The Respiratory Chain of Anaerobically Grown *Escherichia coli*: Reactions with Nitrite and Oxygen

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The reactions of nitrite and oxygen with the cytochrome *d* oxidase of *Escherichia coli* were studied, following growth of cells on glycerol with fumarate as respiratory oxidant. Optical difference spectroscopy was used to investigate the kinetics of product formation during the reaction of the respiratory chain with nitrite. Two kinetically distinct species were formed in the reaction with nitrite; these had spectral features at 438 nm and 630 nm. These observations indicate that the cytochrome *d* does not contribute significantly to absorbance in the Soret region, and that changes elicited by ligand binding in the Soret region are largely attributable to haemoprotein *b*-590. Inhibition of respiratory oxidase activity by nitrite was also investigated. The inhibition was competitive with oxygen (K_i 0.83 mM, pH 7), which allowed analysis of the reaction of the oxidase with oxygen itself. The reaction with oxygen was cooperative with an apparent number of oxygen-binding sites, n , of 1.26 at pH 7, increasing to 1.72 at pH 6. We propose a model for the oxidase in which there are two ligand-binding sites.

INTRODUCTION

Escherichia coli, grown anaerobically on the non-fermentable carbon source glycerol, with fumarate as respiratory oxidant, develops a respiratory chain which contains cytochrome *d* as the major and perhaps sole cytochrome oxidase (Poole & Ingledew, 1987). Under these conditions cytochrome *d* is produced in relatively high abundance in the cytoplasmic membrane. This oxidase contains, in addition to cytochrome *d*, cytochrome *b*₅₅₈ and haemoprotein *b*-590 (Koland *et al.*, 1984), and comprises two subunits, I and II, with M_r values of approximately 70000 and 43000 respectively (Finlayson & Ingledew, 1985). Both the cytochrome *b*₅₅₈ and the haemoprotein *b*-590 are protohaem IX centres, whilst the cytochrome *d* is a chlorin centre (Barrett, 1956; Timkovich *et al.*, 1986). The oxidase contains one or two cytochromes *d*, one haemoprotein *b*-590, and one cytochrome *b*₅₅₈ centre. Coulometric and CO-binding titrations have been used to suggest the presence of two cytochromes *d* per mole of the oxidase (Lorence *et al.*, 1986), but the data can be reinterpreted to support the presence of a single cytochrome *d*. The coulometric method used overestimated the total *b*-cytochrome content by 0.3 (2.3 ± 0.3), and the estimate of the number of cytochromes *d* present was only 1.4 ± 0.3 . The CO-binding data estimated the number of cytochromes *d* as being 1.6 ± 0.1 ; however, as the optical changes elicited in the Soret region by CO were assumed to be due to the cytochrome *d* with little contribution from haemoprotein *b*-590, these values may also be overestimations. On the other hand, analyses of the total iron content of the oxidase indicate that the amount of protohaem IX accounts for only half of the total iron present (Kita *et al.*, 1984), which suggests that there may be two *b*-type cytochromes and two cytochromes *d* present. Cytochrome *b*₅₅₈ is located in subunit I (Green *et al.*, 1984); this subunit is also responsible for

Abbreviations: ETP, electron transport particles; NED, *N*-(1-naphthyl)ethylenediamine; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

ubiquinol-1 oxidation, whilst subunit II is responsible for TMPD oxidation (Kranz & Gennis, 1984).

Reaction of the reduced respiratory chain with nitrite in the absence of oxygen causes bleaching of the 630 nm cytochrome *d* absorbance (Hubbard *et al.*, 1983; Meyer, 1973); there are also changes elicited in the Soret region which may be due to reaction of nitrite with cytochrome *o* (Meyer, 1973) or haemoprotein *b*-590. Electron paramagnetic resonance (EPR) spectroscopy shows that paramagnetic species are formed concurrently with the optical changes discussed above (R. A. Rothery & W. J. Ingledew, unpublished), and that they have EPR signals characteristic of those produced by haem-NO compounds such as cytochrome *a*₃ nitroxide (Mascarenhas *et al.*, 1983) and haemoglobin nitroxide (Yonetani *et al.*, 1972). These reactions with nitrite are distinct from the known *E. coli* nitrite reductase pathways, of which there are three (viz. a membrane bound nitrite reductase, a soluble NADPH-dependent enzyme that functions *in vivo* as a sulphite reductase and a soluble NADH-dependent enzyme; Coleman *et al.*, 1978).

