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Kinetic studies of a soluble $\alpha\beta$ complex of nitrate reductase A from *Escherichia coli*

Use of various $\alpha\beta$ mutants with altered β subunits

Jean BUC, Claire-Lise SANTINI, Francis BLASCO, Roger GIORDANI, María Luz CÁRDENAS, Marc CHIPPAUX, Athel CORNISH-BOWDEN and Gérard GIORDANO

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

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A soluble $\alpha\beta$ complex of nitrate reductase can be obtained from a strain of *Escherichia coli* that lacks the *narI* gene and expresses only the α and β subunits. The β subunit contains four Fe-S centres and the α subunit contains the molybdenum cofactor, which is the site at which nitrate is reduced. Despite the lack of the γ subunit of the complete enzyme, this complex can still catalyse the reduction of nitrate with artificial electron donors such as benzyl viologen, so that it is suitable for studying the transfer of electrons between these two types of redox centre.

To examine whether the electrons from reduced benzyl viologen are initially delivered to the Fe-S centres, or directly to the molybdenum cofactor, or both, we have studied the steady-state kinetics and the binding of benzyl viologen to the $\alpha\beta$ complex and mutants $\alpha\beta$* with altered β subunits.

Reduction of the enzyme by reduced benzyl viologen in the absence of nitrate showed that all four Fe-S centres and the molybdenum cofactor could be reduced. Two classes of site with different equilibrium constants could be distinguished.

The kinetic results suggest that benzyl viologen supplies its electrons directly to the molybdenum cofactor, at a rate showing a hyperbolic dependence on the square of the concentration of the electron donor. A reaction mechanism is proposed for the reduction of nitrate catalysed by the $\alpha\beta$ complex of nitrate reductase with artificial electron donors.

Keywords: nitrate reductase; iron-sulfur centers; molybdenum cofactor; kinetics.

Nitrate can serve as the terminal electron acceptor during anaerobic growth of *Escherichia coli*, and nitrate reductase, the enzyme that catalyses the reduction of nitrate, is a membrane-bound molybdoenzyme with three different subunits designated α, β and γ, coded by the genes *narG*, *narH* and *narI*, respectively. Each of these subunits carries a different redox centre (Blasco et al., 1989; Guigliarelli et al., 1992): the α subunit carries a cofactor containing molybdenum at the active centre for the reduction of nitrate to nitrite; the β subunit contains four Fe-S centres and the γ subunit is a *b*-type cytochrome. The α and β subunits form an $\alpha\beta$ complex that is bound to the membrane by its interaction with the γ subunit embedded in the membrane. The physiological electron source for nitrate reduction is the quinone pool; electrons are transferred initially to the *b*-type haem, and the complete sequence is postulated to be as follows:

quinones → [*b*-type haems (γ) → Fe-S centres (β) → Mo (α)] → nitrate

Detailed EPR studies have unambiguously indicated the presence of one [3Fe-4S] and three [4Fe-4S] centres in the enzyme. These have been named as centres 1, 2, 3 and 4, with redox potentials +80 mV, +60 mV, −200 mV and −400 mV, respectively, centre 2 being the [3Fe-4S] centre (Guigliarelli et al., 1992) (Fig. 1).

A soluble $\alpha\beta$ complex of nitrate reductase can be released from the membrane of the wild-type strain of *E. coli* (Lund and DeMoss, 1976). The complex lacks the γ subunit that is required for quinol oxidase activity, but it still has viologen oxidase activity. [The nitrate reductase activity can be measured using artificial electron donors such as reduced methyl viologen or benzyl viologen instead of quinones, the physiological electron donors (Wissenbach et al., 1990).] Similar results are observed in cells lacking the *narI* gene, which express only the α and β subunits (Blasco et al., 1992), care being taken to check that the biochemical activity and EPR signals of an $\alpha\beta$ complex that had never been attached to the membrane were similar to those of the $\alpha\beta$ complex released from the membrane (Augier et al., 1993a,b).

The sequences of the structural genes of nitrate reductase are known, and the deduced amino acid sequence of the β subunit contains four cysteine-containing clusters of residues identified with coordination of the four Fe-S centres (Blasco et al., 1989; Sodergren and DeMoss, 1988), as illustrated in Fig. 1. Substitution by site-directed mutagenesis of the cysteine residues concerned produces mutant proteins partially or totally devoid of Fe-S centres, with normal, reduced or no activity (Augier et al., 1993a,b).

As a first step towards elucidating the intramolecular pathway of electrons between the quinones and nitrate, especially the role of the Fe-S centres in this transfer of electrons, we have

Correspondence to G. Giordano, 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 91 71 89 14.

Enzyme. Nitrate reductase A (EC 1.7.99.4).

