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Nitrate Reductase Activity in Heme-Deficient Mutants of *Staphylococcus aureus*

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Mutants H-14 and H-18 of *Staphylococcus aureus* require hemin for growth on glycerol and other nonfermentable substrates. H-14 also responds to δ-aminolevulinate. Heme-deficient cells grown in the presence of nitrate do not have lactate-nitrate reductase activity but gain this activity when incubated with hemin in buffer and glucose. Lactate-nitrate reductase activity is also restored to the membrane fraction from such cells by incubation with hemin and dithiothreitol; addition of adenosine 5'-triphosphate has no effect upon the restoration. Cells grown with nitrate in the absence of hemin have two to five times more reduced benzyl viologen-nitrate reductase activity than do those grown with hemin. The activity increases throughout the growth period in the absence of hemin, but with hemin present enzyme formation ceases before the end of growth. There was no evidence of enzyme destruction. The distribution of nitrate reductase activity between membrane and cytoplasm was similar in cells grown with and without hemin; 70 to 90% was in the cytoplasm. It is concluded that heme-deficient staphylococci form apo-cytochrome *b*, which readily combines in vitro with its prosthetic group to restore normal function. The availability of the heme prosthetic group influences the formation of nitrate reductase.

Staphylococcus aureus has a respiratory nitrate reductase system which uses L-lactate as hydrogen donor; studies with inhibitors and with mutants indicate the participation of a *b*-type cytochrome (3). The overall activity with lactate as donor is located in the membrane fraction of lysed cells, but high levels of nitrate reductase are found in the cytoplasm by assay with reduced benzyl viologen (BVH). Nitrate reductase from *Escherichia coli* has been purified to homogeneity and shown to be molybdenum enzyme (17). It is closely associated with cytochrome b_1 in the cytoplasmic membrane, and recently preparations of the enzyme containing the cytochrome component have been purified by agarose gel chromatography in the presence of Triton X-100 (6). Also, antibodies specific for the reductase precipitate cytochrome b_1 from solubilized membrane preparations (15, 16).

Work with heme-deficient mutants of *E. coli* has suggested that functional cytochrome is concerned in regulating the formation and/or assembly of nitrate reductase in the membrane (11, 15). Similar types of mutant of *S. aureus* are readily obtained by exploiting resistance to kanamycin (3, 20). Such mutants apparently form apo-cytochrome when grown without heme, since their ability to reduce nitrate with physiological hydrogen donors can be restored in vitro by addition of hemin (4). The present work concerns the conditions required for reconstruction of lactate-nitrate reductase activity in heme-deficient strains of *S. aurues* using intact cells and membrane preparations. We have also examined the effect of hemin on the development of nitrate reductase activity in growing cultures.

MATERIALS AND METHODS

Organisms and growth conditions. Mutants H-14 and H-18 were derived from the wild-type strain of *S. aureus* SG 511 A on the basis of resistance to kanamycin and have been described before (3). Stock cultures were maintained on chocolate agar. Cells for experiments were grown in semidefined medium containing casein hydrolysate (SB) and supplemented with 50 mM glucose, 10 mM sodium pyruvate, and 0.2 mM uracil (SB-Glu-PU) with other additions as noted in the text. Incubation was at 37 C in flasks filled to the neck but without rigorous exclusion of air. Inocula for bulk cultures were derived as described previously (3). Growth was measured by a Klett colorimeter with a red filter; 100 Klett units was equivalent to 0.21 mg of protein per ml.

Preparation of cell suspensions and cell fractions. Harvested cells were washed in 40 mM potassium phosphate buffer, pH 7.5, and suspended in the same buffer to a density of 5,000 Klett units. Crude lysates were prepared by osmotic lysis of spheroplasts as described previously (3). The membrane fraction was derived from the crude lysate by centrifuging for 1.5 h at 105,000 × *g*; the pellet was rinsed in 40 mM potassium phosphate buffer, pH 7.5, and suspended in this buffer to concentration of about 4 mg of protein per ml.

Measurement of nitrate reductase activity and respiration. Nitrate reductase activity was assayed as described previously (3). Activity with BVH as donor was measured in crude lysates or in cell fractions; activity with DL-lactate as donor was determined in washed cell suspensions or in membrane fractions. Results are expressed as nanomoles of nitrate formed per minute per milligram of protein.

