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Membrane and Cytoplasmic Nitrate Reductase of *Staphylococcus aureus* and Application of Crossed Immunoelectrophoresis

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Specific antiserum to the membrane nitrate reductase of *Staphylococcus aureus* was derived from immunoprecipitates on crossed immunoelectrophoresis plates. Analysis of the cytoplasmic and membrane forms of the enzyme in cells grown with nitrate and azide indicated their identity, and in each case, the major subunit, M_r 140,000, was converted by trypsin to a polypeptide, M_r 112,000, without loss of enzyme activity or immunological reactivity.

Staphylococcus aureus has a respiratory nitrate reductase in the cytoplasmic membrane which can couple with membrane dehydrogenases via cytochrome *b* (1, 2, 6). The enzyme also occurs in the cytoplasm, and the partially purified cytoplasmic form was characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to have a catalytic subunit, M_r 140,000, with evidence of another subunit, M_r 64,000 (3). In this work we compare the membrane and cytoplasmic enzyme by crossed immunoelectrophoresis (CIE), and this technique was also applied to prepare specific antiserum to the membrane form.

The wild-type strain (ATCC 33528) and mutant Chl-1, which lacks nitrate reductase, have been previously described (1). Cells were grown in static culture in supplemented SB medium (Table 1) and converted to spheroplasts as before (3). Methods for protein determination, for assay of nitrate reductase with reduced methyl viologen, and for histochemical staining for the enzyme were also described in previous work.

Cytoplasmic fractions from lysed spheroplasts were prepared as before (3). Membrane fractions were derived from spherophasts by homogenization in the original volume of 50 mM Tris-hydrochloride buffer (pH 7.6) containing 5 mM $MgSO_4$, 1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, and 1 μg each of DNase and RNase per ml. After removal of unlysed cells by centrifugation for 5 min at 1,000 × *g*, membranes were sedimented by centrifugation for 1.5 h at 175,000 × *g*, washed four times by centrifugation, and finally suspended in 50 mM Tris-hydrochloride buffer (pH 7.6) containing 5 mM benzamidine. Unless otherwise stated, solubilized membranes were obtained by treatment with 4% Triton X-100 (final concentration by volume, 15 to 20 mg of protein per ml) for 1 h at 0°C, followed by centrifugation for 45 min at 17,300 × *g*.

Antisera against whole membranes from cells induced with nitrate were raised in rabbits and applied to the analysis by CIE with methods essentially similar to those of Owen and Salton (9). Specific antiserum was derived from nitrate reductase immunoprecipitates on CIE plates. For this purpose, solubilized membranes from cells grown with nitrate and azide (Table 1) were centrifuged for 1.5 h at 170,000 × *g* and run by CIE against whole-membrane antiserum which had been absorbed with solubilized membranes from mutant Chl-1. Each plate received 25 μg of membrane protein, and the top two-thirds of enzyme immunoprecipitate was removed,

TABLE 1. *Nitrate reductase activity and cytochrome b in wild-type S. aureus grown without and with inducers*[a]

Addition to medium	Nitrate reductase (U/mg of protein)			Cytochrome *b* (nmol/mg of protein)
	Membrane	Unfractionated lysate	Cytoplasm	
None		0.17		0.12
Nitrate, 8 mM	0.55		0.29	0.12
Azide, 0.1 mM	0.86		0.59	0.13
Nitrate, 8 mM and azide, 0.1 mM	4.87		2.87	0.31

[a] The fractions were derived from lysed spheroplasts of cells grown in SB medium supplemented with 20 mM glucose, 10 mM pyruvate, and 0.2 mM uracil, with additions as shown. Nitrate reductase was assayed with reduced methyl viologen as the electron donor and is expressed as micromoles of nitrite per min per milligram of protein in the appropriate fraction. Cytochrome *b* was determined in the membrane fraction in the presence of hemin (7), and the values represent apo and holocytochrome calculated from the α-band maximum, Σ mM 17.5 (11).

