Purification and Properties of Nitrate Reductase from Escherichia coli K12*

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CAROLYN H. MACGREGOR, CARL A. SCHNAITMAN, AND DAVID E. NORMANSELL
From the Department of Microbiology, The University of Virginia, Charlottesville, Virginia 22901

MARTIN G. HODGINS
From the Department of Physics, The University of Virginia, Charlottesville, Virginia 22901

SUMMARY

The membrane-bound enzyme nitrate reductase from Escherichia coli has been solubilized and purified 112-fold. The purified enzyme appeared homogeneous by polyacrylamide gel electrophoresis and in the analytical ultracentrifuge. The molecular weight of the enzyme, as measured on an agarose column, was 720,000 and, as measured in the ultracentrifuge, was 773,600. The $s_t$ value was determined by sedimentation analysis to be 23.0 S. Electron micrographs showed the enzyme to be spherical which was in agreement with the intrinsic viscosity as measured in the magnetic visco-densimeter. Sodium dodecyl sulfate polyacrylamide gel electrophoresis indicated that the enzyme is composed of two different subunits with molecular weights of 142,000 and 58,000 and that these are present in roughly equal amounts. As determined by activation analysis, the enzyme contained $3.2 \pm 0.5$ moles of molybdenum per mole of enzyme. These data are consistent with a structure composed of four large polypeptides, four small polypeptides, and four molecules of molybdenum per molecule of enzyme.

Nitrate reductase in Escherichia coli is a membrane-bound enzyme which can be induced in large amounts by growing the organism anaerobically in the presence of nitrate (1). This enzyme is apparently linked via cytochrome $b$ to formic dehydrogenase and as a terminal electron acceptor (2). Mutants lacking nitrate reductase have recently received much attention due to the fact that they are pleiotropic for other defects in cytoplasmic membrane functions as well as for the loss of several proteins present in easily detectable amounts in the cytoplasmic membrane of wild type (nitrate reductase positive) organisms (3-5).

The enzyme was shown by Taniguchi and Itagaki (6) to be a very large molecule containing molybdenum and non-heme iron. Studies on other molybdenum-containing enzymes have indicated that the molybdenum is directly involved in electron transfer and undergoes a change in state during this process (7, 8); therefore, it is of interest to determine accurately the amount of molybdenum present in relation to the subunit structure of the enzyme. In order to examine the physical and chemical parameters of this large protein as well as to be able to investigate how parts of this enzyme have been modified in the mutants lacking nitrate reductase activity, the enzyme has been purified and characterized in regard to molecular weight, shape, subunit composition and molybdenum content.

MATERIALS AND METHODS

Escherichia coli strain RK7 (4) was grown in the basal medium of Lester and DeMoss (9) supplemented with 1 mM Na$_2$MoO$_4$, 1 mM Na$_2$SeO$_4$, 1% KNO$_3$, 7.4 # M thiamine, and 5% glucose. The medium was made anaerobic by continuous bubbling with a mixture of 95% N$_2$ and 5% CO$_2$.

Nondenaturing gels for analysis of the native enzyme contained 6% acrylamide, 0.2% $N$, $N'$-methylenebisacrylamide, and 10% glycerol (for density). After thorough mixing of the above components in a buffer containing 11.5 g of Tris and 7 ml of HCl per liter, polymerization was carried out by the addition of 5 mg of ammonium persulfate and 20 # 1 of $N$, $N$, $N'$, $N'$-tetramethylthylenediamine to 50 ml of gel solution. In order to remove the persulfate from the polymerized gels, a 4-hour pre-electrophoresis was run at 4°C and 1.5 ma per gel in the same buffer. During electrophoresis of the sample, the top buffer contained 5.62 g of Tris and 6.02 g of $N$-tris(hydroxymethyl)-methylglycine (Tricine) per liter and the bottom buffer contained 12.1 g of Tris and 4.15 ml of HCl per liter. The sample was layered on the gel in 10% glycerol and run at 1.5 ma per gel and 4°C. Protein or enzyme bands were detected in three ways: (a) the gel was stained with Coomassie blue (10), (b) it was sliced into 1.2-mm pieces and each slice was assayed for nitrate reductase activity, or (c) the intact gel was immersed in the assay solution to detect bands of nitrate reductase activity.

