Kinetic Analysis of Respiratory Nitrate Reductase from *Escherichia coli* K12†

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ABSTRACT: Purified respiratory nitrate reductase from *Escherichia coli* is able to use either reduced viologen dyes or quinols as the electron donor and nitrate, chlorate, or bromate as the electron acceptor. When reduced viologen dyes act as the electron donor, the enzyme follows a compulsory-order, "Theorell–Chance" mechanism, in which it is an enzyme–nitrate complex that is reduced rather than the free enzyme. In contrast, if quinols are used as the electron donor, then the enzyme operates by a two-site, enzyme-substitution mechanism. Partial proteolysis of the cytochrome *b* containing holoenzyme by trypsin results in loss of cytochrome *b* and in cleavage of one of the enzyme's subunits. The cytochrome-free derivative exhibits a viologen dye dependent activity that is indistinguishable from that of the holoenzyme, but it is incapable of catalyzing the quinol-dependent reaction. The quinol-dependent, but not the viologen dye dependent, activity is inhibited irreversibly by exposure to diethyl pyrocarbonate and reversibly by treatment with 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide. We conclude that the holoenzyme has two independent and spatially distinct active sites, one for quinol oxidation and the other for nitrate reduction.

Nitrate reductase (EC 1.7.99.4) is a terminal respiratory enzyme present in *Escherichia coli* when the organism grows anaerobically in the presence of nitrate. Energy is conserved by this membrane-bound enzyme, and it has been shown to catalyze the translocation of protons across the cytoplasmic membrane coupled to the oxidation of ubiquinol by nitrate (Garland et al., 1975). The enzyme, which contains molybdenum, iron–sulfur centers (Vincent & Bray, 1978), and cytochrome *b* (Enoch & Lester, 1975), possesses three types of polypeptide subunits, α, β, and γ (approximate M_r 155 000, 60 000, and 20 000, respectively). The latter, smallest subunit is the apoprotein of cytochrome *b*, cytochrome *b*-556$^{NO_3^-}$ (Enoch & Lester, 1975; Chaudry & MacGregor, 1983). The enzyme, consistent with its proton-translocating ability, occupies a transmembranous location within the cytoplasmic membrane. The γ subunit (cytochrome *b*) has been located at the periplasmic face of the membrane whereas the α and β subunits are exposed at its cytoplasmic face (Boxer & Clegg, 1975; MacGregor & Christopher, 1978; Graham & Boxer, 1980, 1981).

From their work with intact cells and spheroplasts a mechanism for the respiratory-driven proton translocation catalyzed by nitrate reductase has been proposed by Jones et al. (1980). Two protons are released at the periplasmic face coupled to the oxidation of ubiquinol by cytochrome *b*. The two electrons also produced by this reaction are passed across the membrane through the enzyme to the nitrate reduction site at the cytoplasmic side of the membrane. Here nitrate is reduced to nitrite and water with the obligatory consumption of two protons. This mechanism requires that ubiquinol oxidation and nitrate reduction occur at separate sites on the enzyme that are situated at opposite sides of the membrane.

In this paper we describe the kinetic properties of the purified cytochrome *b* containing enzyme and of a cytochrome-free derivative that has been prepared by limited proteolysis of the isolated enzyme. Our results show that the site of ubiquinol oxidation is associated with the cytochrome *b* moiety of the enzyme and that it is physically distinct from the site of nitrate reduction.

Materials and Methods

Glass-distilled water was used throughout. All substrates were of the highest grade available and were purchased from BDH Chemicals, Poole, Dorset, U.K., except for benzylviologen and duroquinone, which were products of Sigma (London) Chemical Co., Kingston on Thames, Surrey, U.K. Ubiquinone 1 was a gift from Professor P. B. Garland of this department.

Preparation of Nitrate Reductase. The enzyme was prepared by a procedure modified from that described by Enoch & Lester (1975). All operations were performed at 4 °C, and all buffers contained 5 mM 2-mercaptoethanol. *E. coli* (strain EMG2, National Collection of Industrial Bacteria, Aberdeen) were grown anaerobically in 1000-L batch cultures in a supplemented minimal medium containing 1% (w/v) $NaNO_3$ (Cohen & Rickenberg, 1956). The harvested bacteria were stored at −80 °C before use. A total of 800 g wet weight of cell paste was thawed, washed once in 50 mM Tricine[1]–KOH, pH 8.2, containing 5 mM benzamidine hydrochloride, and resuspended in 1.5 L of the same buffer. The cells were broken by two passages through a precooled Manton–Gaulin homogenizer (A.P.V. Ltd., Crawley, U.K.) at 8000 psi. Unbroken cells were removed by sedimentation at 10000*g* for 15 min, and the membrane fraction was sedimented by centrifugation at 78000*g* for 2 h. The pellets were suspended in the 50 mM Tricine buffer, and sodium deoxycholate (1 mg/mg of protein) was added from a 10% (w/v) aqueous solution to a slowly stirred membrane suspension to give a final protein concentration of 10 mg/mL. The mixture was stirred for 10 min, brought to 20% saturation in $(NH_4)_2SO_4$ by the addition of 100% saturated $(NH_4)_2SO_4$ in 50 mM Tricine–KOH, pH 8.2, stirred for a further 1 h, and then centrifuged for 20 min at 40000*g*. The supernatant was adjusted to 34% saturation in $(NH_4)_2SO_4$, stirred, and centrifuged as above. The resulting supernatant was further adjusted to 50% saturation in $(NH_4)_2SO_4$, stirred, and centrifuged in the same manner. A dark

