Limnol. Oceanogr., 44(7), 1999, 1609–1615

Ferredoxin and flavodoxin as biochemical indicators of iron limitation during open-ocean iron enrichment

Deana L. Erdner[1] *and Donald M. Anderson*
Department of Biology, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543

Abstract

Substitution of the noniron protein flavodoxin for the iron–sulfur protein ferredoxin is an iron-stress response employed by a variety of unicellular organisms, including many phytoplankton. The relative abundance of these two proteins has been shown to vary with the severity of growth limitation by iron in marine diatoms. During the IronEx II mesoscale iron-enrichment experiment, large volume (100–600 liters) phytoplankton samples were collected for analysis of community ferredoxin and flavodoxin abundance using a high-pressure liquid chromatography (HPLC) technique. In addition, three pennate diatom species isolated from the fertilization-induced phytoplankton bloom were used for follow-up laboratory experiments, which examined their iron physiology.

Prior to iron enrichment, biomass levels were insufficient to obtain any ferredoxin or flavodoxin signals. Measurements were successful after iron enrichment, with unexpected results. The strength of the HPLC signal tracked the biomass levels of the IronEx II phytoplankton bloom. Chromatographic peaks were evident on the fifth day following enrichment and persisted throughout the experiment before they declined and eventually disappeared following the last iron infusion. The main chromatographic peak was identified as flavodoxin; there was no evidence of ferredoxin in any of the samples. Pennate diatom clones isolated from the fertilization-induced bloom and grown in the laboratory retain the ability to make ferredoxin when iron-replete and induce flavodoxin when iron-stressed. When iron-limited, they are able to completely repress flavodoxin expression in about 1 d in response to iron resupply. Thus, the unexpected absence of ferredoxin and the persistence of flavodoxin during IronEx II, despite the observed increases in biomass and photosynthetic efficiency, suggest that the iron additions were insufficient to completely relieve physiological iron limitation.

Ever since Martin proposed the "iron hypothesis," considerable effort has been invested in the study of the relationship between iron availability and primary production (Martin 1990). Initial evidence for iron limitation of phytoplankton growth was derived primarily from the results of shipboard nutrient addition bottle bioassays (e.g., Buma et al. 1991; Coale 1991). More recently, two mesoscale iron-fertilization experiments performed in the eastern equatorial Pacific provided direct proof of iron limitation of production in that area (Martin et al. 1994; Coale et al. 1996). Interpretation of bottle bioassay results is complicated by potential artifacts due to manipulation and enclosure of the natural population (e.g., Venrick et al. 1977), and mesoscale enrichments are logistically difficult and prohibitively expensive. The difficulties inherent in these nutrient addition methods illustrate the need for a test of iron limitation that does not entail excessive manipulation or incubation of the phytoplankton community.

One of the most promising candidates for a specific assay of iron stress is the ferredoxin/flavodoxin system of proteins (LaRoche et al. 1995; Doucette et al. 1996). Under conditions of iron stress, many organisms are able to replace the iron–sulfur redox protein ferredoxin with its noniron-containing functional equivalent, flavodoxin. Whereas this adaptation has been studied in freshwater algae and bacteria for >30 yr (Smillie 1965; Zumft and Spiller 1971; Peleato et al. 1994), its characterization in marine phytoplankton has been relatively recent (Entsch et al. 1983; LaRoche et al. 1993; Doucette et al. 1996).

Results of laboratory studies of the ferredoxin/flavodoxin response support its use as an indicator of iron stress. Induction of the flavodoxin protein is a common response to iron limitation in a diversity of marine phytoplankton (LaRoche et al. 1995; Erdner et al. in press). Flavodoxin expression is also specific to iron stress, observed only in iron-limited cells and not in those growing under nitrate, phosphate, silicate, zinc, or light stress (LaRoche et al. 1993; Erdner et al. in press). Furthermore, flavodoxin protein expression is extremely sensitive to iron limitation, evident in cells growing at only 10–20% less than their maximum rate (McKay et al. 1997; Erdner et al. in press). These characteristics make flavodoxin alone an excellent diagnostic of the presence or absence of iron limitation. The comparative abundance of flavodoxin and ferredoxin together, however, can potentially indicate not only the presence but also the severity of iron stress. In *Thalassiosira weissflogii* grown under different degrees of iron limitation, the relative proportion of ferredoxin and flavodoxin varies with the extent of growth impairment by iron (Erdner et al. in press).

The aforementioned laboratory studies aimed to characterize this method for detection of iron limitation in natural waters. The true test of this system, however, is its appli-

[1] Present address: University of Warwick, Department of Biological Sciences, Coventry CV4 7AL, Great Britain.