For analysis of subunits, the enzyme was reduced and diluted to 1 mg of protein per ml in a solution containing 3% sodium dodecyl sulfate, dialyzed into a solution containing 0.1% sodium dodecyl sulfate in 8 M urea, boiled 5 min then run on 6% sodium dodecyl sulfate polyacrylamide gels and stained as previously described (10).

Nitrate reductase was assayed by measuring the reduction of nitrate to nitrite with methyl viologen as the electron donor. As described (10), 0.1 ml of I M sodium phosphate buffer (pH 7.1), 0.1 ml of 1 M sodium nitrate, 0.2 ml of 0.05% methyl viologen, 10 # 1 of 10 ml Cleland's reagent, and 1.1 ml of water plus enzyme. To start the reaction, 0.1 ml of a solution containing 0.8% sodium bicarbonate and 0.8% sodium hydrox sulfite was added to the assay mixture and it was gently swirled until uniformly blue. After incubation for 5 min at 23°C, the reaction was...
stopped by mixing rapidly in a Vortex mixer until the blue color had disappeared. Nitrite was then determined by the following modification of the diazo coupling procedure of Nicholas and Nason (11). Two milliliters of a 1% solution of sulfanilic acid in 20% HCl was added to the assay mixture and it was mixed thoroughly. Then, 2 ml of a 0.125% solution of N-1-naphthyl-ethylenediamine diHCl was added and, after 10 min, the absorbance at 540 nm was measured. Of the above reagents, the hydroxysulfite-bicarbonate solution and the methylviologen were prepared fresh daily and the Cleland's reagent was kept frozen. All other reagents were stable indefinitely. A unit of activity is defined as the production of 1 nmole of NO₂⁻ per min at 23°. Specific activity is expressed in units per mg of protein.

Assays for β-galactosidase (12), phosphatase a (13), and catalase (assayed as described by supplier, Sigma Chemicals) are described elsewhere.

Protein was determined by the method of Lowry et al. (14).

For neutron activation analysis, the enzyme sample, the buffer in which the enzyme was dialyzed, and a molybdenum standard in dialysis buffer were irradiated in the University of Virginia reactor for 2 hours. To allow interfering isotopes to decay, counting was postponed for 5 days, after which the samples were counted in a Ge(Li) detector with a 2 keV resolution for 1 hour. The amount of molybdenum was measured as Tc⁹⁹m.

Sedimentation analysis was carried out in a model F analytical ultracentrifuge with electronic speed control, using a Kel F double sector centerpiece. S,\text{av} values were calculated by standard procedures (15). Prior to the run, the sample was dialyzed 3 days against 50 mM potassium phosphate buffer (pH 7.3), containing 0.1 mM sodium chloride, 0.1 mM Cleland's reagent, and 1 mM sodium nitrate.

Density and viscosity were determined simultaneously as a function of protein concentration at 20° in a magnetic viscosimeter (16, 17). This method allows viscosity to be obtained at very low shear stress (~10⁻³ dynes per cm²) in a sealed cell so that the intrinsic value may be considered to be independent of any non-Newtonian behavior which might occur with this protein at the higher stresses (~10 dynes per cm²) normally utilized in capillary viscometers (18). Further, measurements can be made on small volumes of solution (0.2 ml per determination) minimizing the amount of enzyme required. The quantity \( \rho \) (isopotential specific volume) was obtained from the variation of density, \( \rho \), with protein concentration, \( c \), when water and salts were at the same chemical potential, \( \mu \), throughout the range of protein concentration employed. Thus

\[
\frac{\Delta \rho}{\Delta c} = \frac{1 - \sqrt{\mu \rho_0}}{\mu}
\]  

