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Ferric Iron Reductase of *Rhodopseudomonas sphaeroides*

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Ferric iron reductase activity was examined in the facultative photosynthetic bacterium *Rhodopseudomonas sphaeroides*. The specific activities of extracts from cells grown under phototrophic and aerobic conditions were similar and not affected by the concentration of iron in the growth media. The activity was resolved by ion-exchange column chromatography into two fractions, designated iron reductase A and iron reductase B, with molecular weights of 41,000 and 32,000, respectively. Both of these soluble cytoplasmic enzymes required the presence of flavin mononucleotide for activity and utilized NADH to reduce iron supplied as ferric citrate. Iron reductase B was responsible for the majority of activity in crude extracts and was purified 556-fold by conventional protein purification techniques. The apparent K_m values of iron reductase B for NADH, Fe^{3+}, and flavin mononucleotide were determined to be 18.2, 8.3, and 3.2 μM, respectively.

The transition element iron is vital for all organisms studied to date (with the possible exception of *Lactobacilli* spp. [18]). The importance of iron in biochemical processes is due in part to its ability to exist at two redox states, ferric (Fe^{3+}) and ferrous (Fe^{2+}). Iron found in iron-sulfur proteins or heme proteins has been reported to have oxidation reduction potentials between −500 and +300 mV (22). This wide range of redox potentials allows iron to function on a wide variety of electron transfer reactions (see reference 18 for review).

Under aerobic conditions at near neutral pH, iron in the environment is in the oxidized (Fe^{3+}) form, which may form insoluble polymers of hydroxides, carbonates, and silicates (21). Microorganisms under these conditions are, therefore, presented with the problem of obtaining the iron they need for growth. A common strategy for bacteria to use in obtaining iron from their environment is to excrete compounds (siderophores) which bind and solubilize ferric iron (see reference 19 for review). For the iron held in the iron-siderophore complex to be used by the cell, it first must be removed from the siderophore. Currently there are two possible mechanisms envisioned for the removal of iron from siderophores: hydrolysis of the siderophore followed by iron removal, or in situ reduction (siderophores have a relatively low affinity for ferrous iron) and release of the ferrous iron (19, 22).

Enzymes which reduce iron supplied as ferrisiderophores have been reported for *Pseudomonas aeruginosa* (3), *Bacillus megaterium* (1), *Bacillus subtilis* (12), and *Agrobacterium tumefaciens* (11). There is also evidence which suggests that reduction is obligate for the transport of iron supplied as ferric citrate and ferrichrome A to *Ustilago sphaerogena* (6, 7). There is also some evidence, based upon experiments with gallium, that the uptake of iron supplied as ferric citrate by *Rhodopseudomonas sphaeroides* requires reduction (17). Previously, we have shown that *R. sphaeroides* can use iron supplied as ferric parabactin as an iron source for growth (16, 17). This is the only phenolate siderophore that has been directly examined in this organism.

An additional reason for interest in enzymes which may reduce ferric iron is that ferrochelatase, the enzyme which catalyzes the insertion of iron into protoporphyrin IX to form heme, only utilizes ferrous iron (4). It has been reported that in *Aquaspirillum itersonii*, iron reductase activity may be coupled to heme synthesis and that the rate of iron reduction could possibly regulate the synthesis of heme (5). In a facultative, phototrophic organism such as *R. sphaeroides*, the insertion of ferrous iron into protoporphyrin by ferrochelatase is the step which commits protoporphyrin to heme. Thus, it is conceivable that the supply of reduced iron may be involved in controlling the flow of tetrapyrroles at the branch point between heme and bacteriochlorophyll synthesis.

As an approach to the study of this possibility, iron metabolism in *R. sphaeroides* has been examined in this laboratory (4, 15–17). In the present study, we have examined the iron reductase system of *R. sphaeroides*; we report here the purification and kinetic properties of an enzyme which catalyzes the reduction of ferric iron.

