JOURNAL OF BACTERIOLOGY, Feb. 1978, p. 621-625
0021-9193/78/0133-0621$02.00/0

Vol. 133, No. 2
Printed in U.S.A.

sn-Glycerol-3-Phosphate Dehydrogenase and Its Interaction with Nitrate Reductase in Wild-Type and *hem* Mutant Strains of *Staphylococcus aureus*

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Received for publication 27 September 1977

Staphylococcus aureus has membrane-associated *sn*-glycerol-3-phosphate dehydrogenase activity that is strongly activated by detergents. The enzyme can be measured spectrophotometrically in intact cells in assay systems containing lauryldimethylamine oxide (Ammonyx LO). The dehydrogenase activity was located exclusively in the membrane fraction of cells grown with glycerol under aerobic conditions or under anaerobic conditions with the addition of nitrate; there was no evidence of multiple forms. Development of *sn*-glycerol-3-phosphate dehydrogenase activity was studied with suspensions of cells grown previously under semianaerobic conditions with glucose and nitrate. The wild-type strain rapidly formed the enzyme when incubated with glycerol under aerobic conditions or under semianaerobic conditions in the presence of nitrate. Under similar conditions, suspensions of *hem* mutant H-14 required the addition of hemin. Induction of the enzyme was strongly repressed by glucose with both organisms. A procedure was established to obtain cells of mutant H-14 with *sn*-glycerol-3-phosphate dehydrogenase and nitrate reductase activities, but which could not link the systems unless supplemented with hemin. The coupled activity could also be reconstructed in vitro by the addition of hemin to the depleted membranes.

The dissimilation of glycerol has been extensively investigated in enteric bacteria by Lin (7). The *glp* operon of *Escherichia coli* includes an inducible *sn*-glycerol-3-phosphate (G3P) dehydrogenase associated with the cytoplasmic membrane. The enzyme has been purified from deoxycholate-solubilized preparations and shown to be a flavoprotein with flavine adenine dinucleotide as the prosthetic group (16). This enzyme is necessary for aerobic growth on glycerol and also serves for anaerobic growth with nitrate as the terminal acceptor. Anaerobic growth with fumarate as the acceptor requires a second form of G3P dehydrogenase; this enzyme is less firmly associated with the membrane than is the aerobic form, and it is strongly stimulated by flavins (4, 5). The exploitation of mutants lacking one or other of the dehydrogenases has shown that the anaerobic enzyme couples with either nitrate or fumarate but not with oxygen; the aerobic enzyme links with oxygen or with nitrate but not with fumarate.

Staphylococcus aureus grows aerobically with glycerol as the bulk energy source and can also grow anaerobically with glycerol and nitrate. The utilization of glycerol under each condition is linked obligatorily to a cytochrome system as shown by the dependence of *hem* mutants upon hemin for growth on this substrate (1, 2). Presumably, this requirement derives from the G3P dehydrogenase. Membrane vesicles prepared from gluconate-grown *S. aureus* have G3P dehydrogenase, which can link with oxygen via cytochromes and which can be coupled to active transport of amino acids (10–12). The present work concerns the membrane-associated G3P dehydrogenase in wild type and *hem* mutants of *S. aureus* and includes studies of its regulation by induction and catabolite repression. One aim was to obtain heme-depleted cells with both nitrate reductase and G3P dehydrogenase activities to examine the role of cytochromes in the coupling of the systems.

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 previously (1, 2). Stock cultures were maintained on nutrient and chocolate agar, respectively. The semidefined base medium (SB) was as described previously (3) with the addition of 0.1 mM sodium molybdate; supplements were added as indicated in the text. Cultures were grown aerobically in 500-ml volumes in Fernbach flasks on a reciprocal shaker, or they were grown semianaerobically without shaking in flasks filled to the neck. Incubation was at 34 to 37°C.

Preparation of cell fractions. Cells were harvested from early stationary cultures, washed, and suspended in 40 mM potassium phosphate buffer (pH 7.5) to an absorbance at 540 nm of 40 (approximately 10 mg of protein per ml). Spheroplasts were made from the washed cells, converted to crude lysates, and divided into membrane and cytoplasmic fractions as previously described (1).