(1)

where the superscript zero refers to vanishing protein concentration. All densities and viscosities were determined in triplicate and the average value at each concentration was used for the curve analysis. The same sample was used for ultracentrifugate measurements and those made with visco-densimeter. Dilutions were prepared by weight on the analytical balance using the dialysate as the diluent. Volumes were then calculated by applying the relevant density values. For viscosity measurements, the magnetically suspended microbuoy was allowed to rotate sufficiently long so that only in the case of the most dilute solution was the difference in the rotation time (which is proportional to the viscosity) between solution and dialysate as low as 2 s (overall uncertainty ~0.05 s). The precision in the measurements for density was better than 5 \times 10⁻⁴ g per ml.

RESULTS

**Purification Scheme**—Cells were broken by freezing and thawing in the presence of lysozyme and EDTA. The wet packed cells were suspended in 2 ml of the following per g of cells: 0.1 M Tris buffer (pH 8.0) containing 1 to 5 mg of DNAse and RnAse per 200 ml, 5 mM EDTA, and 0.5 mg per ml lysozyme. The suspended cells were rapidly frozen in an ethanol bath, cooled with Dry Ice, then thawed in warm water. This extremely viscous material was treated briefly in a Sorvall Omni-Mixer at low speed then poured quickly into 10 volumes of water containing 1 mM MgCl₂ at 23°C. The diluted cell extract was centrifuged for 5 min at 5000 \( \times \) g, after which the pellet was again suspended in the Tris buffer solution and the above process was repeated. After the second freeze-thaw procedure, about 90% of the cells had been broken. All further steps in the purification were carried out at 4°C unless otherwise stated.

After breakage of the cells, the envelope fraction was isolated by centrifugation at 25,000 \( \times \) g for 2 hours. This fraction was washed by suspending 60 g wet weight of envelope in 1500 ml of sodium phosphate buffer, pH 7.3, and centrifuging as above. Two washes were sufficient to remove the majority of the soluble proteins. Virtually all of the nitrate reductase could be solubilized by suspending the envelope fraction in a protein concentration of 0.4 mg per ml in 5 mM sodium phosphate buffer, pH 8.3, containing 1 mM potassium nitrate and heating at 60° for 7 min. Maximum solubilization occurred if this heated material was allowed to stand overnight at 4°C before continuing the procedure. To remove most of the particulate material, the heated extract was spun for 45 min at 25,000 \( \times \) g. The enzyme was then concentrated from this supernatant by adsorption onto DEAE-cellulose. The DEAE-cellulose (1 g of Whatman DE52 per 20 mg of supernatant protein) was equilibrated with the above 5 mM phosphate buffer, added to the soluble enzyme extract and stirred slowly for 45 min. After being packed into a small column, the DEAE-cellulose was washed with 2 column volumes of 0.08 M sodium chloride in the 5 mM phosphate buffer. To remove the enzyme from the DEAE-column, 1 column volume of 0.3 M sodium chloride was applied. The enzyme was concentrated by centrifugation at 200,000 \( \times \) g for 3 to 4 hours, then applied to a Bio-Gel A-15m column and eluted with 50 mM potassium phosphate buffer containing 1 mM nitrate and 0.1 mM Cleland's reagent. Fractions from this column were concentrated to 4 to 5 mg of protein per ml by again centrifuging at 200,000 \( \times \) g as above and sodium sulfate fractionation was carried out on this concentrated material. The salt was added at 25°C with evacuation of air and replacement with a hydrogen atmosphere after each addition of salt. When the salt concentration reached 95% saturation, the precipitated protein was removed and the purified enzyme remained in the supernatant. At this point the enzyme had been purified 112-fold (see Table I).

