Effects of Iron Starvation on the Physiology of the Cyanobacterium Agmenellum quadruplicatum

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The effects of iron starvation on the growth and physiology of the unicellular cyanobacterium Agmenellum quadruplicatum were studied. Uptake of iron from the medium did not occur at a constant rate. The majority of the iron was removed at two different times, when the cells were initially inoculated into the medium and after the cultures had become quite dense and had stopped growing. Iron became limiting for growth 16 h after transfer to an iron-deficient medium, but cultures retained full viability for at least 212 h. Once iron became limiting, c-phycocyanin and chlorophyll a were degraded concurrently. This was followed by an accumulation of intracellular glucose in place of the c-phycocyanin. Nitrate and nitrite reductase activities were elevated through 50 h, after which they decreased steadily. The photosynthetic unit size also increased through 50 h and then decreased. Once iron was restored to the culture medium, growth resumed. The intracellular pigment levels increased rapidly as the glucose level diminished.

The growth and metabolic activity of cyanobacteria in natural environments may be limited by any of a variety of elements, depending on the ecosystem studied. Nitrogen, carbon, phosphorous, and iron (among others) have been shown to be limiting in specific instances (10, 15). Of these, iron has been shown to be especially important with regard to the ability of cyanobacteria to compete with the other microflora present (22, 23, 44). This appears to be the case, even though some cyanobacteria are unusually efficient iron assimilators (3, 23).

Relatively little is known about the effects of iron starvation on cyanobacterial metabolism. Iron is involved in nitrogen assimilation, since ferredoxin is required as the electron donor for both nitrate and nitrite reductase activities (12, 30, 44). Iron is also important in photosynthesis. It affects the synthesis of the major photosynthetic pigments chlorophyll a (CHL) and c-phycocyanin (CPC) (24), and its incorporation into ferredoxin and the iron-sulfur proteins on the acceptor side of photosystem I makes it a vital component of photosynthesis (5, 32, 34). Iron also affects cytochrome synthesis since it is required for heme synthesis (17, 42, 45). These observations suggest that iron starvation could lead to nitrogen starvation or carbon starvation or both in cyanobacteria, although such a possibility has never been investigated.

Agmenellum quadruplicatum strain PR-6 is a unicellular, halotolerant cyanobacterium. Although little is known about the iron in this organism (3), there have been several studies on nitrogen and phosphorous metabolism in A. quadruplicatum (27, 36, 37), including the physiological effects of nitrogen or phosphorous starvation (36, 37). A. quadruplicatum was able to withstand long periods of nitrogen or phosphorous starvation while maintaining full viability. The organism accomplished this by undergoing a distinct series of physiological changes and intracellular structural alterations.

The present study was undertaken to ascertain the effects of iron starvation on certain physiological characteristics of A. quadruplicatum. The purpose of this investigation was threefold: (i) to assess cell viability throughout prolonged iron starvation, (ii) to assess whether iron starvation may lead to nitrogen starvation or carbon starvation or both, and (iii) to compare the effects of iron starvation with those described previously for nitrogen and phosphorous starvation. We describe here a series of physiological changes which took place in response to prolonged iron starvation and which took place while the organism retained full viability.

MATERIALS AND METHODS

Organism, medium, and growth. A. quadruplicatum strain PR-6 was isolated and described by Van Baalen (43). Cells were grown on medium A of Provasoli et al. (31), as modified by Van Baalen (43) and Stevens et al. (10). Of these, iron has been shown to be especially important with regard to the ability of cyanobacteria to compete with the other microflora present (22, 23, 44). This appears to be the case, even though some cyanobacteria are unusually efficient iron assimilators (3, 23).

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(38). The iron-sufficient medium contained 3.89 mg of FeCl₃ · 6H₂O per liter (0.8 μg of Fe per ml) as a source of iron; the iron-deficient medium contained 0.5 μg of this compound per liter (0.008 μg/ml). Liquid cultures were incubated at 39°C in a water bath. Continuous agitation and carbon were provided by bubbling sterile 5% (vol/vol) CO₂ in air through the cultures. Illumination was provided by four (two on each side of the bath) type F24T12 CW/HO fluorescent lamps (3.8 × 10⁻² μEinsteins cm⁻² s⁻¹ incident on each tube).

