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Characterization of a soluble ferric reductase from *Neisseria gonorrhoeae*

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Summary. An NADH-dependent ferric reductase was identified in extracts of *Neisseria gonorrhoeae*. Enzyme activity was measured in an assay using ferrozine as the ferrous iron acceptor. Ferric reductase activity was enhanced by Mg^{2+} and flavine nucleotides. The enzyme reduced both citrate- and diphosphate-bound ferric iron as well as ferric hydroxide (Imferon). However, no activity was observed with either 30%-iron-saturated transferrin or with the gonococcal iron-binding protein, Fbp. The ferric reductase was found primarily within the cytoplasmic cell fraction. The soluble ferric reductase was purified 110-fold by ammonium sulfate precipitation, gel and anion-exchange chromatography. Results obtained following gel chromatography and SDS/polyacrylamide gel electrophoresis suggested that the enzyme had a molecular mass of about 25 kDa.

Key words: Neisseria gonorrhoeae - Gonococci - Ferric reductase

Introduction

Environmental iron exists in its oxidized (Fe^{3+}) state under aerobic conditions, at near neutral pH. Ferric ions have a high affinity for hydroxy ions with which they form insoluble polymers (Neilands 1981b). This limits the availability of free iron to levels below that required to support microbial growth (Bullen et al. 1978). Bacteria have evolved high-affinity iron-acquisition mechanisms designed to surmount this iron restriction. One such mechanism involves the secretion of low-molecular-mass compounds termed siderophores, that bind and solubilize external iron, followed by the binding of the iron-siderophore complex to a cell-surface receptor and its subsequent internalization (Neilands 1981a).

In contrast, *Neisseria gonorrhoeae*, a mucosal pathogen, does not produce classic hydroxyamate- or phenolate-type siderophores (West and Sparling 1985). Instead, this organism uses a receptor-mediated mechanism in which iron is removed from human transferrin and lactoferrin (McKenna et al. 1988) after it binds to specific outer-membrane receptors (Lee and Schryvers 1988); gonococci are also able to utilize heme and hemoglobin as sources of iron (Deyer et al. 1987). Although the sources of iron have been defined, very little is known of the mechanisms by which gonococci acquire and utilize exogenous iron.

When growing aerobically, bacteria acquire iron as Fe^{3+} and use reductants generated by metabolism to provide Fe^{2+} for the synthesis of protoheme (Dailey and Lascelle 1977). Gonococci face a similar situation in that they must reduce the Fe^{3+} bound to transferrin and lactoferrin before it can be utilized by the cell.

In the present study, we describe the partial purification and properties of a gonococcal ferric reductase. This enzyme reduces Fe^{3+} bound to various chelators and may function to provide the cell with Fe^{2+}.

Materials and methods

Organisms and media. N. gonorrhoeae strain FA171 and the isogenic β-lactamase-producing strain FA421 were obtained from P. F. Sparling (University of North Carolina, Chapel Hill, NC). *N. gonorrhoeae* strain FA171 was grown in a liquid medium containing Proteose peptone, no. 3 (Difco laboratories, Detroit, MI) 15 g/l, KH_2PO_4 1 g/l, K_2HPO_4 5 g/l, glutamine 0.25 g/l, thiamin diphosphate 10 μM and cysteine 0.25 mM. Imferon (0.1 ml/l), a soluble complex of ferric hydroxide and high-molecular-mass dextrans, was used as an exogenous iron source (Le Faou 1985). For limiting the quantity of iron available, ethylene diamine di(*o*-hydroxyphenylacetate) (EDDA), was added to culture medium (Mietzner et al. 1984). Strain FA421 was grown in the same medium but containing ampicillin (10 mg/l). All glassware was acid-washed to reduce levels of contaminating iron.

