JOURNAL OF BACTERIOLOGY, July 1979, p. 120-125
0021-9193/79/07-0120/06$02.00/0

Vol. 139, No. 1

Partial Purification and Some Properties of the *Staphylococcus aureus* Cytoplasmic Nitrate Reductase

KATHLEEN A. BURKE AND JUNE LASCELLES*

Department of Microbiology, College of Letters and Science, University of California, Los Angeles, California 90024

Received for publication 28 February 1979

The cytoplasmic nitrate reductase in heme mutant H-14 of *Staphylococcus aureus* was partially purified by steps which included ammonium sulfate fractionation and chromatography on Bio-Gel A 1.5m and ion-exchange columns. The active fractions from the ion-exchange columns showed two forms of the enzyme upon electrophoresis in nondenaturing gels of polyacrylamide; these corresponded to proteins of R_f 0.16 and 0.28. Each form contained a predominant polypeptide of molecular weight 140,000, as shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The R_f 0.16 form contained another major polypeptide of molecular weight 57,000, but the R_f 0.28 form contained several other polypeptides. The sedimentation properties of the enzyme were examined after partial purification on Bio-Gel A 1.5m. In sucrose gradients containing Triton X-100 the enzyme sedimented as a homogeneous peak with an estimated molecular weight of 225,000; without detergent a heterogeneous profile was observed of molecular weight greater than 250,000. Treatment of the enzyme with trypsin increased the specific activity, and the enzyme sedimented as a homogeneous peak in sucrose gradients without Triton X-100, with an estimated molecular weight of 202,000. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated that trypsin treatment converted the polypeptide of molecular weight 140,000 to a polypeptide of molecular weight 112,000. We conclude that the cytoplasmic nitrate reductase of *S. aureus* has a large subunit of molecular weight 140,000, which can be modified by trypsin to a polypeptide of molecular weight 112,000 without loss of catalytic activity.

Respiratory nitrate reductase systems have been investigated extensively in enteric bacteria and in denitrifying bacteria (13, 14). In general, the nitrate reductase in these organisms has been found almost exclusively in the cytoplasmic membrane, and purification has usually required solubilization with detergents or organic solvents. Different forms of the enzyme from *Escherichia coli* have been described, varying with the method of release from the cytoplasmic membrane. Preparations solubilized with detergent are associated with cytochrome *b* (3, 6, 9, 10), but the cytochrome is not found in preparations released by heat treatment (8, 11). Nitrate reductase released by heat treatment has been purified to homogeneity and shown to have two subunits in a ratio of 1:1. The large subunit has a molecular weight estimated to be from 142,000 (11) to 150,000 (8), and the small subunit has an estimated molecular weight of 55,000 (8) to 58,000 (11).

We have been concerned with the respiratory nitrate reductase system in wild-type and Hem mutants of *Staphylococcus aureus* (1, 2, 7). The reductase in the cytoplasmic membrane links with L-lactate or *sn*-glycerol-3-phosphate dehydrogenase via cytochrome *b*, but the organism also has large amounts of cytoplasmic nitrate reductase. The cytoplasmic enzyme accounts for at least 50% of the total activity measured in crude lysates with artificial electron donors. In this paper we describe the partial purification and some properties of the cytoplasmic enzyme from Hem mutant H-14, with a future view toward comparing it with the enzyme in the cytoplasmic membrane. The mutant was chosen as source since it has higher nitrate reductase activity than the wild type when grown without hemin (2).

MATERIALS AND METHODS

Organism and growth conditions. Hem mutant H-14 of *S. aureus* was used; its properties have been described previously (1, 2, 7). Cells were grown in static culture at 34 to 37°C in SB medium supplemented with 50 mM glucose, 10 mM sodium pyruvate,

20 mM KNO_3, 0.1 mM uracil, and 0.1 mM sodium molybdate (1, 2, 7).

