

Localization and Regulation of Synthesis of Nitrate Reductase in *Escherichia coli*

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The nitrate reductase of *Escherichia coli* K-12 was localized in a particulate fraction of the cell and it sedimented as if it were bound to a large substructure that is subject to fragmentation during cell disruption procedures. Soluble enzyme, exhibiting a homogenous profile in sucrose gradients, was released from this fraction by an alkaline-heat treatment. Less than 1.5% of total active nitrate reductase apparently occurred in this soluble form during the course of formation of the particulate enzyme. Enzyme synthesis was repressed by aeration in the presence or absence of nitrate. Under anaerobic conditions, nitrate reductase was synthesized at a rate that could be increased 20-fold by the addition of nitrate. When enzyme synthesis was initiated by induction with nitrate or anaerobiosis, biphasic kinetics were obtained. We interpreted the results as evidence for the existence of a redox-sensitive repressor which mediates nitrate reductase regulation.

The complex of respiratory enzymes found in *Escherichia coli* K-12 appears to be an ideal subject for the study of the synthesis and regulation of organized elements in cells. Subject to elaborate control mechanisms, the synthesis of many of these enzymes can be initiated or repressed by varying the culture conditions. Because these enzymes are nonobligatory for the growth of a facultative anaerobe, the isolation and study of mutants are facilitated. We initiated such a study with the investigation of nitrate reductase. This is the last enzyme in an electron-transport chain which enables *E. coli* to utilize nitrate instead of oxygen as a terminal electron acceptor (11, 12), carrying out what has been termed "anaerobic respiration."

Nitrate reductase has been studied in several different higher plants and bacteria, including some strains of *E. coli*. These studies have been reviewed by Taniguchi (14) and by Nason (7). The functional properties and physical characteristics of the enzyme found in different organisms vary widely. Nitrate reductase in *E. coli* (Yamaguchi) is principally membrane-bound and is linked functionally to cytochrome b_1 (2, 3, 4). This enzyme was solubilized and purified by Taniguchi and Itagaki (15), who reported its sedimentation constant as 25S and calculated its molecular weight to be 1 million. A soluble nitrate reductase not linked to cytochrome has been obtained by Nicholas and Nason (8) from *E. coli* B grown on a synthetic medium.

Nitrate reductase has been studied in strains of

E. coli grown aerobically with (2) and without (16) nitrate and anaerobically on both complex (15) and synthetic media (1, 5). Various factors seem to affect the synthesis and regulation of the enzyme. Because the varied and sometimes conflicting findings on the nature of nitrate reductase and the effects of environmental factors on its synthesis have been obtained with different strains of *E. coli*, we undertook a study of the physical state and mode of regulation of the enzyme in a single strain, *E. coli* K-12.

MATERIALS AND METHODS

Bacterial strains. Experiments on the effects of various cell disruption procedures were performed with *E. coli* K-10 obtained from M. Santer. Otherwise, the following two strains of *E. coli* K-12 were utilized—strain 3300, which is constitutive for β -galactosidase, and strain 3000, which is inducible for this enzyme. Results obtained in strain K-10 were confirmed in strain K-12.

Media. All liquid media employed the minimal salts base of Sypherd and Strauss (13), supplemented with 50 μ g of thiamine HCl per ml (Calbiochem, Los Angeles, Calif.) and 1% glucose. Broth medium, utilized throughout except as noted, consisted of the synthetic medium enriched by the addition of 4 g of Nutrient Broth powder per liter (Difco). For the induction of nitrate reductase, potassium nitrate (Fisher Scientific Co., Fairlawn, N.J.) was added to a final concentration of 1%. For the experiments described in Fig. 1, a highly enriched medium was employed consisting of 2% glucose, 2% Nutrient Broth, 0.1% yeast extract (Difco), 0.1% Casamino Acids (Difco), and 0.2% dibasic potassium phosphate.

Chemicals. Reagents were obtained as follows: alumina grade A-305, Alcoa Chemicals Div., Pittsburgh, Pa.; methyl viologen, British Drug Houses, Poole, England (recrystallized once from ethyl alcohol before use); 5% CO₂ in nitrogen and argon, Matheson Co., Inc., Newark, Calif.; isopropyl- β -D-thiogalactopyranoside (IPTG) and *o*-nitro- β -D-galactopyranoside (ONPG), Calbiochem; *N*-1-naphthylethylenediamine-2HCl, Eastman Organic Chemicals, Rochester, N.Y.; sodium hydrosulfite, Fisher Scientific Co. All chemicals used in enzyme assays or buffers were free from contamination by nitrite.

Enzymes. Commercial enzymes were obtained from the following sources: lysozyme (three times recrystallized) and bovine albumin from Pentex, Inc., Kankakee, Ill., deoxyribonuclease (B grade) from Calbiochem, and catalase from Worthington Biochemical Corp., Freehold, N.J.