MATERIALS AND METHODS

Organisms and growth conditions. *R. sphaeroides* L was obtained from J. Lascelles (University of California, Los Angeles, Los Angeles, Calif.) and was maintained in broth cultures or agar deeps in yeast extract-malate-glutamate (YEMG) medium as previously described (10). Cells for most experiments were grown aerobically (300 ml of medium per 1-liter Erlenmeyer flask) in the dark in YEMG medium or malate-glutamate medium as previously described (16).

Purification of iron reductases. Unless otherwise noted, all operations were performed at 5°C. Four liters of YEMG medium was inoculated with a mid-log photosynthetic starter culture of *R. sphaeroides* L (2 ml of inoculum per 300-ml culture) and incubated aerobically in the dark at 29°C. After 18 h of growth, these cells were harvested by centrifugation (10,000 × *g* for 10 min), washed in 700 ml of buffer A (10 mM Tris acetate [pH 8.1], 0.5 mM dithiothreitol, 10 μg of phenylmethylsulfonyl fluoride per ml), and then sus-

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pended in buffer A to approximately 130 ml. These cells were lysed in the presence of DNase by two passes through a French pressure cell at 16,000 lb/in^2. To facilitate the action of the DNase, $MgSO_4$ was added to a final concentration of 6.2 mM. These extracts were centrifuged at 100,000 × *g* for 120 min, and the soluble fraction was decanted and applied to a DEAE-Sephacel column (3 by 27 cm) which had been equilibrated with buffer B (10 mM Tris acetate [pH 8.1], 10% [wt/vol] glycerol, 10 μg of phenylmethylsulfonyl fluoride per ml). The DEAE column was washed with 500 ml of 0.09 M KCl in buffer B. Elution of the column with 1 liter of 0.175 M KCl in buffer B resulted in the fractionation of iron reductase activity into two peaks, designated iron reductase A and iron reductase B. Fractions containing iron reductase A were pooled (volume 58 ml) and frozen at −20°C for later use. Fractions containing iron reductase B were pooled (usual volume, approximately 95 ml) and used throughout the remainder of this purification.

The pooled iron reductase B fractions were concentrated to 8 ml with an Amicon CF25 filter cone. This material was diluted with 10 ml of buffer B to reduce the KCl concentration, and applied to a second DEAE-Sephacel column (1.5 by 4 cm) which had been equilibrated with buffer B. The column was washed with 60 ml of 0.09 M KCl in buffer B, and the iron reductase B was eluted with a 200-ml KCl gradient (0.09 to 0.25 M) in buffer B. The fractions containing iron reductase B activity were pooled for a total volume of 26 ml.

Ten milliliters of this material was adjusted to 0.5 M KCl and applied to a phenyl-Sepharose CL4B column (2.5 by 5 cm) which had been equilibrated with buffer B containing 0.5 M KCl. This column was washed with 100 ml of 0.5 M KCl, and the enzyme was eluted with 100 ml of buffer B. Fractions containing iron reductase B activity were pooled (19 ml), and the volume was reduced to 1 ml with a CF25 filter cone. This concentrated enzyme was applied to a Sephadex G-75-120 column (1.5 by 75 cm) which had been equilibrated with buffer B containing 7 μM flavin mononucleotide (FMN) (buffer C). All manipulations of buffers containing flavins were performed in the dark. The column was then eluted with buffer C, and fractions containing iron reductase B were pooled (volume, 14 ml) and frozen at 20°C for later use.

Iron reductase A was further purified by concentrating the 10 ml of pooled DEAE fractions with a CF25 filter cone to 1 ml. This material was applied to a Sephadex G-75 column (1.5 by 75 cm) which had been equilibrated with buffer B, and the iron reductase A activity was eluted with buffer B.

Assays and determinations. Protein was determined by using the BCA protein assay reagent from Pierce Chemical Company, Rockford, Ill. Concentrations of NADH, NADPH, FMN, flavin adenine dinucleotide (FAD), and riboflavin were determined by using published extinction coefficients. Concentrations of iron were determined by using ferrozine as a ferrous iron trap (15) and ascorbic acid as a reductant. Iron reductase activity was assayed aerobically and anaerobically as described previously with solutions of NADH and NADPH prepared in 10 mM Tris acetate buffer (pH 8.1) as the reductant (5, 15).

