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Analysis of Ferredoxin and Flavodoxin in *Anabaena* and *Trichodesmium* Using Fast Protein Liquid Chromatography

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Title: Analysis of Ferredoxin and Flavodoxin in *Anabaena* and *Trichodesmium* Using Fast Protein Liquid Chromatography.

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Iron is an essential nutrient for growth of photosynthetic microorganisms such as cyanobacteria and algae. Iron is required for proteins involved in the

important processes of carbon and nitrogen assimilation. Low concentrations of iron in cultures or natural waters can lead to iron limitation which affects many aspects of algal metabolism. In natural waters, iron limitation can have effects on the patterns and rates of primary productivity.

The cellular content of certain proteins can be affected by media iron concentrations. Methods have been used that assay components of the cell as an indirect measure of iron nutritional status. For example, spectroscopy can be performed to determine the cellular concentration of iron-containing proteins involved in photosynthesis. Organisms grown in media that imitate natural conditions, or organisms collected from their natural habitat are usually dilute. Methods that assay iron nutritional status such as spectroscopy and column chromatography require large sample sizes which are difficult to obtain from natural samples. In addition, methods that utilize techniques such as immunology or radioactive labelling are complex and time-consuming. These considerations led to the necessity of developing a technique that would be simple, rapid and effective on dilute samples. The method developed here utilized fast protein liquid chromatography (FPLC), which fulfilled these requirements. A complete analysis could be done within two to three hours with minimal sample treatment.

The FPLC was simple to operate and was effective on a sample containing less than 100 µg of protein.

Some photosynthetic organisms, when iron-depleted, can produce the flavin-containing protein flavodoxin (Flv). This protein substitutes for the iron-containing protein ferredoxin (Fd) in Fd-dependent reactions such as the light-induced reduction of NADP. The FPLC technique identified and quantified, in relative terms, Fd and Flv in the cell. Optical spectroscopy was used to verify FPLC retention time assignments. The results illustrated how the FPLC could be used to observe the changes in relative Fd and Flv content as a function of media iron concentration in cultures of the cyanobacterium *Anabaena* grown in the laboratory. It was found that Fd content decreased and Flv content increased with decreasing media iron concentration. In addition, samples of the cyanobacterium *Trichodesmium* collected from the ocean near Barbados were analyzed using FPLC to assay relative Fd and Flv content. By analogy with *Anabaena*, Fd and Flv retention times were identified. Using this technique conclusions could be drawn regarding the changing iron nutritional status of *Trichodesmium* in its natural habitat.

ANALYSIS OF FERREDOXIN AND FLAVODOXIN IN *ANABAENA* AND *TRICHODESMIUM* USING FAST PROTEIN LIQUID CHROMATOGRAPHY

by

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INTRODUCTION

There is interest in the role of iron in cyanobacterial and algal physiology and the ecological implications of physiological responses to iron nutrition. The requirement for iron, in photosynthetic microorganisms, can be seen in the important processes of carbon assimilation, via photosynthesis, and nitrogen metabolism. In particular, photosynthetic electron transport components such as ferredoxin (Fd) and cytochromes have iron-containing chromophores.

Physiological responses to varying iron concentrations have been studied with a variety of methods. Chlorophyll content, components of the photosynthetic apparatus and nutrient uptake rates are examples of indices of iron nutrition that have been studied.

The environmental implications of iron nutrition have also been studied. The dynamics of the amount and types of photosynthetic microorganisms present in natural waters influences primary productivity and, consequently, local and global environmental conditions. It has been found that iron nutrition is a factor in the competition between cyanobacteria and eukaryotic algae. Response to

iron availability has also been found to be important to primary productivity in oceans.

Interest in iron nutrition has led to the use and development of a number of biochemical indices of iron nutritional status. Most methods used are complex or require a large sample size. Measurements of biogenic iron in natural waters are complicated by experimental difficulties; for example, the terriginous iron content is many times higher than the biogenic iron content and the chemistry of iron in natural waters is complex. These considerations have necessitated the development of a simple, sensitive measure of cellular iron that would produce immediate results for analysis of dilute natural samples.

Under iron-depleted conditions, certain organisms can induce production of flavodoxin (Flv), a flavin containing protein, that substitutes for Fd in reductive processes (1). This mechanism enables these organisms to thrive under moderately iron-limited conditions. The method described here makes use of fast protein liquid chromatography (FPLC), which has proved to be fast and effective on low concentrations of protein. Using this method will allow the investigator to make on-site measurements of Fd and Flv that function as preliminary indicators of cyanobacterial or algal nutrition.

Cyanobacteria and Algae

Eukaryotic algae are plants that are usually found in water. They are categorized into three divisions, the red, brown and green algae. Red and brown algae are found in marine environments, whereas green algae are freshwater organisms. In contrast to the eukaryotic algae, cyanobacteria are photosynthetic prokaryotes that contain no membrane-bound organelles. Cyanobacteria are different from other photosynthetic prokaryotes in that they function aerobically and contain a photosynthetic apparatus that is analogous to that of plants. In fact, cyanobacteria resemble the chloroplasts of plants and algae. An in-depth discussion of cyanobacterial classification and physiology can be found in a review of the subject (2).

Functions of Iron in Cyanobacteria and Algae

Iron is involved in the processes of carbon assimilation and nitrogen metabolism. A more detailed discussion of the functions of heavy metals in plants can be found in a review by Sandmann and Böger (3). In carbon assimilation, iron is required in the components of photosynthesis and the electron transport chain. In particular, iron is needed for the Fe-S centers of proteins. These proteins can be found in the membrane bound fraction of the photosynthetic apparatus and in

soluble Fd. Fd functions as a low-potential electron carrier in such processes as the light-induced reduction of NADP, mediated by ferredoxin-NADP reductase (FNR) (4). Also involved in electron transfer are the iron-containing hemoproteins called cytochromes. The production of pigments involved in photosynthesis, chlorophyll and phycobillin, requires iron since an enzyme that contains iron is involved in the synthesis of these pigments (5).

Iron is required for several processes in nitrogen metabolism (6,7). The processes of nitrate and nitrite reduction involve iron directly or require Fd as a cofactor. Some organisms have the ability to assimilate nitrogen under nitrate-limited conditions using nitrogen fixation. This process requires nitrogenase which contains large amounts of iron (8).

Literature Review of Iron Nutrition

The importance of iron nutrition to cyanobacterial and algal physiology and primary productivity can be best understood from a review of the literature. The following discussions are of studies representative of the literature concerned with iron nutrition. These studies are most conveniently categorized into those that are concerned with physiological responses to iron nutrition and those that study iron nutrition with respect to its influence on ecology and the environment.

Physiological Responses to Iron Nutrition. The ultrastructure of the cyanobacterium *Agmenellum quadruplicatum* has been studied (9). The effect of iron starvation was observed using thin sectioning and transmission electron microscopy. It was found that polysaccharides accumulated, and ribosomes and thylakoid membranes were degraded as a result of iron starvation. These changes were reversible upon the addition of iron. This study was done to compare changes in ultrastructure as a result of iron depletion to the changes due to nitrogen, phosphorous and carbon limitation. The results indicated that the sequence of changes induced by iron starvation is different from that of nitrogen, phosphorus or carbon limitation.

Another study of the changes in ultrastructure as a result of iron deficiency was done on the cyanobacterium *Anacystis nidulans* (10). Under iron-limited conditions, a decrease in the amount of membranes, phycobilisomes and carboxysomes and an increase in the amount of glycogen storage granules were observed using electron microscopy. Restoration of the normal structure was observed, over time, after the addition of iron.