Culture conditions. Aerobic cultures were grown at 37 C in graduated cylinders with vigorous aeration. For complete repression of nitrate reductase, culture vessels were aerated at a rate by which the volume of the unaerated medium was doubled. Anaerobic cultures were grown at 37 C either in completely filled rubber-stoppered flasks or in graduated cylinders sparged with a mixture of 5% CO₂ in nitrogen.

Preparation of extracts. Washed cells were suspended in 0.1 M phosphate buffer, pH 7.2, for disruption. For the studies on the distribution of nitrate reductase in centrifugal fractions, cells were mixed with alumina, frozen, and then ground for 5 min. For further grinding, the cell-alumina paste was refrozen and was ground again for 5 min. Alumina and whole cells were removed by centrifugation for 10 min at 2,000 \times *g*. Sonic extracts were prepared by exposure of cell suspension to 10-kc vibrations for 3 min with a Branson Sonifier. Lysozyme lysates were prepared by suspending cells in 0.1 M tris(hydroxymethyl)aminomethane, pH 7.2, with 50 μ g of lysozyme and 2 μ g of deoxyribonuclease per ml. The suspensions were repeatedly frozen in a dry ice-acetone slurry and thawed until there was no further decrease in turbidity (usually twice). Extracts which were analyzed on sucrose gradients or were utilized to solubilize nitrate reductase by alkaline-heat treatment were prepared as follows. Cells were harvested by centrifugation and were washed with 0.1 M phosphate buffer, pH 7.2. The pellet was resuspended in the same buffer at a concentration no greater than 20% of the wet weight of cells and was disrupted by a single passage through an Aminco French pressure cell, mounted on a Carver laboratory press, with a force of approximately 18,000 psi. The lysate was centrifuged at 2,000 \times *g* for 10 to 20 min to remove whole cells, and the supernatant fluid, termed the crude extract, was utilized in our experiments.

For the kinetic studies, samples were collected and washed with 0.1 M phosphate buffer, pH 7.2, on 47-mm Millipore filters with a pore size of 0.45 μ ; then these samples were resuspended in the same buffer. More than 95% of the cells were recovered from the filter. Extracts were prepared by three 15-sec treatments with a Branson Sonifier.

Oxygen measurements. Oxygen tension was adjusted by varying the rate of sparging of a culture with air; it was measured in growing cultures with a Clark-type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) inserted through a hole near the bottom of the cylindrical culture vessel. Zero oxygen tension was set by bubbling the CO₂-N₂ mixture through the culture fluid; 100% saturation was set by sparging the culture fluid with air. Under these conditions, the oxygen concentration was proportional to the output voltage of the electrode which was amplified and recorded on a moving chart (PRR Recti-Riter Recorder, Texas Instruments Inc., Houston, Tex.).

Sucrose gradients. Linear 5 to 20% sucrose gradients, used throughout, were made in 0.1 M phosphate buffer, pH 7.2, and were allowed to equilibrate at least 2 hr at 4 C before use. After centrifugation in a Spinco model L or L-2 ultracentrifuge, a hole was punched in the bottom of the tube and fractions were collected by gravity.

Assays and determinations. β -Galactosidase was assayed by the procedure of Sypherd and Strauss (13).

For the assay of nitrate reductase, extracts were preincubated for 5 min at 37 C in a 2.4-ml volume of the following reaction mixture: 0.1 M phosphate buffer (pH 7.2), 0.1 M potassium nitrate, and 10⁻⁴ M methyl viologen. The open tubes were flushed with argon to remove oxygen. Immediately before starting the reaction, 50 mg of sodium hydrosulfite was dissolved in 10 ml of 0.01 M NaOH; this solution was shaken gently to avoid oxidation of the hydrosulfite. The reaction was started by adding 0.1 ml of the solution to the reaction mixture. After 10 min, the reaction was stopped by shaking the tube to oxidize the remaining hydrosulfite and reduced methyl viologen. To determine nitrite, two parts of 4% sulfanilamide in 25% HCl solution was mixed with one part of 0.08% *N*-1-naphthylethylene-diamine dihydrochloride; 0.75 ml of this mixture was added to 2.5 ml of the nitrite-containing sample. After 10 min, the absorbancy of the mixture was read in the Klett-Summerson colorimeter with a 540-m μ filter. An absorbancy of 7 Klett units is yielded by 1 m μ mole of nitrite; the determination is linear to 350 Klett units.

Protein. The total protein content of extracts was measured by the method of Lowry et al. (6), with crystalline bovine albumin as a standard. Extracts were precipitated with 5% trichloroacetic acid and centrifuged; the pellet was dissolved in 1 N NaOH for the determination. Protein in growing cultures was estimated from the absorbancy of the culture as measured in a Klett-Summerson colorimeter with a 540-m μ filter. One Klett unit was equal to a cell protein concentration of 2 μ g/ml.

