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Iron Transport and Its Relation to Heme Biosynthesis in *Rhodopseudomonas sphaeroides*

MARK D. MOODY AND HARRY A. DAILEY*

Department of Microbiology, University of Georgia, Athens, Georgia 30602

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The uptake of iron supplied as ferric citrate or ferric parabactin was examined in aerobically grown whole cells and vesicles of *Rhodopseudomonas sphaeroides*. Inner and outer membrane fractions from *R. sphaeroides* contained no membrane proteins which were inducible by growth in low-iron medium. Vesicles composed of the inner membrane and devoid of outer membrane and periplasmic proteins were able to transport iron supplied as ferric citrate and ferric parabactin. This uptake required the presence of NADH. When the electrical component of the proton motive force was depleted in whole cells, the uptake of iron supplied as ferric parabactin was completely inhibited. The uptake of iron supplied as ferric citrate was inhibited by gallium citrate; however, Ga^{3+} was not transported. The relationship between iron uptake and heme synthesis was examined by treating whole cells with *N*-methylprotoporphyrin which inhibits ferrochelatase, the enzyme which inserts ferrous iron into protoporphyrin to form heme. This treatment reduced ferrochelatase activity by 82% but had no effect on iron uptake, indicating that iron uptake and heme synthesis are not directly coupled. The fate of transported iron was investigated by measuring intracellular concentrations of heme and nonheme iron. It was determined that newly transported iron exists primarily as nonheme iron.

Microorganisms existing under aerobic conditions at a neutral pH are presented with the problem of obtaining iron, which is virtually insoluble under these conditions (37). To obtain the iron necessary for growth, many microorganisms excrete ferric iron binding compounds (siderophores) which serve to solubilize iron so that it may be transported into the cell (29). The iron then is actively transported into the cell as free iron or as the intact iron-siderophore complex.

Although many of the details of iron acquisition are now understood in *Escherichia coli* (4, 17, 28, 31, 33), the facultative photosynthetic bacteria have received very little attention with respect to iron acquisition. This is unfortunate, since these organisms, particularly *Rhodopseudomonas sphaeroides*, have been the focus of a great deal of research pertaining to porphyrin and heme biosynthesis (see reference 12 for review). When *R. sphaeroides* shifts from aerobic dark growth to anaerobic photosynthetic growth, the total tetrapyrrole content of the cell increases about 200-fold. Although the heme content increases only about two-fold, the majority of the tetrapyrrole increase is due to bacteriochlorophyll production (23). Since the insertion of ferrous iron into protoporphyrin by ferrochelatase is the step which commits protoporphyrin to heme, the supply of iron could be the limiting factor in heme synthesis and could thus control the branch point between heme and bacteriochlorophyll synthesis.

Because of this possibility and to better understand the relationship between iron uptake and heme synthesis, we investigated aspects of iron metabolism in *R. sphaeroides*. Previously, investigators in this laboratory purified and characterized ferrochelatase (6), measured iron reductase activity present in cell extracts (26), and showed that *R. sphaeroides* is able to utilize iron supplied as the chelate of citrate or parabactin (a catachol-type siderophore produced by *Paracoccus denitrificans*) but does not produce a siderophore of its own (27). In the present study, the iron uptake system of *R. sphaeroides* was examined under aerobic growth conditions to determine what membrane components are necessary for iron uptake, how iron uptake is energized, and whether iron uptake is coupled to heme synthesis.

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MATERIALS AND METHODS

Organisms and growth conditions. *R. sphaeroides* L was obtained from J. Lascelles, University of California, Los Angeles, and was maintained photosynthetically in broth cultures or agar deeps of malate-glutamate (MG)-yeast extract medium as described previously (22, 27).

Cells for all experiments were grown aerobically at 29°C in the dark in iron-limited or iron-sufficient MG medium as described previously (27).