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[1] Abbreviations: HQNO, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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brown precipitate was obtained, which was suspended into a final volume of 48 mL of 40 mM potassium phosphate, pH 7.0, 0.2 M NaCl, 5 mM benzamidine hydrochloride, and 2% (w/v) Triton X-100 and dialyzed overnight against 2 L of the same suspension buffer except with 0.5% (w/v) Triton X-100. After dialysis the contents of the dialysis bag was centrifuged for 30 min at 40000*g* to give a large cream pellet, which was discarded, and a dark brown supernatant. This was concentrated to 33 mL by pressure dialysis, recentrifuged as before, and applied to a Sepharose CL-6B column (95 × 2.6 cm i.d.) equilibrated in the dialysis buffer. The brown, nitrate reductase containing fractions of the eluate were combined and dialyzed against 40 mM potassium phosphate, pH 7.0, 5 mM benzamidine, and 0.1% (w/v) Triton X-100. The material was applied to a DEAE-Sepharose CL-6B column (30 × 3.5 cm i.d.) equilibrated with the same buffer. At least two column volumes of equilibrating buffer was passed through the column after the sample, before 1.5 L of a 0–250 mM NaCl linear gradient in the same buffer was applied. Nitrate reductase eluted as a single cytochrome *b* containing peak. The fractions through the activity peak were analyzed by polyacrylamide gel electrophoresis prior to their combination. An overall yield of about 20% of the enzyme present in the broken cells was obtained.

If the enzyme was not pure as assessed by polyacrylamide gel electrophoresis, then a further step involving sucrose density gradient centrifugation was performed as described by Clegg (1976). The procedure described has been successfully used on a smaller scale with as little as 35 g wet weight of cells. Cell breakage was carried out in a French press in these cases. The enzyme could be stored as rapidly frozen beads in liquid N_2 for several months without loss of activity. The specific activity of the purified enzyme was normally in the range 45–80 μmol of NO_3^- reduced min^{-1} (mg of protein)$^{-1}$. The M_r of the native enzyme was taken as 235 000. This is a minimum value and was calculated as the sum of the molecular weights of the three subunits.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed in 7.5% (w/v) polyacrylamide gels as described by Laemmli (1970). Cytochrome *b* was estimated from the reduced minus oxidized difference spectrum by employing an extinction coefficient (em) of 19000 M^{-1} cm^{-1} at 560 nm minus 575 nm. Protein was determined by the method of Lowry et al. (1951).

Enzyme Assays. All assays were carried out in 0.1 M potassium phosphate buffer, pH 6.8 at 25 °C. The buffer was purged with oxygen-free nitrogen for several minutes before use and sealed from the air. Aliquots of buffer were removed via the rubber seal with a needle and syringe.

Quinols as the Electron Donor. Duroquinol and ubiquinol were prepared by reduction of an acidic methanolic solution with sodium borohydride. Before use, the pH of the solution was adjusted to about 6. To avoid autoxidation, the quinols were prepared only when needed and stored at 4 °C under a nitrogen atmosphere. The oxidation of duroquinol and ubiquinol 1 was followed spectrophotometrically at 275 nm with a molar extinction coefficient for oxidized minus reduced quinol of 12500 M^{-1} cm^{-1} (Lawford & Garland, 1973). Assays were initiated by addition of quinol to the anaerobic assay buffer containing nitrate reductase and an electron acceptor. In the absence of nitrate reductase autoxidation was not detectable over the 3–4 min required for an assay.

Reduced Bipyridylium Compounds as the Electron Donor. Reduced methyl- and benzylviologens were prepared and assayed as described by Kemp et al. (1975). Care was taken when sodium dithionite was added to avoid any excess, since this resulted in an apparent lag in the assay. In a control experiment sodium dithionite that had been deliberately aged by leaving for 12 h at room temperature (19–23 °C) was used to reduce benzylviologen for assay. Within the limits of experimental error the K_m for nitrate was the same with both freshly made and aged sodium dithionite. Thus the breakdown products of sodium dithionite oxidation do not interfere with the assay. Under the conditions of assay the nonenzymic reduction of nitrite by reduced bipyridylium compounds was negligible (<0.5% of the measured rate).

Initial Rate Kinetic Experiments. The kinetic coefficients of eq 1 (Dalziel, 1957) were estimated by the direct method

$$\frac{e}{V_0} = \phi_0 + \frac{\phi_1}{[S_1]} + \frac{\phi_2}{[S_2]} + \frac{\phi_{12}}{[S_1][S_2]} \quad (1)$$

of Eisenthal & Cornish-Bowden (1974). For presentation purposes, data are shown in double-reciprocal form.

To avoid complications arising from variation of the specific heme content of the preparations, the steady-state parameters for the enzyme, using viologen dyes as the reductant, were normalized to those consistent with an enzyme preparation exhibiting a reduced methylviologen:nitrate oxidoreductase specific activity of 80 μmol of nitrate reduced min^{-1} (mg of protein)$^{-1}$. This facilitated comparison with the data obtained when reduced quinols were used as the reductant.

In eq 1 e is the molar concentration of enzyme active sites. S_1 and S_2 are the reducing and oxidizing substrates, respectively, and V_0 is the specific initial velocity. Initial rate experiments were performed in duplicate and were in general reproducible to within 5% or 10% at the worst. Two complete experiments were performed with each substrate pair. When reduced methylviologen was the reductant, the parameters of eq 1 were reproducible for several enzyme preparations to within 20%; however, with quinols as reductants they were only reproducible to within 40% (one preparation).