Gel electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed as described by Laemmli (9), using a Hoeffer "Protean" electrophoresis apparatus. Protein bands were visualized with silver stain (14). Native gel electrophoresis and activity staining were performed as described previously (15).

Chemicals. Sephadex G-75-120, phenyl Sepharose CL4B, DEAE-Sephacel, cytochrome *c* (from horse heart), NADH, NADPH, FMN, FAD, riboflavin, 2,6-dichlorophenol indophenol, dithiothreitol, and phenylmethylsulfonyl fluoride were obtained from Sigma Chemical Co., St. Louis, Mo. Parabactin A was prepared by the method of Tait (25). The siderophores rhodotorulic acid and Desferal were obtained from Porphyrin Products, Logan, Utah, and Ciba-Geigy, Summit, N.J., respectively. All other chemicals were of the highest grade available.

RESULTS

Iron reductase activity in extracts of *R. sphaeroides*. Investigation of the iron reductase activity of *R. sphaeroides* revealed that the specific activity of crude extracts or soluble fractions of aerobically grown cells was not influenced by the iron content of the medium, and similar rates of iron reduction were seen when cells were grown photosynthetically. Cells grown under low-iron conditions (0.6 μM) had activity identical to that of those grown in 20 μM iron. The activity in cell extracts for either was 1.7 nmol of Fe^{2+} formed per min per mg of protein. This activity is located in the soluble fraction rather than the membrane fraction of the cell. The specific activity of the iron reductase of the soluble fraction of aerobically grown cells is 4.7 to 5.2, while this activity in photosynthetic cells is 8.0. The reason for this slightly higher activity is presently unknown.

Effect of different cofactors, iron sources, and assay conditions on iron reductase activity. To further define the iron reductase activity, both anaerobic and aerobic assays were performed with NADH, NADPH, or succinate as an electron donor; crude extracts, soluble fractions, or membrane fractions (or combinations of these) were used as enzyme sources. These assays were conducted in both Tris acetate and KPO_4 buffers at pH values ranging from 7.5 to 8.1. The data obtained from these assays demonstrated that the soluble fraction of *R. sphaeroides* can reduce iron supplied as ferric Tris or ferric citrate but does not reduce iron supplied as ferric parabactin A, ferric rhodotorulate, or ferric Desferal. The reduction of iron supplied as ferric citrate or ferric Tris required the presence of NADH, and NADPH was unable to serve as a reductant. Membrane fractions did not display any iron reductase activity with ferric citrate.

Ferric parabactin A can serve as an iron source for *R. sphaeroides* (16, 17); the ability of this compound to serve as a substrate for iron reductase activity was investigated thoroughly. Assays were done with iron supplied as ferric parabactin with both soluble and membrane fractions and combinations of the two. NADH, NADPH, and succinate were tried as reductants aerobically and anaerobically. For the membrane fraction, additional assays were carried out in the presence of 1 mM KCN to block the terminal oxidase. It was not possible to detect iron reduction by the membrane fraction or the soluble fraction under any of these conditions.

Identification of two proteins which function as iron reductases. When soluble fractions from either aerobically or photosynthetically grown *R. sphaeroides* are subjected to polyacrylamide gel electrophoresis under nondenaturing conditions, and these gels are stained with an activity stain which detects ferric iron reduction, two bands of activity are seen (15). The resolution of these two forms of iron reductase was possible with DEAE columns of suitable dimensions (3 by 27 cm) (Fig. 1). The two resolved peaks of activity were designated iron reductase A and iron reductase B. Fractions from these peaks were pooled, and the total

