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Purification and Some Properties of Nitrate Reductase (EC 1.7.99.4) from *Escherichia coli* K12

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1. Nitrate reductase was purified 134-fold from *Escherichia coli* K12. The purification procedure involves the release by Triton X-100 of the enzyme from the cell envelope. 2. The purified enzyme exists in aqueous solution either as a monomer (mol.wt. about 220000) or as an associated form (probably a tetramer; mol.wt. about 880000). 3. The purified enzyme has three subunits with apparent mol.wts. of 150000, 67000 and 65000. An additional subunit of apparent mol.wt. 20000 is present in a haem-containing fraction that is also produced by the preparative procedure described. 4. None of the enzyme subunits is present in the cell envelope of cells grown in the absence of nitrate. 5. Reversible changes in the activity of nitrate reductase *in vitro* with $FMNH_2$ as reductant can be induced under circumstances which are without effect on the reduced Benzyl Viologen–NO_3^- activity.

The respiratory nitrate reductase of *Escherichia coli* is noteworthy for several reasons; it is an inducible membrane-bound enzyme whose concentration in the inner membrane of *E. coli* can vary between 0 and 15% of the total protein of that membrane; it is the terminal oxidoreduction enzyme of a proton-translocating respiratory chain; it is a metalloenzyme, containing both iron and molybdenum, and catalyses a two-electron transfer probably from a *b*-type cytochrome (Ruiz-Herrera & De Moss, 1969) to inorganic nitrate by an unknown mechanism.

A prerequisite for an intended programme of investigation into the structural and functional integration of nitrate reductase in the inner membrane of *E. coli* was a knowledge of the size and subunit composition of this enzyme. The purpose of the present paper is to describe the purification and characterization of nitrate reductase which was undertaken to provide this information.

During the course of this work two independent reports were published describing preparations of *E. coli* nitrate reductase (MacGregor *et al.*, 1974; Enoch & Lester, 1974). Although both contained data on the previously unreported subunit composition of the enzyme, these data were not entirely in agreement. Only one of these two papers, namely that of MacGregor *et al.* (1974), evaluated the molecular weight of the native enzyme. The result was different from all previous published values, which were themselves all different from one another (Forget, 1974; MacGregor *et al.*, 1974; Taniguchi & Itagaki, 1960; Showe & De Moss, 1968). These reports therefore left uncertainties over the native and subunit sizes of nitrate reductase.

The present results characterize the native and subunit molecular weights of haem-free nitrate reductase released from the cytoplasmic membrane of *E. coli* under mild conditions by using Triton X-100. Preliminary reports of some parts of this work have been published (Clegg, 1975*a*,*b*; Haddock & Clegg, 1975).

Materials and Methods

Organism

Escherichia coli strain A1002 (K12Y *mel*, *ato*$^-$, *fadR*^{c}, *ilv*$^-$, *lacI*$^-$, *metE*$^-$, *rha*$^-$) was a generous gift from Dr. H. U. Schairer, Max-Planck-Institut für Biologie, 73-Tubingen, West Germany.

Cell growth

Cultures (20 litres) in the medium described by Forget (1974) were grown anaerobically as described by Kemp *et al.* (1975) to mid- or late-exponential phase. The inoculum was 1 litre of culture in the same medium. Sodium azide was sometimes omitted from the medium, without influencing the properties of the enzyme purified from cells grown in this way.

Cell harvesting

Cultures were centrifuged at room temperature (20°C) and washed twice in 50mM-potassium phosphate buffer, pH 6.8. Between 30 and 50g packed wet wt. of cells was obtained per 20 litres of culture.

Assays

Continuous spectrophotometric assay of nitrate reductase by using reduced Benzyl Viologen or $FMNH_2$ as reductant was carried out by the method of Kemp *et al.* (1975). Protein was assayed by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Cytochrome *b* concentration was determined from reduced-minus-oxidized difference spectra, assuming a millimolar extinction coefficient of 19 litre·mmol^{-1}·cm^{-1} at 560nm.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and radioactivity-counting procedure

Sample preparation, disaggregation, electrophoresis in polyacrylamide gels, gel densitometry, slicing and radioactive counting were all performed as described by Clegg & Skyrme (1973). Gels of between 5 and 10% (w/v) acrylamide concentration were used. Plots of mobility (relative to Bromphenol Blue) against log(mol.wt.) were obtained by using the following proteins (subunit mol.wts. in brackets): de-branching enzyme [165000, Taylor *et al.* (1975)]; phosphorylase kinase [α, 145000; β, 130000, Cohen (1973)]; phosphorylase (100000); bovine serum albumin, (67000); carbonic anhydrase (29000); horse heart cytochrome *c* (12500).

Analytical ultracentrifugation

This was done in a Beckman model E analytical ultracentrifuge with an AN-D rotor and a double-sector cell, at 18°C.

Reagents

FMN (sodium salt), deoxyribonuclease I (EC 3.1.4.5; type DN-100) and ribonuclease A (EC 3.1.4.22; type 1-A) were purchased from Sigma Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K., and β-galactosidase (EC 3.2.1.23, from *E. coli*) was from Boehringer (U.K.) Ltd. London W5 2TZ, U.K. Other chemicals were from BDH Chemicals Ltd., Poole, Dorset, U.K. Radiochemicals were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Phosphorylase (EC 2.4.1.1), phosphorylase kinase (EC 2.7.1.38) and de-branching enzyme (EC 3.2.1.68) from rabbit muscle were gifts from Dr. Philip Cohen, Department of Biochemistry, University of Dundee, Carbonic anhydrase (EC 4.2.1.1) was a gift from Dr. John Kernohan, Department of Biochemistry, University of Dundee.

