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Iron Reductases from *Pseudomonas aeruginosa*

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Cell-free extracts of *Pseudomonas aeruginosa* contain enzyme activities which reduce Fe(III) to Fe(II) when iron is provided in certain chelates, but not when the iron is uncomplexed. Iron reductase activities for two substrates, ferripyochelin and ferric citrate, appear to be separate enzymes because of differences in heat stabilities, in locations in fractions of cell-free extracts, in reductant specificity, and in apparent sizes during gel filtration chromatography. Ferric citrate iron reductase is an extremely labile activity found in the cytoplasmic fraction, and ferripyochelin iron reductase is a more stable activity found in the periplasmic as well as cytoplasmic fraction of extracts. A small amount of activity detectable in the membrane fraction seemed to be loosely associated with the membranes. Although both enzymes have highest activity with reduced nicotinamide adenine dinucleotide, reduced glutathione also worked with ferripyochelin iron reductase. In addition, oxygen caused an irreversible loss of a percentage of the ferripyochelin iron reductase following sparge of reaction mixtures, whereas the reductase for ferric citrate was not appreciably affected by oxygen.

Siderophores are produced by microorganisms for the purposes of binding extracellular iron and mediating the transport of Fe(III). However, the iron must also be released from complex with the siderophore to allow synthesis of iron-containing cellular constituents. An esterase enzyme has been described from *Escherichia coli* which hydrolyzes the cyclic trimer of 2,3-dihydroxybenzoylserine (enterochelin) and has a theoretical function in iron release, since mutants lacking the esterase activity accumulate ferrienterochelin, but cannot grow unless supplied with an alternative source of iron (11, 16). Another mechanism of iron release was demonstrated in *Mycobacterium smegmatis* for iron complexed with mycobactin, a siderophore produced by mycobacteria (1). Ferrimycobactin reductase catalyzed the reduction of Fe(III) and release from the siderophore of Fe(II), and was assayed by using a chelator that formed a colored complex with Fe(II). The enzyme was found in the soluble portion of cell extracts and utilized electrons from both NADH and NADPH. Another enzyme from *Paracoccus denitrificans* used electrons from NADH or NADPH to reduce Fe(III) from a catechol siderophore produced by this bacterium (18). Enzymes from *Neurospora crassa* and *Aspergillus fumigatus* in the soluble portions of cell extracts were described as reducing iron from complex with various hydroxamate siderophores (6).

E. coli is capable of utilizing ferric citrate as well as ferrienterochelin in the transport of iron (8). Iron reductase activity for ferric citrate has also been reported in the membrane fractions of *E. coli* as well as *Spirillum itersonii* and *Staphylococcus aureus* (3). The enzyme from *S. itersonii* reduced iron using electrons from NADH or succinate via reactions of the respiratory chain. The enzyme found in *S. aureus* used electrons from NADH or L-glycerol 3-phosphate to reduce iron from ferric citrate (12). The activities of both enzymes were reversibly inhibited by oxygen, which drained the electrons used anaerobically by the iron reductase from the respiratory chain. The alternative electron acceptor, nitrate, also inhibited iron reductase activity in membrane preparations from cells of *S. aureus* induced for nitrate respiration (12).

Pyochelin is a siderophore which can be isolated from culture media of *Pseudomonas aeruginosa* (2). This is probably the same compound studied by Liu and Shokrani and called by them pyochelin A (13). Both ferripyochelin and ferric citrate are used as substrates for iron transport in *P. aeruginosa* (manuscript submitted for publication). The present paper describes iron reductase activities which reduce iron from complexes with pyochelin and citrate. The activities appear to reside on separate proteins and will be referred to as ferripyochelin iron reductase and ferric citrate iron reductase.

METHODS AND MATERIALS

Bacteria and culture conditions. *P. aeruginosa*, strain PAO-1 (ATCC 15692), was obtained from the American Type Culture Collection and was maintained on slants of brain heart infusion agar with monthly transfers. Bacteria were grown in succinate minimal medium containing 20 mM sodium succinate,

4 mM NH_4Cl, 0.4 mM $MgSO_4$, 1.0 mM K_2SO_4, 1 μM $ZnCl_2$, 1 μM $MnCl_2$, 1 μM $CaSO_4$, and 4 mM potassium phosphate buffer (pH 7.4). Some studies involved cells grown in 0.5% Casamino Acids containing 0.4 mM $MgSO_4$. The media were autoclaved without phosphate buffer or minerals, which, along with $FeCl_3$ and ferric citrate, were made bacteria free by passage through a membrane filter of 0.45-μm pore size (Millipore) and added to media before inoculation. Cells were harvested in late logarithmic phase of growth by centrifugation at room temperature. The cells were washed in 0.01 M Tris-hydrochloride (pH 7.6) with 1 mM $MgCl_2$ (TM buffer).