Chlorophyll and membrane content in *A.nidulans* and *Synechococcus cedrorum* were studied during iron starvation (11). The pigments phycocyanin and chlorophyll were analyzed using optical and fluorescence spectroscopy.

Spectral changes indicated that depletion of these pigments was a result of iron stress. Electrophoresis was performed and led to the conclusion that iron starvation resulted in a decrease in chlorophyll-proteins and cytochromes.

The membrane composition of *A.nidulans* has been monitored as a function of iron-deprivation (12). This study utilized radioactive labeling and electrophoretic techniques to observe the difference between normal and iron-depleted cells. Incorporation of ^{35}S sulfate into the membrane proteins and subsequent electrophoresis was used to monitor protein biosynthesis. ^{125}I labeling was performed to determine the exposed surface area of the membrane proteins. These experiments showed that iron deprivation resulted in the depressed biosynthesis of membrane proteins and an increase in the exposed protein surface area. Using similar experiments, recovery from iron deficiency was also studied. A stepwise assembly of the membrane protein structure was observed as a result of restoring normal levels of iron to the organism.

The change in pigmentation and photosynthesis in *A. nidulans* as a result of iron deficiency has been studied (13). This was done using time course studies of photosynthetic oxygen evolution, photo-oxidation of P700 and cytochrome c, photo-reduction of NADP and light-induced oxidation of P700. It was found that iron

deficiency resulted in decreased photo-oxidation of cytochrome c by photosystem I (PSI) particles, decreased photosynthetic oxygen evolution and reduced photo-oxidation of NADP by broken cells. Light-induced oxidation of P700 was also suppressed in iron-depleted samples.

Changes in the components of the photosynthetic electron-transport chain in response to iron-deficiency have been studied in the cyanobacteria *Aphanocapsa* (14). Relative amounts of the photosynthetic Fe-S centers were analyzed using EPR spectroscopy. Fd and Flv concentrations were determined with optical spectroscopy, as were the concentration of other redox components such as plastocyanin and cytochrome f-556. The amount of the Fe-S and non-iron redox components, except Flv, were found to decrease under iron-limited conditions. The Flv concentration increased as a function of iron depletion. Inhibition of electron transport reactions by iron-limitation was observed by monitoring oxygen evolution by intact thylakoids.

Work has been done to study the preliminary effects of iron deficiency (15). The effects of iron-limitation on electron transfer activities and the redox components were studied in *Aphanocapsa*. Photosynthetic oxygen evolution and electron transfer reactions such as PSI and PSII activity and respiratory activity were measured.

Also, the amounts of Fe-S centers in PSI and the cytochrome b6-f complex were assayed using ESR spectroscopy. Optical difference spectroscopy was used to assay components such as P700. The investigators reported that, although respiration and pigmentation were only slightly affected by moderate iron-limitation, electron transfer was suppressed due to decreased amounts of iron containing components of the photosynthetic apparatus.

An immunological assay was used to study the biosynthesis of Fd in *Chlamydomonas reinhardii* (16). Using highly specific antibodies against Fd, it was found that, under iron-limited conditions, the synthesis of Fd was inhibited by apoferredoxin at the transcriptional level.

Other assays of Fe-S proteins that could be used to quantify response to iron nutrition are the extrusion of Fe-S clusters and fluorine NMR as described by Mortenson and Gillum (17) and Mössbauer spectroscopy which has been used to study Fe-S clusters of the photosystem I reaction center in the cyanobacteria *Chlorogloea fitschii* (18). Biochemical studies of the redox components of photosynthesis involved assaying Fd or Flv by monitoring the reduction of NADP in the presence of FNR, light and Fd or Flv (4).

Environmental Considerations of Iron Nutrition. The distribution of trace metals (Fe, Cu and Mn), their

control of primary productivity and biological adaptations to trace metal stress is discussed in detail by Huntsman and Sunda (19). Iron can be taken up as solubilized Fe(II).or Fe(III). Iron limitation could be studied by assaying soluble iron, but analysis of iron in natural waters is complicated by several factors. An example of experimental difficulties can be seen in the method of measuring soluble iron as that which will pass through a 0.4 μm filter. Some insoluble forms of iron can pass through such a filter and some soluble species adsorb to the filter. It is not enough to measure total iron in natural waters as an indicator or iron availability because of complex iron chemistry. For example, iron availability is affected by the presence of biologically-produced iron chelators and other chelators present, such as humic acids. In addition, natural waters are usually stratified producing an aerobic surface layer and an anoxic lower layer and sediment. These environments affect the speciation of iron.

Some microorganisms can produce iron chelators, called siderophores, under iron-depleted conditions. Siderophores are usually hydroxamates or catechols that have a high affinity for Fe(III)(20). The induction of siderophore production as a response to iron depletion in *Anabaena* and its effect on copper toxicity has been studied (21). *Anabaena* has been shown to use the

hydroxamate schizokinen as a siderophore. Cyanobacteria that are considered to be a problem in lakes and reservoirs have been controlled by treatment with copper sulfate which can be toxic to these microorganisms in higher than ambient concentrations. A problem with this form of control is that organisms can develop a tolerance to copper. In addition, the toxic effect of copper can be reduced when chelated by natural materials such as humic acids or siderophores. Cyanobacterial blooms may be iron depleted, so the concentration of siderophores might be high, and the organisms would be more resistant to copper toxicity. These studies indicate that iron nutrition must be a consideration in the control of nuisance cyanobacteria.

The trace metal effects on cyanobacterial dominance in lakes have been considered (22). These are analyzed with respect to the competition between cyanobacteria and algae. Factors that are involved in cyanobacterial dominance are the use of limiting light, ability to fix nitrogen and siderophore production. Since iron has a role in all these processes, it is thought that iron plays a role in the amount, spatial distribution and seasonal patterns of primary productivity.

The effect of iron nutrition on *Anabaena* and the green alga *Scenedesmus bijugatus* was studied to better understand the selective stimulation of *Anabaena* growth

over *Scenedesmus* in mixed populations by increased iron concentrations (23). The two organisms were grown separately and the effects of iron concentration on nitrate reductase, nitrite reductase and glutamine synthetase were monitored using chemical assays. The results showed that iron additions could stimulate *Anabaena* growth via increased nitrate metabolism. Although the iron additions also stimulated growth of *Scenedesmus* it was not via increased nitrate metabolism. The investigators concluded that iron stimulation of nitrate metabolism could be partly responsible for the competitive advantage of *Anabaena* over *Scenedesmus* under iron enriched conditions.

The role of iron in the green alga *Scenedesmus quadricauda* has been studied to consider the influence of iron availability on primary productivity in natural waters (24). It is well known that light and nitrogen availability are important to patterns of productivity. The investigators report that it is likely that iron is also important, since the results showed that nitrogen metabolism and photosynthesis were affected by iron availability.

