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THE ROLE OF FERRICHROME REDUCTASE IN IRON METABOLISM OF *USTILAGO SPHAEROGENA*

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Summary

Ferrichrome, the ferric ionophore for *Ustilago sphaerogena*, can serve as a source of iron for the enzyme ferrochelatase (protoheme ferrolyase, EC 4.99.1.1) in this organism, but only after enzymatic removal of the iron from its carrier. *U. sphaerogena* contains a specific ferrichrome reductase (NADH:ferrichrome oxidoreductase) which catalyzes cellular dissociation of the complex by reduction of the metal to the ferrous state. A spectrophotometric assay was developed based on trapping of the ferrous ion produced by ferrozine. There is an apparent inhibition by oxygen which is thought to be due to re-oxidation of the metal under the assay conditions. The close structural analogue, ferrichrome A, is not a substrate, nor is the ester type siderochrome ferric hexahydro-*N*,*N*′,*N*″-triacetylfusarinine C. Aluminum desferriferrichrome is inhibitory. The importance of this enzyme for the metabolism of iron in this organism is discussed.

Introduction

Virtually all microorganisms examined have been found to utilize low molecular weight iron chelates, or siderophores, as specific ferric ionophores [1]. Some of these compounds, such a mycobactin, appear to be species specific, while others, such as ferrichrome, are utilizable by a wide variety of microorganisms, including bacteria and fungi. Siderophores have an extraordinarily high affinity for ferric ion, and binding constants of the order of 10^{30} are not

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uncommon. After uptake of the siderophore, the cell is faced with the difficult problem of dissociation of the metal from the complex.

Two basic strategies have been proposed for removal of the iron from siderophore complexes. The first is chemical breakdown of the ligand itself. The three ester bonds of enterobactin, the siderophore of *Escherichia coli*, are hydrolyzed by enterobactin (enterochelin) esterase, an intracellular enzyme of that organism [2]. The hydrolytic products are much weaker chelators of iron than the intact siderophore. Similar enzymes, which are capable of hydrolyzing the ester bonds of fusarinine type siderophores, have been found in *Fusarium roseum* and *Penicillium* sp [3]. All attempts to find specific peptidases in organisms producing peptide type siderophores have been unsuccessful. In fact, siderophores such as ferrichrome are notable for their resistance to proteolytic enzymes.

A second way in which iron may be removed from siderophores is by its reduction to the ferrous state. Siderophores have little affinity for ferrous iron and this mechanism would be generally applicable. A ferrimycobactin reductase has been described in *Mycobacterium smegmatis* [4], and more recently an enzyme capable of reducing a variety of siderophores has been found in *Neurospora crassa* [5].

Ferrichrome is the siderophore obtained from cultures of the smut fungus, *Ustilago sphaerogena*. It is a cyclic hexapeptide consisting of three glycyl residues and three δ-*N*-hydroxy-δ-*N*-acetylornithyl residues. The latter residues comprise the trihydroxamate iron binding site. The present investigation describes a ferrichrome reductase from *Ustilago sphaerogena* and its relationship to iron metabolism in that organism.

Methods

Cell preparations. Ustilago sphaerogena (ATCC 12421) was grown under iron deficient conditions as described by Emery [6]. Cell homogenates were prepared using a Braun MSK homogenizer (Quigley-Rochester, Rochester, NY) with 0.45—0.50 mm glass beads at 4000 rev./min for 1—2 min. Whole cells and cellular debris were removed by two 10 min periods of centrifugation at 1500 × *g*, after dilution with about two volumes of buffer. The crude extracts typically contained 20—40 mg protein per ml.

Cell suspensions (1—5 mg/ml, dry weight, in 50 mM Tris-HCl, pH 7.5, containing 50 mM $MgCl_2$) were treated with 0.01 volume of 10% toluene in ethanol (v/v) as described by Kornberg and Reeves [7]. Residual toluene was removed by flushing the suspensions at 4 C with N_2 for 10—30 min. This treatment makes membranes permeable to substrates of intracellular enzymes.