Acknowledgments

The authors thank Elizabeth Mann and Penny Chisholm for the isolation and use of the three pennate diatom clones. We are especially grateful to Kenneth Coale and the organizers of the IronEx II experiment for both the opportunity to participate and for their help during the cruise. This work was supported by a Graduate Fellowship for Global Change (D.L.E.) and an Exploratory Research Agreement RP8021-05 from the Electric Power Research Institute (D.M.A.). Contribution 9901 from the Woods Hole Oceanographic Institution.

cation in the environment. The use of relative flavodoxin/ferredoxin abundance to assess temporal changes in iron stress is not without precedent. Jones (1988) used fast protein liquid chromatography (FPLC) to measure ferredoxin and flavodoxin in *Trichodesmium* collected near Barbados using a plankton net. Increases in the ferredoxin : flavodoxin ratio of this species tracked wind events associated with increased aeolian dust inputs. The *Trichodesmium* samples used by Jones were basically monospecific and could be collected in large quantities by a plankton net. In general, most oceanic samples will contain a greater mixture of phytoplankton species, particularly if they are to be collected in the quantities necessary for analysis by chromatographic techniques.

The IronEx II mesoscale iron-enrichment experiment (Coale et al. 1996) offered an opportunity to perform a test of the ferredoxin/flavodoxin indicator system as well as an HPLC method for their analysis and detection (Doucette et al. 1996). During the experiment, a patch of the eastern equatorial Pacific Ocean was enriched with iron and monitored for several weeks. Timing and magnitude of iron addition were controlled, allowing direct correlation with changes in phytoplankton physiology. The experimental patch was also intensively sampled for a variety of physical, chemical, and biological parameters, allowing interpretation of the ferredoxin/flavodoxin results in the context of other physiological data.

During the experiment, large volume phytoplankton samples were collected to monitor changes in the community ferredoxin and flavodoxin abundance. If the resident phytoplankton were indeed limited by iron, iron addition was expected to stimulate growth with a corresponding increase in the ferredoxin to flavodoxin ratio of the phytoplankton community. Proteins were extracted from filters containing a mixed phytoplankton assemblage (0.7–63-μm particles) and analyzed using an HPLC detection method. In addition, three pennate diatom species isolated during IronEx II were used for follow-up laboratory experiments, which examined their iron physiology. The results of the IronEx II analysis report the physiological response of the phytoplankton to iron addition. The combined results of IronEx II and laboratory studies highlight the role of ferredoxin and flavodoxin in cellular adaptation to iron stress in the environment.

Materials and methods

Sample collection—IronEx II—A description of the IronEx II in situ fertilization experiment and its results is given by Coale et al. (1996). Briefly, a 72-km^2 patch of the equatorial Pacific Ocean near 3.5°S, 104°W was enriched with iron and monitored for 19 d. Three separate iron infusions of ca. 2, 1, and 1 nM occurred on days 0, 3, and 7 of the experiment, respectively. For analysis of ferredoxin and flavodoxin, phytoplankton were collected from large volumes of seawater (100–600 liters) using a pump and filter system. An air-powered double-diaphragm pump (Aro) was used to draw water through a length of reinforced polyvinyl chloride tubing (0.75-inch ID) whose intake was positioned approximately 4 m away from the ship at a depth of about 3 m. Seawater was pumped through a 142-mm-diameter filter stack (Oceanic Industries) containing a 63-μm mesh Nitex screen and a Whatman GF/F glass-fiber filter in series. The glass-fiber filters containing the 0.7–63-μm plankton were then frozen in liquid nitrogen for later analysis in the laboratory.

Protein extraction—IronEx II samples—Filters were removed from liquid nitrogen and allowed to thaw partially on ice. While still frozen, the glass-fiber filters were minced with a razor blade. The minced filter was placed in a 50-ml chamber with 35 ml of ice-cold 0.5-mm-diameter glass beads, 25 ml of ice-cold chloroform, and 25 ml of ice-cold extraction buffer (0.1 M sodium phosphate, 0.1 M NaCl, 1 mM ethylenediamine-tetraacetic acid [EDTA], pH 7.0 with 13 mM beta-mercaptoethanol, and 1 μg ml^{-1} each pepstatin, leupeptin, and aprotinin). The filters were homogenized using a beadbeater (Bio-Spec Products) for two 1-min cycles in an ice and water bath with a 1-min cooling period in between. The filter slurry was transferred to a Pyrex glass bottle and centrifuged for 10 min at 2,000 $\times$ *g*, during which the organic and aqueous phases separated. After centrifugation, the aqueous phase was removed and concentrated to approximately 2 ml in a 3,000-MW cutoff ultrafilter (Centricon-3, Amicon). The concentrated samples were filtered (0.2 μm) prior to analysis by HPLC.

Ferredoxin and flavodoxin analysis—Samples were analyzed using a previously described HPLC method (Doucette et al. 1996). Ferredoxin and flavodoxin in cell extracts were separated by anion-exchange HPLC. Detection was performed with a Hewlett-Packard model 1050 diode array detector, which also allowed identification of the proteins by their absorption spectra. Quantification of peak areas was performed by Hewlett-Packard ChemStation software in autointegration mode.