Enzyme units and specific activities. One unit of enzyme is defined as the amount of enzyme which will transform 1 m μ mole of nitrate to nitrite in 10 min. Specific activity is defined as enzyme units per microgram of protein. Total enzyme per milliliter of growing culture was calculated by multiplying the enzyme specific activity of a sample by the number of milligrams of protein per milliliter of culture.

RESULTS

Localization of nitrate reductase. Experiments performed to determine the localization of nitrate reductase were carried out on cells which were fully induced for the enzyme (see studies below on regulation of nitrate reductase synthesis) by growth in a nitrate-containing medium under anaerobic conditions. When cells grown either in broth or synthetic media were disrupted by various means and separated into a "soluble" and a "particulate" fraction by centrifugation at 15,000 to 20,000 × g for 30 min, the partition of nitrate reductase activity between the two fractions depended on the method of cell disruption used (Table 1). We observed that lysozyme lysis produced the smallest percentage of "soluble" activity of any of the breakage procedures employed. From these data, it seemed likely that the "soluble" enzyme activity could be attributed to fragments of a larger particulate structure and the variability in the amount of "soluble" activity, to differences in the susceptibility of the structure to fragmentation.

Without a more rigorous definition of solubility than sedimentation at a given centrifugal force, it is difficult to assess the significance of "soluble" and "particulate" activities since "soluble" activity may simply be small, randomly

broken pieces of a larger structure. Therefore, we have chosen to define soluble enzyme as that activity which is not sedimented with the membrane fraction and which exhibits a symmetrical profile after zone centrifugation in a sucrose gradient. Soluble enzyme, defined in this way, can be derived from the particulate fraction of *E. coli* by a modification of a procedure described by Taniguchi and Itagaki (15). Particles were prepared by disrupting fully induced bacteria with a French pressure cell and centrifuging the crude extract at 81,000 × g for 30 min. The pellet was resuspended in 0.1 M phosphate buffer, pH 8.3. One portion was incubated at 60 C for 7.5 min to solubilize nitrate reductase. A second portion was adjusted to pH 7.1 and was exposed to ultrasonic vibration for three 15-sec intervals. Samples of each treated suspension and of the particle suspension which received no treatment were layered on sucrose gradients with 0.1 ml of a marker catalase solution and were centrifuged for 2 hr in a Spinco SW-39 rotor at 39,000 rev/min. Fractions were collected and assayed; these results are presented in Fig. 1. The peaks of nitrate reductase activity at the top of the gradients show that all three samples contained some soluble enzyme. However, almost all of the activity of the untreated particles sedimented into the pellet at the bottom of the centrifuge tube (no attempt was made to recover this en-

TABLE 1. Partition of nitrate reductase activity in ultracentrifugal fractions as a function of cell disruption procedures

Disruption procedure	Total enzyme activity in fractions (μmoles/10 min)			
	Crude extracts	High-speed supernatant fluid	High-speed pellet	Percentage in pellet ^a
Alumina (ground once)	— ^b	120 ^c	61	34
Alumina (ground twice)	— ^b	69,000 ^c	3,100	4.6
Sonic treatment (3 min)	900 ^d	500 ^e	—	44
Lysozyme (freeze-thaw)	11,500 ^d	1,250 ^e	—	89

^a In each case, it was assumed that all the activity of the crude extract was recovered in the pellet and supernatant fraction. Subsequent experiments showed that this was the case in extracts prepared by sonic treatment and freeze-thaw lysozyme treatment.

- ^b Centrifuged for 10 min at 2,000 × g.
- ^c Centrifuged for 30 min at 20,000 × g.
- ^d Centrifuged for 30 min at 2,000 × g.
- ^e Centrifuged for 30 min at 15,000 × g.

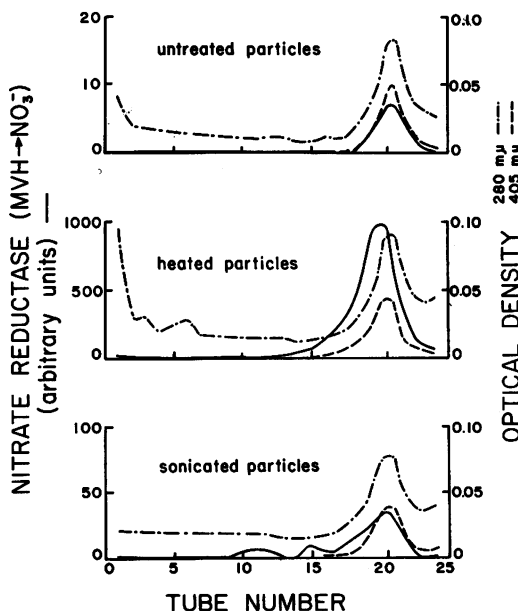


FIG. 1. Sucrose gradient profiles of nitrate reductase particle preparations.

zyme); only 0.3% of the untreated preparation of particles became soluble (apparently during the experimental manipulation). Sonic treatment greatly increased the amount of enzyme released from the particles; this consisted of both soluble enzyme and small fragments (6.8% of the applied activity was recovered). The alkaline incubation solubilized 36% of the activity applied to the gradient.