Limited Proteolysis by Trypsin. Nitrate reductase (approximately 30 mg of protein) was incubated with an equal weight of trypsin for 3 h at 37 °C or at 4 °C for 24 h at pH 7. The incubation mixture was centrifuged and then applied to a Sepharose CL-6B column (130 × 1.8 cm i.d.). Native nitrate reductase eluted at 175 mL and trypsin-cleaved nitrate reductase at 210 mL. Fractions were assayed that were devoid of heme and, on a sodium dodecyl sulfate denaturing gel containing a modified β subunit, were concentrated by pressure dialysis and either used immediately or stored as rapidly frozen beads in liquid N_2. The M_r of the trypsin-modified enzyme was taken as 198 000.

Reaction with Diethyl Pyrocarbonate. Diethyl pyrocarbonate from Sigma was assayed with 10 mM imidazole at pH 6.5 as described by Dickenson & Dickinson (1975). The reaction of nitrate reductase with diethyl pyrocarbonate was carried out in 50 mM potassium phosphate, pH 7 at 25 °C; under these conditions the reagent has a half-life of about 13 min (Dickenson & Dickinson, 1975). The reagent was added from a 44 mM stock solution in ethanol; the final concentration of ethanol in the reaction mixture was 5%.

RESULTS

Cytochrome b Containing Nitrate Reductase. The purification procedure described routinely produces nitrate reductase of comparable specific activity (with reduced viologen dyes as the electron donor) to that found by other workers (Adams & Mortenson, 1982; DeMoss et al., 1981). The recovery of the membrane fraction by centrifugation rather than by ammonium sulfate precipitation (Enoch & Lester,

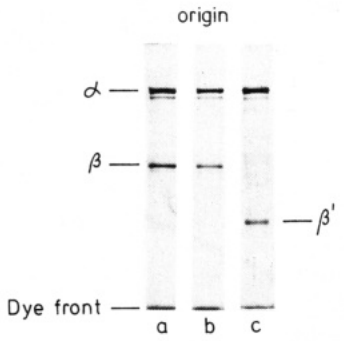


FIGURE 1: Denaturing polyacrylamide gel analysis of nitrate reductase. Photographs of polyacrylamide gels that have been stained for protein (see Materials and Methods for details) are shown. Approximately 15 μg of protein was applied to each gel. (a) Native cytochrome *b* containing nitrate reductase; (b) material from peak fraction of the first-eluting nitrate reductase activity peak from the Sepharose CL-6B column described in Figure 3; (c) as in (b) but of the second-eluting nitrate reductase activity peak.

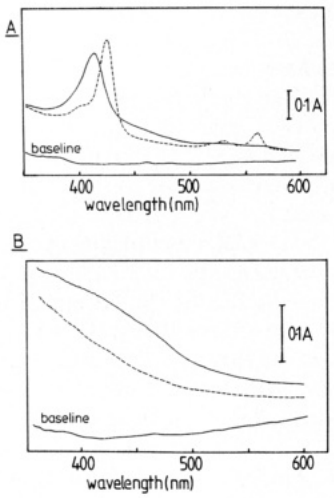


FIGURE 2: Absorption spectra. (A) Absorption spectra of native nitrate reductase as prepared. The spectra were recorded at room temperature. The enzyme (0.6 mg of protein/mL) was in 0.1 M potassium phosphate, pH 7. (—) Oxidized enzyme (as prepared); (---) enzyme reduced with a slight excess of sodium dithionite. (B) Absorption spectra of trypsin-modified nitrate reductase (1.15 mg of protein/mL). Conditions were as in (A).

1975) was important for the recovery of pure enzyme. About 150 mg of pure enzyme was obtained from 800 g wet weight of bacteria. Analysis of the enzyme by sodium dodecyl sulfate–polyacrylamide gel electrophoresis revealed two bands of approximate M_r 155 000 and 60 000, corresponding to the α and β polypeptides, respectively (Figure 1a). We are often unable to detect apocytochrome *b* that migrates as a polypeptide of M_r 20 000, even though the enzyme contains cytochrome *b* (0.8–1.7 mol of cytochrome *b* per mole of enzyme). We note that Chaudhry & McGregor (1983) find that apocytochrome *b* forms high molecular weight aggregates, and our data are consistent with their observation. The absorption spectrum of the oxidized and reduced forms of the enzyme is shown in Figure 2A. The cytochrome *b* absorption peaks can be clearly distinguished, and the remainder of the spectrum is consistent with the presence of iron–sulfur centers in the enzyme.

