JOURNAL OF BACTERIOLOGY, July 1975, p. 308-316

Vol. 123, No. 1
Printed in U.S.A.

Nitrate Reductase System in *Staphylococcus aureus* Wild Type and Mutants

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Received for publication 3 February 1975

Respiratory nitrate reductase with lactate as a hydrogen donor has been studied in cells and spheroplast preparations of wild type and heme-deficient mutants of *Staphylococcus aureus*. The activity is rapidly induced when suspensions of aerobically grown cells are incubated without aeration in a complete medium with nitrate. In ruptured spheroplast preparations, the activity with lactate as the donor is located in the membrane fraction, whereas at least 50% of the activity assayed with reduced benzyl viologen is in the cytoplasm. The reductase is inhibited by azide and cyanide, and the lactate-linked system is also sensitive to oxamate, 2-heptyl-4-hydroxyquinoline-*N*-oxide, dicoumarol, and *p*-chloromercuribenzoate. An inactive form of the reductase is apparently made during induction with tungstate; this can be activated by subsequent incubation with molybdate in the presence of chloramphenicol. Nitrate reductase activity with reduced benzyl viologen as the donor is induced in suspensions of heme-deficient mutants in the presence or absence of heme. The proportion of cytoplasmic activity is increased in the absence of heme. The staphylococcal nitrate reductase has many of the characteristics commonly associated with the respiratory enzyme in other organisms, but the apparent predominance of cytoplasmic activity is unusual.

The respiratory nitrate reductase system of *Escherichia coli* has been studied in detail in several laboratories (21). It functions in the cytoplasmic membrane as a complex of formate dehydrogenase, cytochrome b_1, and nitrate reductase. Recently, the latter component has been purified to homogeneity and shown to be a large molecule (molecular weight 773,600) containing molybdenum (20).

Mutants deficient in respiratory nitrate reductase activity can be selected readily on the basis of their resistance to inhibition by chlorate (2). Analysis of such mutants of *E. coli* has shown complex interrelationships between the components of the membrane complex. Many of them are pleiotrophic and lack the nitrate-inducible type of formate dehydrogenase and cytochrome b_1 in addition to nitrate reductase (23).

Staphylococcus aureus has a respiratory type of nitrate reductase, but this has received little attention since the work with a heme-deficient mutant (4). *S. aureus* does not have the metabolic versatility of *E. coli*, and this is particularly evident under anaerobic conditions of growth. Sugars are fermented essentially to lactic acid and it is restricted biosynthetically, requiring supplements of uracil and acetate or pyruvate (14). Anaerobically grown cells are also deficient in components of the membrane-associated respiratory apparatus, including cytochromes and various lipids; these develop rapidly in response to aeration (9). We have begun studies with *S. aureus* of nitrate reductase and other enzyme systems linked functionally to the cytochrome system, and this paper describes physiological aspects and some of the properties of nitrate reductase in wild type and heme-deficient mutants. One aim has been to establish an induction system with concentrated cell suspensions, which might be convenient for study of membrane assembly.

MATERIALS AND METHODS

Organisms and growth conditions. The wild-type strain of *S. aureus*, SG 511 A, has been described previously (7). Heme-deficient mutants H-14 and H-18 were derived from the wild type by exploitation of resistance to kanamycin (26). Approximately 2×10^8 cells was spread on plates of yeast extract-tryptone (YT) (11) agar supplemented with 10 mM glucose and 10 μM kanamycin. Potential respiratory mutants appeared as small, pale colonies after 3 to 5 days of incubation at 37 C. These were tested for growth on YT agar without and with 1 μM hemin, and those responding to hemin were purified by replating on YT-hemin agar. Some of the isolates respond also to δ-aminolevulinate; H-14 represents this group,

whereas H-18 grew with hemin only. The chlorate-resistant mutants ChlR-1 and ChlR-10 originated from cultures of the wild type in YT broth containing 10 mM glucose and 10 mM sodium chlorate; these were sown with 10^8 organisms per ml and incubated without shaking. Growth was evident after 2 to 3 days, and the strains were purified by plating on YT agar with 10 mM glucose.

Stock cultures of the wild type and the chlorate-resistant mutants were maintained on slants of nutrient agar; the heme-deficient mutants were kept on chocolate agar.

