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TECHNICAL PROGRESS REPORT

NITROGEN CONTROL OF CHLOROPLAST DEVELOPMENT AND DIFFERENTIATION

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PROJECT ABSTRACT

This project concerns the role of nitrogen availability in regulation of photosynthetic gene expression in the nuclear and chloroplast compartments. These studies exploit a continuous culture system wherein *Chlamydomonas reinhardtii* is maintained at a steady-state of nitrogen-limited growth. Nitrogen deficiency leads to alteration of light-harvesting capabilities, augmentation of chlororespiratory electron transport flow by thylakoid membranes and redirection of photosynthetic carbon metabolism toward the accumulation of storage carbohydrates. Recovery from nitrogen-deficiency with accompanying changes in gene expression rapidly follows provision of ammonium to the cells. Previously, we found that expression of nuclear genes encoding apoproteins of light-harvesting complexes and, to a lesser extent, the small subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase, are strongly regulated at the level of mRNA accumulation. Chloroplast activities are also strongly influenced by nitrogen availability but the effect is primarily due to restrictions of the translation of some, but not all, organellar mRNAs. No qualitative or quantitative effects on chloroplast (or mitochondrial) transcripts have been detected in northern analyses performed to date nor is there an appreciable effect of nitrogen limitation on the abundance of organellar DNA. In the course of further studies of this system, we hope to 1) clone and characterize additional nuclear gene loci that are subject to negative or positive regulation by nitrogen availability; 2) establish whether the effects on nuclear transcripts are due to transcriptional or posttranscriptional events; 3) characterize nitrogen-regulated genes through sequence analysis, enabling identification of the functions of their protein products and potential regulatory sequences for nitrogen responses in flanking regions of these genes; 4) extend analyses of chloroplast transcription to reveal previously undetected nitrogen control; 5) characterize the genetic and protein components for chlororespiration, an activity that is especially prevalent in nitrogen-limited cells.

BACKGROUND AND SIGNIFICANCE

A premise of our work on the regulation of photosynthetic gene expression by nitrogen is that this nutrient, more than any other environmental component, commonly limits growth in eukaryotic algae and vascular plants. However, adaptive mechanisms must exist to enable photoautotrophic physiology to be sustained under conditions of nitrogen limitation. Thus, we reason that plants and eukaryotic algae have nitrogen-dependent devices for modifying photosynthetic electron transport and carbon metabolism pathways in order to accommodate restrictions on amino and nucleic acid as well as chlorophyll synthesis. This concept is supported from the classical acknowledgement of the chlorotic phenotype that connotes nitrogen deficiency. However, this nutrient and/or its assimilation products plays more complex regulatory roles than as a simple mediator of metabolic change. In vascular plants, cell division patterns and differentiation are also influenced by nitrogen availability (MacAdam et al., 1989). In unicellular algae (and fungi), deficiency of nitrogen or other nutrients can promote gametogenesis in the sexually-competent species.

Together, the classical perceptions of nitrogen effects support a concept that somehow it also functions as an important component for regulation of gene expression at the transcriptional to the posttranslational levels. Our studies so far indicate that nitrogen assimilation products truly are significant regulatory factors for chloroplast development. Nitrogen availability alters cellular functions at the levels of gene expression, photosynthetic electron transport, and photosynthetic carbon metabolism. However, there is limited knowledge concerning how these changes are brought about, beginning with the initial signal and its transmission to the nuclear and chloroplast compartments. A thorough understanding of the regulatory effects of nitrogen and other nutrients is necessary for a complete comprehension of how environmental signals mediate plant growth and development.