Trypsin Modification of Nitrate Reductase Produces a Cytochrome-Free Derivative. DeMoss (1977) reported that treatment of nitrate reductase with high amounts of trypsin selectively cleaves the β subunit to produce a fragment (β') of M_r 43 000, which remains associated with the α subunit. The αβ' derivative was fully active (with viologen dyes as the

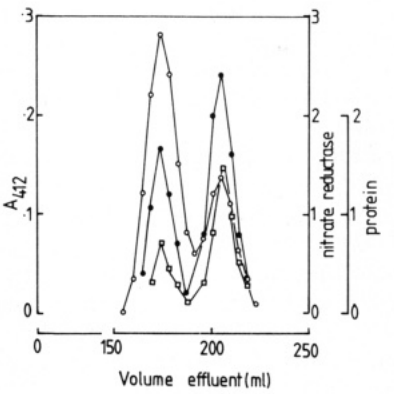


FIGURE 3: Gel filtration of trypsin-treated nitrate reductase. Nitrate reductase was treated with trypsin and applied to a Sepharose CL-6B column and the eluate analyzed as described under Materials and Methods. (○) Absorbance at 412 nm; (●) nitrate reductase activity (arbitrary units); (□) protein concentration (arbitrary units).

electron donor) and was incapable of self-association. The "native" enzyme as prepared by DeMoss was free of cytochrome *b* so that no information regarding the effect of trypsin cleavage of the β subunit on cytochrome *b* was obtained. We exposed cytochrome *b* containing nitrate reductase to trypsin and analyzed the products by gel filtration (Figure 3). Two peaks of nitrate reductase activity were found. The V_e of the first eluted peak corresponded to that of the unmodified enzyme. The enzyme in the first eluted peak possessed a normal cytochrome *b* content (monitored in Figure 3 by absorption at 412 nm) while the second was devoid of cytochrome *b* (Figure 2B). The absorption spectrum in other respects was similar to that of the native enzyme. The cleavage of the β subunit to form a fragment of M_r 43 000 had only taken place in the proportion of nitrate reductase that eluted in the second, lower molecular weight peak (Figure 1b,c). In these experiments no modification of the α subunit was apparent; under other conditions, such modification can also occur (Abraham et al., 1981; DeMoss et al., 1981). As was found by DeMoss, the activity (with viologen dyes as reductant) is fully retained by the αβ' derivative.

Kinetic Characterization of Cytochrome b Containing Nitrate Reductase. (*i*) *Reduced Viologen Dyes as the Electron Donor.* Primary and secondary double-reciprocal (Lineweaver–Burk) plots with reduced methylviologen as the electron donor and nitrate as the electron acceptor are shown in parts A and B of Figure 4. Linear plots were obtained over the concentration ranges of methylviologen and nitrate used, demonstrating that the data conform to eq 1. The primary plot of e/V_0 against the reciprocal of the nitrate concentration (Figure 4A) for fixed reduced methylviologen concentrations within the range used gave a set of parallel lines, indicating that ϕ_{12} is zero or very small. The values for the other constants, estimated from Figure 4, are listed in Table I. From these the turnover number ($1/\phi_0$) and the K_m values for reduced methylviologen (ϕ_1/ϕ_0) and for nitrate (ϕ_2/ϕ_0) were estimated at 455 s^{-1}, 182 μM, and 420 μM, respectively.

Similar analyses were carried out with reduced benzylviologen as the electron donor. Lowering of the reduced benzylviologen initial concentration to 10 μM did not alter the rate of nitrate reduction. Nitrate reductase, therefore, has a much lower K_m for reduced benzylviologen than for the methylviologen. The K_m was too low to be estimated by our spectrophotometric assay. A fixed concentration of reduced benzylviologen of 0.2 mM was used in our kinetic analyses. Since this is very much greater than the K_m for reduced benzylviologen, reasonable estimates for the kinetic parameters

Table I: Kinetic Coefficients Describing the Reduction of Various Electron Acceptors by Reduced Methyl- and Benzylviologens Catalyzed by Nitrate Reductases[a]

Analysis of Unmodified, Cytochrome-Containing Nitrate Reductase with (a) Reduced Methylviologen as Reductant							
electron acceptor	ϕ_0 (s)	ϕ_1 (μM s)	ϕ_2 (μM s)	ϕ_{12} (μM^2 s)	$\phi_1\phi_0$ (μM)	$\phi_2\phi_0$ (mM)	$\phi_{12}\phi_1$ (mM)
nitrate	2.2×10^{-3}	0.4	0.93	0	182	0.42	
chlorate	1.42×10^{-3}	0.26	1.6	42	183	1.13	0.168
bromate	1.3×10^{-2}	1.4	175	1930	107	13.5	1.38

Analysis of Unmodified, Cytochrome-Containing Nitrate Reductase with (b) Reduced Benzylviologen as Reductant			
electron acceptor	ϕ_0^{app} (s)	ϕ_2^{app} (μM s)	$\phi_2^{app}/\phi_0^{app}$ (mM)
nitrate	3×10^{-3}	1.0	0.33
chlorate	1.3×10^{-3}	1.3	1.0
bromate	1.5×10^{-2}	156	10.4

Analysis of Trypsin-Modified, Cytochrome-Free Nitrate Reductase with (c) Reduced Methylviologen as Reductant					
electron acceptor	ϕ_0 (s)	ϕ_1 (μM s)	ϕ_2 (μM s)	ϕ_1/ϕ_0 (μM)	ϕ_2/ϕ_0 (μM)
nitrate	2.6×10^{-3}	0.47	0.75	181	288

[a] The kinetic coefficients listed are those in the reciprocal initial-rate equation $e/V_0 = \phi_0 + \phi_1/[S_1] + \phi_2/[S_2] + \phi_{12}/([S_1][S_2])$, where e is the enzyme concentration, S_1 is the electron donor (reduced methyl- or benzylviologen), and S_2 is the electron acceptor. ϕ_1/ϕ_0 is the Michaelis constant for S_1, and ϕ_2/ϕ_0 is the Michaelis constant for S_2. All measurements were made at 25 °C in 0.1 M potassium phosphate buffer, pH 6.8.