Cells were loaded with iron by incubating washed cells in fresh culture medium with ferrichrome for 45—90 min. Intracellular iron released from ferrichrome was estimated by measuring the amount of desferriferrichrome which had been returned to the medium (see Ref. 8) as follows: after incubation with ferrichrome, a sample of the cell suspension was collected by centrifugation. Equal portions of the resulting supernatant were placed into two 3-ml cuvettes. The solutions were acidified by adding equal volumes (approx. 0.1 ml) of HCl to each cuvette. To the reference cuvette was added approx. 50 μl 10% $FeCl_3$

(w/v); to the sample no additions were made. The absorbance of each was measured against water at 480 nm. The amount of intracellular iron was evaluated by dividing the absorbance of the sample by that of the reference. The ratio of absorbances provides a measure of the fraction of intact ferrichrome remaining in the supernatant, and, by difference, the amount of ligand returned to the medium after intracellular iron release.

Materials. Ferrichrome ($\epsilon_{425} = 2.90\ mM^{-1} \cdot cm^{-1}$) and ferrichrome A ($\epsilon_{440} = 3.36\ mM^{-1} \cdot cm^{-1}$) were isolated from supernatants of *U. sphaerogena* cultures [9]. They were crystallized from methanol or water, respectively, or were purified by chromatography on DEAE cellulose (Cellex-D, Bio Rad Laboratories, Richmond, CA) [8]. Hexahydro-*N,N',N''*-triacetylfusarinine C and its ferric complex ($\epsilon_{425} \simeq 2.9\ mM^{-1} \cdot cm^{-1}$) were prepared as previously described [10]. Ferrioxamine B was prepared from desferriferrioxamine B mesylate (Desferal), obtained from Ciba Pharmaceutical Co., Geneva, Switzerland [9].

Desferriferrichrome was prepared by chemical removal of the metal [11]. Concentrations of desferriferrichrome solutions were quantitated by pipetting 1 ml water and an appropriate volume, typically 20 µl, of the desferriferrichrome into a 2.00 ml volumetric flask. To this was added 0.08—0.20 ml of a ferric citrate solution, prepared by mixing equal volumes of neutralized 0.12 M citrate in 0.1 M Tris-HCl, pH 7, and 0.04 M $Fe(NH_4)_2(SO_4)_2$, followed by vigorous aeration to oxidize the metal. The amount of ferric citrate solution added was adjusted to assure a 10-fold excess of iron over desferriferrichrome. The contents of the flask were brought to volume with buffer and the absorbance of the resulting ferrichrome solution was read at 425 nm against a blank containing all components except desferriferrichrome. Careful control studies have shown this method to be accurate to ±0.5%.

Aluminum desferriferrichrome was prepared by adding solid $AlCl_3$ to solutions of desferriferrichrome and adjusting to pH 7. The amount of metal added was calculated to be present in 10% excess over the ligand (1 : 1 complex).

Deuteroporphyrin IX was obtained from Porphyrin Products, Logan, Utah. NADH, hemin, and rotenone were purchased from Sigma Chemical Co., St. Louis, MO. Ferrozine [disodium 3-(2-pyridyl)-5,6-bis(4-phenylsulfonate)-1,2,4-triazine] was obtained from Hach Chemical Co., Ames, IA. All N_2 was passed through alkaline pyrogallol solutions to remove traces of O_2. All other chemicals were reagent grade or better.

Analytical methods. Absorbance measurements were made using a Beckman DU spectrophotometer fitted with a Gilford power supply and photomultiplier. Spectra and continuously monitored enzyme assays were recorded on a Cary 15 or Beckman Acta V recording spectrophotometer. Protein was estimated by the method of Lowry et al. [12]. Bovine serum albumin was used as the standard.