Fig. 1. Properties of mutant forms of the $\alpha\beta$ complex in relation to coordination of Fe-S centres. The native β subunit contains four Fe-S centres numbered 1 to 4 with midpoint potentials from +80 to −400 mV, as indicated. Each of these is coordinated by a cysteine-containing cluster of residues in the primary structure, and mutations in these clusters are considered. Mutations of residues coordinating Fe-S centres 2, 3 or 4 (C217A, C184A or C244A, respectively) result in loss of all four Fe-S centres, and hence loss of catalytic activity; however, mutations of residues coordinating centre 1 (C16A and C19A) lead to loss of that centre only, and leave a catalytically active $\alpha\beta$ complex. Introduction of the missing cysteine in the cluster coordinating centre 2, a [3Fe-4S] centre, has little effect on the catalytic activity.

studied the dimeric complex $\alpha\beta$. As this involves only the Fe-S centres and the molybdenum cofactor, it is suitable for studying the transfer of electrons between these two types of redox centres, especially as various $\alpha\beta$* mutant complexes are available (Augier et al., 1993a,b). In addition, the solubility of these $\alpha\beta$ complexes facilitates physical studies. The simplified system has the disadvantage, however, of requiring the use of artificial electron donors such as dithionite or reduced viologens, because the natural quinone donors require the missing γ subunit. Nevertheless, such artificial electron donors are still extensively used for the study of redox proteins or enzymes, even though the activities obtained with them are very different from those measured under more physiological conditions (Lemma et al., 1990; Sambasivarao and Weiner, 1991), and some authors, such as Trieber et al. (1994), have suggested that the electrons originating from benzyl viologen could follow a different route from those originating from quinones. This is an important point that needs to be resolved. Furthermore, the $\alpha\beta$ complex is potentially useful for the industrial detection of nitrate and consequently a detailed kinetic characterization is important.

There was an earlier kinetic study of purified nitrate reductase containing cytochrome *b*, and of a cytochrome-free derivative prepared by limited proteolysis of the isolated enzyme (Morpeth and Boxer, 1985). However, the use of an $\alpha\beta$ complex obtained by proteolysis, together with the fact that simple Michaelis-Menten kinetics was observed with respect to benzyl viologen and methyl viologen, in spite of the fact that two molecules of these 1-electron donors are required by the stoichiometry of the reaction, led us to reconsider this problem. In this study we examine whether the electrons from the artificial donor are delivered directly to the molybdenum cofactor, or if they pass via the Fe-S centres, or if both routes operate simultaneously. Steady-state kinetic studies with $\alpha\beta$ and mutant $\alpha\beta$* complexes suggest that reduced benzyl viologen delivers the electrons directly to the molybdenum cofactor.

EXPERIMENTAL PROCEDURES

Bacterial strains and plasmids. The *Escherichia coli* strains and plasmids used in this study are listed in Table 1. Plasmid pVA50 carries the *narG* and *narH* genes under the control of the *tac* promoter *(Ptac)*. Plasmid pVA14, a pACYC184 derivative, carries the *narJ* and *narI* genes under the control of the nitrate reductase promoter *(Pnar)*. These plasmids have been described by Augier et al. (1993a), and have been used to transform either the mutant LCB79 (*narA*::miniMu) or the double mutants LCB162A (*moa*::Mu*cts*, *narA*::Mu*cts*62) and LCB162B (*mob*::Mu*cts*, *narA*::Mu*cts*62) (Santini et al., 1992). In these strains, over-expression of soluble nitrate reductase complexes has been obtained from the two separate transcriptional units pVA50 and pVA14 (Augier et al., 1993a).

Site-directed mutagenesis was used to alter residues involved in coordinating the Fe-S centres of the β subunit of nitrate reductase (Augier et al., 1993a). Mutants C16A and C19A concern cysteine residues coordinated to centre 1 that are replaced by alanine residues; similarly, W220C and W220F affect centre 2, and C244A affects centre 4 (Fig. 1).

Growth conditions. Strains were grown anaerobically on basal medium as described previously (Giordano et al., 1978). Complex medium was prepared by adding Difco yeast extract and Difco bactopeptone (2 g/l of each) to the basal medium. The carbon source was glucose (11 mM, 2 g/l) and 1 μM sodium molybdate was routinely included. For the induction of nitrate reductase, potassium nitrate was added to a final concentration of 9.9 mM (1 g/l). Strains carrying plasmids were grown by add-

Table 1. Strains and plasmids used in this study. Ap^R, ampicillin-resistant, Cm^R, chloramphenicol-resistant, Tet^R, tetracyclin-resistant, *tac*P, tac promoter.