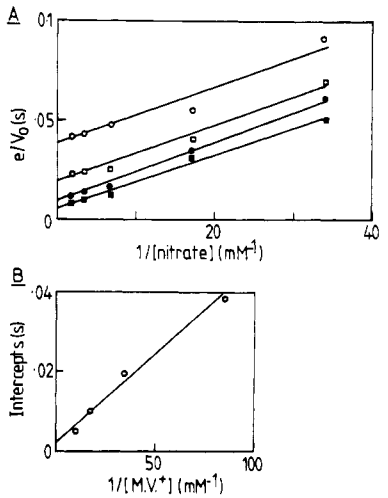


FIGURE 4: Initial-rate measurements of the reduction of reduced methylviologen by nitrate. (A) Primary plots showing the variation of the reciprocal of the initial specific rate at 25 °C and pH 6.8 with the reciprocal of nitrate concentration between 0.03 and 0.60 mM at several concentrations of reduced methylviologen. The reduced methylviologen concentrations were (■) 200, (●) 59, (□) 29.5, and (○) 12 μM. (B) Secondary plots showing the variation of the intercepts of the primary plots with the reciprocal of the reduced methylviologen concentration.

should be obtained. A linear Lineweaver–Burk plot was obtained (not shown), and apparent values of ϕ_0 and ϕ_2 were estimated (Table Ib). Similar values for the turnover number (330 s^{-1}) and K_m for nitrate (0.33 mM) to those found with reduced methylviologen as reductant were obtained.

Clearly, for the reduction examined above, the kinetics of the enzyme are described by eq 1, but there are no significant ϕ_{12} terms, resulting in parallel double-reciprocal plots. Parallel plots of this nature are usually associated with enzyme substitution or "ping-pong" mechanisms, which require that ϕ_{12} is equal to zero. If, however, ϕ_{12} is simply very small in relation to the other terms rather than zero, other mechanisms are also possible. The use of alternative substrates can sometimes illuminate this problem. We examined the kinetics of the enzyme using alternative electron acceptors.

(ii) Chlorate and Bromate as the Electron Acceptor. Chlorate can replace nitrate as the electron acceptor for nitrate reductase (Forget, 1974). The double-reciprocal plots obtained when chlorate was used as electron acceptor were linear under the conditions tested, in accordance with eq 1. However, in contrast to that found for nitrate as the electron acceptor, both intercept and slope were functions of the reduced methylviologen concentration, indicating that ϕ_{12} has a nonzero value. The secondary plots from which the kinetic constants listed in Table I were estimated were also linear. Interestingly, there is a higher turnover number with chlorate (700 s^{-1}) than with nitrate as substrate.

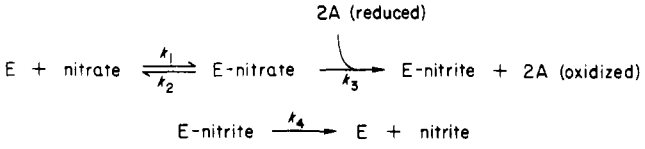
We found that nitrate reductase was able to reduce bromate. This has not previously been reported. Similar kinetic experiments were performed with bromate as the electron acceptor (data not shown). Linear double-reciprocal plots were obtained that were qualitatively similar to those described for chlorate as the electron acceptor; namely, a significant ϕ_{12} term was present. The derived kinetic constants are tabulated in Table I. A much lower turnover number and higher K_m for the electron acceptor were found with bromate than with chlorate or nitrate as the electron acceptor.

The presence of ϕ_{12} terms in the kinetic equation when chlorate and bromate are substrates for nitrate reductase rules out a simple enzyme substitution or ping-pong mechanism. However, Palmer & Massey (1968) have shown for a special case of the ping-pong mechanism known as a "ternary complex" mechanism that ϕ_{12} terms are expected and are frequently vanishingly small. In the ternary complex mechanism it is a complex of reduced enzyme and oxidized electron donator that reacts with the electron acceptor, rather than free, reduced enzyme.

However, characteristic of enzyme-substitution mechanisms, whether ping-pong or ternary complex, is that the parameter of eq 1 describing reduction, ϕ_1, is independent of the nature of the oxidizing substrate [see Palmer & Massey (1968) and Morpeth & Massey (1982)]. From Table I this is clearly not so. With nitrate as the electron acceptor ϕ_1 is equal to 0.4 μM s, and with chlorate and bromate it is equal to 0.26 μM s and 1.4 μM s, respectively. Therefore, such mechanisms cannot be involved.

Further inspection of Table I shows that though ϕ_1 is highly dependent on the oxidizing substrate, ϕ_2, the parameter describing the behavior of the oxidant, is independent of the nature of the reductant. Thus for nitrate, ϕ_2 is equal to 0.93 μM s with reduced methylviologen and 1 μM s with reduced benzylviologen. The ϕ_2 parameters with chlorate and bromate are also independent of the reductant. This suggests that a compulsory-order mechanism is in operation in which the substrate that oxidizes the enzyme binds before the reducing substrate (Dalziel, 1957). In Scheme I we show our proposed

Scheme I: Proposed Mechanisms for Oxidation of Reduced Bipyridylium Compounds by Nitrate, Chlorate, and Bromate Catalyzed by Nitrate Reductase



mechanism for nitrate reductase with reduced methyl- or benzylviologens as the reductant. This is a special case of the compulsory-order mechanism first suggested for liver alcohol dehydrogenase by Theorell & Chance (1951). In the Theorell–Chance mechanism there are no kinetically significant ternary complexes though they must exist, however, transiently. The invariance of ϕ_0 with changing electron donator is also consistent with such a mechanism and suggests that reduced product release from an oxidized enzyme product complex is rate limiting. For a fuller discussion and a well-documented case of the Theorell–Chance mechanism see Dalziel & Dickinson (1966).