Ferrochelatase assay. Ferrochelatase (protoheme ferrolyase, EC 4.99.1.1) was assayed after Porra et al. [13]. The complete assay contained in a final volume of 2.5 ml: cell extract (5—20 mg protein) or toluene treated cells (1—5 mg, dry weight), 200 µmol Tris-HCl (pH 7.8), 1.8 µmol $MgCl_2$, 10 µmol KCl, 10 µmol reduced glutathione, 0.1 µmol Fe(II) (as $FeSO_4$ or Fe(II)-citrate), 0.05—0.10 µmol deuteroporphyrin IX, and 1—2 mg Triton X-100. The assay was run in the dark under N_2 in sealed serum vials (16 mm, internal diameter)

at 35°C. The reaction was initiated by injection of the porphyrin, and was terminated by the injection of 0.5 ml of 0.2 M iodoacetamide. The heme produced was quantitated by the addition of 1.0 ml pyridine and 0.5 ml of 1 N NaOH to form the pyridine hemochrome, and determining the reduced minus oxidized spectrum between 500 and 600 nm as described in Falk [14].

Ferrichrome reductase preparation. Ferrichrome reductase was obtained from cells of *U. sphaerogena* grown for 2—5 days under iron deficient conditions [6]. Extracts were made as described above using 50 mM Tris-HCl, pH 7.5, containing 50 mM $MgCl_2$ and 0.10 M glycerol.

Ammonium sulfate fractionation of the crude extract was done by adding solid $(NH_4)_2SO_4$ to the solution over the range 10—100% saturation in 10—20% increments. Protein precipitates were dissolved in a minimal volume of the original buffer. Enzyme activity was never found in material precipitating at less than about 60% saturation. Therefore, later preparations were made by bringing the extract to 50% saturation, discarding the precipitate, and to 100% saturation in a second step.

Ferrichrome reductase assay. An assay was developed for the measurement of the production of ferrous iron by utilizing ferrozine to trap the reduced iron. The complete assay contained per ml: cell extract (3—30 mg protein), 0.1 μmol NADH, 1 μmol ferrozine, 0.1—1.0 μmol ferrichrome, and 25—40 μmol Tris-HCl (pH 7.5). The reaction was carried out under N_2 at 27—30°C in the dark or in the recording spectrophotometer, and was initiated by the addition of either NADH or ferrichrome. Controls excluded the NADH. Formation of Fe(II)-ferrozine complex was monitored by measuring the absorbance of the assay solution against the appropriate control solution at 562 nm (ϵ = 29.1 $mM^{-1} \cdot cm^{-1}$) [15] which effectively subtracts out any color formation not initiated by NADH. One unit of enzyme will reduce 1 nmol ferrichrome iron per minute. A similar ferrireductase assay system was developed by Dailey and Lascelles [16].

Results

Ferrochelatase activity in U. sphaerogena

Ustilago sphaerogena is known to produce relatively large quantities of cytochrome *c* when grown on iron-containing medium (yeast extract) [17]. Ferrochelatase was examined in this organism to see if ferrichrome is able to act as a substrate for the enzyme. Ferrochelatase could never be demonstrated in cell-free extracts, however. It was shown that the lack of activity was probably due to inactivation of the enzyme during homogenization of the cells rather than due to the presence of an inhibitor (data not shown). The enzyme activity was therefore sought in toluene treated cells. Toluene treatment has been shown to remove membrane permeability barriers for substrates of intracellular enzymes [7].

