JOURNAL OF BACTERIOLOGY, July 1989, p. 4031–4037
0021-9193/89/074031-07$02.00/0

Vol. 171, No. 7

Ferric Reductase Activity in *Azotobacter vinelandii* and Its Inhibition by Zn^{2+}

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Received 12 January 1989/Accepted 12 April 1989

Ferric reductase activity was examined in *Azotobacter vinelandii* and was found to be located in the cytoplasm. The specific activities of soluble cell extracts were not affected by the iron concentration of the growth medium; however, activity was inhibited by the presence of Zn^{2+} during cell growth and also by the addition of Zn^{2+} to the enzyme assays. Intracellular Fe^{2+} levels were lower and siderophore production was increased in Zn^{2+}-grown cells. The ferric reductase was active under aerobic conditions, had an optimal pH of approximately 7.5, and required flavin mononucleotide and Mg^{2+} for maximum activity. The enzyme utilized NADH to reduce iron supplied as a variety of iron chelates, including the ferrisiderophores of *A. vinelandii*. The enzyme was purified by conventional protein purification techniques, and the final preparation consisted of two major proteins with molecular weights of 44,600 and 69,000. The apparent K_m values of the ferric reductase for Fe^{3+} (supplied as ferric citrate) and NADH were 10 and 15.8 μM, respectively, and the data for the enzyme reaction were consistent with Ping Pong Bi Bi kinetics. The approximate K_i values resulting from inhibition of the enzyme by Zn^{2+}, which was a hyperbolic (partial) mixed-type inhibitor, were 25 μM with respect to iron and 1.7 μM with respect to NADH. These results suggested that ferric reductase activity may have a regulatory role in the processes of iron assimilation in *A. vinelandii*.

The presence of iron in many enzymes such as nitrogenase and ribonucleotide reductase, and in many cellular structures such as cytochromes and ferredoxins, make it an essential element for almost all forms of life (26). The extreme usefulness of this particular metal is related to its ability to exist in two stable redox states, Fe^{3+} and Fe^{2+}. The large range of reduction potential connecting these states allows iron to participate in electron transfer reactions in iron sulfur proteins and in cytochromes that have redox potentials of between −500 and +300 mV (26). However, even though iron is the fourth-most-abundant metal on the earth's surface, it is not generally available for biological assimilation in aerobic environments. This is because of the tendency of Fe^{2+} to spontaneously oxidize to Fe^{3+}, which then hydrolyzes and polymerizes to form insoluble ferric hydroxides and oxyhydroxides at neutral pH. The result is that Fe^{3+} has a K_{sol} of $\approx 10^{-38}$, which corresponds to a concentration of about 10^{-17} M, a level much too low to sustain life (36).

In general, the response of iron-requiring aerobic organisms to this situation of iron limitation has been the production of siderophores. These low-molecular-weight compounds have a high affinity for Fe^{3+} and, once excreted by the cells, serve to chelate and solubilize the iron essential for growth (27). *Azotobacter vinelandii*, the organism of interest in this study, produces three siderophores: a yellow-green fluorescent peptide called azotobactin (11), azotochelin [2-*N*,6-*N*,di(2,3-dihydroxybenzoyl)-L-lysine] (6), and aminochelin (33). After the ferrisiderophores are assimilated by the cell, a process that occurs through the use of specific membrane receptors (28), the iron must be removed. Separation of iron from the siderophores is the final step in the process of iron assimilation and is accomplished through enzymatic systems that either hydrolyze the ferrisiderophores or reduce the iron (8).

Enzymatic reduction of siderophore-bound iron and transfer to an acceptor or directly into metabolic processes is a practical and generally applicable method of iron release, since siderophores have a very low affinity for Fe^{2+} (1). Iron reductases have been described in a variety of organisms, including *Agrobacterium tumefaciens* (20), *Bacillus megaterium* (2), *Bacillus subtilis* (14, 19), *Mycobacterium smegmatis* (4, 22), *Pseudomonas aeruginosa* (7), and *Rhodopseudomonas sphaeroides* (25). This list is by no means complete, and the apparent ubiquity of this method of iron reduction may result in iron reductases being described in most aerobic iron-requiring organisms. However, although ferric reductases are suggested to be involved in the transfer of iron from the ferrisiderophores to iron-requiring metabolic systems, the exact role that they play in the process of iron assimilation is still unclear.