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Spheroplast formation. Cells were grown at 37° C for 3 h in a gyratory shaker ($A_{690} \approx 0.6$) and collected by centrifugation ($8000 \times g$, 10 min). The cell pellet was carefully resuspended in a solution containing 10% (mass/vol.) 1-methylamino-D-glucitol (meglumine), 50 mM Tris/HCl pH 8, 10 mM EDTA and 0.5 mg lysozyme/ml (Bergström et al. 1978). After incubation at room temperature for 5 min, $CaCl_2$ was added to a final concentration of 10 mM and spheroplasts were collected by centrifugation at $17000 \times g$ for 10 min. The resulting supernatant (fraction I) was saved and the pellet was suspended in 50 mM Tris/HCl pH 8 to lyse spheroplasts (fraction II). Fractions I and II were dialyzed against 50 mM Tris/HCl pH 8, containing 20 mM $MgSO_4$ and 20 μM ferric citrate, for 20 h at +4° C and centrifuged at $150000 \times g$ for 60 min. The resulting supernatants and pellets yielded four fractions that have been designated: I-supernatant (I-S), I-pellet (I-P), II-supernatant (II-S), II-pellet (II-P).

Preparation of cell-free extract. Cells were disrupted following spheroplast formation as described above. The particulate material was removed by centrifugation at $48000 \times g$ for 60 min. Solid ammonium sulfate was added to 35% of saturation and the precipitate removed by centrifugation. Additional ammonium sulfate was added to 65% of saturation and the precipitated proteins collected by centrifugation at $17000 \times g$ for 15 min. The resulting pellet was resuspended in 50 mM Tris/HCl pH 8.0 and dialyzed overnight against the same buffer containing 20 mM $MgSO_4$ and 10 μM ferric citrate. After dialysis, the suspension was centrifuged twice at $150000 \times g$ for 1 h. The resulting supernatant was used for kinetic studies and enzyme purification.

Enzyme purification. Ferric citrate (10 μM) was added to all buffers to stabilize the ferric reductase activity. The dialyzed cell extract was applied to a gel filtration column (35 × 2.6 cm) of Sephacryl S300, equilibrated with 50 mM Tris/HCl pH 8.0 containing 50 mM NaCl, and eluted with the same buffer. The fractions containing ferric citrate reductase activity were pooled and subjected to an ion-exchange chromatography on a column (7.5 × 1.6 cm) of DEAE-Sephacel. The DEAE-Sephacel was equilibrated with 50 mM Tris/HCl pH 8.0. After washing with 150 ml buffer, the column was eluted with a 0.1–0.6 M gradient of NaCl in 50 mM Tris/HCl pH 8.0. Ferric reductase eluted at 0.2 M NaCl as a single peak of activity.

Molecular mass estimation. After ion exchange chromatography, the fractions containing ferric reductase activity were pooled and concentrated (UM10 membrane, Amicon corp. Danvers MA). The concentrated material was used for molecular mass determination of ferric reductase by gel filtration and polyacrylamide gel electrophoresis under non-denaturing conditions (Moody and Dailey 1983). The reddish band corresponding to the ferric reductase activity was carefully cut out and the proteins eluted with 10% (mass/vol.) sodium dodecyl sulfate (SDS) at 100° C. The eluted proteins were subjected to SDS/polyacrylamide gel electrophoresis (Laemmli 1970). Protein standards were electrophoresed simultaneously for molecular mass estimation. Proteins were visualized by silver staining (Mietzner et al. 1984).

Enzyme assays. Ferric reductase activity was measured by a modification of the method of Dailey and Lascelles (1977). The assay mixture (final volume 1 ml) contained: 0.1 mM NADH, 0.01 FMN, 10 mM $MgSO_4$, 50 mM Tris/HCl pH 8.0, 1 mM ferrozine and cell extract. The reaction was initiated by adding 0.4 mM ferric citrate. The chromophore formed by ferrozine and Fe^{2+} has a molar absorption coefficient of 28000 M^{-1} cm^{-1} at 562 nm (Moody and Dailey 1983). Enzyme activity was expressed as the rate of reduction of Fe^{3+}.