The enzyme behaved anomalously at various steps during the purification. First, if the activity of the crude extract (Table I, Step 1) or the envelope fraction (Table I, Step 2) was measured without prior heat treatment, both fractions contained less total activity than was found in the preparation after heat extraction.

| Step        | Total units | Total protein | Specific activity | Recovery | Purification |
|-------------|-------------|---------------|------------------|----------|--------------|
| Crude extract | 3.0 \times 10⁶ | 4368          | 682              | 100      | 1            |
| Envelope    | 3.1 \times 10⁶ | 882           | 3,514            | 100      | 5            |
| Heat-extracted supernatant | 2.9 \times 10⁶ | 483           | 6,004            | 100      | 9            |
| DEAE eluant | 3.1 \times 10⁶ | 142           | 21,851           | 100      | 32           |
| Bio-Gel column | 2.5 \times 10⁶ | 50            | 50,000           | 81       | 73           |
| Sodium sulfate supernatant | 1.6 \times 10⁶ | 21            | 76,190           | 52       | 112          |
(Table I, Step 3). Consequently, fractions from Steps 1 and 2 were always heated to 60° for 7 min before measuring enzyme activity. Another phenomenon which occasionally occurred was the loss, then reappearance of activity. In some instances, the recovery of activity dropped to about one-half after the Bio-Gel step. After the sodium sulfate step, however, the activity returned to the expected level and all activity could be accounted for.

Sodium sulfate was used in the salt fractionation instead of ammonium sulfate because the latter inactivated the enzyme. The protein precipitating at lower sodium sulfate concentrations (80 to 85% saturation) appeared, by its dark color, to contain a large amount of enzyme. When examined on sodium dodecyl sulfate gels, the precipitate was found to contain mostly the enzyme subunits along with smaller amounts of contaminating material, but it had very little activity. It appears then, that the salt fractionation separates inactive enzyme from active enzyme.

Criteria of Homogeneity—On 6% polyacrylamide gels run under non-denaturing conditions the enzyme appeared as a single band (Fig. 1). Often, very small high molecular weight bands were also present at the top of the gel; however, when these gels were sliced and assayed for enzyme activity, these small bands were found to have activity. Thus, they are probably aggregates of the enzyme formed by the extreme concentration of the protein when it enters these gels. Other proteins were run along with the purified enzyme to be certain that heterogeneous mixtures of proteins could be separated on this gel system. Early stages in the purification of the enzyme gave a complex protein pattern. Moreover, bovine serum albumin dimer (mol wt 138,000), catalase (mol wt 250,000), phosphorylase a (mol wt 370,000) and β-galactosidase (mol wt 520,000) could all be separated from nitrate reductase. (Separation on these gels is highlycharge dependent as well as size dependent.)

In the ultracentrifuge, the enzyme appeared as a sharp peak which remained sharp throughout the run; however, in every run, a small peak of lower molecular weight material was present (Fig. 2). We feel that this small peak is a component of the enzyme which dissociates upon handling. Preparations of the enzyme analyzed immediately after purification had almost none of this component. Preparations which had lower specific activities due to extensive handling or aging before sedimentation analysis showed up to 40% contamination with this component. After these “contaminated” samples were centrifuged for 3 hours at 200,000 × g in the preparative ultracentrifuge, the pellet material showed much less of this low molecular weight component in the analytical ultracentrifuge. Since such a centrifugation was carried out three times in the preparation of the enzyme, it is unlikely that any of this lower molecular weight material would remain with the enzyme if it were not a component of it. This low molecular weight material has an 80,0 value of 13.0 S.

Determination of Molecular Weight—Molecular weight was determined from the 80,0 value as well as on a gel filtration column. For the gel filtration measurement, a Bio-Gel A-15m column was calibrated with the following globular proteins of known molecular weight: catalase (mol wt 250,000), phosphorylase a (mol wt 370,000), apoferritin (mol wt 480,000), β-galactosidase (mol wt 520,000) and turnip yellow mosaic virus (mol wt 5,000,000). All enzymes were detected by assay; other proteins, by absorbance at 280 nm. As shown in Fig. 3, all the standards fell on a straight line when the log of the molecular weight was plotted against the elution volume. Nitrate reductase eluted from this column in the position of a globular protein with a molecular weight of 720,000. No proteins in the molecular weight range from 500,000 to 1,000,000, were available. Both thyroglobulin (mol wt 670,000), and γM immunoglobulin (mol wt 950,000) were tried but neither fell on the calibrated line, probably because of their high carbohydrate content.