Respiration was measured with a Rank oxygen electrode (Rank Bros., Bottisham, Cambs., England). The reaction vessel contained cells suspended to a final concentration of about 0.5 mg of protein per ml of 40 mM potassium phosphate buffer, pH 7.5, with DL-sodium lactate (final concentration, 20 mM) as substrate. Measurements were at 25 C, and results are expressed as nanomoles of oxygen consumed per minute per milligram of protein.

Spectrophotometric measurements. Oxidized (ferricyanide)-reduced (dithionite) spectra were made with a Cary spectrophotometer model 14R equipped with 0- to 0.1-absorbance slide wire.

Protein determinations. Protein was determined by the Folin method (14), with bovine serum albumin as standard.

Materials. Hemin and protoporphyrin were from Porphyrin Products (Logan, Utah). Stock solutions of hemin (2 mM) were in 0.02 N NaOH in 50% ethanol; protoporphyrin was dissolved in a minimal volume of glacial acetic acid to give a concentration of about 2 mM. The pyrroles were diluted in sterile water immediately before addition to cultures. δ-Aminolevulinic acid and dithiothreitol were from the Sigma Chemical Co.

RESULTS

Growth characteristics. Mutants H-14 and H-18 grew on SB-Glu-PU without hemin; the growth yields were not affected by aeration and were similar to the wild type grown anaerobically. Neither mutant grew on SB with glycerol or mannitol as energy source unless hemin was added. Both responded to low concentrations, and maximum growth rates were attained with 0.5 μM hemin (Fig. 1). Mutant H-14 also responded to δ-aminolevulinate (δ-ALA) but at concentrations of 100 to 1,000 times that of hemin (Fig. 1). Protoporphyrin at a final concentration of 4 μM was ineffective with both mutants.

Restoration of respiratory activity to deficient cells. Cell suspensions of the mutants grown on SB-Glu-PU with addition of nitrate did not respire and lacked lactate-nitrate reductase activity (Table 1). Both activities were restored by incubation of the cells in buffer with hemin and glucose in the presence of chloramphenicol. Glucose was needed for maximum activity, but omission of chloramphenicol was without effect. Table 1 shows data with H-18; similar results were obtained with H-14.

Restoration of lactate-nitrate reductase activity to deficient membranes. Membrane fractions from cells grown in SB-Glu-PU with