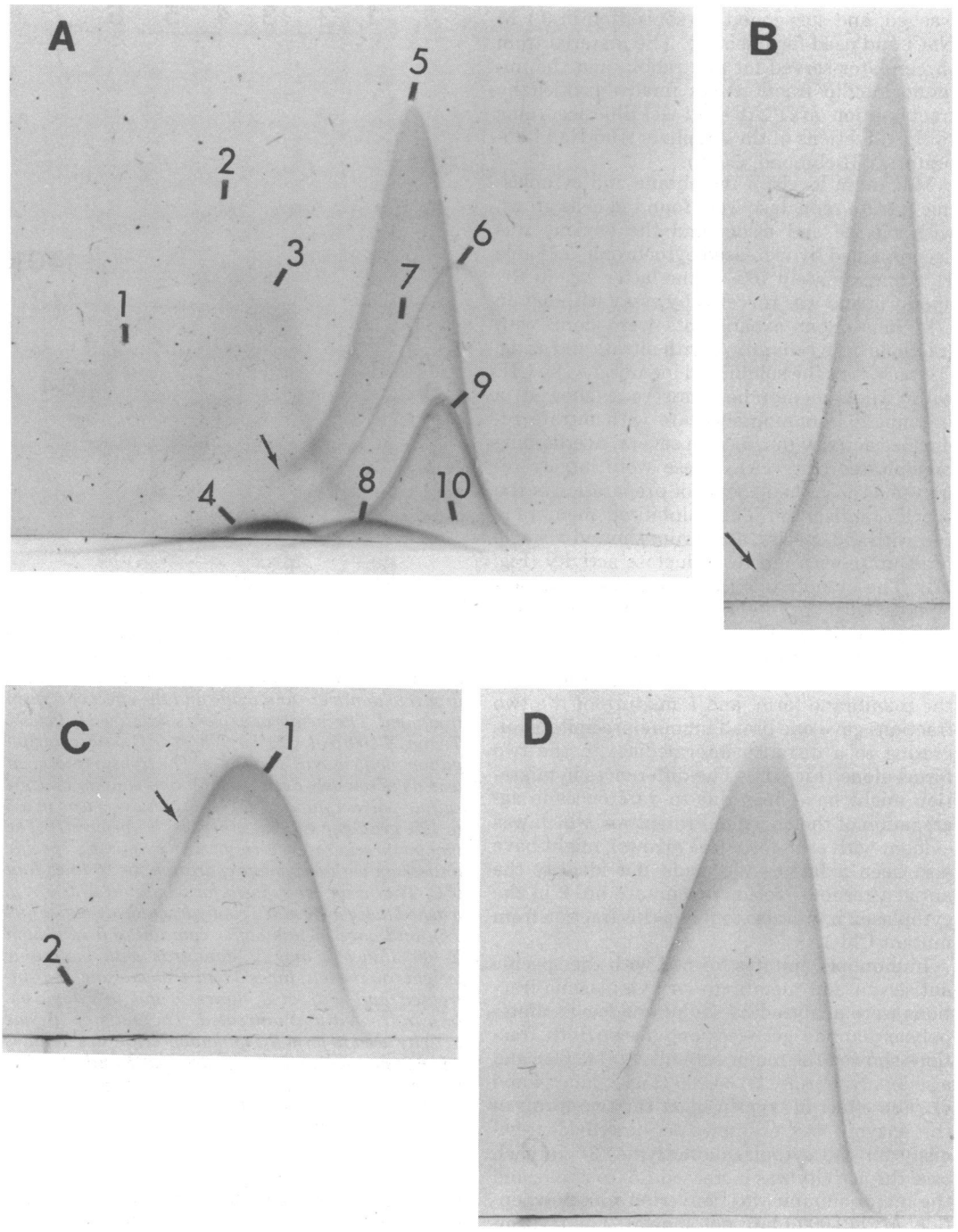

FIG. 1. *CIE patterns of solubilized membrane and cytoplasmic fractions from the wild type grown with nitrate and azide. The second dimension was with 0.2 ml of induced membrane antiserum in (A) and with 0.02 ml of specific antiserum in (B), (C), and (D). The first dimensions were run with: (A) membrane, 12.5 μg of protein; (B) membrane, 23 μg of protein; (C) cytoplasm, 147 μg of protein; (D) membrane, 11 μg of protein, and cytoplasm, 73 μg of protein. In (C), immunoprecipitate no. 1 had enzyme activity, but no. 2 had no activity. The arrows denote proteolysis. The anode is at the top right.*

washed, and suspended by sonication in 0.1 M NaCl and used for injection. The material from three plates served for one rabbit, and the immunoglobulin fraction was finally purified by fractionation on DEAE-Affi-Gel Blue according to the directions of the suppliers (Bio-Rad Laboratories, Richmond, Calif.).

Maximum levels of membrane and cytoplasmic nitrate reductase were found in cells grown with nitrate and azide, and the enzyme was accompanied by increased cytochrome *b* (Table 1). Approximately 70% of the latter was in the apo form and was revealed by assay with hemin (7). Subsequent experiments were done with fractions from cells grown with nitrate and azide.

Analysis of the solubilized membranes by CIE with whole-membrane antiserum showed a prominent immunoprecipitate with nitrate reductase activity (no. 5) with several overlapping precipitates (Fig. 1A). These were largely removed as described above for preparation of the specific antiserum. The solubilized membrane run with the specific antiserum showed a single precipitate with nitrate reductase activity (Fig. 1B). The cytoplasmic fraction showed one precipitate with enzyme activity and a precipitate without activity (Fig. 1C, no. 1 and 2, respectively). The cytoplasmic enzyme migrated slightly more slowly in the first dimension than the membrane form, and a mixture of the two fractions gave one broad immunoprecipitate migrating to a distance intermediate to the two forms alone (Fig. 1D). The differences in migration might have been due to differences in aggregation of the enzyme. Proteolysis, which was evident with each form (see arrows), might have also been a factor. We could not identify the antigen responsible for precipitate no. 2 in the cytoplasm; it was also found in this fraction from mutant Chl-1.