Strain/plasmid	Genotype	Reference
MC4100	*ara*D139*Δ*(*lac*IPOZYA-*arg*F)*rps*L.*thi*	Casadaban (1976)
LCB79	MC4100 with ϕ79 (*nar-lac*)	Pascal et al. (1982)
LCB162	F⁻*thr leu lac*Y *tonA thi str nar*G162::Mu*cts*62	Bonnefoy-Orth et al. (1981)
LCB162A	Same as LCB162, but *moa*203::Mu*cts*	Santini et al. (1992)
LCB162B	Same as LCB162, but *mob*207::Mu*cts*	Santini et al. (1992)
pACYC184	Cm^R,Tet^R	Biolabs
pJF119EH	*tac*P,rrnB,lacI^Q,Ap^R	Fürste et al. (1986)
pVA14	pACYC184 Cm^R (*narJ*)	Augier et al. (1993a)
pVA50	pJF119EH Ap^R (*narGH*)	Augier et al. (1993a)
pVA50-C16A	pJF119EH Ap^R (*narGH*[C16A])	Augier et al. (1993b)
pVA50-C19A	pJF119EH Ap^R (*narGH*[C19A])	Augier et al. (1993b)
pVA50-C244A	pJF119EH Ap^R (*narGH*[C244A])	Augier et al. (1993a)
pVA50-W220F	pJF119EH Ap^R (*narGH*[W220F])	Augier et al. (1993a)
pVA50-W220C	pJF119EH Ap^R (*narGH*[W220C])	Augier et al. (1993a)

Table 2. Characteristics of the enzyme complexes used in this study. The amounts of Fe-S centres and Mo cofactor are from previous work (Augier et al., 1993a,b; Johnson et al., 1991; Santini et al., 1992). The specific activity with reduced benzyl viologen was determined as described in Experimental Procedures. n.d., not determined.

Strain	Enzyme structure	No. of		Activity
		Fe-S centres	Mo cofactors	
				μmol min^{-1} mg^{-1}
MC4100	$\alpha\beta\gamma$	4	1	49
LCB79	none	0	0	< 0.1
LCB79/pVA14+pVA50	$\alpha\beta$	4	1	49
LCB79/pVA14+pVA50(C16A)	$\alpha\beta$(C16A)	3	1	8.5
LCB79/pVA14+pVA50(C19A)	$\alpha\beta$(C19A)	3	1	6.9
LCB79/pVA14+pVA50(W220C)	$\alpha\beta$(W220C)	4	1	27
LCB79/pVA14+pVA50(W220F)	$\alpha\beta$(W220F)	4	1	56
LCB79/pVA14+pVA50(C244A)	$\alpha\beta$(C244A)	0	0	< 0.1
LCB162A/pVA14+pVA50	$\alpha\beta$(moa)	n.d.	0	< 0.1
LCB162B/pVA14+pVA50	$\alpha\beta$(mob)	n.d.	1[a]	< 0.1

[a] Immature molybdenum cofactor.

ing appropriate antibiotics (ampicillin, 50 μg/ml; chloramphenicol, 10 μg/ml and, when needed, isopropyl thio-β-D-galactopyranoside (0.2 mM) to induce the *tac* promoter (*Ptac*).

Preparation of subcellular fractions. The cells were harvested during the exponential phase of growth, suspended in 50 mM Tris/HCl, pH 7.6, and disrupted in a French press at 540 MPa. The soluble and membrane fractions were obtained as described by Saracino et al. (1986). All procedures were performed at 4°C.

Quantification of nitrate reductase. The nitrate reductase antigen present in nitrate reductase preparations was quantified by rocket immunoelectrophoresis (Graham et al., 1980). The reference curves were determined with fully purified nitrate reductase A and immunoadsorbed anti-nitrate-reductase-A serum. The samples (6 μl) were subjected to electrophoresis at 2 mA overnight on 4 cm×4 cm (1% mass/vol.) agarose plates buffered with 20 mM sodium barbital at pH 8.6 containing (1% mass/vol.) Triton X-100 and 0.05% (mass/vol.) sodium azide. Antiserum (100 μl) was included in the agarose medium.

Partial purification of nitrate reductases. Wild-type and mutant nitrate reductases were purified as previously described by Guigliarelli et al. (1992) and Augier et al. (1993a,b). The characteristics of these different forms of the enzyme are listed in Table 2, including literature values for the content of molybdenum cofactor and Fe-S centres of each (Augier et al., 1993a,b; Johnson et al., 1991; Santini et al., 1992).

