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Reduction of Ferric Iron by L-Lactate and DL-Glycerol-3-Phosphate in Membrane Preparations from *Staphylococcus aureus* and Interactions with the Nitrate Reductase System

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Membrane fractions with L-lactate dehydrogenase, *sn*-glycerol-3-phosphate (G3P) dehydrogenase, and nitrate reductase activities were prepared from *Staphylococcus aureus* wild-type and *hem* mutant strains. These preparations reduced ferric to ferrous iron with L-lactate or G3P as the source of reductant, using ferrozine to trap the ferrous iron. Reduction of ferric iron was insensitive to 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) with either L-lactate or G3P as reductant, but oxalate and dicumarol inhibited reduction with L-lactate as substrate. The membranes had L-lactate- and G3P-nitrate reductase activities, which were inhibited by azide and by HQNO. Reduction of ferric iron under anaerobic conditions was inhibited by nitrate with preparations from the wild-type strain. This effect of nitrate was abolished by blocking electron transport to the nitrate reductase system with azide or HQNO. Nitrate did not inhibit reduction of ferric iron in heme-depleted membranes from the *hem* mutant unless hemin was added to restore L-lactate- and G3P-nitrate reductase activity. We conclude that reduced components of the electron transport chain that precede cytochrome *b* serve as the source of reductant for ferric iron and that these components are oxidized preferentially by a functional nitrate reductase system.

The acquisition of ferric iron from the environment by aerobes and facultative anaerobes is a fertile field of study, but little is known about the subsequent processing of the iron for incorporation into hemes and iron-containing proteins (13). Reduction of ferric iron to the ferrous state may be important in such processing, and we have previously investigated this problem in relation to the synthesis of protoheme in *Spirillum itersonii* (7). Succinate and reduced nicotinamide adenine dinucleotide were effective sources of reductant, and the observations suggested that reduction of ferric iron could occur at several sites on the respiratory chain. The reduction of ferric iron was inhibited by oxygen; this was attributed to preferential diversion of reduced components of the respiratory chain into the terminal oxidase system when oxygen was available as the ultimate acceptor.

We have now extended this work to *Staphylococcus aureus*, which can grow fermentatively or by the respiratory mode with nitrate or oxygen as terminal (electron) acceptor. This paper concerns the reduction of ferric iron by membrane preparations from *S. aureus* with L-lactate and glycerol-3-phosphate (G3P) as reductants and gives particular attention to interactions with the nitrate reductase system. The membranes used for the work were taken from cells grown and induced under conditions that gave preparations with high levels of nitrate reductase in addition to the pyridine nucleotide-independent L-lactate and G3P dehydrogenases.

MATERIALS AND METHODS

Organisms and growth conditions. The wild-type strain of *S. aureus*, SG 511 A, and the *hem* mutant H-14 have been described in previous work (4, 5, 11). The cells were grown aerobically in semidefined basal (SB) medium (11) with 20 mM glycerol and supplemented with 0.2 μM hemin for mutant H-14.

Induction of nitrate reductase in cell suspensions. Cells were harvested from the glycerol medium in the early stationary phase and suspended to an absorbance at 540 nm between 2 and 3 in SB medium with 25 mM glucose, 10 mM pyruvate, 0.2 mM uracil, and 8 mM KNO_3. The suspensions were incubated at 37°C in flasks filled to the neck for a total period of 2 h; sodium azide (0.1 mM) was added to suspensions of the wild-type strain after 30 min. The addition of azide increases by three- to fivefold the level of nitrate reductase attained during induction of the wild type, but it does not increase the enzyme activity developed by mutant H-14 (unpublished data).