Cells for experiments were grown in a semidefined medium containing casein hydrolysate (SB) (6) and supplemented with glucose and other compounds as stated in the text. Aerobic cultures were in Fernbach flasks (2.5-liter capacity) containing 500 ml of medium and shaken on a reciprocal shaker. Anaerobic cultures were in completely filled glass-stoppered bottles or screw-capped tubes. Bulk cultures were sown with 0.1% inocula grown aerobically overnight in YT broth with 10 mM glucose. All cultures were incubated at 34 to 37 C.

Induction conditions. The cells were grown aerobically in SB with 10 mM glucose, with the further addition of 10 mM sodium pyruvate and 0.2 mM uracil for the heme-deficient mutants. They were harvested in the stationary phase after 16 h of growth and were then washed with and suspended in 40 mM potassium phosphate buffer (pH 7.5) to a density of 5,000 Klett units (approximately 10 mg of protein per ml).

For induction, the stock suspension of cells was diluted 20-fold in SB supplemented with 50 mM glucose, 10 mM sodium pyruvate, 0.2 mM uracil, and 20 mM potassium nitrate. The suspensions were incubated at 37 C without shaking in flasks filled to the neck. After 60 to 90 min, chloramphenicol (0.2 mM final concentration) was added, and the cells were harvested, washed, and suspended in 40 mM potassium phosphate buffer (pH 7.5) to a density of approximately 5,000 Klett units.

Preparation of cell fractions. Lysates were prepared from spheroplasts by procedures based on those described by Konings et al. (13). Samples of suspensions prepared as above were centrifuged, and the cells were suspended in the original volume of 25% NaCl in 40 mM potassium phosphate buffer (pH 7.5). Lysostaphin was added to give a final concentration of 50 μg/ml, and the mixtures were incubated for 30 min at 37 C. The preparations were centrifuged for 15 min at 27,000 $\times$ g, and the pelleted spheroplasts were lysed by homogenization in the original volume of 10 mM potassium phosphate buffer (pH 7.5) with addition of 2 mM magnesium chloride deoxyribonuclease and ribonuclease (approximately 20 μg/ml, final concentration). These mixtures were incubated for 10 min at 37 C and then dialyzed for 3 h at 5 C against 10 mM potassium phosphate (pH 7.5). The dialyzed material was centrifuged for 10 min at 3,000 $\times$ g to remove unbroken spheroplasts and cell fragments, and the resulting supernatant was designated "crude lysate." The crude lysates were fractionated by centrifuging for 1 h at 200,000 $\times$ g or for 2 h at 70,000 $\times$ g. After removal of the supernatant, designated the "cytoplasmic fraction," the pelleted membranes were rinsed with 40 mM potassium phosphate buffer (pH 7.5) and suspended in the same buffer to a protein concentration of 2 to 5 mg/ml.

Enzyme assays. Nitrate reductase activity was assayed with lactate or with reduced benzyl viologen (BVH) as reductant. The lactate assay, used with intact cells or cell-free preparations, contained in a final volume of 1 ml: potassium phosphate buffer (pH 7.5), 40 mM; potassium nitrate, 20 mM; DL-sodium lactate, 50 mM; and cells or cell fraction; and protein, 0.2 to 2 mg. The assay with BVH contained 0.1 mM benzyl viologen instead of lactate; the reduced dye was generated at the beginning of the reaction by addition of dithionite (8). Reactions were terminated after 10 min at 37 C by addition of zinc acetate-ethanol, and nitrite was estimated as described previously (8). Nicotinamide adenine dinucleotide (NAD)-linked lactate dehydrogenase activity was assayed with pyruvate and NADH (7). The non-NAD-linked activity was measured spectrophotometrically at 600 nm with 2,6-dichlorophenolindophenol as acceptor; the reaction mixture contained in 1 ml final volume: potassium phosphate buffer (pH 7.5), 25 mM; potassium cyanide, 2 mM; 2,6-dichlorophenolindophenol, 0.1 mM; and sodium DL-lactate, 20 mM. The spectrophotometric assays were at 25 C, and all assay values were expressed as nanomoles of product formed per minute per milligram of protein.

Protein determination. Protein was determined by the Folin method (17) with bovine serum albumin as standard.