Iron is essential in several metabolic processes including carbon assimilation, which is responsible in part for atmospheric CO_2 levels and, consequently, global climate. Phytoplankton are a major contributor to the

removal of CO_2 in the atmosphere. In this context, the effect of iron deficiency in phytoplankton found in the north-east Pacific subarctic has been studied (25). Although levels of nutrients such as phosphate, nitrate and silicon dioxide were found to be in excess, growth was limited by inadequate iron supply. Iron additions to phytoplankton collected increased the amount of nitrate utilized and also increased the chlorophyll concentrations. The iron nutritional status of these phytoplankton could contribute,in the long run, to carbon assimilation rates and thus atmospheric CO_2 levels

Interest in the effects of iron availability on primary productivity in tropical oceans has led to a study of the cyanobacterium *Trichodesmium* found in the north Tropical Atlantic near Barbados (26). This organism is responsible for about one fourth of the nitrogen fixation in the ocean. Geochemical cycles in the ocean could be affected by the changes in availability of fixed nitrogen and carbon. *Anabaena* was used as an experimental model in this study. Carbon fixation rates were determined by monitoring the uptake of ^{14}C and nitrogen fixation rates were assayed by measuring acetylene reduction capacity. Iron additions to the iron-depleted *Anabaena* resulted in increased carbon and nitrogen fixation rates and an increase in chlorophyll concentration. Similar responses to iron additions in *Trichodesmium* indicated that it was

iron-limited under natural conditions. The response of *Trichodesmium* to iron availability may be a way to study patterns of primary productivity in tropical oceans.

The cyanobacterium *Phormidium* , found in coral reefs, was iron-limited as indicated by the presence of Flv (27). The amounts of Fd and cytochrome were also assayed and compared to that of iron-limited *Scenedesmus* grown in the laboratory. Techniques used included optical spectroscopy of proteins purified with column chromatography and flavin fluorescence spectroscopy of Flv in low concentrations. The low levels of iron-containing proteins found in *Phormidium* were considered to be a result of iron-deprivation. Such studies can elucidate the effects of iron nutrition on primary productivity in tropical oceans. Analysis of the response of the marine diatom *Thalassiosira weissflogii* to trace metal availability has also been performed to help elucidate the effect of environmental variations on primary productivity (28).

The ecological implications of the effect of iron on bioluminescence have been considered (29). Some symbiotic bacteria have the ability to emit light through bioluminescence, which is maximized by iron limitation. Understanding the influence of nutrients, including iron, on this process could elucidate the role of light emission for the various bacteria in their diverse environments.

Statement of Research Problem

The methods commonly used to observe iron nutrition are often complicated, time consuming and require a large sample size. These methods are usually not portable. Cells growing in their natural environment are usually dilute and much time is required to collect large samples. It would be advantageous to be able to acquire information regarding iron nutrition in natural samples, quickly and on-site. This lead to the necessity of developing a rapid, sensitive and simple assay. The method described here fills these requirements.

Since Flv can be produced by iron-limited cyanobacteria as a substitute for Fd in Fd-dependent reactions, it is possible to estimate the iron nutritional status of an organism by measuring the relative amounts of Fd and Flv present in the cell. The FPLC can readily identify, by retention time, and quantify, by peak area, Fd and Flv in a culture sample. A complete analysis can be performed on several samples within two hours and the FPLC can detect Fd and Flv in a sample containing less than 100 µg of soluble protein. The samples require minimal preparation using a microcentrifuge. and the only major piece of equipment needed is the FPLC apparatus. This method could be used to provide information that

would enable the investigator to draw preliminary conclusions before studying the system in more detail.

Anabaena grown in different concentrations of iron were analyzed using this method. The results were used to identify the retention times of Fd and Flv. The method was then applied to natural samples of *Trichodesmium* collected from Barbados.

METHODS AND MATERIALS

Growth of *Anabaena* 7120

Anabaena 7120 was grown in the synthetic freshwater medium FRAQUIL (30) at 25°C with continuous illumination of 100 μEinsteins m^{-2} sec^{-1}. The culture media were passed through a Bio-Rad, Chelex-100 column at pH 7.5 and filtered through a 0.4 μm pore size, acid washed, polycarbonate, Nucleopore filter. Media were made to 10^{-8} M, 10^{-7} M and 10^{-6} M total iron concentration as FeEDTA. Three to four liters of culture were grown in polycarbonate carboys, continually swirled at 50 rpm to keep cells in suspension.

Acetone Powders

The culture was centrifuged and the resulting pellet was slowly added to 400 mL of vigorously stirring acetone kept between -10 and -20 °C and left for 45 mins. The suspension was filtered on a Whatman GF/A 1.6 μm filter and rinsed with cold acetone. The concentrated slurry was poured into a second volume of cold acetone and the process repeated. The sample was then filtered until the pellet was mostly dry.

Collection of *Trichodesmium*

Trichodesmium colonies were collected two to three km west of the Bellairs Research Institute in Barbados, W.I.

(approximately 13°12'N, 59°40'W). Samples were collected using net tows according to a previously published method (26). Acetone powders were prepared as described above. Several species of this organism were collected at the same time. Samples are not distinguished on the basis of species, so all references to this organism are as *Trichodesmium*.

General Procedures

All buffers used in this work, except where noted, refer to a solution of 25 mM tris(hydroxymethyl)aminomethane-HCl (Tris) from Sigma, in nanopure water at pH 8.3. Other reagents used were Safeway sucrose and Sigma NaCl. Cellex D, DEAE-cellulose from Bio-Rad and Sephadex G75 were used as ion exchange and gel filtration column materials, respectively. Procedures were carried out at 4°C whenever possible.

Purification of Spinach Fd

Fd was purified from Safeway spinach using column chromatography. Spinach (8 to 10 bundles) was ground in a blender in one liter of buffer and filtered through Miracloth. This mixture was stirred overnight in 100 g, dry weight, of DEAE-cellulose, to bind the Fd. An ion-exchange-Fd column was poured into a plastic 2" by 8" column. The protein was eluted with a 0 to 0.8 M NaCl gradient and the brown band dialyzed overnight against buffer. The dialyzed Fd was loaded onto a 0.75" by 3.5" ion-exchange column and eluted with 0.8 M NaCl.

The pinkish brown band was then passed over a 2.5' by 1.25" gel filtration column and eluted with buffer. The final step involved eluting the Fd from a 0.75" by 3.5" ion-exchange column with a 0 to 0.8 M NaCl gradient. The purified Fd was stored at -80°C. Absorbance at 420 nm was used to assay the concentration, using 9700 L $mole^{-1}$ cm^{-1} (4) as the molar absorptivity.

Fast Protein Liquid Chromatography (FPLC)

Sample Treatment. To extract soluble protein, the acetone powders were left for two to three hours in a known amount of buffer (about 1 mL each), with occasional mixing. To remove solids the solution was quantitatively transferred to capped, 1 mL centrifuge tubes and spun at about 9000 rpm for five minutes. The supernatant was decanted and centrifuged again. This process was repeated until no solid remained after centrifugation.

Intrumentation. A Pharmacia FPLC unit was equipped with two A-500 pumps, a GP-250 gradient programmer, a Mono Q, anion-exchange column and pre-filter, a single path monitor UV-1 optical unit and control unit, a fraction collector and a Hewlett Packard 3390A integrator. Before use, the salt buffers used to elute the column were filtered with a 0.2 µm filter using a Millipore apparatus. The sample (500 µL) was injected into the column and the eluent was monitored at 280 nm or 465 and 420 nm simultaneously. There was a 100 µL

volume between the column and fractionater. The delay caused by this dead volume was taken into account when collecting fractions for optical spectroscopy. The FPLC was programmed to wash the column with 0.1 M NaCl for two minutes and the column was eluted with a linear gradient of 0.1 M to 1.0 M NaCl for 16 mins. A schematic representation of the elution scheme is shown in Figure 1.