The physical significance of the kinetic coefficients of eq 2 for the Theorell–Chance mechanism of Scheme I is

$$\phi_0 = \frac{1}{k_4} \qquad \phi_1 = \frac{1}{k_3} \qquad \phi_2 = \frac{1}{k_1} \qquad \phi_{12} = \frac{k_2}{k_1 k_3} \qquad (2)$$

Nitrate reductase was unable to utilize iodate as the electron acceptor.

(iii) Quinols as the Electron Donor. The quinols used in our work were duroquinol and ubiquinol 1. They were selected for their comparatively high aqueous solubility. The series of double-reciprocal plots from experiments where nitrate concentration was varied at different set concentrations of ubiquinol 1 were linear and parallel (data not shown). The secondary plot of the intercept against the reciprocal of ubiquinol 1 concentration was linear. Similar experiments were performed with chlorate and bromate as electron acceptors and a second series with duroquinol as the electron donor. In all experiments reported in this section the double-reciprocal plots were linear over the concentration range of substrates used.

The kinetic behavior was consistent with eq 1, and in all cases significant ϕ_{12} terms were absent. In Table II we have summarized the results of our detailed steady-state experiments with each of the quinols as substrate. As found with the viologens as electron donators, iodate was not a substrate for the enzyme.

Certain points in Table II are of particular interest. First, ubiquinol is a better substrate than duroquinol, having a smaller ϕ_0 and ϕ_1 with the same oxidizing substrate. Second, ϕ_0 for either the ubiquinol series or duroquinol series was, within the limits of error, independent of the nature of the oxidizing substrate.

Table II: Kinetic Coefficients Describing the Reduction of Various Electron Acceptors by Quinols Catalyzed by Nitrate Reductase[a]

electron acceptor	ϕ_0 (s)	ϕ_1 (μM s)	ϕ_2 (μM s)	ϕ_1/ϕ_0 (μM)	ϕ_2/ϕ_0 (μM)
			Duroquinol as Reductant		
nitrate	1.6	56	3.1	35	1.94
chlorate	2.1	39	370	18.6	176
bromate	1.5	47	470	31.3	313
			Ubiquinol 1 as Reductant		
nitrate	0.139	10.8	0.26	77.7	1.9
chlorate	0.132	9.8	2.1	74.2	15.9
bromate	0.10	7.7	11.1	77	111

[a] The kinetic coefficients shown are those in the reciprocal initial-rate equation $e/V_0 = \phi_0 + \phi_1/[S_1] + \phi_2/[S_2]$, where e is the enzyme concentration, S_1 is the quinol, and S_2 is the electron acceptor. ϕ_1/ϕ_0 is the Michaelis constant for the quinol, and ϕ_2/ϕ_0 is the Michaelis constant for the electron acceptor. All measurements were made at 25 °C in 0.1 M potassium phosphate buffer, pH 6.8.

The parameter ϕ_0 is also larger (and so the turnover number is smaller) with both quinols than when reduced viologen dyes are used as the reductant. With the viologen dyes, reduced product release is probably rate limiting (see above). Thus with quinols a step either in the reduction of nitrate reductase by the quinol or in quinone release is presumably rate limiting.

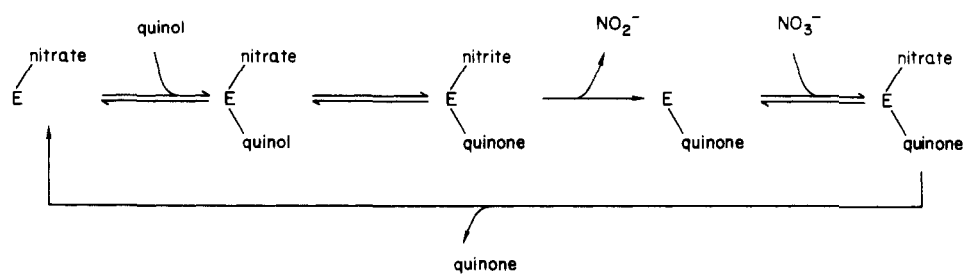
When quinols are the reductants of nitrate reductase, the dependence of the parameters of eq 1 on varying substrates is now reversed in comparison to the reduced bipyridylium compounds. Table II shows that ϕ_1 is now independent of the nature of the oxidizing substrate while ϕ_2 is dependent on the nature of the reducing substrate. Thus, with analogues of reducing substrate in vivo, the mechanism of nitrate reductase has changed from that seen with the viologen electron donors.

The results of the next section indicate that the oxidizing and reducing substrates interact with the enzyme at spatially distinct sites. Thus, classical Michaelis–Menten kinetics do not apply. This type of situation was first considered by Northrop (1969). More recently Coughlan & Rajagopalan (1980) have suggested that oxidation/reduction enzymes of this type are most likely to work by a two-site, ping-pong mechanism with random addition of substrates and products at the two sites.

