Active Transport of Ferric Schizokinen in *Anabaena* sp.

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Received 27 October 1981/Accepted 13 February 1982

The cyanobacterium *Anabaena* sp. strain ATCC 27898 was found to utilize the siderophore schizokinen to accumulate iron from the environment. This organism had previously been shown to produce schizokinen under low-iron conditions, and we observed that the iron-transport capability is also increased in response to iron limitation. Uptake activity was specific for ferric schizokinen and did not occur with ferrioxamine B. The uptake of ferric schizokinen displayed kinetics typical of a protein-mediated process with an apparent K_m of 0.04 μM and saturation at high concentrations of substrate. Light-driven transport was blocked by uncouplers and by ATPase inhibitors. Transport in dark-adapted cells was additionally blocked by inhibitors of respiration. We conclude that ATP serves as an energy source for the cellular uptake of ferric schizokinen.

The efficient utilization of iron by microorganisms requires the production of high-affinity chelating agents known as siderophores to solubilize iron in a biologically available form (16, 21). A wide variety of bacteria and fungi produce siderophores as well as membrane transport proteins that facilitate uptake of hydrophilic siderophore complexes across nonpolar cell membranes (21, 23). Most siderophore transport systems require the input of cellular energy for iron uptake into cells (8, 9, 20, 22).

Cyanobacteria also produce siderophores to satisfy their iron requirements. Field experiments by Murphy et al. showed that under iron limitation, *Anabaena flos-aquae* produces a hydroxamate siderophore which suppresses the growth of eucaryotic algae and promotes "bloom" formation (19). The production of a siderophore by *Anabaena flos-aquae* has been confirmed in an independently isolated axenic culture and extended to *Anabaena cylindrica* (17). In addition to freshwater species, the marine cyanobacterium *Agmenellum quadruplicatum* has shown siderophore activity (2). Furthermore, the experiments of Lange on the ability of microorganisms to grow in the absence of added chelator suggest that many other cyanobacteria may eventually be found to produce siderophores (15).

The only cyanobacterial siderophore which has been structurally characterized is schizokinen, a dihydroxamate produced by *Anabaena* sp. strain ATCC 27898 (24). Schizokinen is a member of the citrate-hydroxamate family of siderophores, which includes aerobactin and

arthrobactin (16). The present study was undertaken to elucidate the role of schizokinen in facilitating iron uptake in *Anabaena* sp. strain ATCC 27898. We have been able to show that this organism contains an iron uptake system which has a high affinity for ferric schizokinen and whose activity increases in response to iron limitation. In addition, we have found that this iron transport requires metabolic energy. It has been possible to probe the nature of this energy requirement by using a variety of inhibitors.

MATERIALS AND METHODS

Growth of *Anabaena* sp. *Anabaena* sp. strain ATCC 27898 was maintained in axenic culture at 20°C and 800 lx on 1.5% agar slants of BG-11 medium (26) containing (per liter): 1.5 g of NaNO_3 , 40 mg of K_2HPO_4 , 20 mg of Na_2CO_3 , 75 mg of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 36 mg of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 6 mg of citric acid, 6 mg of ferric ammonium citrate, 1 mg of EDTA, 2.9 mg of H_3BO_3 , 1.8 mg of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.2 mg of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 mg of $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, 0.08 mg of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, and 0.4 mg of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (pH 7.1). Liquid cultures were grown on BG-11 medium without ferric ammonium citrate, and iron was added from a stock solution of 0.5 mM FeCl_3 in 10 mM HNO_3 , unless otherwise stated. The inoculum for the cultures was grown in 0.1 μM iron. Cultures were incubated at 34°C and continuous light (2,500 lx) from cool-white fluorescent lights (Sylvania Electric Products Inc.). Growth was monitored in a Klett-Summerson colorimeter fitted with a red no. 66 filter (100 Klett units = 0.46 mg [dry weight] per ml).