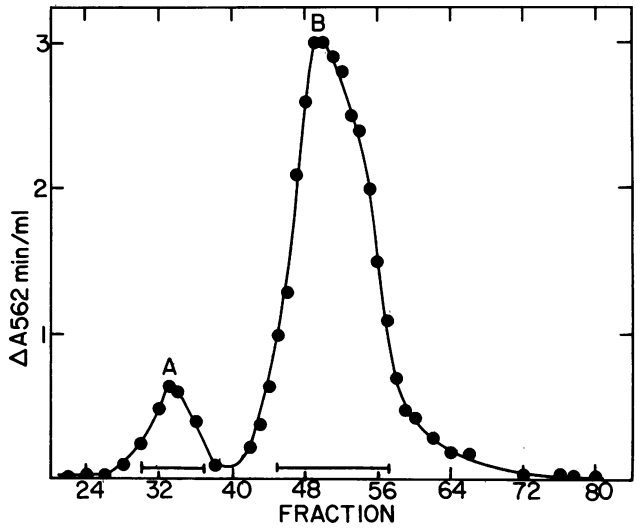

FIG. 1. Separation of two iron reductases by DEAE-Sephacel chromatography. An 88-ml portion of a crude soluble fraction from 8 liters of aerobically grown *R. sphaeroides* was applied to a DEAE-Sephacel column (3 by 27 cm) and eluted as described in the Methods section. Fractions (7.4 ml) were collected and assayed as described in Methods. No activity was seen in runoff or wash fractions. Solid bars represent fractions pooled.

activity of the pooled fractions accounted for 85% of the total activity in the crude extract (Table 1). Since iron reductase B accounted for the majority of the activity in the cell, it was chosen for further purification.

Purification of iron reductase B. The purification scheme described above and shown in Table 2 is for cultures (4 to 8 liters) of aerobically grown *R. sphaeroides* harvested at late log phase. During the development of this procedure, other steps such as $(NH_4)_2SO_4$ fractionation and ethyl agarose chromatography were included in the purification scheme, but while these additional steps did produce a more pure enzyme preparation, they also caused a dramatic loss of enzyme activity. In the purification procedure described above, a French pressure cell, instead of a sonicator as described previously (15), was used to lyse cells.

Molecular weight determination of iron reductase B. The molecular weight of iron reductase B was estimated by gel filtration chromatography to be approximately 32,200. The molecular weight of iron reductase A was determined to be approximately 41,000 by this same method.

By using SDS-polyacrylamide gel electrophoresis, the molecular weight of iron reductase B was estimated to be approximately 32,000. The protein sample used in this estimation was prepared in the following manner. Purified iron reductase B was concentrated 20-fold with a CF25 filter cone; this material was then applied to a 2-mm 10% nondenaturing acrylamide gel which was stained for iron reductase activity. The band which appeared in the activity stain was carefully removed and macerated in a solution of SDS and β-mercaptoethanol (9) and heated at 100°C for 1 min. This sample was then applied to a 12.5% SDS–polyacrylamide gel. When this gel was stained for protein (silver stain), two bands were observed. The apparent molecular weights of these bands were 32,000 and 16,800. Since the molecular weight of 32,000 corresponds to the molecular weight obtained for iron reductase B by gel filtration, we feel that the 32,000-molecular-weight band indicates that iron reductase B is composed of a single subunit and that the lower-molecular-weight protein is either a contaminant or proteolytic digestion product. However, the possibility exists that this protein is actually composed of two 16,800-molecular-weight subunits and the 32,000-molecular-weight band in this gel is a contaminant.

TABLE 1. Iron reductase activity of DEAE peaks A and B

Enzyme	Total activity[a]	% of total activity
Soluble fraction	30.8[b]	100
DEAE-iron reductase A	2.7	9
DEAE-iron reductase B	23.5	76

[a] Total activity equals micromoles of Fe^{2+} formed per minute.
[b] Total activity in the soluble fraction from an 8-liter aerobic *R. sphaeroides* culture.

TABLE 2. Purification scheme for iron reductase B of *R. sphaeroides*

Fraction	Protein (mg/ml)	Sp act[a]	Purification (fold)	Recovery (%)
Soluble	7.3	17.6	1	100
DEAE-Sephacel	0.21	328.8	22	72
2nd DEAE-Sephacel	0.48	565.4	32	53
Phenyl-Sepharose CL4B	0.02	5,032	286	41
Sephadex G-75	0.01	9,790	556	31

[a] Nanomoles of Fe^{2+} formed per minute per milligram of protein.