Pennate diatom isolates—Three pennate diatom clones were isolated from the iron-fertilization–induced bloom during IronEx II by E. Mann. They were rendered clonal by D.E. via plating on ESNW seawater medium (see below) with 1% agar followed by single-cell isolations into liquid medium. They are identified here by their clone numbers, A3-30, 10-40A, and 7-47B, pending further taxonomic identification.

Iron-replete and -deplete culturing of diatom isolates—Cultures were grown in 0.2-μM–filtered Vineyard Sound, Massachusetts, seawater (31‰) enriched with ESNW nutrients according to Harrison (1980) with several modifications. Na_2HPO_4 was substituted in equimolar amounts for Na_2glyceroPO$_4$, and selenium (as H_2SeO_3) was added to a final concentration of 10^{-8} M. Trace metal additions were made according to Brand et al. (1983). Seawater was autoclave sterilized then enriched with sterile nutrients. Macronutrient (nitrate, phosphate, and silicate) stocks were sterilized by autoclaving, while iron, trace metal, selenium, EDTA, and vitamin stocks were sterile filtered (0.2 μm). Iron and EDTA were added to iron-replete cultures at 5 and

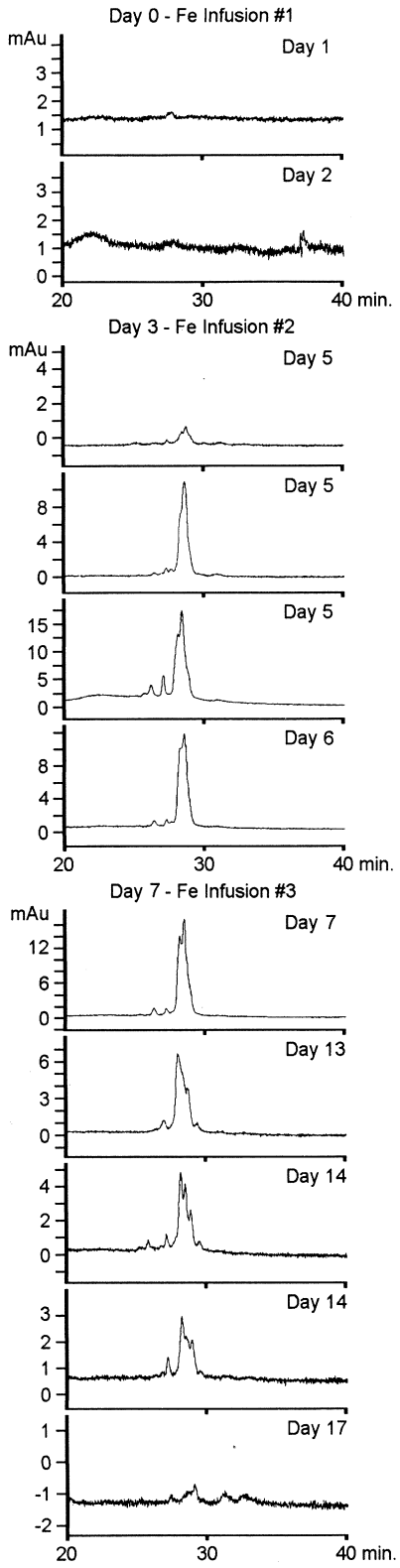

Fig. 1. HPLC chromatograms of samples collected in the IronEx II iron-enriched experimental patch. Collection date is given as days after the first iron infusion. On day 5, three samples were collected, at dawn, midday, and dusk. On day 14, two samples were again collected, one before dawn and another at midmorning. Ferredoxin and flavodoxin from marine phytoplankton elute between 25 and 30 min in this system, and the chromatograms have been formatted to cover the relevant temporal region.

50 μM, respectively. EDTA was added to iron-deplete cultures at 1 μM, and no iron was added.

Cultures (2-liter volume) were grown in acid-washed polycarbonate 2.8-liter Fernbach flasks, maintained at 26°C on a 14 : 10 light : dark (LD) cycle at an irradiance of ca. 200 μE m^{-2} s^{-1}. Cells were harvested by filtration onto 3-μm polycarbonate filters (47-mm diameter). Despite numerous attempts, it was not possible to obtain growth rates from any of the pennate diatom clones, as the cells formed large sticky aggregates that could not be dispersed.

Protein extraction—pennate diatom cultures—The polycarbonate filters containing the cells were minced and placed in a 2-ml screw-capped eppendorf tube with 1 ml of extraction buffer (recipe above) and ice-cold 0.5-mm-diameter zirconium beads. Cells were lysed by three 50-s cycles in a minibeadbeater (Bio-Spec Products) and cooled on ice for at least 1 min between cycles. The cell lysate was centrifuged for 1 h at 105,000 $\times$ *g*, and the resulting supernatant was filtered (0.45 μm) before analysis by HPLC as described above for the field samples.