Examination of membrane proteins. Aerobic cultures of *R. sphaeroides* were grown in either low-iron (~0.6 μM Fe) or high-iron (12 μM Fe) MG medium. A 2-liter culture was harvested by centrifugation (10,000 × *g* for 10 min) at mid-log phase (0.3 mg [dry weight] per ml). These cells were washed with 400 ml of a 0.1-M sodium phosphate buffer (pH 7.6) which contained 10 mM EDTA and suspended in 60 ml of the same buffer. The cell suspensions were lysed by sonication in the presence of DNase with a Heat Systems Cell Disrupter (four times for 30 s each at 60 W). Whole cells and debris were removed by two 10-min centrifugations at 10,000 × *g*. Inner and outer membrane fractions were obtained from these extracts by the method of Ding and Kaplan (8). The isolated inner and outer membrane fractions were resuspended to 1.5 ml in a pH 8.1 buffer containing 10 mM Tris-acetate, 10% (wt/vol) glycerol, and 10 μg of phenylmethylsulfonyl fluoride per ml.

Vesicle preparation. Vesicles were prepared from aerobically grown *R. sphaeroides* by the lysozyme-EDTA method described by Kaback (19), except that the vesicles were resuspended and washed in 25 mM potassium phosphate

* Corresponding author.

(pH 7.0) and the washes were performed by centrifuging the vesicles at 30,000 × *g* for 30 min onto an 80% (wt/wt) sucrose cushion. The vesicles were harvested, collected after the final wash with a 35-min centrifugation at 4,200 × *g*, and then resuspended to a density of 0.5 mg of protein per ml in 25 mM potassium phosphate (pH 7.0). Vesicle formation was confirmed by light microscopy and polyacrylamide gel electrophoresis.

Transport experiments. The uptake of $^{59}Fe^{3+}$, $^{67}Ga^{3+}$, or [^{14}C]alanine was followed at 23 to 25°C as described previously (27), with the following modifications. Cells were harvested at densities corresponding to mid-log phase (0.45 to 0.58 mg [dry weight] per ml), except that those cells used in the ferrochelatase inhibition experiment were harvested at late-log phase (0.94 mg [dry weight] per ml). These cells were washed once in 10 mM potassium phosphate buffer (pH 7.5) which contained 11 mM L-glutamic acid and 20 mM DL-malic acid ($MGKPO_4$ buffer) (the same concentration of glutamate and malate as that found in the growth media). This buffer was prepared with distilled deionized water and treated with Chelex 100 to remove trace amounts of iron. The washed cells were resuspended in $MGKPO_4$ buffer to densities ranging from 0.38 to 0.44 mg (dry weight) per ml, and 2.5 ml of this cell suspension was added to 2.5 ml of $MGKPO_4$ buffer containing the isotopes and other components of the uptake assay. The uptake of iron by vesicles was performed as described above, except that the assay buffer was 25 mM potassium phosphate (pH 7.0) containing 8.0 mM NADH (added as described previously [15]) and the vesicles were at a concentration of 0.25 mg of protein per ml.

Inhibition of ferrochelatase. *N*-Methylprotoporphyrin (NMPP) was prepared as described by Kunze and Ortiz de Montellano (20). A fresh 1.0 mM solution of NMPP was prepared in 50% dimethyl sulfoxide; this was filter sterilized with a Gelman acrodisk filter, and 0.5 ml was added to a 500-ml early-log-phase culture (0.22 mg [dry weight] per ml) of *R. sphaeroides* growing aerobically in MG medium containing 0.7 μM Fe. A control culture was treated with 0.5 ml of filter-sterilized 50% dimethyl sulfoxide. These cultures were harvested by centrifugation (10,000 × *g* for 10 min) at late-log phase (0.94 mg [dry weight] per ml), washed with 400 ml of $MGKPO_4$ buffer (pH 7.5), and suspended in the same buffer to a density of 0.99 mg (dry weight) per ml. Samples of these cells were used immediately in ^{59}Fe and [^{14}C]alanine uptake experiments. The remaining cells were harvested by centrifugation and suspended in 65 ml of a pH 8.1 buffer containing 10 mM Tris-acetate, 0.5 mM dithiothreitol, and 10 μg of phenylmethylsulfonyl fluoride per ml. These cells were cooled to 5°C and then lysed by sonication (see above). Unbroken cells and debris were removed by centrifugation for 10 min at 10,000 × *g*. Membranes and soluble fractions were isolated by centrifugation for 90 min at 100,000 × *g*. The isolated membranes were suspended to 4.5 ml in the Tris-acetate buffer.

