

## Kinetics of membrane-bound nitrate reductase A from *Escherichia coli* with analogues of physiological electron donors

### Different reaction sites for menadiol and duroquinol

Roger GIORDANI, Jean BUC, Athel CORNISH-BOWDEN and María Luz CÁRDENAS

Laboratoire de Chimie Bactérienne, Institut Fédératif 'Biologie Structurale et Microbiologie', Centre National de la Recherche Scientifique, Marseille, France

(Received 16 July/1 October 1997) – EJB 97 1013/4

We have compared the steady-state kinetics of wild-type nitrate reductase A and two mutant forms with altered  $\beta$  subunits. To mimic conditions *in vivo* as closely as possible, we used analogues of the physiological quinols as electron donors and membranes with overexpressed nitrate reductase A in preference to a purified  $\alpha\beta\gamma$  complex. With the wild-type enzyme both menadiol and duroquinol supply their electrons for the reduction of nitrate at rates that depend on the square of the quinol concentration, menadiol having the higher catalytic constant. The results as a whole are consistent with a substituted-enzyme mechanism for the reduction of nitrate by the quinols.

Kinetic experiments suggest that duroquinol and menadiol deliver their electrons at different sites on nitrate reductase, with cross-inhibition. Menadiol inhibits the duroquinol reaction strongly, suggesting that menadiol may be the preferred substrate *in vivo*. To examine whether electron transfer from menadiol and duroquinol for nitrate reduction requires the presence of all of the Fe-S centres, we have studied the steady-state kinetics of mutants with  $\beta$  subunits that lack an Fe-S centre. The loss of the highest-potential Fe-S centre results in an enzyme without menadiol activity, but retaining duroquinol activity; the kinetic parameters are within a factor of two of those of the wild-type enzyme, indicating that this centre is not required for the duroquinol activity. The loss of a low-potential Fe-S centre affects the activity with both quinols: the enzyme is still active but the catalytic constants for both quinols are decreased by about 75%, indicating that this centre is important but not essential for the activity.

The existence of a specific site of reaction on nitrate reductase for each quinol, together with the differences in the effects on the two quinols produced by the loss of the Fe-S centre of +80 mV, suggests that the pathways for transfer of electrons from duroquinol and menadiol are not identical.

**Keywords:** nitrate reductase; quinol; menadiol; duroquinol.

A respiratory system constituted by formate dehydrogenase and nitrate reductase is induced in *Escherichia coli* under anaerobic conditions in the presence of nitrate (Pichinoty, 1969; Ruiz-Herrera and DeMoss, 1969). It allows the flow of electrons to nitrate from the formate that is produced when pyruvate is converted to acetyl-CoA by pyruvate-formate lyase. The electron transfer is strongly dependent on the presence of a quinone, and either ubiquinone (a benzoquinone) or menaquinone (a naphthoquinone) can fulfil the role (Wallace and Young, 1977). Nitrate reductase A (EC 1.7.99.4) is the principal isoenzyme and catalyses the reduction of nitrate by quinols under physiological conditions. It is a membrane-bound molybdoenzyme composed of three subunits designated  $\alpha$ ,  $\beta$  and  $\gamma$ , coded by the genes *narG*, *narH* and *narI* respectively, which form part of a single operon (Sodergren and DeMoss, 1988; Sodergren et al., 1988; Blasco

et al., 1989). The  $\alpha$  and  $\beta$  subunits are located on the cytoplasmic side of the membrane and form an  $\alpha\beta$  complex that binds to the membrane by interacting with the  $\gamma$  subunit, a very hydrophobic protein embedded in the membrane (Sodergren et al., 1988).

Each subunit carries a different set of redox centres (Blasco et al., 1989; Guigliarelli et al., 1992). The 139-kDa  $\alpha$  subunit contains the active site for the reduction of nitrate to nitrite and carries a Mo cofactor (molybdopterin guanine dinucleotide); the 58-kDa  $\beta$  subunit contains four Fe-S centres (Fig. 1); the  $\gamma$  subunit is a cytochrome of type *b* carrying two haems (Hackett and Bragg, 1982).

The membrane of *E. coli* contains three principal types of quinone, menaquinone, demethylmenaquinone and ubiquinone, in proportions that vary in *E. coli* and other bacteria according to the conditions of growth (Polglase et al., 1966; Kröger et al., 1971; Wallace and Young, 1977; Unden, 1988; Wissenbach et al., 1992). Ubiquinone greatly predominates in aerobically grown *E. coli*, but the concentrations of ubiquinone and the naphthoquinones change in opposite directions in anaerobic conditions, ubiquinone and menaquinone existing in roughly equal proportions in the presence of nitrate. They appear to behave as a pool in the membranes of *E. coli* and some other bacteria. Ubiquinone as well as menaquinone can serve as electron carrier in nitrate respiration (Wallace and Young, 1977), whereas fuma-

Correspondence to M. L. Cárdenas, Laboratoire de Chimie Bactérienne, Centre National de la Recherche Scientifique, 31 chemin Joseph-Aiguier, B.P. 71, F-13402 Marseille Cedex 20, France

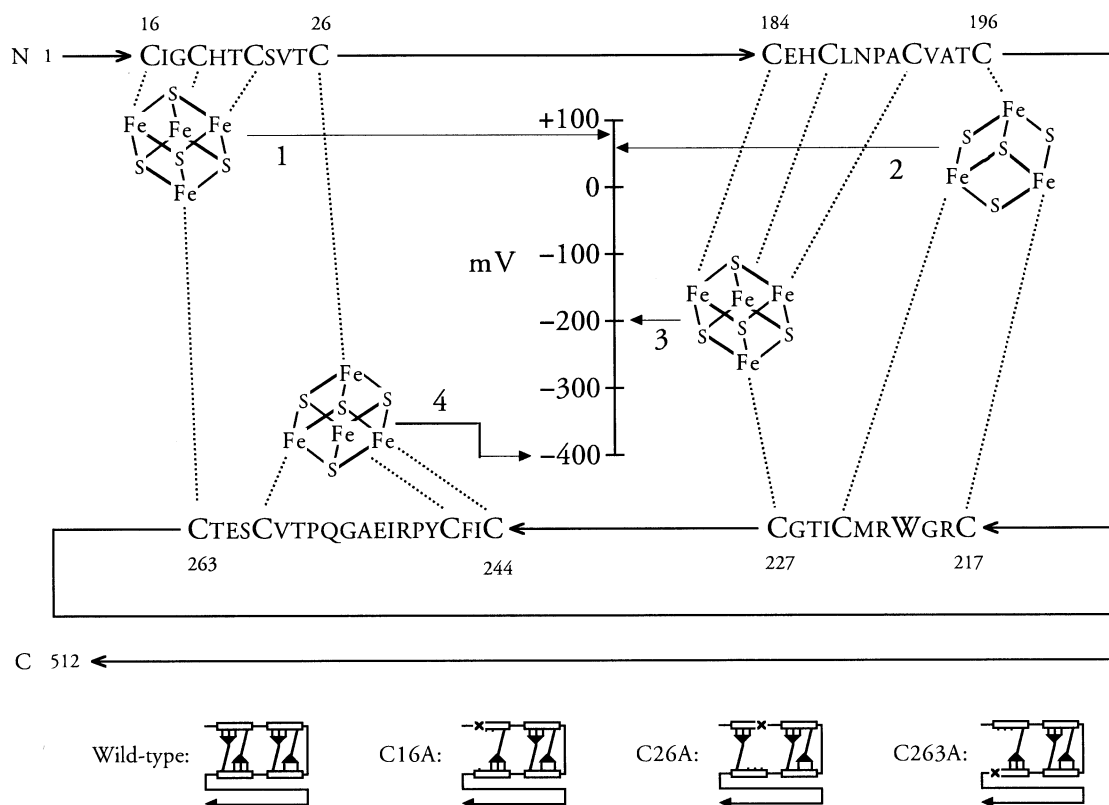
Fax: +33 491 71 89 14.

E-mail: cardenas@ibsm.cnrs-mrs.fr

URL: <http://ir2lcb.cnrs-mrs.fr/lcbpage/lcb.htm>

Abbreviation. [C26A] $\beta$ , mutant of nitrate reductase with Cys26 of the  $\beta$  subunit replaced by Ala.

Enzyme. Nitrate reductase A (EC 1.7.99.4).



**Fig. 1. Arrangement of Fe-S centres in the  $\beta$  subunit of nitrate reductase.** The native  $\beta$  subunit contains four Fe-S centres with midpoint potentials from +80 to  $-400$  mV, as indicated. Each of these is coordinated by four Cys residues. Mutations C16A and C263A of Cys16 and Cys263, which coordinate the +80-mV Fe-S centre, and mutation C26A of Cys26, which coordinates the  $-400$ -mV Fe-S centre, lead to active  $\alpha\beta$  complexes that lack these Fe-S centres. Although to permit clear separation of the four Cys clusters the N-terminal half of the molecule is not drawn to scale, the long extension drawn in the C-terminal gives the correct impression that all four are located in the N-terminal half. The lower part of the figure illustrates the structural characteristics of the mutants considered by reference to a similar diagram for the wild-type enzyme that is a schematic representation of the main scheme.

rate, trimethylamine *N*-oxide and dimethylsulphoxide respiration depend on the presence of naphthoquinones (Guest, 1977; Kröger, 1978; Meganathan, 1984; Unden, 1988). Unlike some more selective reductases and dehydrogenases, therefore, nitrate reductase accepts both menaquinol and ubiquinol as substrates, though apparently not demethylmenaquinol (Wissenbach et al., 1990, 1992).