When a preparation of enzyme solubilized from particles by alkaline incubation was centrifuged for 8 hr in a sucrose gradient at 39,000 rev/min, the bulk of the enzyme was located in a symmetrical peak that sedimented 0.9 the distance of the 12S catalase, and it was assigned a nominal sedimentation constant of 11S (Fig. 2).

For a comparison of the solubilized nitrate reductase from particles with the naturally occurring "soluble" enzyme, a French pressure cell extract of fully induced *E. coli* K-12 was prepared. This extract was clarified to remove heavy particulate activity by centrifugation at $10,000 \times g$ for 15 min, and a sample was layered on a sucrose gradient and was centrifuged for 90 min. The profiles of absorbancy and nitrate reductase activity are shown in Fig. 3. The enzyme sedimented as a heterogenous assortment of particles. Most of the activity was associated with very heavy material; the light activity appeared to be associated with increasingly subdivided fragments of a larger structure rather than a major symmetrical peak of soluble protein, such as was obtained by alkaline incubation of a particle preparation.

The experiments described above demonstrate that the nitrate reductase of *E. coli* K-12 is principally particulate in nature. We wanted to set an upper limit on the amount of soluble enzyme present, thinking it might be a precursor to the membrane-bound form. Therefore, an anaero-

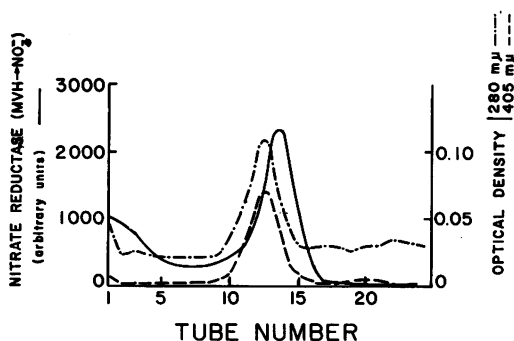


FIG. 2. Sucrose gradient profiles of nitrate reductase solubilized from a particle preparation. The preparation was sedimented with a catalase marker for 8 hr at 39,000 rev/min in an SW-39 rotor.

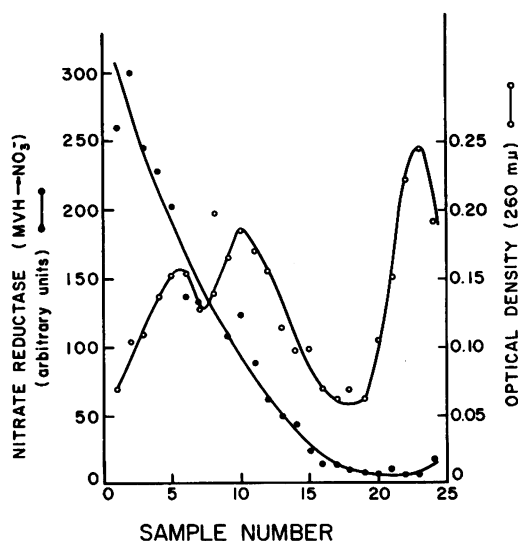


FIG. 3. Sucrose gradient profile of a crude extract of *Escherichia coli*. Bacteria were grown anaerobically in a medium containing 1% peptone, 1% KNO_3 , and 1% glucose. The extract was prepared in a French pressure cell and was clarified by centrifugation at $10,000 \times g$ for 15 min; 0.4 ml of the extract was then layered on a 4.6-ml sucrose gradient and was centrifuged for 90 min at 39,000 rev/min in an SW-39 rotor.

bically growing culture in broth medium was induced with nitrate and was harvested after 15 min. At this time, the total enzyme in cells was low, but synthesis was proceeding at a maximal rate; the proportion of soluble enzyme to particulate enzyme should have been increased if the former was a precursor to the latter. The bacteria were disrupted with a French pressure cell, and whole cells were removed by centrifugation at $2,500 \times g$ for 15 min. This supernatant liquid, the crude extract, was given a preliminary centrifugation for 15 min at $81,000 \times g$ to minimize the size of the pellet on the bottom of the gradient tube. The high-speed supernatant fluid was layered on a sucrose gradient and was centrifuged 135 min at 25,000 rev/min in a Spinco SW25.2 rotor. The resulting enzyme activity and optical density profiles are shown in Fig. 4; the distribution of nitrate reductase activity in the centrifugal fractions of the crude extract is presented in Table 2. The activity in the five upper "soluble" fractions corresponds to 1.5% of the total in the crude extract, but in this case, as in the previous, much of the activity seems to be associated with small fragments rather than homogeneous soluble enzyme.