Determination of intracellular heme and nonheme iron. MG medium containing either 0.46 or 20 μM iron (300 ml of media per 1-liter Erlenmeyer flask) was inoculated with 2 ml of a culture of *R. sphaeroides* (0.8 mg [dry weight] per ml) which had been transferred four times in 0.46 μM Fe–MG medium. These cultures were incubated aerobically in the dark at 30°C. After 14 h the culture growing in 20 μM Fe was harvested (0.65 mg [dry weight] per ml) at 5°C by centrifugation (10,000 × *g* for 10 min) and washed with 100 ml of 5°C $MGKPO_4$ buffer (pH 7) containing 10 mM EDTA. The washed cells were then suspended to 12 ml in $MGKPO_4$ (pH 7), quick frozen with dry ice and acetone, and saved for heme and nonheme iron determinations. The 0.46 μM Fe cultures were allowed to grow for a longer time due to their slower growth rate. After 15 h, 4 mM Fe^{3+}–80 mM citrate was added to a 0.46 μM Fe culture to a final concentration of 20 μM Fe. After an additional 30 min of growth, this culture and a 0.46 μM Fe culture with no added iron were harvested (0.57 mg [dry weight] per ml), washed, and frozen as described above.

Heme iron was determined by measuring cytochrome *c* and cytochrome *b* in whole cells. Cell suspensions were diluted in $MGKPO_4$ buffer (pH 7) to concentrations of 3.6 to 5.7 mg (dry weight) per ml. Cytochrome concentrations were calculated from dithionite reduced minus oxidized difference spectra as described previously (5, 35). To ensure that the cytochromes were fully oxidized, buffers and cell suspensions were sparged with O_2.

Total iron was determined on wet ashed residues (1) by plasma emission spectroscopy. The values given were adjusted for background iron by subtracting the iron concentration found in a blank containing only the $MGKPO_4$ buffer and the acids used in the wet ash procedure.

Assays and determinations. Succinate dehydrogenase was assayed in inner and outer membrane fractions by the method of Singer (36). The protein composition of the inner and outer membrane fractions and of the vesicle preparations was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis performed as described by Laemmli (21). Proteins were visualized with silver stain (25).

Ferrochelatase was assayed in membrane preparations of NMPP-treated cells as previously described (6). Iron reductase activity was measured in the soluble fraction of NMPP-treated cells as previously described (26).

Protein was determined by the method of Lowry et al. (24). Protein determinations of whole cell suspensions were performed by the Lowry method, modified as previously described (14).

Gallium concentrations were determined with dichlorohydroxyquinoline (34).

Materials. Parabactin was prepared as described previously (38). $^{59}Fe^{3+}$, $^{67}Ga^{3+}$, and [^{14}C]alanine were purchased from Amersham Corp. Triphenylmethylphosphonium bromide (TPMP) was obtained from Phaltz and Bauer. All other reagents were of the highest quality available.

RESULTS

Effect of iron concentration on membrane protein composition. Since it has been reported previously that culture iron concentration has an effect on the membrane protein composition of *E. coli* (30), we chose to examine the membrane protein composition of *R. sphaeroides*. Inner and outer membrane preparations were isolated as described above from *R. sphaeroides* grown aerobically in MG medium with low or high iron concentration. Succinate dehydrogenase, which was used as a marker enzyme for the inner membrane fraction, was enriched 4.9- and 7.5-fold in the inner membrane preparations from high-iron and low-iron cultures, respectively (Table 1). However, inner membrane fractions from low-iron-grown cultures had only about one-third the succinate dehydrogenase specific activity of inner membrane fractions from high-iron-grown cultures. The inner membranes from cultures grown in low iron were a much lighter reddish-brown color than were the inner membranes from cells grown in high iron, indicating a lower cytochrome content.