The two-site, ping-pong mechanism, like all enzyme-substitution mechanisms, predicts the nonvariance of ϕ_1 with changing substrates (Coughlan & Rajagopalan, 1980; Morpeth & Massey, 1982).

Thus, the mechanism shown in Scheme II is likely to be in operation when nitrate reductase is oxidizing quinols in the steady state. From the discussion above, this is consistent with

Scheme II: Proposed Mechanism for Nitrate Reductase Catalyzed Oxidation of Quinols[a]



[a] The mechanism shown is not the full two-site, enzyme-substitution mechanism but the preferred pathway, deduced from the data discussed in the text. Nitrate is shown to bind before quinone release. This is a reasonable assumption based on (1) the known tight binding of nitrate to the enzyme and (2) the fast binding of nitrate, deduced from the high turnover number observed with viologen dyes as the reducing substrate.

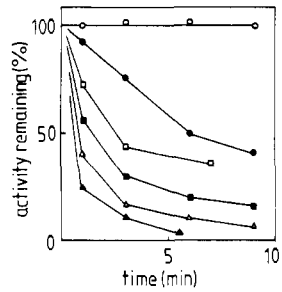


FIGURE 5: Effect of diethyl pyrocarbonate on nitrate reductase activity. Cytochrome *b* containing nitrate reductase (19.2 mM) was incubated with diethyl pyrocarbonate. The plots show the percentage of the initial activity remaining as a function of incubation time: (○) shows the effect of 2.2 mM diethyl pyrocarbonate on the activity of nitrate reductase when assayed with reduced benzylviologen as the reductant. In all the other plots the activity refers to the ubiquinol:nitrate oxidoreductase activity (see Materials and Methods). The following concentrations of diethyl pyrocarbonate were used: (●) 0.22, (□) 0.56 (+ 23 μM ubiquinone 1), (■) 0.56, (△) 1.1, and (▲) 2.2 mM.

an "enzyme-substitution" type of mechanism in which the two substrates are not present simultaneously on the enzyme during its turnover. Furthermore, whereas the K_m for nitrate (ϕ_2/ϕ_0), when viologens are the reductant, is quite high (Table Ia,b), the K_m for nitrate, when analogues of the in vivo quinol reductant are employed, is much lower, 1.9 μM (Table II).

Kinetic Analysis of Trypsin-Treated Nitrate Reductase. The results of steady-state kinetic studies on trypsin-modified, cytochrome-free nitrate reductase are given in Table Ic. Clearly, when either reduced benzylviologen or reduced methylviologen is the reductant, loss of cytochrome *b* and cleavage of the β subunit do not affect the kinetic properties of nitrate reductase. This is in agreement with EPR studies on native and trypsin-modified nitrate reductases, which suggest that the molybdenum sites are identical in both enzymes (F. F. Morpeth, D. H. Boxer, G. N. George, and R. C. Bray, unpublished observations).

However, with either reduced duroquinone or reduced ubiquinone 1 as the electron donor the modified cytochrome-free enzyme was inactive. This is the most direct evidence that quinols donate electrons to nitrate reductase through cytochrome *b* only. Thus it strongly supports the mechanism for proton translation by nitrate reductase suggested by Jones & Garland (1977).

Inhibition of Nitrate Reductase Activity. (i) Reaction of Nitrate Reductase with Diethyl Pyrocarbonate. The above results suggest that quinols and reduced bipyridylium compounds interact with nitrate reductase at distinct sites. In Figure 5 we show clear evidence to confirm this. Nitrate reductase rapidly loses all activity when assayed with ubiquinone 1 as the reductant, on reaction with diethyl pyrocarbonate at 25 °C in 50 mM potassium phosphate, pH 7. If, however, nitrate reductase is assayed with reduced benzylviologen, full activity is retained even under conditions where ~90% of the ubiquinol activity is lost within 3 min. This shows that the reduced viologens and ubiquinol do indeed interact with the enzyme at distinct sites, but only the ubiquinol site is affected by the diethyl pyrocarbonate modification. The presence of ubiquinone in the reaction seems to protect the enzyme against the loss of quinol activity as shown in Figure 5. The reagent had no effect on the benzylviologen-dependent activity of trypsin-modified enzyme. Thus, the diethyl pyrocarbonate is presumably reacting at the quinol active site. Quinols are assumed to interact with the cytochrome moieties of nitrate reductase; thus if diethyl pyrocarbonate is reacting at the quinol oxidation site, this should affect the optical spectrum of nitrate reductase, which is dominated by the cytochromes. Indeed after reaction with diethyl pyrocarbonate there are small but distinct changes in the oxidized and reduced spectra pertaining to the cytochrome *b* component of nitrate reductase with respect to the unmodified enzyme (data not shown).

The nature of the residue or residues that are reacting with diethyl pyrocarbonate that lead to the loss of the quinol oxidizing activity is not known. Diethyl pyrocarbonate usually modifies thiol or imidazole groups (Miles, 1977). An imidazole group does not seem to be involved here, since on inactivation of nitrate reductase, there is no increase in absorbance at 240 nm, which would be characteristic of formation of the *N*-carbethoxyhistidyl derivative. Also, the inactivation cannot be reversed by hydroxylamine, which would lead to breakdown of an *N*-carbethoxyhistidine and reactivate histidine. Since long-term incubation (12 h) with 20 mM iodoacetamide or iodoacetic acid, at 25 °C and pH 7, leads to no increased loss of benzylviologen or quinol activity over a control, it also seems unlikely that diethyl pyrocarbonate is causing loss of activity by modifying a thiol group, but this cannot be completely ruled out without further studies with a wider range of thiol-modifying reagents.