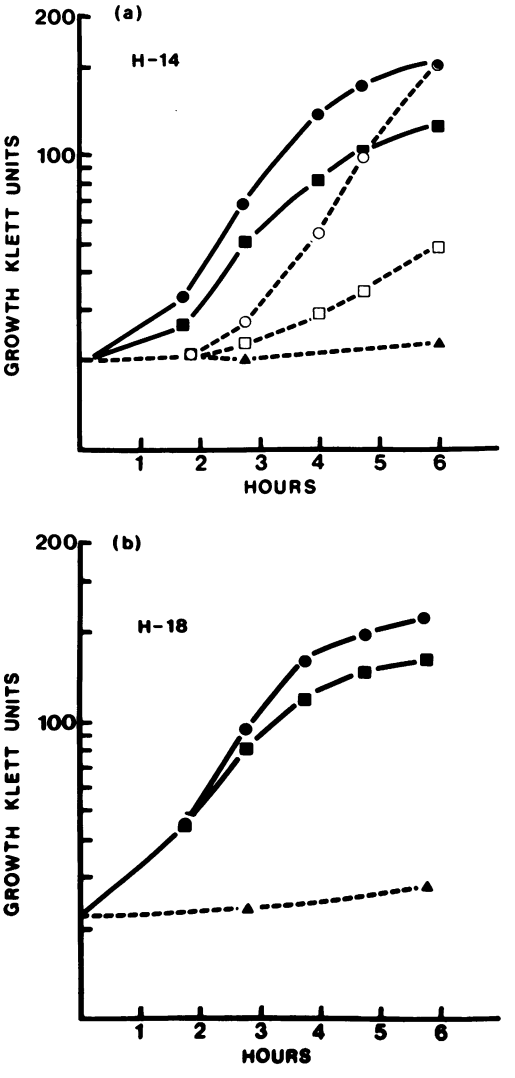

FIG. 1. *Response of mutants to hemin and δ-ALA. Cultures were incubated with aeration in SB medium with 20 mM glycerol without supplement (▲), with hemin (solid lines), or with δ-ALA (broken lines). (a) Mutant H-14 with hemin at final concentrations of 0.1 and 0.5 μM (■, ●); ALA at final concentrations of 20 and 200 μM (□, ○). (b) Mutant H-18 with hemin at final concentrations of 0.1 and 0.5 μM (■, ●).*

nitrate had BVH-nitrate reductase activity, but they did not use lactate as hydrogen donor (Tables 2 and 3). Activity with lactate was restored by incubation of the membranes in buffer with hemin alone. Dithiothreitol stimulated the activity by degrees which varied from preparation to preparation. Addition of adenosine 5'-triphosphate with or without Mg^{2+} did not affect the restoration with membranes from either mutant. The results shown in Table 2 for prepa-

rations from H-18 are similar to those obtained with membranes from H-14.

The reconstructed activity was inhibited by dicoumarol and 2-heptyl-4-hydroxy-quinoline-*N*-oxide at concentrations which inhibited lactate-nitrate reductase activity in membranes from the wild-type organism (3). The reduced-minus-oxidized difference spectrum of the membranes treated with hemin showed maxima at 428 and 558 nm, characteristic of *b*-type cytochromes (Fig. 2).

TABLE 1. *Restoration of lactate-nitrate reductase activity and respiration to heme-deficient cells of H-18*[a]

Pretreatment		Lactate-nitrate reductase activity (nmol of nitrate/mg of protein/min)	Respiration (nmol of oxygen/mg of protein/min)
Heme (μM)	Glucose (mM)		
0	0	0.9	ND[b]
0	10	0.9	ND
0.2	0	2.8	ND
0.2	10	6.4	60
1.0	0	19	16
1.0	10	52	92

[a] Cells were harvested in stationary phase after growth in SB-Glu-PU with 20 mM KNO_3, and the washed cells were suspended to a density of 250 Klett units in 40 mM potassium phosphate buffer, pH 7.5, with 0.2 mM chloramphenicol. Samples were pretreated by incubation for 10 min at 37 C with addition of hemin and glucose as shown (final concentration). After pretreatment the cells were centrifuged, washed once, and suspended in 40 mM potassium phosphate buffer to a density of 5,000 Klett units for determination of nitrate reductase and respiratory activity.

[b] ND, Not detectable (less than 7).

Effect of hemin on the development of nitrate reductase activity. High BVH-nitrate reductase activity was found in the mutants when grown without hemin (Table 3). This high activity was apparent in both the cytoplasmic and membrane fractions derived from the crude lysates. Cells grown with hemin had lactate-nitrate reductase activity but considerably less BVH-nitrate reductase activity than the heme-deficient cells (Table 3). The distribution of this

TABLE 2. *Restoration of lactate-nitrate reductase activity to heme-deficient membranes of H-18*[a]

Heme (μM)	Dithiothreitol (mM)	Mg-ATP[b]	Lactate-nitrate reductase activity (nmol of nitrite formed/mg of protein/min)
0	0.3	−	2.7
0.1	0.3	−	143
0.4	0.3	−	181
1.0	0.3	−	194
1.0	0	−	104
1.0	0.3	+	192

[a] The membrane fraction was prepared from cells grown as in Table 1. Samples containing 0.15 mg of protein were incubated at 37 C in a final volume of 1 ml in 40 mM potassium phosphate buffer, pH 7.5, with additions at the final concentrations shown. After 5 min, sodium DL-lactate and KNO_3 (20 mM each, final concentration) were added, and incubation was continued for 10 min longer. The reaction was terminated with zinc acetate-ethanol, and the nitrite formed was determined by standard procedures (3). The BVH-nitrate reductase activity of the membranes was 2,000 nmol of nitrite/mg of protein per min.

[b] 10 mM $MgCl_2$ and 5 mM adenosine 5'-triphosphate (ATP).