When the protein composition of the membrane fractions from high- and low-iron cultures was compared by sodium

TABLE 1. Succinate dehydrogenase activity of *R. sphaeroides* membrane fractions

Membrane fraction	Succinate dehydrogenase activity[a] in cells grown in:	
	12 μM Fe	0.6 μM Fe
Inner	187	59.5
Outer	37.8	7.9

[a] Micromoles of dichlorophenol-indophenol reduced per minute per milligram of protein.

dodecyl sulfate-polyacrylamide gel electrophoresis, there were no obvious visible differences (Fig. 1). To exclude the possibility that 10 mM EDTA which was used in the membrane fraction isolation procedure could have solubilized any membrane proteins, crude membranes were obtained from *R. sphaeroides* grown in MG medium with high and low iron concentrations. A 10 mM potassium phosphate buffer (pH 7.5) containing 10 μg of phenylmethylsulfonyl fluoride per ml was used throughout the isolation procedure. When these crude membrane preparations were compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, there were no differences in protein composition (data not shown).

Uptake of iron by vesicles. To determine whether iron can be transported by inner membrane vesicles in the absence of outer membrane, periplasmic, or intracellular components, vesicles were prepared from aerobically grown *R. sphaeroides* and their ability to accumulate iron was monitored. These vesicles were observed to accumulate $^{59}Fe^{3+}$ supplied as either ferric citrate or ferric parabactin (Fig. 2). This uptake was dependent on the presence of NADH (8 mM), which presumably acted to donate electrons to the electron transport system and thus energize the vesicle. Although the iron supplied to the vesicles was in the ferric form, the presence of 8 mM NADH in the assay mixture caused concern that the iron may have been reduced nonenzymatically before uptake. To show that the untransported iron in the assay mixture was in the ferric form, ferrozine, a chromophoric ferrous iron chelator (26), was added to the assay mixture after the uptake assay was completed. No increase in absorbance was seen unless the iron in the assay mixture was reduced by the addition of dithionite, indicating

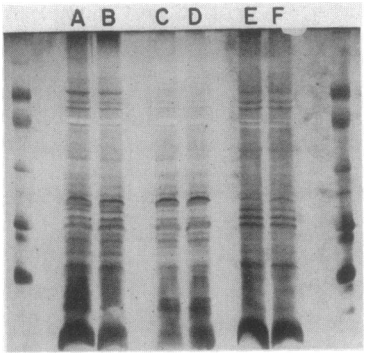

FIG. 1. Effect of iron on membrane protein composition. Inner and outer membrane fractions were obtained from aerobic cultures of *R. sphaeroides* grown in MG medium containing 0.6 or 12 μM Fe. Lanes: A and B, crude membrane fraction; C and D, inner membrane fraction; E and F, outer membrane fraction. Membranes were from cells grown in 12 μM Fe (lanes A, C, and E) or in 0.6 μM Fe (lanes B, D, and F).