Enzyme assays and kinetic studies. Nitrate reductase activity was measured spectrophotometrically (Jones and Garland, 1977) by nitrate-dependent oxidation of the reduced benzyl viologen; 1 U nitrate reductase activity is defined as the amount catalysing the production of 1 μmol nitrite/min. Proteins were estimated by the technique of Lowry et al. (1951).

Steady-state kinetic studies were performed with a Hitachi U-2000 spectrophotometer connected to a PC-compatible computer. Nitrate reductase activity was measured by following the oxidation of reduced benzyl viologen at 600 nm, the measurements being made under anaerobic conditions in 1.6-ml cuvettes. The benzyl viologen was reduced by addition of a stoichiometric amount of dithionite, and the amount of reduced benzyl viologen concentration at the beginning of the reaction was determined by measurement of the absorbance at 600 nm, not assumed from the nominal concentrations in the stock solutions; they typically varied from the nominal values by about 10%. (It is important to avoid excess dithionite, which would interfere with the rate measurements by re-reducing the oxidized benzyl viologen produced in the reaction.) The phosphate buffer (100 mM, pH 6.5) and dithionite solution were kept anaerobic by constant bubbling of nitrogen.

Reduction of the enzyme by reduced benzyl viologen in the absence of nitrate. Aliquots of the enzyme solution (5–10 μl) were added to an anaerobic cuvette containing benzyl viologen previously reduced with dithionite. The change in absorbance is due to oxidation of benzyl viologen and dilution. A series of control measurements were performed under the same experimental conditions with buffer instead of enzyme. The difference of absorbance between sample and control provides a measure of the binding and oxidation of benzyl viologen by the enzyme. Each measurement was made at least five times.

Analysis of data. Calculations were made on a PC-compatible computer, fitting experimental data to various equations by non-linear least squares. The kinetic data were fitted by means of the program Leonora (Cornish-Bowden, 1995), which assesses the appropriate weighting function from internal evidence in the data and minimizes effects of outliers by use of the biweight method.

RESULTS AND DISCUSSION

Reduction of $\alpha\beta$ complexes by benzyl viologen. *Reduction of the $\alpha\beta$ complex of nitrate reductase of the strain LCB79/pVA14+pVA50 by reduced benzyl viologen.* Benzyl viologen has a midpoint redox potential of −330 mV. To verify that the artificial donor can reduce the different redox centres of the $\alpha\beta$ nitrate reductase, we studied the reduction of this complex in the absence of nitrate by adding different concentrations of enzyme to a solution of benzyl viologen that had been previously reduced with dithionite under anaerobic conditions. This reduction can be written as follows:

$$E_{ox} + nBv_{red} \rightleftarrows E_{ox}\text{-}(Bv_{red})_n \rightleftarrows E_{red} + nBv_{ox} \quad (1)$$

E and Bv represent enzyme and benzyl viologen respectively, the subscripts ox and red denoting the oxidized and reduced states respectively, and n is the number of electrons involved in the reduction.

In the absence of nitrate the reduction of the $\alpha\beta$ complex by reduced benzyl viologen is an equilibrium, albeit one that involves chemical transformation as well as binding, and it can accordingly be analysed in the same way as a pure binding experiment. The resulting Scatchard plot (Scatchard, 1949) sug-

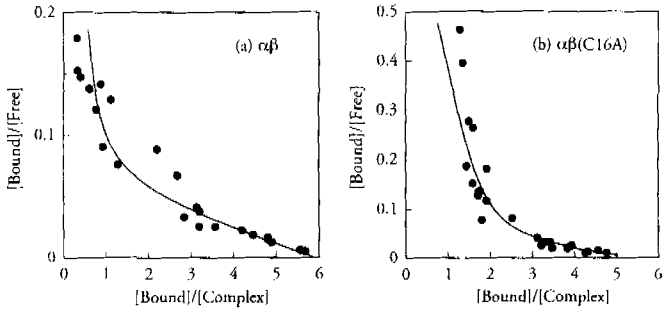

Fig. 2. Reduction of *αβ* complexes of nitrate reductase by reduced benzyl viologen: (a) native *αβ* complex, (b) mutant *αβ*(C16A) complex lacking Fe-S centre 1. The data are shown in the form of Scatchard plots, treating the binding followed by electron transfer as a binding process, as discussed in the text. The lines are calculated by minimizing the sum of squares of differences between observed bound values and values calculated on the assumption of two independent classes of binding sites.

gests the presence of a total of about six sites of reduction falling into at least two classes with different equilibrium constants (Fig. 2a). This total is compatible with the interpretation that all the redox centres in the *αβ* enzyme can be reduced (the four Fe-S centres plus the two-electron reduction of the molybdenum cofactor). The saturation curve can be expressed with fair accuracy as the sum of two processes with n_1 and n_2 sites and dissociation constants K_1 and K_2, respectively, as follows:

$$[Bv]_{bound} = \frac{n_1[Bv]_{free}}{K_1 + [Bv]_{free}} + \frac{n_2[Bv]_{free}}{K_2 + [Bv]_{free}} \quad (2)$$

The best-fit parameters obtained with this equation are given in Table 3. As the fit obtained is not perfect it is likely that the true model is more complex, with more than two classes of site of reduction. This would seem quite reasonable when we consider that the Fe-S centres are all different from one another and from the molybdenum cofactor.