Ferredoxin induction—One of the three pennate diatom clones, 7-47B, was chosen for analysis of the time course of ferredoxin induction. A 1-liter culture of clone 7-47B in iron-deplete medium (as described above) was inoculated into 19 liters of fresh iron-deplete medium in an acid-washed glass carboy. The cells were allowed to acclimate for 2 d prior to the start of the experiment. At time zero, 5 μM Fe and 50 μM EDTA were added to the carboy. Samples for ferredoxin and flavodoxin analysis were removed at 6-h intervals for the next 48 h, then again at 60 and 72 h. Cells were harvested by filtration, then extracted and analyzed by HPLC as described above.

Results

Open-ocean iron enrichment—Neither ferredoxin nor flavodoxin could be detected in samples collected prior to fertilization and outside of the iron-enriched patch during the experiment. HPLC chromatograms of extracts from samples collected inside the iron-fertilized patch are presented in Fig. 1, and their corresponding peak areas shown in Fig. 2. During the first 2 d of the experiment, following iron infusion 1, no ferredoxin or flavodoxin peaks were evident. Identifiable protein signals emerged following the second iron infusion and persisted through day 14 of the enrichment. Samples collected on day 17 again showed no chromatographic peaks.

The chromatograms from in-patch samples are characterized by one large peak accompanied by one to three much smaller peaks. This large primary peak exhibited a consistent retention time between samples and dominated the community profiles throughout the experiment. It was identified

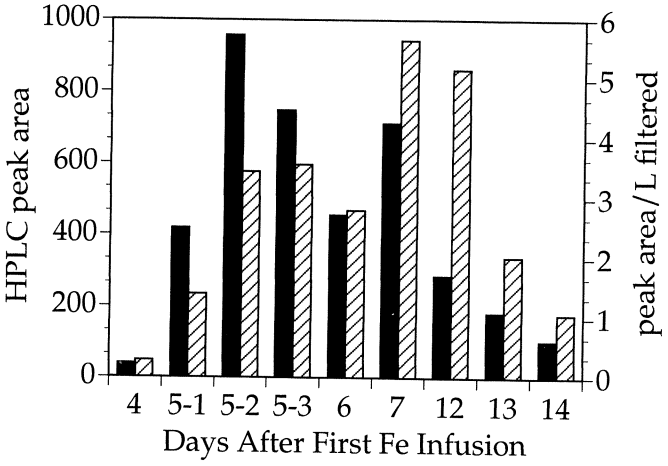

Fig. 2. Total area of flavodoxin peaks from chromatograms in Fig. 1. HPLC peak areas (filled bars) have also been normalized to the total volume of seawater filtered (shaded bars).

as flavodoxin by comparison of its ultraviolet (UV)-visible absorption spectrum (Fig. 3) with the spectra of ferredoxin and flavodoxin from a marine diatom (Erdner et al. in press). No ferredoxin signals were observed during the course of the experiment.

Ferredoxin and flavodoxin in equatorial Pacific pennate diatom cultures—Three pennate diatom clones, A3-30, 10-40A, and 7-47B, were isolated from the iron-fertilization–induced phytoplankton bloom. All three isolates were grown under iron-replete and -deplete conditions to assess their ability to synthesize ferredoxin and flavodoxin. The three organisms expressed only ferredoxin in high iron medium and only flavodoxin in iron-deficient culture (Fig. 4).

Ferredoxin and flavodoxin induction in an equatorial Pacific pennate diatom—Iron was resupplied to an iron-limited culture of clone 7-47B to determine the time required for synthesis of ferredoxin and suppression of flavodoxin expression. Changes in relative ferredoxin and flavodoxin abundance, expressed as the Fd index (ferredoxin peak area/sum of ferredoxin and flavodoxin peak areas), are shown in Fig. 5. At the time of iron resupply, the culture was moderately iron-limited, with an Fd index of approximately 0.5. Over the next 30 h, the Fd index steadily increased until flavo-

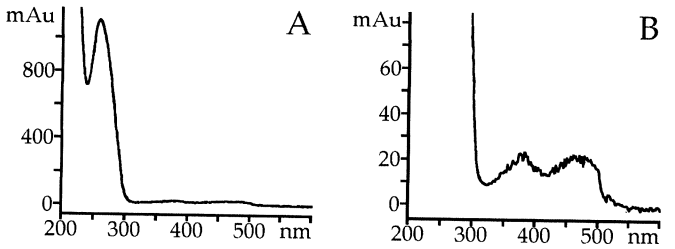

Fig. 3. Representative spectrum of the major peak in the IronEx II chromatograms shown (A) full scale, and (B) enlarged to show features in the 300–600-nm range. This component is clearly identified as flavodoxin.