The 80,0 value, as determined in the analytical ultracentrifuge, was 23.0 S (Fig. 4). This value was used to calculate (Ref. 15, p. 242, Equation 147) a molecular weight of 773,800. To make this calculation, the values for isopotential specific volume, v0, solvent density, ρs, and intrinsic viscosity, [η] (see next section) as measured in a magnetic visco-densimeter, were used. The

Fig. 1 (left). Densitometer tracing of purified nitrate reductase run on a non-denaturing polyacrylamide gel system. Gels were stained with Coomassie blue. A sample of 5 μg of protein was run on a 6% gel for 7 hours.

Fig. 2 (right). Schlieren photograph of sedimenting nitrate reductase. The photograph was taken after 24 min at 60,000 rpm, 20°, with a phase plate angle of 65°. Direction of sedimentation is from left to right. Protein concentration is 6.3 mg per ml. The darker color to the right of the peak is due to the brown color of the concentrated enzyme. Note the small peak of dissociating component to the left of the major enzyme peak.

Fig. 3. Relative elution of nitrate reductase and molecular weight standards from a Bio-Gel A-15m column. A column (0.9 X 60 cm) with a volume of 40 ml and a flow rate of 4.5 ml per hour was used. Both the sample applied and the fraction collected had a volume of 0.4 ml. Nitrate reductase was applied at a protein concentration of 0.3 mg per ml. V0 indicates void volume as determined by blue dextran 2000. Abbreviations are: TYMV, turnip yellow mosaic virus; NR, nitrate reductase; Gal, β-galactosidase; ApF, apoferritin; Pho, phosphorylase a; and Cat, catalase.
quantity \( v_0 \) was obtained by linear least squares fitting of the densities versus protein concentration (Fig. 5). The value of \( v_0 \), using all six concentrations, was 0.721 ml per g with a correlation coefficient of 0.9994. Elimination of data at the two lowest concentrations gives \( v_0 = 0.725 \) ml per g and a correlation coefficient of 0.99999. From past experience with highly dilute solutions, we favor the latter value of \( v_0 \). It may be noted that \( v_0 \) is probably the same as the partial specific volume, \( \tilde{v} \), and the apparent specific volume, \( \phi \), in our case because the plot is linear and the concentration of salts in the solvent medium is quite low (20).

Shape and Size—The greater the deviation of a protein from a sphere, the more inaccurate the molecular weight, as calculated from the above measurements. Nitrate reductase can be shown to be isometric in outline both by viscosity measurements and by electron micrographs. Applying the relation in general use for globular proteins (21):

\[
\frac{\eta^\text{SP}}{c} = [\eta] + k' [\eta]^2 c
\]

(2)