Preparation of extracts. Suspensions of cells were treated for release of periplasmic contents by the method of Stinson et al. (17). Washed cells were suspended (14 mg [dry weight] of cells per ml) in 0.2 M $MgCl_2$ with 0.05 M Tris-hydrochloride buffer at pH 8.5, and the mixture was stirred for 1 h at 24°C. After centrifugation at 5,900 × *g*, the supernatant fluid was saved as "shock fluid," and the cell pellet was suspended in distilled water and stirred for 1 h at 24°C. This suspension was centrifuged at 5,900 × *g*, and the supernatant was combined with the first. The combined shock fluids were concentrated with an Amicon PM-10 membrane and dialyzed against Tris-hydrochloride (0.01 M, pH 7.6) for 20 h at 4°C. Viable bacteria were determined before and after the shock procedure by plating dilutions on 1% tryptic soy agar.

The cell pellet resulting from the preparation of the shock fluid was suspended in TM buffer, and the cells were ruptured at 16,000 lb/in^2 with a French pressure cell (Aminco). The ruptured cell suspensions were centrifuged at 20,000 × *g* at 4°C to remove whole cells and cell debris. The resulting supernatants were centrifuged at 130,000 × *g* for 2 h to obtain fractions rich in membrane constituents (pellet) and in soluble or cytoplasmic constituents (supernatant). The pellets were suspended in minimal volumes of 0.01 M Tris-hydrochloride, and both fractions were dialyzed against the same buffer. The fractions were made cell free by pressurized filtrations through 0.45-μm pore size filters.

Preparations of substrates. Ferric citrate was dissolved in distilled water and made to a final concentration of 10 mg/ml at a pH of 7.5. Ferric chloride was made 0.1 M in distilled water and allowed to equilibrate so that after 1 day there was no detectable Fe(II).

Pyochelin (siderophore without iron) was extracted from culture media into ethyl acetate (2), concentrated by rotary evaporation, and made into the iron chelate (ferripyochelin) by adding $FeCl_3$ until the solution changed to wine red and then to brown. Ferripyochelin was extracted with multiple additions of ethyl acetate, leaving a brown-colored water layer. Unchelated iron was removed by concentrating ferripyochelin in ethyl acetate and extracting the organic solvent phase with 0.01 M HCl. Ferripyochelin was precipitated by adding heptane to the ethyl acetate solution in a 3:2 ratio. The dried precipitate was dissolved in ethanol and purified by preparative thin-layer chromatography on silicic acid (Adsorbosil-5, Applied Sciences Laboratories) in chloroform-acetic acid-ethanol (90:5:5). The red band at R_f 0.22 was scraped and eluted from silicic acid in methanol. Ferripyochelin was dried, dissolved in ethyl acetate, and precipitated and washed with heptane. The precipitate was dried under vacuum for 6 h, weighed, and stored at 4°C.

Iron concentrations in ferric citrate and ferripyochelin solutions were determined by an assay for nonheme iron (5). The concentration of iron in 10 mg of ferric citrate per ml was 28.6 mM, and that in 10 mg of ferripyochelin per ml was 14.6 mM. The molecular weight of ferripyochelin was determined from titrations of pyochelin with iron to be approximately 700. Using this value to determine the molarity of ferripyochelin yielded 14.2 mM in a solution containing 10 mg/ml. This molecular weight estimate has also been confirmed by mass spectroscopy (data not shown).

Enzyme assays. Iron reductase activity was measured by the method of Dailey and Lascelles (3). Iron chelate was added first, followed by the ferrozine reagent in 0.01 M Tris buffer. Spontaneous reduction of iron was measured, and the cell extract was added in 20- to 100-μl volumes. Controls were conducted with varied sequences of reaction constituents. Assays for ferric citrate iron reductase yielded maximal activities when reductant was added before ferric citrate. The reactions at 24°C were monitored at 562 nm on a 0.1 absorbance scale in a model 124 Perkin-Elmer double-beam spectrophotometer. Reference cuvettes contained all of the reaction components except reductant. Nonenzymatic reduction of iron was the slowest in polystyrene cuvettes, but glass Thunberg cuvettes were used in assays to determine effects of oxygen on enzymatic activity. Assays involving large numbers of determinations were conducted by measuring differences in absorbance at times of incubation at 24°C in a Gilford model 240 spectrophotometer with continual reference to reactions containing no extract and reactions containing no reductant.