Glucose-6-phosphate dehydrogenase activity was measured spectrophotometrically in an assay mixture with 1 mM glucose 6-phoshate, 1 mM NAD^+, 50 mM Tris/HCl pH 8.0 and cell extract in a final volume of 1 ml. NADH has a molar absorption coefficient of 6400 M^{-1} cm^{-1} at 340 nm. Activity is expressed as the rate of reduction of NAD^+ (Morse et al. 1974).

β-Lactamase activity was measured spectrophotometrically in an assay mixture containing 26 μg Nitrocefin, 50 mM phosphate pH 7.0 and cell extract in a final volume of 1 ml (O'Callaghan et al. 1972). Enzyme activity is expressed as the rate of change in absorbance at 485 nm (this wavelength corresponded to the maximum absorbance of the chromophore).

Protein concentrations were estimated by the method of Bradford (1976) using bovine serum albumin as a standard.

Chemicals. Fbp was purified from *N. gonorrhoeae* strain F62 as described by Mietzner et al. (1987). Sephacryl-S300 and DEAE-Sephacel were products of Pharmacia Fine Chemicals (Pisataway, NJ). 1-Methylamino-D-glucital (meglumine), lysozyme, NADH, NAD, FMN and ferrozine were purchased from Sigma Chemical Co. (St. Louis, MO). Nitrocefin powder was obtained from BBL, Cockeysville, MD. Imferon was a generous gift from Merell Dow Research Institute (Cincinnati, Ohio). Desferal was a gift of Ciba-Geigy Corp. (Summit NJ). All other chemical reagents were of reagent quality.

Results

Kinetic properties

The synthesis of ferric reductase by *N. gonorrhoeae* was constitutive as supplementation of the growth medium with up to 200 μM EDDA did not change the specific activity of the enzyme in crude cell extracts. Ferric reductase activity was detected as soon as the cell extract was added (Fig. 1). Enzyme activity was linear and was not inhibited by oxygen. Both NADH and NADPH could serve as electron donors but activity was greater with NADH (Table 1). Cysteine, reduced glutathione and succinate could not serve as electron donors. FMN and, to a lesser extent, FAD enhanced enzyme activity. Ferric reductase activity was also increased by the addition of the divalent cation Mg^{2+}. Cyanide, azide and 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) did not inhibit the enzyme activity in crude cell extracts suggesting that the electron transport chain was not directly involved.

Ferric diphosphate ($K_m = 0.035$ mM) as well as ferric citrate ($K_m = 0.01$ mM) served as substrates for the ferric reductase; however, the activity with the former was somewhat higher than with the latter. Imferon was also used as a substrate although the affinity of the enzyme for this substrate was low ($K_m = 4.0$ mM). Attempts to reduce directly the ferric iron bound to 30%-iron-saturated transferrin or to the gonococcal periplasmic iron-binding protein, Fbp, were unsuccessful. Enzyme activity could not be measured in the presence of various ferric salts [e.g. $FeCl_3$, $Fe(NO_3)_3$] as spontaneous reduction of Fe^{3+} was observed. It was difficult to assess which chemical form of the Fe^{3+} salt was present in the assay mixture as ferric hydroxides are readily formed in aerated solutions.

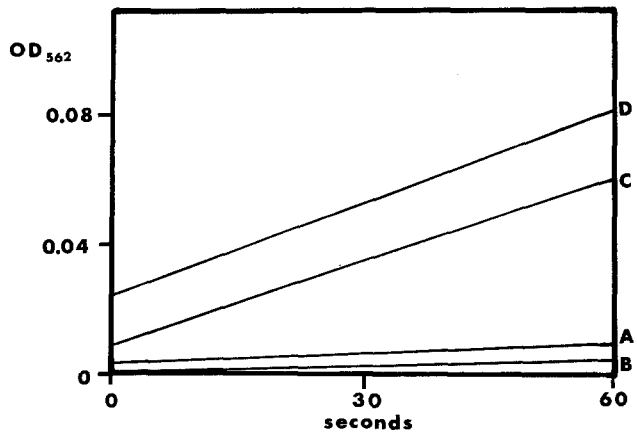

Fig. 1. Ferric citrate activity in cell-free extracts of *Neisseria gonorrhoeae*. Assay conditions were as described in Materials and Methods. (A) Assay conducted with dialyzed cell extracts without NADH; (B) assay conducted with non-dialyzed cell extract without NADH; (C) assay conducted with dialyzed cell extract and NADH; (D) assay conducted with non-dialyzed cell extract with NADH