Assay of nitrate reductase. Nitrate reductase activity was assayed with reduced methyl viologen as electron donor (7). One unit of enzyme is defined as the amount catalyzing the formation of 1 μmol of nitrite per min under the standard conditions of assay.

Polyacrylamide gel electrophoresis. Samples were analyzed by polyacrylamide gel electrophoresis in nondenaturing gels containing 7.5% polyacrylamide. The gels were stained histochemically for nitrate reductase activity with reduced methyl viologen as electron donor (2). Elution of the enzyme for analysis by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was by immersion of the gel slices in 10 mM Tris-hydrochloride buffer (pH 7.5) for 15 h at 5°C. The eluates were concentrated and dialyzed by using an Amicon Dynaflow apparatus under nitrogen with an XM 50 membrane.

Analysis by SDS-polyacrylamide gel electrophoresis was as described previously (1). The gels contained 5% polyacrylamide for determination of molecular weights greater than 100,000; otherwise, 10% gels were routinely used.

Sucrose gradient centrifugation. Linear gradients of 5 to 20% sucrose in 50 mM Tris-hydrochloride buffer (pH 7.6) were prepared by layering a discontinuous gradient of 20, 15, 10, and 5% sucrose with overnight equilibration at 4°C; where stated, Triton X-100 was included in the gradient. Enzyme samples containing 10 μg of catalase as marker were applied in a volume of 0.1 to 0.3 ml to 5-ml gradients, and centrifugation was for 11 h at 34,000 rpm in an SW50.1 rotor at 4°C. Fractions of 3 to 6 drops were collected and assayed for enzyme activity. The molecular weights were estimated as described by Martin and Ames (12).

Trypsin treatment. Samples of enzyme (2 mg of protein in 0.5 ml of 50 mM Tris-hydrochloride buffer, pH 8.1) were mixed with 0.06 ml of 0.1 M $CaCl_2$ and 0.06 ml of trypsin (5 mg/ml in 0.001 N HCl) and incubated at 30°C for 30 min. The reaction was terminated by the addition of 0.12 ml of trypsin inhibitor (5 mg/ml in 0.1 M EDTA). Controls were treated similarly except that water was added instead of trypsin.

Protein determinations. Protein was estimated by a modification of the method of Lowry et al., using bovine serum albumin as standard (5).

Materials. Trypsin was from Worthington Biochemicals Corp. Trypsin inhibitor (soybean), catalase, Triton X-100, and phenylmethylsulfonyl fluoride were from Sigma Chemical Co. The latter compound was prepared immediately before use as a 0.1 M solution in isopropanol. The molecular weights and sources of the polypeptides used as standards for the determination of molecular weight by SDS-polyacrylamide gel electrophoresis were: hemocyanin, 290,000, kindly provided by Mary-Anne Markwell; phosphorylase *a*, 92,500, Sigma; conalbumin, 77,000, Sigma; bovine serum albumin, 68,000, Sigma; catalase, 60,000, Sigma; and ovalbumin, 43,000, Sigma.

Purification of nitrate reductase. The cells from 8 liters of culture were harvested at the end of the logarithmic phase of growth at a culture density of approximately 1.7 at 540 nm. The cells were washed in 50 mM potassium phosphate buffer (pH 7.5) and suspended in approximately 350 ml of this buffer to a protein concentration of 10 mg/ml. They were stored at −20°C until required.