Induction of enzyme activities in cell suspensions. Cells were grown under various conditions as indicated in the text and harvested in the early stationary phase. They were suspended in fresh SB to an absorbance at 540 nm of 2, and appropriate supplements were added as noted in the text. Suspensions were induced for G3P dehydrogenase in volumes of 10 to 15 ml and incubated aerobically in 25-ml flasks shaken at 250 rpm or incubated semianaerobically without shaking in 13-mm-diameter tubes. Nitrate reductase was induced under semianaerobic conditions in flasks filled to the neck and incubated without shaking. All inductions were at 37°C. After induction, the cells were centrifuged, washed, and suspended in 40 mM potassium phosphate buffer (pH 7.5) to an absorbance at 540 nm of 40.

Enzyme assays. G3P dehydrogenase activity was assayed with intact cells or in fractionated lysates as described in the text. The reaction mixture contained the following in a 1-ml final volume: 25 mM potassium phosphate buffer (pH 7.5), 2 mM potassium cyanide, 2 mM phenazine methosulfate, 0.125 mM dichloroindophenol, 0.2% (wt/vol) lauryldimethylamine oxide (Ammonyx LO; LDAO), and 20 mM sodium DL-G3P. The reaction was measured spectrophotometrically at 600 nm at room temperature (approximately 25°C). Nitrate reductase activity was assayed in membrane fractions with reduced benzyl viologen as the hydrogen donor as previously described (1).

Enzyme activities are expressed as nanomoles of product formed per minute per milligram of protein.

Determinations. Protein was estimated by the Lowry method (8), with bovine serum albumin as the standard. Nitrite was determined colorimetrically by the Griess reaction (15).

Materials. Ammonyx LO was generously provided by the Onyx Chemical Co., Jersey City, N.J.; hemin was from Porphyrin Products, Logan, Utah. Stock solutions (2 mM) were in 0.02 N NaOH in 50% ethanol, and dilutions were made in water immediately before use.

RESULTS

Assay of enzyme activity: effect of detergents. The conditions for assay of G3P dehydrogenase activity were examined with membrane preparations from the wild-type strain grown aerobically in SB with glycerol or glucose. The activity was strongly stimulated by detergents, of which LDAO was particularly effective. Under the standard conditions of assay, 0.2% (wt/vol) LDAO increased G3P dehydrogenase activity by 5- to 10-fold (Table 1). Stimulation was also observed with Trition X-100, Brij 35, Brij 58, and cetyltrimethylammonium bromide, but they were less effective than LDAO.

G3P dehydrogenase activity could be assayed with intact cells in the presence of LDAO (Table 2). The whole cell assay gave values similar to those found with crude lysates, and this method was used routinely for the determination of enzyme activity in cultures and cell suspensions.

Location of enzyme activity. Cell fractions were prepared from wild-type organisms grown aerobically in SB with glycerol or semianaerobically in the presence of nitrate. In both types of cells, G3P dehydrogenase activity was located

TABLE 1. *Effect of detergents on G3P dehydrogenase activity in membranes from wild-type S. aureus*[a]

Growth substrate	Detergent in assay (0.2%, wt/vol)	G3P dehydrogenase activity
Glycerol	Nil	238
	LDAO	1,428
	Triton X-100	595
Glucose	Nil	24
	LDAO	238
Glycerol-glucose	LDAO	476

[a] Membranes were prepared from cells grown aerobically in SB with 10 mM glucose or 20 mM glycerol. Dehydrogenase activity was measured as described in the text with appropriate modification of the detergent. The protein concentration was from 0.05 to 0.15 mg/ml, and enzyme activity is expressed as nanomoles per milligram of protein per minute.

TABLE 2. *G3P dehydrogenase activity in cells and cell fractions from wild-type S. aureus*[a]

Enzyme source	Enzyme activity in preparations from cells grown			
	Aerobically		Semianaerobically	
	Per ml	Per mg of protein	Per ml	Per mg of protein
Cell suspension	284	114	ND[b]	ND
Crude lysate	273	99	217	188
Cytoplasm	5	3	14	11
Membranes	277	553	198	867

[a] Cells were grown aerobically in SB with 20 mM glycerol or semianaerobically in SB with 20 mM glycerol–20 mM KNO_3–10 mM sodium acetate–0.2 mM uracil. They were harvested, washed, and suspended in 40 mM potassium phosphate buffer; the protein concentrations of the suspensions were, respectively, 2.5 and 1.2 mg/ml for the aerobic and semianaerobic cells. Crude lysates and derived fractions were prepared from the cell suspensions. The activities are expressed as nanomoles per minute per milliliter of original cell suspension or per milligram of protein.