Electrophoretic procedures. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis with slab gels (1 mm thick) was done by the method of Ames (1). Samples containing 1 mg of protein per ml were prepared for analysis by heating for 3 min at 100 C in a mixture of 40 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 8.0), 3% (wt/vol) sodium dodecyl sulfate, 2% (vol/vol) 2-mercaptoethanol, and 20% (vol/vol) glycerol. Forty to fifty microliters was applied to the gels, which contained 3% (vol/vol) acrylamide in the stacking gel and 10% (wt/vol) acrylamide in the running gel. The gels were run at a current of 15 mA for 4 to 5 h at room temperature. They were stained with 0.04% (wt/vol) Coomassie blue in 25% (vol/vol) isopropanol and 10% (vol/vol) acetic acid.

Nondenaturing polyacrylamide slab gels, 1.5 mm thick, were used for detection of nitrate reductase activity. The stacking gel contained 3% (wt/vol) acrylamide in 0.125 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 6.8), and the running gel contained 10% (wt/vol) acrylamide in 0.75 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 9). The running buffer was 0.2 M glycine adjusted to pH 8.5 with Trizma base. The gels were run at 10 C for 6 to 7 h at a current of 15 mA. Enzyme activity was located on the gels by a modification of the method of Payne and co-workers (22). The slabs were immersed in a mixture containing 0.05 M potassium phosphate buffer (pH 7.5), 0.02 M potassium nitrate, and 1 mM benzyl viologen, reduced with dithionite (8). Incuba-

tion was for 2 to 3 min at room temperature in air. Nitrite formed in the gels was detected by immersion in the reagent used in the nitrate reductase assay (8).

Materials. Dicoumarol and 2-heptyl-4-hydroxyquinoline-*N*-oxide were from the Sigma Chemical Co., hemin was from K & K Laboratories, and lysostaphin was from Schwarz/Mann. Stock solutions of dicoumarol (1 mM) were in 0.02 N NaOH and 2-heptyl-4-hydroxyquinoline-*N*-oxide solutions were in ethanol. Stock solutions of hemin (2 mM) were in 0.02 N NaOH in 50% ethanol; these were stored at −20 C and diluted appropriately in water immediately before use.

RESULTS

Physiological function of nitrate reductase. The growth responses of the wild type and mutant H-18 in the presence of nitrate were compatible with nitrate reduction of the respiratory type, mediated by a cytochrome system (Table 1). In the wild type, addition of nitrate permitted anaerobic growth on a nonfermentable energy source such as glycerol, but the mutant grew under these conditions only upon addition of hemin. High concentrations of nitrite accumulated in these cultures, and the analytical values indicated complete conversion of added nitrate. Higher growth yields were associated with nitrate reduction in cultures containing glucose, suggesting that the system functions physiologically even with fermentable carbohydrate available (Table 1).

Mutant ChlR-1 did not grow anaerobically with glycerol and nitrate. With glucose as energy source, this mutant formed a small amount of nitrite, less than 2% of that found in the corresponding cultures of the wild type (Table 1). Mutants H-14 and ChlR-10 behaved similarly to their counterparts when tested under the same conditions as in Table 1.

Suspensions of wild-type cells grown anaerobically with nitrate and glucose or glycerol actively reduced nitrate to nitrite with lactate as the donor; this activity was strongly repressed in aerobically grown cells (Table 2).

Induction of enzyme activity in the wild type. Wild-type cells harvested after aerobic growth in SB with 10 mM glucose had only traces of nitrate reductase activity. Rapid induction occurred upon subsequent incubation under static conditions in the standard induction mixture. This mixture consisted of SB medium with added glucose, pyruvate, uracil, and nitrate. Omission of any one of these supplements reduced enzyme formation to 10 to 20% of the complete system. With 20 mM nitrate, maximum induction occurred within 90 min (Fig. 1). The development of enzyme activity was completely inhibited by 0.2 mM chloramphenicol, indicating de novo protein synthesis.

Nitrate reductase activity in lysed-cell preparations and fractions from the wild type. Crude lysates were prepared from wild-type cells previously induced for 90 min in the standard system. The membrane and cytoplasmic fractions were prepared by centrifugation as described above.