Purification of nitrate reductase

The preparative procedure was developed from an observation by MacGregor & Schnaitman (1971) that nitrate reductase activity can be purified more than tenfold by ion-exchange chromatography on DEAE-cellulose of that fraction of an *E. coli* cell-envelope preparation soluble in Triton X-100.

All operations, except where otherwise stated, were performed at 0–4°C. Frozen cell pellets (50g wet wt.) were thawed and suspended to a thick slurry in 100ml of 50mM-Tris/HCl/5mM-$MgCl_2$, pH7.8. Portions (25ml) of this slurry were mixed with 50g of glass beads (100 mesh) and shaken for four 30s periods in a Braun MSK cell homogenizer (Shandon Southern Instruments Ltd., Camberly, Surrey, U.K.) operating at its higher speed setting. Deoxyribonuclease and ribonuclease (50μg of each/ml) were added to the resulting viscous suspension, which was then left at 4°C for 15min. The supernatant material was decanted from the settled glass beads, which were then washed twice with 150ml of the above buffer. The washings were combined with the decanted material and this fraction was centrifuged in the 6 × 100ml rotor of an MSE 18 centrifuge for 10min at 3000rev./min. The supernatant fraction was poured off and kept. The pellets were resuspended in 50ml of the above buffer and sonicated with an MSE 150W ultrasonic generator (MSE Scientific Instruments, Manor Royal, Crawley, Sussex, U.K.) for five periods of 30s interspersed with 30s intervals for cooling. The resulting suspension was centrifuged as described above. Supernatant fluid from this centrifugation was combined with those from the previous centrifugation; 0.5M-EDTA solution (neutralized with KOH to pH7.8) was added to give a final EDTA concentration of 7.5mM. This fraction, designated 'crude cell-free fraction', was centrifuged for 90min at 36000rev./min in the 8 × 50ml rotor of an MSE 65 centrifuge. The resulting supernatant fluid was discarded and the pellets were resuspended by homogenization in 100ml of 50mM-Tris/HCl/1mM-EDTA, pH7.8. Large particulate material was removed by centrifugation for 10min at 3000rev./min in the 8 × 10ml rotor of an MSE 18 centrifuge, and the supernatant fluid from this step was centrifuged at 36000rev./min (as described above) to sediment smaller particulate material. Pellets were resuspended by homogenization in 10mM-potassium phosphate buffer, pH7.8, containing 0.1mM-2-mercaptoethanol. This suspension was again centrifuged at 36000rev./min as described above. Pelleted material was resuspended to a protein concentration of 10–15mg/ml in the same phosphate buffer. To this suspension (crude envelope preparation) was added a 20% (v/v) solution of Triton X-100 in the same buffer to a final Triton concentration of 2%. The mixture was stirred at 21°C for 30min, then cooled again to 4°C, and centrifuged at 36000rev./min as described above. The Triton-soluble supernatant fraction, consisting principally of inner-membrane proteins (Schnaitman, 1971), was poured off and kept; the pellet was discarded.

Triton-soluble proteins were adsorbed on to DEAE cellulose (Whatman DE32 or DE52) equilibrated with 10mM-potassium phosphate buffer/0.1mM-2-mercaptoethanol/1% (v/v) Triton X-100, pH7.8, in a column (2.5cm × 25cm). The column was washed sequentially with 100ml of the above buffer, with a linear gradient of 0–0.2M-NaCl in 500ml of the same buffer and then with 100ml of 0.2M-NaCl in the above buffer. Active fractions from the column were pooled, dialysed for a minimum of 1h against the above phosphate buffer and concentrated by adsorption on to a second DEAE-cellulose column (4.5cm diam. × 1.5cm long), followed by elution with 0.1M-potassium phosphate buffer/0.1mM-2-mercaptoethanol/1% (v/v) Triton X-100/0.4M-NaCl, pH7.8. Nitrate reductase-containing fractions from this column were pooled and dialysed against the above 10mM-phosphate buffer containing 0.1mM-2-mercaptoethanol, and 250g of polyethylene glycol/litre (average mol.wt. 6000) until the volume of the sample had diminished to about 2ml. Samples of this concentrated material (up to 1ml) were layered on 5–21% (w/w) linear sucrose gradients [21ml in the above dialysis buffer, containing 1% (v/v) Triton X-100 but omitting polyethylene glycol] and centrifuged in the 3 × 23ml swinging-bucket rotor of an MSE 65 centrifuge for 17h at 25000rev./min. The gradient was fractionated by piercing the bottom of the tube and collecting fractions of equal volume (usually about 1ml) by dripping under gravity. Those containing nitrate reductase activity were pooled as described in the Results and Discussion section. Sucrose and Triton X-100 were removed, when necessary, by adsorbing nitrate reductase on to a small column (0.5cm × 2cm) of DEAE-cellulose and washing with at least 30 column-volumes of 100mM-potassium phosphate buffer/0.1mM-2-mercaptoethanol, pH7.8. Protein was eluted with the same buffer containing 0.4M-NaCl. The enzyme was also concentrated by this step if necessary. Triton X-100 content of enzyme solutions was determined by measuring their E_{280}/E_{410} ratio. [Nitrate reductase itself has an E_{280}/E_{410} ratio of 7.15, and a 1mg/ml solution has an E_{280} of 2.5 (MacGregor *et al.*, 1974). A 0.01% (v/v) solution of Triton X-100 has an E_{280} of 0.2 and zero absorbance at 410nm.] Triton X-100 removal by DEAE-cellulose chromatography as above, measured in this way, was to a final value of below 0.001%. The ionic composition of the purified Triton-free material was adjusted by dialysis as required.