PROGRESS DURING THE PREVIOUS FUNDING PERIOD

Our broad research interests concern resolution of the molecular processes required for chloroplast biogenesis. The model system for the work is *Chlamydomonas reinhardtii*, a unicellular alga that is uniquely amenable to genetic as well as molecular analyses of nuclear-chloroplast interactions. In work supported from other funding agencies as well as by DOE, we have isolated and characterized a broad range of nuclear mutants that have defects in classical and, recently, arcane functions of the photosynthetic membrane. The mutant strains provide a basis for molecular dissection of regulatory functions and interactions that occur during formation of the multisubunit complexes in thylakoids (Jensen et al., 1986; Schmidt et al., 1987; Herrin and Schmidt, 1988). The mutants have served to identify protein components required for photosynthetic function in wild-type cells (i.e. Herrin et al., 1987, Peltier et al., in prep.) in addition to ordering electron transport pathways (Levine, 1968). As important, *Chlamydomonas* is haploid, has a relatively small genome size (1.2 X 10^8 b.p; 24 times that of *E. coli* (Howell and Walker, 1976), and is becoming increasingly tractable for direct molecular

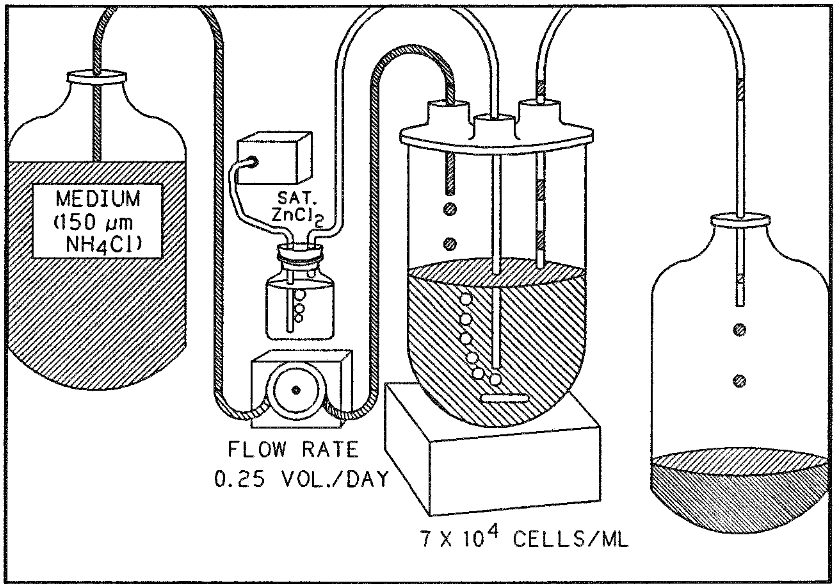


Figure 1 Nitrogen-Limited Continuous Culture System

manipulation via transformation (Boynton et al., 1988). Finally, *Chlamydomonas* can be grown in synchronous and/or continuous culture systems. With regard to the latter, we have analyzed extensively the consequences of nitrogen limitation on biogenesis of chloroplasts using precisely defined chemostat conditions (Fig. 1). By altering either rates of dilution or the concentration of ammonium in the medium reservoir, cells that are limited in growth only by nitrogen can be maintained at a steady state of deficiency. With the standard parameters illustrated, a severely chlorotic phenotype is established, mimicking the nitrogen-deficiency syndrome of vascular plants. Much of our effort has concerned biophysical, biochemical and molecular characterization of cells in the nitrogen-limited state and in cells that are induced to "regreen" upon optimal provisions of nitrogen. Some of the results of these studies are presented in the appendices (Plumley and Schmidt, 1989, Plumley et al., 1989) and can be summarized as follows:

1. Nitrogen-limited cells are highly chlorophyll deficient but are relatively enriched in envelope and/or plastoglobular xanthophylls.

2. The chlorophyll deficiency is largely due to a reduction in the amount of thylakoid membrane per cell but also affects the relative amounts of chlorophyll-protein complexes.

3. Light-harvesting complexes for Photosystem II are depleted but less severely than those for Photosystem I as determined from stained gel profiles of purified thylakoids, immunoanalyses of whole cells and biophysical measurements of light-harvesting efficiency. Thus, there is an increase in the chlorophyll *a* to chlorophyll *b* ratio. In vivo fluorescence and circular dichroic spectroscopy (Plumley and Schmidt, 1989a,b and unpublished) reveal the presence of novel arrays of light-harvesting pigments in the nitrogen limited cells. Recent work indicates that there is virtually no light-harvesting for Photosystem I (Peltier and Schmidt, in

prep.); in part, the reduction of the antenna complex accounts for the increased electrophoretic mobility of the Photosystem I reaction center complex in LDS-polyacrylamide gels run at 4° (Fig. 2, Appendix 1).