The crude lysates and membrane fraction catalyzed nitrate reduction with lactate or BVH as the hydrogen donor, but NADH and formate were ineffective (Table 3). Reduced methyl viologen served as reductant but was less active than BVH. DL-α-Glycerophosphate NADPH and dithionite were inactive.

TABLE 1. *Requirements for anaerobic growth of wild type and mutants*[a]

Strain	Additions to medium (final concn)			Growth (Klett)	Nitrite (μmol/ml)
	Energy source (mM)	Nitrate (mM)	Heme (μM)		
Wild type	Glucose, 10	0	0	120	ND[b]
	Glucose, 10	20	0	171	25
	Glycerol, 20	0	0	10	ND
	Glycerol, 20	20	0	125	26
H-18	Glucose, 10	20	0	122	0.13
	Glucose, 10	20	1	164	25
	Glycerol, 20	20	0	<10	ND
	Glycerol, 20	20	1	136	21
ChlR-1	Glucose, 10	20	0	123	0.24
	Glycerol, 20	20	0	<10	ND

[a] Cultures were in SB medium supplemented with 10 mM acetate, 0.2 mM uracil, and the additions as shown. Incubation was for 24 h without shaking in completely filled screw-capped tubes. Nitrite was estimated in the supernatant medium after centrifugation of the cells.

[b] ND, Not detectable.

TABLE 2. *Nitrate reductase activity in wild-type cells grown aerobically and anaerobically*[a]

Additions to medium (final concn) (mM)	Mode	Nitrate reductase activity
Glucose, 10	Aerobic	59
Glucose, 10	Anaerobic	552
Glycerol, 20	Aerobic	54
Glycerol, 20	Anaerobic	665

[a] SB medium was supplemented with 20 mM KNO_3, 10 mM pyruvate, and 0.2 mM uracil, with additions as shown. Nitrate reductase activity with lactate as the donor was determined in cells harvested in the stationary phase of growth. Activity is expressed as nanomoles of nitrite per milligram of protein.

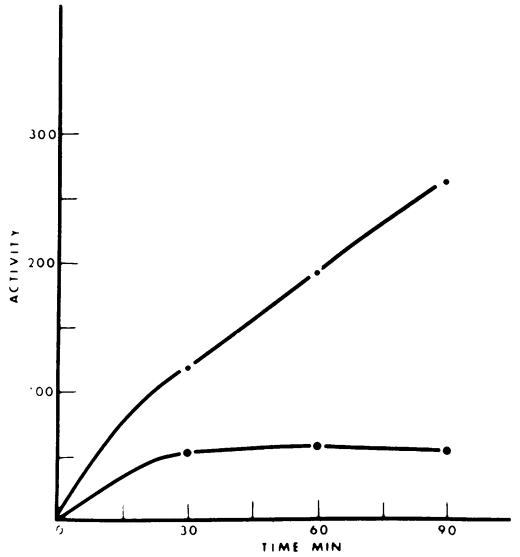

FIG. 1. *Induction of nitrate reductase activity in cell suspensions of the wild type. Suspensions were incubated in the standard system with nitrate (○) or without nitrate (●). Nitrate reductase activity with lactate as the donor was assayed in cell samples removed at intervals and is expressed as nanomoles of nitrite per milligram of protein.*

The cytoplasmic fraction showed no activity with lactate but had high activity with BVH (Table 4). In three preparations, between 52 and 72% of the total nitrate reductase activity with BVH of the crude lysate was recovered in the cytoplasmic fraction.

The cytoplasmic activity is presumed to represent soluble enzyme for the following reasons. (i) It was not sedimented by further centrifugation for 1 h at 200,000 × *g*. (ii) Activity with lactate as donor was not detectable. (iii) Zymograms of the cytoplasmic fraction in 10% polyacrylamide showed activity at an R_f of 0.15, with no activity remaining at the top of the gel. (iv) The same fractionation procedure separated the cytoplasmic NAD-linked lactate dehydrogenase from the membrane-associated dehydrogenase assayed with 2,6-dichlorophenolindophenol; the latter activity was found only in the membrane fraction (Table 4).