In this study, we examined the ferric reductase system of *A. vinelandii*; we report here the purification and kinetic properties of an enzyme that is capable of catalyzing the reduction of a variety of iron chelates. Furthermore, as a continuation of a study indicating that the presence of Zn^{2+} during growth of iron-limited cultures of *A. vinelandii* caused overproduction of the siderophores but did not affect the level of cellular iron (16), the effect of Zn^{2+} on the ferric reductase was examined. The inhibitory action of this ion on the enzyme is described, and the implications of this on the process of iron assimilation in *A. vinelandii* are discussed.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The capsule-negative strain UW of *A. vinelandii* OP was maintained at 30°C on slants of iron-sufficient Burk medium (31) (pH 7.2) containing 1% glucose, 1.1 g of ammonium acetate per liter, and 1.8% agar. The inoculum for each study was pregrown on slants of Burk medium for 2 to 3 days. Cells were washed from the slants with iron-limited Burk medium without glucose and used to inoculate liquid cultures (200 ml per 500-ml Erlenmeyer flask) to an initial optical density at 620 nm of 0.08. Optical density measurements were made with a

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Novaspec spectrophotometer (LKB Instruments, Inc.). Liquid Burk medium did not contain $FeSO_4$ and was prepared by using distilled, deionized water from a Milli-Q water purification system (Millipore Corp.), with glucose autoclaved separately. When required, ferric citrate (filter-sterilized 5 mM stock solution) and $ZnSO_4$ (autoclaved 10 mM stock solution) were added just before inoculation. To minimize iron contamination, all glassware was washed before use with 4 N HCl and 50 mM EDTA (pH 7). Liquid cultures were incubated for 20 h at 30°C with shaking at 225 rpm either in a model G-76 gyratory water bath shaker (New Brunswick Scientific Co., Inc.) or on a Lab-Line Orbit shaker.

Preparation of cell extracts. Cells were harvested from liquid cultures by centrifugation and washed twice with 10 mM Tris hydrochloride (pH 7.6) at 4°C. The cells were broken by sonication (model 350 cell disrupter; Branson Sonic Power Co.), and cell debris was removed by centrifugation at 2,000 × *g*. The soluble cell fraction was separated from the membrane fraction by centrifugation at 100,000 × *g* for 90 min at 4°C.

For determination of the cellular location of the ferric reductase, cells harvested by centrifugation at 10,400 × *g* were washed with water from the Milli-Q water purification system and suspended in 33 mM Tris hydrochloride (pH 8.0) containing sodium citrate, sucrose, and lysozyme (15). After a 10-min incubation at room temperature, Mg^{2+} was added, and the spheroplasts were collected by centrifugation at 400 × *g*, resuspended in Tris-buffered sucrose, and sonicated. The cytoplasm was then separated from the membrane fraction by centrifugation at 100,000 × *g*.

Chemical assays. Protein was routinely determined by the method of Lowry et al. (21), using bovine serum albumin as the standard.

Iron (Fe^{3+}) determination using α,α′-bipyridyl (29) was carried out on the soluble fraction of cell extracts that had been treated with 7% perchloric acid overnight at room temperature and for at least 1 h at 80°C. To determine the level of Fe^{2+} in perchloric acid-treated cell extracts relative to the level of Fe^{3+}, the assay was carried out in the absence of added ascorbate.

Siderophore analysis. After the cells were removed by centrifugation, the culture supernatant was acidified to pH 1.8 with HCl and scanned with a Lambda 3 spectrophotometer (Perkin-Elmer Corp.). Levels of total catechols were estimated from the A_{310}, and levels of azotobactin were estimated from the A_{380} (30).

The *A. vinelandii* siderophores azotochelin and azotobactin were purified as previously described (6, 17). 2,3-Dihydroxybenzoic acid was obtained from Sigma Chemical Co., and Desferal was obtained from Ciba-Geigy Canada Ltd.

Purification of ferric reductase. A 9-liter amount of Burk medium containing 5 μM ferric citrate as the iron source (glucose and ammonium acetate autoclaved separately) in a Magnaferm fermentor (New Brunswick Scientific Co., Inc.) was inoculated to an initial optical density at 620 nm of 0.2 with 1 liter of cell culture obtained from 200-ml liquid cultures prepared as described above. Cultures were grown at 30°C with 8 liters of oxygen per min at an agitation speed of 400 rpm and were harvested after 20 h by using a Pellicon cassette system (Millipore Corp.) equipped with a 0.5 μm-pore-size membrane filter. The resulting cell slurry was centrifuged to pellet the cells. The cells were then washed with 10 mM Tris hydrochloride (pH 7.6) and suspended in 10 mM Tris buffer to a final volume of approximately 120 ml. The cells were broken in the presence of DNase I and RNase with a French pressure cell at 20,000 lb/in^2 and 4°C, followed by treatment with lysozyme (32). The cell extract was centrifuged at 100,000 × *g* for 90 min, and the soluble fraction was used as the source of ferric reductase.