4. Although Photosystem I is altered with regard to peripheral complexes of chlorophyll, the PS I reaction center apoproteins are present at relatively higher levels than are the products of the pcbB and pcbC Photosystem II genes (see Fig. 2 of Appendix 1 and Fig. 8 of Appendix 2). Apparently, a compensation for the absence of LHCP I in nitrogen-limited cells is to accumulate more PS I reaction center apoproteins. This conjecture is supported by results from pulse-labeling studies (Fig. 4, Appendix 1) showing relatively high rates of synthesis of the chloroplast-encoded PS I reaction center apoproteins.

5. The deficiency of light-harvesting complexes in nitrogen-limited cells correlates with diminshed mRNA accumulation, at least for the nuclear-encoded apoproteins of LHCP II (Appendix 1). In contrast, there are modest effects on the total abundance of the RuBPCase small subunit mRNAs although nitrogen-limitation does differentially repress accumulation of the 1.1 kb rbcS1 transcript relative to that of the 0.85 kb species from rbcS2. This effect is similar to that reported by Goldschmidt-Clermont and Rahire (1986) for wild-type cells grown in darkness wherein the rbcS1 transcripts are not found.

6. Alteration of the levels of nuclear transcripts and effects on chloroplast protein synthesis are not a consequence of a generalized stress response: synthesis of heat shock proteins cannot be detected in nitrogen-limited cells (Appendix 2).

7. Chloroplast protein synthesis is differentially affected by nitrogen limitation. Pulse-chase labeling reveals normal rates of synthesis of D1 of PS II and high rates of synthesis of the 65 kDa PS I apoproteins. In contrast, synthesis of the chlorophyll-binding polypeptides of the psbB and psbC as well as of the large subunit of RuBPCase is substantially reduced. The effect is translational inasmuch that in no instance there is a conspicuous reduction or qualitative alteration of chloroplast transcripts that we have examined in dot-blot or northern blot assays. In part, translational regulation might be attributed to diminished synthesis of chlorophyll *a*, an apparent effector of protein synthesis in chloroplasts (Laing et al., 1988). However, other translation factors (Jensen et al., 1986; Gamble et al., 1989.), absent from nitrogen-limited cells, could also be responsible for this effect. We observe that the rate of synthesis of the psbB and psbC proteins does not parallel that of chlorophyll accumulation in cells responding to nitrogen provisions (Appendix 1.)

8. Photosynthetic carbon metabolism is altered toward the accumulation of massive levels of starch and triglycerides in nitrogen limited cells. This is apparent even in electron micrographs (Appendix 2) but, also, these have been analyzed quantitatively and qualitatively (Fig. 2.). When limited cells are provided nitrogen, both the lipid and starch reserves are rapidly metabolized, leading to the normal state wherein triglycerides are not detectable. These results suggest that, in contrast to LHCP II, nitrogen