Samples prepared by the same procedure used to prepare the *αβ* complex, and derived from the same strain (LCB79) used to over-express the *αβ* complex, but devoid of the corresponding plasmid and consequently without nitrate reductase, showed no detectable capacity to oxidize benzyl viologen. This control suggests that all of the reaction observed with the complex was due to it alone and that partially purified proteins could be used in the equilibrium studies without fear of artefacts due to contaminating proteins.

Reduction by benzyl viologen of mutant αβ complexes.* Mutants at cysteine residues 184, 217 and 244 of the *β* subunit, which coordinate Fe-S centres 3, 2 and 4, respectively, have no nitrate reductase activity, because they lack the molybdenum cofactor and all of the Fe-S centres (Augier et al., 1993a). However, a mutant C16A in which a cysteine residue normally coordinating centre 1 was lost produced an active enzyme, even though it had lost the Fe-S centre of highest potential, 80 mV (Augier et al., 1993a). Table 3 includes the results for binding of benzyl viologen to two of these mutants, one active, *αβ*(C16A), and the other inactive, *αβ*(C244A). These confirm and extend the previous observations. Not surprisingly, in the absence of the Fe-S centres and the molybdenum cofactor from the complex *αβ*(C244A) no binding of benzyl viologen could be detected. However, in the absence of Fe-S centre 1 the complex *αβ*(C16A) could still bind benzyl viologen, but one binding site of the low-affinity class was missing (Fig. 2b). The strength of binding at the low-affinity sites was not detectably altered, but binding at the high-affinity sites was much tighter. All of this suggests that Fe-S centre 1 is a low-affinity site.

Reduction by benzyl viologen of nitrate reductase mutants altered in the molybdenum cofactor. All the molybdoenzymes of *E. coli* are thought to contain the same molybdenum cofactor, a molybdopterin guanine dinucleotide (Johnson et al., 1991; Santini et al., 1992). Mutations in the five chlorate-resistant loci designated *moa*, *mob*, *mod*, *moe* and *mog* lead to the pleiotropic loss of all molybdoenzyme activities (Saracino et al., 1986; Boxer, 1989). The *moa* and *moe* loci code for polypeptides required for biosynthesis of the molybdenum cofactor (Johnson and Rajagopalan, 1987; Pitterle and Rajagopalan, 1989).

Experiments similar to those already described were carried out with partially purified nitrate reductase from soluble extracts of the chlorate-resistant mutants LCB162A/pVA14+pVA50 and LCB162B/pVA14+pVA50. Strain LCB162A/pVA14+pVA50 lacks the *moa* gene and produces an inactive complex *αβ*(moa) that lacks one of the early precursors of the molybdenum cofactor. However, strain LCB162B/pVA14+pVA50 lacks the *mob* gene and produces an inactive complex *αβ*(mob) that contains a more mature form of the molybdenum cofactor (molybdopterin) with only the 5′-GMP group missing (Johnson et al., 1991).

No reduction of the complex *αβ*(moa) by reduced benzyl viologen could be detected (Table 3). The presence of molybdopterin thus appears to be absolutely required for reduction, and suggests that this complex does not contain Fe-S centres. Understanding this implied connection between the molybdenum cofactor and the Fe-S centres in more detail will require EPR studies. The results with the complex *αβ*(mob) were quite different: it could be reduced with benzyl viologen, at six sites corresponding to those observed for the reference complex *αβ*, and the similarity of data shown in Table 3 suggests that the absence of 5′-GMP has little effect on any of the binding sites. The fact that the binding at the high-affinity sites is weakened somewhat more than that at the low-affinity sites suggests that the two high-affinity sites may correspond to the molybdenum

Table 3. Equilibrium reduction of the αβ complex of nitrate reductase by benzyl viologen in the absence of nitrate.