Measurement of growth and viability. Growth was measured turbidimetrically with a Bausch & Lomb Spectronic 88 spectrophotometer at 550 nm. A cell suspension with an optical density of 1.0 contained 4.7 × 10⁹ ± 8 × 10⁹ cells per ml, as determined by viable counts, and a dry weight of 0.15 mg/ml. Viability was determined by spread plating in triplicate onto iron-sufficient medium containing 1.5% Bacto-Agar (Difco Laboratories, Detroit, Mich.).

Iron starvation and regrowth of starved cells. The cells used in the iron starvation experiments were first grown in iron-sufficient medium. Exponentially growing cells were then concentrated by centrifugation and washed twice with iron-deficient medium. The washed cells were inoculated into tubes of iron-deficient medium and iron-sufficient medium (as a control) at a starting optical density of 0.01. The cultures were incubated for several days as described above, and samples were removed periodically for biochemical assays.

To study the effects of adding iron back to starved cultures, cells were inoculated into iron-deficient medium as described above. After 200 to 212 h of incubation, 3.89 mg of FeCl₃ · 6H₂O per liter was added back to each tube. Incubation was then continued, and samples were removed periodically for biochemical assays.

Disappearance of iron from the growth medium. Iron concentrations in the growth medium were determined by using the ferrozine assay described by Stokey (35). The absorbance of the final reaction product was read at 562 nm in 1-cm glass cuvettes with a Beckman model DU-8 scanning spectrophotometer. All attempts to measure the rate of iron disappearance were made with iron-sufficient medium because the concentration of iron in the iron-deficient medium was below the detection limit of this assay. It was necessary to omit Na₂-EDTA from the medium because it interfered with the ferrozine assay, producing deceptively low iron values. The medium was filter sterilized (0.45-μm membrane filters; Millipore Corp., Bedford, Mass.) without iron because the filters tended to retain the iron at the normal medium pH (pH 7.8 to 8.0). Sterile FeCl₃ · 6H₂O (3.89 mg/liter) was added after filtration.

Removal of iron from the medium described above was investigated with two physiologically distinct populations of cells. Exponentially growing iron-sufficient cells were used in the first case, whereas cells that had been grown in iron-deficient medium for 90 h were used in the second. In both cases, the filter-sterilized iron-sufficient medium was inoculated to an optical density at 550 nm of 0.1. The preparations were then incubated for 72 h at 91°C and harvested and withdrawn periodically for the ferrozine assay. Cells were removed from the growth medium by centrifugation and filtration (0.45-μm Millipore filters) before the assay was carried out. To prevent loss of iron during this process, it was necessary to lower the pH of the medium to 2.0 with HCl. The samples were monitored frequently by light microscopy to insure that the low pH did not trigger lysis.

Glucose content. For determinations of intracellular glucose content, iron-sufficient and iron-deficient cells were removed from their respective growth media and washed twice by centrifugation in distilled water. The washed cells were placed in Hungate culture tubes and suspended in 4 ml of 0.25 M trifluoroacetic acid. These tubes were sealed tightly and then incubated for 16 to 17 h at 95°C. After incubation, the samples were cooled to room temperature and neutralized with NaOH. Glucose concentrations were determined with a Glucose Assay Kit 15-10 (Sigma Chemical Co., St. Louis, Mo.). The absorbance of the final reaction product was read at 340 nm with a Beckman model DU-8 scanning spectrophotometer.

Pigment levels. Intracellular concentrations of CHL and the accessory pigment CPC were determined as described by Stevens et al., using a Beckman model DU-8 scanning spectrophotometer fitted with a scattered-light detector. Whole-cell absorption spectra taken from 400 to 750 nm and the equations of Sigalat and de Kouchkovsky (35) were used to calculate the absolute amounts of CHL and CPC, as described by Kipe-Noll and Stevens (14).