Preparation and lysis of spheroplasts. After induction, cells were centrifuged, washed in 40 mM potassium phosphate buffer (pH 7.5), and suspended in 40 mM potassium phosphate buffer (pH 7.5) con-

taining 25% (wt/vol) NaCl; the suspension contained approximately 10 mg of protein per ml (absorbance at 540 nm, 40). Lysostaphin and lysozyme were added to respective final concentrations of 25 and 250 μg/ml, and the preparations were incubated for 30 min at 37°C; within this period, at least 90% of the cells were converted to spheroplasts. The mixtures were chilled, and subsequent operations took place at 0 to 5°C. The preparations were centrifuged at 7,000 × *g* for 20 min, and the pellet was suspended with gentle homogenization in 10 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.5) containing 4 mM $MgCl_2$ with the addition of deoxyribonuclease and ribonuclease (1 μg of each per ml). The homogenates were kept for 1 to 2 h at 0°C to allow complete lysis and were then centrifuged for 5 min at 1,000 × *g* to remove large debris. The lysate was centrifuged for 1 h at 60,000 × *g*, the supernatant was removed, and the pellet was homogenized in one-half the equivalent volume of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 20% glycerol (vol/vol). These preparations were designated as "induced membranes" and contained 3 to 5 mg of protein per ml. They were used for experiments within 24 h; storage at 0°C or in the freezer led to loss of activity in systems linked to L-lactate dehydrogenase, but those linked to G3P dehydrogenase were more stable.

Assay of enzyme activities. The reduction of ferric iron to ferrous iron, termed "iron reductase," was measured with ferrozine as the trap for ferrous iron (7). Two methods were used. In the spectrophotometric method, cuvettes contained in a 1-ml final volume: potassium phosphate buffer (pH 7.5), 28 μmol; ferrozine, 0.4 μmol; ferric citrate, 0.12 μmol; and membranes, 0.5 to 2 mg of protein. The reaction was started by the addition of substrate (10 μmol of L-lactate or 20 μmol of G3P), and measurement was made at 562 nm at room temperature in the Cary model 14R spectrophotometer against a reference containing the complete system without substrate. It was not necessary to use anaerobic conditions; in open cuvettes, linear rates were established in less than 1 min after the addition of substrate. Iron reductase was also measured under anaerobic conditions in Warburg vessels with apertures closed with serum caps. The standard reaction mixture was identical with that for the spectrophotometric assay, and the reaction was started by the addition of substrate from the side arm, after the vessels were evacuated and filled with argon. Incubation was at 37°C, usually for 30 min. Reactions were terminated by the addition of 2 ml of water with chilling in an ice bath, and the absorbance at 562 nm was measured in a Zeiss PMQII spectrophotometer against a control without substrate. Results are given in nanomoles of ferrous iron, calculated from the molar extinction coefficient for the ferrous chelate of 28,000 (6).

G3P dehydrogenase activity was assayed spectrophotometrically with phenazine methosulfate-dichloroindophenol as the acceptor system and with lauryl dimethyl amine oxide present (11). A similar assay was used for L-lactate dehydrogenase, except for the omission of detergent and the use of 10 mM L-lactate as substrate. The method used for the assay of nitrate reductase activity was modified slightly from that described previously (4); reduced methyl viologen was used as the electron donor instead of reduced benzyl viologen, and the reaction was terminated by the addition of water instead of the zinc acetate-ethanol mixture. L-Lactate- and G3P-nitrate reductase activities were measured as before (4) except that the reactions were terminated by the addition of water.

Determinations. Protein was estimated by the Folin method (12) with bovine serum albumin as standard. Nitrite was determined colorimetrically by the Griess reaction (16).

Nonheme iron was estimated in membrane preparations with 4,7-diphenyl-1,10-phenanthroline (bathophenanthroline) as described by Brumby and Massey (3), except that samples were treated with 5% (wt/vol) trichloroacetic acid before the addition of dithionite and other reagents (9).

Materials. Lithium L-lactate, sodium DL-glycerol-3-phosphate, and the various reagents and inhibitors were from Sigma Chemical Co. (St. Louis, Mo.). Stock solutions (1 to 5 mM) of 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) and dicumarol were in ethanol and 0.1 M $NaHCO_3$, respectively. Stock solutions of hemin (Porphyrin Products, Logan, Utah) were in 0.01 N NaOH in 50% ethanol.