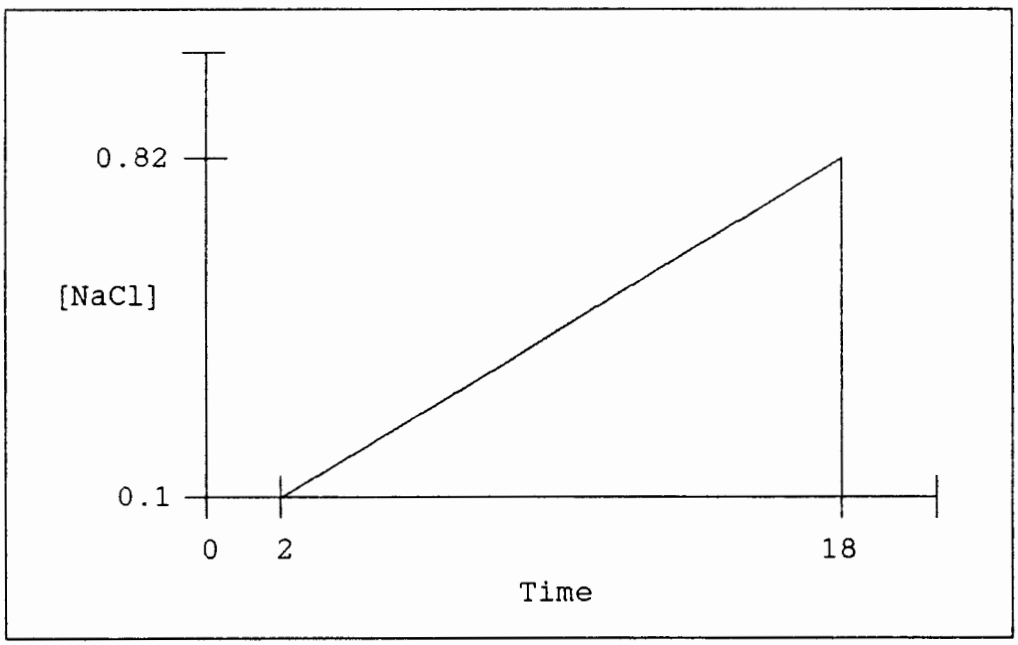

Figure 1. FPLC elution scheme

When the run was complete, the integrator printed out the integrated peak areas when the 280 nm detector was used. The column effluent from 10 to 16 minutes was collected in 0.2 mL portions and the fractions pooled from three samples for use in optical spectroscopy.

Soluble Protein Assay

Before analysis on the FPLC, the solubilized samples were assayed for protein content. The assay was done according to Bradford (31) using the Cary 14 spectrophotometer to measure the absorbance at 595 nm. Spinach Fd was used as a standard for the calibration curve (Figure 2). The equation for the linear portion of the curve was determined using the least squares method with y = ax as the model equation. This equation was used to calculate all subsequent protein concentrations.

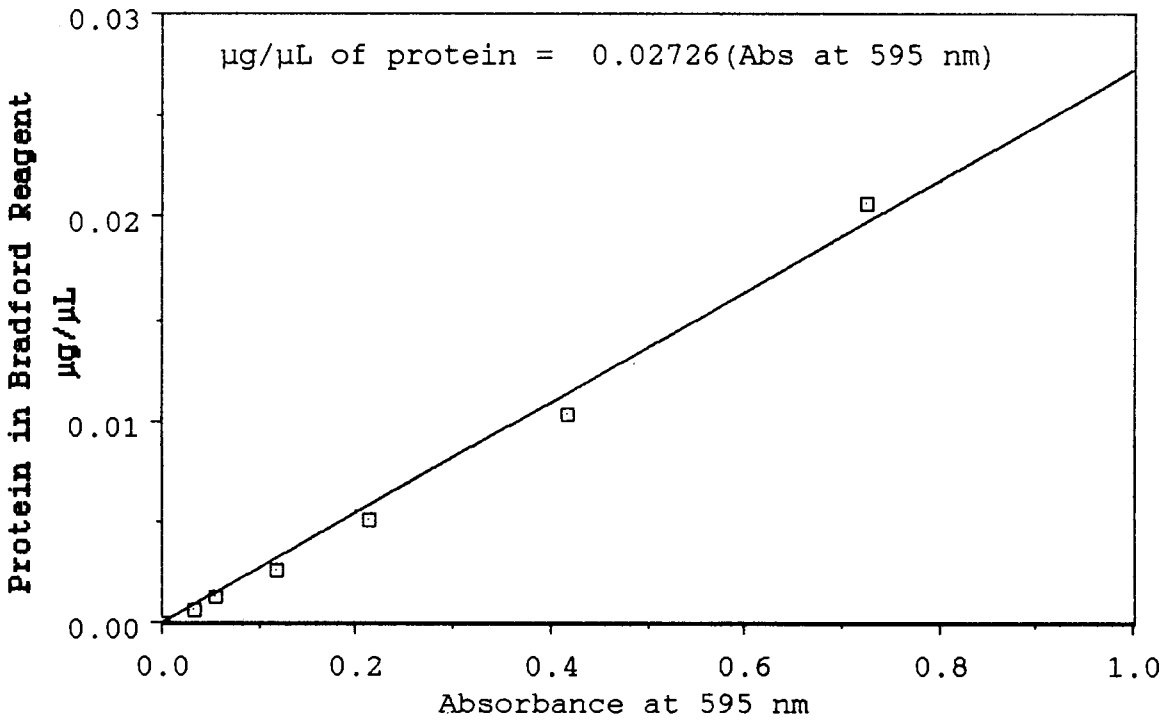

Figure 2. Calibration curve using spinach Fd as a standard for soluble protein concentration. Protein concentration measured according to Bradford

Fd and Flv Identification in *Anabaena*

Purification Attempts. In order to take spectra and rerun the samples on the FPLC, the eluted fractions were dialyzed against buffer overnight to remove the salt and concentrated over a YM-2 membrane in an Amicon concentrator. Desalting and concentration were also performed without dialysis. In addition, concentration of the eluted fractions was attempted using lyophilization.

Visible Spectroscopy. Since the attempts to purify and concentrate the eluted proteins were unsuccessful, identification of Fd and Flv in *Anabaena* was done by performing spectroscopy on the eluted proteins without concentration. An *Anabaena* culture grown in 10^{-7} M Fe medium, specifically for Fd and Flv purification, contained a fraction with the characteristic pink color of Fd. The spectrum of this fraction and a fraction suspected to be Flv was taken on the Cary 14 spectrophotometer. A subsequent sample was analyzed similarly using the Cary 16 spectrophotometer.

Modified Cary 16 Spectroscopy. A Cary 16 spectrophotometer was interfaced to the Macintosh 512KE computer using a Keithley digital multimeter and an IEEE-488 bus controller. The program for data acquisition was written by Dr. John Golbeck and Mr. Martin Corera. Column eluent from 10 to 16 minutes was collected in 0.2 mL portions and the fractions pooled from three samples. The visible spectroscopy

of these fractions was performed using 0.5 mL quartz cuvets and 0.5 M NaCl buffer as a blank and reference. Baselines were subtracted from the the spectra of the 11.5 min and 14 min fractions.

RESULTS

Identification of Retention Times of Fd and Flv

Spinach Ferredoxin. Fd was purified from spinach and analyzed using FPLC and spectroscopy. The results were used as a standard for the subsequently-purified Fd from *Anabaena*. The visible spectrum of Fd, from the Cary 14 spectrophotometer, is shown in Figure 3. The concentration is 66.0 µM.

