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Reduction of Iron and Synthesis of Protoheme by *Spirillum itersonii* and Other Organisms

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Membranes from *Spirillum itersonii* reduce ferric iron to ferrous iron with reduced nicotinamide adenine dinucleotide or succinate as a source of reductant. Iron reduction was measured spectrophotometrically at 562 nm using ferrozine, which chelates ferrous iron specifically. Reduced nicotinamide adenine dinucleotide or succinate was also effective as a source of reductant for synthesis of protoheme with ferric citrate as a source of iron. The effects of respiratory inhibitors suggested that reduction of iron occurs at one or more sites on the respiratory chain before cytochrome *c*. Reduction of iron and synthesis of protoheme with the physiological reductants were also observed with crude extracts of other bacteria, including *Rhodopseudomonas spheroides*, *Rhodopseudomonas capsulata*, *Paracoccus denitrificans*, and *Escherichia coli*. The effect of oxygen upon reduction of iron and formation of protoheme was examined with membranes from *S. itersonii*, using succinate as a source of reductant. Both systems were inhibited by oxygen, but this effect was completely reversed by addition of antimycin A. We conclude that reduced components of the respiratory chain serve as reductants for ferric iron, but with oxygen present they are oxidized preferentially by the successive members of the chain. This could be a mechanism for regulating synthesis of heme and cytochrome by oxygen.

Ferrochelatase (EC 4.99.1.1) is the terminal enzyme of heme biosynthesis and catalyzes the insertion of ferrous iron into the protoporphyrin nucleus. The enzyme is widely distributed in nature: it is located in mitochondria and chloroplasts of eukaryotes and in the cytoplasmic membrane of bacteria (12-15, 20). Ferrochelatase catalyzes the insertion of many divalent cations in addition to ferrous iron, but it does not use ferric iron. It is usually assayed under highly artificial conditions in the presence of detergents and with unnatural porphyrins such as mesoporphyrin as substrate; also, thiols are used to maintain iron in the reduced state (14, 20).

We have previously studied the enzyme in a solubilized and partially purified form derived from membranes of *Spirillum itersonii* (8). Solubilized ferrochelatase requires an artificial reductant such as dithiothreitol, and unnatural porphyrins are more effective as substrates than protoporphyrin. Presumably organisms growing aerobically acquire iron in the ferric form and use reductants generated by metabolism to provide ferrous iron for synthesis of protoheme.

In the present work, provision of ferrous iron for heme synthesis has been studied with membranes from *S. itersonii*, using reductant in the respiratory chain. The organism has an unbranched electron transport chain, containing cytochromes of the *b*, *c*, and *o* types (7, 9, 11, 16). Reduction of ferric iron was examined with ferrozine to indicate ferrous iron, in parallel with studies of protoheme synthesis. Similar activities were also examined in preparations from other facultative aerobes.

MATERIALS AND METHODS

Organisms and growth conditions. The wild-type strain of *S. itersonii* and the heme-dependent mutant H-9 have been described previously (7, 8, 11). *Rhodopseudomonas spheroides* NCIB 8253 has been used in various investigations in this laboratory (15). Wild-type strains of *Rhodopseudomonas capsulata* (St. Louis strain [ATCC 23782]), *Paracoccus denitrificans* (ATCC 19367), *Staphylococcus aureus* (SG 511 A), and *Escherichia coli* K-12 were also used. Stock cultures of *E. coli* and *S. aureus* were on nutrient agar; all other organisms were maintained on malate-glutamate-yeast extract agar (11).

Cells for preparation of extracts were grown in glutamate-glycine-succinate medium with 0.1% yeast extract (7) or in malate-glutamate medium with 0.1% yeast extract; the latter was used for growth of *R. spheroides* and *R. capsulata*. Cultures were grown aerobically at 30°C in 1-liter flasks containing 500 ml of medium; they were shaken at 225 rpm and were harvested in the early stationary phase.

Preparation of cell fractions. Cultures were harvested by centrifugation, washed in 25 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.8), and suspended in this buffer to a density equivalent to 5,000 Klett units (red filter; approximately 10 mg of protein per ml). Except *S. aureus*, extracts were prepared in the French pressure cell at 16,000 lb/in^2; preparations from *S. aureus* were obtained by lysis of spheroplasts (5). A few crystals of deoxyribonuclease were added, and the homogenates were centrifuged for 10 min at 10,000 × *g*; the supernatants were designated as "crude extracts." The membrane fraction from *S. itersonii* was derived from the crude extract by centrifugation for 1.5 h at 100,000 × *g*; the pellet was suspended in 25 M Tris-hydrochloride buffer (pH 7.8) to a protein concentration of 20 to 40 mg per ml.