Column chromatography was carried out at 4°C essentially by the method of Moody and Dailey (25), with slight modifications described below. Approximately one-fourth of the soluble material was applied to the initial DEAE-Sephacel column. Buffer A, used for the DEAE-Sephacel columns and the Phenyl Sepharose CL-4B column, consisted of 10 mM Tris hydrochloride (pH 7.6) and 10% (wt/vol) glycerol. The ferric reductase activity was eluted from the initial DEAE-Sephacel column (2.5 by 28 cm) with 0.35 M KCl in buffer A. Pooled fractions were concentrated by ultrafiltration with a PM-10 ultrafiltration membrane (Amicon Corp.) and reapplied to the DEAE-Sephacel column, which had been equilibrated with 0.1 M KCl in buffer A. The ferric reductase was eluted with a 600-ml KCl gradient (0.15 to 0.5 M) in buffer A. Pooled, concentrated fractions, adjusted to 0.6 M KCl, were applied to a Phenyl Sepharose CL-4B column (1.5 by 14 cm) that had been equilibrated with 0.6 M KCl in buffer A. The column was eluted with 0.6 M KCl in buffer A, followed by buffer A alone. The ferric reductase eluted in the presence of the salt. Pooled fractions were concentrated to approximately 2 ml and applied to a Sepharose 4B column (1.5 by 30 cm) equilibrated with buffer B (10 mM Tris hydrochloride [pH 7.6], 10% [wt/vol] glycerol, 0.5 M KCl, 7 μM flavin mononucleotide [FMN]). The column was eluted with buffer B. All column material was obtained from Pharmacia Fine Chemicals, Inc.

Ferric reductase assay. The reduction of Fe^{3+} to Fe^{2+} was measured by trapping the product as an Fe^{2+}-ferrozine complex, using a slightly modified version of the assay described by Dailey and Lascelles (9). The 2.5-ml assay mixture consisted of 0.4 mM NADH, 0.16 mM ferric citrate, 1 mM $MgCl_2$, and 1 μM FMN in 10 mM Tris hydrochloride (pH 7.6), incubated together in the dark for 10 min before the addition of ferrozine to 0.16 mM. The reaction was started by the addition of enzyme, and the increase in A_{562} was followed spectrophotometrically at room temperature with either a Perkin-Elmer Lambda 3 UV/VIS spectrophotometer or a Pye Unicam SP8-400 UV/VIS spectrophotometer. Activity was defined as the nanomoles of Fe^{2+} formed per minute per milligram of protein.

Gel electrophoresis. Location of ferric reductase activity in nondenaturing discontinuous polyacrylamide gels was carried out essentially as described by Moody and Dailey (24). The gels were run at 15 mA through the 5% stacking gel and at 30 mA through the 10% separating gel at room temperature. Use of the activity stain resulted in the appearance of pink-purple bands and spots in a light pink gel after 20 to 30 min. Bands from the activity gel were cut out, macerated, and boiled for 5 min in 60 μl of sample buffer containing sodium dodecyl sulfate (SDS) and β-mercaptoethanol. These samples were then applied to an SDS-polyacrylamide gel that had a 2.5-cm rather than the usual 1-cm stacking gel. SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli (18) as previously described (16). The protein bands were stained with Coomassie blue in isopropanol and acetic acid (13). The proteins used as molecular weight standards were bovine serum albumin (68,000), immunoglobulin G heavy chain (50,000), ovalbumin (43,000), immunoglobulin G light chain (23,000), and RNase (13,700).

TABLE 1. Effects of iron and zinc on ferric reductase activity and siderophore production

Growth condition (concn [μM])		Siderophore production[a]		Soluble iron (μg of Fe/g of protein)		Ferric reductase activity[b]	Cellular zinc[c] (μg of Zn/g of protein)
Fe	Zn	Azotobactin	Catechol[d]	Fe^{3+}	Fe^{2+}		
0.5	0	1.0	1.0	48.8	47.2	4.96	24.4
	40	3.0	1.0	53.6	53.4	3.70	508
1.25	0	0.2	1.0	90.3	18.7	5.25	23.5
	40	0.5	1.3	119.2	12.5	1.34	1,517
2.5	0	0	0.6	100.2	82.7	6.67	27.7
	40	0.2	1.0	106.5	23.6	1.16	1,652
3.75	0	0	0.2	113.4	168.7	8.11	23.0
	40	<0.1	0.5	173.4	48.5	0.73	NA
5.0	0	0	<0.1	136.6	158.6	7.06	13.6
	40	0	0.2	157.2	49.0	0.56	2,036
10.0	0	0	0	167.2	298.0	7.48	NA
	40	0	<0.1	200.8	98.5	0.53	NA

[a] Shown as relative levels of siderophores; a value of 1.0 is the normal level of catechol (~780 μmol/g of protein) and azotobactin (~20 μmol/g of protein) found in the iron-limited (0.5 μM Fe^{3+}) control.

[b] Expressed as nanomoles of Fe^{2+} formed per minute per milligram of protein.

[c] Determined by atomic absorption spectroscopy. NA, Not assayed.

[d] Mixture of 2,3-DHBA, azotochelin, and aminochelin.