Regulation of nitrate reductase synthesis. Published data show that *E. coli* can synthesize nitrate reductase under aerobic or anaerobic con-

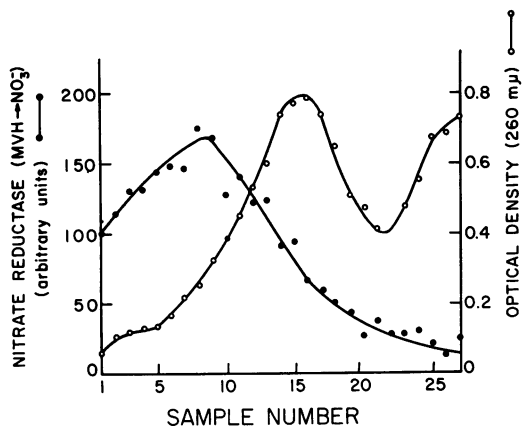


FIG. 4. Sucrose gradient profile of extract from cells early after induction. A 3-ml amount of clarified supernatant liquid was layered on a 50-ml sucrose gradient and was centrifuged at 20,000 rev/min for 135 min in an SW 25.2 rotor.

TABLE 2. Distribution of nitrate reductase activity in ultracentrifugal fractions of crude extracts

Fraction	Total activity (μmoles/10 min)	Percentage of gradient	Percentage of crude extract
2,500 × g Supernatant fluid	30,000	—	100
81,000 × g Supernatant fluid	7,800	—	26
81,000 × g Pellet	17,000	—	57
81,000 × g Supernatant fluid applied to gradient	7,900	100	26
Recovered from gradient	7,200	91	24
Recovered in five top "soluble" gradient fraction	440	5.5	1.5

ditions and in the presence or absence of nitrate (1, 2, 5, 15, 16). We found that the enzyme levels in cultures grown aerobically on a rotary shaker were difficult to reproduce because the nitrate reductase specific activity was dependent on the culture density at which cells were harvested. When oxygen tension in such a culture was monitored with a Clark electrode, the rate of nitrate reductase synthesis could be correlated with the decrease in oxygen concentration which occurred as the culture density increased.

The effects of oxygen tension on the differential rate of nitrate reductase synthesis in the presence or absence of nitrate were examined by growing cultures in tall, thin cylinders with vigorous aeration. The oxygen tension was var-

ied by adjusting the rate of aeration and was measured with a Clark electrode placed at the bottom of the cylinder. The differential rate of nitrate reductase synthesis at each oxygen tension was established by taking several samples to determine the increase in enzyme synthesis relative to the increase in protein. The results of these experiments are shown in Fig. 5. At concentrations of oxygen near saturation, the rate of synthesis of the enzyme was independent of the presence of nitrate; in the experiment depicted in Fig. 5, the specific activity of nitrate reductase was 0.037 in the presence of nitrate and 0.030 in its absence when aeration was maximal. Thus, when aeration was vigorous, nitrate reductase synthesis was repressed to what we shall call its basal level, independent of the presence or absence of nitrate. However, enzyme formation was induced by nitrate at concentrations of oxygen which would repress completely in its absence. The results of the differential rate determinations in cells grown at 0% saturation with oxygen are misleading because there was a very high transient of enzyme synthesis in either the presence or absence of nitrate reductase (Fig. 6). Table 3 gives the specific activity of nitrate reductase in cells grown anaerobically by sparging with a mixture of 95% N₂ and 5% CO₂ for several generations. Similar values were obtained when cultures were grown in filled, rubber-stoppered flasks. We noted that the anaerobic steady-state level was increased about 20-fold by the presence of nitrate.

The experiments described above demonstrate that nitrate reductase synthesis is repressed by

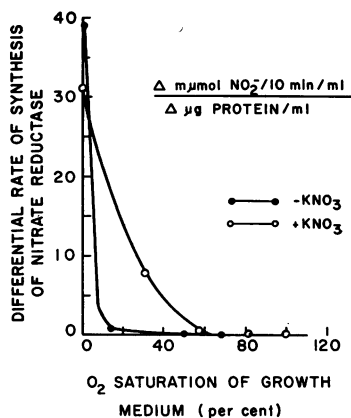


FIG. 5. Dependence of the rate of nitrate reductase synthesis on oxygen tension of the medium. Total enzyme was determined at different times at each oxygen tension. The differential rate of enzyme synthesis was calculated as the increase in enzyme divided by the increase in protein.