The complete sequence of electron transfer is postulated to be as follows: quinols  $\rightarrow$  [*b*-type haems ( $\gamma$ )  $\rightarrow$  Fe-S centres ( $\beta$ )  $\rightarrow$  Mo ( $\alpha$ )]  $\rightarrow$  nitrate. EPR studies indicate that the four Fe-S centres belong to two classes with markedly different redox potentials (Guigliarelli et al., 1992). The midpoint potentials of +120 mV and +20 mV for the haem groups (Hackett and Bragg, 1982), together with those of +180 mV and +220 mV for the couples Mo(IV)/Mo(V) and Mo(V)/Mo(VI) respectively (Vincent, 1979), suggest that likely candidates for involvement in the electron-transfer pathway are the two Fe-S centres of high potential, the [4Fe-4S] centre at +80 mV and the [3Fe-4S] centre at +60 mV.

In a previous study of the roles of the different Fe-S centres (Buc et al., 1995) we worked with the soluble complex  $\alpha\beta$ , which is catalytically active with non-physiological electron donors such as benzyl viologen and methyl viologen. Steady-state kinetic studies with  $\alpha\beta$  and mutant  $\alpha\beta^*$  complexes suggested that reduced benzyl viologen delivers the electrons directly to the Mo cofactor, i.e. electrons derived from benzyl viologen or similar electron donors may follow a different pathway from those derived from quinols (Buc et al., 1995). To obtain physiologically significant results, therefore, it became necessary to work with

the membrane-bound enzyme and with donors more closely related to the physiological quinols. In this work we use membranes with wild-type or mutant nitrate reductase A overexpressed, together with menadiol and duroquinol, analogues of the physiological quinols menaquinol and ubiquinol. We set out to determine the kinetic behaviour of menadiol and duroquinol in nitrate reduction, and the kinetic mechanism of the reaction. Given that menadiol and duroquinol differ considerably in structure, we have examined whether they may interact with different sites on the enzyme. Finally, we have studied whether both of the two Fe-S centres with high midpoint potentials are necessary for activity.

A brief account of part of this work was presented at the 7th International Meeting on BioThermoKinetics (Cárdenas et al., 1996).

## EXPERIMENTAL PROCEDURES

**Reagents and chemicals.** Menadione (2-methyl-1,4-naphthoquinone) and duroquinone (tetramethyl-*p*-benzoquinone) were purchased from Aldrich. Benzyl viologen and carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone were from Sigma. All other chemicals were of the highest grade of purity commercially available, and were supplied either by ProLabo or Merck.

**Bacterial strains and plasmids.** The *E. coli* strains and plasmids used in this study are listed in Table 1. Details on plasmids and on oligonucleotide site-directed mutagenesis of bacterial strains are given by Guigliarelli et al. (1996).

**Table 1. Strains and plasmids used in this study.** Ap<sup>r</sup>, ampicillin-resistant, Km<sup>r</sup>, kanamycin-resistant, *tacP*, *tac* promoter.

Strain/plasmid	Genotype	Reference
MC4100	<i>araD</i> 139 $\Delta$ ( <i>lac</i> POZYA- <i>argF</i> ) <i>rpsL</i> <i>thi</i>	Casadaban (1976)
LCB79	MC4100 $\phi$ ( <i>nar-lac</i> )	Pascal et al. (1982)
LCB333	<i>thi1</i> <i>leu6</i> <i>thr1</i> <i>tonA21</i> <i>lacY1</i> <i>supE44</i> $\Delta$ <i>nar25</i> ( <i>narGH</i> ) Km <sup>r</sup>	Pascal et al. (1982)
pJF119EH	<i>tacP</i> <i>rrnB</i> <i>lacI</i> <sup>q</sup> Ap <sup>r</sup>	Fürste et al. (1986)
pVA700	pJF119EH ( <i>narGHJI</i> ) Ap <sup>r</sup> , <i>tacP</i>	Guigliarelli et al. (1996)
pVA700-C16A	pJF119EH ( <i>narGH</i> [C16A]JI) Ap <sup>r</sup>	Guigliarelli et al. (1996)
pVA700-C26A	pJF119EH ( <i>narGH</i> [C26A]JI) Ap <sup>r</sup>	Guigliarelli et al. (1996)

**Growth of bacteria.** Growth conditions were as described in Guigliarelli et al. (1996). All the strains were grown anaerobically at 37°C on TY medium supplemented with glucose (2 g/l). Expression of nitrate reductase was induced by isopropyl  $\beta$ -thiogalactoside (0.2 mM) (pVA700). The antibiotics ampicillin (50  $\mu$ g/ml) and chloramphenicol (10  $\mu$ g/ml) were used.

**Preparation of subcellular fractions.** Cells were harvested during the exponential phase of growth and suspended in 100 mM Tris/HCl pH 8.3, 1 mM MgCl<sub>2</sub> and disrupted in a French press. The buffer used in this step contained 0.2 mM phenylmethylsulphonyl fluoride. The resulting suspension was centrifuged for 15 min at a maximum of 18000 g to sediment unbroken cells. The supernatant was further centrifuged for 90 min at a maximum of 120000 g, and the new supernatant was discarded while the pellet was retained. All of these procedures were performed at 4°C. The pellets, containing nitrate reductase as a complete membrane-bound  $\alpha\beta\gamma$  complex, were resuspended in a small volume of buffer and stored at -18°C until use.

Some controls were done with partially purified  $\alpha\beta$  nitrate reductase prepared as previously described (Buc et al., 1995).

**Enzyme assays and kinetic studies.** Nitrate reductase activity with benzyl viologen as substrate was measured spectrophotometrically (Jones and Garland, 1977) by nitrate-dependent oxidation of the reduced benzyl viologen, with the precautions described previously (Buc et al., 1995). To avoid any ambiguity between the results with one-electron and two-electron donors, all rates are expressed as rates of reduction of nitrate, even though in all cases the actual spectrophotometric observations were of oxidation of the electron donors.

Nitrate reductase activities with quinols as substrates were measured by a spectrophotometric method based on one described previously (Unden and Kröger, 1986), but optimized for kinetic studies of menadiol and duroquinol, with an exhaustive exploration of the different experimental conditions suitable for these two quinols (Buc and Giordani, 1997). KBH<sub>4</sub> was preferred to dithionite as a reducing agent for menadione and duroquinone, which have mid-point potentials of -1 mV and +35 mV respectively, because dithionite has a strong absorbance in the range 200–360 nm that interferes with the signals of duroquinone and menadione, and it can also reduce nitrate reductase. KBH<sub>4</sub> does not have these inconvenient properties and is specific for aldehydes and ketones. The quinones were directly reduced in the cuvette by a freshly prepared aqueous solution of KBH<sub>4</sub> (10 g/l), renewed every hour to maintain a

high reducing power. Study of the spectra of the oxidized and reduced quinones showed that the best wavelengths were 260 nm for menadione and 270 nm for duroquinone: at these wavelengths the differences between oxidized and reduced forms are maximal, with no detectable spectral interference between the reduced and the oxidized forms. To determine the minimum amount of KBH<sub>4</sub> capable of producing a complete reduction, the spectrum of each quinone was monitored after addition of different quantities of KBH<sub>4</sub> increasing progressively by small amounts. In this way it was possible to avoid an excess of reducing agent. The differences in molar absorbance between oxidized and reduced forms were determined at various concentrations of quinones, yielding the values 21.3 mM<sup>-1</sup> cm<sup>-1</sup> for duroquinone and 17.2 mM<sup>-1</sup> cm<sup>-1</sup> for menadione.

Steady-state kinetic studies were performed with a Hitachi U-2000 spectrophotometer, thermostatted at 30°C and connected to a PC-compatible computer. The absorbance was measured during 300 s at 260 nm (menadione) and 270 nm (duroquinone), under anaerobic conditions. Oxygen was removed from buffer by flushing with N<sub>2</sub>. A 1.6-ml quartz cuvette closed by a teflon cap with a central hole was used to allow the different reagents to be added with a microsyringe, in the following order: 1.6 ml 50 mM potassium phosphate pH 7.5; 2–10  $\mu$ l of a quinone solution in ethanol; KBH<sub>4</sub>; the membrane suspension (5–75  $\mu$ g protein); KNO<sub>3</sub>, added last to start the reaction. The additions (not counting the buffer) add up to 30–60  $\mu$ l. The medium absorbance was monitored for about 1 min before adding the nitrate to verify the stability of the medium and the lack of an activity independent of nitrate. Thorough mixing in the cuvette was achieved by displacement of glass beads. As the membranes were from a strain with nitrate reductase overexpressed about 10-fold, amounts of membrane low enough to avoid turbidity could be used.