RESULTS

Ferric reductase activity in extracts of *A. vinelandii*. Examination of crude, soluble cell extracts from cultures grown with various concentrations of iron indicated that the specific activity of the enzyme was not significantly affected by the iron content of the growth medium (Table 1). Through the use of an experimental protocol designed to separate the cytoplasmic and periplasmic fractions of *A. vinelandii* cells (15), the ferric reductase was found to be located in the cytoplasm.

Cells that were grown in the presence of 40 μM Zn^{2+} showed a general decrease in the activity of the ferric reductase. The degree of enzyme inhibition was related to the zinc content of the cells; this, in turn, was dependent on the iron content of the growth medium, since the amount of Zn^{2+} acquired by the cells increased as the degree of iron limitation decreased (Table 1). Interestingly, the amount of Fe^{2+} in cells in which the ferric reductase was inhibited by Zn^{2+} was much lower than that in cells in which the enzyme was not so inhibited (Table 1). The level of Fe^{3+} was not significantly affected by the presence of Zn^{2+} during cell growth. At all levels of iron tested, the presence of Zn^{2+} caused an increase in siderophore synthesis (Table 1) similar to that previously reported (16).

The soluble fraction of *A. vinelandii* could reduce iron supplied as the ferrisiderophores ferriazotobactin and ferriazotochelin, although the specific activity of the enzyme with respect to ferriazotobactin was low (Table 2). The level of iron in the growth medium did not affect the specific activity of the ferric reductase, indicating that the ferrisiderophores could be reduced regardless of whether they were being synthesized by the cell. However, the presence of 40 μM Zn^{2+} during cell growth caused a relatively greater decrease in the level of ferrisiderophore reduction than of ferric citrate reduction (Table 2). The approximate decrease in reduction of the iron chelates by the Zn^{2+}-grown culture with respect to the Zn^{2+}-free culture was 25% for ferric citrate, 50% for ferriazotobactin, and 75% for ferriazotochelin.

TABLE 2. Effects of iron and zinc on ability of ferric reductase of soluble cell extracts to reduce various iron chelates

Growth condition (concn [μM])		Ferric reductase activity[a]			
		Ferric citrate		Ferriazotobactin (30 μM)	Ferriazotochelin (50 μM)
Fe	Zn	30 μM	50 μM		
0.5	0	4.68	5.74	1.60	6.65
0.5	40	3.34	4.19	0.77	1.65
5	0	5.56	6.93	1.89	4.45

[a] Expressed as nanomoles of Fe^{2+} formed per minute per milligram of protein. All assays contained 0.3 mM NADH, 10 mM $MgCl_2$, 4 μM FMN, and 0.48 mM ferrozine.

Simultaneous examination of the ferric reductase activity of several samples was achieved through the use of nondenaturing polyacrylamide gels. The application of equal portions of soluble cell extracts to the gel, followed by staining for enzyme activity, revealed the presence of one major spot of activity in the center region of the gel, with various very faint bands present immediately above the major spot and also near the top of the gel (data not shown). Growth of cultures with different levels of iron and Zn^{2+} did not change the pattern of bands revealed by the activity stain but did decrease the relative intensities of the bands.

The ferric reductase activity could also be inhibited by adding Zn^{2+} to the enzyme assays. The pattern of inhibition was hyperbolic. The addition of 10 μM Zn^{2+} resulted in a sharp decrease in enzyme activity, whereas a gradual decline in activity was noted with further additions of Zn^{2+} (Fig. 1). These results suggested that Zn^{2+} was unable to eliminate the ferric reductase activity completely.

The possibility that Zn^{2+} might be interfering with the enzyme assay rather than acting to inhibit the enzyme was examined by determining the A_{562} values from enzyme-free ferric reductase assays containing known concentrations of Fe^{2+} [from a freshly made stock of $Fe(NH_4)_2(SO_4)_2$] with

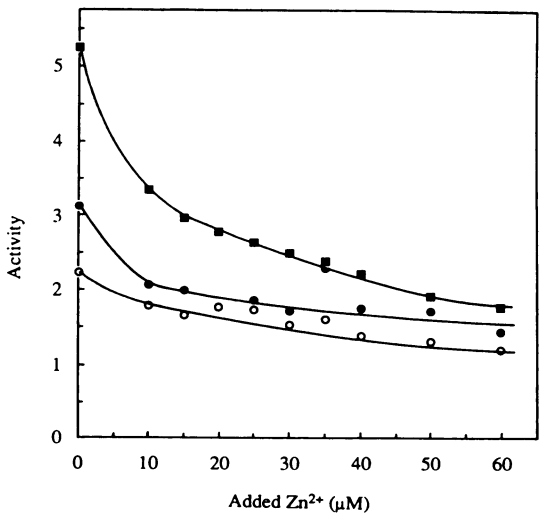

FIG. 1. Activity of ferric reductase in the presence of increasing levels of Zn^{2+}. Samples tested were the soluble cell extracts from cultures grown in the presence of 5 μM ferric citrate (■), 0.5 μM ferric citrate (●), and 0.5 μM ferric citrate with 40 μM $ZnSO_4$ (○). Enzyme activity is expressed as nanomoles of Fe^{2+} formed per minute per milligram of protein.