[b] ND, Not determined.

in the membrane fraction, with less than 10% of the activity in the cytoplasm (Table 2). Addition of 1 mM riboflavine 5'-phosphate and 10 μM flavine adenine dinucleotide to the assay mixtures did not affect enzyme activity in crude lysates or in the derived fractions.

Effect of growth conditions. The staphylococcal dehydrogenase was inducible and subject to catabolite repression. The level of activity in membranes from the wild-type strain grown in SB with glucose present was considerably lower than that found in preparations from organisms grown on glycerol alone (Table 1). The activity in cells grown semianaerobically with glycerol and nitrate was approximately twice that in aerobic organisms (Table 2).

Induction of G3P dehydrogenase in cell suspensions of wild type and mutant H-14. An experimental system was devised that promoted the induction of G3P dehydrogenase activity in cells grown previously to induce nitrate reductase. The wild type and mutant H-14 were grown semianaerobically in SB with glucose, pyruvate, uracil, and nitrate to favor the development of nitrate reductase (2). The cultures were harvested in the stationary phase, and the cells were suspended in SB with pyruvate, uracil, glycerol, and other additions as required to induce G3P dehydrogenase. Suspensions of the wild-type strain rapidly developed this activity when incubated aerobically or under semianaerobic conditions with nitrate (Table 3). Induction of G3P dehydrogenase in cell suspensions of mutant H-14 under either condition occurred only upon the addition of hemin (Table 3).

The development of G3P dehydrogenase activity in cell suspensions of the wild type and mutant was strongly repressed by glucose at concentrations of 2.5 mM or more (Table 3). Addition of 15 mM cyclic AMP did not affect such repression.

Reconstruction of G3P-nitrate reductase activity in mutant H-14. *hem* mutants of *S. aureus*, including H-14, form apo-cytochrome when grown without hemin; the addition of hemin in vitro to depleted membranes restores apparently normal *b*-type cytochrome (2). To determine whether G3P-nitrate reductase activity could be reconstituted, heme-depleted membranes with G3P dehydrogenase and nitrate reductase activities were prepared from mutant H-14 after a two-step induction procedure (Table 4). The mutant was grown aerobically in SB with glycerol and suboptimal concentrations of hemin; G3P dehydrogenase was induced under these conditions. The cells were then induced for nitrate reductase without (A) or with (B) the addition of hemin to the induction system. Membrane fractions were prepared from each type of cell and tested for G3P-nitrate reductase activity (Table 4). The linked activity in membranes A occurred only upon the addition of hemin to the assay system; membranes B had high activity without hemin, although its addition increased the activity.

DISCUSSION

The G3P dehydrogenase activity found in *S. aureus* grown on glycerol is membrane associated and is strongly activated by detergents. The activation might be due to improved access of G3P to the catalytic center of the enzyme. The

TABLE 3. *Induction of G3P dehydrogenase activity in suspensions of wild type and mutant H-14*[a]

Strain	Addition to suspensions			Incubation conditions	G3P dehydrogenase activity
	KNO_3 (mM)	Glucose (mM)	Hemin (μM)		
Wild type	—	—	—	Aerobic	198
	—	—	—	Semianaerobic	32
	20	—	—	Semianaerobic	217
	20	2.5	—	Semianaerobic	79
H-14	—	—	—	Aerobic	53
	—	—	0.5	Aerobic	285
	—	2.5	0.5	Aerobic	129
	20	—	—	Semianaerobic	67
	20	2.5	—	Semianaerobic	88
	20	—	0.1	Semianaerobic	99
	20	—	0.5	Semianaerobic	327
	20	2.5	0.5	Semianaerobic	109