Results and Discussion

Purification of nitrate reductase

Table 1 records the data from a typical purification. The resulting preparation has a specific activity slightly lower than that reported by Forget (1974), but the procedure has the merits of a tenfold better yield and much greater speed and simplicity relative to that of Forget (1974). The elution of protein and nitrate reductase activity from the first DEAE-cellulose column (results not shown) almost exactly resembled that demonstrated by MacGregor & Schnaitman (1971). The distribution of protein and nitrate reductase activity in the preparative sucrose gradient comprising the final purification step is shown in Fig. 1(*a*). The relative sizes of the three peaks of activity (marked I, II and III) varied considerably from one preparation to the next. Peak I is the pure nitrate reductase whose properties are described here. The nature of material in peaks II and III is discussed in the following sections.

Reversible association and molecular weight

If the preparative sucrose-gradient separation was performed on material that had not first been concentrated by dialysis against polyethylene glycol the

Table 1. *Purification of nitrate reductase*

The fractions are those described in the Materials and Methods section, where the purification procedure is also given. Peak-I nitrate reductase is characterized in the text and in Fig. 1. Activity is expressed as μmol of NO_2^- produced/min at 30°C.

	Protein		Activity			
Fraction	Total (mg)	(%)	(units)	(%)	Specific activity (units/mg of protein)	Purification (fold)
Cell suspension	5217	100	8874	100	1.70	1
Cell-free extract	4680	90	7811	88	1.67	0.98
Crude envelope fraction	1072	23	7451	84	6.95	4.1
Triton X-100 extract	381	7.3	7351	83	19.3	11.3
Pooled DEAE-cellulose eluate	83	1.6	6920	78	83.2	49
Polyethylene glycol-concentrated DEAE-cellulose eluate	83	1.6	4144	47	49.2	29
Sucrose-gradient-purified enzyme (peak I)	11	0.2	2516	28	229	134