Immunoprecipitates formed with the specific antiserum and membrane or cytoplasmic fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Both fractions showed the major subunit, M_r 140,000, and also a polypeptide, M_r 64,000 (Fig. 2, lanes 4 and 6). The effect of trypsin upon the two forms of the enzyme was examined as described previously for the cytoplasmic enzyme (3). In each case the activity was increased 1.5- to 2-fold, and the major subunit was converted to a polypeptide, M_r 112,000 (data not shown). The trypsin-treated enzymes were precipitated by the specific antiserum, and analysis of the immunoprecipitates by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed loss of the polypeptide, M_r 140,000, accompanied by the appearance of a polypeptide, M_r 112,000 (Fig. 2, lanes 3 and 5).

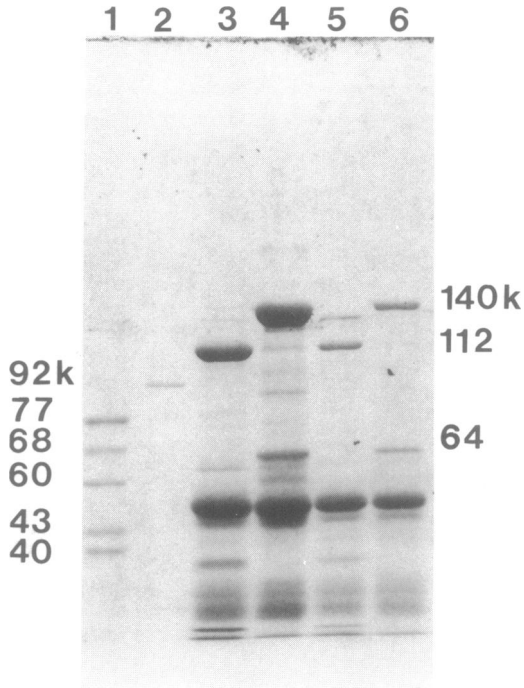

FIG. 2. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of immunoprecipitates of solubilized membrane and cytoplasmic fractions from cells grown with nitrate and azide and the effect of trypsin treatment. The membranes were solubilized with 0.5% Triton X-100 (vol/vol) for 1 h at 0°C with centrifugation for 45 min at 30,000 × g. The trypsin treatment was as previously described, (3) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis was in a 5 to 12% gradient gel, but otherwise as before (1). The immunoprecipitates were formed by mixing specific antiserum with enzyme preparation for 36 to 48 h at 0°C. They were centrifuged for 30 min at 27,000 × g, washed twice in 50 mM Tris-hydrochloride buffer (pH 7.6), and once in this buffer containing 0.5% Triton X-100. Lanes 1 and 2, standards with molecular weights on the left; lanes 3 and 4, membrane, trypsin-treated and untreated; lanes 5 and 6, cytoplasm, trypsin-treated and untreated. The units of enzyme activity used to form the immunoprecipitates in lanes 3 to 6 respectively were: 3.95, 3.55, 0.915 and 0.85. The molecular weights of the polypeptides associated with nitrate reductase are on the right.*

An advantage of CIE exemplified by this work and by recent work with the succinate dehydrogenase of *Bacillus subtilis* (5, 10) is that it permits identification of membrane enzymes, and the immunoprecipitates can serve as an antigen for developing a specific antiserum. The immunological analysis of the nitrate reductase in the membrane and cytoplasm indicated their identity with respect to subunit composition and also with respect to modification by trypsin. The

large segment removed by trypsin from the catalytic subunit (equivalent to a polypeptide, M_r 28,000) is not required for catalytic activity with artificial electron donors, but it may have a role in orienting the enzyme in the membrane for coupling with physiological donor systems.

An appropriate application of the specific antiserum concerns the synthesis and assembly of the nitrate reductase in the membrane of *S. aureus*, particularly in relation to the role of heme and cytochrome *b*. In *hem* mutants of *Escherichia coli*, formation of the holocytochrome is required for incorporation of the enzyme into the membrane (8), but *hem* mutants of *S. aureus* apparently incorporate the enzyme in the absence of the heme prosthetic group (2, 7). Also, wild-type *S. aureus*, under conditions of maximum enzyme formation (with nitrate and azide), accumulates excess apocytochrome which can only be revealed by spectrophotometric assay with hemin (Table 1). These problems of assembly can more readily be pursued by the application of specific antiserum, as demonstrated in work with *E. coli* (4).

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