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INDUCTION OF SPECIFIC PROTEINS IN EUKARYOTIC ALGAE GROWN UNDER IRON-, PHOSPHORUS-, OR NITROGEN-DEFICIENT CONDITIONS[1]

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ABSTRACT

What limits phytoplankton growth in nature? The answer is elusive because of methodological problems associated with bottle incubations and nutrient addition experiments. We are investigating the possibility that antibodies to proteins repressed by a specific nutrient can be used as probes to indicate which nutrient limits photosynthetic carbon fixation in the ocean. The diatom Phaeodactylum tricornutum *Bohlin and the chlorophyte* Dunaliella tertiolecta *Butcher were grown in batch cultures in artificial seawater and f/2 nutrient lacking either phosphorus, iron, or nitrogen. Chlorosis was induced by nutrient limitation in both species with the exception of phosphorus-limited* D. tertiolecta. *The synthesis and appearance of specific proteins were followed by labeling with* ^{14}*C-bicarbonate. Nutrient limitation in general leads to a decrease in the quantum efficiency of photosystem II, suggesting that deficiency of any nutrient affects the photosynthetic apparatus to some degree; however, the effect of nitrogen and iron limitation on quantum efficiency is more severe than that of phosphorus. A crude fractionation of the soluble and membrane proteins demonstrated that the large proteins induced under limitation by phosphorus and iron were associated with the membranes. However, small iron-repressible proteins were located in the soluble fraction. Isolation with anion-exchange chromatography and N-terminal sequencing of iron-repressible, 23-kDa proteins from* D. tertiolecta, P. tricornutum, *and* Chaetoceros gracilis *revealed that these small soluble proteins have strong homology with the N-terminal sequence of flavodoxins from* Azotobacter *and* Clostridium. *The identity of the flavodoxin from* D. tertiolecta *was confirmed by immunodetection using anti-flavodoxin raised against* Chlorella. *Flavodoxin was detected only under iron deprivation and was absent from nitrogen- and phosphorus-limited algae. Flavodoxin is a prime candidate for a molecular probe of iron limitation in the ocean. The requirements to confirm its utility in nature are discussed.*

Key index words: Bacillariophyceae; Chlorophyta; Dunaliella tertiolecta; *flavodoxin; iron; nitrogen; nutrient limitation;* Phaeodactylum tricornutum; *phosphorus; phytoplankton*

In large regions of the oceans, growth of phytoplankton may be limited by the availability of major and minor nutrients (Falkowski et al. 1992). Limitation of phytoplankton carbon fixation has often been attributed to nitrogen or phosphorus. In oceanic basins, where dissolved macronutrients are abundant in the euphotic zone but chlorophyll is low, iron is the suspected limiting nutrient (Martin et al. 1991). The regions presumed to be most affected by iron limitation are the subarctic and equatorial Pacific and the Southern Ocean, where input of trace elements from atmospheric dust is minimal (Duce and Tindale 1991). It has been suggested that iron limitation (or, conversely, the alleviation of this limitation) induces changes in the species composition of some open-ocean phytoplankton assemblages (Chavez et al. 1991, Price et al. 1991). However, direct evidence for iron limitation of rare species and nutrient sufficiency of dominant species has not been provided and is difficult to obtain.

Some of the physiological responses of phytoplankton to nutrient limitation appear to be common for many elements. For example, the photosynthetic apparatus of phytoplankton is strongly affected by nutrient limitation. Chlorosis is commonly observed in response to limitation by several

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essential elements, such as nitrogen, phosphorus, iron, and sulfur (Kolber et al. 1988, Herzig and Falkowski 1989, Greene et al. 1991, Collier and Grossman 1992). Collier and Grossman (1992) demonstrated that chlorosis in cyanobacteria is less pronounced under phosphorus limitation than under nitrogen or sulfur limitation. However, there are quantitative and qualitative differences in the algal response to nutrient deprivation among elements. Growth reduction induced by a limiting nutrient is due to impairment of an essential function. For example, carbon and nitrogen are essential for protein synthesis. Phosphorus is a part of nucleic acids and membranes, and it can act as a carrier of substrate or chemical energy and as a signaling device in the cytoplasm. In addition, it plays an important role in reversible chemical modifications of proteins. Iron is mainly used as a catalyst in enzymes or for the transfer of electrons and is predominantly found in mitochondrial and photosynthetic proteins. The iron atoms in photosystem I, which contains several iron–sulfur protein complexes, account for half of the iron requirements of the photosynthetic apparatus. In addition, iron plays an essential role in the assimilation of nitrate or dinitrogen gas (Orme-Johnson 1985, Raven 1988).

There are currently no adequate methods for determining which nutrient limits growth of phytoplankton in the natural environment. Quantitative differences in commonly measured parameters such as elemental composition, pigment ratios, and photosynthetic parameters do not always provide a definite answer because of inherent interspecific variations (Elrifi and Turpin 1985, Tett et al. 1985). In unialgal cultures, C:P and N:P ratios are among the most useful parameters to distinguish between nitrogen and phosphorus limitation (Perry 1976, Sakshaug and Holm-Hansen 1977, Elrifi and Turpin 1985); however, in nature, departure from Redfield ratios cannot be used because high concentrations of bacteria (Fuhrman et al. 1989) and detritus (Hobson et al. 1973) prevent accurate measurements of the chemical composition of phytoplankton in field samples. In addition, the elemental composition of iron-limited algae does not depart significantly from the Redfield ratio, and this invalidates the use of this ratio to diagnose nutrient limitation in field samples unless iron limitation can be disproved (Greene et al. 1991).

Most of the experimental evidence supporting iron limitation is derived from nutrient addition bioassays (Buma et al. 1991, Martin et al. 1991). In these experiments, water samples are incubated after the addition of one or more suspected limiting nutrients. The interpretation of bioassays is controversial because of contamination problems and confinement effects, as discussed by Cullen (1991).

A potentially unambiguous approach to answering the question of what limits carbon fixation in the ocean would be to look for specific responses to nutrient deprivation (Falkowski et al. 1992). A given stress can induce specific proteins in microorganisms. A few examples are siderophore production under low iron conditions in the marine bacterium *Alteromonas*, accumulation of alkaline phosphate in *Protogonyaulax*, the expression of the PhoE operon under phosphate limitation in pseudomonads, and the expression of the glu operon under low fixed nitrogen conditions (Poole and Hancock 1986, Reddy et al. 1988, Boni et al. 1989, Matin et al. 1989, Reid and Butler 1991). In this study, we looked for the expression of specific proteins in the marine algae *Dunaliella tertiolecta* and *Phaeodactylum tricornutum* limited by nitrogen, phosphorus, or iron. An iron-repressible protein was purified and identified as flavodoxin. In this article, we explore the possibility that antibodies to such specifically expressed proteins could be used as probes of natural samples to determine which nutrient is limiting phytoplankton photosynthesis. In a companion article (Geider et al. 1993), we discuss changes in the structure and function of the photosynthetic apparatus of *Phaeodactylum tricornutum* that accompany nutrient starvation.