In this paper we have studied the reactions of the respiratory chain with nitrite and oxygen in electron transport particles prepared from cells grown anaerobically on glycerol with fumarate as respiratory oxidant.

METHODS

Growth of cells. *Escherichia coli* strain EMG2 (prototroph) was grown at 37 °C in 20 litre bottles on a mineral salts medium (Cohen & Rickenberg, 1956) but with the omission of manganese. The medium was supplemented with 0.1% (w/v) vitamin-free Casamino acids. The major reductant and carbon source was glycerol (1%, w/v) and the respiratory oxidant was fumarate (100 mM, potassium salt). Cells were harvested in late exponential phase (after 24 h growth) using an MSE continuous flow rotor running in an MSE High Speed 18 centrifuge at 18000 r.p.m. with a through flow of 250 ml min⁻¹. The cells were washed twice in ice-cold 50 mM-potassium phosphate buffer (pH 7.5) containing 2 mM-magnesium sulphate, by resuspension in the buffer followed by recentrifugation at 10000 *g* in a Sorvall RC-5B (DuPont) refrigerated centrifuge operating at 4 °C. The pellet was stored at -30 °C before preparation of electron transport particles as described below.

Preparation of electron transport particles (ETP). Frozen cells were thawed and suspended in an equal volume of a 20 mM-TES/KOH buffer (pH 7.5) containing 5 mM-Na₂EDTA at 4 °C. Bovine spleen deoxyribonuclease II and lysozyme (2 µg ml⁻¹ each) were added to hydrolyse any DNA released during cell breakage and to digest the cell wall peptidoglycan respectively. Cell breakage was achieved by passing the suspension of cells twice through a French press operating between 10 and 15 MPa. Cell debris and residual whole cells were removed by centrifuging the lysate at 10000 *g* for 15 min. Membrane fragments from the supernatant were pelleted by centrifugation for 90 min at 125000 *g* in a Prepspin 50 ultracentrifuge operating at 4 °C. This pellet was washed by resuspension in the TES-EDTA buffer and recentrifugation at 125000 *g* for 90 min. Finally, the pellet was suspended in a minimal volume of the buffer and frozen by pipetting dropwise into liquid nitrogen. ETP were stored under liquid nitrogen until required.

Assays for oxidase and nitrite reductase activity. Anaerobic nitrite reductase activity was measured in ETP and whole cells by a modification of the method of Wray & Filner (1970). Cells or ETP were incubated anaerobically in 50 mM-potassium phosphate buffer (pH 7) with 20 mM-sodium lactate and 2 mM-sodium nitrite at 30 °C. Samples were withdrawn into test-tubes containing 3 ml distilled water, 1 ml 1% (w/v) sulphanilamide in 3 M-HCl, and 1 ml 0.02% NED. Suspended cells or membrane fragments were centrifuged out and the absorbance was measured at 528 nm. Nitrite reductase activity at pH 7 in whole cells was 1.87 nmol min⁻¹ (mg protein)⁻¹, whereas in ETP it was 0.068 nmol min⁻¹ (mg protein)⁻¹. This compares with oxidase activities of 153 nmol min⁻¹ (mg protein)⁻¹ with lactate as substrate in ETP.

Rates of oxygen consumption were determined using a Clark type oxygen electrode with a glass incubation vessel (Rank) operating at 30 °C. The Perspex plunger of this electrode was replaced by a ground-glass airtight stopper with a capillary portal through which additions could be made. Lactate (34 mM) was used as reductant. The oxygen electrode was interfaced with a BBC Model B microcomputer (Acorn Computers). Oxygen concentrations were calculated using the data of Chappell (1964). The pH dependence of nitrite inhibition of oxidase activity was investigated by using a range of buffers between pH 5.6 and pH 9.0. Buffers used were as follows: 50 mM-malonnate, pH 5.6; 50 mM-MES, pH 6; 50 mM-TES, pH 7; and 50 mM-Tricine, pH 8 (pH values adjusted using KOH or HCl). All buffers contained 2 mM-magnesium sulphate. Mixtures were used at intermediate pH with a total buffer concentration of 50 mM.