Subunit structure	High affinity		Low affinity	
	number of sites	K_{diss}	number of sites	K_{diss}
		mM		mM
αβ	2	12.1 ± 2.1	4	114 ± 15
αβ(C16A)	2	3.00 ± 0.21	3	114 ± 21
αβ(C244A)	no reduction detected			
None (strain LCB79)	no reduction detected			
αβ(moa)	no reduction detected			
αβ(mob)	2	17.2 ± 1.4	4	90.1 ± 4.9

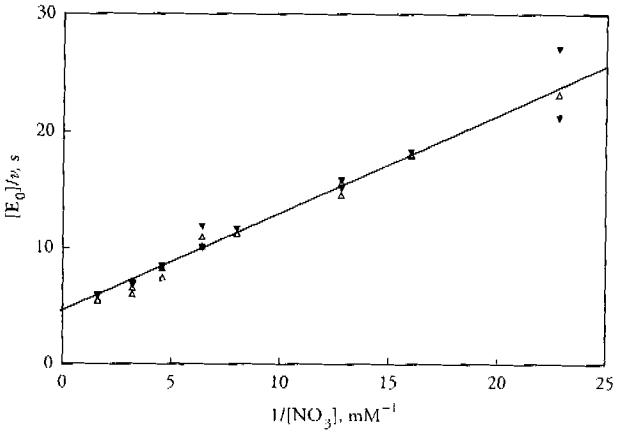

Fig. 3. Evidence that the $\alpha\beta$ complex has the same kinetic behaviour whether obtained by solubilizing and purifying the native enzyme with loss of the γ subunit (△) or by partially purifying it from a strain that lacks the γ subunit (▼). Experiments were performed at a concentration of reduced benzyl viologen of 0.193 mM.

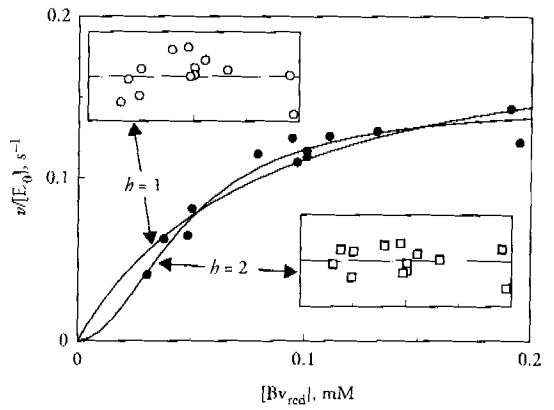

Fig. 4. Evidence that the rate depends on the square of the reduced benzyl viologen concentration. Data obtained at a nitrate concentration of 0.313 mM were fitted to the Michaelis-Menten equation with respect to reduced benzyl viologen (curve labelled $h = 1$) and to the same equation with this concentration raised to the power 2 (curve labelled $h = 2$). The two residual plots shown as insets are left unlabelled to avoid distracting attention from the arrangement of points, but the scales were identical in the two cases: abscissa, reduced benzyl viologen concentration from 0 to 0.2 mM; ordinate, difference between observed and calculated values of $v/[E_0]$ from -0.025 to $+0.025$ s^{-1}.

cofactor whereas the four weaker sites correspond to the Fe-S centres.

Steady-state kinetics. The measurements of the reduction of $\alpha\beta$ complexes by benzyl viologen show that all of the redox centres of this complex can be reduced. This leaves open, however, the question of whether electrons are transferred during the catalytic process first from benzyl viologen to the Fe-S centres then to the molybdenum cofactor, or whether they can be transferred directly to the molybdenum cofactor. The latter possibility would raise the further question of what role the Fe-S centres fulfil. To answer these questions we have compared the steady-state kinetic behaviour of the $\alpha\beta$ complex with that of various complexes with mutations affecting the Fe-S centres.

The $\alpha\beta$ complex can be prepared in two different ways, either separating it from the normal enzyme of the parent strain MC 4100 in which it is attached to the membrane via the γ subunit, or by purifying it from the strain LCB79/

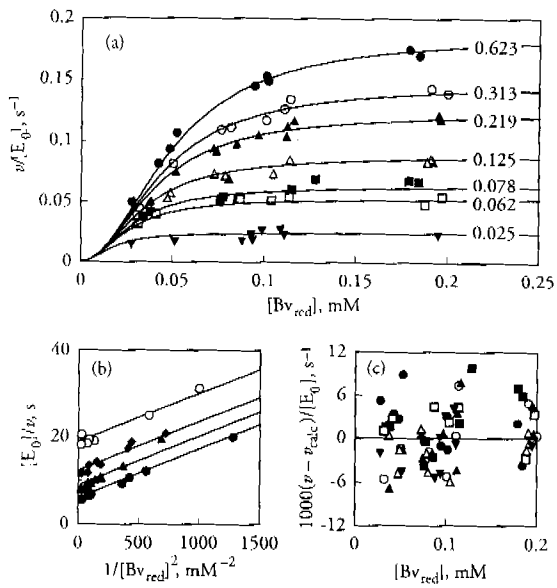

Fig. 5. Dependence of the rate on the two substrate concentrations. (a) Plot of rate against the concentration of reduced benzyl viologen at nitrate concentrations of 0.623 (●), 0.313 (○), 0.219 (▲), 0.125 (△), 0.078 (■), 0.0625 (□) and 0.025 mM (▼), with curves calculated from Eqn (4) with the parameter values shown as the top line of Table 5; (b) double-reciprocal plots at four nitrate 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.