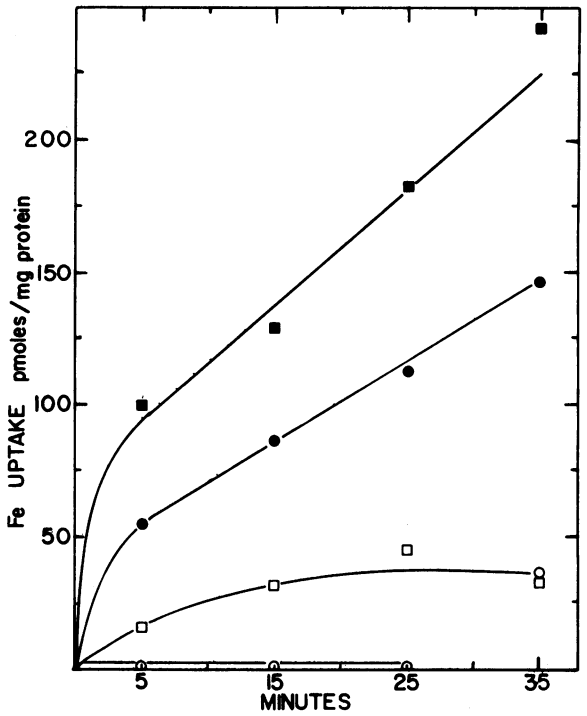

FIG. 2. Uptake of $^{59}Fe^{3+}$ by *R. sphaeroides* vesicles. Assays contained 2.5 μM Fe^{3+} and either 50 μM citrate or 3.0 μM parabactin and were performed with or without 8.0 mM NADH as an electron donor. Symbols: iron supplied as ferric citrate, plus NADH (■) or minus NADH (□); iron supplied as ferric parabactin, plus NADH (●) or minus NADH (○). Values given for Fe uptake in the presence of NADH are the average of six replicate observations.

that the untransported iron in the assay mixture was still in the ferric state.

Energetics of iron uptake. As mentioned above, the uptake of iron by vesicles did not occur unless they were energized with NADH (Fig. 2). This is in agreement with earlier observations that treatment of whole cells with NaN_3 resulted in a 78% reduction in the uptake of iron supplied as ferric parabactin (27) and that cells accumulated iron for a longer period of time when the aerobic uptake assay buffer contained oxidizable substrates such as malate and glutamate (unpublished data). In an effort to determine whether iron uptake proceeds via the proton motive force in *R. sphaeroides*, TPMP, a compound shown to deplete the electrical potential ($\Delta\psi$) while leaving the pH gradient (ΔpH) relatively undisturbed (2), was used in iron uptake experiments. The presence of 5 mM TPMP completely inhibited the uptake of $^{59}Fe^{3+}$ supplied as ferric parabactin (Fig. 3), suggesting that this uptake requires a membrane energized by the proton motive force and in particular $\Delta\psi$. Similar dependence on an energized membrane has been reported for ferrichrome transport by *E. coli* (28). Interestingly, the uptake of iron supplied as ferric citrate does require NADH or an energy source such as malate or glutamate but was not inhibited by NaN_3 (unpublished data) and therefore may not require an energized membrane.

Uptake of $^{67}Ga^{3+}$ by *R. sphaeroides*. Gallium is a trivalent metal which is similar in size and charge to ferric iron, but unlike iron it cannot be reduced to a divalent state. Thus, Ga^{3+} is a ferric iron analog that is experimentally useful to probe biological systems that recognize ferric iron (11). Here

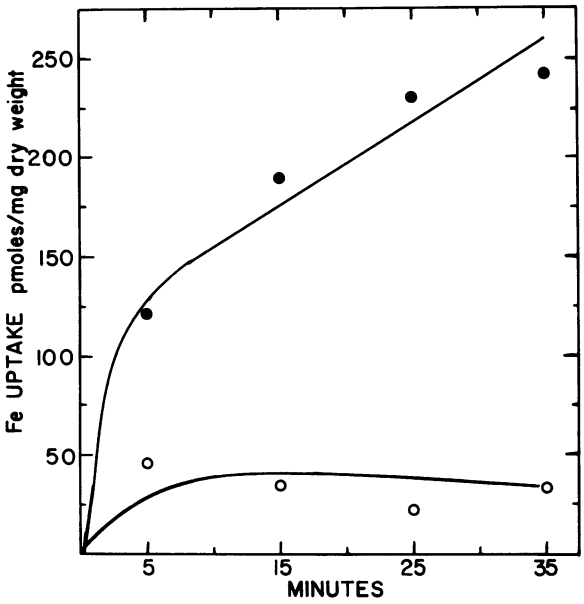

FIG. 3. Effect of TPMP on $^{59}Fe^{3+}$ uptake by *R. sphaeroides*. Assays contained 2.5 μM Fe and 3.0 μM parabactin with or without 5 mM TPMP. Symbols: ●, Fe uptake; ○, Fe uptake in the presence of TPMP.