MacGregor and Schnaitman (19) found cytoplasmic nitrate reductase activity in *E. coli*, but most of this activity became particulate after

TABLE 3. *Hydrogen donors for nitrate reductase in preparations from lysed cells of wild type*[a]

Hydrogen donor (mM)	Nitrate reductase activity (nmol/mg of protein)	
	Crude lysate	Membrane
Nil	<1	<1
DL-Lactate, 5	44	456
L-Lactate, 5	ND[b]	456
BVH, 0.1	384	614
NADH, 1	2	7
Formate, 5	2	5

[a] The crude lysate and membrane fractions were prepared from cells induced for 90 min. Nitrate reductase activity was determined by the standard assay procedure but with different donors at the final concentrations shown. The protein concentration of the crude and membrane fractions in the assays were, respectively, 0.75 and 0.85 mg/ml.

[b] ND, Not determined.

TABLE 4. *Enzyme activities in cell fractions of wild type*[a]

Enzyme	Sp act			Amt in cytoplasm[b]
	Crude lysate	Cytoplasm	Membrane	
Nitrate reductase				
Lactate	61	<1	538	
BVH	470	376	846	62
Lactate dehydrogenase[c]	31	<0.5	99	<10
Lactate dehydrogenase (NAD)	8,944	12,130	787	108

[a] Cells were induced for 90 min in the standard system; the lysate and fractions were prepared as described in the text. Nitrate reductase was assayed with BVH as donor, and the lactate dehydrogenases were determined spectrophotometrically. The activities are expressed as nanomoles per milligram of protein.

[b] Activity in cytoplasm as a percentage of the total in the crude lysate.

[c] 2,6-Dichlorophenolindophenol.

incubation with Mg^{2+}. This and other data suggested that the cytoplasmic enzyme was derived from the membrane during cell breakage. In similar experiments with staphylococci, only minor amounts (less than 15%) of the cytoplasmic activity became particulate.

Polyacrylamide gel electrophoresis of fractions. Preparations from uninduced and induced cells were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The membrane and cytoplasmic fractions from induced wild type showed three protein bands, 2, 8 and 11, that were lacking in the uninduced cells (Fig. 2). Band 11 has the same R_f as the largest protein standard used, conalbumin, molecular weight 76,000. Analysis of fractions from mutants ChlR-1 and ChlR-10 indicate that band 2 is a protein essential for nitrate reductase activity. These mutants were subjected to the same induction procedure as the wild type but did not develop soluble or membrane-bound nitrate reductase activity, nor did they show band 2 in any of the cells fractions (Fig. 3), although bands 8 and 11 are present in the mutants.

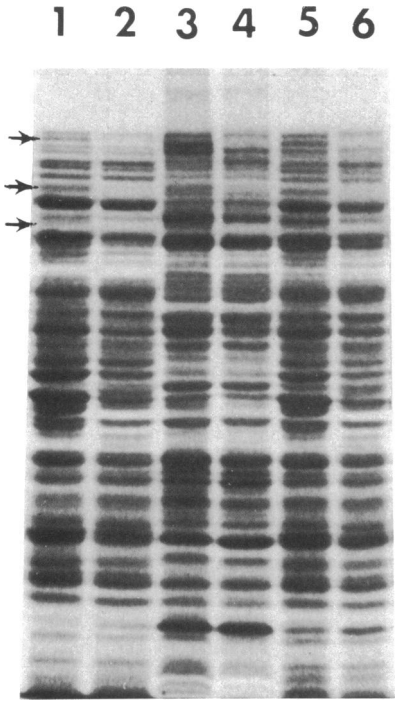

FIG. 2. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis slab gel of crude cell extract, membranes, and supernatants prepared from cells uninduced and induced for nitrate reductase. Sample 1, Crude extract induced; sample 2, crude extract uninduced; sample 3, membranes induced; sample 4, membrane uninduced; sample 5, supernatant induced; sample 6, supernatant uninduced. Samples 1, 2, 3, and 4 contained 40 μg of protein; samples 5 and 6 had 50 μg of protein. Arrows indicate three bands (1, 8, 11, respectively) that are present in all three induced samples and absent in uninduced samples. The band numbering in the membrane fraction is not identical to that of the crude fraction, but it can be seen that bands corresponding to 8 and 11 in the induced crude are absent in the uninduced membrane fraction. Band 11 is very faint in this photograph and can be more clearly distinguished in Fig. 3.*

Fractions from induced wild-type cells were also analyzed for enzymatic activity after polyacrylamide gel electrophoresis without detergent. The membrane fraction showed activity only at the top of the gel. In contrast, the cytoplasmic fraction had a single band of activity with an R_f of approximately 0.15.