A variety of enzyme assays were used in the determination of the location of iron reductase activities in cell fractions. Diaphorase activities measured in these extracts were equivalent in rates with NADH and with NADPH and appear similar to the DT diaphorase described by Ernster (7). Sensitive assays were conducted by fluorometric determination of resazurin reduction in dialyzed extracts as described by Guilbault and Kramer (9). The activities of diaphorase are expressed in nanomoles of resorufin accumulating per minute per milligram of protein, using NADH as electron donor. Malate dehydrogenase (EC 1.1.1.37) was used as another marker of the soluble fraction of cell extracts and was measured by fluorometric determination of NADH accumulation using the assay conditions of Yoshida (21). Succinate dehydrogenase (EC 1.3.99.1) was used as an indicator of the presence of membrane-bound enzymes and was determined by the spectrophotometric method of Veeger et al. (19). Ferricyanide was measured at 420 nm, and an extinction coefficient of 1.03×10^3 M^{-1} cm^{-1} was used to measure activity. The assay conditions for glucose dehydrogenase (EC 1.1.99.10) in particulate fractions of pseudomonads by Hauge (10) measured dichlorophenolindophenol reduction. Alkaline phosphatase has been found in the periplasm of *P. aeruginosa* (4) and was assayed using the conditions employed by Malamy and Horecker (15) with the exception that 1 M Tris

buffer (pH 8.0) with 1 mM $MgCl_2$ was used.

Protein was determined according to the method of Lowry et al. (14) in particulate and soluble fractions and by the Coomassie blue assay (BioRad) in soluble fractions. Reduced cofactors, glutathione, and ferrozine were obtained from Sigma.

Measurements of enzyme stability were made by adding extract to buffer and ferrozine reagent preheated to 60°C and incubating the mixtures for specific times. At the end of the incubation times, the solutions were rapidly cooled in an ice bath and equilibrated to 24°C in a water bath. Substrate and reductant were added, and the reactions were measured spectrophotometrically.

Chromatography of reductase activities was conducted at 4°C on a G-100 Sephadex superfine column (2.6 by 60 cm) equilibrated with 0.05 M Tris-hydrochloride (pH 7.6) with 0.1 M KCl. Flow was against gravity at a rate of 1.5 ml per 10 min, and Dextran Blue 2000 was used to mark the void volume elution. Column fractions were measured for absorbance at 280 nm, and then the volumes in the tubes were split equally and separate assay mixtures were added to the tubes: ferric citrate to one set and ferripyochelin to the other. Each set of tubes was measured for changes in absorbance at 562 nm between 10 and 70 min of incubation. The differences in these readings were taken as estimations of the respective activities of the two reductase enzymes in the fractions.

RESULTS

Assay of iron reductases. Several iron-containing compounds were used in iron reductase assays. The insoluble nature of Fe(III) at pH 7.4 imposed limitations on the concentrations of unchelated iron that could be used in assays, but no reduction was detected when 0.2 μmol of $FeCl_3$ per ml was incubated with NADH and cell-free extract of *P. aeruginosa* (curve a, Fig. 1). Utilization of iron from ferripyochelin (2) indicated the presence of a mechanism for release of the iron from this complex, and there was reduction of the Fe(III) and release of Fe(II) from ferripyochelin when mixed with fresh, dialyzed extracts (curve b, Fig. 1). No reduction was apparent without added NADH (curve c, Fig. 1). Anaerobic conditions were not required for maximal activity of this enzyme, but a 5-s sparge with oxygen (arrow, curve b, Fig. 1) caused a decrease in specific activity from 3.54 to 2.83 nmol/min per mg of protein. This effect was reproducible, with an average inhibitory effect of 25.1%, and was irreversible.