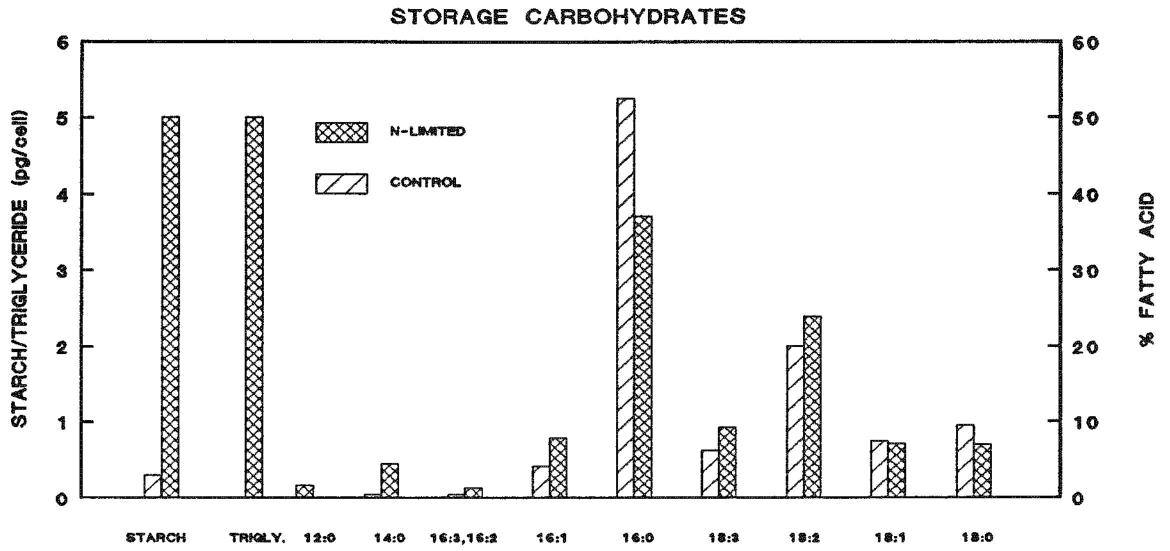


Figure 2: STARCH AND LIPIDS OF N-LIMITED CELLS

may suppress expression of genes that encode enzymes for starch and triglyceride synthesis. To begin assessment of this possibility, part of our efforts during the past year have led to isolation and partial characterization of mutants that are blocked in starch synthesis. In one case, a mutant is capable of starch synthesis if grown in the nitrogen-limited chemostat (Berry-Lowe and Schmidt, in prep.). Thus, nitrogen deficiency could activate expression of unique gene loci for formation of storage carbohydrates or enhance the activation of constitutive enzymes.

9. Nitrogen limited cells have "chlororespiratory" activities that are almost ten-fold greater than that of control cells (Peltier and Schmidt, in prep.). The evidence for the enhancement of this activity includes 1) kinetics for fluorescence induction under anaerobic versus aerobic conditions; 2) kinetics of light-inhibition of oxygen uptake as measured with a bare platinum electrode system in conjunction with strobe flashes; 3) evaluation of thylakoid state transitions (nitrogen-limited cells are in state II in darkness unlike control cells); 4) assays of NADH and NADPH oxidoreductase activities associated with thylakoid membranes and preliminary characterization of these after their isolation by sucrose density gradients of detergent extracts as well as in non-denaturing polyacrylamide gels. We suppose that this pathway develops preferentially under conditions of N-limited growth because cell growth and division is severely restricted and reduction of carbon dioxide to carbohydrates becomes a sated, inconsequential process. Dissipation of energy toward ATP accumulation could become a means for ensuring cell viability/motility until more suitable environmental conditions are encountered. Part of our proposed research effort is to more thoroughly define the components of chlororespiration and to study their biogenesis. A propitious supplement for this project is a mutant, isolated some time ago in our laboratory, with electron transport anomalies that were enigmatic: the strain was found

to be high fluorescent but possessed intact photosynthetic electron transport. Dr. Gilles Peltier, a visiting scientist in our laboratory from C.E.N. de Cadarache, St-Paul-lez-Durance, has determined that the strain is missing chlororespiratory function. Together, studies of the mutant and nitrogen-limited cells should provide insight as to the significance of chlororespiration as an alternative electron transport pathway and, perhaps, a role for the NADH dehydrogenase-like loci of chloroplast genomes (Ohyama et al., 1988).

SCIENTIFIC BACKGROUND FOR FUTURE STUDIES

The regulatory factors that have been studied intensively with regard to photosynthetic gene expression have included light, phytohormones, cell differentiation, diurnal rhythm and the effects of inhibitors of pigment synthesis (see Mullet, 1988 for review). In contrast, relatively little effort has been made to examine the effects of nitrogen on these processes. We have shown that light-harvesting genes are especially sensitive to nitrogen availability and we will continue to study the basis of these effects. In addition, chloroplast activities are affected mostly, but not universally, at the translational level. Although the means by which translational restrictions are imposed is of considerable interest to us, it will be a subject of study in a separate project related to nuclear gene products that selectively influence utilization of plastid mRNAs by ribosomes. However, we have not eliminated the possibility that certain chloroplast genes are transcriptionally regulated by nitrogen assimilation products and therefore will examine this further. Of particular interest is a recent finding that chlororespiratory activity is especially high in nitrogen-limited cells. Because no surveys of chlororespiration have appeared, background information about this process, at the expense of reiteration of reviews about more familiar aspects of chloroplast biogenesis, may be of use for reviewers of this proposal.