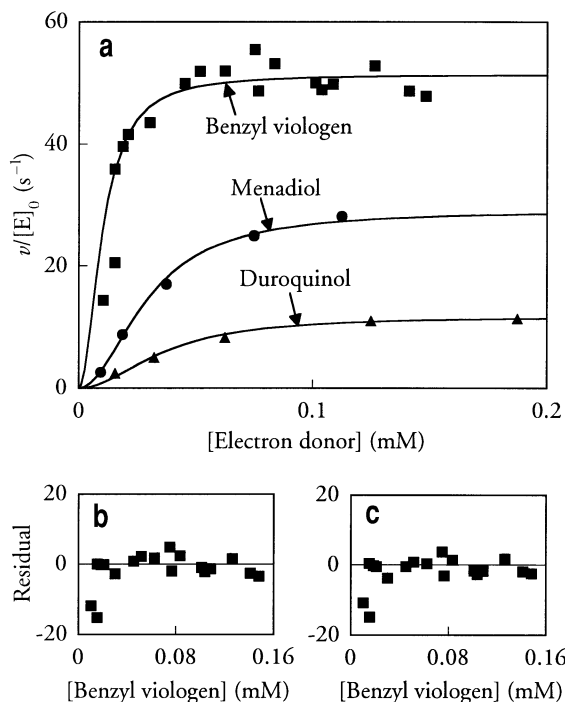
**Competition studies.** For experiments with the two types of quinones present at the same time the reaction was followed at 250 nm, the isosbestic point, which was determined by comparing the difference spectrum of the reduced and oxidized forms of menadione with that of duroquinone. The difference in molar absorbance at this wavelength is 8.6 mM<sup>-1</sup> cm<sup>-1</sup>.

**Quantification of nitrate reductase.** The nitrate reductase concentration was estimated by reference to the percentage of enzyme in the total protein, measured by determining the percentage of nitrate reductase antigen present in nitrate reductase membranous preparations by rocket immunoelectrophoresis (Graham et al., 1980) as described previously (Buc et al., 1995). Proteins were estimated by the technique of Lowry et al. (1951). The amount of overexpressed enzymes was about ten times that of the parent strain MC4100. With the plasmids used here, which express the genes stoichiometrically, about 90% of the enzyme is membrane-bound. For more details see Guigliarelli et al. (1996).

**Analysis of data.** A PC-compatible computer was used to fit experimental data to appropriate equations by non-linear least squares. The kinetic data were fitted by means of the program Leonora (Cornish-Bowden, 1995a), which assesses the appropriate weighting function from internal evidence in the data and uses the biweight method to minimize effects of outliers.

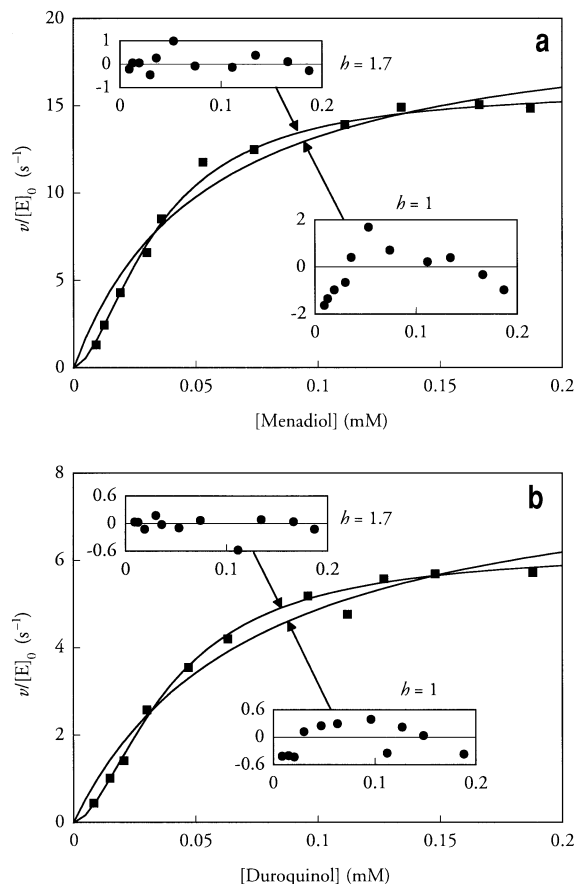
## RESULTS

**Kinetic behaviour with respect to the concentration of menadiol or duroquinol.** Menadiol and duroquinol can both reduce nitrate reductase in the absence of nitrate (Guigliarelli et al., 1996) and are substrates for the complete reaction when nitrate is present, menadiol giving the higher rate (Fig. 2a). In



**Fig. 2. Dependence of the rate on the electron donor concentration.** (a) Comparative rates with the different electron donors benzyl viologen (■), menadiol (●) and duroquinol (▲). Data obtained at a nitrate concentration of 12.5 mM were fitted to the Michaelis-Menten equation with respect to the electron donor concentration raised to the power of 2. The electron donor concentration corresponds to the amount of quinone added initially, which is assumed to be fully reduced to the quinol before the nitrate reductase reaction begins. This applies to all figures. (b) Plot of residual error as a function of benzyl viologen concentration for the benzyl viologen line in (a). (c) Plot of residual error as a function of benzyl viologen concentration for the benzyl viologen data fitted to an equation that allowed substrate inhibition. Note that the residual plots in (b) and (c) offer very little support for the visual impression of substrate inhibition given by the data in (a).

contrast, if a soluble  $\alpha\beta$  complex is used instead of membranes enriched with nitrate reductase, no activity is detected with either menadiol or duroquinol; unlike benzyl viologen, therefore, they cannot reduce the complex. As has been widely described, the membrane-bound nitrate reductase preparation was active with benzyl viologen or methyl viologen as substrates. The substantial difference in the maximal rates for benzyl viologen and the quinols (Fig. 2a) can probably be explained in terms of differences in reaction pathway, as benzyl viologen can short-circuit the complete mechanism by reducing the Mo cofactor directly (Buc et al., 1995). Although menadiol and duroquinol are less active than benzyl viologen, they are sufficiently active for kinetic studies in the conditions of the assay, especially menadiol. No menadiol oxidase or duroquinol oxidase activity was detected in the absence of nitrate, nor in membranes obtained from cells that did not express nitrate reductase; thus there are no unwanted reactions that could interfere with the kinetic studies. Similarly, the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone had no effect on the activity at a concentration of 10  $\mu$ M. (This control was important for the kinetic studies, because the membranes tend to form inverted vesicles, and so the possibility of microgradients of pH that could affect the rate in certain conditions could not be ruled out *a priori*, even though the buffer should largely eliminate such gradients.)



**Fig. 3. Evidence that the rate depends on the square of the quinol concentration.** Data with (a) menadiol and (b) duroquinol at a nitrate concentration of 12.5 mM were fitted to the Michaelis-Menten equation with respect to the quinol concentration (curve labelled  $h = 1$ ) and to the same equation with this concentration raised to the power that gives the best fit to the data (curve labelled  $h = 1.7$ ). In the residual plots shown as insets, which are left unlabelled to avoid crowding, the abscissa shows the quinol concentration in mM, and the ordinate shows the difference between observed and calculated values of  $v/[E_0]$  in  $s^{-1}$ .

For benzyl viologen (a one-electron donor) and the soluble  $\alpha\beta$  complex, two molecules of benzyl viologen are required for each ion of the two-electron acceptor nitrate (Buc et al., 1995). Similar behaviour was obtained with the membrane-bound enzyme (Fig. 2a), a Hill coefficient of 2.0 giving the best fit. The quinols are in principle two-electron donors and so they are not bound by the same constraints as benzyl viologen. Nonetheless, the sigmoidal dependence of rate on the concentration of menadiol (Fig. 3a) or duroquinol (Fig. 3b) indicates the involvement of two molecules of quinol for each ion of nitrate.

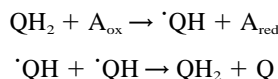
For both experiments the rate was calculated both as a normal Michaelis-Menten function and as a Michaelis-Menten function with the quinol concentration raised to a power  $h$ ; in both cases a power around 2 gives a better fit (see the residual plots shown as insets). The possibility that the sigmoidicity is due to the differential partitioning of the quinols between the membrane and the bulk solution cannot be ruled out. Nevertheless, considering that even without the isoprene sidechains menadiol and duroquinol have low solubility in water (the oxidized forms being even less soluble), the quinols may be expected to be more soluble in the phospholipid bilayer than in water, and so the partition between the two phases should favour the phospholipid bilayer over the aqueous phase.

The kinetic order of the reaction need not correspond exactly to the stoichiometry, because the relative values of the rate con-

stants of the different steps involved in the reaction have also to be considered, and the order is not necessarily an integer; the best fits were in fact obtained in this case with exponents of 1.7 both for menadiol and for duroquinol (Fig. 3) and, in general, in the different experiments the values obtained varied between 1.7–2.0.

The behaviour suggests that either quinol supplies only one electron in any redox step, i.e. when duroquinol or menadiol reacts it delivers only one electron at a time, the second electron coming either from the semiquinone produced or from a second molecule of quinol. (We cannot distinguish spectrophotometrically between these two possibilities.) The delivery of only one electron/step would be compatible with the fact that the haem groups of the cytochrome ( $\gamma$  subunit) can only accept one electron in each redox step.