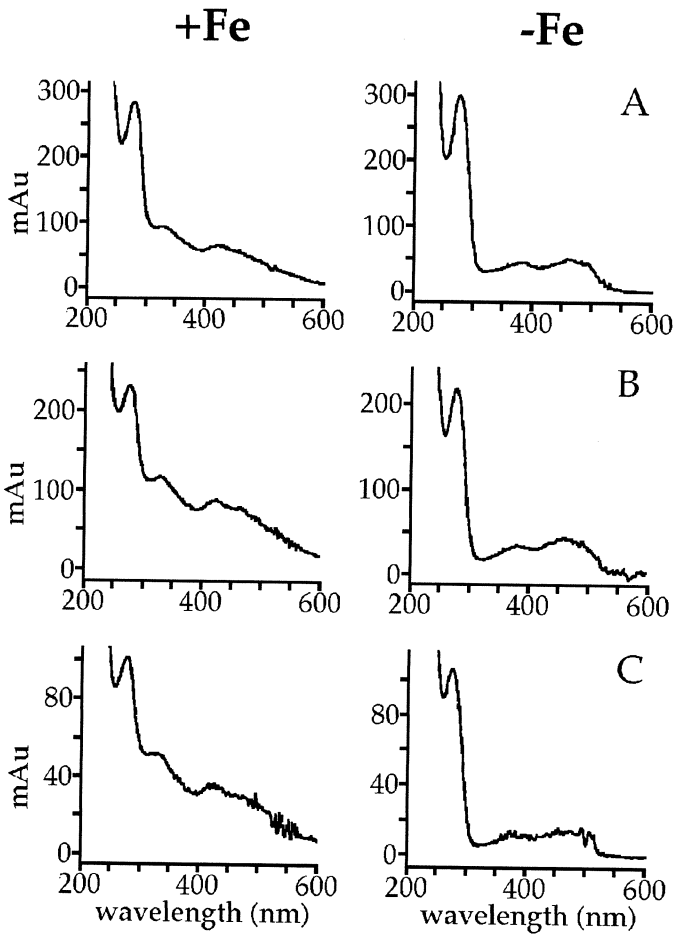

Fig. 4. UV-visible absorption spectra from HPLC analysis of pennate diatom clones (A) 10-40A, (B) 7-47B, and (C) A3-30 grown under iron-replete (+Fe) and -deplete (−Fe) conditions.

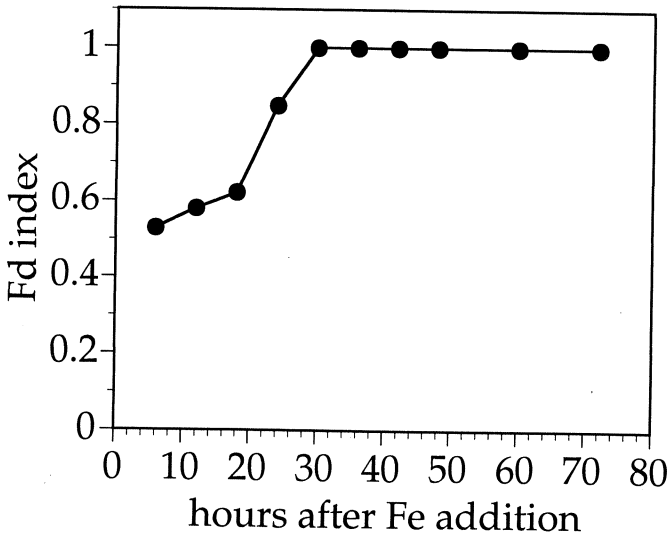

Fig. 5. Changes in the relative ferredoxin and flavodoxin abundance in iron-limited culture of clone 7-47B after resupply of iron. Ferredoxin and flavodoxin content is expressed as the Fd index (ferredoxin peak area/sum of ferredoxin and flavodoxin peak areas).

doxin expression was completely absent (Fd index = 1). Flavodoxin was undetectable after 30 h of incubation.

Discussion

Laboratory studies suggest that relative cellular ferredoxin/flavodoxin content, as measured by HPLC, is a sensitive and reliable indicator of iron limitation in cultured marine phytoplankton (Erdner et al. in press). The IronEx II mesoscale iron-enrichment experiment provided an invaluable opportunity to test both this biomarker system and its HPLC detection method in a well-characterized field situation. During IronEx II, HPLC analysis was successfully used to detect the iron nutritional status of the phytoplankton community during the iron-induced bloom. Several follow-up laboratory studies utilizing pennate diatom cultures isolated from the fertilization-induced phytoplankton bloom further substantiate our interpretation of the IronEx II data. The results of this analysis tell us not only about the response of the phytoplankton community to iron fertilization but also about the adaptive role the ferredoxin and flavodoxin proteins play in the environment.

HPLC of ferredoxin and flavodoxin in natural samples—The HPLC detection method worked well for samples collected from the IronEx II phytoplankton bloom, provided that sufficient biomass could be obtained. Samples collected prior to fertilization and outside of the patch during the experiment were analyzed but showed no chromatographic peaks. This complete lack of a signal indicates that the ferredoxin and/or flavodoxin levels in these samples were below the detection limits of the method, even though 300–600 liters had been filtered for each. The detection limit for this method is determined by the amount of protein that can be reliably identified by its UV-visible absorption spectrum using the diode array detector. This requires about 200 and 300 pmol of flavodoxin and ferredoxin, respectively, although smaller amounts will yield detectable (but not identifiable) peaks (data not shown). Use of the absorption spectrum of a peak rather than retention time for identification is preferable when analyzing mixed samples such as those from IronEx II or in situations where no appropriate standards are available.