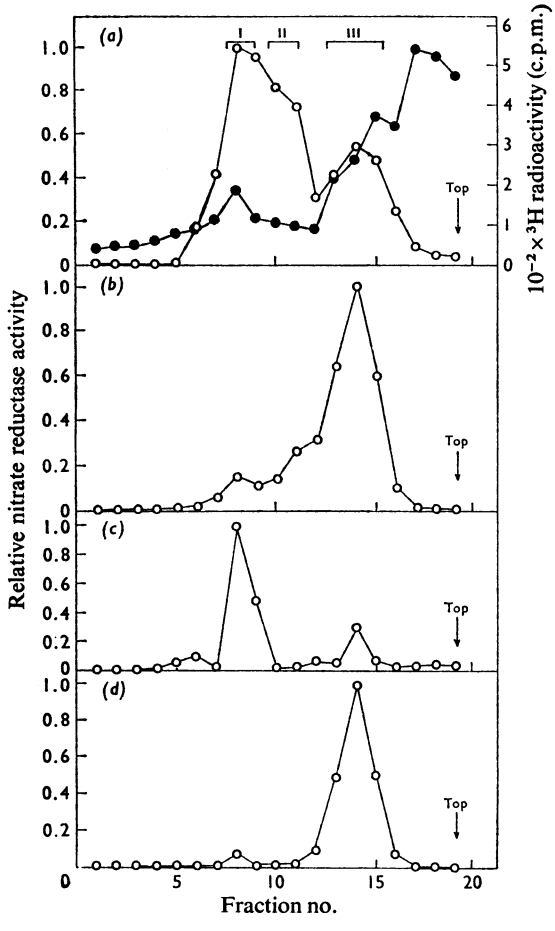


Fig. 1. *Sedimentation of nitrate reductase in sucrose gradients*

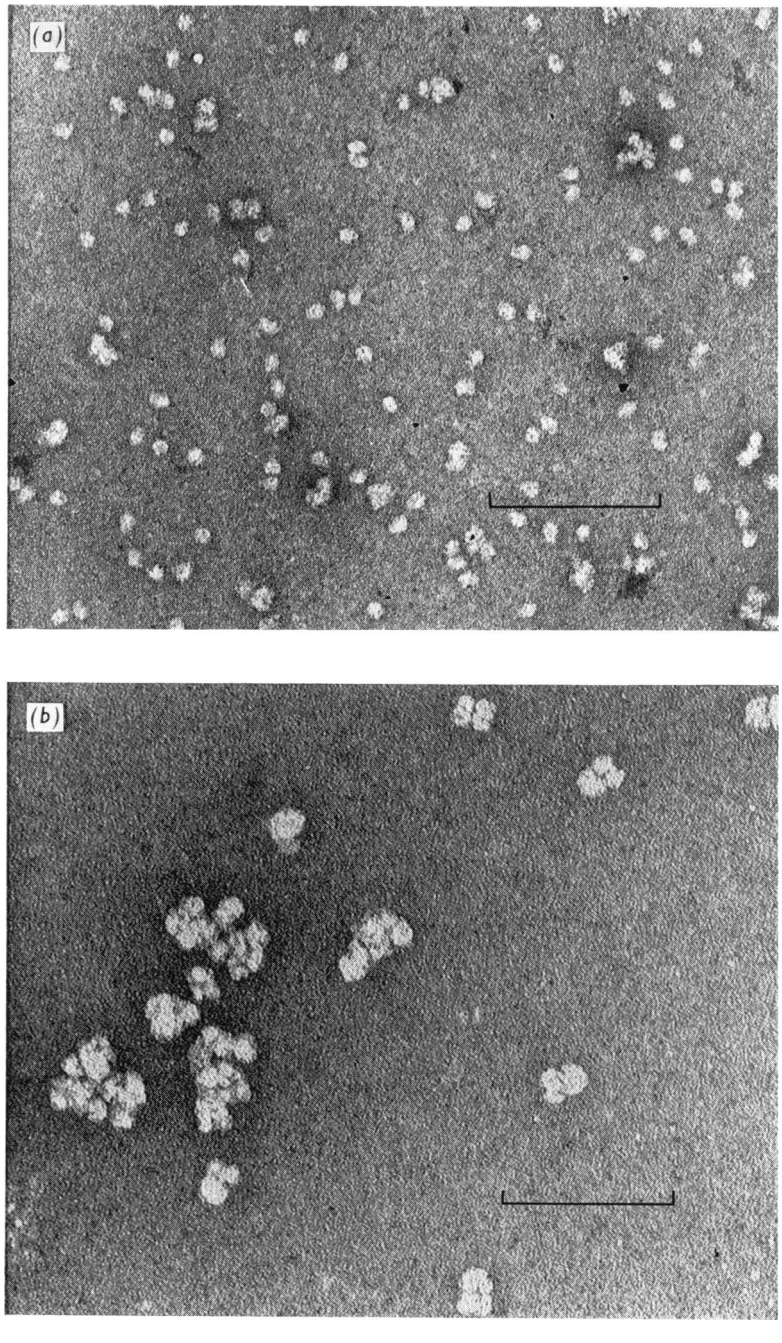
Gradients were constituted and run as described in the Materials and Methods section. The sample loaded in gradient (*a*) was derived from cells grown in the presence of 0.05μCi of [^{3}H]valine/ml. Protein was measured as filter-bound ^{3}H radioactivity (c.p.m.) after precipitation of a portion of each fraction with 10% (w/v) trichloroacetic acid and filtering each through a glass-fibre (Whatman GFA) filter disc. Samples were as follows: (*a*) 0.25ml (7.2mg of protein) of polyethylene glycol-concentrated DEAE-cellulose eluate; (*b*) 0.25ml (0.2mg of protein) of pooled DEAE-cellulose eluate before polyethylene glycol concentration; (*c*) 0.25ml (0.8mg of protein) of peak-I fractions pooled from a gradient similar to that in (*a*) above; (*d*) 0.25ml (2.1mg of protein) of peak-III fractions pooled from a gradient similar to that in (*a*) above. Nitrate reductase activity (○) is expressed relative to that of the most active fraction in each gradient. ●, ^{3}H radioactivity.

activity distribution shown in Fig. 1(*b*) resulted. This is markedly different from that shown in Fig. 1(*a*) in that almost no peak-I activity is discernible. It is also noteworthy that this procedure yields no further significant purification of nitrate reductase. Material from Peaks I and III (Fig. 1*a*) when re-centrifuged under identical conditions after dialysis against the buffer in which the gradients were run, showed the activity distributions shown in Fig. 1(*c*) and Fig. 1(*d*) respectively. The two activity peaks in Fig. 1(*c*) had the same specific activity and polypeptide composition (see below), both characteristic of the original peak-I material. If material from peak III (Fig. 1*a* or 1*b*) was first subjected to any one of a number of concentrating or lipid-removing procedures, and then centrifuged in sucrose gradients, the activity distribution was similar to that shown in Fig. 1(*a*).

Chromatography of peak-I material on Sepharose 4B also yielded two eluting peaks of activity having apparent mol.wts. of 290000 and 620000 (see Fig. 2). Estimation of apparent mol.wts. by the method of Martin & Ames (1961) from the sucrose-gradient data by using β-galactosidase as a standard, gave values of 740000 and 230000 for peaks I and III respectively. These empirical estimates are based on the assumption that nitrate reductase has the same shape and partial specific volume as the standard. Its ready solubility in dilute aqueous buffers suggests an absence of bound lipid, which would profoundly alter $\bar{v}$.

The most simple interpretation of these data is that a protein–protein interaction is occurring, transforming the native protein (mol.wt. between 2×10^5 and 3×10^5) into an associated form. More precise molecular-weight measurements are necessary to determine the degree of association (see below). This conclusion in some measure clarifies the disparity between published values of the native molecular weight of this enzyme (Taniguchi & Itagaki, 1960; Showe & De Moss, 1968; Forget, 1974; MacGregor *et al.*, 1974). The equilibrium of this association is such that the associated form is favoured at high protein concentration and low detergent concentration. Dilution and the addition of Triton X-100 to a detergent-free nitrate reductase solution does generate some monomeric material (see Fig. 1*c*), but the depolymerization is much more efficiently (>90%) caused by 0.2% deoxycholate at pH8.0 (results not shown).

More accurate values of molecular weight than those above were calculated from analytical-ultracentrifuge data. Sedimentation coefficients ($s_{20,w}$) of 22.4 and 9.9S were obtained for the associated and monomeric forms of the enzyme. The empirical relationship, $\text{mol.wt.} = 8000 \cdot (s_{20,w})^{3/2}$ [which gives results accurate to within ±10% for a wide range of well characterized soluble proteins (P. Cohen, personal communication)] was used to derive mol.wts. of 9×10^5 and 2.5×10^5 for the two forms of nitrate reductase. Sedimentation-equilibrium measurements in the presence of 0.2% deoxycholate gave a mol.wt.