MATERIALS AND METHODS

Algal strains were *Dunaliella tertiolecta* (CCMP1320, formerly DUN), *Phaeodactylum tricornutum* (CCMP1327, formerly Phaeo), *Chaetoceros gracilis* (unknown source), and *Pycnococcus provasolii* (CCMP1203, formerly Ω48-23). All algae were grown under continuous light (250 μmol quanta$\cdot$m$^{-2}\cdot$s^{-1}) in artificial seawater (ASW; Goldman and McCarthy 1978) supplemented with f/2 nutrients and trace metals (Guillard and Ryther 1962), except for Fe, which was added at a final concentration of 250 nM. This represented media with complete nutrient addition. For nutrient starvation experiments, cells were initially grown in nutrient-replete media to a density of 5×10^5 cells$\cdot$mL^{-1}, and 1 L of culture was added to 8 L of media lacking either iron, phosphorus, or nitrogen. The culture media for the nutrient limitation experiments were modified by adjusting the ratio of dissolved inorganic phosphate, nitrate, or iron to approximately 1 order of magnitude lower than that in the nutrient-replete media. The medium for nutrient-replete cultures was that of f/2 but with iron adjusted to 250 nM instead of the 11 μM Fe of the original formulation. The ratio of dissolved inorganic nutrients in the nutrient-replete media was, thus, N:P:Fe of 3520:145:1, with an N:P ratio of 24. In media containing f/2 nutrients, phosphate is the limiting nutrient in the stationary growth phase. By transferring a fixed volume of the nutrient-replete culture, the nutrient ratios for nitrogen- and phosphorus-limited cultures were set to a N:P:Fe of 391:145:1 and 3520:16:1, respectively. Thus, the N:P ratios were 2.6 for nitrogen-limited and 220 in phosphorus-limited media. The iron-limited medium was set to a N:P:Fe of 32,000:1318:1. Nutrient starvation experiments were replicated three times, but we only show the results from one experiment. After the transfer to nutrient-limited media, cells were grown for 5 days, and cell density, chlorophyll *a* (chl *a*) concentrations, and particulate carbon, nitrogen, and phosphorus concentrations were measured every day. On day 5 of starvation, the limiting nutrient was added to the respective culture, and a sample of the algae in recovery from nutrient limitation was taken 24 h later. One liter of the culture grown in nutrient-replete media was harvested as a control. Bacteria were not detected in aliquots of the cultures subjected to both transmission electron microscopy and DAPI staining followed by epifluorescence microscopy

TABLE 1. *Elemental composition (molar ratios) of algae in nutrient-replete and nutrient-limited cultures (day 5) and 24 h after nutrient addition (day 6).*

Species	*P. tricornutum*			*D. tertiolecta*		
	C:N (SD)	C:P (SD)	N:P (SD)	C:N (SD)	C:P (SD)	N:P (SD)
Replete	7.6 (0.9)	47.8 (6.4)	6.3 (.80)	7.4 (0.2)	50 (2.9)	6.8 (0.4)
N-limited	24.3 (2.4)	42.9 (4.2)	1.8 (.30)	20.2 (0.9)	58.4 (2.7)	2.9 (0.2)
N addition	9.3 (0.6)	56.1 (6.7)	6.0 (.50)	7.9 (0.8)	57.9 (7.8)	7.4 (1.0)
P-limited	14.3 (1.7)	578.0 (37.0)	40.0 (4.0)	9.0 (0.4)	560.0 (14.0)	62 (2.2)
P addition	10.5 (0.6)	84.0 (15.0)	8.3 (1.5)	6.1 (0.3)	183 (32.0)	29.9 (5.5)
Fe-limited	9.4 (1.2)	85.0 (14.0)	9.0 (1.9)	6.5 (0.4)	49.3 (4.6)	7.6 (0.8)
Fe addition	8.1 (0.9)	142.0 (18.0)	17.4 (2.7)	6.4 (0.4)	71.2 (14.5)	11.2 (2.4)

(data not shown). Pigment concentrations were determined in 90% acetone extracts using the equations of Jeffrey and Humphrey (1975). Cells were counted using a hemocytometer. Total particulate carbon and nitrogen were measured on cells collected on precombusted GF/C filters and analyzed with a Perkin Elmer 240B elemental analyzer. Particulate phosphorus was determined following Solorzano and Sharp (1980). *In vivo* fluorescence measurements were made using a pump-and-probe fluorometer (Kolber et al. 1988) on cells that were dark-adapted for 30 min prior to measuring the minimum fluorescence (F_o). The maximum fluorescence (F_m) was measured after a flash of saturating intensity. The ratio of variable to maximum fluorescence (F_v/F_m, where $F_v = F_m - F_o$) was calculated every day during our experiments.

Incorporation of C^{14} into proteins. Each day, ^{14}C-bicarbonate (50 or 100 μCi) was added to a 250-mL subsample and incubated for 24 h next to the parent cultures at the same irradiance and temperature. At the end of the incubation, triplicate subsamples were filtered through Whatman GF/C filters, washed with 5 mL of ASW, and transferred to scintillation vials for determination of total particulate organic ^{14}C activity. To drive off inorganic ^{14}C, 0.5 mL of 1% HCl was added, and the sample was allowed to evaporate to dryness at room temperature before addition of 5 mL of scintillation cocktail. Triplicate 5-mL subsamples were filtered through GF/C filters and washed with 5 mL of ASW for ^{14}C fractionation, using a modification of the procedure of Li et al. (1980). Each filter was homogenized in 4 mL of 2:1 chloroform : methanol using a Teflon tissue grinder, transferred to a 15-mL centrifuge tube, and extracted for 1 h at −20° C. The sample was centrifuged, and the supernatant was discarded. The filter was washed with an additional 4 mL of chloroform : methanol extract. The filter was resuspended in 2 mL 5% trichloroacetic acid (TCA) and incubated at 80° C for 1 h. The samples were centrifuged, and the supernatant was removed and the filter was washed once with 2 mL of cold 5% TCA. The filter was suspended in 2 mL 0.1% Triton-X100 with a 2-mL wash and transferred to a scintillation vial. Samples were evaporated to dryness at room temperature and redissolved in 5 mL of scintillation cocktail. Following Li et al. (1980), the TCA-insoluble material is considered to be protein. The results of this fractionation assay were used to calculate the fraction of the total particulate ^{14}C incorporated into proteins. This was necessary because preliminary experiments demonstrated clearly that ^{14}C-bicarbonate is heavily incorporated into carbohydrates and lipid fractions, especially in nitrogen-limited cultures. Without this correction factor, it was not possible to obtain autoradiograms of uniform intensity for the three nutrients. The remaining culture (~220 mL) was harvested by centrifugation, rinsed once with seawater, and stored at −80° C as a cell pellet in microcentrifuge tubes until further analysis.