TABLE 3. Purification scheme for ferric reductase of *A. vinelandii*

Fraction	Sp act[a]	Purification (fold)	Protein recovered (mg)
Soluble	11.1	1.0	338
DEAE-Sephacel			
First	32.1	2.9	108
Second	42.7	3.8	64.5
Phenyl Sepharose CL-4B	153.2	13.8	8.28
Sepharose 4B	244.5	22.0	2.53

[a] Expressed as nanomoles of Fe^{2+} formed per minute per milligram of protein. Each assay contained 0.4 mM NADH, 10 mM $MgCl_2$, 0.16 mM ferric citrate, 4 μM FMN, and 0.48 mM ferrozine.

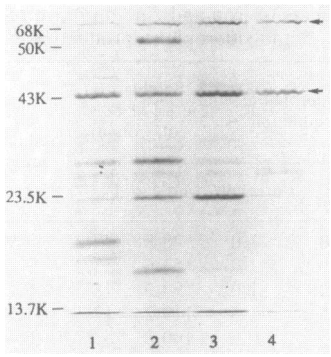

FIG. 2. SDS-polyacrylamide gel electrophoresis of the proteins associated with ferric reductase activity during enzyme purification. Samples of protein (~200 μg) were taken from the soluble cell extract (lane 1), from the first DEAE-Sephacel column (lane 2), from the second DEAE-Sephacel column (lane 3), and from the Sepharose 4B column (lane 4); ferric reductase activity was located on a nondenaturing gel, and the protein content of the major spot of activity was determined by SDS-polyacrylamide gel electrophoresis. Positions of molecular weight standards (in thousands [K]) are shown at the left. Arrows on the right indicate proteins with molecular weights of 69,000 (top) and 44,600 (bottom).

0.16 and 0.8 mM ferrozine. Rather than deleteriously affecting the assay, the addition of Zn^{2+}, at levels ranging from 40 μM to 1 mM, appeared to cause a slight increase in the A_{562} (data not shown). Of particular significance was the observation that the levels of Zn^{2+} which had been noted to affect ferric reductase activity did not affect the formation of the Fe^{2+}-ferrozine complex. The inhibitory effect of Zn^{2+} on the enzyme itself was confirmed by determining the activity of a partially purified enzyme preparation in the presence and absence of added 40 μM Zn^{2+}, using both 0.16 and 0.8 mM ferrozine in the assay. The latter excess amount of ferrozine was used to overcome any potential competition between Zn^{2+} and Fe^{2+}. In each instance, enzyme activity decreased by 40 to 50% in the presence of Zn^{2+}.

Conditions for optimization of the assay for ferric reductase activity. The ferric reductase activity of the soluble fraction of *A. vinelandii* was examined over the pH range 5.6 to 8.8, using the buffers 2-(*N*-morpholino)ethanesulfonic acid (MES; pH 5.6, 6.0, and 6.8), 3-(*N*-morpholino)propanesulfonic acid (MOPS; pH 6.8, 7.2, and 7.6), and Tris hydrochloride (pH 7.6, 8.0, and 8.8). A single pH optimum was found at pH 7.5.

A partially purified enzyme preparation obtained by passage of the soluble cell extract through a DEAE-Sephacel ion-exchange column was used to examine the effect of Mg^{2+} on ferric reductase activity. The addition of up to 10 mM $MgCl_2$ to the ferric reductase assays caused a sixfold increase in enzyme activity. Similar results were obtained with added $CaCl_2$, and $SrCl_2$ was approximately half as effective as $MgCl_2$ in stimulating enzyme activity (data not shown).

Determination of ferric reductase activity under anaerobic conditions was accomplished by sparging the assays with nitrogen gas for >1 min before addition of the enzyme solution. This typically resulted in up to a threefold increase in enzyme activity. However, the background level of nonenzymatic reduction of iron also increased markedly, and widely varying levels of enzyme activity were obtained. We were unable to resolve these problems and therefore used conditions that were not strictly anaerobic throughout the study.

Purification of the ferric reductase. In each of the columns used to purify the enzyme, all of the ferric reductase activity eluted as a single peak. Final purification of the enzyme with this procedure was 22-fold, and enzyme protein accounted for 0.75% of the starting material (Table 3). Loss of enzyme activity as a result of degradation or storage did not appear to be a problem, since no decrease in activity was detected for samples at any stage of the purification procedure after storage for several months at −20°C or after repeated freeze-thaw cycles.