TABLE 3. *Nitrate reductase activity in cells grown with and without hemin*[a]

Strain	Hemin in growth medium (μM)	Nitrate reductase activity (nmol of nitrite/mg of protein/min)			
		Lactate	BVH		
			Crude lysate	Cytoplasm	Membrane
H-14	0	Less than 10	2,300	ND[b]	ND
	0.5	150	640	ND	ND
H-18	0	Less than 10	1,240 (8,000)	1,490 (6,687)	4,790 (1,386)
	0.5	75	150 (1,185)	120 (840)	390 (293)

[a] Cells were harvested in the stationary phase after growth in SB-Glu-PU with 20 mM KNO_3 and supplemented with hemin as shown. Nitrate reductase activity was assayed with lactate in whole cells or with BVH in crude lysates or fractions. The values in parentheses are for total units per milliliter of fraction.

[b] ND, Not determined.

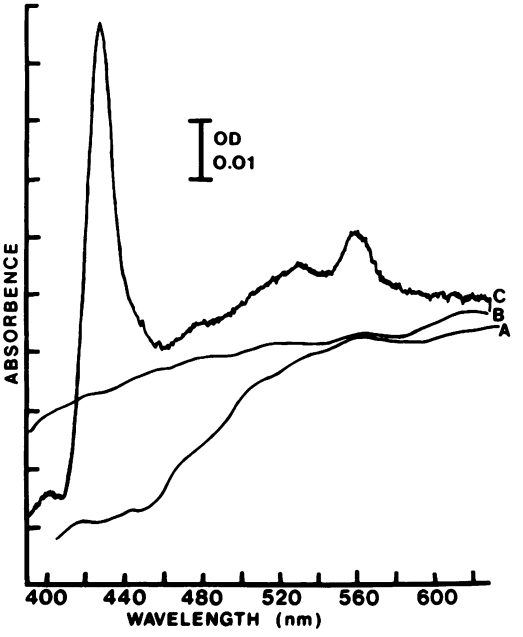

FIG. 2. *Reduced-minus-oxidized difference spectra of membranes from H-14. The membrane fraction was prepared from cells grown in SB-Glu-PU with 20 mM KNO_3. Samples containing 2.5 mg of protein per ml were incubated for 5 min in 40 mM potassium phosphate buffer, pH 7.5, with or without 2 μM hemin; they were diluted threefold for measurement of the dithionite-minus-ferricyanide difference spectra. (A) Membranes treated without hemin; (B) base line; (C) membranes treated with hemin.*

activity between cytoplasm and membrane did not differ significantly in cells grown with and without hemin. In both types of cell 70 to 90% of the total BVH-nitrate reductase activity was located in the cytoplasmic fraction.

Membrane and cytoplasmic fractions were also analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. No qualitative difference was observed in preparations from cells grown with or without hemin, and in each case the protein band associated with nitrate reductase activity was evident (3).

The effect of hemin on the development of nitrate reductase activity was examined more closely in growing cultures of H-14, to which nitrate was added to induce enzyme formation. Nitrate reductase activity with BVH and lactate as donors was determined in samples removed at stages of the growth curve, and the development of these activities is shown in relation to time (Fig. 3a and b) and also as a differential plot (Fig. 3c). The culture medium was also analyzed for nitrite (Fig. 3a).

In culture I, containing nitrate as the only addition, BVH activity increased at a constant rate throughout the growth period. Lactate-nitrate reductase activity was not detectable in such cells, nor was nitrite found in the culture medium.

Addition of hemin increased the growth rate, and nitrite formation was observed (Fig. 3a, culture II). Nitrate reductase activity increased initially in the cells growing with hemin, but enzyme formation apparently ceased before the end of growth (Fig. 3a and b). The differential plot of BVH-nitrate reductase suggests that the decline in specific activity occurring in later phases of growth could be attributed to dilution rather than to inactivation.

The concentration of nitrite in the cultures with hemin increased throughout the growth period until quantitative conversion of the nitrate had occurred (Fig. 3a). This raised the possibility that nitrite formed by the functional enzyme system was influencing the further development of BVH-nitrate reductase activity. However, this was not supported by the observations made with culture III, to which nitrite (10 mM) was added after nitrate. The growth rate was slightly retarded, but BVH-nitrate reductase activity increased at a rate similar to that observed in culture I (Fig. 3b and c).