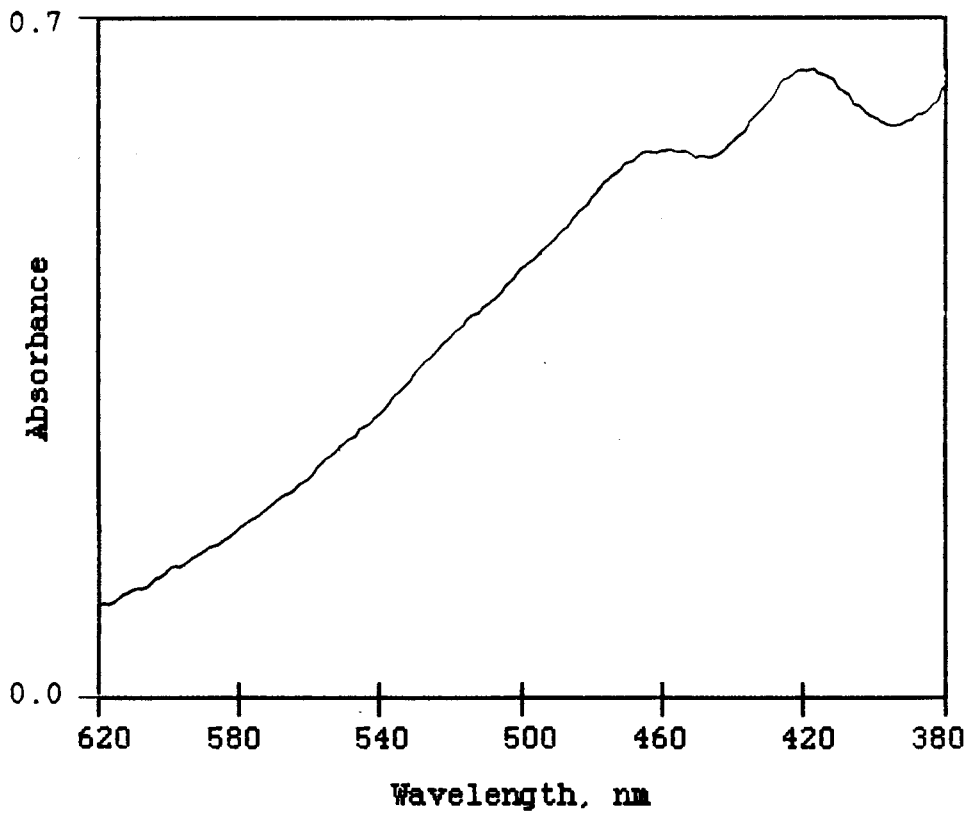

Figure 3. Visible spectrum of spinach Fd taken on a Cary 14 spectrophotometer.

Spinach Fd eluted at about 13.5 minutes (Figure 4) on the FPLC under the specified conditions.

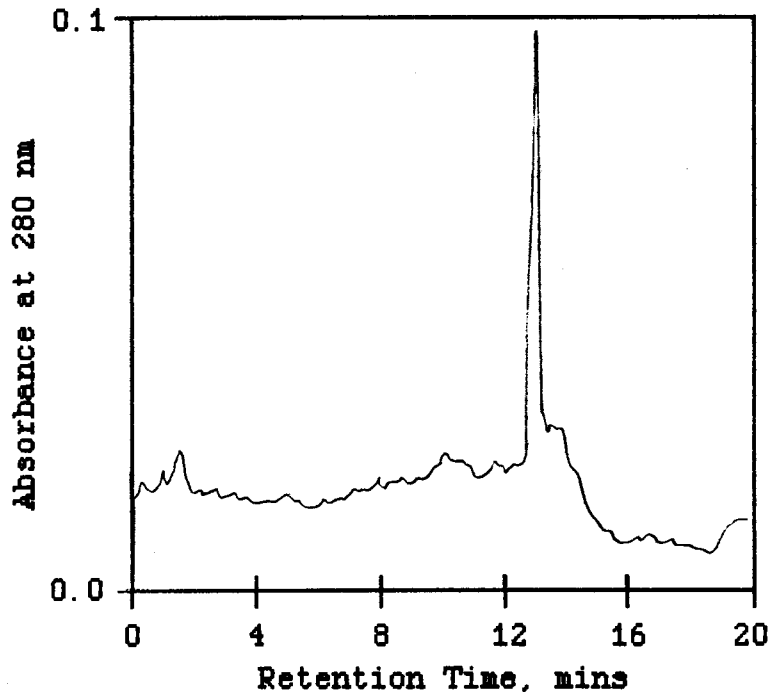

Figure 4. Elution of spinach Fd (500µL at 3.4µM) from FPLC column.

Fd and Flv in *Anabaena*. Both Fd and Flv absorb at 470 and 435 nm. Flv has an absorption maximum at 465 nm and Fd has an absorption maximum at 420 nm. Preliminary results from the FPLC using 470 and 435 nm filters to monitor the eluent indicated that Fd and Flv could be found in the fraction eluting between 11 and 15 minutes in *Anabaena* (Figure 5). Since, according to previous studies, Flv should elute prior to Fd (32), the peaks found at about 11.5 and 14 minutes were suspected to be Flv and Fd, respectively.

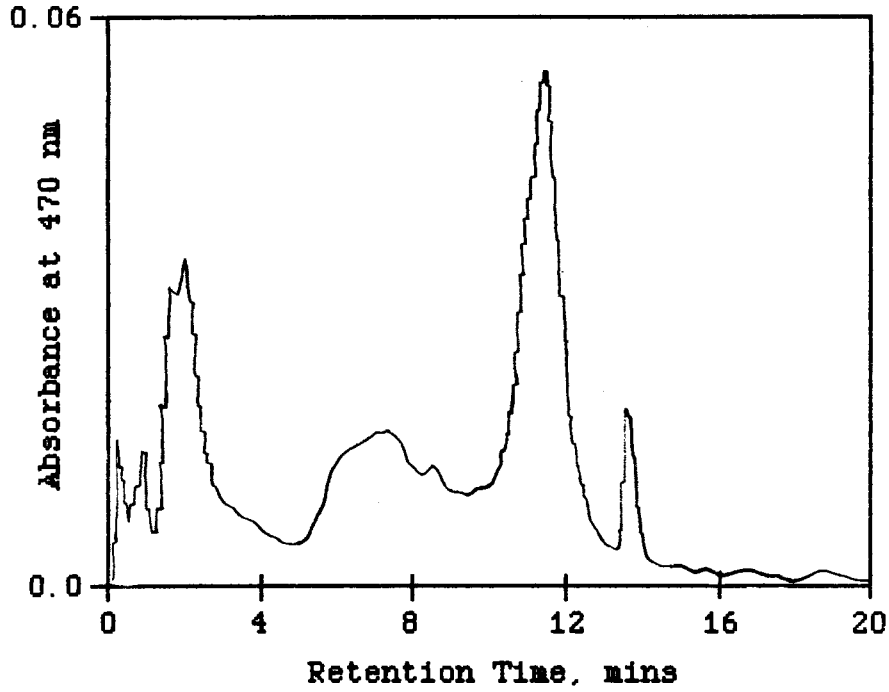

Figure 5. Elution of soluble proteins in *Anabaena* (500 µL) from a FPLC column.

To verify these retention time assignments, attempts were made to purify the eluted proteins for further analysis. The initial attempts to purify these proteins from *Anabaena* were unsuccessful. Fractions collected, dialyzed, concentrated and run on the FPLC a second time show significant loss of protein. Desalting and concentration without dialysis was not successful for the same reason. Spectra of resolubilized, lyophilized fractions indicated that no intact Fd or Flv remained.