Growth media were sterilized by passage through 0.22- μm filters (GS, Millipore Corp.). All glassware was soaked in 6 N HCl and rinsed in glass-distilled demineralized water (Milli-Q system, Millipore) to minimize iron contamination. Milli-Q water was also used in the preparation of growth media. Liquid BG-11 culture medium with no added iron was analyzed by

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atomic absorption spectroscopy and found to contain less than $0.08 \mu\text{M}$ iron. *Anabaena* cultures were periodically checked for contaminating bacteria by inoculation into nutrient agar and also by microscopic examination of aged cultures.

Purification of schizokinen. Schizokinen was isolated from culture supernatants of *Bacillus megaterium* ATCC 19213. Cultures were grown in 500-ml batches in 2-liter Erlenmeyer flasks in the medium described by Mullis et al. (18). The medium was purged of iron by passage through a column of Chelex 100 (Bio-Rad Laboratories) before the addition of calcium and magnesium salts. Maximum yields were obtained when 10^{-7} M FeCl_3 was added to the medium. Cultures were harvested after incubation for 72 h at 37°C with heavy aeration.

Schizokinen was extracted from 10 liters of supernatant fluid after rotary evaporation to 250 ml as previously described (18). The aqueous extract was applied to a column (2.5 by 20 cm) of Bio-Rad AG-2-X10 in the acetate form ($\text{pH} \geq 6.0$), rinsed with 100 ml of distilled water, and eluted with a linear gradient of 0.1 to 1.0 M NH_4Cl . Schizokinen was detected by absorbance at 490 nm after 0.1 ml of each fraction was added to 0.9 ml of FeCl_3 in 0.1 M HClO_4 (4). Peak fractions were lyophilized. Subsequent gel filtration on Bio-Gel P2 (1.5 by 90 cm) produced pure schizokinen in the first half of the ferric iron reactive peak. After lyophilization and dissolution in D_2O , this material yielded an ^1H nuclear magnetic resonance spectrum which was identical to those previously published for schizokinen (18, 24).

Schizokinen concentrations were determined by titrating a sample with a standardized solution of ferric chloride in dilute HCl (followed by immediate readjustment to $\text{pH} 7.0$) to an endpoint in the absorbance at 390 nm. The titration was performed at neutral pH because schizokinen acetyl groups are labile upon prolonged exposure to acid.

Siderophore assays. The production of schizokinen by *Anabaena* sp. was measured by two different techniques. Hydroxamate groups in culture filtrates were assayed by the method of Csaky (7) with *N*-(1-naphthyl)ethylenediamine used as the color-forming agent (27) after a 4-h acid hydrolysis at 120°C (12). The presence of intact siderophores was measured using a bioassay with *Arthrobacter flavescens* JG-9 (ATCC 25091), an auxotrophic mutant requiring siderophore activity for growth. Tube cultures of JG-9 were grown in the medium described by Estep et al. (10) at 30°C on a rotary shaker set at 200 rpm. Growth was measured after 24 h and found to be proportional to the concentration of added purified schizokinen. The half-maximal response of 0.04 to 0.05 μg of schizokinen per ml was identical to that reported by Lankford for schizokinen (16).

Iron transport assay. *Anabaena* filaments were harvested from the exponential phase (0.2 to 0.4 mg [dry weight] per ml) by collection on 0.45- μm filters (HA, Millipore). The filaments were washed with, and suspended to a similar cell density in, Chelex 100-treated uptake medium containing (per liter): 1.10 g of NaNO_3 , 50 mg of NH_4Cl , 250 mg of K_2HPO_4 , 531 mg of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 58 mg of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ($\text{pH} 7.0$). Stock solutions of ^{55}Fe ferric schizokinen were prepared by mixing $^{55}\text{FeCl}_3$ in 0.1 M HCl (New England Nuclear Corp.; 25.9 mCi/mg) with a twofold molar