where \( \eta \) is specific viscosity, [\( \eta \)] is intrinsic viscosity, and \( k' \) is the Huggins constant reflecting interactions of protein and solvent components as the concentration, \( c \) (in g per ml), increases, a parabolic least squares fit for [\( \eta \)] gives a value of 4.46 ml per g (Fig. 6). This value for the intrinsic viscosity is somewhat higher than would be expected for a hydrated sphere but is not appreciably greater than that for isometric viruses in solution (17). Because of the number of subunits, which is small for this rather large enzyme, the hydrodynamic volume per unit mass of dry protein, even if isometric, could tend to be a little greater than for the isometric icosahedral viruses. Electron micrographs (Fig. 7) are consistent with the viscosity data showing only molecules with a spherical outline. No subunit structure can be determined from these. As measured from the micrographs, the diameter of the protein is 9.3 nm. This measurement is predictably lower than the actual diameter of the enzyme due to penetration of the stain into the molecule, making the unstained region (the enzyme) smaller. The hydrodynamic volume assuming spherical symmetry, however, is 1.784 ml per g of the dry protein (i.e. [\( \eta \)]/2.5). By assigning the value of \( \phi \) to the density of the solvent in the hydrodynamic domain, the specific volume of the solvated unit becomes 0.864 ml per g having a molecular weight of \( 1.597 \times 10^6 \), i.e. 773,600/[1 + (1.784 - 0.725)\( \rho \)]. The diameter of the equivalent sphere for this mass is 16.4 nm. It is conceivable that the maximum distance between the center of mass and the extreme protuberances of the isometric reductase approaches half this value for the hydrodynamic diameter, and that this length is not seen in the electron micrographs because of the staining method employed. It must be underscored, however, that the value for the hydrodynamic volume depends heavily upon the values assigned to the concentrations; hence, a substantial revision of this hydrodynamic diameter may be required if the definition of the dry mass of the reductase differs from that applied here.

Subunit Composition—Molecular weights of the enzyme subunits were determined on sodium dodecyl sulfate polyacrylamide gels. To calibrate these gels ovalbumin (subunit mol wt 43,000), bovine serum albumin (subunit mol wt 69,000), transferrin (subunit mol wt 77,000), \( \beta \)-galactosidase (subunit mol wt 130,000), and bovine serum albumin dimer (mol wt 138,000) were each run individually with cytochrome c (subunit mol wt 12,000) as a marker. Relative mobility of each protein was calculated from densitometer tracings of stained gels by measuring the distance which the standard (S) and the marker (M) moved from the top of the gel and dividing S by M (Fig. 8).
Fig. 9 shows densitometer tracings from five separate enzyme preparations. In each case, there is a single band at the top of the gel (Peak A) which has a molecular weight of 142,000. The lower molecular weight components are slightly different in each preparation. For comparison between scans, the 58,000 molecular weight peak in each is marked Peak B. In preparation I, there is only a single low molecular weight component (Peak B). In preparations IV and V, one sees two broad peaks in this region, each peak composed of two or three very close bands. The shoulders to the left of Peak B in preparations II and III indicate that these too have several low molecular weight components. Comparing specific activities of each of these preparations (Fig. 9), one can see no correlation between activity and number of peptides in the 58,000 region. We have considered all of the peptides in this region to be functioning interchangeably as the smaller of two subunits which make up the native enzyme.

To determine the ratio of the amount of large polypeptides (Fig. 9, Peak A) to the amount of the smaller polypeptides (all lower molecular weight peaks), the area under each peak was measured. Although the results were somewhat variable, in preparations with the highest specific activity there was 2.2 to 2.4 times the protein in the high molecular weight region than in the low region. This would indicate that there is a 1:1 ratio of large to small peptides in the enzyme. An attempt was made to measure this ratio more accurately by running the reduced and alkylated enzyme on a Sephadex G-200 column equilibrated in 0.1% sodium dodecyl sulfate. This column gave the same peptide pattern as the sodium dodecyl sulfate gels. Unfortunately, the separation of these peptides on the column was much poorer than on the gels, preventing any accurate measurement of their amount. The column did reveal the presence of a very small peptide (~10,000 mol wt) which was not seen on gels of the enzyme before separation on the column, due to its scarcity in comparison to the other peptides. Whether this is actually part of the enzyme cannot be determined at this point.

**Metal Content**—The molybdenum content, as measured by activation analysis, was 3.2 ± 0.5 moles of molybdenum per mole of enzyme, assuming a molecular weight of 773,600.