Ferrochelatase activity was demonstrated in toluene treated cells, and suitability of ferrichrome and intracellular iron derived from ferrichrome ('endogenous iron') as substrates for the enzyme was examined and compared to $FeSO_4$. The results are shown in Table I. The incorporation of iron is enzymatic, since boiled cells and incubation in the presence of iodoacetamide

TABLE I

FERROCHELATASE IN TOLUENE TREATED CELLS USING EXOGENOUS $FeSO_4$, FERRICHROME, OR ENDOGENOUS IRON AS SUBSTRATES

Cells *	Iron substrate added (μmol/assay)	nmol deuteroheme formed $h^{-1} \cdot mg^{-1}$ cells
—Fe	$FeSO_4$ (0.1)	0.64
—Fe	$FeSO_4$ (0.5) + 33 mM iodoacetamide	0
—Fe, boiled	$FeSO_4$ (0.5)	0
—Fe	Ferrichrome (0.1)	0.25
—Fe	None	0
+Fe	None	0.51
+Fe	$FeSO_4$ (0.1)	0.71

* Cells were iron-free (—Fe) or were incubated for 1 h at room temperature with 0.1 mM ferrichrome (+Fe). Intracellular iron derived from ferrichrome in these cells was present at 0.065 μmol/mg. Each assay contained 3 mg (dry weight) of toluene treated cells. 25 nmol deuteroporphyrin were used per assay.

showed no activity. Ferrichrome was less than one half as effective as either $FeSO_4$ or endogenous iron as a substrate for the enzyme. These results suggest that ferrichrome itself is not a substrate for the enzyme, but that intracellular iron derived from ferrichrome is used for heme biosynthesis. Based on these results, a search for an activity capable of removing the iron from ferrichrome was initiated.

Ferrichrome reductase in U. sphaerogena

In their characterization of ferrimycobactin reductase, Brown and Ratledge developed an assay in which ethylenediaminetetraacetate (EDTA) was utilized to trap the Fe(II) produced by the enzymatic reaction [4]. We found that under the conditions of their assay EDTA is capable of non-enzymatically removing iron from ferrichrome at an initial rate of about 45 nmol/h. This non-enzymatic transfer continued until the thermodynamic equilibrium had been reached. It should be noted that the ferrichrome 'reduction' observed in extracts of *N. crassa* may have in fact been this simple chemical exchange reaction [5]. Ferrozine was found to be an effective and specific ferrous ion trap in our assay system, which enabled us to evaluate enzymatic activity on the basis of Fe(II) produced rather than the less desirable disappearance of substrate. Upon mixing solutions of $Fe(NH_4)_2(SO_4)_2$ and ferrozine in 0.075 mM Tris-HCl, pH 7.5, color formation was 98% complete within 10 s, and reached its calculated maximum in 30—60 s. It was also observed that mixing solutions of inorganic ferric salts or ferric citrate with ferrozine resulted in an apparent photoreduction of the iron. Similar photoreduction of ferrichrome iron was not observed. As a precautionary measure, reference solutions used as controls always included all components of the assay except enzyme or NADH in order to subtract any non-enzymatic iron reduction.

Crude extracts of *U. sphaerogena* were found to catalyze NADH-dependent ferrichrome reduction (Fig. 1). The reaction under the conditions used was biphasic, showing a lag phase of about 10 min followed by a linear phase lasting up to an hour. We also observed that the lag phase could be eliminated by pre-