Preparation of lysates and cytoplasmic fraction. The thawed cell suspensions were centrifuged, and the cells were suspended in approximately 350 ml of 50 mM potassium phosphate buffer (pH 7.5) containing 25% NaCl. Lysozyme and lysostaphin were added (1 and 0.025 mg/ml of suspension, respectively), and the mixture was incubated for 30 min at 37°C; in this time at least 90% of the cells were converted to spheroplasts. The preparations were centrifuged for 15 min at 27,000 × *g*, and the pellet was homogenized in 40 to 50 ml of 50 mM potassium phosphate buffer (pH 7.5) containing 10% (wt/vol) glycerol, 5 mM $MgSO_4$, and 1 mM phenylmethylsulfonyl fluoride. DNase and RNase (1 μg of each per ml) were added, and the preparation was passed through a French pressure cell at 18,000 lb/in^2. The lysate was centrifuged for 2 h at 150,000 × *g* to remove particulate matter. The supernatant, designated the crude cytoplasm, was the starting material for the subsequent purification steps, which were carried out at 0 to 5°C.

Step 1. The cytoplasmic fraction was brought to 30% saturation by the addition of saturated ammonium sulfate in 50 mM potassium phosphate buffer (pH 7.5), and after 30 min the mixture was centrifuged for 15 min at 27,000 × *g*. The supernatant was brought to 60% saturation with ammonium sulfate, stirred for 30 min, and then centrifuged for 15 min at 27,000 × *g*. The supernatant was discarded, and the pellet was dissolved in 50 mM potassium phosphate buffer (pH 7.5) containing 10% (wt/vol) glycerol and 1% (wt/vol) Triton X-100 to a protein concentration of 10 to 20 mg/ml.

Step 2. The solution from step 1 was layered on a column (2 by 92 cm) of Bio-Gel A 1.5m equilibrated with 50 mM potassium phosphate buffer (pH 7.5) containing 0.1 M NaCl, 10% glycerol, and 1% Triton X-100. The column was eluted with the same solution at a flow rate of 25 ml/h, and 3-ml fractions were collected. Active fractions eluting behind the void volume were combined, concentrated by using an Amicon XM 50 membrane, and dialyzed for 18 h against 20 mM Tris-hydrochloride buffer (pH 7.5) containing 10% glycerol and Triton X-100.

Step 3. The solution from step 2 was applied to a column (2 by 13 cm) of epichlorohydrin triethanolamine-cellulose previously equilibrated with 20 mM Tris-hydrochloride buffer (pH 7.5) containing 10% glycerol and 1% Triton X-100. The column was washed with 1 column volume of the same buffer and then eluted with a linear gradient of 0 to 0.35 M KCl in this buffer. Fractions of 3 ml were collected over a total gradient volume of 450 ml. Active fractions were pooled, concentrated by using an Amicon XM 50 membrane, and dialyzed against 20 mM Tris-hydrochloride buffer (pH 7.8) containing 10% glycerol and 1% Triton X-100.

Step 4. The solution from step 3 was applied to a column (1 by 12 cm) of Whatman DEAE-cellulose equilibrated with 20 mM Tris-hydrochloride buffer

(pH 7.8) containing 10% glycerol and 1% Triton X-100. The column was washed with 1 column volume of the same buffer and then eluted with a linear gradient of 0 to 0.45 M KCl in 200 ml of the same buffer. Fractions (1 ml) were collected.

RESULTS

Purification. The procedure summarized in Table 1 gave an 18-fold purification of the cytoplasmic nitrate reductase with an overall recovery of 13%. The final step on DEAE-cellulose gave two areas of enzyme activity; the fractions including each area were pooled and designated peaks I and II (Fig. 1). Preparations of the enzyme were kept in the presence of 10% glycerol and could be stored for at least 6 months without loss of activity.

The major loss of activity occurred during chromatography on ion-exchange celluloses. No significant loss was observed during the dialysis and concentration of the material from the Bio-Gel column (step 2). Purification was also attempted in the absence of Triton X-100. Large amounts of activity eluted in the void volume of the Bio-Gel column, but inclusion of the detergent in the buffer resulted in elution of the enzyme as one peak. However, in subsequent steps on ion-exchange columns heterogeneity was observed with or without Triton X-100. Such heterogeneity frustrated attempts to improve upon the purification by isoelectric focusing, by chromatography on DEAE-cellulose before epichlorohydrin triethanolamine-cellulose, and by varying the elution gradients. Heterogeneous forms of the enzyme were consistently observed, and significant increases in specific activity were not obtained. Phenylmethylsulfonyl fluoride (1 mM) was routinely included in preparations of the crude lysates to inactivate proteases. The inhibitor was also included in the elution buffers in several purifications, but it did not affect the heterogeneity or yield.