Preparation of total proteins for sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Total protein preparation was as described in Sukenik et al. (1988). Cell pellets were resuspended in buffer A (100 mM Na_2CO_3/2% sodium dodecyl sulfate [SDS]/200 μM phenylmethylsulfonyl fluoride [PMSF]) and sonicated for 1 min on ice using a microprobe sonicator (Kontes) at maximum energy output. Duplicate aliquots were taken for total protein determination using bicinchoninic acid (Smith et al. 1985). Dithiothreitol, glycerol, and bromophenol blue were added to the remaining protein extract to give final concentrations of 100 mM, 10 and 0.05%, respectively. Aliquots were taken for measurements of radioactivity. The protein samples were denatured by heating at 95° C for 2 min, cooled, and quick-frozen in liquid nitrogen. Proteins were separated on 15 or 17% SDS–polyacrylamide gels and transferred to nitrocellulose by electroblotting. Equal amounts of proteins or cpm in the protein fraction were loaded in each well.

Cell fractionation into soluble and membrane proteins. Frozen cell pellets were resuspended in 1 mL of buffer B (50 mM Tris-maleate pH 7.0/10 mM NaCl/100 μM PMSF) and sonicated for 1 min on ice. Cell debris was collected by centrifugation at 3000 × *g* for 5 min at 4° C. The supernatant was adjusted to 50 mM $MgCl_2$ and centrifuged at 150,000 × *g* for 1 h. The supernatant was precipitated with TCA (10% final concentration) for at least 1 h on ice. The proteins were collected by centrifugation, and both soluble and membrane pellets were washed three times with buffer B containing 50 mM $MgCl_2$. The proteins were solubilized in buffer A and processed as for the total protein samples. Equal amounts of soluble proteins were separated on 15% SDS–polyacrylamide gels and transferred to nitrocellulose. The blots were challenged with polyclonal antisera raised against *Chlorella* flavodoxin (see acknowledgments) followed by goat anti-rabbit IgG and horseradish peroxidase conjugate. The reaction was developed colorimetrically using 4-chloro-1-naphthol, as described in Harlow and Lane (1988).

Purification of flavodoxin from iron-limited phytoplankton. Iron-limited algae were grown in 8 L of ASW deficient in iron for 5 days, as already described, and harvested by centrifugation. The cell pellet was frozen in liquid N_2 prior to processing of the samples because this improved the disruption of the cells. Approximately 3 g of cells (wet weight) were collected from 8 L of culture. Cells were resuspended in breaking buffer and disrupted by sonication or by three passages through a precooled Yeda press at 140 kg-force·cm^{-2} of pressure. The soluble proteins were obtained as already described. The flavodoxin isolation protocol followed published methods (Zumft and Spiller 1971, Crespi et al. 1972). The supernatant containing the soluble proteins was fractionated by adding ammonium sulfate to a final concentration of 70% (w/v) followed by centrifugation. The pellet was discarded, and ammonium sulfate was added to the supernatant to a final concentration of 100%. The 70–100% fraction enriched in flavodoxin was dissolved in buffer B and dialyzed overnight against 1 L of buffer B. The dialyzed fraction was added to diethylaminoethyl (DEAE)–sephacel and eluted first by adding buffer B with 0.4 M NaCl, followed by elution with buffer B containing 0.55 M NaCl. This last fraction containing flavodoxin was dialyzed again. The concentration was determined as already described (Smith et al. 1985) and assayed for purity on SDS–polyacrylamide gel electrophoresis.

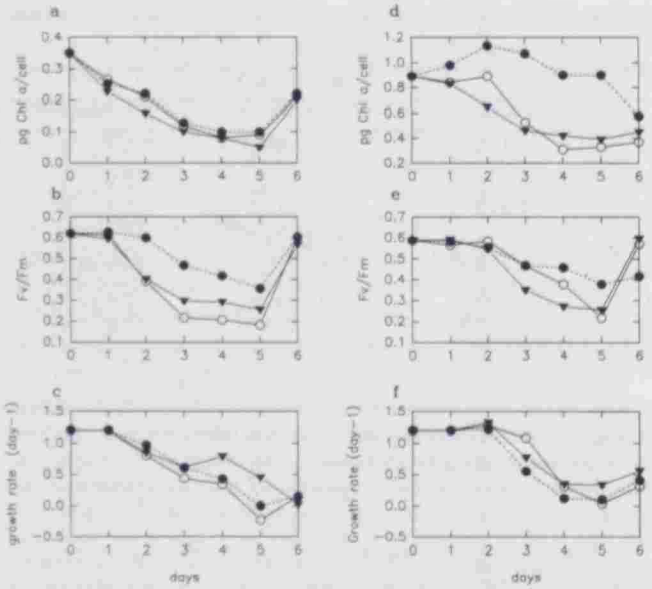

FIG. 1. Change in a, d) chl $a \cdot \text{cell}^{-1}$, b, e) F_v/F_m, and c, f) growth rate during nutrient limitation in batch cultures of a–c) *Phaeodactylum tricornutum* and d–f) *Dunaliella tertiolecta*. The cultures were transferred to media missing either nitrogen (▼), iron (○), or phosphorus (●) on day 0. The limiting nutrient was added to the culture on day 5.

For rapid screening of several algal species, the 70–100% ammonium-precipitated protein fraction, which is enriched in flavodoxin, was resuspended in buffer B, and an aliquot was TCA-precipitated as described for the total soluble protein fraction.

N-terminal sequencing. Partially purified proteins from iron-limited phytoplankton were transferred to polyvinyl pyrrolidone membranes (Immobilon-P, Millipore) in 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid, 10% methanol, pH 11, and stained with Coomassie brilliant blue (Matsudaira 1990). The 23–24-kDa bands were excised, and N-terminal ends were sequenced as described in LaRoche et al. (1990).