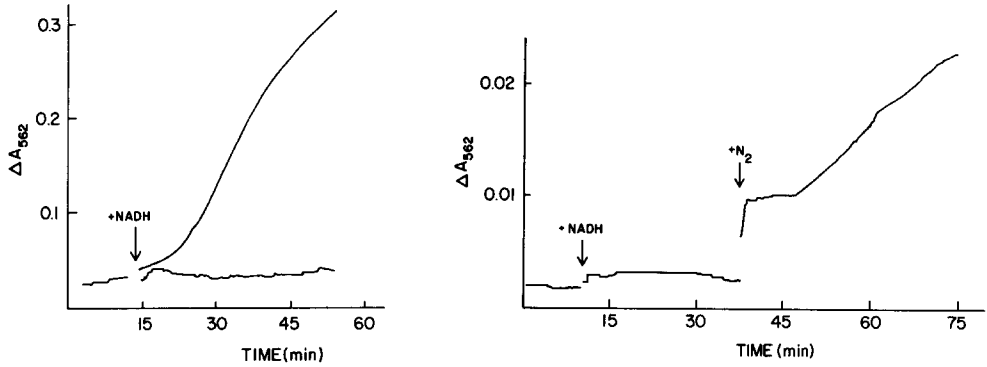

Fig. 1. Enzymatic reduction of ferrichrome by crude extracts of *U. sphaerogena*. The assay contained in a final volume of 3 ml: 2.7 ml crude extract (approx. 30 mg protein/ml), ferrichrome (0.04 mM), and ferrozine (0.3 mM). At the arrow NADH (93 mM) was added to initiate the reaction. The lower trace is that of an assay using extracts which had been heated at 100° C for 10 min prior to assay. The reference (control) contained all components except NADH.

Fig. 2. Effect of O_2 on ferrichrome reductase assay. The concentrations of ferrichrome and NADH were 0.04 mM and 3.3 mM, respectively. At the arrow the solutions were chilled to 4 C and N_2 was passed over the solutions for 5 min. Assay solutions were re-quilibrated to ambient temperature for 3 min and were replaced in the spectrophotometer.

incubating the enzyme with NADH for 5—10 min and initiating the reaction with the addition of ferrichrome.

The activity was concentrated and partially purified from crude extracts by preparing a 50—100% ammonium sulfate precipitate. The resulting precipitate, after dissolving in buffer and removing insoluble material by centrifugation, was found to contain the bulk of the ferrichrome reductase activity. These preparations were generally stable for several days at 4° C. Repeated freezing and thawing caused rapid decrease in activity, complete loss occurring after 3 or 4 cycles. Furthermore, dialysis overnight against fresh buffer caused a marked decrease in activity. The enzyme could be partially stabilized by the inclusion of 0.1 M glycerol and 0.05 M $MgCl_2$ in the buffer solution. Therefore, ammonium sulfate precipitates were dissolved in 0.05—0.10 M Tris-HCl, pH 7.5, containing these additions. The resulting solutions were stored at 4° C or frozen at —20° C in small volumes.

Properties of the enzyme

Ferrichrome reductase activity is dependent upon added NADH, ferrichrome, and enzyme. The rates of reduction are directly proportional to the amount of enzyme added between 0 and 30 mg protein. Enzyme activity shows a fairly sharp pH optimum at 7.5 in 0.1 M Tris-HCl buffer. At pH values greater than 8.5, a slight increase in activity was noted. This is most likely due to partial hydrolysis of the metal and concomitant weakening of complexation by the ligand, resulting in some nonspecific reduction of the iron.

The effect of O_2 on ferrichrome reductase was studied. Using crude extracts, we found that under aerobic conditions no ferrichrome reduction was observed. Upon flushing the assay mixture for 5 min with N_2 at 4° C and reequilibration of the assay for 3 min to room temperature, reduction began immediately (Fig. 2). Similar results were obtained with ammonium sulfate preparations.

TABLE II

EFFECTS OF INHIBITORS ON FERRICHROME REDUCTASE

Inhibitor	Relative activity *
None	100
$HgCl_2$ (10^{-4} M)	17
$HgCl_2$ (10^{-3} M)	0
Iodoacetamide (3.3×10^{-2} M)	45
NaN_3 (10^{-4} M)	49
Rotenone (10^{-6} M)	200
$CuSO_4$ (10^{-6} M)	94
$ZnSO_4$ (10^{-3} M)	64
Cycloheximide (10^{-5} M)	100
Al(III)-desferrichrome (0.35 mM) **	39
Al(III)-desferrichrome (0.035 mM) **	118

* In each case the enzyme was pre-incubated with the inhibitor 10 min prior to initiating the reaction.
** In these assays, [NADH] = 0.09 mM and [ferrichrome] = 0.45 mM.