we have made use of these features of gallium in an attempt to discriminate between simple uptake versus uptake coupled to ferric iron reduction. In competition experiments in which unlabeled gallium citrate (42 μM Ga^{3+} and 2.3 mM citrate) was present in a concentration 17 times that of labeled iron citrate (2.5 μM $^{59}Fe^{3+}$ and 50 μM citrate), there was strong inhibition of iron uptake (Fig. 4). Since these are short-term experiments in which gallium would not be expected to be toxic, the decrease in iron uptake is most likely due to competition between Fe^{3+} and Ga^{3+} for the transport system, indicating that the iron transport system recognizes ferric iron. The lower rate of iron uptake seen in the control of Fig. 4 as compared with that of Fig. 3 is due to the amount of citrate (2.3 mM) needed to solubilize the gallium. Citrate at this concentration was found to reduce the amount of iron transported, and it was necessary to include it in the control assay. To determine whether it is the trivalent or divalent form of iron which is actually transported into the cell, the uptake of $^{67}Ga^{3+}$ was examined. *R. sphaeroides* was unable to accumulate $^{67}Ga^{3+}$ citrate (data not shown), suggesting that reduction of ferric iron may be an obligate step for the uptake of iron supplied as ferric citrate.

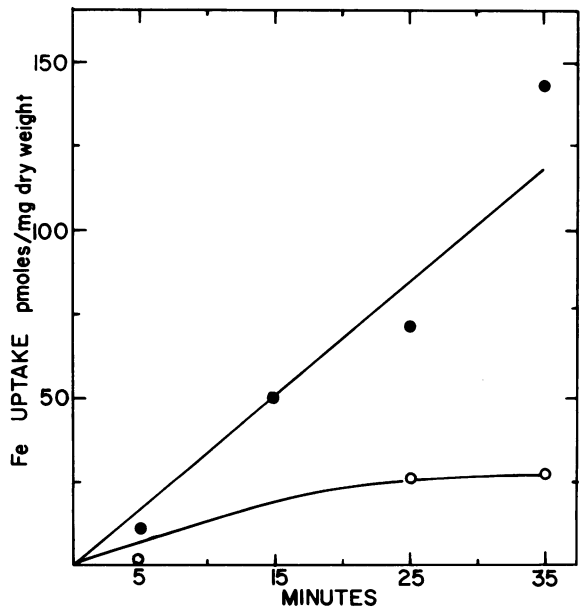

FIG. 4. Effect of Ga^{3+} on the uptake of $^{59}Fe^{3+}$ by *R. sphaeroides*. Assays contained 2.3 mM citrate and 2.5 μM Fe^{3+} with or without 42 μM Ga^{3+} Symbols: ●, Fe^{3+} uptake; ○, Fe^{3+} uptake in the presence of Ga^{3+}.

Effect of ferrochelatase inhibition on iron transport. NMPP is a tight binding competitive inhibitor of ferrochelatase with respect to protoporphyrin (7). NMPP previously has been demonstrated to inhibit heme synthesis in photosynthetic cultures of *R. sphaeroides* (16). Thus, treatment of cultures with NMPP provides a means to inhibit ferrochelatase and observe the relationship between heme synthesis and iron transport. Treatment of aerobically grown whole cells with 1.0 μM NMPP resulted in an 83% reduction of ferrochelatase specific activity when compared with that in control cells. When the uptake of $^{59}Fe^{3+}$ (supplied as ferric citrate) was examined in these same cells, there was no significant change in the rate of iron uptake (Fig. 5), demonstrating that a decrease in heme synthesis does not affect iron transport. To ensure that NMPP did not affect the ability of the cell to energize membrane transport systems, the uptake of [^{14}C]alanine was followed a control (Fig. 5).

Since the reduction of iron to the ferrous form is an intermediate step between the transport of iron and heme formation, it was necessary to determine whether enzymatic iron reduction might have been affected by NMPP. Iron reductase activity was measured in the cytoplasmic fraction of NMPP-treated cells and found not to differ from activity in the control cells (data not shown).