EXPLANATION OF PLATE 1

Electron micrographs of negatively stained nitrate reductase

The bar represents 100nm on both photographs and both preparations were negatively stained in saturated aq. uranyl acetate. In (*a*) unfixed protein at 40μg/ml was mounted as described by Valentine *et al.* (1968). In (*b*), protein at a concentration of 80μg/ml in 10mM-potassium phosphate buffer, pH 7.8, was fixed by the addition of 1% (v/v) glutaraldehyde for 20min at 0°C. The mounting was then performed as in (*a*).

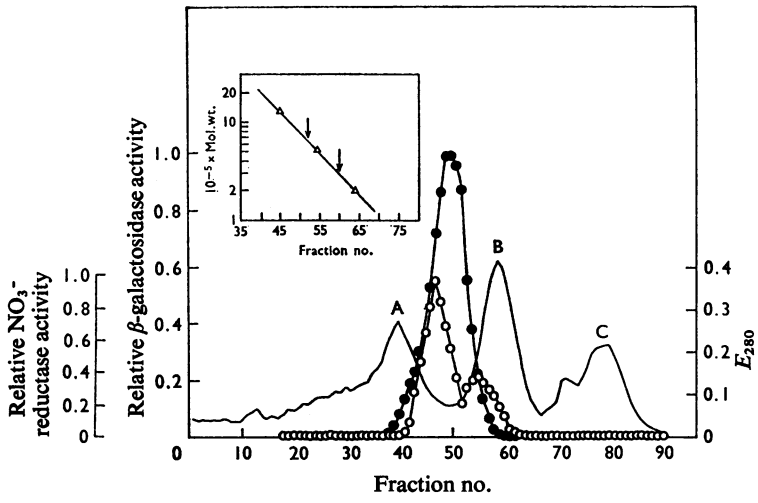


Fig. 2. *Chromatography on Sepharose 4B of nitrate reductase*

The sample (1 ml) contained 6 mg of phosphorylase (mol.wt. 200000), 6 mg of phosphorylase kinase (mol.wt. 1.28×10^6), 0.2 mg of β-galactosidase (mol.wt. 520000), and 0.07 mg of peak-I nitrate reductase. Fraction volume was 1.88 ml and the column void volume was 60 ml. Fraction 1 was collected after 36 ml had emerged from the column (1.5 cm × 110 cm), which was equilibrated and developed in 50 mM-sodium glycerophosphate buffer, pH 7.0, containing 1 mM-EDTA and 1.5 mM-2-mercaptoethanol. The E_{280} (——) of each fraction was measured; peaks A and B are phosphorylase kinase and phosphorylase respectively. Peak C is bovine serum albumin, which is added as a stabilizing agent to the commercial preparation of β-galactosidase. Nitrate reductase activity (○) was assayed in each fraction. β-Galactosidase activity (●, determined spectrophotometrically by the rate of *o*-nitrophenyl-β-D-galactoside hydrolysis) was also measured. Each enzyme activity is expressed relative to that of the most active fraction eluted. The inset shows the logarithmic plot of molecular weight against peak elution position used to derive molecular weights for the two nitrate reductase species (arrows).

of 220×10^3 for the monomeric protein. These measurements indicate that a monomer–tetramer association occurs with *E. coli* nitrate reductase, as with that of *Klebsiella aerogenes* (Van't Riet & Planta, 1975).

Electron microscopy

The slightly aspherical nitrate reductase molecules seen in unfixed negatively stained electron-microscopic preparations (Plate 1*a*) are probably the monomers (mol.wt. about 220000) characterized above; their size is consistent with this interpretation. After light cross-linking, those units which are not grossly aggregated appear to be tetramers of the molecules seen in unfixed preparations (Plate 1*b*). Each monomeric unit appears to have a cleft partially transversing its short axis.

Subunit composition

Electrophoresis in sodium dodecyl sulphate/polyacrylamide gels showed that the enzyme has three subunits (see Fig. 3*a*). Their apparent mol.wts. were as follows: α, 150000; β_1, 67000; β_2, 65000. These values are in reasonable agreement with those reported by MacGregor *et al.* (1974). Like these authors, I found two closely migrating peaks in the 65000-mol. wt. region, which were not resolved when radioactively labelled enzyme was detected by gel slicing and counting for radioactivity. In these experiments radioactively labelled enzyme was prepared in the following way. *E. coli* was first grown in the presence of ^{3}H- or ^{14}C-labelled isoleucine, valine or methionine to label cell protein. The resulting radioactively labelled cells were then used as starting material for a small-scale preparation of nitrate reductase. A markedly different content of any one of these amino acids in the α and $(\beta_1+\beta_2)$ subunits would have resulted in an erroneous estimate (from relative c.p.m.) of relative protein quantification, since this method in fact equates the ratio of the content of one amino acid in the α and $(\beta_1+\beta_2)$ subunits with the ratio of protein in these subunits. In practice the same ratio (2.3) in a given gel separation of detected radioactivity (c.p.m.) in the α subunit to detected radioactivity (c.p.m.) in the $(\beta_1+\beta_2)$ subunit was obtained by using any of the above amino acids as a radioactive protein precursor. This is the same as the ratio of the mol.wts. of α to $(\beta_1+\beta_2)$ subunits and leads to the conclusion that the prepared enzyme has α and $(\beta_1+\beta_2)$ subunits in a 'molar' ratio of 1:1. It