PSU determination. Cells were harvested for isolation of P700-CHL-protein complexes and subsequent photosynthetic unit (PSU) determinations by the procedure of Perry et al. (29). The P700-CHL-protein complexes were then isolated by using the procedure of Shiozawa et al., (33). The CHL contents of the final, detergent-extracted samples were determined by measuring their levels of absorbance between 350 and 725 nm with an Aminco model DW-2a spectrophotometer. The CHL concentrations were calculated by using the equation of Thornber (41). The P700 contents of the samples were determined from the reversible light-induced absorbance change at 697 nm by using an isobestic point of 725 nm as a reference (33). This was done with the Aminco model DW-2a spectrophotometer in which an actinic light source was turned on and off repeatedly. The reactions were facilitated by adding 100 μM sodium ascorbate and 10 μM methyl viologen (29). The PSU was calculated as the molar ratio of CHL to P700.

Nitrate and nitrite reductase activities. The nitrate reductase activities of iron-sufficient and iron-starved cells were analyzed by measuring the amount of NO₂⁻ produced from NO₃⁻. A cell suspension containing 4 × 10⁹ cells was pelleted by centrifugation and suspended by vortexing for 1 min with 0.4 ml of 50 mM Tris-hydrochloride buffer (pH 8.0) containing 0.025% Nonidet P-40 (Bethesda Research Laboratories, Bethesda, Md.). Each sample was incubated for 2 min at room temperature, after which 1.6 ml of reagent mixture was added. The reagent mixture, which was sparged with oxygen-free nitrogen gas before use, contained 1 ml of 1 M Na₂CO₃/NaHCO₃ (pH 10.0), 2 ml of 0.5 M NaNO₃, and 4 ml of 30 mM methyl viologen. The reaction was allowed to proceed for 1 min, and then 0.5 ml of the cell-reagent mixture was pipetted into tubes containing 0.1 ml of 1 M barium acetate and 1.9 ml of 95% ethanol at 0°C. These samples were vortexed until the blue color disap-
pared and then centrifuged to remove precipitates. The supernatants were prepared for the NO$_2^-$ assay by mixing 1 ml with 0.5 ml of sulfanilic acid reagent (0.58 g of sodium sulfanilate in 5 ml of concentrated HCl and 20 ml of distilled water) for 3 min at room temperature, after which 0.5 ml of a N-1-naphthylethylene diamine dihydrochloride solution (0.32 mg/ml) was added. After incubation for an additional 20 min, the absorbance of each sample at 540 nm was measured against a reagent blank with a Beckman model DU-8 scanning spectrophotometer.

The nitrite reductase activities of iron-sufficient and iron-starved cells were analyzed by measuring the disappearance of NO$_2^-$ as it was converted to ammonia. A cell suspension containing $7 \times 10^8$ cells was pelleted and washed twice by centrifugation in 0.1 M KH$_2$PO$_4$ buffer and then disrupted in a French pressure cell at 12,000 lb/in$^2$. Cell debris was removed by centrifugation, and a reaction mixture containing 0.2 ml of 0.5 M Tris-hydrochloride buffer (pH 7.0 to 7.5), 0.3 ml of a sodium dithionite solution (0.313 g of Na$_2$S$_2$O$_4$ in 5 ml of 0.23 M NaHCO$_3$), 0.3 ml of 5.0 mM methyl viologen, and 0.2 ml of 20 mM NaNO$_2$ was added to 2.0 ml of the supernatant. All reagents were sparged with oxygen-free nitrogen before use. Each reaction mixture was incubated for 5 min, and then 0.5 ml was removed and mixed with 1 ml of 0.01 M MgCl$_2$ at 0°C. This mixture was vortexed and centrifuged. The resulting supernatant was added to a tube containing 0.2 ml of 1.0 M barium acetate and 5 ml of 95% ethanol at 0°C, incubated for 5 min, and centrifuged again. The supernatant was then prepared for the NO$_2^-$ assay by adding 0.5 ml to a solution containing 0.5 ml of 1% sulfanilamide (in 3 M HCl), 0.5 ml of 0.02% N-1-naphthylethylene diamine dihydrochloride, and 3.5 ml of distilled water. After incubation for 20 min, the absorbance of each sample at 540 nm was measured against a reagent blank with a Beckman model DU-8 scanning spectrophotometer.