CHLORORESPIRATION

A conceptual framework for this pathway includes the notion that if plastids are evolutionary derivatives of photosynthetic prokaryotes, it is possible that the capacity for oxidative and reductive electron transport chains can function simultaneously on the thylakoid membrane (see Figure 3). In the dark and when NAD(P)H or other low potential donors are in excess of the requirement for carbon, sulfur and nitrogen reduction in the organelle, electron donation to the plastoquinone pool can occur. In addition to the activity of PS I, oxidation of the PQ pool occurs through additional electron carriers (presumably cytochrome complexes) to oxygen as the final acceptor. Various kinds of evidence in support of the existence of chlororespiration has accumulated since the original studies of Kok (1949) on nonlinearity in the progression of net oxygen exchange in *Chlorella* as a function of light intensity. However, the original interpretation of the "Kok effect" that light might directly depress mitochondrial respiration was subsequently found instead to be due to chlorophyll-dependent effects on the activities of the photosynthetic membrane (Goedheer, 1963; Hoch et al., 1963). Later, Healy (1970), Ben-Amotz and Gibbs (1975) and Godde and Trebst (1980) detected DCMU-insensitive and rotenone-insensitive NADH photooxidation in studies of hydrogen photoevolution. The NADH dehydrogenase of *Chlamydomonas* thylakoids was partially purified and biochemically characterized in Triton X-100 solubilized preparations by Godde (1982) who also determined that oxidized plastoquinone is required for the activity since DBMIB, blocking cytochrome b complexes, was an effective inhibitor of NADH oxidation. Godde's preparation possessed spectral properties of a flavoprotein containing iron-sulfur groups. Concurrently, Bennoun (1982) reported evidence for a thylakoid respiratory chain through studies of the fluorescence induction kinetics of cells subjected to anaerobic adaptation. In the absence of oxygen as an alternative electron acceptor for reduced plastoquinone in a mutant lacking PS I, electron transport from the PS II reaction center is strongly impaired, yielding a fluorescence rise toward the maximal levels normally observed when cells are treated with DCMU. Inhibitors of the oxidation of the plastoquinone pool (by NAD(P)H) in darkness include carbon monoxide, cyanide, and dicyclohexylcarbodiimide, indicating the involvement of cytochrome b and cytochrome oxidase homologues; SHAM (salicylhydroxamic acid) was shown to be ineffective as an inhibitor. Bennoun also showed directly that NADPH and NADH could reduce the plastoquinone pool in cell free extracts from the PS I mutant strain. Finally, delayed fluorescence (luminescence) analyses indicated the generation of a pH gradient as a consequence of chlororespiration. In a subsequent report, Bennoun (1983) studied several photosynthetic electron transport mutants and demonstrated mutant ac21, missing cytochrome b_6/f, still possesses active chlororespiration. Thus, the only shared electron transport component for chlororespiration and photosynthesis appears to be plastoquinone whose oxidation state is dependent upon both activities. The interface of these pathways is diagramed in Fig. 3.