[a] Cells were grown semianaerobically in SB with 20 mM glucose–10 mM pyruvate–0.2 mM uracil–20 mM KNO_3. The cell suspensions were in SB with 10 mM pyruvate–0.2 mM uracil–20 mM glycerol, with additions as shown (final concentration); incubation was for 1 h. G3P dehydrogenase activity was assayed in whole cells and is expressed as nanomoles per minute per milligram of protein; the initial activity in wild type and H-14 was 24 and 60, respectively.

effect appeared to be specific for the G3P dehydrogenase because it was not found with L-lactate, succinate, and dihydroorotate dehydrogenases assayed with the same artificial hydrogen acceptors. The dipolar ionic detergent LDAO was active with intact cells or with membranes. Its inclusion in the assay mixture permitted assay of the dehydrogenase in cells, thus avoiding cumbersome procedures for the preparation of cell extracts. The detergent has also been used to solubilize the dehydrogenase from whole cells as a first step in the purification of the enzyme (Maria Tomio, personal communication).

There was no evidence of other forms of G3P dehydrogenase in *S. aureus*. The apparent lack of a form of G3P dehydrogenase analogous to the anaerobic form in *E. coli* is consistent with the absence of fumarate reductase in staphylococci (6). The anaerobic G3P dehydrogenase in *E. coli* seems to be particularly associated with fumarate reductase both in function and in location in the cytoplasmic membrane (7, 9). Electron transfer between the dehydrogenase and fumarate reductase is not linked obligatorily to cytochromes, because *hem* mutants of *E. coli* can grow without supplement on glycerol and fumarate, and the cytochrome-deficient cells can transport amino acids by means of the coupled system (13, 14). The G3P dehydrogenase in *S. aureus* resembles the aerobic form in *E. coli*, at least in respect to function. In both organisms, the enzyme couples with oxygen or with nitrate by a cytochrome-linked system.

TABLE 4. *Reconstruction of G3P-nitrate reductase activity in membranes of mutant H-14*[a]

Addition to assay system[b]		Nitrite formed (nmol)	
Hemin (μM)	G3P (mM)	Membranes A	Membranes B[c]
0	20	47	3,465
0.5	20	2,299	4,442
0.5	0	44	12

[a] Cells were grown aerobically in SB with 20 mM glycerol–0.1 μM hemin. They were suspended in SB with 25 mM glucose–10 mM pyruvate–0.1 mM uracil–20 mM KNO_3, and the suspension was divided into A and B. Hemin (0.5 μM) was added to B only, and the suspensions were incubated semianaerobically for 2 h to induce nitrate reductase. Membrane fractions were prepared from the induced cells and tested for G3P-nitrate reductase activity.

[b] Membranes (4.5 mg of protein) were in a total volume of 1 ml of mixture containing: 40 mM potassium phosphate buffer (pH 7.5), 20 mM KNO_3, 0.3 mM dithiothreitol, and hemin and DL-G3P as shown. The mixtures were incubated for 5 min before the addition of G3P; the reaction was terminated 20 min after the addition of G3P and analyzed for nitrite. The results are expressed as nanomoles of nitrite formed.

[c] The G3P dehydrogenase activity in cells A and B before preparing the membrane fractions were 304 and 262 nmol/mg of protein, respectively. Nitrate reductase activities in membranes A and B were 948 and 1,200 nmol/mg of protein, respectively.

One aim of this work was to establish conditions that provided heme-depleted cells with nitrate reductase and G3P dehydrogenase activities. The direct approach of growing the mutant with limiting hemin on glycerol and nitrate was not feasible due to poor growth yields. The nitrate reductase can be induced gratuitously in the mutant by incubation with nitrate, with glucose as the energy source (1, 2). However, the G3P dehydrogenase could only be induced under nongratuitous conditions with hemin present to permit energy production by the respiratory mode. The provision of glucose as the energy source caused severe catabolite repression of the dehydrogenase (Table 3). A two-step procedure was finally adopted in which mutant cells were grown aerobically with glycerol and low concentrations of hemin and then induced for nitrate reductase without hemin. Membrane preparations from such cells lacked G3P-nitrate reductase activity, but were restored by the addition of hemin. This suggests that apo-cytochrome is formed by the mutant, which can readily combine in vitro with the heme prosthetic group to reconstitute functional cytochrome. The reconstructed system is also capable of coupling the L-lactate dehydrogenase with nitrate reductase (2).

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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