2f was isolated as a high fluorescence mutant but, unlike other strains of this phenotype, no defects in photosynthetic electron transport could be discerned. However, the light-harvesting apoproteins of this strain appeared to migrate slightly differently than those of wild-type cells. As this could be due to a posttranslational modification, we studied *in vivo* phosphorylation of the LHC proteins. Whereas LHC proteins normally are phosphorylated in a light-dependent manner, those of 6.2f are phosphorylated also in darkness. We reasoned this could be due to a block at the level of terminal chlororespiratory electron transport, causing a reduction of the plastoquinone pool to activate the LHC protein kinase. Hence, an assessment of the chlororespiratory activity in the mutant was made to find that in fact it is diminished. Coincidentally, there is a deficiency of one of the novel cytochromes, h2, but it is not completely absent. The leakiness of 6.2f is also apparent in nitrogen-limiting growth conditions: its growth is diminished but the cultures can be sustained. This experience indicates that it may be possible to isolate tight chlororespiratory mutants on the basis of elevated chlorophyll fluorescence. However, there is no obvious means to efficiently discriminate between chlororespiratory vs. photosynthetic mutants except by brute, biochemical methods. Consequently, the mutant hunt is not ongoing. In the future, it may be possible to generate chlororespiratory mutants directly via transformation with *in vitro* mutated genes encoding subunits of the complexes. This awaits characterization of the chlororespiratory proteins and their genes.

A second year graduate, Jeff Blanchard, has embarked upon purification of the chlororespiratory NADH dehydrogenase and also has initiated characterization of the h1 and h2 cytochromes. With non-ionic detergents, solubilized thylakoids from N-limited cells yield membrane complexes that have been fractionated by sucrose gradient centrifugation. Cytochromes h1 and h2 co-sediment as high-molecular weight complexes that pellet with Photosystem I particles. The dehydrogenase is of smaller size and co-sediments with the residual light-harvesting complexes of N-limited thylakoids. Although these complexes are present in low yields from control cells grown mixotrophically, we have decided that an expedient means to purify the complexes away from photosynthetic contaminants is through the use of *Chlamydomonas* mutants that are missing LHC, PSI or both. Consequently, it should be possible to obtain intact complexes suitable for spectroscopic analyses and protein purification. An alternative method is to employ affinity chromatography, with immobilized cytochrome c to enrich for cytochrome oxidase. Finally, we have obtained or requested antibodies to subunits of cytochrome oxidase, some of which have been shown to cross-react with the corresponding proteins of bacteria and cyanobacteria. These can be used for immunoaffinity purification as well as measurements of immunological relatedness via immunoblotting. We have reported that the major subunits of the dehydrogenase have molecular sizes that are close to that of cyanobacteria (Peltier, G., and G.W. Schmidt, 1991, Proc. Natl. Acad. Sci. USA, 88:4791-4795) and hence, we expect the chlororespiratory components to be structurally related to the complex of the prokaryotic plasma membrane. Following purification of the major subunits, they will be subjected to proteolytic fragmentation and microsequencing. Oligonucleotide probes to nondegenerate amino acid sequences will be employed to screen the genomic lambda library and differentially regulated genes that have been described above.

The final problem we have assessed, the roles of pigment synthesis in the expression of pigment-binding proteins, has been completed and is described in the accompanying preprint. We have shown that N-limitation leads to chlorophyll deficiency, loss of the major transcripts encoding the nuclear-encoded LHC apoproteins and an apparent attenuation of the translation of chloroplast mRNAs that encode light-harvesting and reaction center proteins (Plumley, F.G. and G.W. Schmidt, 1989, Proc. Natl. Acad. Sci. USA, 86:2678-2682). It has not been clear whether these effects are due to a direct activity of nitrogen-signal systems or whether it is more closely related to impaired pigment synthesis. Since this problem is nearly intractable in studies with N-limited cells, we recruited a series of *Chlamydomonas* mutants that are blocked in chlorophyll or carotenoid synthesis. In addition, the involvement of light in the expression of the genes encoding these proteins was examined. It is obvious that regulation of nuclear LHC gene expression and chloroplast mRNA translation are not affected by pigment synthesis in the alga since normal levels of the transcripts are present and normal rates of chloroplast protein synthesis are obtained. However, both chlorophyll and carotenoid are required for stabilization of the chloroplast-encoded proteins, which when synthesized in their absence, are degraded following thylakoid insertion. It also appears degradation is a major means of regulating the accumulation in thylakoids of the LHC apoproteins in the absence of the pigments since their synthesis can be detected. However, the nuclear-encoded LHCs seem to be synthesized at reduced rates, raising the possibility of translational controls elicited by pigment-deficiency. Translational regulation is even more conspicuous in dark-grown, chlorophyll-less cells but, under these conditions, the level of LHC transcripts is also altered in a manner similar to, but less severe than, that of N-limited cells. Darkness also affects the translation of the chloroplast-encoded *psbA* mRNA. However, this effect is likely due to differences in the rate of selective turnover of this protein in the light which, in turn, somehow results in enhanced translation of the *psbA* mRNA. Overall, these studies show that nitrogen control of nuclear gene expression and also that the nitrogen effects on chloroplast protein synthesis are unrelated to pigment synthesis. In the latter case,

N-limitation, seems to affect chloroplast protein synthesis at the translational, as opposed to the post-translational, level.

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