According to Kröger and Klingenberg (1973) the transfer of single electrons in the oxidation of quinols is likely, especially as a cytochrome is the most probable electron acceptor. As the disproportionation of semiquinones is much faster than their formation by electron transport (Kröger and Klingenberg, 1973), the expected steady-state concentration of semiquinone radical is rather low. The complete process of quinol oxidation could then be represented in two steps as follows:

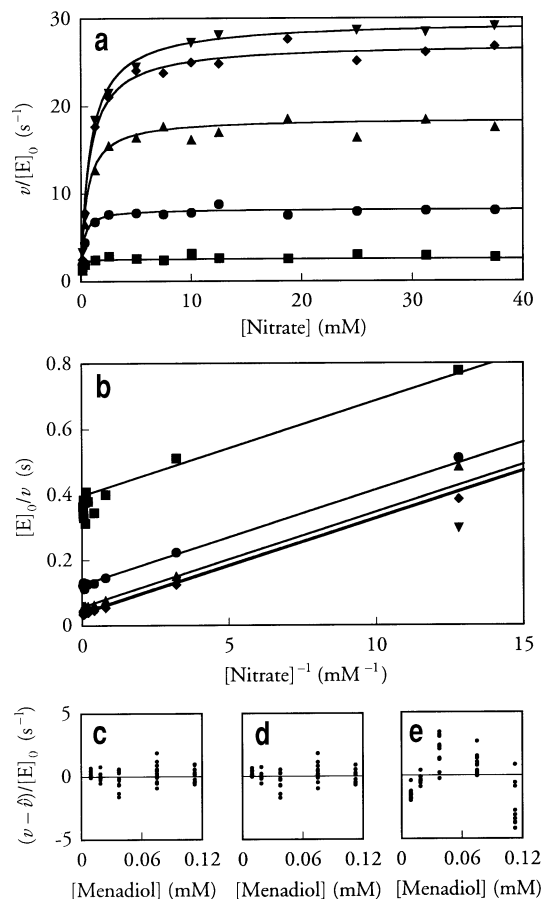


where  $\text{QH}_2$ ,  $\cdot\text{QH}$  and  $\text{Q}$  represent quinol, semiquinone and quinone respectively, and  $\text{A}_{\text{ox}}$  and  $\text{A}_{\text{red}}$  are the oxidized and reduced forms of the acceptor.

**Kinetic behaviour with respect to both quinol and nitrate: type of mechanism.** Two types of mechanisms can be considered for a reaction with two substrates, either a ternary-complex mechanism in which both substrates need to be bound to the enzyme before the reaction can occur and a substituted-enzyme mechanism in which the first substrate reacts with the enzyme to give the first product before the second substrate binds. Chemically it is far easier to visualize the latter for nitrate reductase, given that the substrates react at sites very far from one another, but the kinetics characteristic of a ternary-complex mechanism could still occur if there were very strong interaction between the three subunits. However, the fact that a soluble complex  $\alpha\beta$  can be obtained by heat or proteolysis that is still active with artificial donors such as benzyl viologen (DeMoss, 1977; Morpeth and Boxer, 1985), argues against the formation of a ternary complex. The ternary-complex type of mechanism thus appears *a priori* the less probable.

In agreement with this expectation, Morpeth and Boxer (1985) reported that a purified  $\alpha\beta\gamma$  complex of nitrate reductase followed a substituted-enzyme mechanism when duroquinol was the substrate (even though they had concluded that when benzyl viologen was the substrate the mechanism proceeded through a ternary complex). However, the fact that they interpreted their results in terms of simple Michaelis-Menten kinetics with respect to duroquinol casts doubts on their conclusions and, as they also worked with a purified  $\alpha\beta\gamma$  complex rather than a membrane-bound enzyme, it was worthwhile to reexamine this question.

When the initial rate of nitrate reduction was measured as a function of nitrate at several fixed concentrations of menadiol (Fig. 4) or duroquinol (not shown), the corresponding double-reciprocal plots produced reasonably parallel lines (Fig. 4b) indicating that the apparent specificity constant for each substrate is independent of the concentration of the other, a characteristic of the substituted-enzyme mechanism. Consequently, although



**Fig. 4. Dependence of the rate on the two substrate concentrations.** (a) Plot of rate against the concentration of nitrate at menadiol concentrations of 0.1125 mM ( $\nabla$ ), 0.075 mM ( $\blacklozenge$ ), 0.0375 mM ( $\blacktriangle$ ), 0.01875 mM ( $\bullet$ ) and 0.00936 mM ( $\blacksquare$ ), with curves calculated from Eqn (1) with the parameter values shown as the top line of Table 2. (b) Double-reciprocal plots at different menadiol concentrations showing the pattern of parallel lines characteristic of a substituted-enzyme mechanism, with symbols as in (a). (c) Residual plot of all the data. (d) Residual plot after fitting the data to an equation with a constant term in the denominator of the rate expression (characteristic of a ternary-complex mechanism); note that there is no significant improvement in fit, and the best-fit value of the additional parameter was close to zero. (e) Residual plot after fitting the data to Eqn (1) but with the menadiol concentration raised to the power of 1 rather than 2.

the quality of the data leaves in doubt the question of whether the lines are strictly parallel, the fact that they tend to agree with the more plausible model makes it reasonable to retain the substituted-enzyme mechanism. This implies that, in the reduction of nitrate by quinols catalysed by nitrate reductase, the first half of the reaction involves reduction of the enzyme by the quinol, followed by reoxidation of the enzyme by nitrate. This model leads to the following rate equation:

$$v = \frac{k_{\text{cat}}[\text{E}]_0 [\text{Q}]^2 [\text{NO}_3^-]}{K_{\text{m,N}} [\text{Q}]^2 + K_{0.5,\text{Q}}^2 [\text{NO}_3^-] + [\text{Q}]^2 [\text{NO}_3^-]} \quad (1)$$

where  $v$  is the initial rate at total enzyme concentration  $[\text{E}]_0$  and concentrations  $[\text{Q}]$  and  $[\text{NO}_3^-]$  of quinol and nitrate respectively, and the other symbols represent kinetic parameters:  $k_{\text{cat}}$  is the catalytic constant,  $K_{\text{m,N}}$  is the Michaelis constant with respect to nitrate, and  $K_{0.5,\text{Q}}$  is the half-saturation concentration of quinol at saturating nitrate (not a Michaelis constant because the reaction does not follow Michaelis-Menten kinetics with respect to either quinol). For both quinols a much better fit was obtained

**Table 2. Kinetic parameters obtained for reduction of nitrate by menadiol and duroquinol.** All the parameter values shown were obtained by fitting Eqn (1) to different sets of data.

Enzyme	Electron donor	$k_{cat}$	$K_{m,N}$	$K_{0.5,Q}$	$k_{cat}/K_{m,N}$
		$s^{-1}$	mM	$\mu M$	$mM^{-1} s^{-1}$
Wild type	Menadiol	$31.9 \pm 0.3$	$0.916 \pm 0.031$	$31.9 \pm 0.7$	$34.8 \pm 1.0$
Wild type	Duroquinol	$12.25 \pm 0.13$	$0.746 \pm 0.040$	$35.9 \pm 1.3$	$16.4 \pm 0.8$
[C26A] $\beta$	Menadiol	$8.40 \pm 0.05$	$0.847 \pm 0.033$	$31.5 \pm 0.5$	$9.91 \pm 0.35$
[C26A] $\beta$	Duroquinol	$2.73 \pm 0.03$	$1.139 \pm 0.031$	$32.1 \pm 0.7$	$2.39 \pm 0.05$
[C16A] $\beta$	Menadiol		undetectable activity		
[C16A] $\beta$	Duroquinol	$8.77 \pm 0.10$	$0.390 \pm 0.051$	$29.2 \pm 0.9$	$22.5 \pm 2.8$

with the exponent 2 (Fig. 4c) shown in Eqn (1) than with an exponent of 1 (Fig. 4e). There was no indication that the exponent varied with the nitrate concentration, consistent with the interpretation that the sigmoidicity is due to the stoichiometry. In cases where the sigmoidicity in the saturation curve is due to kinetic factors rather than the stoichiometry or cooperative binding, the degree of sigmoidicity of one of the substrates often varies with the concentration of the other (Cornish-Bowden and Cárdenas, 1987). Despite the relatively high concentrations of both quinols and especially of nitrate, there was no evidence of substrate inhibition.

Inclusion of a constant term in the denominator of the rate equation led to no significant improvement in fit (Fig. 4d), and the best-fit value of the additional parameter was close to zero, as expected for a substituted-enzyme mechanism. The data therefore provide no basis for preferring a ternary-complex mechanism over a substituted-enzyme mechanism.

A similar type of mechanism was found for the soluble  $\alpha\beta$  complex with the artificial donor benzyl viologen (Buc et al., 1995). The details of the mechanism were simpler, as the donor appeared to reduce the  $\alpha$  subunit without direct participation of the Fe-S centres, which is not the case with quinols and the whole enzyme.

**Comparison between duroquinol and menadiol as electron donors.** Table 2 shows the parameter values for the membrane-bound nitrate reductase (wild type) with menadiol and duroquinol as electron donors, obtained by fitting the data to Eqn (1). The catalytic activity  $k_{cat}$  for menadiol is more than double that for duroquinol (see also Fig. 2a); however, the two quinols have similar  $K_{0.5}$  values. The value of  $K_m$  for nitrate is also essentially the same with both electron donors, which means, of course, that the values of  $k_{cat}/K_m$  for nitrate differ by a factor of about two: this is inconsistent with the simplest type of substituted-enzyme mechanism, and similar behaviour led Morpeth and Boxer (1985) to exclude such a mechanism and to suggest that nitrate reductase might in some circumstances follow the Theorell-Chance mechanism, a limiting case of a ternary-complex mechanism. However, memory effects can cause departure from the simplest expectation, and the majority of enzymes for which the operation of a substituted-enzyme mechanism is not in doubt do in fact display such effects if one looks for them (Jarabak and Westley, 1974; Katz and Westley, 1979, 1980).