Protein was determined by the Lowry method modified by the inclusion of 1% (w/v) SDS in the incubation mixture to solubilize membrane-bound protein.

Data analysis. Where appropriate, data collected from the oxygen electrode were transferred to the University's mainframe computer for analysis. Rates of oxygen consumption were determined using a noise-averaging differentiating algorithm. For each concentration of nitrite used, the $V_{\max}$ of the oxidase reaction was determined by measurement of the gradient of a plot of s/v versus s (the Hanes plot). Other parameters were determined by analysis of a plot of v versus v/s (the Eadie-Hofstee plot). In cases where positive cooperativity is observed, the value of v/s reaches a maximum, and the coordinates of this maximum can be used to determine the K_m for substrate binding and the apparent number of substrate-binding sites, n (Segel, 1975).

Spectroscopy. Optical difference spectra were obtained using a split beam spectrophotometer constructed in the workshop of the Department of Biochemistry and Microbiology at the University of St Andrews. Cytochrome *d* concentrations were determined using the differential extinction coefficient quoted by Reid & Ingledew (1980) as $5.3 \text{ mm}^{-1} \text{ cm}^{-1}$ for the wavelength pair 630–610 nm. Lactate was used as reductant (10 mM) and difference spectra were recorded anaerobically of lactate reduced + nitrite *minus* lactate reduced. The kinetics of the reaction of ETP with nitrite were investigated by following the reaction at a fixed wavelength in the spectrophotometer. All optical spectra were recorded at 30 °C.

Chemicals. Tricine, MES, TES, malonic acid, lysozyme and deoxyribonuclease II were obtained from Sigma. Sulphanilamide and NED were obtained from BDH. Sodium nitrite was obtained from Fisons. Other chemicals were obtained from BDH or Sigma.

RESULTS

Changes in the optical absorption spectra of cytochromes elicited by nitrite

A reduced *minus* oxidized difference spectrum of ETP is shown in Fig. 1 (top). Noticeable in this spectrum are the broad absorption differences in the Soret region (around 435 nm) and the prominent peaks at 560 nm (cytochrome *b*, alpha band) and 630 nm (cytochrome *d*, alpha band). The trough at 650 nm arises because the oxidized form of cytochrome *d* has an absorbance maximum in this region, whilst the reduced form has an absorbance maximum around 630 nm. In addition to the *b* and *d* type cytochromes, a small peak at approximately 590 nm can be distinguished. This absorption is attributed to haemoprotein *b*-590 (Poole *et al.*, 1986). The effect of nitrite on the cytochrome spectra can be seen in Fig. 1 (*a*–*d*). Trace BL (baseline) is a reduced *minus* reduced spectrum of the membrane preparation used, and traces (*a*)–(*d*) are reduced + nitrite *minus* reduced spectra taken at successive times after addition of nitrite (15 s, 14 min, 21 min and 51 min, respectively). It can be seen that a reaction resulting in an apparent bleaching of the cytochrome *d* alpha band (inverse of the reduced *minus* oxidized spectrum) goes to completion within the time-span taken to produce spectrum (*a*). This rapid reaction is not accompanied by any other detectable change of comparable rate. A relatively slow absorption change in the Soret region (maximum at 438 nm) can be discerned in the spectra of Fig. 1; this change is quite large compared with the control reduced *minus* oxidized spectrum (top). A parallel change is barely discernable at 590 nm, and this link is strengthened by a kinetic analysis of this respiratory chain from which it has been suggested that the 438 nm component is part of the spectrum of haemoprotein *b*-590 (D. S. Wariabharaj & W. J. Ingledew, unpublished). The broad absorbance change at approximately 560 nm can be attributed to the beta band of haemoprotein *b*-590 (Poole *et al.*, 1986).