Obviously, the primary limitation on the use of this HPLC technique for analysis of environmental samples is its insensitivity of detection. Large amounts of biomass are required but may be obtained by filtration, as shown here. The problem in this case was the inefficiency of protein extraction from the filters. The glass-fiber filters, which allow the filtration of large volumes of seawater, also hinder cell breakage and protein extraction. Calculations based on laboratory data for cellular flavodoxin and ferredoxin content estimate that only about 5–10% of these proteins were successfully extracted (*see* Erdner 1997). Improvements in extraction techniques should allow signals to be obtained from smaller samples, such as those obtained before iron addition. These improvements are vital if this technique is to be used to monitor phytoplankton populations at their natural abundance.

Ferredoxin and flavodoxin during IronEx II—The iron fertilization induced a "massive phytoplankton bloom" that was dominated primarily by pennate diatoms (Coale et al. 1996). Thus, the HPLC results are presumed to reflect the cellular response of these organisms. The strength of the HPLC signal parallels the growth of the fertilization-induced phytoplankton bloom. Extracted chlorophyll concentrations in the experimental patch, a proxy for phytoplankton biomass, had doubled by day 2, before increasing to >10 times the initial value on day 6. Values remained elevated and gradually subsided to background levels on day 16 (Cavender-Bares et al. 1999). Protein peaks in the HPLC chromatograms show a similar pattern (Figs. 1, 2). The first two in-patch samples were below the detection limit because phytoplankton biomass was still too low. The first identifiable peaks appeared on day 5, similar to huge increases in chlorophyll concentrations. A large signal persisted throughout the experiment but, like chlorophyll, declined then disappeared between days 14 and 17.

The chromatograms from in-patch samples are characterized by one large main peak, identified as flavodoxin by its absorption spectrum (Fig. 3). There is no clear evidence of ferredoxin induction in any of the samples. This result is somewhat unexpected, as we anticipated that ferredoxin would be resynthesized when the iron-starved cells were supplied with iron. There are several potential explanations for the observed lack of ferredoxin during the IronEx II enrichment despite the tremendous increase in biomass generated by fertilization: (1) ferredoxin may not be extractable or detectable in natural communities, (2) the endemic phytoplankton, having evolved in a chronically low iron environment, may lack the ability to make ferredoxin, (3) there was insufficient time for ferredoxin resynthesis, and (4) there was no significant induction of ferredoxin despite iron addition. Each of these possibilities is explored below.

HPLC detection of ferredoxin in natural samples—It is possible that ferredoxin, unlike flavodoxin, is difficult to extract from natural samples. Alternatively, it may be unstable once isolated and thus not detectable. This is not the case, however, as ferredoxin has been observed in both estuarine and coastal marine phytoplankton using this same HPLC technique (Erdner 1997).

Ferredoxin and flavodoxin in equatorial Pacific pennate diatoms—Several pennate diatom clones isolated from the IronEx II bloom were cultured under iron-replete and -deplete conditions to assess their ability to synthesize ferredoxin and flavodoxin (Fig. 4). Ferredoxin expression was evident in all three species when grown in high iron medium. When iron-limited, all of the organisms expressed only flavodoxin. These results are consistent with those obtained from a number of other marine phytoplankton (Erdner et al. in press). Flavodoxin expression is somewhat variable among species, but all marine phytoplankton species examined to date are able to express ferredoxin. Thus, the lack of ferredoxin induction during IronEx II cannot be attributed to the inability of the native phytoplankton to synthesize ferredoxin.

Kinetics of ferredoxin and flavodoxin protein expression—One of the three diatom isolates, clone 7-47B, was used to determine the time scale in which cells can alter their ferredoxin and flavodoxin expression. Iron was resupplied to an iron-limited culture of clone 7-47B, and its relative ferredoxin and flavodoxin content was measured every 6 h afterward using HPLC. Before iron addition, the ferredoxin : flavodoxin ratio of the culture was approximately 0.5, indicating only mild iron limitation. Although no iron was added to the culture medium, it was made with natural seawater, which can vary in its composition. The particular batch of seawater used in this instance seemed to contain unusually high amounts of bioavailable iron, as judged from the response of this and several other unrelated cultures. Nonetheless, the response of this culture to iron addition still provides information on the time scale of the protein response. Within 30 h of iron addition, this organism was able to induce ferredoxin synthesis and degrade any residual flavodoxin (Fig. 5). These findings are consistent with the results of LaRoche et al. (1995), who found that natural populations of diatoms could switch on flavodoxin synthesis in about 1 d. Thus, the IronEx II experiment, which was monitored for 19 d, provided ample time for cells to respond to iron addition by modifying their protein expression.