RESULTS

Chlorosis and Decrease in Photosynthetic Activity Induced by Nutrient Limitation

The relative concentration of essential nutrients in the culture media determines which nutrient becomes limiting first. In our experiment, nitrogen, phosphorus, or iron limitation was achieved by a 10-fold decrease of the appropriate nutrient. Comparing the elemental ratio of C, N, and P for each culture 4 days after transfer to the limiting nutrient to that of the nutrient-replete culture shows that the C:N ratio in nitrogen-limited cells was approximately 3 times higher than that in the nutrient-replete conditions, indicating nitrogen deficiency, whereas the C:P ratio in phosphorus-limited cells was 10 times higher than the nutrient-replete cells, indicating phosphorus deficiency (Table 1). Upon addition of the limiting nutrient, the ratios decreased to values comparable to nutrient-replete cells. The elemental ratios in the iron-limited cultures did not depart significantly from the nutrient-replete cultures except for the C:P ratio in iron-limited *P. tricornutum* which were 1.8 times higher than nutrient-replete cells. However, the addition of iron but not of phosphate (results not shown) led to an increase in chl $a \cdot \text{cell}^{-1}$ and photosynthetic activity (F_v/F_m), indicating that iron was the limiting nutrient under the growth conditions. These experiments were replicated three times with similar results. An independent replication of the nutrient-limited cultures was also conducted by Olaizola (1993).

In *P. tricornutum*, a similar degree of chlorosis was achieved with time in nutrient-limited batch cultures, regardless of the limiting nutrient (Fig. 1a). Addition of the limiting nutrient to the respective nutrient-limited culture on day 5 led to an increase in chl $a \cdot \text{cell}^{-1}$ on day 6 (Fig. 1a). The quantum efficiency of photosystem II, approximated by measurements of F_v/F_m, also decreased with time in nutrient-limited *P. tricornutum* batch cultures (Fig. 1b) and recovered to its nutrient-replete value (day 6) upon addition of the limiting nutrient on day 5. Division rates decreased with time and upon the addition of the limiting nutrient, cell division rate increased only in phosphorus- and nitrogen-limited cultures (Fig. 1c).

The physiological response of *D. tertiolecta* to nitrogen, phosphorus, and iron limitation is different from that of *P. tricornutum*. Although chl $a \cdot \text{cell}^{-1}$ decreased in iron- and nitrogen-limited cultures, no increase was detected after addition of the limiting nutrient (Fig. 1d). This contrasts with the results for *P. tricornutum* (Fig. 1a), described earlier. In addition, phosphorus-limited *D. tertiolecta* cultures did not become chlorotic. However, a decrease and recovery of F_v/F_m in response to nutrient limitation and addition of the limiting nutrient, respectively, was observed for nitrogen- and iron-limited cultures (Fig. 1e). Similar decreases in division rates were observed for nitrogen-, phosphorus-, and iron-limited cultures of *D. tertiolecta* (Fig. 1f). Addition of the appropriate limiting nutrient led to at least a doubling in division rates for all cultures (Fig. 1f).

Synthesis of Specific Proteins during Nutrient Limitation

Nutrient limitation led to a decrease in the synthesis of several proteins, as indicated by the profiles of ^{14}C-labeled proteins (Fig. 2). However, visual examination and scanning of autoradiograms by laser densitometry showed the induction and synthesis of unique proteins during starvation by nitrogen, phosphorus, and iron (Fig. 2). Protein bands appeared at around 26 kDa in nitrogen-limited *P. tricornutum* and *D. tertiolecta*. Large proteins were induced in both *D. tertiolecta* (>200 kDa) and *P. tricornutum* (56 kDa) by phosphorus limitation. Iron limitation induced two proteins of 116 and 90 kDa and, tentatively, 36 and 22 kDa protein (Fig. 2A) in *D. tertiolecta* and proteins of 47 and 24 kDa in *P. tricornutum* (Fig. 2B). Whereas some of the proteins were detected in Coomassie blue-stained gels (e.g. the 24-

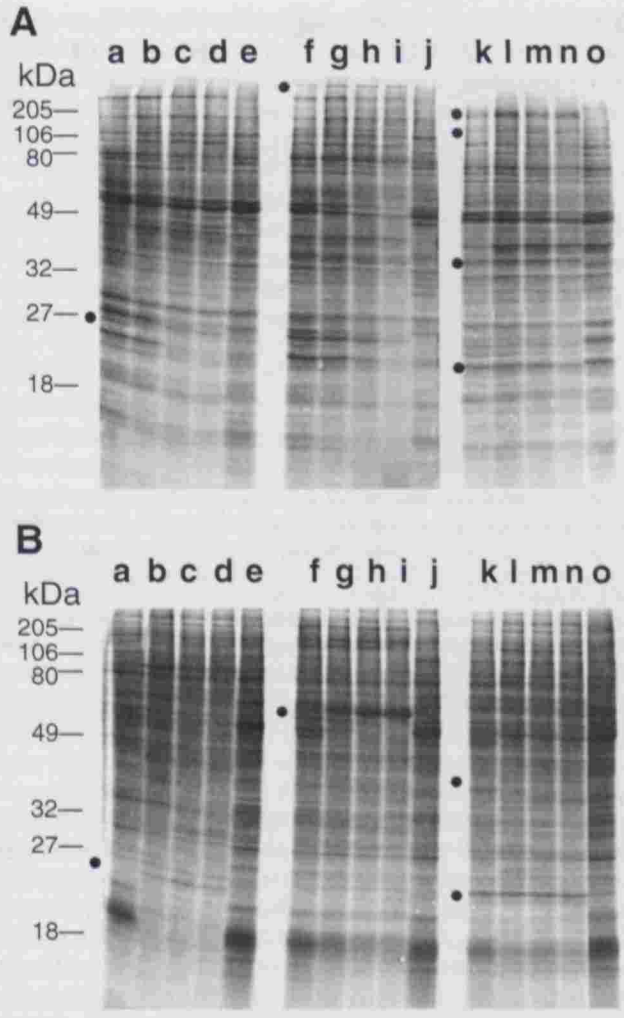

FIG. 2. Autoradiogram of total proteins labeled with ^{14}C-bicarbonate and separated on a 15% SDS–polyacrylamide gel showing the induction of protein synthesis with increased time of starvation in A) *D. tertiolecta* and B) *P. tricornutum*, 1–5 days after transfer to media lacking nitrogen (lanes a–d), phosphorus (lanes f–i), or iron (lanes k–n). Lanes e, j, and o represent the addition of nitrogen, phosphorus, or iron to nitrogen-limited, phosphorus-limited, or iron-limited cultures, respectively. The amounts of radioactivity in the protein extracts were normalized using the ratio of ^{14}C incorporation into proteins : total incorporation into particulate carbon. Samples were loaded on a basis of equal radioactivity in proteins. Dots on the left-hand side of a block of lanes mark proteins accumulated under either nitrogen, phosphorus, or iron limitation.