Determination of molecular weight of ferric reductase. Samples of the enzyme, removed after each step in the purification scheme, eluted at the void volume of Sephadex G-75 and G-100 gel filtration columns. Increasing the ionic strength of the column buffer with KCl (μ = 0.5) did not change the elution pattern. Similarly, the enzyme eluted just after the void volume on a Sepharose 4B column, which indicated a molecular weight range of 6×10^4 to 20×10^6.

A sample of the enzyme from each step in the purification scheme was applied to a nondenaturing acrylamide gel and stained for ferric reductase activity. The spot corresponding to the major region of activity was located in the center region of the gel, exactly as observed with the unpurified material. The spot was cut out of the gel, applied to an SDS-polyacrylamide gel, and stained for protein (Fig. 2). The final enzyme preparation from the Sepharose 4B column contained two proteins, with apparent molecular weights of 69,000 and 44,600, that were more concentrated than the other proteins originally present (Fig. 2).

Properties of the ferric reductase. The apparent K_m values of the ferric reductase for Fe^{3+} (supplied as ferric citrate) and for NADH were 10 and 15.8 μM, respectively. The families of parallel lines obtained from plotting $1/v$ against either 1/[NADH] or $1/[Fe^{3+}]$ at increasing concentrations of the other substrate (Fig. 3) were consistent with a Ping Pong Bi Bi mechanism whereby a product is released between additions of the substrates (35).

The specificity of the ferric reductase with respect to several different substrates and cofactors was also examined. Enzyme activity in the presence of NADPH was 46% of that obtained with NADH (specific activity, 25.1 nmol of Fe^{2+}/min per mg of protein), suggesting that NADH was the substrate of choice. The presence of a flavin was also necessary for maximum ferric reductase activity. FMN was preferred as a cofactor over flavin adenine dinucleotide since the presence of 4 μM FMN stimulated the ferric reductase activity by a factor of 15, whereas an equal amount of flavin adenine dinucleotide produced only a 1.8-fold increase in activity. Although enzymatic activity during the purification

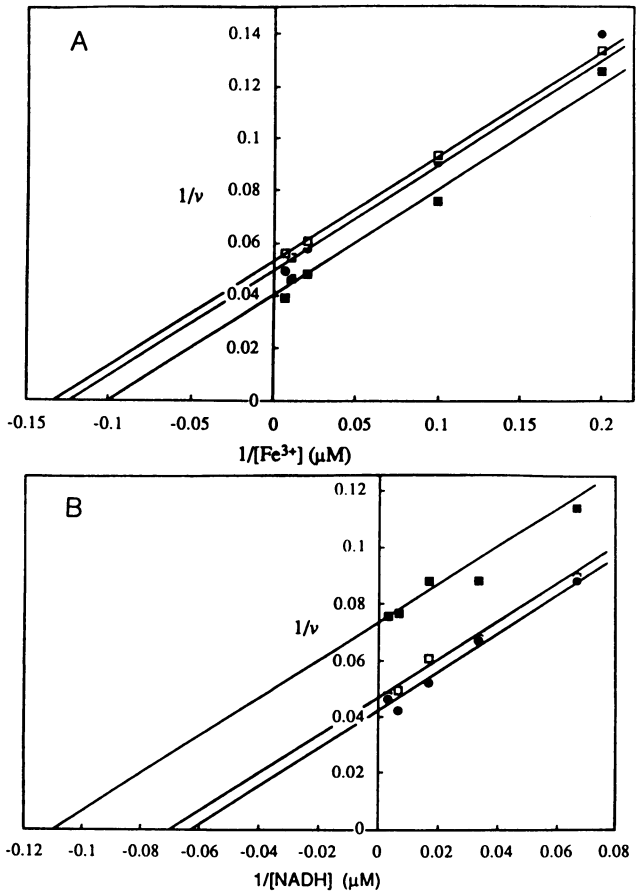

FIG. 3. Lineweaver-Burk plots of ferric reductase activity with respect to Fe^{3+} (A) and NADH (B) concentration. $1/v$ is expressed as (nanomoles of Fe^{2+} formed per minute per milligram of protein)$^{-1}$. Each assay contained 0.48 mM ferrozine, 4 μM FMN, and 10 mM $MgCl_2$, with Fe^{3+} supplied as ferric citrate. NADH was present at concentrations of 60 (□), 120 (●), and 300 (■) μM (A); ferric citrate was present at concentrations of 15 (■), 50 (□), and 100 (●) μM (B).