excess of schizokinen from *B. megaterium*, adjusting the pH to 7.0 with NaOH , and passing the solution through a 0.45- μm filter. Assays were initiated by the addition of ferric schizokinen (10 to 20 nM in ^{55}Fe) and incubated at 34°C and 2,500 lx unless otherwise noted. Samples were removed at timed intervals, rapidly filtered through 0.45- μm filters (HA, Millipore), and washed with 1 volume of ice-cold uptake medium. Samples of filtrates were dispersed in Ready-Solv HP scintillation fluid (Beckman Instruments, Inc.) and counted in a Beckman LS-9000 liquid scintillation counter. Uptake was calculated from the loss of counts relative to filtrates of cell-free solutions containing the same initial concentration of ^{55}Fe ferric schizokinen. For K_m determinations, the iron concentration was increased by adding unlabeled iron, and the resultant uptake values were corrected for the dilution of the radioactive label. The twofold molar excess of schizokinen to iron was utilized to ensure that all of the added iron was coordinated. Even a 10-fold excess of schizokinen caused no significant decrease in uptake activity.

Chemicals. Desferal (methane sulfonate salt of deferriferrioxamine B) was purchased from CIBA Pharmaceutical Co. *N,N'*-Dicyclohexylcarbodiimide (DCCD) and carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP) were obtained from Sigma Chemical Co.

RESULTS

Growth and siderophore production. *Anabaena* cultures were exposed to a range of iron concentrations to study the behavior of this organism in response to iron limitation. Growth curves for these cultures are shown in Fig. 1. In cultures receiving no added iron (i.e., with less than $0.08 \mu\text{M}$ iron present) or $1 \mu\text{M}$ added iron, an initial period of reduced growth was ob-

FIG. 1. Growth of *Anabaena* sp. in BG-11 medium as a function of added iron. Iron was added from a freshly prepared solution of filter-sterilized FeSO_4 as follows: □, 1.0 mM; ■, 100 μM ; ●, 10 μM ; ▲, 1.0 μM ; and ○, no added iron.

served, indicating an iron-limited state. However, once growth commenced, the rates approximated those observed with the 10 and 100 μM iron cultures, and a higher cell density was actually achieved with the iron-limited cultures. Only the 1 mM iron culture exhibited lack of growth due to iron toxicity.

It has been previously demonstrated by Simpson and Neilands (24) that growth of *Anabaena* sp. under iron-limiting conditions results in the production of Csaky assay-detectable (7) extracellular hydroxamates and that the hydroxamate-containing siderophore schizokinen is present in the supernatant of iron-limited cultures. To obtain a more direct measure of siderophore production in response to iron availability, we determined the siderophore activity of *Anabaena* filtrates by using the siderophore auxotroph *Arthrobacter flavescens* JG-9 as a test organism. We found that siderophore concentrations for cells grown on BG-11 medium minus EDTA and citrate increased linearly during days 1 through 5 of growth and that the amount of siderophore released was inversely proportional to the amount of iron in the *Anabaena* growth medium. After 5 days of growth, all of the *Anabaena* cultures showed a marked drop in the *Arthrobacter* assay response. A similar mid-log-phase drop in the production of *Arthrobacter*-active siderophore has been reported for the marine cyanobacterium *Agmenellum quadruplicatum* (2), which was also grown on nitrate-containing medium.

Subsequent experiments on *Anabaena* filtrates showed that *Arthrobacter* growth factor activity could be restored by organic extraction of the schizokinen or by omitting nitrate from the *Anabaena* growth medium (Table 1). Thus, siderophore levels continued to increase in log-phase *Anabaena* sp., and they were inversely proportional to the amount of iron in the growth medium. The apparent drop in growth factor activity after 5 days was most likely due to the production of a substance which was inhibitory to *Arthrobacter flavescens* JG-9, which was eliminated during an organic extraction, and which was particularly produced by cells grown in nitrate. Although nitrite would be a candidate for this inhibitor, we found that the concentration of nitrite was negligible according to Csaky assays performed without the addition to iodine.

Greatly enhanced growth factor activity was observed for *Anabaena* sp. grown on BG-11 medium minus EDTA with 0.1 mM citrate. For cells grown in 0.1 μM added iron, the schizokinen concentration in extracted filtrates increased to 42 μM by the end of the log phase. Medium blanks containing this amount of citrate had no significant growth factor activity with *Arthrobacter flavescens* JG-9.