Fe(III) from ferric citrate was also not reduced in cell-free extracts in the absence of reductant (curve a, Fig. 2), but there was reduction after addition of NADH (first arrow, curve b, Fig. 2). Anaerobic conditions were not required for maximal activity, and a 5-s sparge with oxygen caused an apparent increase in activity. However, repeated analyses indicated that there was no detectable effect with oxygen, and the apparent increase in activity was an event occurring with or without oxygen over the subsequent minutes of assay. The aeration was strenuous in both Fig. 1 and Fig. 2, and the period of time for the return to steady rates was apparently due to bubbles leaving solution. Both ferric citrate and

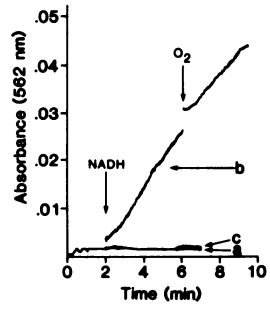

FIG. 1. *Tracing of a ferripyochelin iron reductase reaction conducted with a dialyzed cell-free extract from P. aeruginosa. The reaction mixture contained 58.3 μg of protein per ml with (a) 0.2 mM $FeCl_3$ and 1 μmol of NADH; (b) 0.31 mM ferripyochelin and 1 μmol of NADH; or (c) 0.31 mM ferripyochelin lacking NADH. All reactions were initiated with nitrogen-flushed buffer in Thunberg cuvettes containing 2.4 ml composed of 100 μmol of Tris-hydrochloride (pH 7.4) and 2 μmol of ferrozine. NADH was added to assays (curves a and b) at the first arrow, and a 5-s oxygen sparge was conducted at the second arrow on curve b. Cell-free extract was obtained from bacteria grown in succinate minimal medium without added iron.*

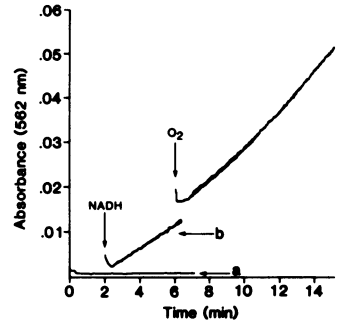

FIG. 2. *Tracing of a ferric citrate iron reductase reaction conducted with a dialyzed cell-free extract of P. aeruginosa. The reaction mixture contained 58.3 μg of protein per ml with (a) 600 μM ferric citrate and no NADH or (b) 600 μM ferric citrate with 1 μmol of NADH. All reactions were initiated in nitrogen-flushed buffer in Thunberg cuvettes containing 2.4 ml composed of 100 μmol of Tris-hydrochloride (pH 7.4) and 2 μmol of ferrozine. NADH was added at the first arrow, and a 5-s oxygen sparge was conducted at the second arrow. Cell-free extract was obtained from bacteria grown in succinate minimal medium without added iron.*

ferripyochelin iron reductases were affected by oxygen in ways that were different from those reported for ferric citrate reductases from other bacteria (3).

Locations of activities. There was no loss of viability in populations of bacteria subjected to osmotic shock for the release of components of the periplasm. Alkaline phosphatase activity was present in highest activity in the periplasmic fraction (Table 1). The absence of diaphorase activity from this fraction indicated that there was no contamination of periplasm with cytoplasmic components. Malic dehydrogenase was found in highest activity in the cytoplasmic fraction, but there was also activity in the other fractions. Glucose and succinate dehydrogenase activities were used as markers for the membrane-rich fraction, and although high activities were found in this fraction, approximately 10% of both activities were in the soluble fraction.

Ferripyochelin iron reductase was present in the periplasmic fraction where negligible ferric citrate iron reductase was detected. Both reductase activities were in high levels in the cytoplasmic fraction. The presence of both activities in low amounts in the membrane fraction was further studied by washing the membranes in buffer and again collecting the membrane fractions by centrifugation. Both activities appeared again in the supernatants (soluble fractions), indicating a possible loose attachment to the membranes. The activities of the enzymes in membrane and soluble fractions were identical; the apparent K_m values were 56 μM for ferripyochelin and 303 μM for ferric citrate.

Specificity of the reductant. Table 2 displays the abilities of various reduced compounds and succinate to serve as electron donors for the two iron reductase activities in soluble and membrane fractions. NADH was routinely the best reductant; NADPH worked approximately 30 to 40% as well. Reduced glutathione was also very effective in donating electrons to the ferripyochelin iron reductase, but not to ferric citrate iron reductase. The periplasmic activity reducing iron from ferripyochelin was identical in specificity for reductant to the soluble activity. Many other amino acids, carbohydrates, and carboxylic acids were tested and demonstrated no electron-donating function for the reductases. Other reducing agents (ascorbate and dithiothreitol) reduced iron from the complexes as rapidly with extract as without extract.