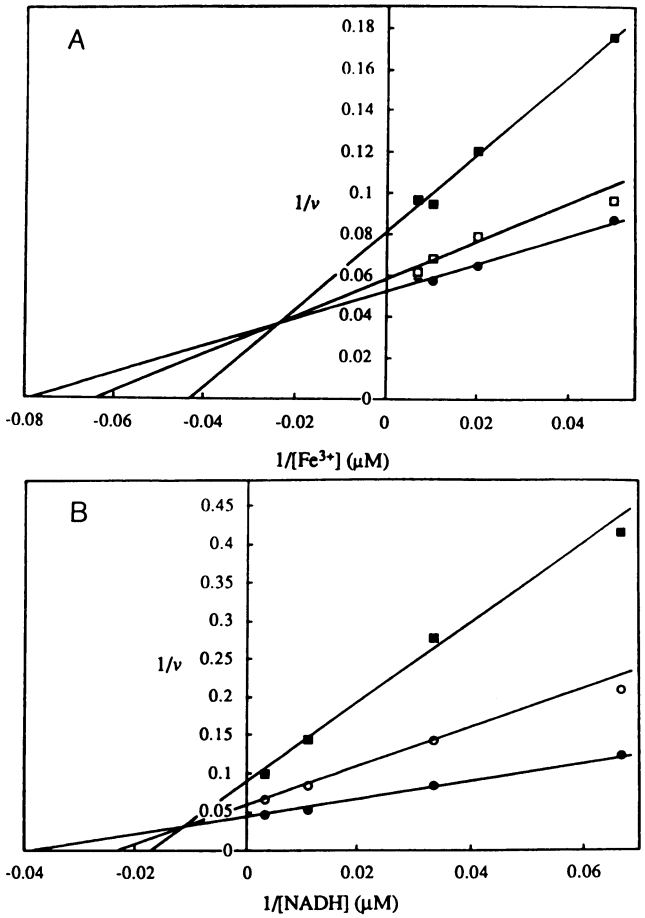

FIG. 4. Lineweaver-Burk plots of ferric reductase activity in the presence of the inhibitor Zn^{2+} with respect to Fe^{3+} (A) and NADH (B) concentration. $1/v$ is expressed as (nanomoles of Fe^{2+} formed per minute per milligram of protein)$^{-1}$. Each assay contained 0.48 mM ferrozine, 4 μM FMN, and 10 mM $MgCl_2$, with Fe^{3+} supplied as ferric citrate. (A) Assays carried out with 0.3 mM NADH. $ZnSO_4$ was added to concentrations of 10 (●), 20 (□), and 100 (■) μM. (B) Assays carried out with 0.15 mM ferric citrate. $ZnSO_4$ was added to concentrations of 4 (●), 20 (○), and 100 (■) μM.

procedure was based on ferric citrate reduction, the purified enzyme was able to reduce iron supplied as a number of different iron chelates. The enzyme reduced iron bound to low-affinity chelators, such as 30 to 50 μM citrate and 150 μM 2,3-DHBA (specific activities, 20.6 and 15.9 nmol of Fe^{2+}/min per mg of protein, respectively), more readily than iron bound to the high-affinity siderophores 30 μM azotobactin and 50 μM azotochelin (specific activities, 1.02 and 3.45 nmol of Fe^{2+}/min per mg of protein, respectively) and to 150 μM Desferal (specific activity, 5.63 nmol of Fe^{2+}/min per mg of protein).

Kinetic analysis of ferric reductase with added Zn^{2+} as an inhibitor indicated that Zn^{2+} affected both the V_{max} and K_m values of the reaction (Fig. 4). Thus, Zn^{2+} was acting as a mixed-type inhibitor (35). The intersection points on the abscissas of the plots of $1/v$ versus 1/[Fe] and $1/v$ versus 1/[NADH] in the presence of different fixed concentrations of Zn^{2+} (Fig. 4) were used to calculate K_i values for Zn^{2+}, giving K_i values of approximately 25 μM with respect to iron and approximately 1.7 μM with respect to NADH.

DISCUSSION

A ferric reductase was identified in crude, soluble cell extracts of *A. vinelandii*. The enzyme was active under aerobic conditions; however, in common with most ferric reductases from other microorganisms (1), the creation of a partially anaerobic environment did result in increased activity. The activity of the enzyme was not affected by the iron concentration of the growth medium; therefore, the enzyme appeared to be constitutive. A lack of correlation between the iron status of the cells and the activity of ferric reductases is a typical characteristic. So far, only the ferric reductase from *Neurospora crassa* has been distinguished as an exception to this general rule (12). The location of the ferric reductase of *A. vinelandii* within the cytoplasm is another common characteristic. However, this is by no means a universal trait, since the iron reductase activity in *Spirillum itersonii* is associated with the respiratory chain (9), and the ferripyochelin iron reductase of *P. aeruginosa* is a periplasmic enzyme (7).