Absence of ferredoxin induction during IronEx II—The final, and most plausible, explanation for the absence of ferredoxin expression during IronEx II is that the cells simply did not synthesize ferredoxin to any significant degree. Although no ferredoxin peaks were identified in any of the samples, it is possible that ferredoxin was present below the identification limit of about 300 pmol per sample. The largest IronEx II sample analyzed contained the biomass from 1,063 liters of water collected during the height of the phytoplankton bloom (chromatogram not shown). The area of its flavodoxin peak corresponds to approximately 1,100 pmol of flavodoxin (using a standard curve for flavodoxin from *Chlorella fusca*). Thus, during IronEx II, there were less than three molecules of ferredoxin for every 11 molecules of flavodoxin. If ferredoxin was present, it was at an extremely low level.

Unfortunately, the absence of ferredoxin during IronEx II means that changes in flavodoxin expression cannot be quantitatively assessed. The two proteins, when measured in the same sample, act as internal references for one another. Normalization of flavodoxin content to other common parameters for the purpose of quantitation is problematic for a number of reasons. Because of the differences in extraction efficiency, flavodoxin content cannot be compared to chlorophyll extracted from the same filter. Comparison to total or soluble extracted protein is also likely to be inaccurate. Total protein is derived from all organisms, heterotrophic and autotrophic, that were captured on the glass-fiber filters, whereas the flavodoxin would be derived only from the autotrophs. As a result of the lack of ferredoxin expression, the flavodoxin results presented here must be considered to be qualitative.

Persistence of iron limitation during IronEx II—There is no doubt that phytoplankton growth in the equatorial Pacific is limited by iron, based on the unequivocal biological response to iron fertilization (Behrenfeld et al. 1996; Coale et al. 1996). Because we were unable to obtain signals prior to fertilization, the flavodoxin data here tell us only about the iron status of the population after iron addition. The conclusion, however, is rather unexpected—the persistence of flavodoxin indicates that the iron addition was insufficient to completely relieve physiological iron limitation. The presence of flavodoxin has been shown to be a sensitive and specific indicator of iron limitation (LaRoche et al. 1995, 1996; McKay et al. 1997; Erdner et al. in press). Flavodoxin expression in the absence of ferredoxin is symptomatic of severe iron limitation (Erdner et al. in press), a conclusion that is consistent with the relatively low growth rates observed during the experiment. From the results of dilution experiments, the maximal diatom growth rate during IronEx II was calculated to be about 1.8 divisions d^{-1} (Coale et al. 1996). This is slow relative to the 3.3 divisions d^{-1} measured by Fryxell and Kaczmarska (1994) for similar pennate diatoms in iron-enrichment bottles.

The apparent contradiction between the lack of ferredoxin resynthesis and the substantial biological response observed during IronEx II emphasizes the role of ferredoxin and flavodoxin in cellular adaptation to iron stress. Flavodoxin substitution provides a significant decrement in cellular iron requirements. Iron contained in ferredoxin may account for approximately 30–40% of the Fe quota of an iron-replete *T. weissflogii* cell (Erdner 1997). Thus, replacement of ferredoxin with flavodoxin allows cells to easily and significantly lower their iron requirements. For diatoms, which exhibit extremely high maximum growth rates, utilization of flavodoxin allows rapid growth under suboptimal iron supply.

Cellular ferredoxin and flavodoxin content is also extremely sensitive to iron stress. In *T. weissflogii,* flavodoxin synthesis is induced when cells are only mildly limited by iron. Ferredoxin content decreases and flavodoxin levels rise when cells are grown at 90% of their maximum rate (Erdner et al. in press). Thus, ferredoxin may be one of the first cellular components to respond to iron limitation, representing an internal labile iron pool for phytoplankton. Conversely, it may be one of the last components to be reconstituted when iron is resupplied. More essential compounds such as light-harvesting pigments and photosynthetic reaction centers, which do not have equivalent substitutes, are apparently resynthesized before ferredoxin. This is evident from the increases in cellular chlorophyll content (Coale et al. 1996) and photochemical efficiency (Behrenfeld et al. 1996) observed during IronEx II in the absence of ferredoxin induction.

The results presented here also indicate that the adaptive role of flavodoxin that has been observed in the laboratory is relevant to conditions in natural waters. Flavodoxin expression is an extremely sensitive indicator of iron limitation, responding immediately when iron availability drops below that required for maximal growth (McKay et al. 1997; Erdner et al. in press). This may be irrelevant for populations in areas such as the equatorial Pacific; it is unlikely that they receive iron inputs that are even a fraction of that required for fully replete growth. The IronEx II iron additions of 2, 1, and 1 nM were slightly more than what would be expected

from natural inputs, which suggests that organisms in the study area may always be reliant upon flavodoxin. For these organisms, flavodoxin substitution is an essential adaptation that allows them to persist in iron-poor environments, respond rapidly to periodic inputs, and grow quickly on very small amounts of iron.

References

BEHRENFELD, M. J., A. J. BALE, Z. S. KOLBER, J. AIKEN, AND P. G. FALKOWSKI. 1996. Confirmation of iron limitation of phytoplankton photosynthesis in the equatorial Pacific Ocean. Nature **383:** 508–511.