The enzyme is stable for brief periods (approx. 30 min) at temperatures up to about 40°C. Heating the enzyme in the absence of substrates at 50°C for 10 min resulted in a 40% decrease in activity. It was observed that when a mixture containing enzyme, ferrichrome, and ferrozine was heated to 50°C, rapid ferrichrome reduction occurred. After cooling and adding NADH no enzymatic ferrichrome reduction was observed. This is probably due to a partial denaturation of proteins in the solution, resulting in ferrichrome reduction by exposed sulfhydryl groups. The resulting oxidation of sulfhydryl groups on the reductase enzyme may in turn destroy its enzymatic activity (see below).

The effects of chemical and metabolic inhibitors on ferrichrome reductase activity are summarized in Table II. Under the conditions of these assays, the sulfhydryl reagents $HgCl_2$ and iodoacetamide are inhibitory, Hg(II) strongly so. Azide (10^{-4} M) inhibits the reaction about 50%. Rotenone at low concentration increases the NADH-dependent reduction of ferrichrome iron. At higher concentrations inhibition was observed, but this was traced to the volume of ethanolic rotenone solution added. With 10% (v/v) ethanol, protein denaturation caused the observed inhibition, rather than an inhibition by rotenone itself (data not shown). The approximately 1% (v/v) ethanol used in toluene treatment of whole cells was not inhibitory. The substrate analogue Al(III)-desferri-

TABLE III

ACTIVITY OF FERRICHROME REDUCTASE WITH VARIOUS FERRIC COMPLEXES

Substrate	Relative activity
Ferrichrome (0.04 mM)	100
Fe(III)-hexahydro-*N,N',N''*-triacetylfusarinine C (0.09 mM)	0
Fe(III)-hexahydro-*N,N',N''*-tracetylfusarinine C (0.8 mM)	14
Ferrioxamine B (0.06 mM)	73
Ferrichrome A (0.04 mM)	0
Fe(III)-citrate (0.1 mM)	>200
Fe(III)-EDTA (0.1 mM)	180

ferrichrome was found to be inhibitory, about 60% inhibition occurring when the ferric and aluminum complexes were approximately equimolar (Table II).

The activity of ferrichrome reductase with various ferric complexes is compared in Table III. The total lack of activity with ferrichrome A correlates well with its low rate of transport by *U. sphaerogena* [8] and its lack of growth factor activity in *Arthrobacter* JG-9 [18]. Ferric hexahydro-*N,N'N''*-triacetylfusarinine C is a poor substrate; ferrioxamine B shows some substrate activity with the enzyme. The non-siderophore complexes, Fe(III)-citrate and Fe(III)-EDTA, are both reduced rapidly and linearly with time by this system.

Preliminary kinetics were run using ammonium sulfate preparations. With NADH as variable substrate and the ferrichrome concentration fixed at 0.06 mM, straight lines were obtained in double reciprocal plots of $1/v$ vs. 1/[NADH] at low NADH concentrations (<0.05 mM). With different preparations, the V was quite variable. With all preparations used, however, the apparent K_m for NADH was in the range of 5 to 20 μM. At NADH concentrations greater than about 0.05 mM, a distinct upward curvature of the double-reciprocal plots was seen. Double-reciprocal plots with ferrichrome as the variable substrate and NADH concentrations fixed at 0.05 mM tended to be non-linear, and no meaningful kinetic constants could be derived. The fixed NADH concentration used was approximately that which gave maximal velocities in the $1/v$ vs. 1/[NADH] studies.

Discussion

The active transport of ferrichrome by *U. sphaerogena* far exceeds the organism's iron requirements [8]. A portion of the transported ferrichrome iron is removed from the siderochrome and retained by the cells, and the resulting ligand is returned to the medium intact. It has been observed, however, that up to one-half of the transported iron remains associated with the ferrichrome [19]. Excretion of the ligand may provide the thermodynamic drive to keep the siderochrome from re-forming. If iron reduction were involved in metal release, maintenance of iron in the ferrous state, for which hydroxamates have little affinity, would contribute to this drive. These combined activities could explain the ability of this organism to attain high intracellular concentrations of iron.