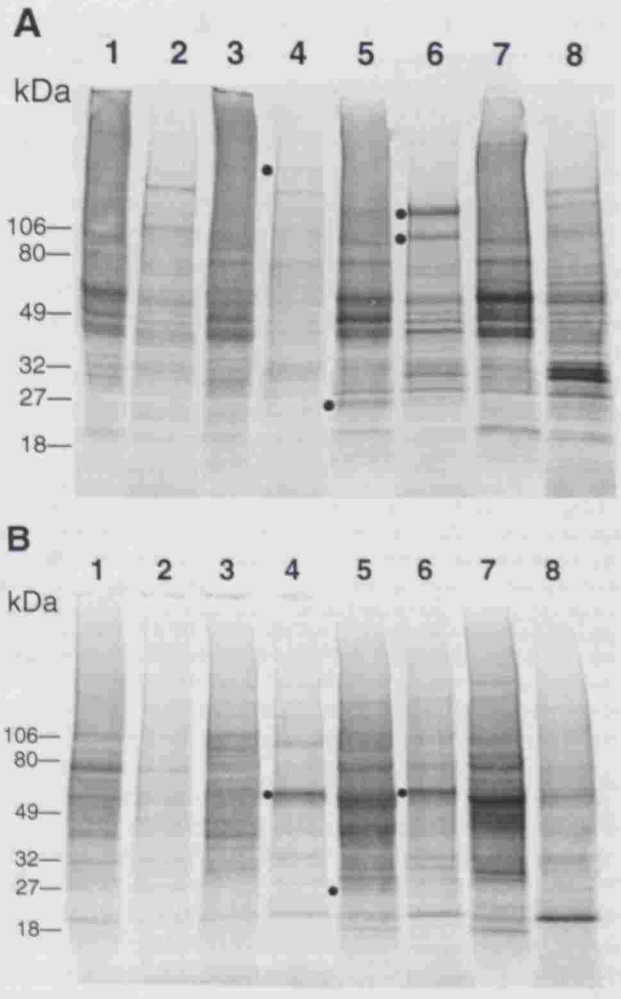

FIG. 3. Soluble and membrane proteins from nutrient-limited A) *D. tertiolecta* and B) *P. tricornutum*. One hundred-mL aliquots were collected 4 days after transfer to the media lacking either nitrogen, phosphorus, or iron and labeled with ^{14}C-bicarbonate. Proteins were fractionated into soluble and membrane fractions and separated on a 4–20% polyacrylamide gel containing SDS. Equal amounts of radioactivity were loaded in each lane. Lanes 1 and 2 represent nitrogen-limited, soluble and membrane proteins; lanes 3 and 4, phosphorus-limited, soluble and membrane proteins; lanes 5 and 6, iron-limited, soluble and membrane proteins; and lanes 7 and 8, soluble and membrane proteins from nutrient-replete cells. Dots on the left-hand side of a lane indicate proteins induced by nutrient deprivation.

kDa iron-induced protein from *P. tricornutum*; results not shown), some induced proteins (e.g. the phosphorus-limited induced 56-kDa protein in *P. tricornutum*) were not detected without ^{14}C labeling, probably because of the presence of other proteins of similar molecular weight in the background.

Protein samples from cells grown for 4 days under phosphorus, nitrogen, or iron deprivation were fractionated into soluble and membrane proteins by differential centrifugation (Fig. 3). Although we could not detect all of the proteins marked in Figure 2, we determined that the 200- and 56-kDa proteins accumulated under phosphorus limitation in *D. tertiolecta* and *P. tricornutum*, respectively, were present in the membrane fraction. Likewise, the 116- and 90-kDa proteins accumulated under iron limitation in *D. tertiolecta* were found in the membrane fraction (Fig. 3A). This indicated that large proteins detected in phosphorus- and iron-limited cultures were membrane-bound whereas the iron-repressible, 24-kDa protein of *P. tricornutum* was recovered in the soluble fraction (Fig. 3B). In addition, this analysis confirmed the presence of a 22-kDa soluble protein in iron-limited *D. tertiolecta* not clearly detected in the autoradiogram of ^{14}C-labeled total protein gels (Fig. 2A).

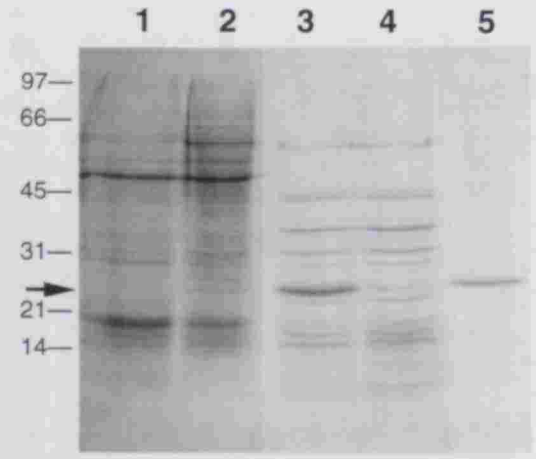

FIG. 4. Purification of flavodoxin from an iron-limited *P. tricornutum* culture. Lanes 1 and 2 show total proteins from nutrient-replete and iron-limited cells, respectively. Lanes 3–5 show proteins from iron-limited *P. tricornutum* cultures recovered from a 70–100% ammonium sulfate precipitate, enriched in the 24-kDa flavodoxin (lane 3), from the fraction that does not bind to DEAE–sephacel (lane 4), and the fraction bound to DEAE–sephacel that is eluted between 0.4 and 0.55 M NaCl, containing flavodoxin (lane 5).

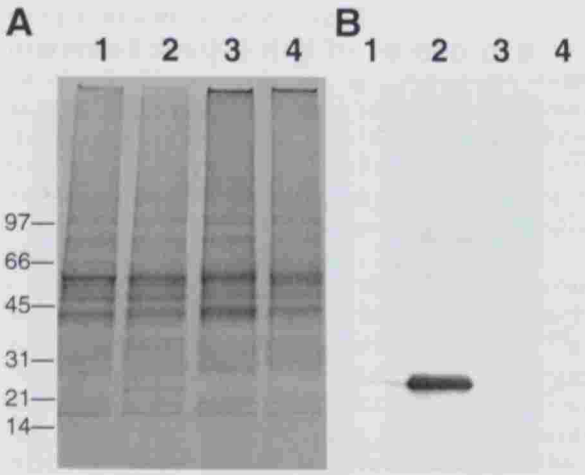

FIG. 6. Western blot of soluble proteins from *D. tertiolecta* showing the specific accumulation of flavodoxin in iron-limited cultures. A) Coomassie stain of soluble proteins (25 $\mu g \cdot lane^{-1}$) from nutrient-replete (lane 1), iron-limited (lane 2), phosphorus-limited (lane 3), and nitrogen-limited (lane 4) cultures, separated on a 4–20% gradient gel. B) Western blot of the same samples challenged with anti-flavodoxin polyclonal antisera from *Chlorella* showing the specific cross-reactivity with flavodoxin only in iron-limited cultures (lane 2). Lanes 1, 3, and 4 correspond to nutrient-replete, phosphorus-limited, and nitrogen-limited cells, respectively.