Verification of the identities of the proteins eluting at 11.5 and 14.5 minutes was made by performing optical spectroscopy directly on eluted fractions. The fraction

collected between 14 and 15 minutes in an acetone powder from 10^{-7} M Fe *Anabaena* culture, grown specifically for protein purification, was a brownish pink color characteristic of spinach Fd. The spectrum of this fraction is characteristic of that of Fd. Spectra of proteins collected at 11.5 and 14.5 minutes from a subsequent culture (Figures 6 and 7) were homologous to the spectra of Flv and Fd, respectively.

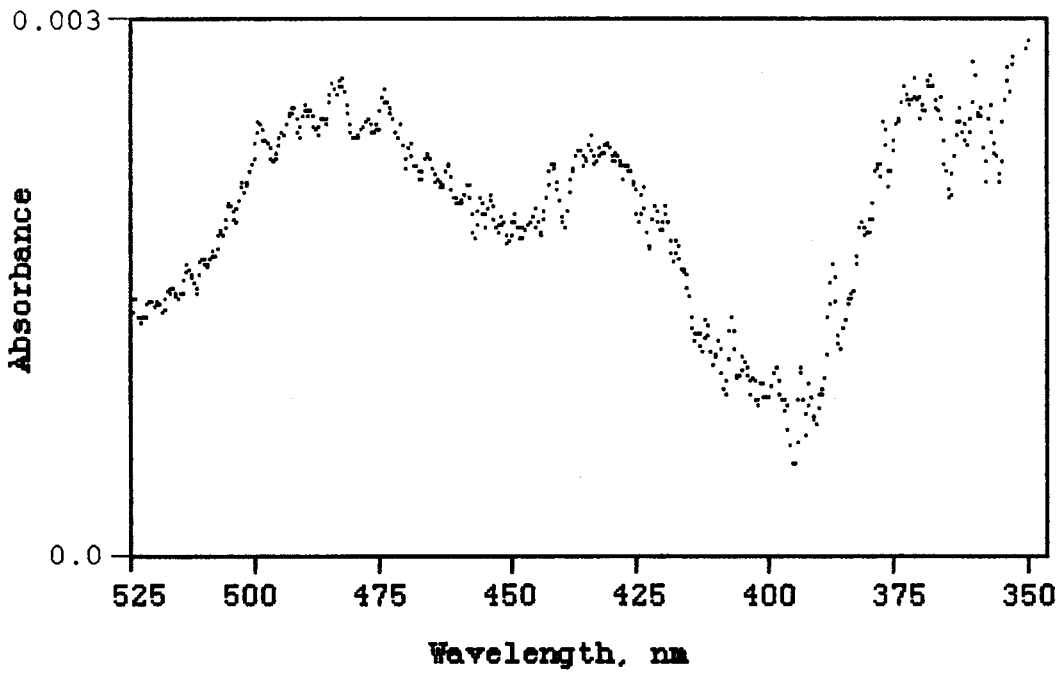

Figure 6. Visible spectrum, from Cary 16, of protein collected at 11.5 minutes (Flv) from *Anabaena*.

The protein eluted at 11.5 minutes has absorption peaks at 377, 440 and 490 nm that are typical of flavodoxins from other organisms (1, 33 and 34). The spectrum of the protein eluting at 14.5 min has an absorption maximum at 420 nm and a shoulder

at 465 nm, which is identical to that of ferredoxins previously studied (34) and that of spinach Fd (Figure 3).

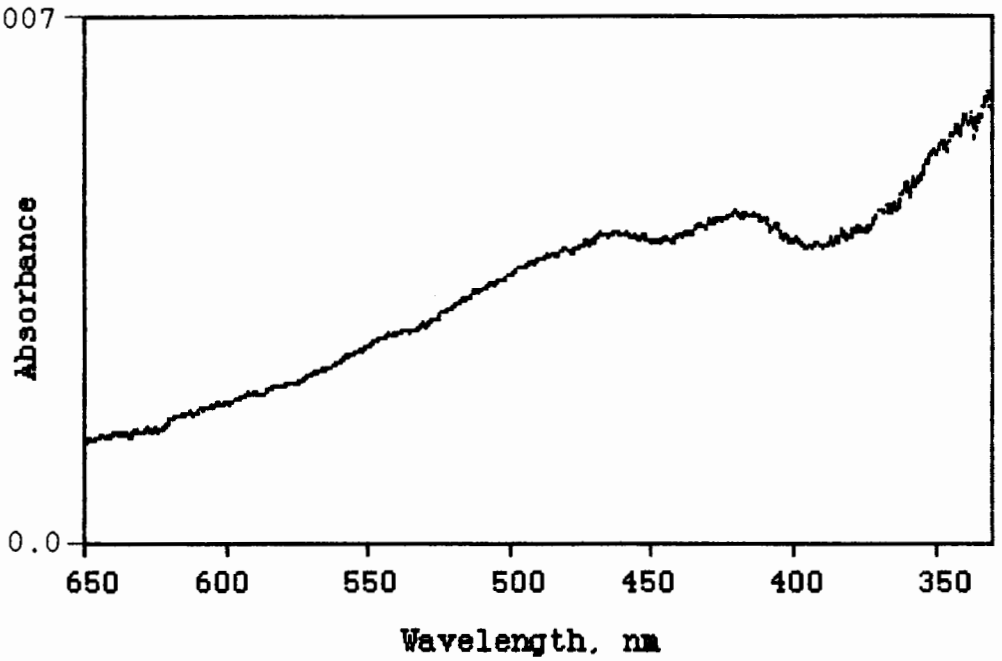

Figure 7. Visible spectrum, from Cary 16, of protein collected at 14.4 minutes (Fd) from *Anabaena*.

Fd and Flv Response to Iron Depletion

Analysis of *Anabaena*. *Anabaena* grown at different iron concentrations were analyzed with the FPLC. The *Anabaena* growth medium does not duplicate the complicated chemistry in natural waters, but the concentrations of iron in the samples are expected to be similar to that of natural waters. Table I lists the Fd and Flv to soluble protein ratios for different Fe concentrations from a representative set of samples. Soluble protein content in each sample was about 1.5 mg. Fd content, relative to soluble protein, decreases as a function

of Fe concentration. The highest concentration of Fd was in the sample grown in 10^{-6} M iron. Flv is only detectable in the samples grown in 10^{-7} and 10^{-8} M iron, with the highest concentration in the sample grown in 10^{-8} M iron.

TABLE I

RELATIVE FD AND FLV CONTENT IN *ANABAENA* AS A FUNCTION OF IRON CONCENTRATION IN CULTURE MEDIUM

Fe concn,M [a] μg	Sol Protein,[b]	Fd [c] Sol Protein	Flv [c] Sol Protein	Flv [d] Fd
10^{-6}	1450	0.53	e	0.0
10^{-7}	1800	0.36	0.73	2.0
10^{-8}	1700	0.24	1.3	5.4

(a) The concentration of Fe in the culture medium. (b) Soluble protein in culture determined by Bradford method. (c) Integrated peak area of protein, $x10^{-3}$, divided by the soluble protein. (d) Integrated peak are of Flv divided by integrated peak area of Fd. (e) Not measurable.