TABLE 1. *Purification of nitrate reductase*

Step	Fraction	Total Protein (mg)	Total Activity (U)	Sp act[a]	Yield (%)
	Crude cytoplasm	619	931	1.5	100
1	60% Ammonium sulfate precipitate	322	876	2.7	94
2	Bio-Gel A 1.5m	86	662	7.7	71
3	ECTEOLA-cellulose[b]	25	311	12.4	33
4	DEAE-cellulose				
	Peak I	2.8	60	21.4	5.5
	Peak II	3.1	83	26.8	7.5

[a] Expressed as micromoles of nitrite formed per minute per milligram of protein.

[b] ECTEOLA-cellulose, Epichlorohydrin triethanolamine-cellulose.

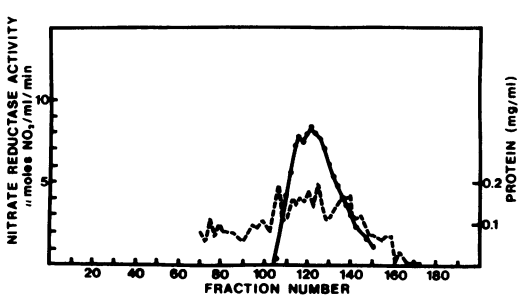

FIG. 1. *Elution profile from DEAE-cellulose column. Solid line, nitrate reductase; dashed line, protein. Fractions 108 to 119 and 120 to 133 were pooled, concentrated, and designated peaks I and II, respectively.*

Analysis of enzyme fractions by polyacrylamide gel electrophoresis. Preparations of nitrate reductase at various stages of purification were analyzed by electrophoresis on slab gels containing 7.5% polyacrylamide; the gels were stained histochemically for enzyme activity and for protein with Coomassie brilliant blue (Fig. 2A). The crude cytoplasm and the fraction from the Bio-Gel step showed one band of activity corresponding to a protein band of R_f 0.16. After chromatography on the ion-exchange columns a second band of enzyme activity was revealed on the gels, corresponding to a protein band of R_f 0.28. Peak I from the DEAE-column showed a predominance of enzyme activity associated with this protein (Fig. 2A, channel 4), whereas peak II showed two areas of enzyme activity corresponding to proteins of R_f 0.16 and 0.28 (Fig. 2A, channel 3).

The two forms of enzyme found on the histochemical gels were analyzed by SDS-polyacrylamide gel electrophoresis. Peaks I and II from the DEAE-cellulose column were first run on the nondenaturing gels; the bands of enzyme were then eluted and subjected to SDS-polyacrylamide gel electrophoresis (Fig. 2B). Both the R_f 0.16 form (channel 4) and the R_f 0.28 form (channels 1 and 3) showed a predominant polypeptide of molecular weight 140,000. In addition, the R_f 0.16 form showed one other polypeptide of molecular weight 57,000. The R_f 0.28 form from both peaks I and II showed an array of polypeptides ranging in molecular weight from 77,000 to 43,000.