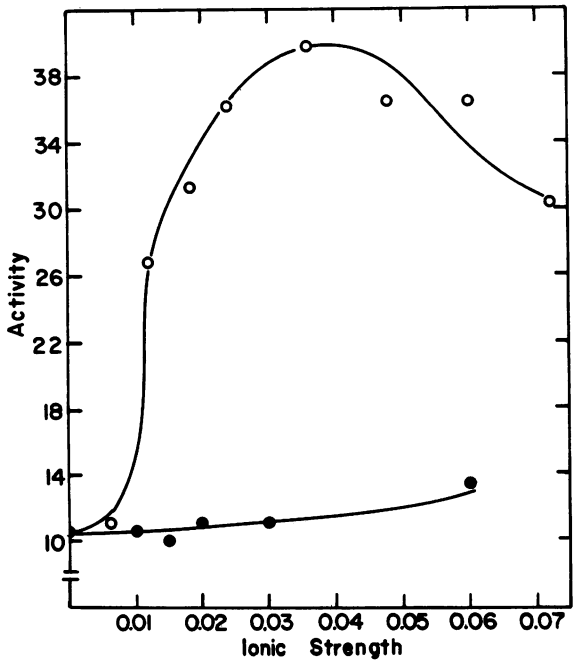

FIG. 2. Effect of magnesium on iron reductase B activity. Assay conditions: 50 μl of 1.9 mM NADH; 50 μl of 3.3 mM Fe^{3+} supplied as ferric citrate; 100 μl of 10 mM ferrozine; 100 μl of 16.5 μM FMN; 600 μl of 10 mM Tris acetate (pH 7.9) containing 0 to 40 mM $MgCl_2$ (○) or 0 to 100 mM NaCl (●). Reactions were initiated by the addition of 100 μl of an enzyme solution containing 320 ng of protein per μl. Activity is expressed as nanomoles of Fe^{2+} formed per minute per milligram of protein.

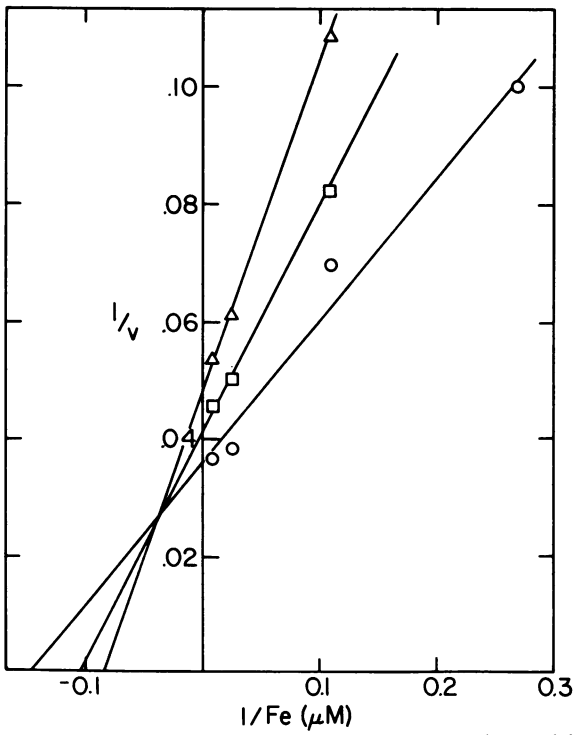

FIG. 3. Lineweaver-Burke plot of iron concentration and iron reductase B activity. Assay conditions: 100 μl of 10 mM ferrozine; 100 μl of 106 μM FMN; 600 to 650 μl of 20 mM Tris acetate buffer (pH 7.5), which contained $MgCl_2$ for a final concentration of 13.5 mM; Fe^{3+} supplied as ferric citrate; NADH at concentrations of 30 μM (△), 61 μM (□), and 318 μM (○). Reactions were initiated by the addition of 50 μl of an enzyme solution containing 7 ng of protein per μl.

Effect of ionic strength and pH on iron reductase B activity. When NaCl was added to iron reductase B assays to yield an ionic strength of 0.06, there was little change in enzyme activity. However, when $MgCl_2$ was added, iron reductase activity was stimulated almost fourfold (Fig. 2).