**Kinetic properties of membrane-bound nitrate reductase with mutations in the Cys clusters of the  $\beta$  subunit.** The  $\beta$  subunit of nitrate reductase contains four Fe-S clusters bound to four groups of Cys residues (Blasco et al., 1989; Guigliarelli et al., 1992, 1996), as illustrated schematically in Fig. 1.

Mutant forms of nitrate reductase with  $\beta$  subunits that lack a low-potential centre (Cys26 $\rightarrow$ Ala: [C26A] $\beta$ ), or even the highest-potential centre ([C16A] $\beta$ , [C19A] $\beta$  and [C263A] $\beta$ ) sug-

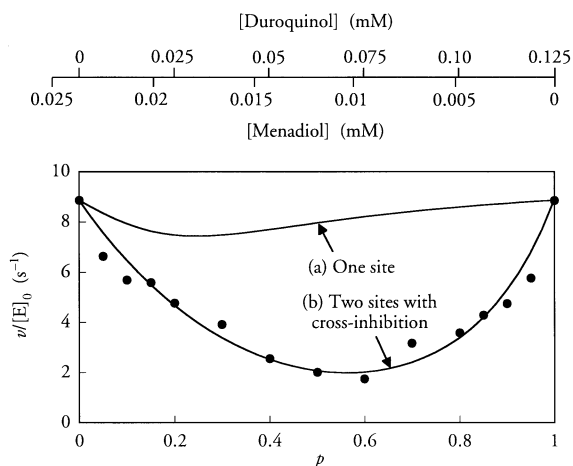
gested to be involved in the complete pathway of transfer of electrons from quinols, are active with the artificial electron donors methyl viologen and benzyl viologen (Augier et al., 1993a, b; Buc et al., 1995; Guigliarelli et al., 1996). The cells that produce these mutant enzymes are able to grow in minimal medium supplemented with glycerol and nitrate under anaerobic conditions although much more slowly (Guigliarelli et al., 1996). The molecular masses of the mutant enzymes are similar to those of the control enzyme and the cellular location is not affected, indicating that the enzyme structure is not greatly disturbed. (Some other site-directed mutations induce not only the loss of the target Fe-S centre, but of all four, and even of the Mo cofactor.)

In the mutant [C16A] $\beta$  that has lost the +80 mV Fe-S centre (Augier et al., 1993b), the mutant enzyme has no detectable activity with menadiol in our experimental conditions, though it remains active with duroquinol (Table 2), with kinetic parameters within a factor of 2 of those of the wild type. Thus although the Fe-S centre of +80 mV appears essential for the activity with menadiol, it is not essential with duroquinol. The conclusion that the loss of this Fe-S centre rather than the replacement of Cys16 *per se* brings about loss of activity with menadiol is supported by observations with other mutants, like [C19A] $\beta$  and [C263A] $\beta$ , that also lack the +80 mV Fe-S centre. For example, [C263A] $\beta$  showed no activity with menadiol at the single concentration of 130  $\mu M$  examined, though it had 72% of wild-type activity with 150  $\mu M$  duroquinol (Guigliarelli et al., 1996).

Mutant [C26A] $\beta$  has substantially decreased activity with both duroquinol and menadiol but it is far from negligible ( $k_{cat}$  decreased by about 75% from the wild type for both quinols), as shown in Table 2. EPR studies with the  $\alpha\beta$  complex of this mutant have shown that it lacks an Fe-S centre of low potential, probably the Fe-S centre of -400 mV (Guigliarelli et al., 1996). Our results thus suggest that this Fe-S centre is not required for catalysis with duroquinol or menadiol as electron donor, but that its presence is required for full activity. The lower activity may be due to the fact that although EPR signals of the high-redox-potential centres were hardly changed in this mutant, their redox potentials are significantly lowered (Guigliarelli et al., 1996).

Although the  $K_{0.5}$  for the quinol remains unchanged in the two mutants, the  $K_m$  for nitrate is significantly altered (Table 2): for [C26A] $\beta$  it is increased, whereas in [C16A] $\beta$  it is decreased. Because of the complexity of the mechanism, involving many redox steps, it is difficult to interpret these effects, but they suggest that the conformational change in the  $\beta$  subunit induced by the mutations is transmitted to the  $\alpha$  subunit. Interestingly, there was a similar decrease in the  $\alpha\beta$  complex from mutants [C16A] $\beta$  and [C19A] $\beta$  when benzyl viologen was the electron donor, which supports this interpretation (Buc et al., 1995).

The requirement of the +80 mV Fe-S centre for the activity with menadiol could be due either to its direct participation in the electron transfer or simply to its importance for maintaining



**Fig. 5. Competition plot showing oxidation of menadiol and duroquinol at different sites.** The plot shows total oxidation rate of mixtures of menadiol and duroquinol at concentrations  $0.0237(1-p)$  mM and  $0.125p$  mM respectively, at 3.125 mM nitrate. The fitted curve (b) was calculated from Eqn (2) with the parameter values shown in Table 2. Curve (a) was calculated from Eqn (3) and applies to the case where menadiol and duroquinol react at the same site, i.e. only one quinol site exists.

the  $\beta$  subunit (and the whole enzyme) in a correct conformation. The suggestion of a structural role for the Fe-S centres comes from the fact that each Fe-S centre connects two Cys clusters (Guigliarelli et al., 1996) and hence two different parts of the polypeptide chain (Fig. 1); thus a structural role in the folding of the protein and its stability is highly probable. On the other hand, the absence of the +80 mV Fe-S centre from the [C16A] $\beta$  and [C263A] $\beta$  mutants and of the -400 mV Fe-S centre from the [C26A] $\beta$  mutant appears to have little effect on the spectral properties of the three remaining Fe-S centres in the soluble  $\alpha\beta$  complex; this suggests that these two Fe-S centres do not greatly affect the protein conformation in the surroundings of the other Fe-S centres. Nevertheless, even though this change in the conformation of the mutants is small, it does appear to have kinetic effects, as the catalytic constant is decreased; a similar effect is also observed in the  $\alpha\beta$  complex with benzyl viologen as electron donor.

Whichever is the correct interpretation for the role of the +80 mV Fe-S centre, the different behaviour with menadiol and duroquinol when it is lost suggests that the sequence of the reactions with menadiol and duroquinol is not the same. If alternative electron pathways exist the question arises of whether there may be two different active sites for quinol interaction; this is examined below.

#### Kinetic studies with mixtures of duroquinol and menadiol.

The question of whether menadiol and duroquinol react at the same site of nitrate reductase was tested in experiments to produce competition plots (Chevillard et al., 1993): in these the total rate (sum of rates for the two substrates mixed together) is plotted against a parameter  $p$  that varies over 0–1; it specifies the concentrations  $p[\text{DQ}]_0$  and  $(1-p)[\text{MD}]_0$  of duroquinol and menadiol, respectively, in terms of reference concentrations  $[\text{DQ}]_0$  and  $[\text{MD}]_0$  that were chosen to give the same rates at  $p = 0$  and  $p = 1$ .

If the two substrates react at the same site, the competition plot gives a horizontal straight line, i.e. the total rate is independent of  $p$ , regardless of the values of  $K_m$  and  $V_{max}$  for the two substrates. Reactions at completely independent sites produce a curve with a maximum. However, the height of the maximum is

decreased if the sites are separate but there is cross-inhibition by binding at the 'wrong' sites, and it may be converted into a minimum if the cross-inhibition is very strong.

If the reaction follows cooperative kinetics the interpretation is complicated by the fact that cooperativity alone can produce a curve with a minimum (Chevillard et al., 1993). However, the deepest minimum attributable to cooperativity when there is competition for a single site can be calculated, and is  $2^{1-h}$ . For nitrate reductase, therefore, reaction of duroquinol and menadiol at the same site with similar Hill coefficients in the range 1.7–2.0 would produce a minimum no deeper than 50–62% of the reference rate. Even this is not attainable unless the experiments are performed at extremely small concentrations of both substrates; at ordinary concentrations the minimum is less deep.