Table 1. Effect of cofactor and electron donors on the activity of gonococcal ferric reductase

Addition	Relative activity
NADH	100
NADH + FAD	300
NADH + FMN	678
NADH + Mg^{2+}	155
NADH + FMN + Mg^{2+}	1126
NADPH + FMN + Mg^{2+}	813
FMN + Mg^{2+} + Cysteine	nd
FMN + Mg^{2+} + GSH	nd
FMN + Mg^{2+} + succinate	nd

nd = not detected

Enzyme localization

The localization of ferric reductase activity within *N. gonorrhoeae* strain FA421 was determined by comparing the distribution of ferric reductase with that of glucose-6-phosphate dehydrogenase and the β-lactamase. Enzyme activities were measured in the four fractions obtained during spheroplast formation and lysis. The results (Table 2) indicated that the majority of the ferric reductase activity (70%) was found in the cytoplasm-containing fraction (fraction II-S); 26% of the activity was found in association with the membrane-containing fraction (fraction II-P). However, at least half of this activity could be solubilized by washing the particulate fraction with buffer, suggesting that a portion of the enzyme activity was loosely associated with the cell membranes. A small but consistent amount of enzyme activity (3%) was associated with the periplasmic fraction (fraction I-S). The observation that a low level of glucose-6-phosphate dehydrogenase activity was also present in the periplasmic fraction suggested that the ferric reductase present in this fraction may be due to cell lysis. β-Lactamase activity was distributed between the periplasm (15%) and the cytoplasmic cell fraction (84%).

Table 2. Distribution of ferric reductase activity in the different fractions of *Neisseria gonorrhoeae* obtained after spheroplast formation

Fraction	Activity (% total) of		
	ferric reductase	glucose-6-phosphate dehydrogenase	β-lactamase
I-S	3	1	15
I-P	1	nd	nd
II-S	70	98	84
II-P	26	1	1
Total	109	2031	2422

Enzyme activities were measured as described in Materials and Methods. The activity for each fraction is expressed as percentage of the total activity. nd = not detected

Purification and molecular mass determination

Preliminary experiments revealed two peaks of ferric reductase activity following Sephacryl S-300 column chromatography. The addition of 10 μM ferric citrate to the elution buffer resulted in a single peak of activity (Fig. 2). Therefore, 10 μM ferric citrate was added to buffers used during all purification steps to prevent a loss of ferric reductase activity and to stabilize the enzyme.

The data in Table 3 indicate that a combination of ammonium sulfate precipitation, gel exclusion and ion-exchange chromatography resulted in a 110-fold purification of the ferric reductase with a recovery of 19% of the total activity.

The molecular mass of the ferric reductase was estimated to be 25 kDa by gel exclusion chromatography on Sephacryl S-300. In order to confirm this value, a portion of the partially purified enzyme preparation was subjected to polyacrylamide gel electrophoresis under non-denaturing conditions and the gel stained for ferric reductase activity as described in Materials and Methods. The band containing the ferric reductase activity was eluted from the gel and subjected to SDS/polyacrylamide gel electrophoresis. After staining with silver, a major band of 25 kDa and two minor bands of 32 kDa and 39 kDa were observed (data not shown).