RESULTS

Enzyme activities in membranes from wild-type *S. aureus* and effects of inhibitors. The membranes were prepared from cells grown aerobically with glycerol and were then induced to form nitrate reductase in concentrated cell suspension. The preparations had high levels of L-lactate dehydrogenase (696), G3P dehydrogenase (1,318), and nitrate reductase (2,157), and they also had L-lactate- and G3P-nitrate reductase activities of 320 and 87, respectively. The effect of various inhibitors upon the latter activities was examined (Table 1). Reduction of nitrate with either L-lactate or G3P was strongly inhibited by HQNO, indicating the participation of cytochrome *b*. Azide, a competitive inhibitor of nitrate reductase (14, 17), also inhibited the linked systems. The L-lactate–nitrate reductase system was extremely sensitive to dicumarol, suggesting that vitamin K_2 is an obligatory component. In contrast, the G3P-nitrate reductase activity was only slightly inhibited by dicumarol. Oxalate is a competitive inhibitor of the L-lactate dehydrogenase in *S. aureus* (15); as expected, it inhibited the L-lactate–nitrate-reductase system but not the system with G3P as electron donor.

Reduction of ferric iron and the effect of inhibitors. The induced membranes reduced ferric iron at a linear rate for at least 40 min when incubated anaerobically with L-lactate or G3P as the source of reductant (Fig. 1); no activity was detectable in the absence of reductant. Reduced nicotinamide adenine dinucleotide (but not reduced nicotinamide adenine di-

TABLE 1. *Effect of inhibitors on enzyme activities in membranes from wild-type S. aureus*[a]

Inhibitor	Enzyme activity[b]			
	Nitrate reductase		Iron reductase	
	G3P	L-Lactate	G3P	L-Lactate
None (control)	100	100	100	100
HQNO (10 μM)	<1	<1	108	105
Dicumarol (4 μM)	96	24	100	75
Dicumarol (40 μM)	84	4	92	42
Sodium oxalate (25 mM)	—[c]	—[c]	100	19
Sodium azide (10 mM)	3	2	100	100

[a] The assays were performed with induced membranes prepared as described in the text; the final concentration of protein in each assay system was approximately 0.5 mg/ml.

[b] Results are given as percentage of the control without inhibitor. The control values (in nanomoles per minute per milliliter of reaction mixture) were: G3P-nitrate reductase, 84; L-lactate-nitrate reductase, 133; G3P-iron reductase, 0.75; L-lactate-iron reductase, 0.86. The iron reductase activities were determined by spectrophotometric assay.

[c] —, Not tested.

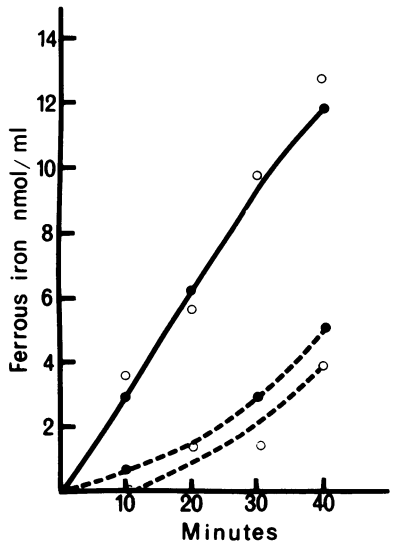

FIG. 1. *Effect of aeration on iron reductase activity in induced membranes from wild-type S. aureus. Membranes (0.5 mg of protein) were in 1 ml of standard reaction mixture with 10 mM* L-*lactate* (○) *or 20 mM G3P* (●). *Incubation was at 37°C under argon* (——) *or in air with shaking at 150 rpm* (---).

nucleotide phosphate) was also an effective reductant. Iron reductase activity was severely inhibited by aeration, achieved by conducting the reaction in shaken tubes (Fig. 1).

Linear rates of iron reductase activity were also observed with the spectrophotometric assay, and this method was used to examine the effect of inhibitors (Table 1). With L-lactate as the source of reductant, iron reductase activity was inhibited by dicumarol and by oxalate but was unaffected by HQNO or by azide. The system with G3P was insensitive to all of the inhibitors tested.

Effect of nitrate upon iron reductase. The rate of iron reductase activity with L-lactate as substrate was decreased by 50% or more when nitrate was added to the reaction mixture (Fig. 2a, line D). This effect of nitrate was overcome by the addition of azide, an inhibitor of nitrate reductase (Fig. 2a, line C). These observations suggested that the nitrate reductase system competed effectively with iron reductase for electrons from the electron transfer chain. This possibility was supported by experiments with azide and HQNO, in which the reduction of nitrate and ferric iron was monitored (Table 2). Reduction of ferric iron with L-lactate and G3P was enhanced by the inhibitors, whereas electron transport to the nitrate reductase system was blocked.