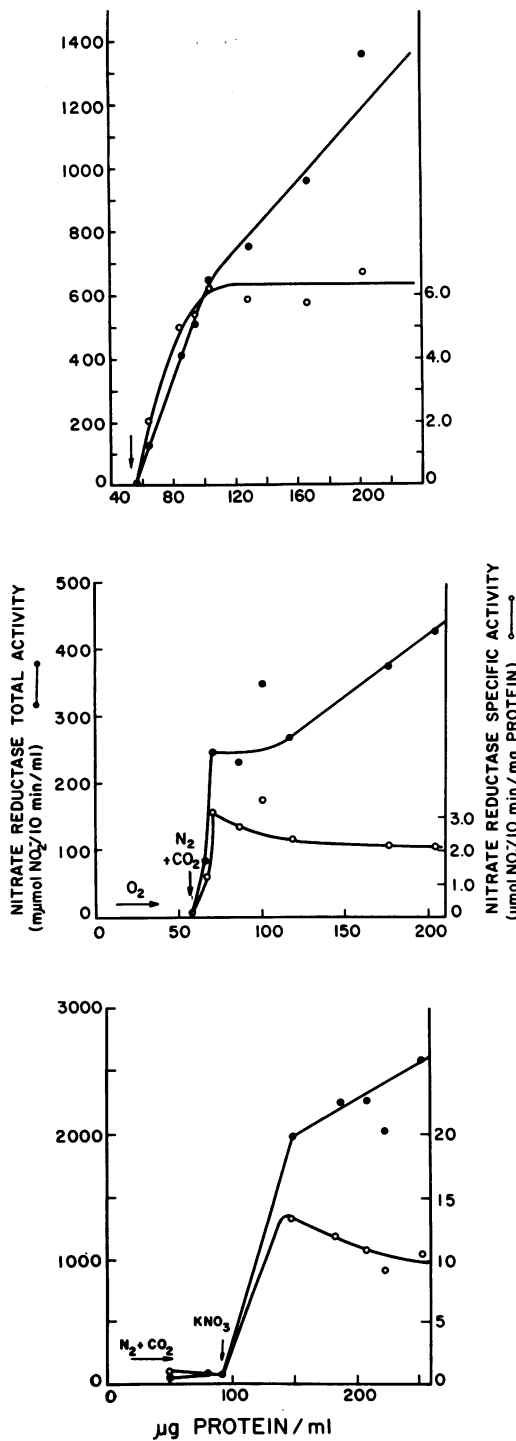


FIG. 6. Induction and derepression of nitrate reductase. Top: a culture growing aerobically in broth medium with 0.8% KNO_3 was made anaerobic (at arrow) by sparging with nitrogen and carbon dioxide.

TABLE 3. Effect of nitrate on nitrate reductase levels in anaerobic cultures

Medium ^a	Specific activity of nitrate reductase ^b	
	- KNO_3	+ 1% KNO_3
Glucose (1%)	0.91	21.0
Glucose (1%), Nutrient Broth (0.4%)	0.90	18.0

^a Synthetic medium with indicated additions.
^b Activity measured in extracts prepared by sonic oscillation.

aeration and induced by nitrate. The rate of enzyme synthesis can be increased by (i) derepression, a shift from aerobic to anaerobic growth, (ii) induction, the addition of nitrate to an anaerobic culture, or (iii) a combination of the two, e.g., the shift from aerobiosis to anaerobiosis of a culture growing in nitrate. Figure 6 shows the kinetics of nitrate reductase synthesis under these three sets of conditions. In each case, a high initial rate of synthesis which lasted less than one doubling time was followed by synthesis at a much lower rate.

At no time during derepression in the presence of nitrate was the amount of soluble enzyme greater than 1.5%. Therefore, the formation of a highly active soluble precursor to the membrane-bound enzyme was not responsible for the initial burst of activity. Moreover, induction was dependent on protein synthesis; if chloramphenicol (200 $\mu g/ml$) was added to a culture 3 min before induction or before derepression was begun, no enzyme activity was produced when aeration was ceased. Therefore, the initially high rate of synthesis is not due to activation of an inactive precursor.

The induction and derepression kinetics indicated that some sort of feedback control is in operation. To test for the possibility of end-product repression by nitrite, the following experiment was performed. A culture growing anaerobically was divided in half; one half was permitted to continue growing as before, without nitrate or nitrite, while sodium nitrite, at a final concentration of 0.11 M, was added to the other half. This concentration of nitrite is about twice that present when a culture finally stops growing in a nitrate medium. After a generation of further anaerobic growth, both cultures were

Center: as above, except that the medium contained no nitrate. Bottom: an anaerobically growing culture was induced for nitrate reductase by the addition of 1% potassium nitrate to the medium.

harvested and the nitrate reductase specific activity was determined. That of the control culture was 0.9, whereas the culture to which the presumptive co-repressor was added had a specific activity of 1.6. Thus, nitrite does not repress nitrate reductase synthesis.