Csaky assays also showed increased hydroxamate production in low-iron media. However, in this case the apparent siderophore concentrations from cells grown on N_2 , NH_4^+ , or NO_3^- were approximately 10 times higher than those obtained by the growth factor assay on extracted filtrates. When filtrates for the Csaky assay were extracted as described in Table 1, there was some reduction (ca. over twofold) in the Csaky response. Since schizokinen recoveries of about 80% are generally observed after organic extraction, the excess Csaky assay-positive material in unextracted filtrates must be due to a substance other than schizokinen. Since this Csaky assay-positive material was also present at high levels in cells grown in 10 μM iron, it is unlikely to be a siderophore. These results indicate that a preliminary purification of siderophores by organic extraction or column chromatography is required to obtain meaningful data on siderophore levels in the growth media of cyanobacteria with the above assays.

Specificity of iron transport. The ability of *Anabaena* sp. to take up iron from the medium was studied by exposing cells to ^{55}Fe under conditions in which all iron in the assay medium was bound to schizokinen. To ensure that the *Anabaena* cells were responding primarily to added [^{55}Fe]ferric schizokinen, we used an uptake medium which was devoid of iron chelators and depleted of endogenous iron, and we added 2 mol of schizokinen per mol of ^{55}Fe . After exposure to [^{55}Fe]ferric schizokinen, the cells were filtered and washed to remove loosely bound iron. The amount of radioactivity remaining in the filtrate was then determined. The

TABLE 1. Concentration of schizokinen in *Anabaena* filtrates^a

Nitrogen source ^b	Schizokinen concn (μM) with added iron:			
	No Fe		1.0 μM Fe	
	Filtrate	Extract ^c	Filtrate	Extract ^c
N_2	0.9	2.1	0.2	0.8
NH_4Cl , 1 mM	3.6	5.1	0.1	0.3
NaNO_3 , 13 mM	0.5	6.6	0.1	2.2

^a The schizokinen concentration was determined by growth factor activity for *Arthrobacter flavescens* JG-9 relative to a standard curve obtained with purified schizokinen. Errors were $\pm 30\%$ for values above 3 and $\pm 70\%$ for values below 2.

^b *Anabaena* sp. grown to the late log phase on BG-11 medium (minus citrate, EDTA, and NaNO_3) with nitrogen added as indicated.

^c *Anabaena* filtrates (1 to 2 ml) were extracted three times with 5 ml of chloroform-phenol (1:1 by weight); then 80 ml of ethyl ether was added, and the schizokinen was back-extracted twice with 5 ml of distilled water. The water layer was lyophilized, and the material was redissolved in the original sample volume.

uptake of ferric schizokinen by iron-starved cells was linear with time until the substrate became limiting (Fig. 2). The fraction of iron taken up was generally 75 to 90% of the amount added in the concentration range studied (10 to 50 nM Fe). The ability of *Anabaena* sp. to accumulate iron against a concentration gradient indicates that a transport system is present and that it is driven by metabolic energy. Furthermore, the transport system was found to be selective for iron bound to schizokinen. Another siderophore, ferrioxamine B, showed no support of iron uptake in concentrations of up to 0.055 μM in Fe and 0.11 μM in deferriferrioxamine B.

Control of iron transport. Cells grown in the presence of three different concentrations of iron were suspended to identical cell densities and tested for their ability to transport [^{55}Fe]ferric schizokinen. Ferric schizokinen was taken up by all cultures, but the rates of uptake were significantly faster in the iron-starved cultures (Fig. 3). The lower the concentration of iron in the growth medium, the greater the uptake rate. This pattern of inducible uptake activity indicates that the schizokinen-dependent iron uptake system is subject to regulatory control.

Iron transport kinetics. Further evidence for the involvement of specific carriers in the transport of ferric schizokinen was obtained by studying the kinetics of iron uptake as a function of the concentration of ferric schizokinen. Results demonstrate that uptake occurs via a saturable system (Fig. 4, inset). Double-reciprocal plots as in Fig. 4 yield an apparent K_m of 0.04 μM and a

FIG. 3. Rate of uptake of ferric schizokinen as a function of the iron concentration in the growth medium. *Anabaena* sp. was grown in 0 ($\circ$), 0.1 μM ($\square$), and 1.0 μM ($\triangle$) added iron. Cells were suspended to 52 Klett units in uptake medium containing 0.014 μM ^{55}Fe and 0.028 μM schizokinen.