In the experiments on nitrate reductase with mixtures of the two quinols all the observed rates were below the base line (Fig. 5). The deep minimum at about 30% of the rate at the two extremes indicates that there are two different active sites for menadiol and duroquinol. The data fit the following equation quite closely:

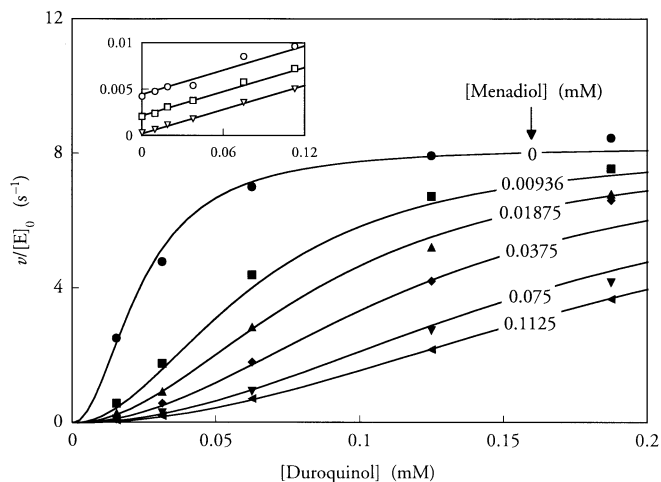
$$v_{\text{tot}} = \frac{V_{\text{MD}} \cdot \frac{(1-p)^2 [\text{MD}]_0^2}{K_{0.5,\text{MD}}^2}}{1 + \frac{(1-p)^2 [\text{MD}]_0^2}{K_{0.5,\text{MD}}^2} + \frac{p [\text{DQ}]_0}{K_{i,\text{DQ}}}} + \frac{V_{\text{DQ}} \cdot \frac{p^2 [\text{DQ}]_0^2}{K_{0.5,\text{DQ}}^2}}{1 + \frac{(1-p) [\text{MD}]_0}{K_{i,\text{MD}}} + \frac{p^2 [\text{DQ}]_0^2}{K_{0.5,\text{DQ}}^2}} \quad (2)$$

This is derived from a model for two separate sites with cross-inhibition, and defines a curve with a deep minimum in the competition plot (Fig. 5). Similar results were obtained at a different nitrate concentration chosen to give a different reference rate. For comparison, we also calculated a best-fit curve assuming competition between duroquinol and menadiol for a single site at which both react, with Hill coefficients of 2.0 (the largest value observed experimentally), as expressed by the following equation:

$$v_{\text{tot}} = \frac{V_{\text{MD}} \cdot \frac{(1-p)^2 [\text{MD}]_0^2}{K_{0.5,\text{MD}}^2} + V_{\text{DQ}} \cdot \frac{p^2 [\text{DQ}]_0^2}{K_{0.5,\text{DQ}}^2}}{1 + \frac{(1-p)^2 [\text{MD}]_0^2}{K_{0.5,\text{MD}}^2} + \frac{p^2 [\text{DQ}]_0^2}{K_{0.5,\text{DQ}}^2}} \quad (3)$$

Unlike Eqn (2), this equation completely failed to account for the experimental results (curve a in Fig. 5).

Thus although the interpretation is complicated by the cooperative kinetics of nitrate reductase with respect to both duroquinol and menadiol, the minimum expected from cooperativity alone if duroquinol and menadiol reacted at the same site would be much shallower than the minimum actually observed. Cooperativity alone cannot therefore explain the magnitude of the deviation observed from a horizontal line, and the deep minimum suggests that each quinol has a separate reaction site, but that both can bind at both sites, acting as inhibitors at the sites where they do not react. Inhibition constants were obtained of 38  $\mu\text{M}$  for duroquinol and 0.27  $\mu\text{M}$  for menadiol. This implies that menadiol is a very powerful inhibitor of the duroquinol reaction. Notice that the inhibitory terms in the equation are not raised to the power of 2, implying that the inhibition results from binding of complete molecules. This is what one would expect, but to check it we also fitted curves with these terms raised to the power of 2 and found the fits to be less good. It might appear



**Fig. 6. Inhibition of duroquinol oxidation by menadiol in mutant [C16A] $\beta$ .** The plot shows the rate against the concentration of duroquinol at menadiol concentrations of 0.1125 mM ( $\blacktriangleleft$ ), 0.075 mM ( $\blacktriangledown$ ), 0.0375 mM ( $\blacklozenge$ ), 0.01875 mM ( $\blacktriangle$ ), 0.00936 mM ( $\blacksquare$ ) and 0 mM ( $\bullet$ ), with curves calculated from Eqn (4) with the parameter values shown for this mutant in Table 2. The plot shown as inset, left unlabelled to avoid crowding, has the menadiol concentration as abscissa and [duroquinol][E] $_0$ /v as ordinate. Data are shown at three menadiol concentrations: 0.3125 mM ( $\triangle$ ), 0.125 mM ( $\square$ ) and 0.1875 mM ( $\circ$ ); the parallel arrangement of points in this type of graph is characteristic of competitive inhibition (Cornish-Bowden, 1974), with  $K_i = 1.53$  mM.

surprising that menadiol has an inhibition constant much lower than its  $K_{0.5}$  but it is reasonable in view of the differences in type between these constants, the more severe constraints for substrates than for inhibitors, and the steric effects that result from the greater bulk of menadiol.

If there are two sites with cross-inhibition, the duroquinol activity of the mutant [C16A] $\beta$  should still be inhibited by menadiol even though it is not a substrate for this mutant. In accordance with this expectation, menadiol does inhibit the oxidation of duroquinol (Fig. 6), showing the behaviour characteristic of competitive inhibition according to the following equation:

$$v = \frac{V_{DQ} \cdot \frac{[DQ]^2}{K_{0.5,DQ}^2}}{1 + \frac{[MD]}{K_{i,MD}} + \frac{[DQ]^2}{K_{0.5,DQ}^2}} \quad (4)$$

Inclusion of an uncompetitive term in this rate expression leads to no significant improvement in fit, indicating that the inhibition is purely competitive.

The inhibition constant of 1.53  $\mu$ M for menadiol with this mutant is an order of magnitude higher than that with the wild-type enzyme, the weaker inhibition possibly being due to differences in conformation produced by the mutation.

## DISCUSSION

The precise detail of the electron transfer pathway through the nitrate reductase molecule is not known, partly because of the difficulty of studying kinetics with the membrane-bound enzyme, and also because the detailed structure of the enzyme is not known. The finding that the mutant [C263A] $\beta$  can transfer electrons from duroquinol to nitrate despite loss of the +80 mV Fe-S centre raised doubts about the obligatory participation of this centre in the electron transfer; it was suggested that parallel electron pathways exist in the enzyme, each involving one of the

high-potential Fe-S centres (Guigliarelli et al., 1996). Moreover, enzyme lacking the +80 mV Fe-S centre still had 30% of the normal capacity to accept electrons from formate via the physiological quinones embedded in the membrane, indicating that this centre is not essential for the reaction with at least one type of quinol. Our kinetic studies now show that menadiol and duroquinol react at different sites on nitrate reductase, and have different kinetic responses to the [C16A] $\beta$  mutation, differing in their requirement for the +80 mV Fe-S centre. These results support the hypothesis of alternative pathways, which offers the possibility of great flexibility of regulating nitrate reduction in different metabolic states.

A problem, however, is how to relate this kinetic model to the structure of nitrate reductase. As the quinols are in the membrane, the two reacting sites for menadiol and duroquinol are most probably in the  $\gamma$  subunit itself (cytochrome *b*) or in the interface with the  $\beta$  subunit (the  $\alpha\beta$  complex has no activity with the quinols). As the  $\gamma$  subunit has two haem groups, it is tempting to postulate a specific interaction of each quinol with a site close to a particular haem group, with each of these having a different Fe-S centre as redox target: the haem reduced by menadiol would be part of an electron-transfer pathway involving the Fe-S centre of +80 mV, whereas the haem reduced by duroquinol would form part of another one involving the Fe-S centre of +60 mV.

This hypothesis would explain our results and also why it is possible to reduce the cytochrome *b* with menadiol and duroquinol in the absence of nitrate in the wild type and in the mutant [C263A] $\beta$ , even though the latter has no menadiol activity. It would also explain the qualitative observation that cytochrome *b* reduced by menadiol cannot be reoxidized with nitrate, whereas this reoxidation is possible if the reducing agent is duroquinol (Guigliarelli et al., 1996). In effect, this experiment indicates that the two quinols are not equivalent in relation to the reduction of the haem groups and argues against electron transfer between the haem groups in this mutant. The capacity of menadiol to reduce cytochrome *b* ( $\gamma$  subunit) in the wild type as well as in the mutant [C263A] $\beta$  (Guigliarelli et al., 1996) indicates that the reacting site for menadiol has not been lost as a consequence of the loss of the +80 mV Fe-S centre, but does not exclude the possibility that it may have been altered. Characterization of the reduction of the haem groups is needed to establish whether they are both reduced and, in particular, whether the reduction is fast enough to constitute part of the catalytic process.

For an electron transfer to be successful the distance between redox centres and their orientation is important, although energetic factors are also important for defining rate and directional specificity (Bertrand, 1991; Moser et al., 1992). One must ask, therefore, whether the distance between the haem groups and the Fe-S centres in nitrate reductase is compatible with the kinetic model. First of all, although the  $\alpha\beta$  complex is mainly localized at the cytoplasm, it could also be partially inserted in the membrane. The masses of the  $\gamma$ ,  $\beta$  and  $\alpha$  subunits increase approximately in geometric progression,  $\beta$  having twice the mass of  $\gamma$  and  $\alpha$  having twice the mass of  $\beta$ ; so, if 20–25% of  $\beta$  were inserted, this would still leave most of it on the cytoplasmic side, though it could bring an Fe-S centre sufficiently close to the periplasmic haem group. Several observations of different groups support the possibility that  $\alpha\beta$  may be partially inserted in the membrane. For example in mutants that lack the  $\gamma$  subunit, a fraction of  $\alpha\beta$  appears to be associated with the membrane fraction (Dubordieu and DeMoss, 1992); although this is unspecific binding, it shows that partial insertion is possible.



The apparent alignment of the haem groups in the  $\gamma$  subunit (Berks et al., 1995) may allow the electrons to pass from one haem to the other; the distances from edge to edge and from iron to iron obtained by modelling are compatible with electron transfer between the haems. The two haem groups would then be equally dependent on the existence of the Fe-S centre of +80 mV unless, of course, binding of menadiol induced a conformational change sufficient to modify this expectation. However, the modelling was based on the analysis of primary structure and the exact positions of the different amino acid residues may vary from the model as a result of interactions with lipids or other proteins such as the  $\beta$  subunit.