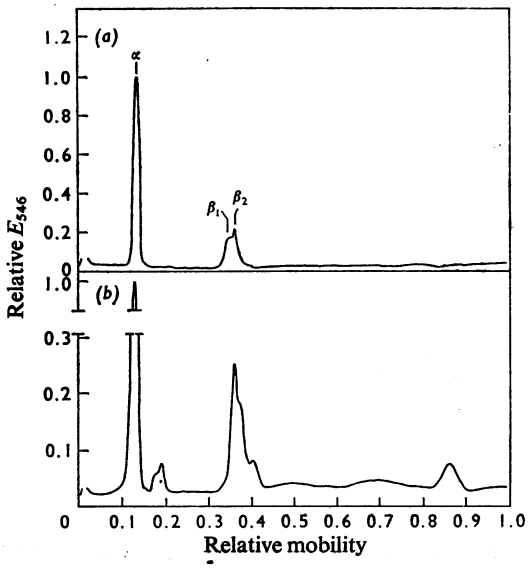


Fig. 3. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of nitrate reductase preparations*

Densitometer traces of Coomassie Blue-stained 7.5% polyacrylamide gels are shown. Mobility is plotted relative to that of a Bromophenol Blue marker dye and E_{546} relative to that of the highest peak in the scan. Samples were: (*a*) 60 μg of peak-I nitrate reductase from a sucrose gradient as in Fig. 1(*a*); (*b*) 92 μg of peak-II nitrate reductase.

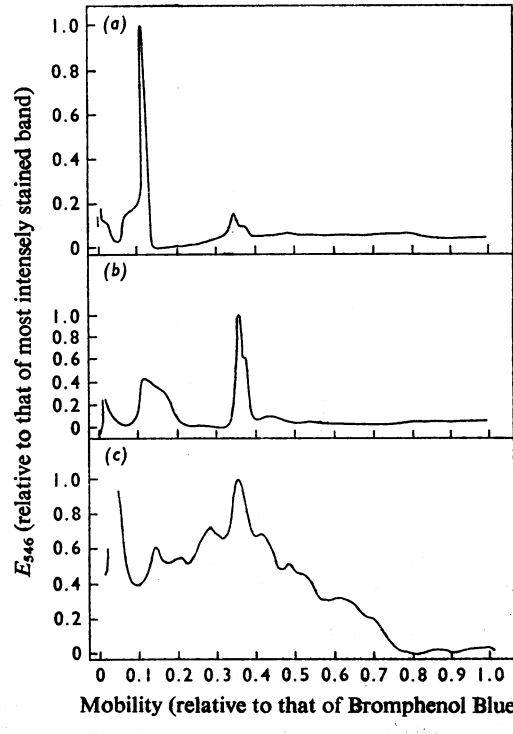


Fig. 4. *Effect of pretreatments on the sodium dodecyl sulphate/polyacrylamide-gel-electrophoretic profile of nitrate reductase*

Densitometer traces of Coomassie Blue-stained 7.5% polyacrylamide gels are shown. Samples of purified nitrate reductase (those for *a* and *b* were stored for 5 weeks at 4°C before use) were treated as follows before electrophoresis: (*a*) 31 μg, disaggregated as normal at 100°C for 5 min in 10 mM-sodium phosphate buffer, pH 7.2/1% (w/v) sodium dodecyl sulphate/1% (v/v) 2-mercaptoethanol; (*b*) 30 μg, disaggregated as above but with the 2-mercaptoethanol concentration increased to 10% (v/v); (*c*) 200 μg, oxidized with performate (Weber *et al.*, 1972); variation in disaggregation conditions as above was without affect on the electrophoretic profile. In control experiments, increasing the concentration of 2-mercaptoethanol to 10% (v/v) had no effect on the mobility of standard proteins in either the presence or the absence of nitrate reductase.

is noteworthy that because of the difficulty in resolving the subunits β_1 and β_2 from one another, their protein contents are summed for the purpose of this calculation. Therefore this 'molar' ratio is not a true ratio of numbers of molecules since 1 'mol' of $\beta_1+\beta_2$ contains approximately a $\frac{1}{2}$mol fraction each of β_1 and β_2. This means that the minimum association of subunit molecules allowed by this 'molar' ratio is $2\alpha:1\beta_1:1\beta_2$.

Integration of the areas under the peaks of densitometer tracings of Coomassie Blue-stained gels also gives a 'molar' ratio, $\alpha/(\beta_1+\beta_2)$ of 1:1. In this case it was assumed that equal weights of protein in the α and $(\beta_1+\beta_2)$ regions of the gel are stained with equal intensity by Coomassie Blue.

Molecular weight of the enzyme calculated from subunit composition

The subunit data for nitrate reductase are difficult to reconcile with the native-molecular-weight determinations. Assuming equimolar concentrations of subunits β_1 and β_2 then the minimum mol.wt. imposed by the measured subunit molar abundancies is $[(2\times150)+67+65]\times10^3=432\times10^3$; this value does not correspond to that of either the monomeric or tetrameric native enzyme. One possible explanation for this discrepancy is that the α subunit may not be a single polypeptide but may be a homologous or heterologous dimer of β subunits. In this case the native monomeric enzyme would consist of three or perhaps four β subunits; if it were three, then of course the molar concentrations of β_1 and β_2 subunits could not be equal, as supposed above.