BRAND, L. E., W. G. SUNDA, AND R. R. L. GUILLARD. 1983. Limitation of marine phytoplankton reproductive rates by zinc, manganese and iron. Limnol. Oceanogr. **28:** 1182–1198.

BUMA, A. G. J., H. J. W. DE BAAR, R. F. NOLTING, AND A. J. VAN BENNEKOM. 1991. Trace metal enrichment experiments in the Weddell–Scotia Seas: Effects of iron and manganese on various plankton communities. Limnol. Oceanogr. **36:** 1865–1878.

CAVENDER-BARES, K. K., E. L. MANN, S. W. CHISHOLM, M. E. ONDRUSEK, AND R. R. BIDIGARE. 1999. Differential response of equatorial Pacific phytoplankton to iron fertilization. Limnol. Oceanogr. **44:** 237–246.

COALE, K. H. 1991. Effects of iron, manganese, copper, and zinc enrichments on productivity and biomass in the subarctic Pacific. Limnol. Oceanogr. **36:** 1851–1864.

———, AND OTHERS. 1996. A massive phytoplankton bloom induced by an ecosystem-scale iron fertilization experiment in the equatorial Pacific Ocean. Nature **383:** 495–501.

DOUCETTE, G. J., D. L. ERDNER, M. L. PELEATO, J. J. HARTMAN, AND D. M. ANDERSON. 1996. Quantitative analysis of iron-stress related proteins in *Thalassiosira weissflogii:* Measurement of flavodoxin and ferredoxin using HPLC. Mar. Ecol. Prog. Ser. **130:** 269–276.

ENTSCH, B., R. G. SIM, AND B. G. HATCHER. 1983. Indications from photosynthetic components that iron is a limiting nutrient in primary producers on coral reefs. Mar. Biol. **73:** 17–30.

ERDNER, D. L. 1997. Characterization of ferredoxin and flavodoxin as molecular indicators of iron limitation in marine eukaryotic phytoplankton. Ph.D. thesis, MIT/WHOI.

———, N. M. PRICE, G. J. DOUCETTE, M. L. PELEATO, AND D. M. ANDERSON. In press. Characterization of ferredoxin and flavodoxin as markers of iron limitation in marine phytoplankton. Mar. Ecol. Prog. Ser.

FRYXELL, G. A., AND I. KACZMARSKA. 1994. Specific variability in Fe-enriched cultures from the equatorial Pacific. J. Plankton Res. **16:** 755–769.

HARRISON, P. J., R. E. WATERS, AND F. J. R. TAYLOR. 1980. A broad spectrum artificial seawater medium for coastal and open ocean phytoplankton. J. Phycol. **16:** 28–35.

JONES, K. L. 1988. Analysis of ferredoxin and flavodoxin in *Anabaena* and *Trichodesmium* using fast protein liquid chromatography. M.Sc. thesis, Portland State Univ.

LAROCHE, J., P. W. BOYD, R. M. MCKAY, AND R. J. GEIDER. 1996. Flavodoxin as an *in situ* marker for iron stress in phytoplankton. Nature **382:** 802–805.

LAROCHE, J., R. J. GEIDER, L. M. GRAZIANO, H. MURRAY, AND K. LEWIS. 1993. Induction of specific proteins in eukaryotic algae grown under iron-, phosphorous-, or nitrogen-deficient conditions. J. Phycol. **29:** 767–777.

LAROCHE, J., H. MURRAY, M. ORELLANA, AND J. NEWTON. 1995. Flavodoxin expression as an indicator of iron limitation in marine diatoms. J. Phycol. **31:** 520–530.

MARTIN, J. H. 1990. Glacial–interglacial CO_2 change: The iron hypothesis. Paleoceanography **5:** 1–13.

———, AND OTHERS. 1994. Testing the iron hypothesis in ecosystems of the equatorial Pacific Ocean. Nature **371:** 123–129.

MCKAY, R. M. L., R. J. GEIDER, AND J. LAROCHE. 1997. Physiological and biochemical response of the photosynthetic apparatus of two marine diatoms to Fe stress. Plant Physiol. **114:** 615–622.

PELEATO, M. L., S. AYORA, L. A. INDA, AND C. GÓMEZ-MORENO. 1994. Isolation and characterization of two different flavodoxins from the eukaryote *Chlorella fusca.* Biochem. J. **302:** 807–811.

SMILLIE, R. M. 1965. Isolation of phytoflavin, a flavoprotein with chloroplast ferredoxin activity. Plant Physiol. **40:** 1124–1128.

VENRICK, E. L., J. R. BEERS, AND J. F. HEINBOKEL. 1977. Possible consequences of containing microplankton for physiological rate measurements. J. Exp. Mar. Biol. Ecol. **26:** 55–76.

ZUMFT, W. G., AND H. SPILLER. 1971. Characterization of a flavodoxin from the green alga *Chlorella.* Biochem. Biophys. Res. Commun. **45:** 112–118.

Received: 8 October 1998
Accepted: 28 April 1999
Amended: 19 May 1999