Assay of enzyme activities. The reduction of ferric to ferrous iron, termed "iron reductase," was assayed spectrophotometrically in anaerobic Thunberg cuvettes with ferrozine [3-(2-pyridyl)-5,6-bis-(4-phenylsulfonic acid)-1,2,4-triazine] to bind the ferrous iron. This agent forms a highly colored chelate with ferrous iron (molar extinction coefficient, 28,000 at 562 nm). Ferrozine does not react with ferric iron and is water soluble (6). Reagents and buffers for assay were prepared in glass-, doubly distilled water; buffers were degassed in vacuo and sparged with argon. Each cuvette contained (micromoles in final volume of 2.4 ml): Tris-hydrochloride buffer (pH 7.6), 100; sodium succinate, 5, or reduced nicotinamide adenine dinucleotide (NADH), 1; ferrozine, 2.0; ferric citrate, 0.4. Enzyme preparations containing 1 to 7 mg of protein were in the side arm. The cuvettes were evacuated and filled with argon, and the reaction was started by addition of enzyme. The increase in absorbance at 562 nm was measured at room temperature (25°C) in a spectrophotometer (Cary model 14R) with the 0 to 0.1 slide wire against a reference cuvette containing the complete assay system without ferrozine. Results are expressed as nanomoles of ferrous complex formed per milligram of protein per hour.

Heme synthase activity was measured by incorporation of ^{59}Fe into protoporophyrin; protoheme was isolated for counting by extraction with acid methyl ethyl ketone (10). The complete reaction mixture contained (micromoles in 2-ml final volume): Tris-hydrochloride buffer (pH 7.6), 70; sodium succinate, 5, or NADH, 1; protoporphyrin, 0.1; [^{59}Fe]ferric citrate, 0.1 μCi, 0.2 μmol; and enzyme preparation containing 1 to 7 mg of protein. The reaction was carried out under an atmosphere of argon in small tubes (10-mm ID) sealed with serum caps. The tubes were evacuated and filled with argon by insertion of a hypodermic needle through the cap. Iron was added by injection through the cap after the tube had been incubated for 10 min at 37°C. The reaction was terminated after 30 min of further incubation by addition of 1 ml of 0.2 N HCl and 2.5 ml of methyl ketone; the tubes were thoroughly mixed, chilled in ice, and centrifuged to separate the phases. One milliliter of upper phase was removed and added to tubes containing 1 ml of water, 0.5 ml of 0.2 N HCl, and 0.5 ml of methyl ethyl ketone; the upper phase was separated as before, and 0.2 ml was removed for counting by the liquid scintillation procedure. Ready-To-Use II (Eastman Kodak Co.) scintillation fluid was used, with a Beckman counter operated in the ^{32}P minus ^{3}H channel. The observed radioactivity was converted to nanomoles of protoheme by calculation from reference assays with a solubilized preparation of ferrochelatase (8). These assays were set up in duplicate tubes, each containing enzyme in reaction mixture similar to that described above but with dithiothreitol (5 μmol) as reductant. One sample was extracted for counting as described, and the other was treated with alkaline pyridine for spectrophotometric determination of protoheme as the pyridine hemochromogen derivative (10). Results are expressed as nanomoles of protoheme formed per milligram of protein per hour.

Succinate and NADH oxidase activities were measured at 25°C with an oxygen electrode (Rank Bros., Bottisham, Cambs, England). The reaction vessel contained, in a 2-ml final volume: membrane fraction, 1 to 2 mg of protein in 25 mM potassium phosphate buffer (pH 7.5); NADH, 1 μmol, or sodium succinate, 10 μmol. Results are expressed as nanomoles of oxygen consumed per milligram of protein per minute.

Protein determination. Protein was determined by the Folin method (17) with bovine serum albumin as standard.