DISCUSSION

Mutants H-14 and H-18 respond to remarkably low concentrations of hemin; maximum growth rates were attained with concentrations of 0.5 μM. Mutant H-14 also grew with δ-ALA but at concentrations 100 times higher than hemin. This difference considerably exceeds the 8:1 molar ratio required to form heme from δ-ALA and it might be partly attributed to differences in permeation.

The ability to use exogenous hemin is not usual among bacterial mutants that grow with δ-ALA. Hemin does not replace δ-ALA for growth of mutants of *E. coli* (19), *Salmonella typhimurium* (18), *Proteus mirabilis* (5), and *Rhodopseudomonas spheroides* (12). Those organisms that can use hemin require concentrations of 5 μM or more and include mutants of *Spirillum itersonii* (13), *Bacillus subtilis* (1), and *E. coli* (2). This could be due to differences in penetration of hemin through the surface layers to the assembly sites of hemoproteins.

Restoration of lactate-nitrate reductase activity by addition of hemin to deficient cells or membranes suggests that synthesis of apocytochrome by the staphylococci occurs independently of the heme prosthetic group. Maximum restoration occurred within 5 min, and the activities attained were consistently comparable

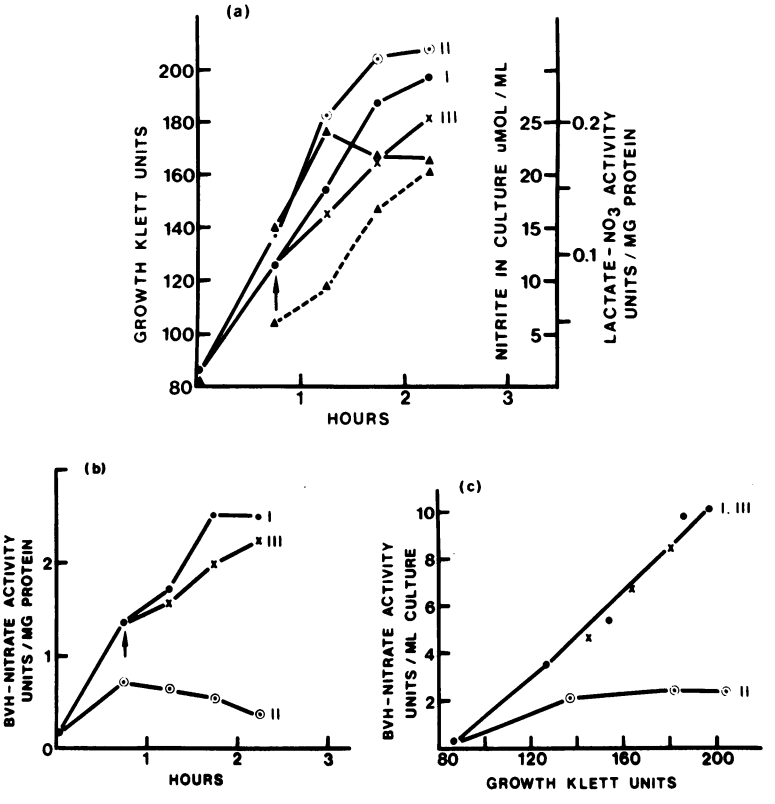

FIG. 3. *Effect of hemin and nitrite on growth and nitrate reductase activity in cultures of mutant H-14. A culture of H-14 growing in SB-Glu-PU was divided into parts I, II, and III at zero time. Potassium nitrate (final concentration, 20 mM) was added to each, and hemin (final concentration, 0.5 μM) was added to culture II only. After 0.75 h of incubation, KNO_2 (final concentration, 10 mM) was added to culture III (arrow). Samples were removed at intervals for determination of growth, nitrite production, and nitrate reductase activity. (a) Growth (●, I; ⊙, II; X, III); lactate-nitrate reductase activity, measured in intact cells from culture II (▲, solid line); nitrite production in culture II (▲, broken line). (b) Specific activity of BVH-nitrate reductase in crude lysates. (c) Differential plot of BVH-nitrate reductase activity as enzyme units per milliliter of culture. Enzyme activity is expressed as micromoles of nitrite per minute.*