Isolation and Identification of Soluble Iron-Repressible Proteins

An iron-repressible, soluble protein was initially isolated from *P. tricornutum* by binding to DEAE–sephacel and eluting with 0.55 M NaCl (Fig. 4). The protein was identified as flavodoxin after N-terminal sequencing followed by a sequence similarity search in Genbank. N-terminal sequences for flavodoxins isolated from *C. gracilis*, *P. tricornutum*, and *D. tertiolecta* (Fig. 5) show strong similarity among these proteins. In addition, N-terminal sequences of flavodoxins from the diatoms are similar to those of several bacterial flavodoxins. The amino acid sequence TGNTE is found in both species of diatoms, in several species of bacteria, such as *Clostridium* and *Desulphovibrio*, and in the red alga *Chondrus crispus*. In addition, polyclonal antisera raised against the flavodoxin from *Chlorella* cross-reacted with the flavodoxin from *D. tertiolecta*, further confirming its identity (Fig. 6). Immunoblotting showed that, in *D. tertiolecta*, flavodoxin is present only under iron-limited growth. No traces were detected in cells grown in nutrient-replete, nitrogen-limited, or phosphorus-limited media (Fig. 6). This was observed also in *P. tricornutum*, *C. gracilis*, and *P. provasolii* (results not shown), where soluble proteins of 22–24 kDa in size were detected in iron-limited but not in iron-replete cultures. Polyclonal antisera against *Azotobacter* and *Anabaena* flavodoxin (see acknowledgments) all failed to cross-react with protein extracts from the marine algae *P. tricornutum*, *C. gracilis*, *D. tertiolecta*, and *P. provasolii*. Although the *Chlorella* polyclonal antisera cross-reacted faintly with the flavodoxin of *P. provasolii*, none of the polyclonal antisera cross-reacted with flavodoxins from the diatom species.

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                                10        20
Chaetoceros gracilis            AGVA-VYYDTSTGNTEDVADY
                                |||   ||   |||||  |   |
Phaeodactylum tricornutum       AGVK-IYYSSATGNTEQVPEY
                                |     |  ||    ||  |   |
Dunaliella tertiolecta          AVG--IFYSTSQRNT-QIAMY
                                   | |      ||   |
Clostridium sp.                 MK---IVYWSGTGNTEKMAEL
                                      ||||     |
Chondrus crispus                KIG---FFSTSTGNTTEVADF
                                      |  |||||    |
Desulfovibrio vulgaris          MSKVLIVFGSSTGNTESIAQK
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FIG. 5. Comparison of N-terminal amino acid sequences (single letter code) of flavodoxin from three species of eukaryotic algae obtained from this study and other known flavodoxins from *Clostridium* sp. (Wakabawashi et al. 1989), *Chondrus crispus* (Burnett et al. 1974), and *Desulfovibris vulgaris* (Tanaka et al. 1977).

DISCUSSION

Nutrient-Limited Growth

Evidence for nutrient-limited growth during our experiments is threefold. First, the ratios of dissolved phosphate, nitrate, and iron in the culture media were adjusted to depart from the Redfield ratios such that they would lead to depletion of the selected nutrient in stationary phase. The relative decrease in dissolved iron in the culture media necessary to achieve iron limitation was determined from preliminary experiments (results not shown) and the minimum and maximum C:Fe ratios estimated by Greene et al. (1991). Second, the molar ratios for phosphorus- and nitrogen-limited cultures (Table 1) show strong departures from the Redfield molar ratio of 106 C:16 N:1 P. Our results, like others

(Glover 1977, Greene et al. 1991), show that C:N:P ratios do not depart from the Redfield prediction of 106:16:1 at a low, iron-limited growth rate (Sakshaug and Holm-Hansen 1977). Third, addition of the limiting nutrient to a nutrient-limited culture led to the recovery of one or more of the physiological parameters depressed by nutrient limitation (Fig. 1). Interspecific variation in the physiological response of each species observed in this study indicated that measurements of a single diagnostic parameter may be misleading.

Chlorosis Induced by Nutrient Limitation

Our results show that nitrogen, phosphorus, and iron limitation can induce chlorosis in *P. tricornutum*, whereas chlorosis was induced only by nitrogen and iron limitation and not by phosphorus limitation in *D. tertiolecta*. In *P. tricornutum*, the changes in chl $a \cdot \text{cell}^{-1}$ and F_v/F_m were qualitatively similar for cells deprived of iron, nitrogen, and phosphorus. Different quantitative responses found for each nutrient in our experiment may be a function of the maximum and minimum cell quota tolerated by each species for a given nutrient. Although detailed physiological studies of each species under a variety of nutrient stresses may identify noticeable quantitative differences among the nutrients (e.g. Collier and Grossman 1992, Geider et al. 1993), detection of those differences in natural samples would require accurate measurements of cellular quotas, photosynthetic capacity, and pigment concentrations in order to identify the limiting nutrient. Similarly, analysis of photosynthetic proteins will require a quantitative approach since residual amounts of ribulose bis-phosphate carboxylase/oxygenase (RUBISCO) proteins, light-harvesting complex proteins (LHCPs), and D1 proteins will always be present in obligate photoautotrophs.

Expression of Specific Proteins during Nutrient Limitation

We have shown that specific proteins in both *P. tricornutum* and *D. tertiolecta* are synthesized when growth is limited by either nitrogen, phosphorus, or iron. Although several of the newly synthesized proteins are detectable in total protein samples stained with Coomassie blue or labeled with ^{14}C-bicarbonate, additional proteins were identified when the samples were fractionated into soluble membrane protein fractions. Fractionation into soluble and membrane proteins demonstrated a distinct pattern of large membrane proteins and small soluble proteins. This is in agreement with general observations in nutrient-limited prokaryotes (Riethman and Sherman 1988, Matin et al. 1989, Scanlan et al. 1989). For example, several nutrient-repressible systems in bacteria are composed of large membrane proteins usually involved in the transport of the limiting nutrient (Poole and Hancock 1986, Matin et al. 1989).

Proteins specific to iron and phosphorus limitation were easily detected in *P. tricornutum* and *D. tertiolecta* by the simple methods used in this study; however, this approach may not be successful when looking for proteins specifically expressed under nitrogen limitation, because very little ^{14}C-bicarbonate is incorporated into proteins in nitrogen-limited cultures. A more detailed study of the nutrient-repressible proteins could be performed by examining protein samples using two-dimensional gel electrophoresis. Coleman et al. (1988) found only 1 out of approximately 500 proteins, which increased more than 10-fold (14-fold) in nitrogen-limited *Euglena*, using two-dimensional gel analysis, although they did not identify the enriched protein. Although a more detailed analysis of the protein pattern may reveal more proteins that are specifically accumulated under limitation by a given nutrient, our approach is useful in identifying the proteins that are the most strongly accumulated under nutrient limitation. Those proteins are likely to be good candidates for designing immunological probes for identifying nutrient limitation.