Discussion

Gonococcal ferric reductase is a constitutive oxygen-insensitive enzyme that is located primarily in the cytoplasm of the cell. NADH is the preferred reductant and activity is enhanced by flavin compounds (e.g. FMN)

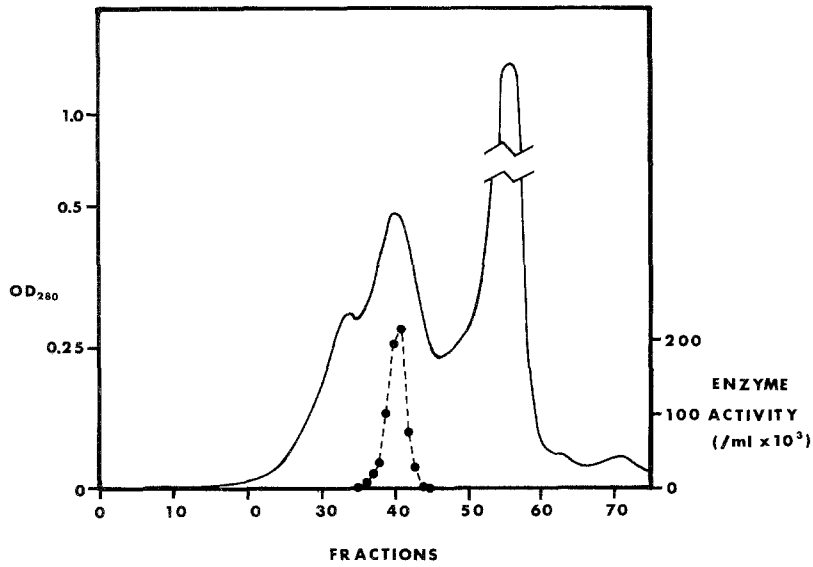

Fig. 2. Partial purification of gonococcal ferric reductase by column chromatography (Sephacryl S-300). The addition of 10 μM ferric citrate to the elution buffer resulted in a single peak of activity

Table 3. Partial purification of gonococcal ferric reductase

Step	Activity (U/ml)	Proteins (mg/ml)	Specific activity (U/mg)	Recovery (%)
Crude extract	42.4	6.05	7	100
Soluble extract[a]	18.7	3.10	6	85
After $(NH_4)_2SO_4$ precipitation	39.1	4.44	9	61
Sephacryl S300	12.2	0.27	45	35
DEAE-Sephacel	6.9	0.01	767	19

One unit (U) of ferric reductase is the amount catalyzing the reduction of 1 μmol $Fe^{3+} \times min^{-1}$

[a] After centrifugation at 48000 × *g* for 60 min

and Mg^{2+}. The gonococcal enzyme has a broad substrate specificity and is even able to reduce solubilized ferric hydroxide (Imferon).

Two types of ferric reductase have been described in bacteria. One type consists of membrane-bound enzyme such as those described in *Spirillum itersonii* (Dailey and Lascelles 1977) and *Staphylococcus aureus* (Lascelles and Burke 1976). The other type consists of cytoplasmic enzymes such as those described in *Pseudomonas aeruginosa* (Cox 1980), *P. fluorescens* (Halle and Meyer 1989), *Bacillus megaterium* (Arceneaux and Byers 1980), *Bacillus subtilis* (Gaines et al. 1981), *Mycobacterium smegmatis* (Brown and Ratledge 1975; McReady and Ratledge 1977), *Agrobacterium tumefaciens* (Lodge et al. 1982), *Rhodopseudomonas sphaeroides* (Moody and Dailey 1985). However about ca. 25% of the activity of these enzymes is often found in association with the membrane fraction. The preferred reductant for most of these enzymes is either NADH or NADPH: the ferric reductase from *S. aureus* utilizes lactate as a reductant, whereas the enzyme from *S. itersonii* utilizes either NADH or succinate. In these latter two organisms Fe^{3+} reduction is linked to the respiratory chain.

Gonococcal ferric reductase activity was enhanced by FMN (and a lesser extent by FAD) and by divalent cations such as Mg^{2+}. FMN may be essential for enzyme activity. Thus, the cofactor requirements of the gonococcal ferric reductase resemble those of previously described enzymes. In addition, the K_m of the gonococcal enzyme for ferric citrate (10 mM) is similar to the K_m values of 8.3 μM for the *R. sphaeroides* enzyme (Moody and Dailey 1985), 10 μM for the *A. vinelandii* enzyme (Huyer and Page 1989) and 12 μM for *S. itersonii* enzyme (Dailey and Lascelles 1977).