Kinetics of the nitrite-induced spectral change at 630 nm

The time-course of the nitrite-induced change in alpha band absorption was studied in greater detail by monitoring absorption at a fixed wavelength of 630 nm. The rate of reaction is dependent on both the concentration of nitrite and the pH. Progress curves of the 630 nm change at different nitrite concentrations are shown in Fig. 2(*a*). The rate of reaction is more rapid at lower pH values (Fig. 3*a*), but the extent of the reaction is unaltered. The relationship between the velocity of the reaction and proton concentration at two different concentrations of unreacted cytochrome *d* is shown in Fig. 3(*a*). Above pH 8 the reaction is first-order with respect to protons, whilst it is less than first-order below this pH. Fig. 3(*b*) shows the relationship between the velocity of the reaction and the concentration of unreacted cytochrome *d* for a range of concentrations of nitrite. The order of the reaction with respect to cytochrome *d* decreases with increasing nitrite concentration. At low nitrite concentrations the reaction tends towards

Fig. 1. Optical difference spectra taken during the reaction of membranes with nitrite. Spectra of suspensions of ETP (7 mg protein ml^{-1}) showing reduced *minus* oxidized (top), baseline BL, and reduced + nitrite *minus* reduced spectra taken at different times after addition of 10 mM-nitrite to ETP reduced with 10 mM-lactate at pH 7: (a) 15 s; (b) 14 min; (c) 21 min; (d) 51 min. The reaction with nitrite (a–d) causes absorbance changes in the 438 nm, 560 nm, 590 nm, and 630 nm bands of the reduced *minus* oxidized spectrum (top). The change at 630 nm is essentially complete within the time taken to record the first spectrum (a).

Fig. 2. Progress curves of the optical changes induced by nitrite. Nitrite was added to ETP (7 mg protein ml^{-1}) reduced with 10 mM-lactate. The curves were recorded with lactate-reduced ETP in the reference cuvette. (a) 630 nm reaction; (b) 438 nm reaction.

Fig. 3

Fig. 4

Fig. 3. Orders of the 630 nm reaction with respect to nitrite, protons and cytochrome *d*. Data were collected by analysis of progress curves recorded as in Fig. 2(a). (a) Velocity of the 630 nm reaction versus concentration of protons: ■, log rate of reaction with 0.007 A_{630} (3.77 μM) cytochrome *d* remaining; ▲, log rate of reaction with 0.02 A_{630} (3.77 μM) cytochrome *d* remaining. Reactions were initiated by adding 1 mM-nitrite to a suspension of ETP (7 mg protein ml^{-1}) reduced with 10 mM-lactate. (b) Velocity of the reaction versus concentration of cytochrome *d*. Reaction order was determined at pH 8 with different concentrations of nitrite: ●, 0.5 mM; ▲, 1 mM; ■, 2 mM; ○, 3 mM; △, 4 mM; □, 10 mM. Protein concentration 7 mg ml^{-1} . (c) Velocity of the reaction versus the concentration of nitrite. ■, 0.005 A_{630} (0.94 μM) cytochrome *d* remaining; ▲, 0.035 A_{630} (6.60 μM) cytochrome *d* remaining; ●, 0.025 A_{630} (4.72 μM) cytochrome *d* remaining. Protein concentration 8.9 mg ml^{-1} ; pH 7.0.

Fig. 4. Effect of pH on the optical changes. (a) Spectra after completion of reaction at different pH values. Reduced + nitrite minus reduced difference spectra are illustrated between pH 6.5 and 8. ETP reduced with 10 mM-lactate were reacted with 10 mM-nitrite. Protein concentration 7.0 mg ml^{-1} . (b) Extent of the 438 nm reaction versus pH.

first-order, whilst with increasing nitrite concentration it approaches an order of 0.25. Fig. 3(c) shows the relationship between the concentration of nitrite and the rate of reaction. The reaction is first-order with respect to nitrite and there is no change in order with increasing nitrite concentration.

Kinetics of the nitrite-induced spectral change in the Soret region

The reaction of nitrite with the *E. coli* respiratory chain was monitored in the Soret region at a fixed wavelength of 438 nm. This reaction is considerably slower than that considered in the previous section. Its initial rate under the conditions used for Fig. 2(b) at 10 mM-nitrite was only 0.02 absorbance units min⁻¹, whilst the rate of the 630 nm change was 0.20 absorbance units min⁻¹ (both rates measured at pH 7 with a protein concentration of 7 mg ml⁻¹). The pH dependence of the reaction rate was similar to that observed at 630 nm although in this case the extent of the reaction was also pH-dependent. Fig. 4(a) shows spectra taken after completion of the reaction of membranes with nitrite and Fig. 4(b) shows the effect of the pH on the extent of the 438 nm change. A pH-dependent equilibrium is suggested with a pK_a of 7.3. The kinetics of the 438 nm reaction are therefore more complex than those of the 630 nm change.