The effect of pH on iron reductase B activity was examined by using buffers from pH 5.8 to 8.7. The optimal pH was found to be approximately 7.1 and the buffer composition had no significant effect. The buffers used were Tris acetate, sodium phosphate, and sodium morpholinoethanesulfonate.

Kinetic properties. The apparent K_m values of iron reductase B for NADH, Fe^{3+} (supplied as ferric citrate), FMN, and FAD are 18.2, 8.3, 3.2, and 14.1 μM, respectively. The apparent K_m values of iron reductase A for NADH and Fe^{3+} are 20.4 and 4.2 μM, respectively. Previously, we have reported that FMN is more effective than FAD in stimulating the activity of iron reductase B by a factor of 1.8 (15). FMN is also required for the activity of iron reductase A (data not shown). Here, we show that the apparent K_m for FMN is lower than that for FAD, indicating that the enzyme has a greater affinity for FMN. Riboflavin was also tested as a cofactor, but the rates of iron reduction were too low to allow accurate measurement and kinetic analysis. The apparent K_m of iron reductase B for iron was dependent on the concentration of NADH (Fig. 3), and similarly, the apparent K_m for NADH was dependent on the iron concentration (Fig. 4).

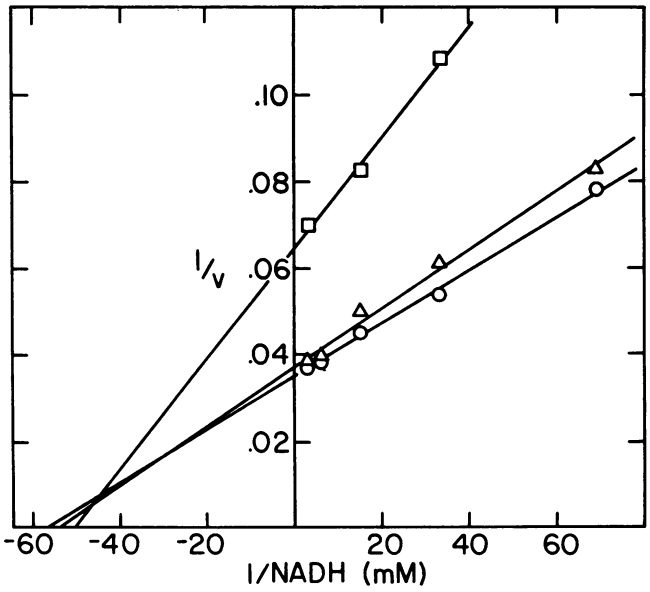

FIG. 4. Lineweaver-Burke plot of NADH concentration and iron reductase B activity. Assay conditions: 100 μl of 10 mM ferrozine; 100 μl of 106 μM FMN; 600 to 650 μl of 20 mM Tris acetate buffer (pH 7.5), which contained $MgCl_2$ for a final concentration of 13.5 mM; NADH; Fe^{3+} supplied as ferric citrate at concentrations of 9.3 μM (□), 41.2 μM (△), and 82.5 μM (○). Reactions were initiated by the addition of 50 μl of an enzyme solution containing 7 ng of protein per μl.

DISCUSSION

In the present study, we have described two flavoproteins from *R. sphaeroides* that utilize NADH to reduce ferric iron. These iron reductases were present in both aerobically and phototrophically grown cells, and their specific activity in crude extracts was not affected by the concentration of the iron in the media. We have reported a 556-fold purification of the enzyme responsible for the majority of the activity in crude extracts. To our knowledge, this represents the highest purification reported for a bacterial iron reductase.

Enzymes which function in the reduction of iron have been reported for a number of microorganisms and can generally be divided into two classes. One class of iron reductase activity is represented by *Aquaspirillum itersonii*, in which the activity appears to be linked to the respiratory electron transport chain (5). The second class of iron reductase activity consists of soluble enzymes which utilize reduced nicotinamide cofactors. Iron reductases of this class have been reported in *Mycobacterium smegmatis* (13), *Pseudomonas aeruginosa* (3), *Bacillus megaterium* (1), and *B. subtilis* (12). These enzymes, with the exception of the ferrimycobactin reductase from *M. smegmatis* (for which no location was suggested), were all found to reside in the cytoplasm.