$V_{\max}$ of approximately 10 nmol/min per g of cells (dry weight) (for *Anabaena* sp. exposed to 0.1 μM Fe). The K_m value is similar to reported values for the uptake of ferrienterobactin and

FIG. 2. Rate of uptake of ^{55}Fe by *Anabaena* cells. *Anabaena* sp. was grown in 0.1 μM added iron and suspended to 96 Klett units in uptake medium containing 0.012 μM ^{55}Fe and 0.024 μM schizokinen ($\circ$) and 0.008 μM ^{55}Fe and 0.016 μM Desferal ($\triangle$).

FIG. 4. Kinetics of uptake of ferric schizokinen. *Anabaena* sp. was grown in uptake medium containing 0.1 μM added iron and suspended to 47 Klett units in uptake medium containing a twofold excess of schizokinen (SK) to Fe.

ferrichrome by *Escherichia coli* (20, 28), and it gives an indication of the extremely high affinity of the transport system for its substrate.

Energy dependence. Once it was shown that *Anabaena* sp. is capable of accumulating ferric schizokinen against a concentration gradient, it was of interest to determine the energy requirements of the process. Since *Anabaena* sp. is an obligate phototroph, light is the ultimate source of all its metabolic energy. To determine whether ferric schizokinen uptake is energized directly by light-associated processes, uptake activity in the light and in the dark was measured. We found that uptake activity was only slightly decreased in the dark, ranging from 80 to 95% of light controls; dark adaptation periods from 15 min to 2 h had little effect.

To discriminate between possible sources of energy for iron uptake, the effect of various inhibitors on iron transport was tested (Table 2). Both light uptake and dark uptake were sensitive to the uncoupler CCCP, which dissipates the transmembrane, electrochemical hydrogen ion gradient. In addition, DCCD, an inhibitor of H^+ -translocating ATPases in bacteria, mitochondria, and chloroplasts (1), caused almost complete inhibition of iron uptake both in the light and in the dark. The sensitivity of iron uptake to DCCD implies that ATP is required for this process. The inhibition of iron uptake by arsenate, which inhibits ATP synthesis by acting as a substrate analog, supports this conclusion. The fact that ferric schizokinen uptake in the light is less sensitive than uptake in the dark to arsenate inhibition may possibly be due to a higher overall rate of ATP formation in the light.

The inhibition of ferric schizokinen uptake by cyanide in the dark strongly suggests that dark

uptake in *Anabaena* sp. is driven by respiratory electron transport. Cyanide is a well-known inhibitor of electron transport in bacteria and mitochondria due to strong binding of CN^- to the oxygen-binding site in the terminal cytochrome oxidase of the electron transport chain. The lack of significant cyanide inhibition of uptake in the light indicates that *Anabaena* sp. depends on photophosphorylation for light uptake and on oxidative phosphorylation for dark uptake.

DISCUSSION

Active transport of nutrients across bacterial cell membranes is generally facilitated by membrane permeases, periplasmic binding proteins, or intracellular modifications leading to group translocation (30). In the case of iron uptake in *E. coli*, ferrichrome transport has been shown to occur in inner membrane vesicles via a permease system which responds to the electrical component ($\Delta\psi$) of the membrane potential (20), possibly via a symport process with Ca^{2+} or Mg^{2+} bound to the ferrichrome molecule to produce a divalent cation (14). Ferrienterobactin uptake also requires an energized membrane state (22). Ferrienterobactin receptors with possible permease activity are present in both the outer and the inner membranes (23), with the inner membrane system presumably accounting for the $\Delta\mu_{H^+}$ dependence. In addition, there appears to be an ATP requirement for ferrienterobactin uptake in *E. coli*. However, in this heterotrophic organism, it is difficult to rule out the possibility that the primary target of ATP inhibitors is glucose uptake rather than iron uptake (22).