The presence of non-heme iron was indicated by the absorption spectrum, the main characteristic of which was a broad shoulder with a midpoint at approximately 410 nm. It is the presence of this non-heme iron which causes the enzyme to have a brown color and is responsible for its unusually high absorbance at 280 nm. (1 mg of protein has an absorbance at 280 nm of 2.5). The ratio of the absorbance at 410 to the absorbance at 280 nm is 0.140.

**Stability**—As described in the purification procedure, this enzyme, at early stages of purification is rather heat stable. The enzyme after Step 6 (Table I) also showed no inhibition when heated at 50° for 1 hour at a protein concentration of 0.1 mg per ml. Conversely, it was rapidly inactivated by freezing at this same concentration. Although small amounts (0.1 nm) of Cleland's reagent stabilized the enzyme, a concentration of 1 mM caused inactivation. The purified enzyme was also inactivated by azide and ammonium salts.

**DISCUSSION**

From the above data, the most likely structure for nitrate reductase is an octamer composed of four large and four small subunits. Assuming a molecular weight of 142,000 for the large subunit and 58,000 for the small one, the molecular weight of the native enzyme with this proposed structure would be 800,000 (very close to our calculated molecular weight of 773,600). These subunits are probably associated in the form of a double tetrahedron (Fig. 10). This would be consistent with the spherical shape indicated by the viscosity and electron microscope data. Fig. 10 shows that this structure would have a globular outline. The micrographs reveal no hole in the enzyme (eliminating a ring structure) and show a small diameter which, together with the calculated diameter, also indicates that the subunits are tightly packed. Such a structure would produce the largest number of subunit contacts and, therefore, should be the most stable structure.

Nitrate reductase may be similar in structure to glutamate synthetase from E. coli. Miller and Stadtman (22) have char-

![Fig. 8. Relative mobilities of nitrate reductase subunits and those of molecular weight standards on 6% sodium dodecyl sulfate polyacrylamide gels. Ten micrograms of each protein standard was mixed with 10 μg of cytochrome c before placing on the gel. Mobilities, relative to cytochrome c, were measured from densitometer tracings of Coomassie blue-stained gels. Abbreviations are NR<sub>A</sub>, nitrate reductase subunit A; BSA<sub>2</sub>, bovine serum albumin dimer; Gal, β-galactosidase; Tran, transferrin, BSA, bovine serum albumin monomer, NR<sub>B</sub>, nitrate reductase subunit B; and Ova, ovalbumin.](image)

![Fig. 9. Densitometer tracings of purified nitrate reductase from five consecutive preparations (I-V), run on 6% sodium dodecyl sulfate polyacrylamide gels stained with Coomassie blue. The top of the gel is on the left. Peaks labeled A and R refer to NR<sub>A</sub> and NR<sub>B</sub> in Fig. 8. Numbers in parentheses indicate specific activities. To optimize comparison among preparations, all gels were run simultaneously in the same bath and each contained 6 μg of protein.](image)
characterized this enzyme as an octamer consisting of two different kinds of subunits. Its total molecular weight is 800,000 and its two subunits have molecular weights of 135,000 and 53,000.

The ratio of molybdenum molecules to subunits of the enzyme is of interest because, as there is evidence that the molybdenum interacts directly with the substrate, one might use this ratio to determine the number of active sites on the enzyme. Since the amount of molybdenum measured per molecule of enzyme was close to four, this suggests that there is one molybdenum molecule associated with each pair (large plus small) of subunits. Although our measured value for molybdenum was slightly lower than four, it is likely that some of the molybdenum is lost during purification, causing the final enzyme preparation to be slightly lower in molybdenum than the enzyme in vivo. A variability in metal content related to specific activity has been observed in other metal-containing enzymes (8, 24).

This proposed model depends on the assumption that all the small polypeptides seen on sodium dodecyl sulfate gels are functionally equally as the smaller of the two subunits in the native enzyme. The variation in size of these peptides might be explained in the following way. A protease could be activated during the purification, probably at the heat extraction step, which could clip off small pieces of the exposed ends of the small subunits. The presence of extensively hydrolyzed subunits in the native enzyme would cause inactivation of the enzyme, explaining why we see significant amounts of this in our sodium sulfate precipitate.