That the biphasic enzyme kinetics are not a nonspecific effect of anaerobic shock on the synthesis of inducible enzymes is illustrated by the experiment described in Fig. 7. An aerobic culture was simultaneously induced for β -galactosidase by the addition of IPTG and was derepressed for nitrate reductase by sparging with a mixture of nitrogen and carbon dioxide. Nitrate reductase synthesis exhibited biphasic kinetics while β -galactosidase was synthesized at a constant rate. The graph of β -galactosidase activity extrapolates back to a point shortly after that at which induction was begun, whereas the steady-state rate of nitrate reductase synthesis has a positive ordinal intercept.

If nitrate reductase synthesis is regulated by a repressor which is sensitive to the intracellular redox potential, then the biphasic induction kinetics could be explained by the nongratuitous nature of the induction when it is carried out in the presence of nitrate. A mutant which is unable to reduce nitrate was used to test this possibility. This mutant (Ni-6) derived from strain 3300 possesses a normal nitrate reductase activity as assayed with methyl viologen in extracts, but it has only 5% of wild-type formate dehydrogenase activity and 10% of wild-type formate-nitrate re-

ductase activity (*unpublished data*). Nitrite does not accumulate in the culture fluid of this mutant as it does in the culture fluid of the wild-type organism grown with nitrate. Thus, although it possesses a normal nitrate reductase, the mutant cannot metabolize nitrate *in vivo* because the physiological pathway for nitrate reduction which uses electrons from the oxidation of formate for nitrate reduction is not functional. In this strain, nitrate should act as a gratuitous inducer.

Figure 8 shows the kinetics of nitrate reductase production by the mutant upon induction with nitrate. The differential rate of synthesis is linear and proceeds at the steady-state rate of the parental strain. Thus, the biphasic kinetics of nitrate reductase formation during induction by nitrate are caused by the metabolism of nitrate; in the presence of an active formate-nitrate reductase system, nitrate is a nongratuitous inducer.

DISCUSSION

The nitrate reductase of *E. coli* K-12 appears localized strictly in a particulate fraction of the cell. Because of the fragility of the subcellular structure to which the enzyme is bound, we have found it necessary to define soluble enzyme as that which sediments as a homogeneous peak in a sucrose gradient with a sedimentation constant of 11S. This value is less than half of that given by Taniguchi and Itagaki (15). This difference could be attributed to structural protein bound specifically to their enzyme, to lipid bound to ours, or, less probably, to strain differences between *E. coli* K-12 and *E. coli* Yamaguchi. But whatever the accurate size of soluble nitrate reductase is, it should sediment as a symmetrical peak in a sucrose gradient if soluble enzyme is physiologically significant in the cell. Some confusion undoubtedly exists in the literature about the localization of particulate enzymes because of failure

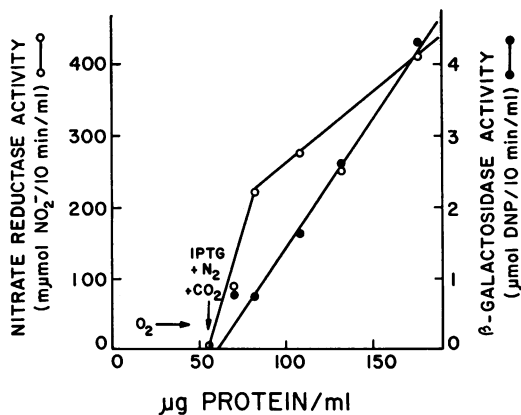


FIG. 7. Induction of β -galactosidase and derepression of nitrate reductase in *Escherichia coli* 3000. A culture was grown aerobically on broth medium with 0.11 M sodium pyruvate substituting for glucose. The culture was derepressed for nitrate reductase by sparging with 95% N_2 and 5% CO_2 , and simultaneously induced for β -galactosidase by the addition of isopropylthio- β -D-galactoside in a final concentration of 1 mM.

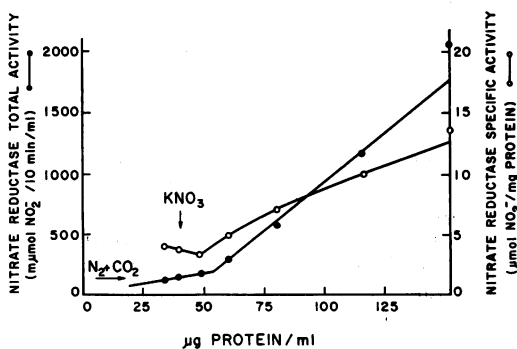


FIG. 8. Induction of mutant Ni-6. Experimental procedure as in Fig. 6 (bottom), except that the mutant substituted for the parental wild-type strain.

to apply sufficiently rigorous criteria for solubility to rule out artifacts caused by cell disruption.

In cultures of *E. coli* K-12 induced in a rich medium, nitrate reductase activity which is soluble by the above criterion never exceeds 1.5% of the total. This is an upper limit because small fragments tail into the region which would be occupied by the soluble enzyme. This figure may rise as high as 10% for cells grown on a synthetic medium (*unpublished data*), but we now believe that the higher figure is caused by greater ease of release of the enzyme from the membrane of these cells.