Effect of nitrite on oxygen consumption in ETP

In the absence of nitrite, there is a slight initial acceleration in the rate of oxygen consumption by *E. coli* ETP with decreasing oxygen concentration. The rate of the oxidase reaction increases by 7% when the oxygen concentration decreases from 0.18 mM to 0.06 mM at pH 7. This effect is not a function of the oxidase reaction, since it occurs at oxygen tensions where the lactate dehydrogenase activity is rate-limiting. Nitrite inhibits oxygen consumption by ETP and its use allows analysis of the oxidase reaction itself. The oxidase reaction has a very low K_m for oxygen (0.23 μ M, Kita *et al.*, 1984) and this reaction can be more easily studied by artificially increasing its K_m using a competitive inhibitor. The nitrite-inhibited enzyme exhibits cooperative steady-state kinetics (Fig. 5a, b) and there is an increase in the apparent number of sites (n) with decreasing pH, from 1.26 at pH 7 to 1.72 at pH 6 (Fig. 6). This increase is linear over the pH range studied with a gradient of -0.46 sites per pH unit.

pH dependence of nitrite inhibition

The oxidase reaction in ETP with lactate as reductant is pH-dependent with an optimum at pH 6.8 in the presence of 0.098 mM-oxygen. This compares with a reported optimum of pH 7.5 for the purified cytochrome *d* complex (Lorence *et al.*, 1984). Nitrite (16 mM) alters the apparent pH optimum of the oxidase reaction to pH 7.8 and becomes a more potent inhibitor of the oxidase reaction with decreasing pH from pH 7.8. The K_i values for nitrite were calculated assuming that the K_m for the oxidase reaction was 0.23 μ M (Kita *et al.*, 1984). The pK_i ($-\log_{10} K_i$) for nitrite increases from 3.1 at pH 7 to 4.7 at pH 6 (Fig. 6); this variation is linear with respect to pH with a gradient of -1.6. A small nitrite-induced activation of the reaction rate is observed at low oxygen concentrations above pH 7.8 (data not shown). This is not an ionic effect, but the reasons for it are unknown.

DISCUSSION

The reactions of anaerobic *E. coli* membranes with nitrite show complex kinetics which are both concentration- and pH-dependent. Two optically resolvable species can be detected: a nitroxide adduct to the cytochrome *d* which partially bleaches the 630 nm absorbance of reduced ETP, and a nitroxide adduct to the haemoprotein *b*-590 which bleaches the 438 nm absorbance of reduced ETP. Parallel EPR experiments also indicate that two species are formed when ETP are reacted anaerobically with nitrite (R. A. Rothery & W. J. Ingledew, unpublished). The cytochrome *d* species appears through a range of pH values between 6 and 8.5, whilst the haemoprotein *b*-590 species appears only at lower pH values.

Analysis of the kinetics of the formation of the 630 nm species reveals that the reaction shows constant order only with respect to nitrite concentration (first-order). The order of the reaction

Fig. 5

Fig. 6

Fig. 5. Eadie-Hofstee (a) and Hanes (b) plots for the nitrite-inhibited oxidase reaction. Data were obtained from differentiated oxygen electrode progress curves at pH 6.0 using 34 mM-lactate as reductant and with a protein concentration of 0.27 mg ml⁻¹.

Fig. 6. Effect of pH on the pK_i of nitrite ($\square$) and the apparent number of sites, n ($\circ$). The rate of change of pK_i is -1.6 , whilst that of the apparent number of sites was -0.46 sites per pH unit.

with respect to cytochrome *d* varies between 0.25 at high nitrite concentrations and approximately one at low nitrite concentrations. Therefore, only a single cytochrome *d* is involved kinetically in the formation of the 630 nm species. The rate of formation of the 630 nm species is pH-dependent, and this is consistent with nitrous acid being the reactive species. However, this does not explain the increase in reaction order above pH 8 and this may be due to the ionization of a functional group with a pK_a approaching 8. The formation of the nitroxide adduct to the cytochrome *d* is much as expected, and appears to be monophasic with a reaction of one cytochrome *d* with one nitrous acid.