Another hypothesis would be that, although both quinols reduce cytochrome *b* in equilibrium conditions in the absence of nitrate (Guigliarelli et al., 1996), only duroquinol reduces it at a significant rate in the steady-state reaction, whereas menadiol would reduce another receptor site, maybe in the interface between the  $\gamma$  and  $\beta$  subunits. A plausible candidate for this site could be a quinone that has been recently reported to exist in nitrate reductase (Brito et al., 1995), which could be situated at the interface between the  $\gamma$  and  $\beta$  subunits and act as an acceptor, reducing the +80 mV Fe-S centre. This second hypothesis would not be constrained by the distance between the periplasmic heme and an Fe-S centre, but it implies that the reduction of the cytochrome by menadiol is not part of the normal reaction.

It appears very difficult to conceive of a model that can reconcile all the data without some degree of parallel pathways. Unfortunately, it has not been possible to obtain a mutant that lacks only the +60-mV centre, which according to our hypothesis ought to have activity with menadiol but not with duroquinol. (Site-directed mutations intended to produce the loss of this centre lack all four Fe-S centres and also the Mo cofactor and suggest that it has an essential structural role.) Consequently it is not possible at present to reject the possibility that the wild-type enzyme could possess a single pathway with two different points of entry and that the loss of the Fe-S centre of +80 mV simply disturbs the normal electron transfer from one of these.

Several reductases can act with only one type of quinol and there are cases where the specific requirements apparently cannot be explained on the base of redox potentials alone (Kröger, 1978). This supports the idea that the capacity of nitrate reductase to react with either involves the presence of two different sites. Dimethylsulphoxide reductase, which is similar in organization and sequence with nitrate reductase, reacts only with menaquinol, not with ubiquinol (Weiner et al., 1992); as it has no haem groups in its  $\gamma$  subunit this would agree with the hypothesis that menadiol may not reduce the haem groups of nitrate reductase in steady-state conditions.

The differences in the kinetic behaviour of the artificial donors menadiol and duroquinol raise the question of whether menaquinone and ubiquinone likewise act differently from one another, though the absence of the isoprene tails from menadiol and duroquinol may make them an unreliable guide to the differences between the physiological quinones. Nevertheless, the observation that only menadiol activity requires the Fe-S centre of +80 mV deserves a molecular explanation, and this may contribute to understanding the electron-transfer pathway with the physiological quinones. This interpretation is supported by results obtained in other systems, such as photosynthetic reaction centre proteins from bacteria, which indicate that the quantitative effect of complete absence of the isoprenic tail is no more than an order of magnitude. For example, binding studies with purified proteins of this kind (Diner et al., 1984; McComb et al., 1990) indicated that the native tail with 10 isoprene units does not contribute strongly to quinone binding affinity at the quinone binding sites. Quantum yield measurements with qui-

none head groups at one of the sites of this centre showed that the complete absence of a tail structure did not decrease the rate of forward electron transfer by more than 5–10-fold (Gunner and Dutton, 1989; Warncke et al., 1994).

All of this suggests that duroquinol and menadiol could adequately represent the reaction *in vivo*, albeit with lower rates. It is likely, therefore, that isolated membranes overexpressing mutant nitrate reductase A [C16A] $\beta$  would be inactive with menaquinol as they are with menadiol. However, the situation in the whole cell may be more complex, as preliminary studies *in vivo* (Magalon, A. and Blasco, F., personal communication) show that a double mutant lacking the +80 mV Fe-S centre and incapable of producing ubiquinone is nonetheless able to grow. A possible explanation could be that in the absence of this Fe-S centre the reaction with menadiol becomes too slow to be detected in the short time of the experiment, as the electron transfer would occur just through the protein medium, but it is not eliminated entirely; with menaquinol the reaction could still be very slow, but an order of magnitude higher. As the nitrate reductase is overexpressed about ten times in these *in vivo* experiments, even a very small residual level of activity could suffice for growth; in many systems *in vivo* it is possible to decrease the activity of an arbitrarily chosen enzyme by large amounts without effects on growth because flux control is generally shared by all the enzymes in the system (Kacser et al., 1995; Cornish-Bowden, 1995b). As the flux control coefficient of nitrate reductase must be very small in overexpression conditions, a large decrease in activity could occur without affecting growth.

The competition experiments show that when menadiol and duroquinol are both present, menadiol is the preferred substrate, because of its high capacity to inhibit the duroquinol reaction. If similar behaviour occurs with menaquinol and ubiquinol, nitrate reductase *in vivo* would use menaquinol preferentially or even exclusively. It will be interesting to know whether formate dehydrogenase has similar properties, as formate is the main physiological electron donor for nitrate reduction, and the same question can be posed for nitrate reductase Z, the other isoenzyme. In a transition from aerobic to anaerobic conditions in the presence of nitrate, the ubiquinol site could make nitrate reductase functional if it started to be induced before sufficient menaquinol was available to provide the necessary reducing capacity. A switch from one quinol to the other would then occur progressively as the level of menaquinol increased. Although nitrate reductase Z is never present in large amounts, it is constitutive (Iobbi et al., 1987) and thus can provide the possibility of reducing nitrate in the early stages of induction of nitrate reductase A.

We thank Dr J. Pommier for help in the initial phase in perfecting the assay of nitrate reductase with quinol substrate; the technical assistance of S. Stuyck is also acknowledged. We are greatly indebted to Drs A. Magalon and F. Blasco for kindly providing us with the bacterial strains and for help in the membrane preparation. We thank also Drs D. Richardson and B. Guigliarelli for helpful discussions, and Drs M. Chippaux and J. DeMoss for useful criticisms of earlier drafts of the paper. The helpful comments of Dr G. Giordano throughout this work have been greatly appreciated.

## REFERENCES

- Augier, V., Guigliarelli, B., Asso, M., Frixon, C., Giordano, G., Chippaux, M. & Blasco, F. (1993a) Site-directed mutagenesis of conserved cysteine residues within the  $\beta$  subunit of *Escherichia coli* nitrate reductase. Physiological, biochemical, and EPR characterization of the mutated enzymes, *Biochemistry* 32, 2013–2023.
- Augier, V., Asso, M., Guigliarelli, B., More, C., Bertrand, P., Santini, C. L., Blasco, F., Chippaux, M. & Giordano, G. (1993b) Removal of