The results of these experiments show that this method can be used to identify Fd and Flv. In addition, the trends in the relative amounts of Fd and Flv as a function of media iron concentration can be observed. This study also proved that the FPLC can be effective for analysis of small amounts of protein.

The FPLC results for *Anabaena* from Table I are graphed in Figure 8. Because of the complex chemistry of iron, it is not possible to use this graph as a calibration curve for measuring iron availability as a function of physiological response. Figure 8 does, however, illustrate the relative trend as a result of iron stress. These observations make it is possible to assess the relative amounts of iron available to an organism like *Trichodesmium*, by measuring Fd content, Flv content or the ratio of Flv to Fd.

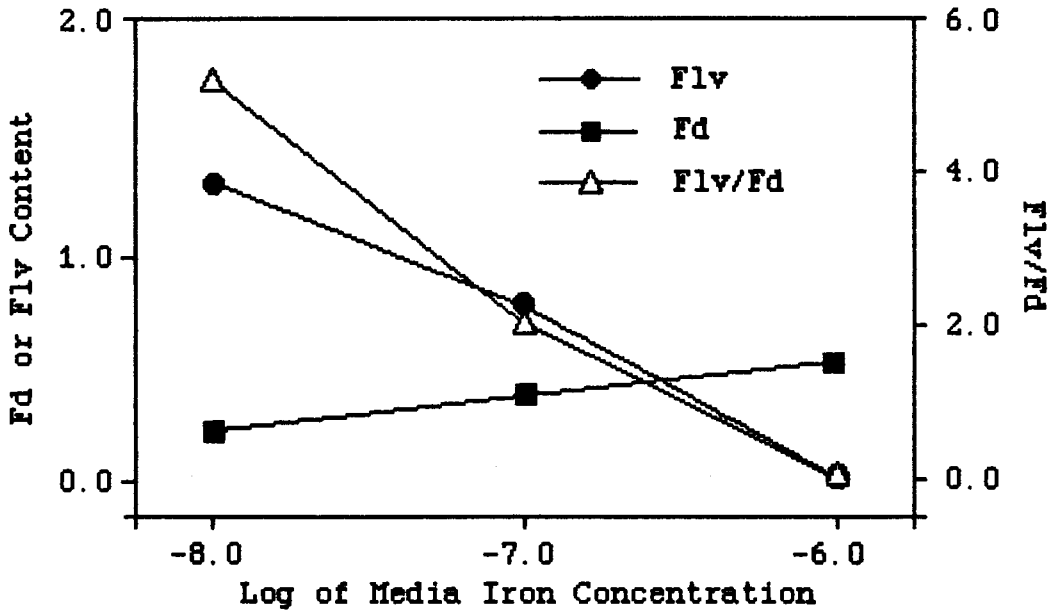

Figure 8. Fd and Flv content (FPLC integrated peak area per soluble protein) and the ratio of Flv content to Fd content as a function of media iron concentration in *Anabaena*. Data is reported in Table I

Analysis of *Trichodesmium*. Figure 9 is representative of the peaks found in *Trichodesmium* collected in Barbados.

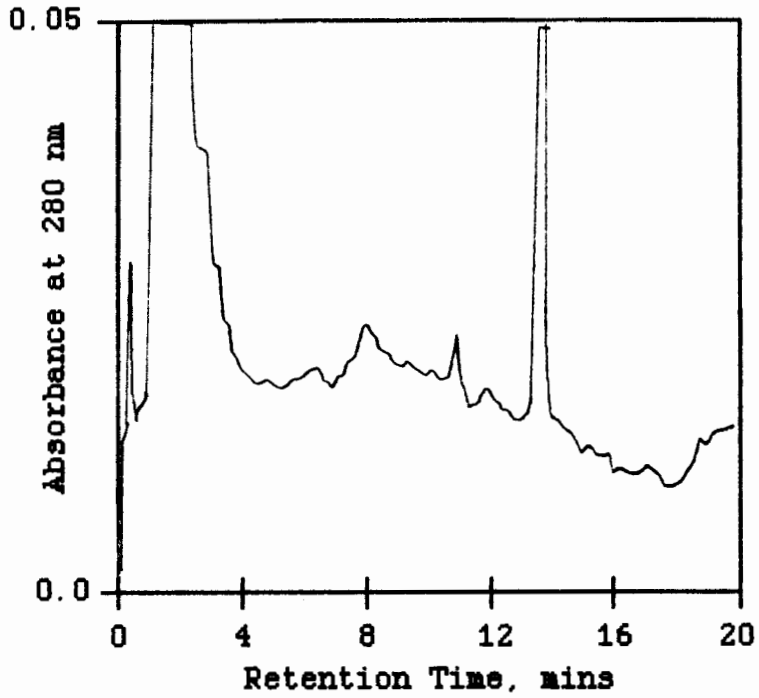

Figure 9. Elution of *Trichodesmium* collected in Barbados on 2-15-88.

By analogy with *Anabaena*, the proteins that eluted at 11.5 and 14 minutes were thought to be Flv and Fd, respectively. Table II lists the FPLC results. Cell Fe content is reported in terms of pmole Fe per colony and Fe per cell nitrogen. These two measures of cell Fe content do not agree, so it is not possible to make a good correlation between these data and Fd and Flv content. The Fd and Flv content alone may be a better indication of the Fe nutritional status of the organism. This experiment could be augmented by comparing the Fe concentration in the organism's environment with Fd and Flv content.

TABLE II

Fd AND Flv PER SOLUBLE PROTEIN, IRON CELL CONTENT AND SOLUBLE PROTEIN IN BARBADOS *TRICHODESMIUM*

Date [a]	Fd [b] Sol Prot	Flv [b] Sol Prot	Flv [c] Fd	pmole Fe [d] Colony	Fe/N [e] $x10^{-3}$	Sol [f] Protein
1-25-88	410	90	0.22	56	1.2	9
2-1-88	270	75	0.28	g	g	34
2-8-88	500	75	0.15	140	4	10
2-15-88	240	52	0.22	110	4.1	6
2-22-88	280	59	0.21	90	1.4	24
2-29-88	72	18	0.25	g	g	23
3-7-88	240	22	0.09	110	2.1	32
3-14-88	93	32	0.34	225	g	49
3-22-88	160	30	0.19	121	2.2	29

(a) Date *Trichodesmium* collected in Barbados. (b) Integrated peak area divided by soluble protein content. (c) Flv integrated peak area divided by Fd integrated peak area. (d) pmole iron per colony determined in Barbados. (e) Iron per nitrogen in cells determined in Barbados. (f) Soluble protein determined by Bradford method.

These experiments demonstrate the ability of this method to identify and quantify, in relative amounts, Fd and Flv in an environmental sample.

CONCLUSIONS

Summary

Interest in iron nutrition and its influence on primary productivity has led to the need for a sensitive, rapid assay of iron nutritional status in natural samples of cyanobacteria and algae. The method described here made use of FPLC and successfully identified and quantified Fd and Flv in the laboratory-grown *Anabaena* and *Trichodesmium* collected from its natural habitat. Initial assignments of retention times of Fd and Flv were verified by optical spectroscopy. Methods used previously were usually complex and required sample sizes on the order of grams. The FPLC allowed for effective analysis of samples with less than 100 µg of soluble protein. The instrument is simple to operate and analyses can be done rapidly.