**RESULTS**

**Growth and viability.** The turbidimetrically measured growth of *A. quadruplicatum* in iron-sufficient and iron-deficient media is shown in Fig. 1. Growth was exponential, with a doubling time of 2.5 h, in both media for approximately 16 h. After 16 h, the growth rate of iron-starved cultures decreased progressively. Cultures in both media reached their maximum optical densities by approximately 60 h, which then remained fairly constant through 212 h. The addition of pure FeCl$_3$·6H$_2$O to iron-starved cultures after 212 h resulted in a second increase in optical density. The cessation of growth in iron-sufficient culture at 60 h was probably due to light limitation caused by the increase in optical density (note scale), since the iron-sufficient medium contained ample nutrients to support further growth.

The viabilities of iron-sufficient and iron-starved cultures, as determined by plate counts, are also shown in Fig. 1. No appreciable cell death was detected in the iron-deficient medium through 212 h of incubation.
fresh iron-sufficient medium, a considerable amount of the iron in that fresh medium disappeared immediately (35 to 37% and 43 to 44%, respectively). The iron concentration remained fairly constant for the first 35 to 45 h after inoculation, but then decreased steadily until the cultures stopped growing.

Glucose content. The glucose content of cells grown in iron-sufficient medium (data not shown) remained constant at 5% of cellular dry weight, until the cultures entered the stationary phase. By contrast, the glucose content of cells grown in iron-deficient medium (Fig. 1) increased from 5 to 45% as growth tapered off in response to iron starvation; it then remained at approximately 45% through 212 h. Addition of FeCl₃·6H₂O to the cultures at 212 h resulted in a rapid loss of intracellular glucose; the glucose content dropped from 45 to 20% within 26 h and to 12% within 50 h after the addition of iron.

Pigment contents. The intracellular photosynthetic pigment contents of A. quadruplicatum grown in iron-sufficient and iron-deficient media are shown in Fig. 3 and 4, respectively. The concentrations of both CHL and CPC in iron-sufficient cells increased until growth began to taper off and then decreased steadily throughout the stationary phase. In the iron-deficient medium, the concentrations of both pigments increased only until growth started to slow down in response to the lack of iron (at approximately 16 h). The concentrations then began to decrease, although the CHL concentration decreased more rapidly than the CPC concentration between 16 and 90 h. After 90 h, the concentrations of both pigments decreased at approximately the same rate. When FeCl₃·6H₂O was added to starved cultures after 200 h, the concentrations of both pigments increased rapidly.

PSU. The PSU of A. quadruplicatum, as defined by the molar ratio of CHL to P700, fluctuated widely during growth in iron-deficient medium (Fig. 5); it rose from 210 to 1,100 by the time growth ceased in response to iron starvation (at approximately 50 h). The PSU then decreased continuously through 180 h. The PSU of iron-sufficient cells remained constant (at approximately 200) until the cultures entered the stationary phase (data not shown).

Nitrate and nitrite reductase activities. The nitrate reductase activity of A. quadruplicatum grown in iron-sufficient medium remained at 7.9 × 10⁻⁶ nmol of NO₂⁻ produced per cell per min throughout the period of exponential growth (data not shown). In contrast, the nitrate reductase activity of iron-starved cells increased between 0 and 17 h, remained somewhat elevated between 17 and 60 h, and then decreased between 60 and 100 h (Fig. 6).