The reduced *minus* oxidized spectrum of the cytochrome *d* oxidase has an absorption in the Soret region at 438 nm. This is due to the presence of the *b* type cytochromes *b*-590 and *b*₅₅₈ (Poole *et al.*, 1981). Since cytochrome *b*₅₅₈ does not react directly with oxygen, the bleaching of the Soret band at 438 nm by nitrite is most likely to be due to a reaction of haemoprotein *b*-590 with nitrite. This has been confirmed by analysis of the pre-steady-state kinetics of the reaction of the electron transport chain containing the cytochrome *d* oxidase with oxygen (D. S. Wariabharaj & W. J. Ingledew, unpublished) and by the parallel optical change at 590 nm. However, the optical changes associated with the *b*-590 are also consistent with oxidation of this species rather than formation of a nitroxide adduct. Both the extent (Fig. 4b) and the rate of this reaction are pH-dependent. The pH profile of its extent indicates a pH-dependent equilibrium with a pK_a of 7.3. The stability of the haemoprotein *b*-590 species responsible for the 438 nm

optical change is therefore pH-dependent. An explanation for this is that the reaction between the *b*-590 and nitrite is linked via a pH-dependent equilibrium between the two centres; the formation of the 438 nm species proceeds via formation of the 630 nm species and the nitroxide ligand can only be transferred at low pH values. This is consistent with a model for the cytochrome *d* oxidase complex in which the two ligand-binding sites are in close proximity.

The cooperative steady-state kinetics of the oxidase reaction suggest cooperativity between two oxygen-binding centres. The decrease in the apparent number of substrate-binding sites with increasing pH indicates that the affinity of one of these sites has a pronounced pH dependence; the optical data suggest that the ligand-binding behaviour of haemoprotein *b*-590 is responsible for this effect. The steady-state kinetics of the oxidase reaction catalysed by the cytochrome *cd*₁ complex of *Pseudomonas aeruginosa* show negative cooperativity (Ingledew & Saraste, 1979). This enzyme contains two cytochromes *d*₁ and two cytochromes *c* with the former acting as binding sites for oxygen. The negative cooperativity is therefore between the two identical binding sites for oxygen. We suggest that the situation in the cytochrome *d* complex of *E. coli* is different, with two types of oxygen-binding centres, cytochrome *d* and haemoprotein *b*-590, and positive cooperativity.

Inhibition of oxidase activity by nitrite is pH-dependent, nitrite becoming a more potent inhibitor with decreasing pH from about pH 7.4. The pK_i for nitrite as an inhibitor increases with decreasing pH and is consistent with nitrous acid being the inhibitory species. The slope of Fig. 6 is approximately -1.6 and is 0.6 more negative than could be explained purely by the increase in nitrous acid concentration. These results suggest that inhibitor binding is accompanied by binding of a single proton (see Dixon, 1953).

The formation of the optically observed species is distinct from the inhibition of the oxidase activity by nitrite, as they are not detectable in the presence of oxygen. Nitrous acid has been identified as the species responsible for the formation of the 630 nm optical species and for the inhibition of the oxidase reaction. Both the optical and polarographic data presented here point to a model for the oxidase which has two binding sites for oxygen which are in close proximity. It is uncertain whether these two binding sites are cytochromes *d* or whether one of them is cytochrome *d* and the other haemoprotein *b*-590, although the evidence presented here favours oxygen binding to a single cytochrome *d* site and to the haemoprotein *b*-590. The oxidase reaction would only be cooperative if the reaction cycle involves a mechanism which proceeds more rapidly when both adjacent centres are bound to oxygen. For example, the reaction cycle may involve disproportionation between two bound peroxides leading to a residual bound oxygen and two molecules of water. There is evidence that the aerobically oxidized enzyme has an oxygen bound to the cytochrome *d* (Hata *et al.*, 1985; Koland *et al.*, 1984); this would be consistent with cooperativity being observed at oxygen tensions where the oxygen begins to become dissociated from the cytochrome *d*.

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