Separate activities of ferric citrate and ferripyochelin reductases. The apparent differences in location of the ferripyochelin and ferric citrate reductase activities suggested the existence of separate enzymes and not one enzyme with multiple substrates. There was a small but reproducible difference in the elution profile of the two activities from a G-100 Sephadex superfine column. Effluent fractions resulting from crude, dialyzed extract applied to the column were measured for absorbance at 280 nm (solid line, Fig. 3), and then the fractions were split and reagents were added to the two sets of tubes for reductase assays for ferric citrate and ferripyochelin. Both activities appeared in later fractions containing proteins of smaller size, and there was a significant amount of activity for ferripyochelin eluting in the void volume, possibly with particulate material or as an aggregate of activity eluting later.

The separate nature of the two enzymes was also suggested by differences in stabilities of the two activities. Optimal activity of ferric citrate iron reductase was found only in fresh extracts, whereas the activity of ferripyochelin iron reductase could be measured over a period of approximately 1 week before there was significant decay in activity. Heating cell-free extract at 60°C destroyed the ferric citrate iron reduc-

TABLE 1. *Specific activities of marker enzymes and iron reductase enzymes in different cell fractions*

Cell fraction	Sp act of enzymes						
	Alkaline phosphatase[a]	Diaphorase[b]	Malate dehydrogenase[c]	Succinate dehydrogenase[d]	Glucose dehydrogenase[e]	Ferripyochelin iron reductase[f]	Ferric citrate iron reductase[f]
Soluble	5.41×10^{-3}	3.19	28.35	8.0×10^{-3}	1.82×10^{-4}	3.88	3.64
Membrane	ND[g]	0.63	16.14	8.6×10^{-2}	3.57×10^{-3}	0.68	0.28
Periplasm	4.77×10^{-2}	ND	9.20	ND	ND	2.29	0.08

[a] Units per milligram of protein (1 unit = 1 micromole of *p*-nitrophenol formed per hour).
[b] Nanomoles of resorufin formed per minute per milligram of protein.
[c] Nanomoles of NADH formed per minute per milligram of protein.
[d] Units per milligram of protein (1 unit = 1 micromole of succinate oxidized per minute).
[e] Units per milligram of protein (1 unit = 1 micromole of glucose oxidized per minute).
[f] Nanomoles of Fe(II) formed per minute per milligram of protein.
[g] ND, Not detected.

TABLE 2. *Specificity of the reductant for iron reductase activities*

Reductant	Sp act with enzyme substrate[a]:			
	Ferripyochelin		Ferric citrate	
	Soluble fraction	Membrane fraction	Soluble fraction	Membrane fraction
NADH	3.88	0.68	3.64	0.007
NADPH	1.75	0.25	1.56	ND[b]
Reduced glutathione	2.21	0.41	ND	ND
Succinate	ND	0.02	ND	ND

[a] Specific activities are in nanomoles of Fe(II) per minute per milligram of protein.

[b] ND, Not detected.

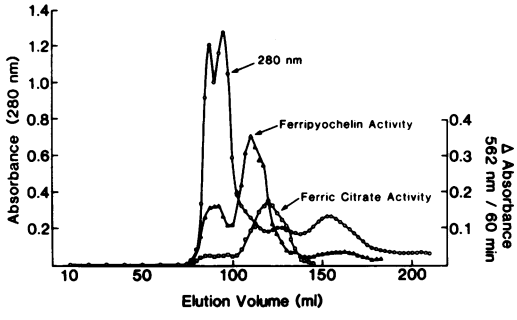

FIG. 3. *Separation of iron reductase activities on a G-100 superfine Sephadex column (2.6 by 60 cm) with reversed flow. Protein in fractions of the column elution was estimated by absorbance at 280 nm* (○). *Ferripyochelin iron reductase activity* (△) *was estimated by adding 2 μmol of ferrozine, 1 μmol of NADH, and 0.01 mM ferripyochelin to one-half the volume of each fraction. Ferric citrate iron reductase* (□) *was estimated by adding 2 μmol of ferrozine, 1 μmol of NADH, and 600 μM ferric citrate to the remaining half of the volume of each fraction. Enzyme activities were recorded as changes in absorbance at 562 nm during 60 min of incubation at 24°C. Dialyzed cell-free extract, 10 mg, was obtained from bacteria grown in succinate minimal medium without added iron.*

tase activity within 1 min, whereas over 80% of the ferripyochelin reductase remained active. Ten minutes of incubation at 60°C was required to destroy the activity for ferripyochelin.