Effect of inhibitors. The inhibitors were examined with membrane preparations by using lactate and BVH as hydrogen donor (Table 5). Cyanide and azide strongly inhibited nitrate reduction with either reductant. The system with lactate was also sensitive to 2-heptyl-4-hydroxyquinoline-*N*-oxide and dicoumarol, indicating the participation of the cytochrome *b* and vitamin K_2 components of the staphylococcal respiratory chain. The linked system was strongly inhibited by oxamate, an inhibitor of the membrane D-lactate dehydrogenase of *E. coli* (3, 12), and by *p*-chloromercuribenzoate (Table 5).

Effect of molybdenum and tungsten on enzyme formation. Participation of molybdenum in the staphylococcal nitrate reductase system was shown in experiments with tungsten, a competitive inhibitor of molybdenum function (5, 15, 16). Addition of sodium tungstate to the standard induction system severely inhibited the development of nitrate reductase activity measured in vitro with BVH or in intact cells with lactate (Table 6; Fig. 4a). This inhibition was completely reversed by addition of sodium molybdate to the induction system.

Formation of enzyme protein presumably occurred during the incubation with tungstate. This was shown by addition of molybdate together with chloramphenicol during the induction (Fig. 4a). Nitrate reductase activity with lactate as the donor rapidly developed and attained levels at least as high as in controls without tungstate. This increase in activity in response to molybdate apparently occurred without de novo protein synthesis since it was not blocked by chloramphenicol. In control experiments, addition of this inhibitor to cells incubated without tungstate prevented the further increase in nitrate reductase activity (Fig. 4b).