The observations suggest that the R_f 0.28 form of the enzyme may have been derived from the R_f 0.16 form during purification on the ion-

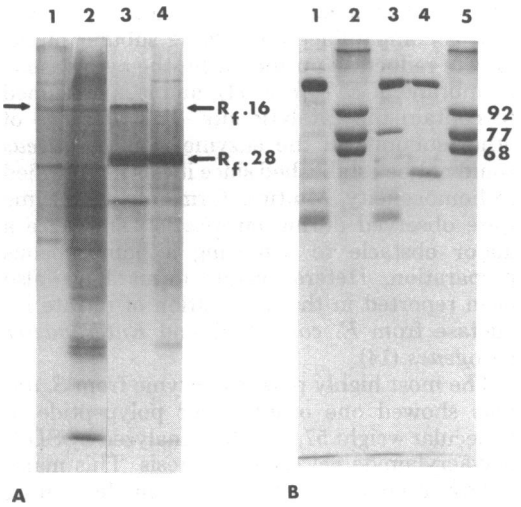

FIG. 2. *(A) Nondenaturing polyacrylamide gel electrophoresis of nitrate reductase preparations at various stages of purification. Slab gels containing 7.5% polyacrylamide were stained histochemically for nitrate reductase and then for protein with Coomassie brilliant blue; the arrows denote bands of enzyme activity. Channel 1, material from Bio-gel A 1.5m (16 μg of protein, 0.227 U of enzyme); channel 2, crude cytoplasm (70 μg of protein, 0.105 U of enzyme); channel 3, peak II from DEAE-cellulose (15 μg of protein, 0.46 U of enzyme); channel 4, peak I from DEAE-cellulose (15 μg of protein, 0.395 U of enzyme). (B) SDS-polyacrylamide gel electrophoresis of R_f 0.16 and R_f 0.28 forms of nitrate reductase. Samples of peaks I and II from the DEAE-cellulose column were run on nondenaturing gels, and the bands of activity were eluted and subjected to SDS-polyacrylamide gel electrophoresis; 10 μg of protein was applied to each channel. Channel 1, R_f 0.28 form from peak I; channel 3, R_f 0.28 form from peak II; channel 4, R_f 0.16 form from peak II; channels 2 and 5, protein standards (phosphorylase a, conalbumin, and bovine serum albumin); the numbers show the respective molecular weights, in thousands, of these standards.*

exchange columns. The polypeptide of molecular weight 140,000 was common to both forms of the enzyme and is presumed to be the catalytic subunit.

Sedimentation of nitrate reductase in sucrose gradients: effect of Triton X-100. In these experiments nitrate reductase was purified by chromatography on Bio-Gel A 1.5m as described above, except for the omission of Triton X-100 from the buffer. The enzyme showed a diffuse sedimentation profile when centrifuged in sucrose gradients without detergent (Fig. 3, control), and the estimated molecular weight was greater than that of the catalase marker (250,000). In contrast, a sharp sedimentation profile was observed when the enzyme preparations were centrifuged in sucrose gradients containing 1% (wt/vol) Triton X-100 (Fig. 3), and the activity sedimented slightly behind the catalase marker. The estimated molecular weight under these conditions was 225,000.

Effect of trypsin. The effect of trypsin treatment was examined with preparations of nitrate reductase purified from the Bio-Gel column in the absence of Triton X-100. Incubation of the enzyme preparations with trypsin for 30 min or longer increased the activity by approximately 1.5-fold and markedly altered the sedimentation pattern in sucrose gradients (Fig. 4). The trypsin-treated material sedimented as a single homogenous peak of activity in gradients without detergent, and the estimated molecular weight was 202,000. Under the same conditions, the control without trypsin showed the diffuse profile described above.

The control and trypsin-treated enzymes were isolated from the sucrose gradients and analyzed by polyacrylamide gel electrophoresis. On nondenaturing gels, the R_f 0.16 form of the enzyme (described above) was found in the control (Fig. 5A, channel 1). The trypsin-treated samples revealed two bands of activity corresponding to proteins of R_f 0.36 and 0.40; no evidence was found of either the R_f 0.16 form or the R_f 0.28 form (Fig. 5A, channel 2). Analysis of the trypsin-treated material by SDS-polyacrylamide gel electrophoresis showed a predominant poly-