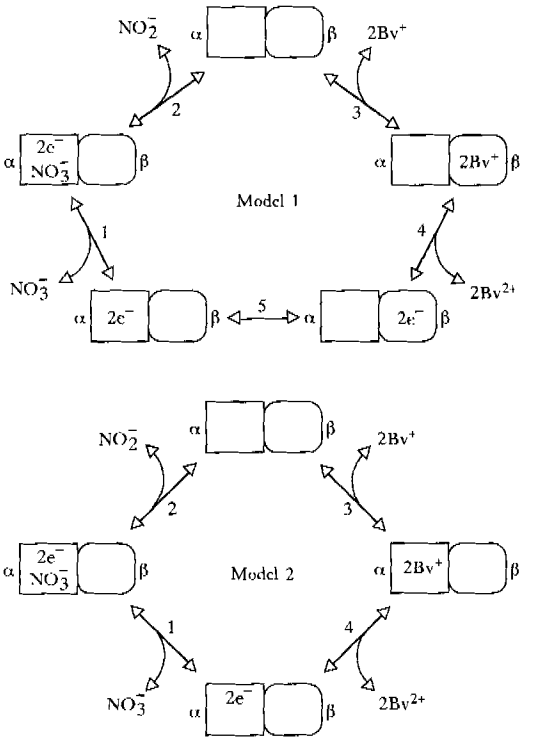

Fig. 6. Models for transfer of electrons between reduced benzyl viologen, the $\alpha\beta$ complex and nitrate. The essential difference between the models is that in model 1 benzyl viologen and nitrate are assumed to react at different subunits (β and α, respectively), so there is a compulsory step involving electron transfer between the two subunits in the complex (step 5), whereas in model 2 both substrates are assumed to react at the α subunit, so no transfer within the complex is necessary. To simplify the presentation, steps rather than rate constants are numbered; in the text, positive and negative subscripts refer to clockwise and anticlockwise rate constants respectively.

Table 4. Definitions of the kinetic parameters in terms of the two models.

Parameter	Model 1	Model 2
k_{cat}	$\frac{k_2k_4k_5}{k_2k_4 + k_2k_5 + k_4k_5}$	$\frac{k_3k_4}{k_3 + k_4}$
$K^2_{0.5,Donor}$	$\frac{k_3(k_{-3} + k_4)k_5}{k_3(k_2k_4 + k_2k_5 + k_4k_5)}$	$\frac{k_2(k_{-3} + k_4)}{k_3(k_2 + k_4)}$
$K_{m,nitrate}$	$\frac{(k_{-1} + k_2)k_4(k_5 + k_{-5})}{k_1(k_2k_4 + k_2k_5 + k_4k_5)}$	$\frac{(k_{-1} + k_2)k_4}{k_1(k_2 + k_4)}$
$\frac{k_{cat}}{K^2_{0.5,Donor}}$	$\frac{k_3k_4}{k_{-3} + k_4}$	$\frac{k_3k_4}{k_{-3} + k_4}$
$\frac{k_{cat}}{K_{m,nitrate}}$	$\frac{k_1k_2k_5}{(k_{-1} + k_2)(k_5 + k_{-5})}$	$\frac{k_1k_2}{k_{-1} + k_2}$

pVA14+pVA50 that lacks the *narI* gene and does not express the γ subunit at all. We have found it more convenient to use the complex prepared in the latter way, but we have first checked that its kinetic properties are essentially the same as those of the complex separated from the holoenzyme (Fig. 3).

Kinetic behaviour with respect to the concentration of benzyl viologen. Fig. 4 shows the dependence of rate on the concentration of benzyl viologen, plotted both as a normal Michaelis-Menten function and as a Michaelis-Menten function in which the concentration of benzyl viologen is replaced by its square. It may be seen that the latter gives a somewhat better fit, consistent with the fact that two molecules of the 1-electron donor benzyl viologen are required by the stoichiometry of the reaction for each ion of the 2-electron acceptor nitrate:

$$2Bv^{+}_{red} + 2H^{+} + NO_3^- \rightarrow 2Bv^{2+}_{ox} + H_2O + NO_2^- \quad (3)$$

In their earlier study of nitrate reductase Morpeth and Boxer (1985) interpreted their results in terms of simple Michaelis-Menten kinetics with respect to reduced benzyl viologen and methyl viologen. They reported linear plots over the ranges of electron donor used, both with unmodified cytochrome-containing nitrate reductase and with trypsin-modified, cytochrome-free nitrate reductase, with very similar parameters for both types of preparation.