Fate of transported iron. *R. sphaeroides* was grown in iron-sufficient (20 μm Fe) or iron-deficient (0.46 μM Fe) MG medium, and the concentrations of heme and nonheme iron

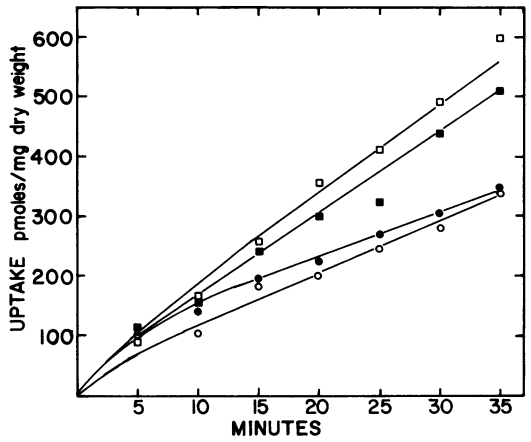

FIG. 5. Uptake of [^{14}C]alanine and $^{59}Fe^{3+}$ by *R. sphaeroides* grown in MG medium (0.7 μM Fe) alone or containing 1.0 μM NMPP. Assays contained either 4.9 μM Fe^{3+} and 109 μM citrate or 18 μM [^{14}C]alanine. Symbols: ○, Fe^{3+} uptake by NMPP-treated cells; ●, Fe^{3+} uptake by control cells; □, alanine uptake by NMPP-treated cells; ■, alanine uptake by control cells.

were determined in these cells (Table 2). Cells grown in medium with sufficient iron contained nearly 3 times as much heme iron (cytochrome *c* and cytochrome *b*) and 8.9 times as much nonheme iron as did cells grown in iron-deficient medium. When iron (20 μM) was added to an iron-deficient culture 30 min before harvesting, there was a 2.5-fold increase in total iron in the cells compared with the cells without added iron. Most of this newly incorporated iron was found to exist as nonheme iron (0.7 nmol/mg of protein) as opposed to heme iron (0.06 nmol/mg of protein) (Table 2).

DISCUSSION

The present study described experiments designed to develop an understanding of iron metabolism in *R. sphaeroides*, specifically what membrane components are necessary for iron uptake, how this uptake is energized, and how iron transport is related to heme synthesis.

E. coli has been shown to transport iron supplied as ferric enterochelin (a siderophore produced by *E. coli*), ferrichrome (a siderophore produced by *Ustilago sphaerogena*), and ferric citrate. The uptake of all of these forms of iron has been shown to require a variety of outer and inner membrane proteins. The synthesis of these transport proteins is regulated by iron, and their synthesis can be induced by growth under iron-limiting conditions (see reference 30 for review). Membrane proteins controlled by iron concentration also have been found in a wide variety of other bacteria (see reference 30 for review). However, when membranes from aerobic cultures of *R. sphaeroides* grown in iron-limited medium (0.6 μM Fe) were compared with membranes isolated from cultures grown in high-iron medium (12 μM Fe), no differences were seen in inner or outer membrane protein composition. Although unusual, this was not completely unexpected, since the rate of iron uptake is not affected by the concentration of iron in the growth media (27; unpublished data). Because there were no obvious candidates for inducible membrane proteins that functioned in iron transport, vesicles were prepared to determine whether iron uptake could occur in a simplified inner membrane system. Vesicles prepared from aerobically grown cells were able to transport iron supplied as both ferric citrate and ferric parabactin, indicating that outer membrane and periplasmic proteins are not obligately involved in iron transport. The outer membrane of gram-negative bacteria functions as a physical barrier for many substances. Because whole cells of *R. sphaeroides* are able to transport iron supplied as ferric citrate and ferric parabactin, these forms of iron must be able to penetrate the outer membrane. Hydrophilic substances within certain size limits have been shown to pass through the outer membrane of certain gram-negative bacteria via water-filled pores (13). Such pores in the outer membrane of *R. sphaeroides*, which have recently been described by Weckesser and co-workers (39), are of sufficient size (radius, 0.62 nm) that ferric parabactin and ferric citrate could pass through the outer membrane.