Our results show that although the iron of ferrichrome is available as a substrate for ferrochelatase and heme synthesis, ferrichrome is a poor source of iron compared to ferrous sulfate or iron previously released from cellular ferrichrome. This rules out ferrichrome as a direct substrate or cofactor for ferrochelatase in *U. sphaerogena*. Siderophores apparently are not substrates for ferrochelatase in *N. crassa* [5], and this is in accord with all data indicating that iron must be in the ferrous state to be incorporated into heme.

An NADH-dependent ferrichrome reductase is active in the release of iron from ferrichrome in *U. sphaerogena*. The enzymatic activity appears to be oxygen sensitive, but we believe that this is due to rapid re-oxidation of the Fe(II) produced in the assay and re-complexation by the ferrichrome ligand. This may also explain the lag when the enzymatic reaction is initiated by addition of NADH. The lag is eliminated when the enzyme is pre-incubated with NADH

and the reaction initiated by addition of ferrichrome. The NADH is thought to interact with NADH oxidase activities present in the crude enzyme preparations which reduce traces of molecular oxygen in the assay medium. This hypothesis is also consistent with the increase in activity caused by rotenone (see below). A time-dependent association of the coenzyme with the enzyme cannot be rule out, however.

The siderophore transport system of *U. sphaerogena*, unlike some other microorganisms, is quite specific for ferrichrome [8] so it is not surprising that the reductase shows considerable specificity for ferrichrome as substrate. This is similar to the specificity observed with *N. crassa* extracts towards its native siderophore, coprogen [5]. The ester type siderochrome derivative, ferric hexahydro-*N*,*N*',*N*''-triacetylfusarinine C, which (like ferrichrome) is a neutral complex and which has an iron affinity comparable to ferrichrome, is inactive as substrate at comparable concentrations. Ferrichrome A is a sister siderochrome of ferrichrome produced by *U. sphaerogena*, but is not a ferric ionophore [8]. Neither does it show any substrate activity with the reductase. The high activity of ferric citrate and ferric EDTA may be due to the presence of other ferrireductase activities in the crude preparations used. The need for activities which can catalyze iron reduction from complexes other than ferrichrome is evident from results which show that a significant fraction of cellular iron is not associated with siderophore (see above). Until further purification of ferrichrome reductase is accomplished, the possibility that these ferric complexes are substrates for the enzyme cannot be ruled out. The inhibition of ferrichrome reductase by Al(III)-desferriferrichrome is not surprising in view of the close structural homology of that complex with ferrichrome. This is also supportive of considerable specificity of the enzyme toward siderophores.

It is intersting that ferrichrome reductase is quite sensitive to the sulfhydryl reagents, mercuric chloride and iodoacetamide. The ornithine esterases of *F. roseum* and *Penicillium* sp., which are believed to be involved in siderophore iron release in those organisms, are believed to have sulfhydryl groups at the active site [3], and the same may be true of ferrichrome reductase. The increase in enzyme activity at low concentrations of rotenone may be due to diversion of reducing equivalents from flavin-linked NADH oxidase activities to ferrichrome reduction. This would imply that ferrichrome reductase may not be a flavoprotein, as has been suggested [1]. Ferrichrome reductase is inhibited by high concentrations of NADH. Double-reciprocal plots of $1/v$ vs. $1/[\text{NADH}]$ consistently showed an upward inflection at [NADH] > 50 μM. The role of NADH in the control of cellular energy metabolism is well known. With adequate iron, *U. sphaerogena* will have a full complement of heme and nonheme iron electron transport components and, hence, high energy charge. Under these conditions reduction of ferrichrome iron would be unnecessary and would waste reducing potential.

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