Although we cannot, at present, rule out the possibility that the proteins enriched under nutrient limitation result from the accumulation of precursors or degradation products for major proteins, this is unlikely considering that the proteins were synthesized throughout the 5 days following transfer to nutrient-limited media. The incorporation of ^{14}C radioactive bicarbonate in protein bands, as detected under conditions that do not favor protein synthesis, such as under nitrogen limitation, strongly suggests a functional role for the induced proteins. In addition, in our experiments, none of the enriched proteins showed cross-reactivity with antibodies to D1, LHCPs and RUBISCO (La Roche et al. 1993); therefore, they are not precursors to these major photosynthetic proteins.

Derepression of Flavodoxin under Iron Limitation

We have identified one of the dominant soluble proteins induced under iron limitation as flavodoxin on the basis of its size, N-terminal sequence, isolation protocol, and mode of expression and, for *D. tertiolecta*, by cross-reactivity with a flavodoxin antibody. The induction of flavodoxin under iron limitation has been observed in several prokaryotes including obligately aerobic, facultative anaerobic, photosynthetic bacteria and cyanobacteria (reviewed by Mayhew and Ludwig 1975). Flavodoxin has been isolated from two eukaryotic algae, *Chlorella fusca* (Zumft and Spiller 1971) and *Chondrus crispus* (Fitzgerald et al. 1978); however, ours is the first report of flavodoxin in diatom species. It has not yet been found in higher plants or in animals.

Several species of microorganisms commonly replace ferredoxin (an Fe-S protein) with flavodoxin when deprived of iron. Flavodoxin, which contains

flavin mononucleotide (FMN) instead of iron, is repressed by high cellular iron concentrations. Flavodoxin can apparently replace ferredoxin in most reactions including ferredoxin–nicotinamide adenine dinucleotide phosphate (NADP) reductase and reduced NADP–cytochrome *c* reductase (Mayhew and Ludwig 1975). Flavodoxin is also involved in dinitrogen fixation; however, some nitrogenases appear to require both ferredoxin and flavodoxin in their redox reactions (Yoch and Valentine 1972, Drummond 1985, Sandmann et al. 1990). Two types of flavodoxins have been described: a short-chain flavodoxin from prokaryotes with molecular weights of 14–16 kDa and a long-chain flavodoxin ranging in size from 18.5 to 23 kDa. The long-chain type has also been isolated from cyanobacteria (Laudenbach et al. 1988) as well as *Klebsiella pneumonia* (Drummond 1985) and *Azotobacter vinelandii* (Tanaka et al. 1977). From three-dimensional structural analysis of the *Synnechococcus* flavodoxin, amino acid residues 9–14, 55–61, and 97–107 have been identified as important in the formation of hydrogen bonding between FMN and apoflavodoxin, and these regions are, in general, highly conserved (Smith et al. 1983). It is, therefore, not surprising that we found similarity among N-terminal sequences for the algal flavodoxins (Fig. 5) because they encompass the highly conserved residues 9–14. The estimated molecular weights of the flavodoxins described in our study range between 22 and 24 kDa, as determined from electrophoresis on SDS–polyacrylamide gels, suggesting that they belong to the large-chain flavodoxin. In contrast, the N-terminal sequences of the first 21 amino acids from three unicellular eukaryotic algae examined in this study (Fig. 5) show stronger similarity with the short-chain flavodoxins of *Clostridium* and *Desulfovibrio* than to the long-chain flavodoxins of cyanobacteria and *Azotobacter*. Some similarity with the flavodoxin from *Chondrus crispus* indicates that more sequence data are needed to determine whether or not flavodoxins from eukaryotic algae and, in particular, those from diatoms have greater similarity to the short- or long-chain flavodoxins.

Physiological Significance of Flavodoxin

The abundance of iron–sulfur proteins in redox reactions originated early during evolution when a reducing atmosphere was present and iron was found at high concentrations as dissolved Fe^{2+} in the oceans (Raven 1988, daSilva and Williams 1991). As a result of photosynthetic oxygen evolution, the supply of dissolved iron became sparse as oxidized Fe^{3+} forms were precipitated from seawater. Why has ferredoxin been retained as an important redox protein in marine algae that inhabit a niche chronically low in iron when it can be replaced by the iron-free flavodoxin (Duce and Tindale 1991)? Replacement of ferredoxin by flavodoxin provides only a small decrement in iron requirement. Calculation of iron requirements for photosynthetic algae and cyanobacteria demonstrates that the replacement of ferredoxin with flavodoxin only reduces the cellular iron requirement by 2 iron atoms out of the 23 atoms required by a complete set of the components of the electron transport chain of O_2-evolving photolithotrophs (Raven 1990). Although the catalytic capacity of ferredoxin is slightly greater than that of flavodoxin (Raven 1984), additional factors may also give a selective advantage to ferredoxin. The molecular weight of flavodoxin is, in general, 1.5–2 times that of ferredoxin, and it may be advantageous to retain the ability to synthesize ferredoxin, which is a smaller protein under nitrogen limitation or energy limitation.

Flavodoxin as an Indicator of Iron Limitation

Our results show that in several eukaryotic marine algae, flavodoxin is expressed only under iron limitation; therefore, it is a prime candidate as a marker protein for iron limitation in natural communities. This approach has been used to infer iron limitation in primary producers on coral reefs (Erntsch et al. 1983). Antibodies raised against flavodoxin from a marine alga may, therefore, be a useful indicator of iron limitation and could be used on total protein extracted from bulk phytoplankton samples (Tanoue 1992) or as a species-specific probe in *in situ* hybridization of preserved phytoplankton samples (Currin et al. 1990, Orellana and Perry 1992). There is an indication that some phytoplankton species (e.g. large diatoms) are more affected by iron limitation than others (e.g. picoplankton) (Brand 1991, Martin et al. 1991). Addition of iron to natural water samples, followed by incubation, often leads to a large increase in diatom species relative to smaller algae (Martin et al. 1991). Diatoms are an important component of the phytoplankton community of the Southern Ocean (Buma et al. 1991). The presence or absence of flavodoxin from diatoms may help resolve whether or not natural populations of Antarctic diatoms are iron-limited. *In situ* hybridization would allow the dissection of communities and indicate which species are limited by iron.