The gonococcal enzyme appeared to be partially membrane-associated as has been described for the enzymes from *B. subtilis* (Gaines et al. 1981) and from *Pseudomonas* (Cox 1980; Halle and Meyer 1989). However the existence of a unique membrane-associated activity is unclear as the enzyme activity in crude extracts of *N. gonorrhoeae* was not affected by inhibitors such as cyanide, azide and HQNO, and a substantial portion of the membrane-associated activity was removed by washing. Further studies will be necessary to elucidate this point.

A small percentage of the ferric reductase activity was found in the periplasm of *N. gonorrhoeae*. Cox (1981) observed a similar phenomenon with *P. aeruginosa*. However, the fact that we detected glucose-6-phosphate dehydrogenase activity in the periplasmic fraction suggests that the presence of ferric reductase may be an artifact due to the cell fractionation technique.

Ferric reductase activities are generally evident under anaerobic conditions and oxygen-insensitive enzymes have only been infrequently observed (Cox 1980; Halle and Meyer 1989; Moody and Dailey 1985). Ferric reductases have been mainly described as ferrisiderophore reductases. Multiple ferric reductase activities have been identified in several bacterial species. In *B. megaterium* two different ferrisiderophore reductases have been identified (Arceneaux and Byers 1980); while in *B. subtilis*, the membrane-associated ferric re-

ductase activity was related to a dichloroindophenol reductase (Gaines et al. 1981). In *P. aeruginosa,* the ferric citrate reductase and the ferrisiderophore reductase could be chromographically separated (Cox 1980). Two ferric citrate reductases with different physicochemical properties and molecular masses of 32 kDa and 41 kDa have been identified in *R. sphaeroides* (Moddy and Dailey 1985). The ferripyoverdine reductase from *P. fluorescens* has been shown to reduce ferric citrate under either aerobic or anaerobic conditions; however, ferripyoverdine was reduced only under anaerobic conditions and after a lag of 10 min (Halle and Meyer 1989). Without further characterization, it is difficult to determine whether one or more enzymes are present in *N. gonorrhoeae*. Gonococci are able to use ferri-aerobactin as a source of iron for growth (West and Sparling 1987). It would be interesting to determine whether gonococcal cell extracts are able to reduce the Fe^{3+} bound to aerobactin and to compare this activity to the oxygen-insensitive gonococcal ferric citrate reductase.

Data obtained by gel filtration column chromatography suggested that molecular mass of the gonococcal ferric reductase was about 25 kDa. This was similar to the molecular mass of the major band observed on SDS/polyacrylamide gels. The presence of three bands on gels suggested that the enzyme may consist of subunits. However, this was unlikely because of the great difference in staining intensity between the major and the two minor bands. Thus, the two minor bands were probably contaminants. Further studies are necessary to obtain a more precise value.

N. gonorrhoeae possesses specific outer-membrane receptors for human transferrin and lactoferrin (Lee and Schryvers 1988). Transferrin has little affinity for Fe^{3+} (Kojima and Bates 1979). Thus, a membrane-associated or periplasmic ferric reductase could be involved in the removal of Fe^{3+} from transferrin. A reductive mechanism for the removal of transferrin-bound iron by *Listeria monocytogenes* has been proposed (Cowart and Foster 1985). However, gonococcal ferric reductase was unable to reduce the transferrin-bound iron in vitro. Gonococci also possess a periplasmic iron-binding protein (Fbp) that is thought to be involved in the transport of Fe^{3+} (Mietzner et al. 1987; Berish et al. 1990). The gonococcal ferric reductase was also unable to reduce the Fe^{3+} bound by Fbp. It is possible that the proper conditions were not used or that intermediate steps may be involved before the Fe^{3+} is reduced. Fe^{3+} reduction could take place before the iron enters the cell although our results suggest that this reduction occurs in the cytoplasm.

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