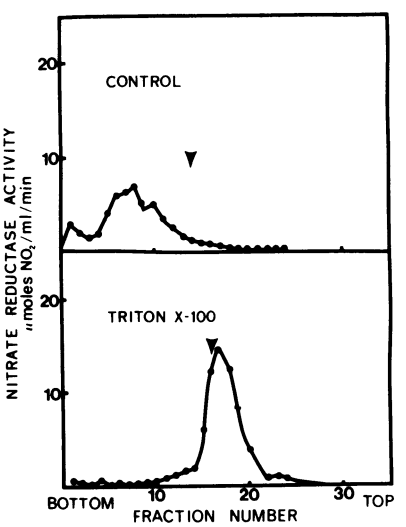

FIG. 3. *Sedimentation of nitrate reductase in sucrose gradients. The gradients were prepared as described in the text without Triton X-100 (control) and with 1% (wt/vol) Triton X-100. The enzyme preparation was from the Bio-Gel A 1.5m column without Triton X-100. The arrows mark the position of the catalase marker.*

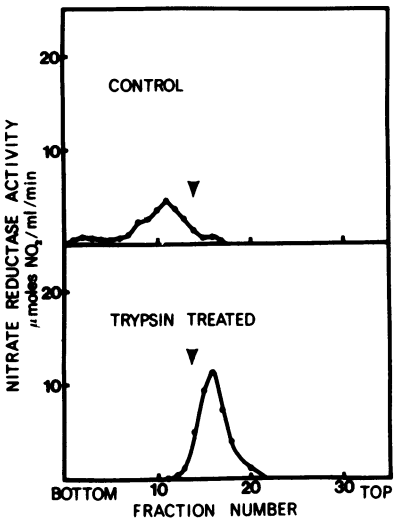

FIG. 4. *Sedimentation in sucrose gradients of untreated and trypsin-treated preparations of nitrate reductase. The enzyme was from the Bio-Gel A 1.5m column without Triton X-100. Control and trypsin-treated samples were prepared as described in the text, and 0.2-ml amounts were applied to the gradients (5 to 20% sucrose in 50 mM Tris-hydrochloride buffer, pH 7.5). The arrows show the elution position of the catalase marker.*

peptide of molecular weight 112,000 (Fig. 5B, channel 2). The polypeptide of molecular weight 140,000 shown in the control (Fig. 5B, channel 3) was not detectable after trypsin treatment.

The modifications described above occurred within 30 min of incubation with trypsin, and further incubation for periods up to 2 h did not alter the polypeptide pattern or the enzyme activity.

Forms of nitrate reductase during the growth cycle. Cell extracts were prepared from organisms harvested in the early, middle, and late logarithmic and the stationary phases of growth. The extracts were separated into membrane and cytoplasmic fractions, and nitrate reductase activity was assayed in each fraction and in the crude extract. The proportion of activity found in the cytoplasmic fraction did not vary significantly throughout the growth cycle and accounted for 50 to 70% of the total activity. The cytoplasmic fraction from each sample was analyzed on histochemical gels containing 7.5% polyacrylamide. The R_f 0.16 form of nitrate reductase was found to predominate throughout the growth cycle, and only traces of the R_f 0.28 form were observed.

DISCUSSION

This work has shown that the cytoplasmic nitrate reductase from *S. aureus* has a large subunit of molecular weight 140,000. This subunit is comparable to the large subunit of the nitrate reductase purified from the cytoplasmic membrane of *E. coli* (8, 11), and it is presumed to contain the catalytic site. The presence of other subunits in the enzyme from *S. aureus* could not be established since it was not purified to homogeneity. Multiple forms of the enzyme were observed during purification and were a major obstacle to achieving a homogeneous preparation. Heterogeneous forms have also been reported in the purification of nitrate reductase from *E. coli* (3, 8) and *Enterobacter aerogenes* (14).