Iron reductase activity in membranes from mutant H-14. Mutant H-14 was grown and induced under conditions that provided membranes deficient in hemes but with L-lactate dehydrogenase, G3P dehydrogenase, and nitrate reductase activities. Such preparations did not couple L-lactate or G3P with nitrate reductase unless hemin was added (Table 3). However, iron reductase activity was observed; it was not affected by hemin (Table 3). Nitrate did not

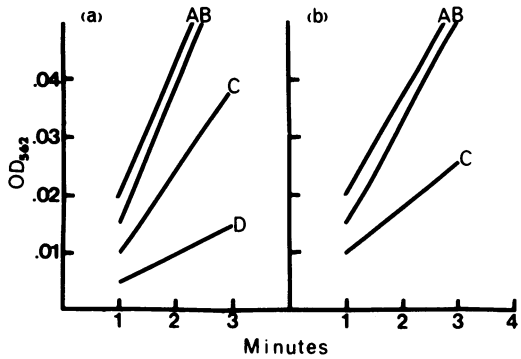

FIG. 2. *(a) Effect of nitrate and azide on rate of iron reductase by induced membranes from the wild-type strain. The spectrophotometric assay was used with membranes (0.5 mg of protein) in a final volume of 1 ml of standard reaction mixture with 10 mM* L-*lactate. (A) control; (B) sodium azide, 20 mM; (C) sodium azide, 10 mM, and KNO_3, 20 mM; (D) KNO_3, 20 mM. (b) Effect of nitrate and hemin on rate of iron reductase by induced membranes from mutant H-14. The details are as in (a), with cuvettes containing 1.1 mg of protein per ml. (A) control; (B) KNO_3, 20 mM; (C) KNO_3, 20 mM, and hemin, 2 μM.*

TABLE 2. *Iron reductase activity in the presence of nitrate and effect of inhibitors of nitrate reductase*[a]

Substrate	Inhibitor	Product formed (nmol/ml)	
		Ferrous iron	Nitrite
L-Lactate (10 mM)	None	8.2	5,618
	HQNO (10 μM)	13.2	389
	Sodium azide (4 mM)	18.2	536
G3P (20 mM)	None	5.0	2,258
	HQNO (10 μM)	11.1	168
	Sodium azide (4 mM)	10.0	116

[a] Induced membranes from the wild-type strain (0.9 mg of protein) were incubated for 30 min under argon in the standard reaction mixture containing 20 mM KNO_3, with substrates and inhibitors as shown. Results are given as nanomoles of product formed in 30 min.

TABLE 3. *Effect of hemin on iron reductase and nitrate reductase in membranes from mutant H-14*[a]

Substrate	Additions		Product formed (nmol/ml)	
	Hemin (μM)	Nitrate (mM)	Ferrous iron	Nitrite
L-Lactate (10 mM)	0	0	5.4	ND[b]
	2	0	5.2	ND
	0	20	5.0	200
	2	20	3.9	2,135
G3P (20 mM)	0	0	7.6	ND
	2	0	7.4	ND
	0	20	7.5	116
	2	20	6.1	1,386

[a] Induced membranes (1.1 mg of protein) were incubated for 20 min under argon with additions as shown. Results are given as nanomoles of product formed in 20 min.
[b] ND, Not determined.

affect the rate of iron reductase in the heme-depleted membranes, but it was inhibitory in systems supplemented with hemin (Fig. 2b, Table 3). The observations with the mutant suggest that the cytochrome *b*-nitrate reductase system interferes with iron reductase by withdrawing electrons derived from dehydrogenation of L-lactate and G3P.

Nonheme iron and substrate-reducible iron. Induced membranes from the wild-type strain were analyzed for nonheme iron, with dithionite as reductant and bathophenanthroline as trap. The membranes were treated with trichloroacetic acid before the addition of the reagents. Such treatment was necessary to obtain maximum values, as found previously with membranes from *Mycobacterium phlei* (9). A mean value of 24 nmol of nonheme iron per mg of membrane protein (range, 17.5 to .31) was obtained with four preparations from *S. aureus*.