Materials. Antimycin A, 2-heptyl-4-hydroxyquinoline-N-oxide (HQNO), and rotenone were purchased from Sigma Chemical Co.; solutions were freshly prepared and dissolved in ethanol. NADH, dithiothreitol, and ferrozine were also from Sigma Ferrichrome and Desferal were gifts provided, respectively, by B. Burnham and Ciba Pharmaceutical Co. The ferric chelates were made by mixing with equimolar proportions of [^{59}Fe]ferric citrate in water. Stock solutions of ferric citrate (2 mM) were prepared by mixing equimolar ferrous ammonium sulfate and sodium citrate; the solution was vigorously aerated to oxidize the ferrous iron to the ferric state. ^{59}Fe (in 0.5 N HCl, 2 to 40 Ci/g) was from New England Nuclear Corp.; it was added to stock solutions of ferric citrate immediately before use to give approximately 1 μCi per ml. Protoporphyrin IX was from Porphyrin Products, Logan, Utah; solutions were freshly prepared as described previously (8).

RESULTS

Iron reductase activity in *S. itersonii*. Conditions were established for assay of iron reductase activity in membranes from *S. itersonii*, using succinate as the reductant. The rate of formation of the ferrous-ferrozine complex was linear with time for at least 5 min and with protein concentrations of at least 4 mg per ml. Failure to remove traces of oxygen from the reaction mixture resulted in a lag period, and linearity was not achieved until oxygen had been consumed (Fig. 1a).

The effect of pH was determined in Tris-hydrochloride buffers at constant ionic strength

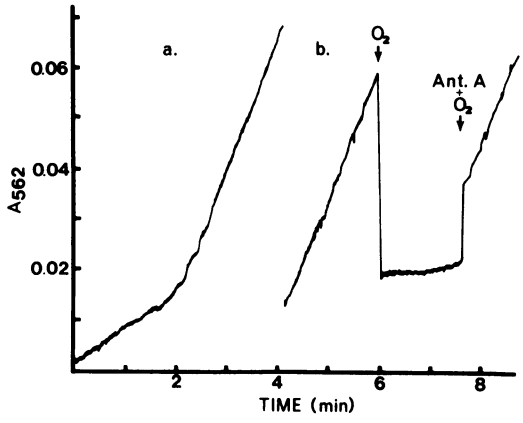

FIG. 1. *Effects of aeration on iron reductase activity in membranes from S. itersonii. The assays were in open cuvettes with succinate as reductant and contained 0.6 mg of membrane protein per ml. In experiment (a), the cuvettes remained undisturbed throughout. In experiment (b), the cuvettes were gently aerated after a linear rate had been established (first arrow); they were again aerated with addition of antimycin A to give a final concentration of 4 μM (second arrow).*

maintained with NaCl ($I = 0.10$). The optimum was pH 7.60, and this was unaffected by variation of ionic strength over the range $I = 0.02$ to 0.05. The apparent K_m for iron as ferric citrate was 12 μM.

The reaction was dependent upon reductant. NADH was more effective than succinate, but NADPH was inactive (Table 1).

Interaction of iron reductase with the respiratory chain in *S. itersonii*. Membranes from *S. itersonii* have NADH and succinate oxidase activities (Table 2). Both were inhibited by HQNO and antimycin A; rotenone inhibited NADH oxidase but did not affect succinate oxidase activity. Neither activity was affected by ferrozine at the concentration used for assay of iron reductase.

Observations with respiratory inhibitors and with oxygen indicate that ferric iron is reduced by interaction with the respiratory chain at site(s) prior to cytochrome *c*. Neither antimycin A nor HQNO affected the reaction with NADH or succinate as reductant; rotenone inhibited reduction with NADH but not with succinate (Table 3). Aeration strongly inhibited iron reductase (Fig. 1a, b). This effect was completely abolished by addition of antimycin A (Fig. 1b), suggesting that the inhibition was due to diversion of reductant into the terminal oxidase system.

Iron reductase in relation to heme synthase in *S. itersonii*. Parallel studies of heme synthase and iron reductase were made with membranes from *S. itersonii*. NADH, succinate, and dithiothreitol were equally effective as reductants for heme synthase (Table 1). With succinate as reductant, heme synthase was insensitive to rotenone, HQNO, and antimycin A; rotenone inhibited the reaction with NADH (Table 3).

Under the standard conditions of assay, heme synthase activity was less than 20% that of iron reductase. For instance, the activities depicted in Fig. 1 and 2 are, respectively, 79 and 9.8 nmol of ferrous iron and of heme per h per mg of protein.