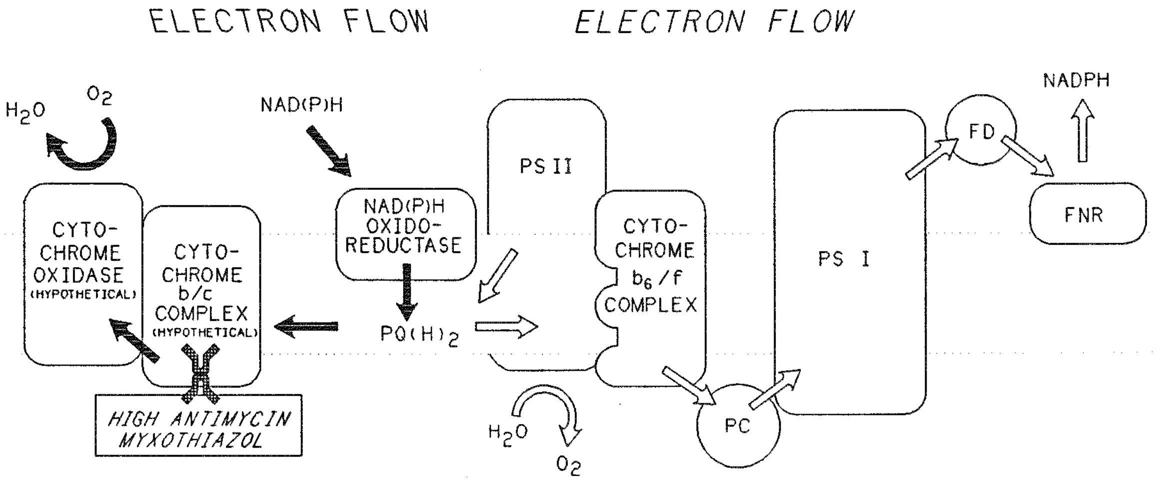
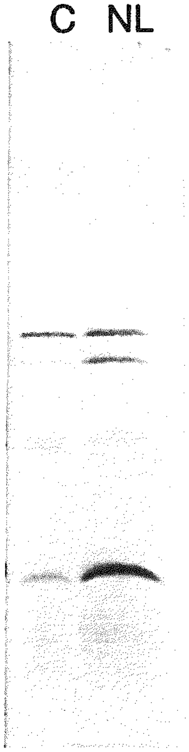


Figure 3. CHLORORESPIRATORY AND PHOTOSYNTHETIC ELECTRON TRANSPORT.

Direct demonstration of chlororespiratory oxygen consumption was demonstrated by Peltier, et al. (1987). They used cells deposited on a bare platinum electrode subjected to saturating light flashes and mass spectroscopy measurements in the presence of $^{18}O_2$. Due to PS I oxidation of the reduced PQ pool (competitively inhibiting respiratory electron flow to oxygen), a transient 20% increase in the stationary oxygen yield was detected. This correlated with an inhibition $^{18}O_2$ consumption as opposed to stimulation of $^{16}O_2$ liberation through water photolysis. Additional studies of these workers showed the requirement of PS I for inhibition of chlororespiration whereas DCMU, SHAM and antimycin A were ineffective.

Genes (ndhA-F) encoding for subunits corresponding to NADH dehydrogenase are apparently ubiquitous in vascular plant chloroplast genomes (Meng et al., 1986) and are transcriptionally active (Matsubayashi et al., 1987; Schantz and Bogorad, 1988; Steinmuller et al., 1989). However, it has not been possible to detect the protein products or establish their function. In contrast, *Chlamydomonas* thylakoids possess NAD(P)H dehydrogenase activity, but the genes for these have were not detected in at least one heterologous probe attempt (Schantz and Bogorad, 1988). As potential components for the plastoquinone oxidation moieties of chlororespiration, novel cytochromes and/or heme-containing proteins are present in thylakoids of *Chlamydomonas* (Lemaire et al., 1986; Rolfe et al., 1987; Peltier and Schmidt, in prep.). Some of these are missing in the chlororespiratory mutant we have isolated (Peltier, Sieburth and Schmidt, in prep.) and are greatly enhanced in thylakoids from nitrogen limited cells as determined by TMBZ staining of polypeptides resolved by gel electrophoresis (Fig. 4). Measurements of NADH dehydrogenase activities, using menadione as an electron acceptor, also support the concept that chlororespiration becomes a major pathway in thylakoids of nitrogen-limited cells (Table 1).

Figure 4. TMBZ Staining of Thylakoid Polypeptides from Control and Nitrogen-Limited Cells



Purified thylakoids from control and nitrogen-limited cells were electrophoresed in 10-20% polyacrylamide gels after heating at 90° for 1 min. Heme-containing polypeptides were visualized by staining with TMBZ and hydrogen peroxides as described by Francis and Becker (1984).

TABLE 1. NADH OXIDASE ACTIVITY OF THYLAKOID MEMBRANES

	nM NADH OXIDIZED/MIN·MG CHLOROPHYLL
CONTROL CELLS (AUTOTROPHIC)	5
CONTROL CELLS (PHOTOHETEROTROPHIC)	7
NITROGEN LIMITED CELLS	29
Anacystis variabilis (From Alpes et al., 1989)	30

Assay conditions modified from Alpes et al, 1989 with menadione as exogenous electron acceptor.