The endogenous nonheme iron in the membranes could contribute to measurements of iron reductase activity, provided that the ferrous form was accessible to ferrozine. This was examined by incubation of membranes under the standard conditions for assay of iron reductase but without the addition of ferric citrate; incubation was continued until there was no further increase in absorbance at 562 nm (1 to 2 h). Substrate-dependent reduction of endogenous iron was observed, as measured by formation of the ferrozine complex (Table 4). The amount of such iron was approximately 15 to 25% of the total nonheme iron.

DISCUSSION

This work has shown that membrane preparations from *S. aureus* reduce ferric iron with reductant derived by dehydrogenation of L-lactate and G3P. Under anaerobic conditions, ferric iron apparently interacts with a reduced component(s) of the electron transfer chain that precedes cytochrome *b*. Thus, iron reductase activity is not inhibited by HQNO at concentrations that abolish electron transfer between L-lactate and G3P dehydrogenases and nitrate reductase. Also, heme-depleted membranes from mutant H-14 reduce ferric iron in the absence of hemin, in contrast to their capacity to link the dehydrogenases with nitrate reductase.

Iron reductase activity with L-lactate as substrate is inhibited by oxalate, a competitive inhibitor of the L-lactate dehydrogenase (15). The L-lactate-dependent activity is also inhibited by dicumarol, suggesting that vitamin K_2 is involved in the transfer of reductant to ferric iron. Dicumarol had little or no effect at similar con-

TABLE 4. *Endogenous iron and iron reductase activity in membranes from wild-type strain*[a]

Additions	Ferrous iron formed (nmol)
None	<1
L-Lactate (10 μmol)	6.1
G3P (20 μmol)	9.3
L-Lactate (10 μmol) and ferric citrate (0.12 μmol)	37.8
G3P (20 μmol) and ferric citrate (0.12 μmol)	35.7

[a] Replicate samples of induced membranes containing 45.6 nmol of nonheme iron and 1.6 mg of protein were in cuvettes containing (in 1-ml final volume): potassium phosphate buffer (pH 7.5), 28 μmol; ferrozine, 0.4 μmol; and additions as shown. Incubation was for 2 h at 25°C under argon.

centrations upon G3P-dependent iron reductase or nitrate reductase. Possibly, vitamin K_2 may not intervene in the systems linked to G3P dehydrogenase, or these systems may not be accessible to the inhibitor. This problem requires further investigation.

Interaction of ferric iron with the respiratory chain may occur by some type of exchange with forms of nonheme iron. Such forms include a variety of iron sulfur proteins, which are abundant in bacterial membranes (1). In preparations from other bacteria, including *Mycobacterium phlei* (10) and *Escherichia coli* (8), only a small fraction of the total nonheme iron is reducible by respiratory substrates such as succinate and reduced nicotinamide adenine dinucleotide. We made similar observations with the membranes from *S. aureus*; approximately 20% of the total nonheme iron was reducible by L-lactate or by G3P. In assessing the role of nonheme iron, consideration should be given to the type of trap used for the ferrous iron. The ferrozine reagent used in this work is hydrophilic and is unlikely to penetrate deeply into the interior regions of the membrane. The ferrous iron measured under our conditions is apparently released close to the membrane surface.

The inhibitory effect of nitrate upon iron reductase activity is attributed to preferential oxidation of reduced components of the respiratory chain by the cytochrome *b*-nitrate reductase system. Evidence to support this interpretation is based upon reversal of the inhibition by blocking electron transfer to the nitrate reductase system. With preparations from the wild-type strain, this effect was achieved with HQNO and azide, whereas starvation for heme was effective with preparations from the mutant strain. Another possibility is that nitrite, the product of an active nitrate reductase, may inhibit the reduction of ferric iron. Our observations do not exclude this possibility.

Preferential withdrawal of reductant into terminal oxidation systems might be significant in regulating the synthesis of cell components that require a source of ferrous iron. We have previously suggested that the provision of ferrous iron for protoheme synthesis may be a critical factor in the repression of cytochrome formation by high aeration (7). The reduction of ferric iron may also be critical in the formation of certain enzymes containing nonheme iron. These include nitrate reductase and nitrogenase, both of which are repressed by aeration (2, 14, 17).

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