TABLE 2. Intracellular iron concentrations of *R. sphaeroides*

Iron concn in media	Intracellular iron concn[a]		
	Heme		
	Cytochrome *c*	Cytochrome *b*	Nonheme[b]
Iron sufficient	0.72	0.34	1.43
Iron deficient	0.21	0.14	0.16
Iron deficient with 30-min exposure to 20 μM Fe	0.23	0.19	0.87

[a] Nanomoles of Fe per milligram of protein.
[b] Nonheme iron concentration equals total iron minus total heme iron.

The transport of iron supplied as ferric parabactin across the inner membrane has been shown to be inhibited by NaN_3, indicating that this uptake is an energy-requiring process (27). This finding was confirmed by the observation that the uptake of iron supplied as ferric citrate and ferric parabactin was energized in whole cells by oxidizable substrates such as malate and glutamate and in vesicles by NADH. Furthermore, the uptake of iron supplied as ferric parabactin was eliminated when the electrical component ($\Delta\psi$) of the proton motive force was depleted.

An important question concerning iron uptake is what is the valence state of iron as it is transported across the inner membrane. Using electron paramagnetic resonance spectroscopy, Ecker and co-workers (9) and Emery (10) showed that the transport of iron supplied as ferric citrate and ferrichrome to *U. sphaerogena* required the reduction of iron to the ferrous state. To examine the possibility that the uptake of ferric iron by *R. sphaeroides* requires the reduction of iron to the ferrous form, we used another method, measuring the uptake of $^{67}Ga^{3+}$, a trivalent metal whose size and charge is similar to ferric iron (11). Because Ga^{3+} cannot be reduced to a divalent state, its uptake would indicate that iron could be transported in its ferric form. Conversely, the lack of Ga^{3+} uptake may indicate that reduction of iron is necessary for transport. *R. sphaeroides* was found to be unable to transport $^{67}Ga^{3+}$ supplied as gallium citrate, although excess Ga^{3+} inhibits the uptake of ferric iron. Although these data are not conclusive, they provide some support for a model in which iron supplied as ferric citrate is recognized by a transport protein, reduced to the ferrous state, and then transported into the cell.

One possible fate of iron which has been transported into the cell and reduced to ferrous iron is that it is inserted into protoporphyrin IX by ferrochelatase to form heme. If heme formation represented a major "sink" for intracellular iron, one might expect that iron transport and heme synthesis are coupled. If this were true and if heme synthesis could be inhibited, the iron transport system might "back up," thus causing a decrease in iron transport. This hypothesis was tested by inhibiting ferrochelatase in whole cells of *R. sphaeroides* with NMPP. Inhibition of heme synthesis with NMPP was found to have no effect on the transport of iron supplied as ferric citrate, indicating that iron transport did not back up with reduced ferrochelatase activity. We feel this provides convincing evidence that iron uptake is not obligately coupled to heme synthesis.

To better understand the fate of transported iron, the concentrations of heme and nonheme iron were determined in cells exposed to different concentrations of iron for different lengths of time. The principal observations from these experiments are that the nonheme iron concentration varies much more than the heme concentration in response to the iron concentration of the growth media and that newly transported iron exists predominantly as nonheme iron. These results are consistent with results from similar experiments with *E. coli* (3), *P. denitrificans* (18), and photosynthetic *R. sphaeroides* (32) cultures. From these observations, it may be assumed that in *R. sphaeroides*, nonheme iron acts as a reservoir of intracellular iron. It is not yet possible to say whether this reserve iron is found in iron

sulfur proteins involved in electron transport and catalysis or whether the iron exists only as a storage product.

The facts that newly transported iron is found as nonheme iron and that the transport of iron is not coupled to heme synthesis suggest that controlling the rate of iron uptake does not serve to regulate the activity of ferrochelatase, although the possibility exists that there may be regulation of the supply of intracellular iron to this enzyme by some unknown mechanism.

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