The rate of heme formation under aerobic conditions was considerably less than that observed under the standard anaerobic conditions with succinate as reductant (Fig. 2). Addition of

TABLE 1. *Effects of reductants on iron reductase and heme synthase activity in membranes from S. itersonii*[a]

Reductant	Activity (units/mg of protein)[b]	
	Iron reductase	Heme synthase
None	0	0
NADH, 0.5 mM	176	2.0
NADPH, 0.5 mM	4	0.1
Succinate, 2.5 mM	64	2.5
Dithiothreitol, 3 mM	NT[c]	2.0

[a] Assays were made with membranes from *S. itersonii*, as described in Materials and Methods, but with varying reductants. These were added to give the final concentrations shown. The protein concentration was 1.5 mg per ml of assay mixture.

[b] Units for iron reductase and heme synthase are nanomoles of product per hour.

[c] NT, Not tested.

TABLE 2. *Effect of ferrozine and respiratory inhibitors on NADH and succinate oxidase activities in membranes from S. itersonii*[a]

Additions	Oxidase activity (units[b]/mg of protein)	
	NADH	Succinate
None	130	42
Ferrozine, mM	129	42
HQNO, 2 μM	4	2
Antimycin A, 2 μM	0	0
Rotenone, 2 μM	3	42

[a] Activities were measured as described in Materials and Methods, with additions to give the final concentration shown. The protein concentration was 1 to 2 mg per ml.

[b] Units are nanomoles of oxygen consumed per minute.

TABLE 3. *Effect of respiratory inhibitors on iron reductase and heme synthase activity in membranes from S. itersonii*[a]

Inhibitor	% Control activity[b]			
	Iron reductase		Heme synthase	
	NADH	Succinate	NADH	Succinate
Rotenone, 4 μM	13	80	34	100
Antimycin A, 4 μM	100	110	100	120
HQNO, 4 μM	81	92	98	100
KCN, 0.7 mM	NT[c]	NT	103	100

[a] Assays were performed under the standard conditions with succinate or NADH as reductant and with addition of inhibitor to the final concentration shown. The concentration of protein was 0.6 and 2.5 mg per ml, respectively, in the iron reductase and heme synthase systems.

[b] Results are expressed as percentage of the control activities. These were: iron reductase, 150 (NADH) and 50 (succinate) nmol per mg of protein per h; heme synthase, 2.5 nmol per mg of protein per h with both reductants.

[c] NT, Not tested.

antimycin A restored the aerobic rate to the anaerobic level (Fig. 2). This response to antimycin A is similar to that observed in assays of iron reductase (Fig. 1b).

Iron reductase and heme synthase activity in other organisms. Crude extracts from bacteria of diverse physiological types were assayed for iron reductase and heme synthase activity (Table 4). All had iron reductase activity with either NADH or succinate as reductant. Heme synthase activity was also detectable in all except *S. aureus* and the heme-dependent mutant of *S. itersonii*. Failure to find heme synthase in the mutant was not surprising, since this organism was previously found to lack ferrochelatase (8). Lack of demonstrable activity in *S. aureus* is probably due to insensitivity of the assay; the organism forms types *b* and *a* cytochromes (albeit in low amounts) when grown under the conditions used in these experiments.

The high levels of heme synthase activity in photosynthetic and denitrifying bacteria is consistent with their capacity to form large amounts of types *b* and *c* cytochromes, particularly when grown anaerobically in the light or with nitrate (11, 12, 15).

DISCUSSION

Ferrozine has several advantages as an agent to trap ferrous iron in the assay for iron reductase. It is specific for the ferrous form and, of the other divalent cations, it reacts only with copper (6). The compound is water soluble, and the chelate is highly colored, whereas the free form is colorless. Particularly important, ferrozine did not inhibit NADH or succinate oxidase activities.

The observations with membranes from *S. itersonii* show that ferric iron is reduced by reductant(s) derived from dehydrogenation of NADH or succinate and that ferrous iron generated in this way is available for synthesis of heme. Similar observations have been made with sonicated mitochondria and with membranes from avian erythrocytes (1, 2). The membrane-associated respiratory chain of *S. itersonii* is outlined in Fig. 3. It is relatively simple, with no evidence of a branched terminal oxidase system (7, 9, 11). Reduction of iron presumably occurs at one or more sites before cytochrome *c*, since it was not inhibited by antimycin A or HQNO. The need for a functional primary dehydrogenase is evident from the inhibition by rotenone with NADH as reductant. Possibly, ferric iron may interact with free or bound forms of nonheme iron, which are present in respiratory preparations from mitochondria and bacteria (3).