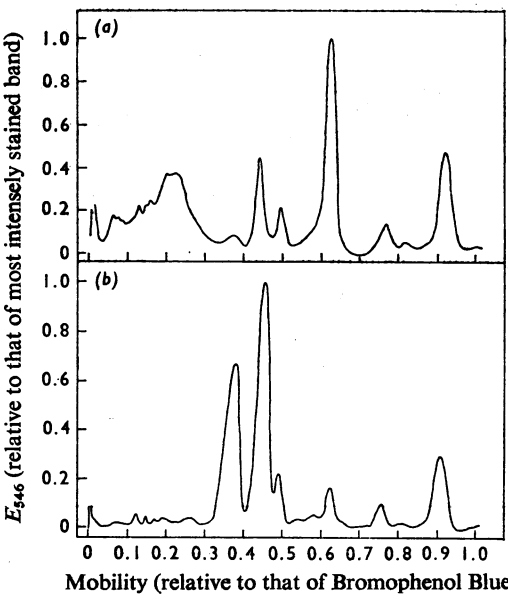


Fig. 5. *Influence of mercaptoethanol concentration during sample disaggregation on the electrophoretic mobilities of outer-membrane polypeptides in a sodium dodecyl sulphate/polyacrylamide-gel system*

Densitometer traces of Coomassie Blue-stained 7.5% polyacrylamide gels are shown. Outer membranes were prepared from *E. coli* by the method of Osborn *et al.* (1972). A sample (200 μg) of outer-membrane protein was disaggregated at 70°C for 20 min in 10 mM-sodium phosphate buffer, pH 7.2, containing 1% (w/v) sodium dodecyl sulphate; 2-mercaptoethanol was present at a concentration of 1% (v/v) for trace (*a*), or 10% (v/v) for trace (*b*). The profile in trace (*b*) could be duplicated by sample disaggregation at 100°C for 5 min; under these conditions, the 2-mercaptoethanol concentration (in the range 1–10%, v/v) had no effect on the electrophoretic profile.

A number of observations support this possibility. First, the relative 'molar' abundance of $\alpha/(\beta_1+\beta_2)$ increases as the enzyme is allowed to age at 4°C aerobically (compare Figs. 4*a* and 3*a*). Secondly, sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of performate-oxidized nitrate reductase yields little α-band material and a major peak in the β-band region; however, the unexplained presence of large numbers of other polypeptides in such samples necessarily limits the confidence that can be placed in this result (Fig. 4*c*). Thirdly, when sodium dodecyl sulphate/polyacrylamide-gel electrophoresis is done on nitrate reductase disaggregated in the presence of 10% (v/v) 2-mercaptoethanol but otherwise as normal, the quantity of α- relative to β-band material is smaller than in a control, disaggregated in the presence of 1% (v/v) 2-mercaptoethanol as normal (Fig. 4*a* and 4*b*). This treatment with high concentrations of reductant, also has an effect on the migration characteristics of outer-membrane polypeptides of *E. coli* (Fig. 5*a* and 5*b*). It is already well established (Inouye & Yee, 1973; Schnaitman, 1973) that sample temperature during disaggregation affects the electrophoretic mobilities of some outer-membrane polypeptides. The changes induced here by reductant are qualitatively the same as those inducible by variation in the temperature of disaggregation.

Other possible explanations for the discrepancy between native and subunit molecular weights of nitrate reductase are that one or more of the apparent subunits is a degradation product of one or more of the others (unlikely because of the nature of the changes in apparent subunit composition induced by storage) or that an allelism is present such that the purified enzyme consists of two populations of molecules, namely α,β_1 and α,β_2. This last possibility is extremely difficult to disprove and should therefore be considered only if rigorous efforts to disprove the other possibilities should fail.

Induction by nitrate

Small-scale enzyme preparations were performed on the mixed yields of a nitrate-induced and a non-induced *E. coli* culture. The induced culture was grown in the presence of [^{3}H]valine and the non-induced culture in the presence of [^{14}C]valine. Analysis of these enzyme preparations by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis demonstrated that neither the α nor the $\beta_1+\beta_2$ subunits contained detectable ^{14}C. Therefore as would be expected, in the absence of nitrate the enzyme subunits are either not synthesized constitutively or they are synthesized and rapidly degraded without being incorporated into the cytoplasmic membrane.

Stability

Slow freezing inactivates the purified enzyme (cf. MacGregor *et al.*, 1974), but rapid freezing in liquid N_2 and thawing at room temperature can be repeated at least ten times without measurable effect on its activity assayed with reduced Benzyl Viologen. This activity is also stable to prolonged storage at 4°C. The $FMNH_2$–NO_3^- reductase activity of the enzyme behaves in a more complex fashion, as shown in Table 2. Triton X-100 solubilization of the crude envelope fraction appears to have an inhibitory action, which is reversed in the final Triton-free purified enzyme. The presence of Triton X-100 itself is not, however, inhibitory, as is shown by the high activity of the immune precipitate resuspended in Triton-containing buffer. On storage at 4°C the pure enzyme loses $FMNH_2$–NO_3^- reductase activity, but the loss can be partially reversed by immune precipitation or

Table 2. *Activation and inactivation of the $FMNH_2$–NO_3^- reductase activity in crude and purified preparations*

Activity is expressed as μmol of NO_2^- produced/min per mg of protein, measured at 30°C. The preparation of the Triton X-100 extract was as described in the Materials and Methods section; no nitrate reductase activity with either reductant was detectable in the Triton-insoluble residue. The activities of the immune precipitates are expressed per mg of bacterial protein in the fraction from which they were precipitated, so as to be comparable with the activities measured in those fractions. Immune precipitation was done [by using rabbit antiserum to purified nitrate reductase (R. A. Clegg, unpublished work)] under conditions which resulted in the quantitative cross-reaction of nitrate reductase