(ii) Reaction with 2-n-Heptyl-4-hydroxyquinoline N-Oxide. 2-*n*-Heptyl-4-hydroxyquinoline *N*-oxide (HQNO) is a quinol analogue that is known to inhibit nitrate respiration in *E. coli* (Jones et al., 1980). With purified cytochrome *b* containing nitrate reductase we found that HQNO does not inhibit the reduction of nitrate by reduced methylviologen. However, it does inhibit nitrate reduction by ubiquinol. The inhibition appeared to be competitive with ubiquinol since at a low nitrate concentration the inhibition was relieved by increasing ubiquinol concentration. Unfortunately, further investigation was hampered by curved double-reciprocal plots in the presence of HQNO. At present we can offer no explanation for this phenomenon.

Clearly it is possible to inhibit one activity of nitrate reductase yet not the other with HQNO. This is further evidence to support the scheme that in vivo the catalytic events, reduction by quinol and oxidation by nitrate, occur at spatially distinct sites.

DISCUSSION

Our results indicate that the mechanism of nitrate reductase action is different with a reduced bipyridylium compound as the electron donor from that found when quinols (analogues of the physiological reductant) are oxidized. Our data for the viologen dye dependent activity are best interpreted by assuming a compulsory-order mechanism in which nitrate (or oxidizing substrate) binds to the enzyme before the reducing substrate and in which there are no kinetically significant ternary complexes. This Theorell–Chance mechanism requires that an enzyme–nitrate complex would be easier for the viologen reductant to reduce than the free enzyme. Vincent & Bray (1978) demonstrated the existence of an enzyme–nitrate complex, and more recent EPR investigations (Morpeth et al., 1983) have shown that nitrate, chlorate, and bromate all form complexes at the molybdenum site in the enzyme. With nitrate as the electron acceptor ϕ_{12} in eq 1, describing the kinetics, appears to be vanishingly small, in contrast to that found for the alternative electron acceptors, chlorate and bromate. Very small ϕ_{12} terms are occasionally observed for compulsory-order mechanisms (Dalziel, 1957) and may arise if k_2/k_1 (the dissociation constant for the enzyme-oxidizing substrate complex) is small in relation to the K_m for the oxidizing substrate. That the dissociation constant for the en-

zyme–nitrate complex is indeed small can be inferred from the demonstration by EPR spectroscopy that the enzyme as prepared displays a molybdenum signal characteristic of the enzyme–nitrate complex, even though the enzyme has been prepared in buffers not containing nitrate (Morpeth et al., 1983). We estimate the K_m for nitrate, employing the viologen dyes as reductant, to be about 0.42 mM. This high value is in agreement with that reported by Adams & Mortenson (1982). A vanishingly small ϕ_{12} term would, therefore, be expected for the enzyme obeying our proposed mechanism with nitrate as the oxidizing substrate. We estimate the dissociation constants (ϕ_{12}/ϕ_1) for the enzyme–chlorate and –bromate complexes to be 0.16 mM and 1.38 mM, respectively.

When quinols serve as the reducing substrate, the kinetic behavior of the enzyme no longer conforms to the above model but is consistent with a two-site, enzyme-substitution mechanism. The steady-state parameters in this case are radically different from those observed with the viologen dyes as substrate. The turnover number is much reduced, and the K_m for nitrate assumes the physiologically more reasonable value of about 2 μM when quinols are the reductant. Significantly, the lowest value for K_m/V_{max} for all the substrates tested was found for ubiquinol 1:nitrate oxidoreductase activity—the closest approximation to the reaction catalyzed by the enzyme in vivo.

This is the first report of a detailed kinetic analysis of isolated nitrate reductase employing quinols as the reducing substrate. We suggest that any physiological argument based on the steady-state kinetic parameters of the enzyme obtained with viologen dyes as the reducing substrate must be interpreted with extreme caution.

It has been proposed that the redox-driven, proton-translocating capacity of nitrate reductase arises from the enzyme's spatially distinct protolytic, quinol oxidation site and its proton-consuming, nitrate reduction site, which are located at opposite faces of the coupling, cytoplasmic membrane (Jones et al., 1980). Our analysis of the properties of the purified enzyme establishes that the quinol and nitrate active sites are indeed distinct. This conclusion is based on the following arguments. First, the kinetic behavior of the ubiquinol:nitrate oxidoreductase activity is consistent with a two-site, enzyme-substitution mechanism. Second, the cytochrome-free derivative of the enzyme does not exhibit ubiquinol:nitrate oxidoreductase activity but possesses unperturbed reduced viologen dye dependent nitrate reductase activity. Third, diethyl pyrocarbonate is a specific, irreversible inhibitor of the ubiquinol:nitrate oxidoreductase activity. Fourth, HQNO, a quinone analogue, also specifically inhibits, in a manner competitive with quinol, the ubiquinol:nitrate oxidoreductase activity but does not affect the viologen dye dependent activity.

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Registry No. HQNO, 341-88-8; reduced methylviologen, 24934-49-4; reduced benzylviologen, 15591-62-5; nitrate, 14797-55-8; chlorate, 14866-68-3; bromate, 15541-45-4; duroquinol, 527-18-4; ubiquinol 1, 52590-98-4; nitrate reductase, 37256-45-4; diethyl pyrocarbonate, 1609-47-8.

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