The activity for ferripyochelin found in the periplasmic fraction appeared to be the same as that of the ferripyochelin iron reductase in the cytoplasm. The elution profile of reductase activity from periplasmic protein loaded on the G-100 column was identical to that for ferripyochelin reductase activity from the cytoplasm (Fig. 3). The apparent K_m found in the periplasmic fraction for ferripyochelin was 49 μM, similar to that found in the cytoplasmic fraction.

Effects of iron supply during growth on the formation of the reductase enzymes. Iron reductase activities were determined in cells grown in succinate minimal medium supplied with increasing concentrations of iron. Although formation of pyochelin by *P. aeruginosa* was inhibited by high concentrations of iron in growth media (2), the activities of the two reductase enzymes were relatively uniform in extracts of strain PAO-1 grown in media containing from 0.01 to 10 μM $FeCl_3$ or from 0.028 to 28 μM ferric citrate.

DISCUSSION

Cell-free extracts of *P. aeruginosa* contain enzymes which reduce Fe(III) in ferric citrate and in the siderophore, ferripyochelin, resulting in the release of Fe(II). The conclusion that there are two enzymes for the two substrates is based on differences in elution patterns from a molecular sieving column, occurrence of the activities in different cell fractions, differences in reductant specificity, and differences in heat stability. It may also be pertinent to the study of iron metabolism that no activity could be detected that reduced Fe(III) supplied as $FeCl_3$. It may be that Fe(III) is not transported uncomplexed but rather is complexed shortly after transport, or that the Fe(III) is incorporated into some iron-containing compounds in *P. aeruginosa*. It is apparent that the reductase activities in *P. aeruginosa* are not similar to the activities for ferric citrate described by Dailey and Lascelles (3) in other bacteria. In contrast to those enzymes that are membrane bound, there were only small fractions of the iron reductases from *P. aeruginosa* in the membrane fraction, and succinate, normally a good reductant for membrane-bound reductases, was not a good reductant for the reductases in *P. aeruginosa*. Oxygen reversibly inhibited the membrane-bound reductase activities in other bacteria (3), but in *P. aeruginosa*, the ferric citrate iron reductase was essentially unaffected and the ferripyochelin iron reductase was irreversibly inactivated by oxygen.

Repeated experiments verified the presence of ferripyochelin iron reductase in the periplasmic fractions of bacteria. If the presence of ferripyochelin iron reductase and malate dehydrogenase in the periplasmic fraction were due to leakage of soluble components during the osmotic shock procedure, then diaphorase and ferric citrate iron reductase activities should also have been apparent. The absence of these activities in the periplasmic fraction was not due to sensitivity of the enzymes to the osmotic shock procedure. The high activity of ferripyochelin

iron reductase in the periplasm raises speculation about the release of reduced iron from the siderophore prior to membrane transport and the subsequent transport of Fe(II). It is unclear how the periplasmic enzyme could interact with reductant, but a fraction of the malate dehydrogenase activity was also found in the periplasmic fraction, and there may be NADH-generating mechanisms available for this reductase.

The survey of possible reductants for the soluble ferripyochelin iron reductase revealed that NADH was most effective, but that reduced glutathione also served as an effective electron donor. The physiological importance of this reductant to enzyme activity is unknown, but fluorometric examination of NADH oxidation in extracts with reduced glutathione and flavin adenine dinucleotide revealed glutathione reductase activity in the soluble fraction of extracts of *P. aeruginosa*.

The lack of control of the reductase activities by iron supplied in high concentrations in growth media was an unexpected finding. *P. aeruginosa* has a high requirement for iron (20), and a relatively high concentration of iron (10 μM $FeCl_3$) was found necessary to inhibit formation of pyochelin (2). Ferric chloride in growth media at pH 7.4 may not constitute a readily available source of iron, and even at high concentrations of $FeCl_3$ bacteria must transport and incorporate extracellular Fe(III). Although this may explain the lack of iron control on the synthesis of ferripyochelin iron reductase, lack of control by ferric citrate, a soluble source of iron, suggests alternatively that reductase enzymes may not be affected by extracellular iron concentrations.

Methods are being investigated for the purification of large amounts of reductase enzymes to answer remaining questions. However, future research must first solve the problem of stability of both enzymes, because activities survive only a few weeks in frozen extracts.

ACKNOWLEDGMENTS

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