Preparation	Reduced Benzyl Viologen–NO_3^- reductase activity	$FMNH_2$–NO_3^- reductase activity	(Rate of nitrate reduction with reduced Benzyl Viologen)/(rate with $FMNH_2$)
Crude envelope	0.886	0.15	6
1%-Triton extract of crude envelope	2.21	0.060	37
Immune precipitate from above crude extract	2.21	0.200	11
Pure enzyme prepared from above crude extract	62.6	10.9	5.8
Pure enzyme aged at 4°C for 60 days	62.0	0.92	66
Aged pure enzyme, after 10 cycles of freeze-thawing	63.0	2.5	25
Immune precipitate from aged pure enzyme	61.8	1.7	36

freeze-thawing. Although the mechanism(s) underlying these effects remains unknown they are noteworthy for indicating that $FMNH_2$ and reduced Benzyl Viologen may reduce nitrate reductase at different sites.

Haem-containing nitrate reductase

The preparative scheme described above yields, in addition to the haem-free enzyme, a haem-containing nitrate reductase (peak II in Fig. 1*a*) having 15 nmol of haem/mg of protein, whose subunit composition is shown in Fig. 3(*b*). It contains, in addition to the α, β_1 and β_2 subunits of haem-free nitrate reductase, a major component of apparent mol.wt. 20000 whose molar abundance identifies it as the haem-bearing polypeptide. The subunit composition of peak-II material (including minor components) is closely similar to that of *E. coli* membrane proteins cross-reacting with rabbit antiserum to purified nitrate reductase (Clegg, 1975*a*; see also MacGregor, 1975).

Comparison of haem-free and haem-containing nitrate reductases with previously described preparations

Nitrate reductase and its functionally associated *b*-type cytochrome are synthesized in large quantities after addition of nitrate to anaerobic *E. coli* cultures (Showe & De Moss, 1968), and both have long been known to be purified together from crude cell extracts (Itagaki *et al.*, 1962). This latter observation has been confirmed by Enoch & Lester (1974), who purified haem-containing nitrate reductase after its release by Triton X-100 from the envelope of *E. coli*. Their enzyme contained subunits of mol.wts. 155000, 63000 and 19000 (cf. the preceding section). The smallest subunit was identified as the haem-bearing one, and could be removed from the others by heat/alkali treatment. Such a treatment (originally described by Taniguchi & Itagaki, 1960) has been the one most widely used to effect the release of nitrate reductase from the *E. coli* envelope (Showe & De Moss, 1968; MacGregor *et al.*, 1974). Enoch & Lester (1974) found no haem-free nitrate reductase in the absence of this treatment and concluded that it was only an artifact resulting from the severity of the treatment. MacGregor (1975) has further characterized the heat/alkali treatment and found that it owes its effectiveness to the action of a proteinase, greatly enhanced under those conditions. She finds (MacGregor, 1975) that material cross-reacting (in the presence of Triton X-100) with antibody raised against nitrate reductase purified from a heat/alkali-treated envelope extract consists of three subunits (detected by gel slicing and counting for radioactivity), essentially those described by Enoch & Lester (1974). She concludes that haem-free nitrate reductase derived from a membrane fraction is a proteolytic artifact induced by heat/alkali treatment and that proteolysis is also responsible for the fine structure in the β region (in the nomenclature of the present paper), which was originally described as consisting of two peaks (MacGregor *et al.*, 1974). This conclusion implies, among other things, that the Triton X-100 extraction procedure, as used in the present study, does not entail proteolytic damage to nitrate reductase. Be this as it may, the data presented above show that this procedure has two previously unrecognised properties. First, it can release haem-free nitrate reductase as well as a related haem-containing enzyme; secondly, in sodium dodecyl sulphate/polyacrylamide-gel electrophoretic analyses, the β region of both such preparations consists of two polypeptides. These two are not resolved from one another by gel-slicing and radio-

activity-counting techniques, but this absence of resolution does not constitute evidence, as proposed by MacGregor (1975), that the β region in the gel consists of only one band.

The native molecular weights (or sedimentation coefficients) of previously described haem-free nitrate reductases from *E. coli* all approximate to those of either the monomeric or tetrameric enzyme. The reasons why some authors observe monomers whereas others observe associated molecules are not clear, but probably are to be found in the experimental details of the preparative procedures [e.g. the use of deoxycholate in the presumably monomeric preparation of Forget (1974)].

The haem-containing nitrate reductases of Enoch & Lester (1974) and MacGregor (1975) have not been characterized with respect to their native molecular weights. The sedimentation results (Fig. 1*a*) of the present study suggest that this complex has a mol.wt. in the region of 500000 and therefore probably contains two nitrate reductase monomers.

Relation of structure to function

Transmembrane proton translocation is associated with electron flow from the quinone pool to nitrate through cytochrome $b_{556}^{NO_3^-}$ and nitrate reductase *in vivo* (Brice *et al.*, 1974; Downie, 1974). For the eventual understanding of the mechanism of the coupled electron and proton-conduction events, it is necessary that the sites of interaction with the enzyme's physiological reductant and oxidant are identified and the topography of these sites determined (cf. Kemp *et al.*, 1975). In this context the recognition of multiple reductant sites (which may perhaps be artifacts) is important. It remains to be determined whether nitrate reductase *in vivo* exists as a tetramer, whose dimensions would allow it easily to span the cytoplasmic membrane, or as a monomer, and whether sites on the enzyme are accessible from one or both sides of that membrane.

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