Iron reductase activity in membranes from *S. itersonii* is extremely sensitive to oxygen. This is attributed to more rapid oxidation by subsequent reactions of the electron transport chain, since the inhibition was abolished by antimycin A.

The effects of reductant, aeration, and respiratory inhibitors upon heme synthase activity

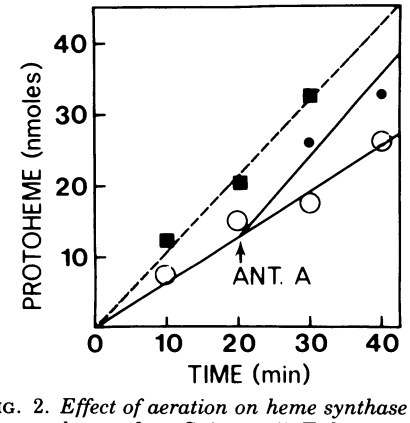

FIG. 2. *Effect of aeration on heme synthase activity in membranes from S. itersonii. Tubes containing the components for assay of heme synthase with succinate were incubated aerobically with shaking at 125 rpm (○), with addition of antimycin A (5 μM final concentration) as shown by the arrow (●); controls incubated under argon are shown as (■). The concentration of membrane protein was 6.5 mg per ml.*

TABLE 4. *Iron reductase and heme synthase activity in crude extracts of various bacteria*[a]

Organism	Activity (units[b]/mg of protein)				
	Iron reductase		Heme synthase		
	NADH	Succinate	NADH	Succinate	Dithiothreitol
S. itersonii (wild type)	80	25	1.3	1.1	1.3
S. itersonii H-9 (heme mutant)	22	2	<0.1	<0.1	<0.1
P. denitrificans	19	17	12.0	12.6	10.0
R. spheroides	74	13	10.3	10.0	8.7
R. capsulata	9	18	6.1	8.0	8.7
E. coli	35	35	0.1	0.1	0.1
S. aureus	44	2	<0.1	<0.1	<0.1

[a] Assays were performed with crude extracts under the standard conditions described in Materials and Methods. The final concentration was 1 to 5 mg per ml of assay mixture.
[b] Units for iron reductase and heme synthase are nanomoles per hour.

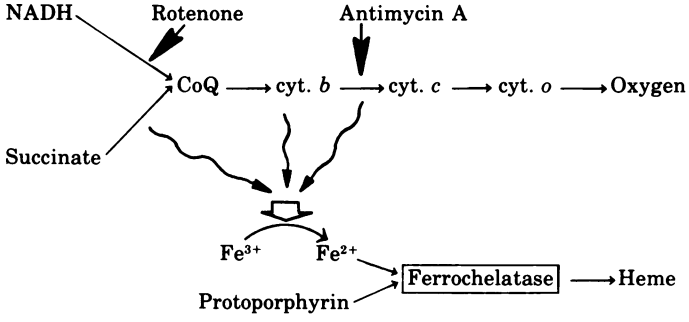

FIG. 3. *Respiratory chain in membranes of S. itersonii and its interaction with ferrochelatase. Cyt., Cytochrome.*

were qualitatively similar to those observed with iron reductase. Provision of ferrous iron for heme formation could be critical in the overall regulation of cytochrome synthesis by oxygen. In most aerobic or facultatively aerobic bacteria, cytochrome formation is repressed by strong aeration, and maximum levels are found only in cells grown with limiting oxygen (7, 12, 15). This can be rationalized physiologically as a response to respiratory deficiency by expansion of oxygen-harvesting capacity.

Many aerobes and facultative anaerobes acquire iron from the environment as ferric chelates (siderophores) and accumulate the ligands when grown in iron-deficient media (18, 19). In the present work, organisms were grown with optimal concentrations of ferric citrate, and presumably siderophores did not participate in iron transport or delivery to functional sites. We have tested ferrichrome and ferric-Desferal as sources of ferric iron in place of ferric citrate in assays for iron reductase and heme synthase with crude extracts of *S. itersonii*, *R. spheroides*, and *P. denitrificans*. The chelates had little or no activity with these organisms. Of these organisms only *P. denitrificans* is known to accumulate siderophores; these have recently been identified as catechol derivatives (22). A major problem concerns the mechanism for release of iron from the chelates within the cell; this is being investigated in several laboratories with chelates native to particular organisms (4, 21, 22).

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