Similar results were obtained for nitrite reductase activity. Activity remained constant (at approximately 4 × 10⁻⁶ nmol of NO₂⁻ consumed per cell per min) throughout the exponential growth period in iron-sufficient medium. In iron-starved cells, the nitrite reductase activity increased slightly between 0 and 17 h, remained elevated between 17 and 45 h, and then dropped between 45 and 80 h (Fig. 6).
DISCUSSION

In this study we documented some of the physiological changes that take place in *A. quadruplicatum* strain PR-6 during iron starvation. There can be little doubt that the long-term effects described here actually did result from a shortage of iron, since growth resumed when iron was added back to iron-deficient cultures. In addition, these effects were not observed when cultures were grown in iron-sufficient medium.

We presume that in this study the phenomena observed early (around 16 h after inoculation into iron-deficient medium) were actually triggered by depletion of iron from the medium. However, it was not possible to determine precisely when the iron was fully depleted. Because the iron concentration of the iron-deficient medium was too low to be measured accurately, we attempted to calculate an iron uptake rate by using iron-sufficient medium. This was unsuccessful because iron was not taken up at a constant rate. A considerable amount of the iron was removed from the medium as soon as the cells were added to it. Similar results were obtained by Parel (26), who used $^{55}$Fe autoradiography to study iron uptake in *Anabaena* and related bacteria. This author noticed that 55% of the $^{55}$Fe was found in clumps attached to the slime layer on the *Anabaena* filaments. Lange (16) has suggested that such slime layers may facilitate nutrient uptake. Perhaps slime-producing organisms can absorb iron in excess of their immediate cellular requirements and then hold the metal in reserve until later needs warrant its mobilization into the cell. This idea is certainly consistent with the results of the present study. After the initial absorption of iron at inoculation, very little iron was removed from the medium until the cultures became quite dense. Iron then started to disappear more rapidly, perhaps because the iron initially absorbed by the slime layer at inoculation was depleted. In this regard, *A. quadruplicatum* has been shown to possess an extracellular slime layer or glycocalyx that is present under a wide variety of environmental conditions (4, 36, 37).

*A. quadruplicatum* began to break down the accessory pigment CPC shortly after the growth rate dropped in response to iron deprivation (at 16 h). This compound was probably degraded early during iron starvation because it is a relatively expendable cellular component. Cyanobacteria can continue to photosynthesize without CPC by using CHL as the primary light acceptor (47), although the rate of photosynthesis is somewhat reduced under these conditions (20). It should be realized, however, that cyanobacteria are generally thought to degrade CPC as a source of endogenous nitrogen (6, 47). In fact, the disappearance of this compound from cells is a well-documented result of nitrogen starvation or limitation in *A. quadruplicatum* (36) and other cyanobacteria (1, 2, 6, 18, 46, 47). This suggests that iron starvation in the present study led to a shortage of endogenous nitrogen, which, in turn, prompted the organism to degrade CPC. Since the activities of both nitrate reductase and nitrite reductase decreased markedly after 50 h, this shortage must have been the result of impaired nitrogen assimilation during the later portions of the starvation period. A similar result was noted for *Anabaena flos-aquae* grown in the absence of iron (44). It is not entirely clear, however, why there may have been a shortage of endogenous nitrogen while the activities of these two enzymes were elevated (16 to 50 h). The most likely explanation is suggested by the results of Armstrong and Van Baalen (3), who showed that *A. quadruplicatum* produces large numbers of iron-chelating siderophores during iron starva-
tion. Since compounds like this can sometimes be the most quantitatively important products of amino acid synthesis (9), *A. quadruplicatum* may not have been able to assimilate sufficient nitrogen for synthesis of both siderophores and the nitrogenous compounds normally needed for growth. Consequently, CPC was degraded as a supplement to any exogenous nitrogen still being assimilated. For that matter, the stimulation of reductase activities itself may have been a direct result of the need for additional nitrogen.