Estimation of the molecular weight of the active ferric reductase was inconclusive because of a lack of correlation

between the results provided by gel filtration chromatography and SDS-polyacrylamide gel electrophoresis. The former method suggested that the enzyme may be extremely large, whereas two proteins of apparent molecular weights of 69,000 and 44,600 were revealed by the latter method. By comparison, the ferric reductase of *R. sphaeroides* has been proposed to be a single-subunit protein with a molecular weight of 32,000 (25), whereas the same activity in *B. subtilis* is associated with an aromatic biosynthetic enzyme complex (14). However, the observed migration of the active *A. vinelandii* enzyme halfway down a nondenaturing gel is not typical of an extremely large protein. It is possible that during purification the enzyme formed aggregates similar to that obtained during the purification of the pyridine-nucleotide transhydrogenase of *A. vinelandii* (37, 38) but was dissociated by the procedures used in polyacrylamide gel electrophoresis. However, it remains a question whether the two proteins found in the purified ferric reductase are both required for enzyme activity.

The properties of the enzyme were similar to those recorded for other ferric reductases in that the enzyme utilized NADH as a reductant, required FMN as a cofactor, and was stimulated by the presence of a divalent group IIA cation. Kinetic analysis of the dependence of the K_m values for NADH and iron on the concentration of the other substrate revealed a pattern typical of a Ping Pong Bi Bi type of reaction mechanism (35). During enzymatic reactions of this kind, a product is released before all substrates have been added; thus, the enzyme oscillates between two different stable forms. Therefore, since NADH was presumably used by the ferric reductase as a source of electrons for the reduction of iron, the enzyme probably bound this substrate first. The Fe^{3+} chelate would then not be bound until after the enzyme released NAD^+. This mechanism is quite different from that of *R. sphaeroides* (25), wherein the ferric reductase apparently carries out an ordered bisubstrate enzymatic reaction which requires that both substrates be bound before products are released.

The pattern of enzyme inhibition resulting from the addition of Zn^{2+} to the ferric reductase of *A. vinelandii* was characteristic of a mixed-type inhibition system (35). This designation was refined by replotting the primary reciprocal plot data as slope versus Zn^{2+} concentration and $1/v$-axis intercept versus Zn^{2+} concentration (data not shown). These replots were hyperbolic and indicated that Zn^{2+} was a hyperbolic (partial) mixed-type inhibitor. In this type of system, which is a mixture of partial competitive and partial noncompetitive inhibition, both the enzyme-substrate complex and the enzyme-substrate-inhibitor complex will form product, but the rates at which they do so differ (35). Therefore, the enzyme activity could not be completely inhibited by Zn^{2+}, a result that was also suggested by the observation that the ferric reductase activity in soluble cell extracts decreased hyperbolically with increasing amounts of Zn^{2+}.

A survey of the effect of various ions, including Zn^{2+}, on ferric reductase activity has been carried out in a few organisms, including *B. subtilis* (14), *N. crassa* (12), and *R. sphaeroides* (24). In each of these systems, Zn^{2+} was not observed to inhibit the ferric reductase; therefore, the inhibitory action of Zn^{2+} on this enzyme may not be ubiquitous. However, it is possible that Zn^{2+} affects the ferric reductase of *Pseudomonas fluorescens*, since the presence of this ion resulted in increased siderophore production (3, 5), as was observed in *A. vinelandii* (this study; 16).

Inasmuch as Fe^{2+}, in a free, intracellular pool, has been proposed to be the major controlling factor for iron uptake (10, 39), lower levels of this ion resulting from the presence of Zn^{2+} could explain the observed increase in siderophore synthesis in *A. vinelandii* (16). The effect of Zn^{2+} on siderophore production (this study; 16) was observed under iron-limited and iron-sufficient growth conditions. The ferric reductase purified in this study was constitutive with respect to the iron nutritional status of the cells, and inhibition of this enzyme activity by Zn^{2+} was coincident with depressed Fe^{2+} levels in the cell cytoplasm except in the most iron-limited cultures. Thus, the ferric reductase may play an important regulatory role in the process of iron assimilation in *A. vinelandii*. This enzyme also had some ability to reduce ferrisiderophores, which suggested that it could also play a role in siderophore-mediated iron uptake. However, the differential sensitivity of the ferric reductase activity in iron-limited cells to Zn^{2+} and the apparent loss of ferrisiderophore reductase activity compared with ferric citrate reductase activity during enzyme purification suggests that a second, perhaps oxygen-sensitive, ferrisiderophore reductase may be present in *A. vinelandii*, as has been found in *P. fluorescens* (23) and *Aquaspirillum magnetotacticum* (34). We are now searching for this enzyme; it will be interesting to determine its sensitivity to Zn^{2+} in light of these results and our previous findings (16), which suggested that azotobactin-mediated iron uptake could escape Zn^{2+} inhibition.

ACKNOWLEDGMENTS

We thank Margaret von Tigerstrom for the preparation of purified siderophores and Park Yee, of the Department of Soil Science, University of Alberta, for performing the atomic absorption analyses.

This study was supported by grants from the Natural Sciences and Engineering Research Council of Canada and the Alberta Heritage Foundation for Medical Research.

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