Toward resolution of the physiological, biochemical and molecular bases for how the photosynthetic capacity of plants is regulated in response to nitrogen deprivation, we employ the unicellular green alga *Chlamydomonas reinhardtii* as a model. With chemostat cultures, steady-state levels of nitrogen can be precisely controlled and N-limited cells exhibit the classical symptoms of deficiency of this nutrient, chlorosis and slow growth rates. They respond to nitrogen provision by rapid greening, providing a superior system for dissection of molecular and metabolic changes that rapidly ensue during the nutrient response. As the photosynthetic apparatus is most conspicuously affected by N-deprivation, the research necessarily confronts issues of the structure and function of photosynthetic complexes and how they are modulated. Complementary studies with *Chlamydomonas* mutants facilitate defining regulatory and physiological interactions of the chloroplast, nuclear and mitochondrial compartments.

Our work has been on three basic aspects of the nitrogen-deficiency problem:

- Consequences of N-limitation on synthesis of pigments and pigment-binding proteins
- Effects of N-limitation on expression of nuclear and chloroplast genes encoding photosynthetic proteins
- Effects of N-limitation on metabolic, electron transport and photoprotection pathways.

For the last, principle components are enhanced mitochondrial respiratory activity intimately associated with oxidation of photosynthesis products. This appears to result from the accumulation of saturating levels of starch and triglycerides in the slowly dividing cells upon adaptation to N-limitation. Consequently, various activities are altered, seemingly to augment oxidation activities on the one hand and protection against photoinhibition on the other. Thus, dark-adapted N-limited cells display a supplemental pathway for development of a strong thylakoid membrane potential. Upon illumination, highly effective mechanisms for quenching of excess excitation energy are detected. These features of N-limited cells are likely to enable cell survival, especially under the combined-condition of irradiance stress. Still, we have limited information concerning the number of components that could participate in protection from photoinhibition and whether they are enhanced in N-limited cells. As an expansion of this aspect of the project, we also initiated a related project on photoinhibition in symbiotic dinoflagellates of reef-building corals and found that there is a striking adverse effect of elevated temperatures on the integrity of the photosystem II reaction center and this seems to be the major cause of coral bleaching.

With steady-state chemostat cultures, the effects of defined concentrations of nitrogen and the effects of its deficiency can be studied. N-limited (NL) cells exhibit the classical symptoms of N
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deficiency, chlorosis and slow growth rates. Cells respond to N provision by rapid greening, providing a superb system for dissection of molecular and metabolic changes that ensue during the nutrient response. Chlorosis overlies modifications of principle metabolic pathways that result when protein and pigment synthesis is restricted. With mutants that are phenocopies of NL cells, we address effects of N-limitation and its recovery on thylakoid biogenesis and activities. A stringent requirement for N availability in the transcription of nuclear genes for light-harvesting complexes (LHCs) coincides with similar control of expression of genes encoding chlorophyll biosynthesis enzymes. Synthesis of most plastid-encoded proteins is regulated at the translational level. Finally, non-photochemical quenching (NPQ) mechanisms, dependent on mitochondrial activities, are also striking features of NL cells.