Two recent preliminary observations support the idea that the enzyme is released from the membrane by proteolytic activity. First, we have used the technique of immune precipitation to examine the subunit composition of the enzyme as it is released from the membrane by heating or by the detergent Triton X-100. This was done by preparing a rabbit antiserum against the purified enzyme. This was mixed with crude heat-solubilized or Triton X-100-solubilized extracts, and the enzyme-antibody complex was precipitated with goat anti-rabbit IgG and analyzed on sodium dodecyl sulfate polyacrylamide gels. The enzyme preparations were from cultures labeled with [3H]leucine. When the heat-solubilized enzyme was examined, the immune precipitate contained both the 142,000 and 60,000 molecular weight subunits but a wide variety of fragments of varying sizes were also present. When the cytoplasmic membrane solubilized with Triton X-100 at room temperature was examined, both of the subunits were present as single sharp peaks, and the fragments noted above were absent. The molecular weight of the smaller subunit was 60,000, slightly greater than in the heat-extracted enzyme. In the Triton X-100-solubilized preparation, the molar ratio of the 142,000 and 60,000 subunits was exactly 1:1, as determined by the amount of leucine label in each peak on the gels.

A second line of evidence concerning the role of proteolytic activity comes from experiments on the effect of the protease inhibitor phenylmethylsulfonyl fluoride on the solubilization of the enzyme. When the envelope is heated to 60°C in the presence of 4 mM phenylmethylsulfonyl fluoride all of the nitrate reductase activity remains associated with the envelope, and neither of the subunits are present in the solubilized material when it is examined on sodium dodecyl sulfate polyacrylamide gels. These data, which will be described in greater detail in a later paper, strongly indicate that the enzyme is removed from the membrane by protease action when the envelope is heated to 60°C, and that the enzyme itself is slightly degraded by the protease as it is released. This explains the variability in the 58,000 molecular weight subunit, and probably also explains why so much inactive enzyme is present in the heat-solubilized material.

No relationship can be demonstrated between the amount of variation in the smaller peptides on sodium dodecyl sulfate gels and the amount of the smaller 13 S component seen in ultracentrifuge runs. Since this enzyme appears homogeneous on nondenaturing gels and because this 13 S component can be removed by procedures employed in the purification of the enzyme, it must be a portion of the intact enzyme which dissociates from it. This dissociation would make molecular weight measurements in the ultracentrifuge by the equilibrium method extremely difficult.

There are several bacterial reductases which have similarities to nitrate reductase from E. coli. Among these are nitrate reductase from Micrococcus denitrificans (24) and the molybdenum-iron component of Azotobacter nitrogenase (25). They differ in molecular weight, the Micrococcus enzyme being 160,000 and the Azotobacter enzyme, 270,000, but both have absorption spectra virtually identical with E. coli nitrate reductase and both contain molybdenum and non-heme iron. Both of the enzymes also have an unusually high absorbance at 280 nm and are not sensitive to heating at 55-60°C. Micrococcus nitrate reductase is particulate while Azotobacter molybdenum-iron apparently is not.

The most striking feature of the E. coli nitrate reductase is its size. With the exception of viruses, very few proteins have been purified which have a molecular weight of greater than 500,000 and fewer still have been characterized in regard to their physical parameters. This enzyme has several properties which make it useful as a high molecular weight standard in gel filtration columns. It is a spherical protein which can be detected in very small amounts, both by a very simple enzyme assay or by virtue of its high absorbance at 280 nm. If applied to a column in reasonably concentrated amounts, it can even be followed by eye due to its color.

It is of interest to note the large amount of this enzyme produced under maximum induction conditions. If one assumes that the cytoplasmic membrane makes up a maximum of one third of the protein of the cell envelope (26), then 15% of the protein in the cytoplasmic membrane is nitrate reductase.

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