Kinetic behaviour of the $\alpha\beta$ complex with respect to both substrates. The initial rate of nitrate reduction was measured as a function of reduced benzyl viologen concentration at several fixed concentrations of nitrate, with the results shown in Fig. 5a. The corresponding double-reciprocal plots produced parallel lines (Fig. 5b), indicating that the apparent specificity constant for each substrate is independent of the concentration of the other.

This type of behaviour is characteristic of a substituted-enzyme type of mechanism in which the first substrate reacts with the enzyme to give the first product and a modified form of enzyme before the second substrate reacts with this modified enzyme to give the second product and regenerate the original enzyme. In the case of nitrate reductase this implies a first half-reaction in which benzyl viologen reduces the enzyme, followed by a second half-reaction in which nitrate reoxidizes it.

We now consider these results in relation to the two reaction schemes shown in Fig. 6. In model 1, binding of the electron donor at the β subunit is followed by reduction of the Fe-S centres, electron transfer to the molybdenum cofactor and finally to nitrate. In model 2, the Fe-S centres play no direct part in the reaction as the electron donor binds to the α subunit where it directly reduces the molybdenum cofactor. Both models are consistent with the behaviour illustrated in Fig. 5, as they lead to rate equations of the same form:

$$v = \frac{k_{cat}[E]_{total}[Donor]^2[NO_3^-]}{K_{m,nitrate}[Donor]^2 + K^2_{0.5,Donor}[NO_3^-] + [Donor]^2[NO_3^-]} \quad (4)$$

where v is the initial rate at total enzyme concentration $[E]_{total}$ and concentrations [Donor] and $[NO_3^-]$ of electron donor and nitrate respectively, and the other symbols represent kinetic parameters: k_{cat} is the catalytic constant, $K_{m,nitrate}$ is the Michaelis constant with respect to nitrate and $K_{0.5,Donor}$ is the half-saturation concentration of electron donor at saturating nitrate (not a Michaelis constant because the reaction does not follow Michaelis-Menten kinetics with respect to benzyl viologen). The electron donor was normally reduced benzyl viologen in our experiments, but data for reduced methyl viologen will be considered in the next section. The expressions for these parameters are shown in Table 4, and the parameter values obtained by fitting the data to Eqn (4) are shown in Table 5.

Comparison between benzyl viologen and methyl viologen as electron donors. Table 5 also includes parameter values for the $\alpha\beta$ complex with methyl viologen as electron donor. It can be seen that the catalytic activity decreases considerably when methyl viologen is used as the electron donor, in agreement with a previous observation (Jones and Garland, 1977) that methyl viologen was oxidized more slowly than benzyl viologen. However, Morpeth and Boxer (1985) reported similar k_{cat} values for both artificial donors. It is noteworthy that the specificity constant for nitrate, i.e. $k_{cat}/K_{m,nitrate}$, differs by a factor of 5 between the two experiments, even though one might expect it to be independent of the identity of the electron donor, as its definition

Table 5. Kinetic parameters.

Electron donor	Complex	k_{cat}	$\frac{k_{cat}}{K^2_{0.5,Donor}}$	$\frac{k_{cat}}{K_{m,nitrate}}$	$K_{0.5,Donor}$	$K_{m,nitrate}$
		s^{-1}	$mM^{-2}\,s^{-1}$	$mM^{-1}\,s^{-1}$	mM	
Reduced benzyl viologen	$\alpha\beta$	0.260 ± 0.006	75.5 ± 2.5	1.071[a] ± 0.023	0.059	0.243
	$\alpha\beta$(C16A)	0.0434 ± 0.0034	14.0 ± 1.6	0.541 ± 0.052	0.055	0.0803
	$\alpha\beta$(C19A)	0.0335 ± 0.0034	15.6 ± 3.0	0.239 ± 0.028	0.046	0.140
	$\alpha\beta$(W220C)	0.139 ± 0.010	41.3 ± 5.5	0.779 ± 0.048	0.058	0.179
	$\alpha\beta$(W220F)	0.289 ± 0.067	460 ± 680	0.838 ± 0.091	0.025	0.345
Reduced methyl viologen	$\alpha\beta$	0.118 ± 0.039	3.88 ± 0.65	0.207[a] ± 0.055	0.551	0.571

[a] Values expected to be equal in the absence of memory effects, as the definitions (Table 4) do not include rate constants for steps involving the electron donor.