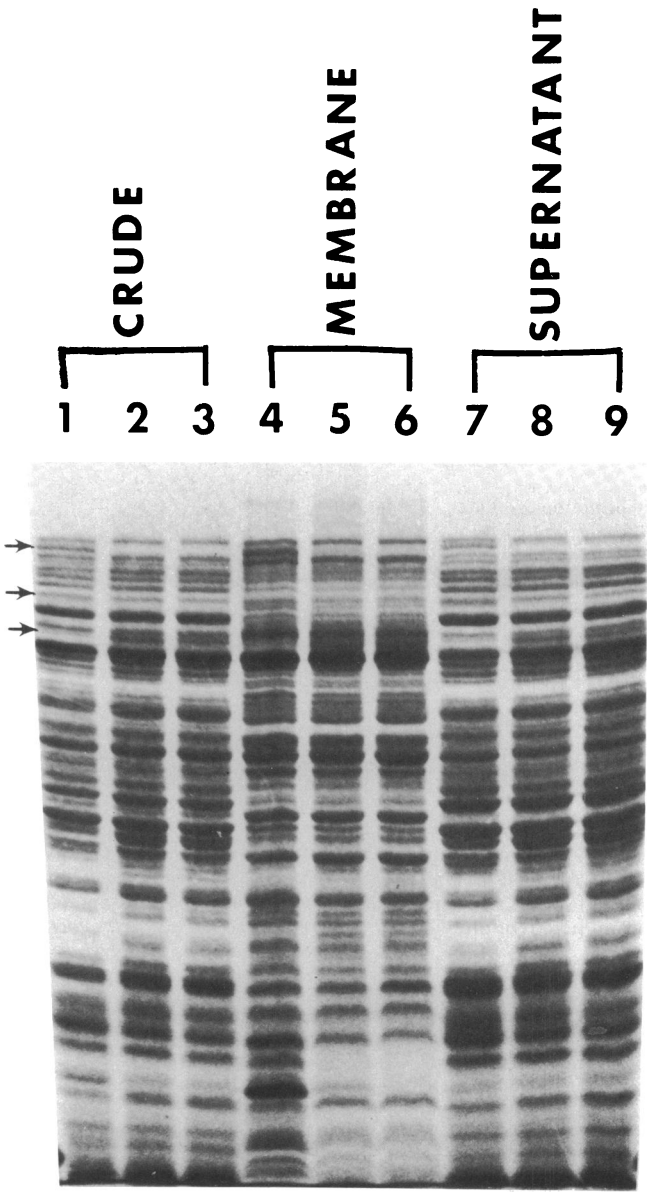

FIG. 3. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis slab gel of crude cell extract, membranes, and supernatants prepared from wild-type A511 and two chlorate-resistant, nitrate reductase-negative mutants, ChlR-1 and ChlR-10, grown under inducing conditions. Samples 1, 4, and 7, A511; samples 2, 5, and 8, ChlR-1; samples 3, 6, and 9, ChlR-10 fractions. The supernatant fractions contained 50 μg of protein, whereas all other fractions contained 40 μg. Arrows indicate band 2, which is missing from all fractions of the Chl mutants, and bands 8 and 11, which are present in A511 and both mutants. Band 11 appears to be a more prominent component of the mutant proteins.*

Activation of nitrate reductase in vitro was attempted by addition of molybdate to crude lysates from tungstate-treated cells. These attempts were unsuccessful. Molybdate also fails to reactivate the assimilatory nitrate reductase in vitro in preparations from *Neurospora* grown with tungstate (21).

Nitrate reductase activity in heme-deficient mutants. The effect of hemin on induction of nitrate reductase activity was examined with strain H-18. Hemin was not required for the induction of activity with BVH as the hydrogen donor, but it was necessary for activity with lactate (Table 7). The repressive

TABLE 5. *Inhibitors of nitrate reductase activity in membranes from wild-type cells*[a]

Inhibitor	% Inhibition of activity with:	
	BVH	Lactate
Sodium azide, 1 mM	97	88
Sodium cyanide, 1 mM	98	82
Dicoumarol, 2 μM	0	100
HOQNO,[b] 1 μM	9	84
HOQNO, 10 μM	8	97
Sodium oxamate, 4 mM	0	93
p-Chloromercuribenzoate, 0.2 mM	19	100

[a] Membranes were prepared from induced cells. The inhibitors at the final concentration shown were tested under standard assay conditions with BVH or lactate.

[b] 2-Heptyl-4-hydroxyquinoline-*N*-oxide.

TABLE 6. *Effect of molybdenum and tungsten on formation of nitrate reductase by the wild-type strain*

Additions to induction system[a] (mM)	Nitrate reductase activity
Nil	224
Sodium molybdate, 1	241
Sodium tungstate, 0.1	70
0.5	38
Sodium molybdate, (1) plus sodium tungstate, 0.5	241

[a] The cells were induced in the standard system for 60 min. The activity was determined in crude lysates with BVH as the donor, and results are expressed as nanomoles of nitrite per milligram of protein.

action of oxygen occurred with or without hemin (Table 7).

The distribution of nitrate reductase activity between cytoplasm and membrane was examined in lysates from H-14 and H-18 after induction with and without hemin (Table 8). The proportion of cytoplasmic enzyme was significantly higher in preparations from the hemin-deficient cells, being at least 80% of the total. The proportion in cells induced with hemin was similar to that in the wild-type strain (Table 8).

DISCUSSION

The staphylococcal nitrate reductase has the characteristics of a respiratory type of enzyme (21). It is inhibited by cyanide and azide and its formation is repressed by aeration.

Inhibition of enzyme development by tungstate and its reversal by molybdate indicate that the latter is needed to form an active enzyme. However, inactive protein apparently accumulates in the presence of tungstate, and this can be activated in the absence of protein synthesis by addition of molybdate to intact cells. These observations with *Staphylococcus* are compatible with the notion of a molybdenum cofactor, possibly common to many molybdenum proteins in addition to nitrate reductase (15, 18, 10).