**Chlorosis.** Severe chlorosis is the classical symptom for N-deficiency but how deprivation of the nutrient attenuates pigment biosynthesis has not been understood. Consequently, we measured expression encoding genes for Chl synthesis components during N-deprivation of the cells and their recovery during N-induced greening. It is unlikely that chlorosis is due to limited amounts of δ-aminolevulinic acid (ALA) as the result of deficiency and/or charging of the pathway's initial substrate, glutamyl tRNA. In *Chlamydomonas*, two chloroplast *trnE* genes (differing in their non-transcribed flanking sequences) give rise to identical transcripts. There are no significant differences in the abundance of *trnE* transcripts between NL and control cells. In contrast, expression of nuclear-encoded glutamate 1-semialdehyde aminotransferase, ALA dehydratase, and coproporphyrinogen oxidase are under strong, but not absolute, N control. Thus, key enzymes for Chl synthesis are subject to N-regulated gene expression. However, there are further controls that are superimposed. A feature of NL cells is that normally abundant mRNAs encoding LHC proteins are replaced by lower amounts of transcripts from other *lhc* gene family members. Transcription analyses also indicate that, similar to the enzymes for Chl synthesis, the genes for normally high abundance *lhc* mRNAs largely are repressed under N limitation. Depletion of LHCs in NL cells is largely due to low *lhc* mRNA levels. This contributes substantially to the chlorotic phenotype. In pigment pulse-labeling studies of mutants deficient in Chl or with incompletely assembled LHCs, we found that the extent of Chl *a* and Chl *b* synthesis is precisely regulated by the rates of synthesis and proper thylakoid insertion of LHC proteins. Thus, endpoints of Chl utilization are apparent determinants for activity of protochlorophyllide reductase, chlorophyllase and/or the enzymes responsible for chlorophyll(ide) *a* to chlorophyll(ide) *b* conversion.

Fine tuning of the Chl synthesis and LHC biogenesis occurs by feedback pathways as deduced from studies of Chl synthesis inhibitors and mutants blocked in the pathways. Accumulation of magnesium protoporphyrin methyl ester by *a*,*a*-dipyridyl treatment of *Chlamydomonas* was found to result in decreases of *lhc* transcripts while blocking formation of ALA or utilization of protoporphyrin IX had no effect on gene expression. However, mutants such as OE9, blocked in utilization of protoporphyrin IX, exhibit over-expression of *lhc* transcripts but these have diminished *lhc* translation. Over-expression of *lhc* genes, but not those encoding porphyrin synthesis enzymes, is also observed in Chl-deficient mutants PA2.1 and pg113. In contrast, pulse-labeling transcription assays with protochlorophyllide accumulating mutants showed rapid degradation of their *lhc* transcripts. Together, the results show that N-availability profoundly regulates the transcription of nuclear genes encoding proteins involved in both Chl synthesis and
assembly. The apparent coordinate control can be uncoupled, however, as a function of accumulation of Chl precursors either as a result of interruption of the pigment synthesis pathway or Chl(ide) incorporation into LHCs. The nature of the plastid to nucleus signaling pathway remains to be defined.

**Photoprotection Processes in N-limited Cells.** In earlier work, NL cells were markedly distinguishable by the extent to which mechanisms for protection against photoinhibition are engaged. Hence, they exhibit low yields of fluorescence as determined by PAM fluorometry. This was attributed to phosphorylation and dissociation of Lhcs though being of low abundance, from reaction centers to maintain thylakoids in low fluorescence State 1. In addition, the cultures displayed an enhanced “Kok effect”. This phenomenon, attributed to chlororespiratory electron transport, was first discovered in assays with *Chlorella* cells that were provided increasing levels of low illumination and unexpectedly yielded non-linear O$_2$ exchange curves. We now report that the Kok effect (low light inhibition of O$_2$ uptake) is predominantly due to effects on mitochondrial oxidative electron transport. This was established with *Chlamydomonas* mutants with deletions of mitochondrial cytochrome b/c and cytochrome c oxidase. Like WT, NL *dum* mutants exhibit a strong Kok effect but are completely insensitive to Myxo which only partially diminishes the discontinuity of the P vs. I curves and maximal rates of O$_2$ evolution obtained with WT. The Kok effect in the *dum* mutants is completely eliminated by SHAM inhibition of the alternative quinol oxidase which becomes highly abundant in mitochondria of NL cells. SHAM treatment inhibits photosynthesis in *dum* mutants. Effects of these inhibitors on the O$_2$ exchange curves of WT are equivalent to those obtained with Myxo.