- the high-potential [4Fe-4S] center of the  $\beta$  subunit from *Escherichia coli* nitrate reductase. Physiological, biochemical, and EPR characterization of site-directed mutated enzymes, *Biochemistry* 32, 5099–5108.
- Berks, B. C., Page, M. D., Richardson, D. J., Reilly, A., Cavill, A., Outen, F. & Ferguson, S. J. (1995) Sequence analysis of subunits of the membrane-bound nitrate reductase from a denitrifying bacterium: the integral membrane subunit provides a prototype for the dihaem electron carrying arm of a redox loop, *Mol. Microbiol.* 15, 319–331.
- Bertrand, P. (1991) Application of electron transfer theories to biological systems, *Struct. Bonding* 75, 1–47.
- Blasco, F., Iobbi, C., Giordano, G., Chippaux, M. & Bonnefoy, V. (1989) Nitrate reductase of *Escherichia coli*: completion of the nucleotide sequence of the *nar* operon and reassessment of the role of the  $\alpha$  and  $\beta$  subunits in iron binding and electron transfer, *Mol. Gen. Genet.* 218, 249–256.
- Brito, F., DeMoss, J. & Dubourdieu, M. (1995) Isolation and identification of menaquinone-9 from purified nitrate reductase of *Escherichia coli*, *J. Bacteriol.* 177, 3728–3735.
- Buc, J., Santini, C. L., Blasco, F., Giordani, R., Cárdenas, M. L., Chippaux, M., Cornish-Bowden, A. & Giordano, G. (1995) Kinetic studies of a soluble  $\alpha\beta$  complex of nitrate reductase A from *Escherichia coli*. Use of various  $\alpha\beta$  mutants with altered  $\beta$  subunits, *Eur. J. Biochem.* 234, 766–772.
- Buc, J. & Giordani, R. (1997) A spectrophotometric method for kinetic studies with quinone-dependent oxidoreductases. Application to detection in membranes of nitrate reductase activity with menadiene and duroquinone as electron donors, *Enzyme Microb. Technol.*, in the press.
- Cárdenas, M. L., Buc, J., Giordani, R., Magalon, A., Blasco, F., Giordano, G. & Cornish-Bowden, A. (1996) in *Biothermokinetics of the living cell*, pp. 15–19, BioThermoKinetics Press, Amsterdam.
- Casadaban, M. J. (1976) Transposition and fusion of the lac genes to selected promoters in *Escherichia coli* using bacteriophage lambda and mu, *J. Mol. Biol.* 104, 541–555.
- Chevillard, C., Cárdenas, M. L. & Cornish-Bowden, A. (1993) The competition plot: a simple test of whether two reactions occur at the same active site, *Biochem. J.* 289, 599–604.
- Cornish-Bowden, A. (1974) A simple graphical method for determining the inhibition constants of mixed, uncompetitive and non-competitive inhibitors, *Biochem. J.* 137, 143–144.
- Cornish-Bowden, A. (1995a) *Analysis of enzyme kinetic data*, Oxford University Press, Oxford.
- Cornish-Bowden, A. (1995b) *Fundamentals of enzyme kinetics*, pp. 239–270, Portland Press, London.
- Cornish-Bowden, A. & Cárdenas, M. L. (1987) Co-operativity in monomeric enzymes, *J. Theor. Biol.* 124, 1–23.
- DeMoss, J. A. (1977) Limited proteolysis of nitrate reductase purified from membranes of *Escherichia coli*, *J. Biol. Chem.* 252, 1696–1701.
- Diner, B., Schenck, C. C. & De Vitry, C. (1984) Effect of inhibitors, redox state and isoprenoid chain length on the affinity of ubiquinone for the secondary acceptor binding site in the reaction centers of photosynthetic bacteria, *Biochim. Biophys. Acta* 766, 9–20.
- Dubourdieu, M. & DeMoss, J. (1992) The *narJ* gene product is required for biogenesis of respiratory nitrate reductase in *Escherichia coli*, *J. Bacteriol.* 174, 867–872.
- Fürste, J. P., Pansegrau, W., Frank, R., Blöcker, H., Scholz, P., Bagdasarian, M. & Lanka, E. (1986) Molecular cloning of the plasmid RP4 primase region in a multi-host-range *tacP* expression vector, *Gene* 48, 119–131.
- Graham, A., Jenkins, H. E., Smith, N. H., Mandrand, M. A., Haddock, B. A. & Boxer, D. H. (1980) The synthesis of formate dehydrogenase and nitrate reductase proteins in various *fdh* and *chl* mutants of *Escherichia coli*, *FEMS Microbiol. Lett.* 7, 145–151.
- Guest, J. R. (1977) Menaquinone biosynthesis: mutants of *Escherichia coli* K-12 requiring 2-succinylbenzoate, *J. Bacteriol.* 130, 1038–1046.
- Guigliarelli, B., Asso, M., More, C., Augier, V., Blasco, F., Pommier, J., Giordano, G. & Bertrand, P. (1992) EPR and redox characterization of iron-sulphur centers in nitrate reductase from *Escherichia coli*. Evidence for a high-potential and a low-potential class and their relevance in the electron-transfer mechanism, *Eur. J. Biochem.* 207, 61–68.
- Guigliarelli, B., Magalon, A., Asso, M., Bertrand, P., Frixon, C., Giordano, G. & Blasco, F. (1996) Complete coordination of the four Fe-S centers of the  $\beta$  subunit from *Escherichia coli* nitrate reductase. Physiological, biochemical, and EPR characterization of site-directed mutants lacking the highest or lowest potential [4Fe-4S] clusters, *Biochemistry* 35, 4828–4836.
- Gunner, M. R. & Dutton, P. L. (1989) Electron transfer from BPH-ion to Q-A in reaction center protein from *Rhodobacter sphaeroides* with different quinones as Q-A, *J. Am. Chem. Soc.* 111, 3400–3412.
- Hackett, N. R. & Bragg, P. D. (1982) The association of two distinct *b* cytochromes with the respiratory nitrate reductase of *Escherichia coli*, *J. Bacteriol.* 154, 719–727.
- Iobbi, C., Santini, C. L., Bonnefoy, V. & Giordano, G. (1987) Biochemical and immunological evidence for a second nitrate reductase in *Escherichia coli* K12, *Eur. J. Biochem.* 168, 451–459.
- Jarabak, R. & Westley, J. (1974) Enzymic memory. A consequence of conformational mobility, *Biochemistry* 13, 3237–3239.
- Jones, R. W. & Garland, P. B. (1977) Sites and specificity of the reaction of bipyridylum compounds with anaerobic respiratory enzymes of *Escherichia coli*, *Biochem. J.* 164, 199–211.
- Kacser, H., Burns, J. A. & Fell, D. A. (1995) The control of flux, *Biochem. Soc. Trans.* 23, 341–366.
- Katz, M. & Westley, J. (1979) Enzymic memory. Steady state kinetic and physical studies with ascorbate oxidase and aspartate aminotransferase, *J. Biol. Chem.* 254, 9142–9147.
- Katz, M. & Westley, J. (1980) Enzymic memory studies with nucleoside-5'-diphosphate kinase, *Arch. Biochem. Biophys.* 204, 464–470.
- Kröger, A., Dadák, V., Klingenberg, M. & Diemer, F. (1971) On the role of quinones in bacterial electron-transport: differential roles of ubiquinone and menaquinone in *Proteus rettgeri*, *Eur. J. Biochem.* 21, 322–333.
- Kröger, A. & Klingenberg, M. (1973) The kinetics of the redox reactions of ubiquinone related to the electron-transport activity in the respiratory chain, *Eur. J. Biochem.* 34, 358–368.
- Kröger, A. (1978) Fumarate as terminal acceptor of phosphorylative electron transport, *Biochim. Biophys. Acta* 505, 129–145.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) Protein measurement with the Folin phenol reagent, *J. Biol. Chem.* 193, 265–275.
- McComb, J. C., Stein, R. R. & Wraight, C. A. (1990) Investigations on the influence of headgroup substitution and isoprene side-chain length in the function of primary and secondary quinones of bacterial reaction centers, *Biochim. Biophys. Acta* 1015, 156–171.
- Meganathan, J. R. (1984) Inability of *men<sup>-</sup>* mutants of *Escherichia coli* to use trimethylamine *N*-oxide as an electron acceptor, *FEMS Microbiol. Lett.* 24, 57–62.
- Morpeth, F. F. & Boxer, D. (1985) Kinetic analysis of respiratory nitrate reductase from *Escherichia coli* K12, *Biochemistry* 24, 40–46.
- Moser, C. C., Keske, J. M., Warncke, K., Farid, R. S. & Dutton, P. L. (1992) Nature of biological electron transfer, *Nature* 355, 796–802.
- Pascal, M. C., Burini, J. F., Ratouchniak, J. & Chippaux, M. (1982) Regulation of the nitrate reductase operon: effects of mutations in *chlA*, *B*, *D* and *E* genes, *Mol. Gen. Genet.* 188, 103–106.
- Pichinoty, F. (1969) Les nitrate-réductases bactériennes. I. Substrats, état particulier et inhibiteurs de l'enzyme, *Arch. Mikrobiol.* 68, 51–64.
- Polglase, W. J., Pun, W. T. & Whithaar, J. (1966) Liponones of *Escherichia coli*, *Biochim. Biophys. Acta* 118, 425–426.
- Ruiz-Herrera, J. & DeMoss, J. A. (1969) Nitrate reductase complex of *Escherichia coli* K-12: participation of specific formate dehydrogenase and cytochrome *b<sub>1</sub>* components in nitrate reduction, *J. Bacteriol.* 99, 720–729.
- Sodergren, E. J. & DeMoss, J. A. (1988) *narI* region of the *Escherichia coli* nitrate reductase (*nar*) operon contains two genes, *J. Bacteriol.* 170, 1721–1729.
- Sodergren, E. J., Hsu, P. Y. & DeMoss, J. A. (1988) Roles of the *narJ* and *narI* gene products in the expression of nitrate reductase in *Escherichia coli*, *J. Biol. Chem.* 263, 16156–16162.
- Uden, G. & Kröger, A. (1986) Reconstitution of a functional electron-transfer chain from purified formate dehydrogenase and fumarate reductase complexes, *Methods Enzymol.* 126, 387–399.
- Uden, G. (1988) Differential role for menaquinone and demethylmenaquinone in anaerobic electron-transport of *E. coli* and their *fnr*-independent expression, *Arch. Microbiol.* 150, 499–503.

- Vincent, S. P. (1979) Oxidation-reduction potentials of molybdenum and iron-sulphur centres in nitrate reductase from *Escherichia coli*, *Biochem. J.* 177, 757–759.
- Wallace, B. J. & Young, I. G. (1977) Role of quinones in electron transport to oxygen and nitrate in *Escherichia coli*. Studies with a *ubiA<sup>-</sup>menA<sup>-</sup>* double quinone mutant, *Biochim. Biophys. Acta* 461, 84–100.
- Warncke, K., Gunner, M. R., Braun, B. S., Gu, L., Yu, C.-A., Bruce, J. M. & Dutton, P. L. (1994) Influence of hydrocarbon tail structure on quinone binding and electron-transfer performance at the Q<sub>A</sub> and Q<sub>B</sub> sites of the photosynthetic reaction center protein, *Biochemistry* 33, 7830–7841.
- Weiner, J. H., Rothery, R. A., Sambasivarao, D. & Trieber, C. A. (1992) Molecular analysis of dimethylsulfoxide reductase: a complex iron-sulfur molybdoenzyme of *Escherichia coli*, *Biochim. Biophys. Acta* 1102, 1–18.
- Wissenbach, U., Kröger, A. & Uuden, G. (1990) The specific functions of menaquinone and demethylmenaquinone in anaerobic respiration with fumarate, dimethylsulfoxide, trimethylamine *N*-oxide and nitrate by *Escherichia coli*, *Arch. Microbiol.* 154, 60–66.
- Wissenbach, U., Kröger, A. & Uuden, G. (1992) An *Escherichia coli* mutant containing only demethylmenaquinone, but no menaquinone: effects on fumarate, dimethylsulfoxide, trimethylamine *N*-oxide and nitrate respiration, *Arch. Microbiol.* 158, 68–73.