Conversely, with the autotroph *Anabaena* sp. we have been able to demonstrate unambiguously that ferric schizokinen uptake is dependent on the availability of ATP. The complete inhibition of light and dark ferric schizokinen uptake by the ATPase inhibitor DCCD, which is not expected to affect electron transport or the development of a membrane potential (5), clearly shows that the energized membrane state alone is not sufficient to drive the transport of iron. Similar results were obtained with arsenate, which blocks ATP formation. Because membrane level phosphorylation is the only known mechanism for ATP synthesis in cyanobacteria (25), it has not been possible to determine whether the inhibition caused by uncouplers and electron transport inhibitors is due only to interference with ATP formation or whether a membrane potential is also required for iron transport.

It is perhaps significant that ferric schizokinen and ferrienterobactin are monovalent and trivalent anions, respectively, at physiological pH.

TABLE 2. Effect of metabolic inhibitors on ferric schizokinen uptake in *Anabaena* sp.

Inhibitor	Concn	Uptake activity (%) ^a	
		Light	Dark
CCCP	8 μ M	10	0
DCCD	40 μ M	5	5
Na ₂ HAsO ₄	20 mM	50	15
KCN	100 μ M	90	20

^a Uptake activity relative to light or dark control in the absence of an inhibitor. Values are $\pm 5\%$ for CCCP, DCCD, and Na₂HAsO₄ and $\pm 10\%$ for KCN. For dark uptake, cells were dark adapted for 10 min before adding the inhibitor. The total time of exposure to the inhibitor before the addition of [⁵⁵Fe]ferric schizokinen was 20 min for CCCP and DCCD and 50 min for Na₂HAsO₄ and KCN. *Anabaena* cultures were grown in uptake medium containing 0.1 μ M added iron.

As a result, both must be transported against the electrical component of the membrane potential (interior negative). The ATP requirement shown by both systems suggests that transport is more complicated than a simple permease-type system, yet whether either system will fit the binding protein model or the group translocation model (or some combination of them) remains to be determined. The involvement of a periplasmic binding protein appears to have been ruled out in the case of ferrienterobactin uptake in *E. coli* (22). A group translocation process acting at the level of intracellular release of iron from ferric Desferal has been implicated in *B. megaterium* (6, 8).

Siderophore transport systems tend to be highly specific for a given ligand, so that if more than one siderophore is transported by an organism, separate transport systems exist for each one (3, 11). One of the most versatile organisms is *E. coli*, which utilizes not only the previously mentioned ferrichrome and enterobactin systems, but also an inducible ferric citrate system (23), a low-affinity Fe(III) uptake system (23), and a plasmid-mediated aerobactin uptake system (29). *B. megaterium* has separate transport systems that recognize ferrioxamine B and ferric schizokinen, but will not accept ferri-aerobactin, which is structurally similar to ferrischizokinen (6, 13). *Anabaena* sp. appears to be somewhat more selective than *B. megaterium* in that ferrioxamine B is not utilized as an iron transport mediator.

The affinity of siderophore transport systems for their substrates is generally higher in bacterial than in fungal systems. For comparison, *Neurospora crassa* accumulates iron via its own siderophore, coprogen, with a K_m of 20 μ M (31). The ferrichrome and ferrienterobactin systems in *E. coli* are characterized by K_m values of 0.15 to 0.25 and 0.10 to 0.36 μ M, respectively (11, 20, 28), whereas the apparent K_m for ferric schizokinen uptake in *Anabaena* sp. is 0.04 μ M. The high affinity of the *Anabaena* siderophore transport system may help to counteract the large potential for dilution of cyanobacterial extracellular products in an aquatic environment.

ACKNOWLEDGMENTS

We are grateful to Jack Myers, Mary Taylor, and Jon Abramson for their advice and discussions. We thank Alden Pritchett for his valuable and good-natured technical assistance.

This work was supported in part by Public Health Service grant GM 18865 from the National Institutes of Health.

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