The most highly purified enzyme from *S. aureus* showed one other major polypeptide of molecular weight 57,000 when analyzed by SDS-polyacrylamide gel electrophoresis. This material was prepared by elution from nondenaturing gels of the R_f 0.16 form, which in turn was derived from the enzyme in peak II from the DEAE-cellulose column (Fig. 1 and 2). The second subunit of the *E. coli* nitrate reductase is of similar molecular weight (estimated to be from 45,000 to 67,000) (3, 6, 8, 11). This subunit is

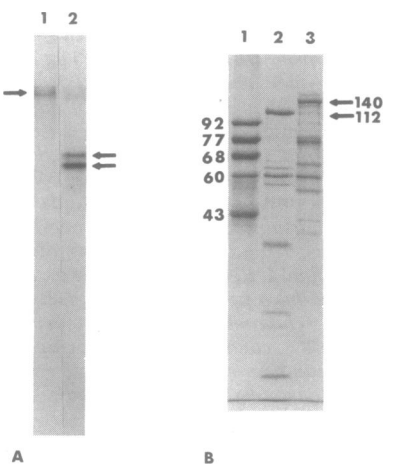

FIG. 5. *Polyacrylamide gel electrophoresis of control and trypsin-treated nitrate reductase from sucrose gradients. Fractions 8 to 13 (control) and 14 to 18 (trypsin treated) from the gradients shown in Fig. 4 were pooled and concentrated. (A) Nondenaturing gels containing 7.5% polyacrylamide, stained for nitrate reductase activity (shown by arrows) and then for protein with Coomassie brilliant blue. Channel 1, control (10 μg of protein); channel 2, trypsin treated (15 μg of protein). (B) SDS-polyacrylamide gel electrophoresis. Each channel received 10 μg of protein. Channel 1, standard proteins (phosphorylase a, conalbumin, bovine serum albumin, catalase, ovalbumin); channel 2, trypsin-treated enzyme; channel 3, control enzyme sample. The numbers show the molecular weights (in thousands); the catalase polypeptide (60,000) is present in both enzyme samples.*

apparently susceptible to degradation during purification, possibly accounting for the discrepancies in the molecular weight estimates (3, 4, 8, 10, 11). The apparent molecular weight of the nitrate reductase from *S. aureus* was 225,000, as estimated by sedimentation in sucrose gradients containing 1% Triton X-100. This value gives some support for a second subunit and approximates the molecular weight (200,000 to 220,000) of the monomeric form of the enzyme from *E. coli* (3, 8). However, the estimated value must be regarded cautiously because of possible binding of the detergent to the enzyme.

The two forms of nitrate reductase revealed by electrophoresis on nondenaturing polyacrylamide gels were found only in preparations purified by chromatography on ion-exchange cellulose columns. The R_f 0.28 form was not detectable in the crude cytoplasm of extracts from cells harvested at various stages of growth, nor was it apparent in preparations from the Bio-Gel columns. We suggest that the R_f 0.28 form was derived from the R_f 0.16 form during purification. The array of polypeptides revealed by SDS-polyacrylamide gel electrophoresis of the R_f 0.28 form (Fig. 2B) may represent degradation products, possibly arising from the putative subunit of molecular weight 57,000.

The presence of Triton X-100 in the sucrose gradients was necessary to obtain a sharp sedimentation profile (Fig. 3). Such effects of detergent have been reported with certain enzymes solubilized from cytoplasmic membranes, including the nitrate reductase (3) and D-lactate dehydrogenase of *E. coli* (15). It is thought that the detergent prevents aggregate formation due to protein-protein interaction between enzyme monomers.