Discussion

The response of *Anabaena* to iron depletion was consistent with the known roles of iron in cyanobacteria. Decreased amounts of iron increased the Flv content and decreased the Fd content. The presence of Flv in *Trichodesmium* indicated that this organism was iron limited in its natural environment. This conclusion could not easily be drawn from other data on cellular iron. The FPLC data is

consistent with a previous study of *Trichodesmium* (26) that indicated that *Trichodesmium* may be iron-limited. Carbon and nitrate uptake rates were monitored to determine iron nutritional status. From this study it was concluded that the response of *Trichodesmium* physiology to the varying external iron input could be studied to better understand the dynamics of primary productivity in tropical oceans. The FPLC method discussed here would be a valuable and effective biochemical index of iron nutritional status in studies such as this.

Directions for Future Work

The FPLC retention times for the Fd and Flv were essentially identified using optical spectroscopy. A more positive identification of Flv could be made by purifying Flv from *Anabaena* using a previously published method for *A. Nidulans* (1). Concentrated Flv would provide a better visible spectrum and could be used as a standard in future studies. In order to better characterize Fd and Flv purified from *Anabaena*, other biochemical techniques, such as electrophoresis, could be used on both proteins prepared this way.

The quantity of Fd and Flv per total cellular protein could be estimated from a calibration of integrated peak area as a function of protein concentration. This could be done using Fd and Flv purified from *Anabaena* as standards. The

concentration of Fd and Flv from this calibration curve could be compared to the total protein content in order to evaluate the percent of total protein that Fd and Flv represented in each sample.

To further study biochemical response to iron, a time course analysis of Fd and Flv as a function of iron stress could be done. For the method described here, *Anabaena* was grown at three different concentrations of iron. Instead, a culture could be started in an iron replete medium and Fd and Flv assayed over time as the organism became iron-depleted. This kind of study could also be done in reverse, by starting a culture in a low iron concentration and measuring Fd and Flv over time, after iron additions. Combined with other information, such as siderophore production and nitrate uptake rates, this study could help elucidate the mechanisms of survival under iron-stressed conditions. It would also be interesting to compare time course studies of different cyanobacteria and algae. By studying the methods by which different organisms cope with iron stress, it might be possible to explain blooms or the dominance of one organism in natural waters.

The method described here could be used as a routine analysis of nuisance blooms of cyanobacteria and algae in natural waters or reservoirs prior to the development of a treatment scheme. For example, if the organism is iron-depleted, it may produce siderophores, which also bind copper

(21). Copper sulfate has been used to control nuisance blooms since it is toxic to cyanobacteria in high concentrations. Chelation of copper has been observed to reduce its toxicity. Effective treatment of blooms with copper might be done before the organism responds to iron limitation with siderophore production. A rapid, sensitive assay of iron stress would help determine when the copper should be added.

Since iron availability may limit carbon and nitrogen assimilation in oceans, studies of iron nutrition in marine, photosynthetic microorganisms would be important to global ecological considerations (26). The method employed here to study *Trichodesmium* could be used to analyze other marine cyanobacteria and algae in order to understand the effect of iron availability on the environment.

REFERENCES

1. Smillie, Robert M. and Barrie Entsch (1971) In: Methods in Enzymology 23, Ed. by Anthony San Pietro, Academic Press, New York, 504-514.

2. Stanier, R. Y. and G. Cohen-Bazire (1977) Ann. Rev. Microbiol. 31, 225-274.

3. Sandmann, G. and P. Böger (1983) In: Encyclopedia of Plant Physiology 15, Ed. by A. Laüchli and R. K. Bielesley, Springer Publications, Berlin, 563-596.

4 Buchanan, Bob B. and Daniel I. Arnon (1971) In: Methods in Enzymology 23, Ed. by Anthony San Pietro, Academic Press, New York, 413-440.

5. Spiller, S. C., Castelfranco, A. M. and P. A. Castelfranco (1982) Plant Physiol. 69, 107-111.

6. Zumft, W.G. (1974) In: Bioenergetics and Metabolism of Green Algae:I, Ed. by J. Cardenas, MSS Information Corp., New York, 14-26.

7. Peschek, Günter (1979) FEMS Microbiology Letters 6, 371-374.

8. Averill, B. A. and W. H. Orme-Johnson (1978) In: Metal Ions in Biological Systems 7, Ed. by H. Siegel, Marcel Dekker, New York, 128-183.

9. Hardie, L. P., Balkwill, D. L. and S. E. Stevens Jr. (1983) Applied and Environmental Microbiology 45, No. 3, 1007-1017.

10. Sherman, Debra M. and Louis A. Sherman (1983) Journal of Bacteriology 156, No. 1, 393-401.

11. Guikema, James A. and Louis A. Sherman (1983) Plant Physiol. 73, 250-256.

12. Guikema, James A. and Louis A. Sherman (1984) Plant Physiol. 74, 90-95.

13. Öquist, Gunnar (1973) Physiol. Plant 30, 30-37.

14. Sandmann, Gerhard and Richard Malkin (1983) Plant Physiol. 23, 724-728.

15. Sandmann, Gerhard (1985) Photosynth. Res.6, 261-271.

16. Huisman, J. G., Touw, I., Liebregts, P. and A. Bernards (1979) Planta 145, 351-356.

17. Mortentson, L. E. and W. O. Gillum (1980) In: Methods in Enzymology 69, Ed. by Anthony San Pietro, Academic Press, New York, 779-793.

18. Evans, Hilary E., Rush, James D., Johnson, Charles E. and Michael C. W. Evans (1979) Biochem J. 182, 861-865.

19. Huntsman, Susan A. and William G. Sunda (1981) In: Physiological Phytoplankton Ecology, 285-328.

20. Simpson, Frank B. and Neilands, J. B. (1976) J. Phycol. 12, 44-48.

21. Clarke, Suzanne E., Stuart, Janice and Joann Sanders-Loehr (1987) Applied and Environmental Microbiology 53, No. 5, 917-922.

22. Rueter, John G. and Richard R. Petersen (1987) N. Z. J. Mar. and Freshwat. Res. 21, 435-445.

23. Verstreate, David R., Storch, Thomas A. and Dunham, Valgene L. (1980) Physol. Plant 50, 47-51.

24. Rueter, John G. and Dennis R. Ades (1987) J. Phycol. 23, 452-457.

25. Martin, John H and Steve E. Fitzwater (1988) Nature 331, No 6154, 341-343.

26. Rueter, John G. (1988) J. Phycol. 24, No. 2, 249-254.

27. Entsch, B., Sim, R. G. and B. G. Hatcher (1983) Marine Biology, 17-30.

28. Harrison, Gail I. and F. M. M. Morel (1986) Limnol. Oceanogr. 31, No. 5, 989-997.

29. Haygood, Margo G. and Kenneth H. Nealson (1984) In: Current Perspectives in Microbial Ecology, Ed. by M. J. Klug and C. A. Reddy, American Society for Microbiology, Washington, D.C., 56-61.

30. Petersen, R.(1982) Environ. Sci. Technol. 16, 443-447.

31. Bradford, M. (1976) Anal. Biochem. 72, 248.

32. Hutber, G. N., Hutson, K. G. and L. J. Rogers (1977) FEMS Microbiology Letters 1, 193-196.

33. Mayhew, S.G. and M. L. Ludwig (1975) In: The Enzymes 12, 3rd ed., Ed. by P.D. Boyer, Academic Press, New York, 57-118.

34. Ragsdale, Steve W. and Lars G. Ljungdahl (1984) Journal of Bacteriology 157, No 1, 1-6.

35. Palmer, G. (1973) In: Iron-Sulfur Proteins 2, Ed. by W. Lovenberg, Academic Press, New York, 285.