Ion reductase A and iron reductase B from *R. sphaeroides* would be placed in this second group on the basis of their cytoplasmic location and requirement for NADH. Another similarity of the iron reductase activity of *R. sphaeroides* and the iron reductases found in *P. aeruginosa* and *B. megaterium* is that the specific activity is not affected by the iron content of the media and the enzymes appear to be constitutive.

As stated above, both iron reductase A and iron reductase

B utilize NADH as a source of electrons to reduce iron. The apparent K_m values for NADH are about 20 μM and compare favorably to the K_m values of other NADH-utilizing enzymes (20, 24). Both iron reductase A and iron reductase B require FMN as a cofactor. The activity of iron reductase B is stimulated to a greater degree by FMN than by FAD, and this fact is reflected in the lower K_m for FMN. We observed that the K_m for NADH was dependent on the concentration of iron and that the K_m for iron was dependent on the concentration of NADH. When the data from these kinetic experiments were analyzed by double reciprocal Lineweaver-Burke plots, they resembled the type of plot predicted by an ordered model for bisubstrate enzymatic reactions (2).

Iron citrate was used as an iron source in most of the iron reductase assays. We did find that iron reductase B can also reduce iron supplied as an iron-Tris chelate. When iron was supplied as ferric siderophores, such as ferric parabactin A, ferric rhodotorulate, or ferric Desferal, no reduction was observed. We have shown previously that whole cells and vesicles of *R. sphaeroides* can transport iron supplied as ferric parabactin A (16, 17). The fact that ferric parabactin A can be utilized by *R. sphaeroides* but cannot serve as a substrate for the iron reductases can be reconciled. The reduction of iron chelated by parabactin A might require the prior hydrolysis of the siderophore, or there may be no actual need for enzymatic reduction of iron in vivo supplied as ferric parabactin A. It has been demonstrated that in hydrophobic environments, such as the lipid bilayer of the cell membrane, the internal oxidation of the catechol groups to the quinone form could reduce iron held by catechol-type siderophores (22).

In conclusion, we must consider a physiological role for iron reductase A and iron reductase B. As discussed above, these enzymes may function in the removal of iron from parabactin A or in the transport of iron supplied as ferric citrate. However, we have no direct evidence to support such a hypothesis. A more likely role for these enzymes may be in providing ferrous iron to the enzyme ferrochelatase for use in heme synthesis. In vitro experiments have shown that iron reduction and heme synthesis may be coupled in *Aquaspirillum itersonii* (5). It has also been shown that the rate of NADH-linked iron reduction is sufficient to support the maximal rate of heme synthesis in crude extracts of aerobically grown *R. sphaeroides*. Previously, we have demonstrated that the majority of newly transported iron in *R. sphaeroides* is present as nonheme iron (17). If iron reductase A or iron reductase B acts to reduce this iron and, thus, to supply ferrochelatase with ferrous iron, the rate of iron reduction could control the flow of tetrapyrroles at the branch point between heme and bacteriochlorophyll synthesis.

In addition to a role in iron metabolism, the iron reductases we have described may also function to control the flow of electrons within the cell. Other workers have described a soluble flavoprotein with a molecular weight of 33,000 from extracts of phototrophically grown *Rhodospirillum rubrum* (8). This enzyme, which is called NADH:heme protein oxidoreductase, utilizes NADH to reduce cytochrome *cc'* and 2,6-dichlorophenol indophenol. It has been suggested that this enzyme functions in the oxidation-reduction buffer system and controls the flow of electrons coupled to oxidative phosphorylation. We have found that both iron reductase A and iron reductase B are able to reduce cytochrome *c* and 2,6-dichlorophenol indophenol (data not shown); it is possible that these enzymes may function to control electron flow as well as to reduce intracellular iron. Further work is required to explain this role of the iron reductases.

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