Treatment of the staphylococcal nitrate reductase with trypsin drastically altered its behavior. The enzyme sedimented in sucrose gradients without detergent as a homogeneous peak, with an estimated molecular weight of 202,000. Also, the specific activity was increased. These changes were correlated with the disappearance of the polypeptide of molecular weight 140,000 and the appearance of a polypeptide of molecular weight 112,000. The observations suggest that the subunit of molecular weight 140,000 can lose a large segment without damage to the catalytic site, as measured with reduced methyl viologen as electron donor. The segment removed by trypsin could be responsible for the tendency of the enzyme to form aggregates in the absence of detergent. The effect of trypsin on the nitrate reductase of *S. aureus* differs markedly from its effect upon the enzyme from *E. coli* (4). In the latter case, the large subunit was not affected, whereas the second subunit was apparently converted from a polypeptide of molecular weight 59,000 to one of molecular weight 43,000.

A major question concerning the cytoplasmic nitrate reductase of *S. aureus* is its relationship to the enzyme in the cytoplasmic membrane which functions physiologically with membrane L-lactate and glycerol-3-phosphate dehydrogenase.

ACKNOWLEDGMENTS

We thank Walter Thomas for his most able assistance.

This work was supported by Public Health Service grant AM-1114 from the National Institute of Arthritis, Metabolism, and Digestive Diseases and by grant PCM 76-84301 from the National Science Foundation.

LITERATURE CITED

1. **Burke, K. A., and J. Lascelles.** 1975. Nitrate reductase system in *Staphylococcus aureus* wild type and mutants. J. Bacteriol. **123**:308–316.
2. **Burke, K. A., and J. Lascelles.** 1976. Nitrate reductase activity in heme-deficient mutants of *Staphylococcus aureus*. J. Bacteriol. **126**:225–231.
3. **Clegg, R. A.** 1976. Purification and some properties of nitrate reductase (EC 1.7.99.4) from *Escherichia coli* K12. Biochem. J. **153**:533–541.
4. **DeMoss, J. A.** 1977. Limited proteolysis of nitrate reductase purified from membranes of *Escherichia coli*. J. Biol. Chem. **252**:1696–1701.
5. **Dulley, J. R., and P. A. Greene.** 1975. A simple technique for eliminating interference by detergents in the Lowry method of protein determination. Anal. Biochem. **64**:136–141.
6. **Enoch, H. G., and R. L. Lester.** 1975. The purification and properties of formate dehydrogenase and nitrate reductase from *Escherichia coli*. J. Biol. Chem. **250**: 6693–6705.
7. **Lascelles, J., and K. A. Burke.** 1978. 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. J. Bacteriol. **134**:585–589.
8. **Lund, K., and J. A. DeMoss.** 1976. Association-dissociation behavior and subunit structure of heat-released nitrate reductase from *E. coli*. J. Biol. Chem. **251**:2207–2216.
9. **MacGregor, C. H.** 1975. Anaerobic cytochrome b_1 in *Escherichia coli*: association with and regulation of nitrate reductase. J. Bacteriol. **121**:1111–1116.
10. **MacGregor, C. H.** 1975. Solubilization of *Escherichia coli* nitrate reductase by a membrane-bound protease. J. Bacteriol. **121**:1117–1121.
11. **MacGregor, C. H., C. A. Schnaitman, D. E. Normansell, and M. G. Hodgins.** 1974. Purification and properties of nitrate reductase from *Escherichia coli* K12. J. Biol. Chem. **249**:5321–5327.
12. **Martin, R. G., and B. N. Ames.** 1961. A method for determining the sedimentation behavior of enzymes: application to protein mixtures. J. Biol. Chem. **236**: 1372–1379.
13. **Payne, W. J.** 1973. Reduction of nitrogenous oxides by microorganisms. Bacteriol. Rev. **37**:409–452.
14. **Stouthamer, A. H.** 1976. Biochemistry and genetics of nitrate reductase in bacteria. Adv. Microb. Physiol. **14**: 315–375.
15. **Tanaka, Y., Y. Anraku, and M. Futai.** 1976. *Escherichia coli* membrane D-lactate dehydrogenase. Isolation of the enzyme in aggregated form and its activation by Triton-X-100 and phospholipids. J. Biochem. **80**: 821–830.