Several key questions remain to be answered, however, before flavodoxin can be used successfully as a probe for iron limitation. A detailed investigation of flavodoxin induction by iron deprivation in a wide taxonomic range of algal species is necessary to assess the diversity of responses in the regulation of flavodoxin by iron. In particular, it is important to assess what proportion of marine algae, if any, do not accumulate flavodoxin under iron limitation or have a constitutive flavodoxin regardless of iron availability. There is a large plasticity in phytoplankton iron requirements. Phytoplankton clones isolated from open ocean waters, which are typically low in dissolved iron, can grow at much lower dissolved inorganic iron concentrations (Brand et al.

1983) and have much lower iron requirements (Brand 1991, Sunda et al. 1991) than do coastal clones. The variability in iron requirements reflects differences in the intracellular allocation of iron that may affect flavodoxin.

The induction of flavodoxin by iron deprivation is common in prokaryotes, but this is not a universal response. Flavodoxin is constitutively expressed in several nitrogen-fixing prokaryotes, where it appears to be an essential electron carrier in the nitrogen fixation reactions, intermediary between ferredoxin–NADP reductase and the nitrogenase enzymes. In some cyanobacteria, flavodoxin is constitutive in heterocysts only (Sandmann et al. 1990). In addition, flavodoxin is believed to be constitutively expressed in *Chondrus crispus*, in which very little ferredoxin can be detected. Although no experimental manipulation was reported, flavodoxin is apparently a constitutive protein in *Chondrus crispus* collected from intertidal zones, where dissolved iron should be present (Fitzgerald et al. 1978). A similar situation may occur in oceanic phytoplankton species. In contrast, physiological studies of freshwater cyanobacteria have demonstrated that certain strains of *Anabaena* do not contain flavodoxin even under severe iron deprivation (Sandmann et al. 1990). In strains that can substitute flavodoxin for ferredoxin, the ferredoxin content decreases at higher external iron concentrations than in those strains that are unable to synthesize flavodoxin. The possibility that flavodoxin is absent from the genome of certain algal species will therefore have to be considered when using flavodoxin as a marker protein for iron limitation.

An ideal probe for nutrient limitation should behave like an on/off switch. That is, synthesis of this protein should occur only below a threshold level that corresponds to the concentration of the nutrient below which the cellular demands are not met. Several metal-binding proteins are regulated by a factor that acts as a repressor of transcription when bound to a metal ion. For example, in *Chlamydomonas*, cytochrome c_6, which can replace the copper-binding plastocyanin, is repressed by high copper concentration. The copper-dependent transcriptional regulation of cytochrome c_6 is reversible. It has been hypothesized that a copper-binding regulator, below a threshold level, competes with plastocyanin for the copper ions and results in a "titration" of the copper regulator (Hill and Merchant 1992). Similar regulation of yeast metallothionin genes has been observed (Thiele 1988) and suggests that the expression of metal-repressible genes may be a quantitative response to cellular metal concentration. If iron repression of flavodoxin were controlled by a similar mechanism, the development of a quantitative assay for iron limitation may be possible. Experiments are underway to look at the accumulation of flavodoxin in iron-limited chemostats where a precise control of growth rate is possible.

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PURIFICATION AND CHARACTERIZATION OF TWO FORMS OF PHOSPHOGLYCERATE KINASE FROM THE GREEN ALGA *SELENASTRUM MINUTUM*[1]

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ABSTRACT

We report the first purification and characterization of a eukaryotic algal phosphoglycerate kinase (PGK). Two forms of PGK (PGK_1 and PGK_2) from the green alga Selenastrum minutum *(Naeg.) Collins were purified to electrophoretic homogeneity with specific activities of 1100 and 1069 units·mg^{-1} protein, respectively. The portion of PGK_1 and PGK_2 (probably the cytosolic and chloroplastic forms, respectively) in this organism was estimated as 32 and 68%, respectively. PGK_1 was more heat-stable than PGK_2. The M_r estimation for PGK_1 and PGK_2 by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and gel filtration indicated that they both were monomeric with a similar M_r of approximately 44 kDa. Antibodies raised against* S. minutum *PGK_1 cross-reacted with PGK_2 as well as PGKs from prokaryotic and eukaryotic sources, suggesting that PGK_1 was structurally and immunologically closely related to PGK_2 and other PGKs, which was consistent with NH_2-terminal sequence analysis.*

Comparative kinetic and regulatory properties of PGK_1 and PGK_2 from S. minutum *were investigated. Both forms exhibited hyperbolic kinetics with respect to both 3-phosphoglycerate (3-PGA) and Mg–adenosine triphosphate^{2-} ($MgATP^{2-}$) under the conditions tested and had similar K_m values for each substrate (PGK_1: K_m ($MgATP^{2-}$) = 0.37 mM, K_m(3-PGA) = 0.59 mM; PGK_2: K_m($MgATP^{2-}$) = 0.32 mM, K_m(3-PGA) = 0.46 mM). PGK_1 and PGK_2, however, differed significantly in several other kinetic properties. PGK_2 had a broad pH optimum between 7.3 and 7.8, as compared to PGK_1, with a pH optimum of 7.3 Mg^{2+} was the most efficient cofactor for both forms; it inhibited PGK_1 but not PGK_2 at higher concentrations (>10 mM). Other divalent cations (Mn^{2+}, Zn^{2+}, Co^{2+}, Cd^{2+}, and Ca^{2+}) only partially replaced Mg^{2+} and were more effective for PGK_1 than for PGK_2. A wide range of metabolites was examined for regulatory properties. Energy charge was the most important factor in regulating the two forms of* S. minutum *PGK. These results were interpreted in light of the regulation of this kinase in response to the cell energy requirement and the need for glycolytic carbon flow to provide carbon skeletons for amino acid biosynthesis.*

Key index words: antibodies; Chlorophyta; energy charge; kinetic studies; metabolite inhibitors; phosphoglycerate kinase; purification; regulation; Selenastrum minutum

Phosphoglycerate kinase (PGK; E.C.2.7.2.3), which participates in glycolysis, gluconeogenesis, and the Calvin cycle, catalyzes the reversible interconversion of 3-phosphoglycerate (3-PGA) and 1,3-diphosphoglycerate (1,3-DPGA) with concomitant utilization or generation of adenosine triphosphate (ATP):

$$1,3\text{-DPGA} + \text{ADP} \leftrightarrow 3\text{-PGA} + \text{ATP}.$$

In glycolysis, PGK generates ATP by converting 1,3-DPGA to 3-PGA. In the Calvin cycle or gluconeogenesis, the kinase consumes ATP in producing 1,3-DPGA. Studies in our laboratory have shown that nitrogen assimilation by the nitrogen-limited green alga *Selenastrum minutum* caused a rapid stimulation in respiration and a transient suppression of photosynthetic CO_2 fixation (Elrifi and Turpin 1986,

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