to those observed in cells grown with hemin. Presumably, apo-cytochrome is oriented in the membrane to permit its association with hemin and to permit interaction with other components concerned in electron transport from lactate to nitrate. However, it cannot be concluded from these experiments that the deficient membranes contain similar quantities of the identical apo-cytochrome made under normal circumstances; the availability of the heme prosthetic group could well have a role in regulating the synthesis and assembly of the protein components. Knowledge of the purified proteins together with analysis by electrophoretic and immunological techniques are needed for further progress (15, 16).

Reconstitution of activity with the membranes was unaffected by adenosine 5'-triphosphate, although intact cells require glucose for restoration of respiration and lactate-nitrate reductase activity. Energy may be needed to transport hemin into the cells, but it is not apparently needed for combination of the prosthetic group with apo-cytochrome in the staphylococcal membranes. This contrasts with the behavior of the *hem*$^-$ mutant of *E. coli* studied by Haddock and Schairer (9, 10). Reconstruction of *b*- and *o*-type cytochromes in deficient membranes was achieved with hemin but only in the presence of adenosine 5'-triphosphate. The *E. coli* mutants also differ from staphylococci in that nitrate reductase activity with physiological reductants is not restored by treatment of deficient membranes with hemin, and such activity is not restored to intact cells by ALA (11). Presumably the apo-protein of cytochrome b_1 is not formed, or it is degraded, when the heme prosthetic group is unavailable.

Evidence for this is provided by observations with another *hem*⁻ mutant of *E. coli* (15). Immunoprecipitates from Triton-solubilized membranes of deficient cells lacked the polypeptide component (molecular weight, 19,500) attributed to cytochrome b_1.

The level of BVH-nitrate reductase activity in the mutant staphylococci was strikingly influenced by growth with hemin. Deficient cells had two to five times more activity than did those grown with hemin. Hemin apparently limited formation of the enzyme; the decline in specific activity occurring in the later stages of growth could be attributed to dilution as the cell mass increased (Fig. 3c). There was no evidence that nitrite affected either enzyme formation or activity.

Previously we have studied the effect of hemin on induction of BVH-nitrate reductase activity in suspensions of cells grown aerobically and then suspended and incubated under semianaerobic induction conditions (3). The behavior of such cells differs in several respects from that observed in the present experiments with cultures growing continuously under semianaerobic conditions. The total level of enzyme activity attained in the suspensions of cells pregrown aerobically was considerably less than that achieved in the semianaerobic cultures, and hemin was without effect. However, the presence of hemin in the suspensions slightly diminished the proportion of cytoplasmic enzyme. Possibly, these differences in behavior might be attributed to the difference in history of the cells prior to induction. Previous aeration might be a critical factor in determining the ability of cells to form the reductase and to incorporate it into the membrane.

In other organisms there are indications of control of enzyme production mediated in some fashion by heme, but a consistent pattern has not emerged. The *hem*⁻ mutant of *E. coli* studied by MacGregor (15) formed excess cytoplasmic enzyme when grown without ALA, indicating that the heme deficiency was influencing formation and integration of the enzyme into the membrane. In contrast, the mutant used by Kemp et al. had less nitrate reductase activity when grown without supplement, and no difference was found in distribution of the enzyme between membrane and cytoplasm (11). Yet another pattern was observed with a *hem*⁻ mutant of *Proteus mirabilis*, which lacked nitrate reductase activity under conditions of heme deficiency (5).

Some of these various effects upon nitrate reductase formation and assembly could be indirect and possibly a consequence of metabolic changes imposed by lack of a functional respiratory chain. Pleiotropic effects upon various membrane components have been observed in other types of respiratory chain mutants. Menaquinone-deficient mutants of *S. aureus* and *B. subtilis* have diminished levels of certain flavoprotein dehydrogenases and cytochromes (7, 8). Respiratory deficiency could also affect lipid biosynthesis, and an altered pattern of membrane lipids might influence the assembly of membrane proteins.

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