As CPC was degraded by *A. quadruplicatum* during iron starvation, intracellular glucose accumulated in its place. Like CPC degradation, glucose accumulation (usually in the form of polysaccharides like glycogen) is a well-documented result of nitrogen starvation in *A. quadruplicatum* (36) and other cyanobacteria (1, 2, 8, 11, 19, 28). This lends further support to the idea that the iron-starved *A. quadruplicatum* cells were deficient in nitrogen. Meisch et al. (21) reported an accumulation of starch in the chloroplasts of the green alga *Chlorella fusca* during iron limitation, but did not speculate on the possible relationship of this accumulation to nitrogen metabolism.

Some of the iron starvation effects observed in the present study were clearly not related directly to nitrogen metabolism. For example, CPC and CHL were degraded extensively at the same time. Similar results have been reported for *Anacystis nidulans* during iron deficiency (24). In contrast, CHL was not degraded during nitrogen starvation in *A. quadruplicatum* (36) or other cyanobacteria (2, 6, 7, 8, 13, 18, 25, 46-48). Both pigments were degraded during phosphorous starvation in *A. quadruplicatum*, but phosphorous starvation also led to the accumulation of cyanophycin granule polypeptide. Cyanophycin granule polypeptide was not detected during iron starvation (unpublished data). Thus, the eventual degradation of CHL was probably a direct result of iron deficiency and probably reflects the importance of iron in chlorophyll synthesis.

The extensive degradation of both CHL and CPC during prolonged iron starvation undoubtedly led to a marked decrease in photosynthetic activity because these compounds serve as the primary light acceptors for photosystems I and II, respectively. This was also quite different from what was observed during nitrogen starvation in *A. quadruplicatum* (36), in which the persistence of CHL probably allowed some photosynthesis to occur throughout the entire starvation period. If so, this would explain why more intracellular glucose accumulated during nitrogen starvation than during iron starvation; in the latter case, CO₂ assimilation ceased earlier because photosynthesis was severely impaired. Eventually, these cells may have become starved for carbon, as well as for nitrogen and iron.

The apparent increase in PSU size through the first 40 h of growth in iron-deficient medium represents further evidence that photosynthetic activity was reduced during iron starvation of *A. quadruplicatum*. An increase in the PSU indicates that the CHL-associated P700 protein is being degraded (CHL is high in concentration compared with fewer P700 molecules), thereby limiting the amount of light energy passing into photosystem I and leading to the impairment of photosystem I-related reactions (such as CO₂ assimilation). The decrease in the PSU observed after 40 h was due to the extensive degradation of CHL that was taking place during the course of iron starvation. The PSU underwent a change in apparent size of approximately fivefold during the course of iron starvation.

*A. quadruplicatum* remained fully viable throughout prolonged iron starvation, despite the fact that it was probably lacking nitrogen and (possibly) carbon in addition to iron. The mechanism for this is not fully understood, but some of our results are suggestive. The intracellular glucose that accumulated during iron starvation was degraded rapidly when iron was added back to starved cultures. At the same time, CHL and CPC were very rapidly restored to their normal intracellular levels. This could mean that the glucose was used as a readily available energy or carbon source (19) once iron was made available to the cells again, thereby enabling them to return to active photosynthetic metabolism quickly. Thus, glucose accumulation and storage may have been important factors in maintaining cell viability throughout the starvation period. This seems reasonable because the same kind of response was observed in *A. quadruplicatum* during nitrogen or phosphorous starvation. In the former case, intracellular glucose (in the form of glycogen) accumulated as it did in the present study (36). Glucose accumulated to some extent during phosphorous starvation as well, but there was considerably more extensive accumulation of cyanophycin granule polypeptide (37). Since the accumulation of glucose in the present study was less extensive than that observed during nitrogen starvation, the possibility remains that one or more undetected substances accumulated along with the glucose during iron starvation. In any event, *A. quadruplicatum* has now been shown to respond to a variety of starvation situations by accumulating one or more intracellular reserve substances. The significance of these compounds with regard to survival and recovery from starvation merits further investigation, as does their possible significance with regard to the survival
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of cyanobacteria in nutrient-poor natural environments.

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