When alternative oxidase inhibitors are applied together with Myxo, photosynthesis of WT is inhibited just as seen in *dum* mutants treated with SHAM alone. The data indicate that mitochondria and chloroplasts are highly interdependent in the intact system: either the conventional or the latent alternative oxidase mitochondrial systems can suffice to sustain photosynthetic electron transport in WT. When these activities are blocked, net O$_2$ evolution is impaired. This might be due to a feedback effect leading to enhanced PS I-driven O$_2$ uptake by the Mehler reaction. Conversely, efficient transfer of reductants from chloroplasts to mitochondria occurs in darkness in NL cells. This pathway is remarkably sensitive to chloroplast activities: flash illumination of dark-adapted cells immediately arrests respiration. These results are consistent with studies of Bennoun who suggested that enhanced protonation of thylakoids occurs when mitorespiration is inhibited.

Development of strong NPQ is another hallmark of the mitochondrial/chloroplast interaction. As shown in our earlier report [15], N-limitation leads to a higher NPQ in both WT and mitochondrial mutant cell types when dark-adapted. Transition to high fluorescence State 1 can be induced by illumination in the presence of DCMU. However, illumination under anaerobic conditions dramatically reduces the extent of state transition of NL but not NS mitochondrial mutant cells, as they remain in a condition where NPQ is quite strong. This is another strong indication that NL cells are much more highly mitochondrial-dependent than are control cultures. By maximizing the oxidation state of thylakoids by activation of PS I in the presence of DCMU, however, anaerobic transitions to State 1 can be partially induced. Unexpectedly, when NL *dum* mutants are returned to the dark and then reilluminated, PS II fluorescence is initially maximal
but then becomes rapidly and almost completely quenched to the minimal F0 levels. Because of the kinetics of the quenching transient, it must be unrelated to antenna/PS II interactions although rapid transitions to State 2 in darkness is superimposed onto the overall quenching phenomenon. Transition to State 2 during a dark, anaerobic incubation period is approximately 5 times more rapid in NL than NS WT and this is true for dum mutants. Comparative analyses with a LHC mutant show that rates of state transitions are inversely proportional to LHC abundance.

The additional NPQ process that is observed upon illumination of briefly dark-adapted dum mutants is without precedent. It is likely to reflect an important process that, although normally obscured by other NPQ mechanisms, could be vital to the integrity of PS II under other stress conditions, especially in conjunction with N-deficiency. We hypothesize that the quenching transient is related to ChlZ+-dependent quenching of P680 as established recently with isolated PS II reaction centers. In vitro, ChlZ+ formation by P680 photooxidation is enhanced by low pH but is inhibited through its reduction by cyt b599. Thus, a cyclic pathway involving either pheophytin or Qb reduction of cyt b599 would be one means, in addition to linear electron flow from the water-splitting complex, by which PS II fluorescence can be kept high (and photoinhibition somewhat attenuated) when the PQ pool is reduced. Conversely, if cyt b599 is oxidized or otherwise unable to participate in the cyclic pathway, transient ChlZ+ would be a highly effective quencher of excitation energy: Schweitzer and Brudvig have found that even if only 17% of the ChlZ population is oxidized, more than 70% of PS II fluorescence is quenched. These findings correlate with those of a redox-sensitive component believed to correspond to cyt b599 in its low potential (LP) form was ineffective in protection of photoinhibition when it was in a reduced state. High levels of cyt b599LP in darkness are predicted if the thylakoid lumen is highly acidified and/or the membrane potential is high as is particularly true of NL mitochondrial mutants. This would enable initial reduction of ChlZ+ upon illumination, allowing high fluorescence. Subsequent ChlZ oxidation and development of fluorescence quenching would follow if cyt b599LP is unable to continue providing electrons. This hypothesis is consistent with the initially high fluorescent yields followed by quenching observed with the mitochondrial mutants but not WT. Thus, either re-reduction of the cyt b599LP by the cyclic pathway described above is impaired or cyt b599LP is oxidized by another mechanism, such as by O2, in the dum mutants. Subsequent recovery of high fluorescence could correlate with consumption of residual O2 or reinstatement of the cyclic pathway mediated by cyt b599LP (in its relatively more stably reduced form in an O2-free atmosphere). These effects would not be apparent in WT cells since intracellular scrubbing of residual O2 would be more efficient by cytochrome c oxidase whose affinity constant is 10 to 20 times higher than that of the alternative oxidase.

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