Because of the low levels of soluble nitrate reductase apparent during the early stages of induction, it seems likely that even the small percentage of soluble enzyme found under these conditions is not a soluble precursor to the membrane-bound species but is entirely an artifact due to the process of cell disruption. It is possible that the enzyme attains an active configuration only after, and by reason of, its incorporation into the electron-transport chain. This hypothesis is supported by preliminary observations showing that nitrate reductase synthesis can be separated from activation by the use of chloramphenicol (*unpublished data*).

The synthesis of the nitrate reductase of *E. coli* K-12 is controlled by two principal growth conditions: the presence of nitrate, which acts to induce, and aeration of the medium, which serves to repress. The control is similar to that found in *Aerobacter aerogenes* by Pichinoty and D'Ornano (10), in that vigorous aeration will repress enzyme synthesis even in the presence of nitrate.

The basal level of enzyme in highly aerated cultures is 2 to 4% of that observed in cultures that are derepressed in enzyme production by anaerobiosis. The fully induced levels, obtained in anaerobic cultures in the presence of nitrate, are 20-fold higher. A single enzyme is apparently responsible for the activity present under these various growth conditions, for a mutation which abolishes one of them abolishes them all (*unpublished data*).

The biphasic kinetics observed during nitrate reductase induction or derepression are most easily explained as a manifestation of the control mechanism which is responsible for the repression of nitrate reductase synthesis during aeration. That such kinetics did not result directly from nitrate metabolism was demonstrated by the fact that similar kinetics were obtained during derepression in the absence of nitrate. The final steady-state rate of enzyme synthesis extrapolates back to a positive ordinal intercept in Fig. 6, showing that all components of the enzyme are initially synthesized at a high rate. Thus, the possi-

bility is eliminated that the biphasic kinetics are caused by rate limitation by a component that is present at steady-state level prior to induction.

We propose that nitrate reductase synthesis is controlled not only by a repressor that is sensitive to nitrate but also by a redox-sensitive repressor. This molecule must be sensitive to the redox potential within the cell, possibly through an equilibrium between an oxidized, active form and a reduced, inactive one, with the equilibrium established either by the electron-transport systems of the cell or by steady-state levels of oxidizable intermediates. When the redox-sensitive repressor is fully active, under conditions of vigorous aeration, nitrate reductase synthesis is repressed to its basal level. Under anaerobic conditions, however, the redox-sensitive repressor is only partly active, reflecting, for example, a shift in its equilibrium concentration to a higher proportion in a reduced state. Under these circumstances, the nitrate-specific repressor maintains nitrate reductase synthesis at a relatively low rate unless it too is inactivated by the addition of nitrate to the culture.

On this model, the biphasic kinetics found during induction and derepression of nitrate reductase exhibit transients in the internal redox potential of the bacteria. These are caused by the synthesis of new enzyme systems (possibly also under control of the redox-sensitive repressor) capable of disposing of the reducing equivalents generated by metabolism. When a culture is derepressed by anaerobiosis, the redox potential should drop abruptly because terminal oxidases can no longer function. In the absence of nitrate, the synthesis of formic hydrogenlyase partially replaces the lost oxidase activity by permitting formate to be oxidized, thus raising the redox potential to a new steady state and reducing the rate of nitrate reductase synthesis accordingly. In the presence of nitrate, the synthesis of nitrate reductase itself performs the same function with a similar effect. The mutant which is defective in both its formate and nitrate metabolism does not show biphasic kinetics of nitrate reductase synthesis because it is unable to synthesize the alternative pathways which permit the wild-type strain to shift its redox potential back toward the aerobic level.

The control of synthesis of redox enzymes has been the subject of some discussion. Pichinoty (9) suggested that oxygen itself was responsible for the repression of enzymes that appear under anaerobic conditions of growth. In contrast, Wimpenny and Cole (17) indicated that the electron potential of the medium is the controlling factor, based on their observations of variation in the levels of a number of enzymes in cells grown anaerobically in the presence and absence of

nitrate. Our findings revealed that the controlling factor may be the effective intracellular redox potential. This potential is a function not only of the potential oxidants and reductants in the cell but also of its catalytic capabilities for electron flow. It depends on the balance between the oxidation and reduction reactions actually taking place in the cell at a given time. The findings of D. C. White (*personal communication*) that levels of the cytochromes of *Haemophilus parainfluenzae* can be shifted by quinoline oxide, an inhibitor of cytochrome oxidation, support this view.

Our investigation has been limited principally to the control of nitrate reductase synthesis. We would like to suggest, however, that redox-sensitive repressors may be responsible for the control of many of the oxidation-reduction enzymes of *E. coli* and other bacteria. Whether or not a single repressor species is responsible for the control of enzymes behaving similarly will be the subject of a future investigation.

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