The staphylococcal nitrate reductase probably functions in vivo with L-lactate as a major

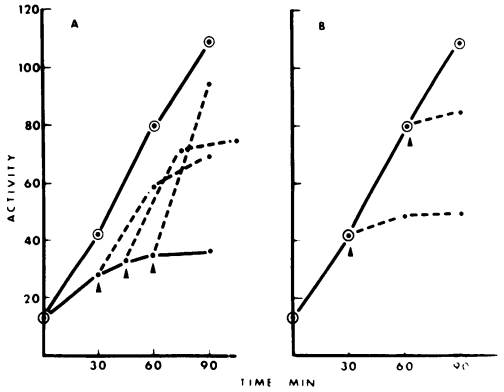

FIG. 4. *Inhibition of nitrate reductase formation by tungstate and reversal by molybdate. (a) Wild-type cells were incubated in the standard system without (○) or with 0.5 mM sodium tungstate (●). At intervals shown by the arrows, 0.5 mM chloramphenicol and 1 mM sodium molybdate were added and incubation continued (broken lines). (b) Controls without tungstate, receiving 0.5 mM chloramphenicol and 1 mM sodium molybdate at intervals. Nitrate reductase activity with lactate as donor was assayed in cell samples removed at intervals and is expressed as nanomoles of nitrite per milligram of protein.*

TABLE 7. *Induction of nitrate reductase activity in mutant H-18*[a]

Incubation conditions	Nitrate reductase activity[b]	
	Lactate	BVH
Static, without hemin	<1	230
Static, with 1 μM hemin	101	124
Aerated, without hemin	<1	23
Aerated, with 1 μM hemin	<1	4

[a] The cells were suspended in the standard incubation mixture with addition of hemin at the final concentration shown. Incubation was for 90 min under static conditions or with aeration in shallow suspensions shaken at 250 rpm.

[b] Nitrate reductase was assayed in whole cells with lactate as donor or in crude lysates with BVH as the donor, and the activity is expressed as nanomoles of nitrite per milligram of protein. No activity was detectable prior to induction.

TABLE 8. *Nitrate reductase activity in cell fractions of heme-deficient mutants*[a]

Strain	Induction system	Nitrate reductase sp act			Amt cytoplasm[b]
		Crude lysate	Cytoplasm	Membrane	
H-14	Standard	230	224	486	84
	Standard + hemin	242	181	646	67
H-18	Standard	283	287	306	81
	Standard + hemin	368	259	671	55

[a] Cells were induced for 90 min in the standard system with addition of 1 μM hemin as shown. The cytoplasmic and membrane fractions were prepared from crude lysates and assayed for nitrate reductase with BVH as the donor. Activity is expressed as nanomoles of nitrite per milligram of protein.

[b] Activity in cytoplasm as a percentage of the total in the crude lysate.

source of reductant. The primary dehydrogenase for this substrate is presumably the membrane-associated L-lactate dehydrogenase, which mediates lactate oxidation by the aerobic respiratory chain (7, 9, 25). The predominance of lactate among the end products of fermentation by *S. aureus* is consistent with its effectiveness as a primary reductant for nitrate.

Electron transfer from lactate to nitrate involves components that are also common to the respiratory path. The lactate-nitrate system was strongly inhibited by dicoumarol and by 2-heptyl-4-hydroxyquinoline-*N*-oxide, indicating participation of vitamin K_2 isoprenologues and cytochrome *b*. These effects confirm observations with mutant strains of *S. aureus* unable to synthesize menaquinones (24) and heme (14).

Nitrate reductase of the respiratory type is usually associated with the cytoplasmic membrane (21). The high activity found in the cytoplasm of the staphylococci might be due to small fragments detached from the membrane during fractionation as occurs in *E. coli* (19). This possibility cannot be excluded, but it is not supported by evidence presented above. Also, extracts were prepared by osmotic lysis of spheroplasts, which should not cause extensive comminution of the membrane.

The relationship of the cytoplasmic enzyme activity to that in the membrane may be clarified by comparison of the properties of the two forms after purification. This work is now in progress.

In *E. coli* formation of nitrate reductase is linked to the synthesis of specific types of formate dehydrogenase and cytochrome *b*. There is no evidence of such sophistication in *S. aureus*. Exploitation of the heme-deficient mutants may contribute to a definitive conclusion about the type(s) of heme protein involved. Unlike similar mutants in *E. coli* (10), the staphylococci respond readily to exogenous heme. They might be valuable in determining possible interrelationships among components of the membrane in the regulation and assembly of nitrate reductase into a functional lactate-nitrate system. The high proportion of cytoplasmic enzyme activity when heme is lacking suggests a role either for the prosthetic group itself or for intact heme protein in this assembly.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AM-1114 from the National Institute of Arthritis, Metabolism, and Digestive Diseases and by grant GB-32113X from the National Science Foundation.

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