The Electron Transport Chain of *Escherichia coli* Grown Anaerobically with Fumarate as Terminal Electron Acceptor: an Electron Paramagnetic Resonance Study

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The electron transport chain of *Escherichia coli* grown anaerobically on glycerol with fumarate as terminal electron acceptor, has been studied using electron paramagnetic resonance (EPR) spectroscopy. The analysis did not include cytochromes, but was confined to the lower potential (dehydrogenase) section of the electron transport chain. Several ferredoxin-type centres were detected and partially characterized, and the possible presence of iron–sulphur centres paramagnetic in their oxidized form ('HiPIP-type') was also noted. An EPR-detectable signal that may have been due to the presence of molybdenum in the electron transport chain is described and assessed. Most of the centres detected were reducible by substrate in the absence of oxidant and some were found to be wholly or partly reduced during steady state oxidation of substrates in the presence of oxygen.

INTRODUCTION

The composition of the electron transport chain of *Escherichia coli*, in terms of its oxidation–reduction enzymes, has been the subject of a number of investigations (e.g. Shipp, 1972; Hendler *et al.*, 1975; Pudek & Bragg, 1976; Reid & Ingledew, 1979; Ingledew *et al.*, 1980; Poole *et al.*, 1980). There are four principle modes of oxidative growth that have been widely studied: aerobic (high aeration), aerobic (low aeration), anaerobic (NO₃ as terminal electron acceptor), and anaerobic (fumarate as terminal electron acceptor). The cytochrome composition of the electron transport chain alters greatly between these different growth conditions, and a rationale for these changes is beginning to emerge. However, there has been only a small number of investigations into the iron–sulphur centres and the effects of alteration of growth conditions on the number and type of these centres. The studies on *E. coli* iron–sulphur centres that have been published are confined to aerobically grown cells (Nicholas *et al.*, 1962; Hamilton *et al.*, 1970; Hendler & Burgess, 1974; Poole & Haddock, 1975; Ingledew *et al.*, 1980) or to a haem-deficient mutant grown fermentatively with glucose (Blum *et al.*, 1980). Ingledew *et al.* (1980), using electron paramagnetic resonance (EPR) spectroscopy, have reported that membrane fragments derived from aerobically grown *E. coli* contain a minimum of four iron–sulphur centres, three of which appear to be very similar to the iron–sulphur centres of succinate dehydrogenase of eukaryotic mitochondria (Ohnishi *et al.*, 1976a, b) and of other prokaryotes (e.g. Ingledew & Prince, 1977) and it was suggested that these centres did belong to the *E. coli* succinate dehydrogenase. This paper describes an EPR study of the iron–sulphur centres and a possible molybdenum signal found in membranes of *E. coli* grown anaerobically with glycerol as carbon and energy source and fumarate as terminal electron acceptor.

Abbreviation: ETP, electron transport particles.

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**METHODS**

*Ori*nusm, *growth* conditions and *preparation* of *cells*. *Escherichia coli* strain EMG-2 (prototroph) was grown at 37 °C in 20 l batches on a mineral salts medium (Cohen & Rickenberg, 1956) but with manganese omitted; the medium was supplemented with 0.1% (w/v) vitamin-free Casamino acids. The major reductant was glycerol (1%, w/v), and the electron acceptor was potassium fumarate (100 mM).

Cells were harvested in late exponential phase using an MSE continuous flow rotor running in an MSE 18 centrifuge at 18000 r.p.m. and a flow rate of approximately 250 ml min⁻¹. The cells were washed twice by resuspension in 50 mm-potassium phosphate buffer (pH 7.5), and centrifugation at 10000 g for 15 min at 4 °C. The cell paste was frozen in small samples in liquid N₂ and stored at −30 °C until required.

**Cell breakage and the preparation of electron transport particles (ETP).** Cells were resuspended in a buffer that contained 20 mm-TES plus 2 mm-EDTA (pH 7.0), to a concentration of approximately 10 mg protein ml⁻¹. The cell suspension was passed twice through a French pressure cell at approximately 120 MPa. Unbroken cells and debris were removed by centrifugation at 10000 g for 15 min and the decanted supernatant centrifuged at 100000 g for 1 h, frozen in liquid N₂ and stored at −30 °C until required.

**Sample preparation, redox titrations and assays.** Samples for EPR were prepared in 3 mm internal diameter quartz tubes and frozen quickly in an iso-pentane/cyclohexane (5:1, v/v) freezing mixture cooled with a liquid N₂ cold-finger. The samples were stored under liquid N₂ until the EPR spectra could be taken. Oxidation–reduction titrations, and the determination of midpoint potentials and equivalencies (n-values) were performed as described by Dutton (1978); computer analysis of the data was as described by Reid & Ingledeuw (1979). Titrations were performed in 50 mm-TES buffer (pH 7.0) with protein, concentrations in excess of 10 mg ml⁻¹. The following oxidation-reduction mediators were added at concentrations of between 25 and 150 μM: phenazine ethosulphonate; vitamin K₃; indigotetrasulphonate; indigodisulphonate; 5-hydroxy-1,4-naphthoquinone; 2-hydroxy-1,4-naphthoquinone; anthraquinone-1,5-disulphonate; anthraquinone-2-sulphonate; anthraquinone-2,6-disulphonate; resorufin; safranine; methylviologen; benzylviologen; duroquinone; pyocyanine and cysteine. The redox titration vessel was purchased from the University of Pennsylvania glass workshop; a combination platinum-calomel reference electrode was used to measure the ambient Eₒ (Russell pH, Auchtermuchty, Fife, U.K.). The vessel was continuously flushed with White Spot N₂ (British Oxygen Company) which had been passed through a Nil-Ox apparatus (Jencons Scientific, Mark Rd, Hemel Hempstead, U.K.) to remove residual O₂. E.p.r. spectra were obtained using a Bruker ER 200 D EPR spectrometer (Bruker Analytische Messtechnik, Silberstreiten, D-7512 Rheinstetten, F.R.G.) fitted with a variable temperature cryostat and liquid helium transfer line (Oxford Instruments, Osney Mead, Oxford, U.K.). Rates of substrate oxidation were determined using a Clark O₂ electrode at 30 °C, and protein concentrations measured by the Lowry method using BSA as the standard.

**Chemicals.** Chemicals for bacterial growth and general media were from BDH, except for Casamino acids which was from Oxoid. Oxidation–reduction mediators were obtained from Aldrich, Koch-Light and Eastman Kodak. BSA (V) and NADH were obtained through Sigma.

**RESULTS AND DISCUSSION**

**EPR spectra and oxidase activities**

Formate is the substrate most rapidly oxidized by freshly prepared ETP [570 ng-atom O min⁻¹ (mg protein)⁻¹], followed by NADH, succinate, lactate and z-glycerophosphate at 260, 140, 62 and 5 ng-atom O min⁻¹ (mg protein)⁻¹, respectively (30 °C, pH 7.0 with 20 mm-potassium phosphate buffer). The z-glycerophosphate oxidation rate in these particles was low because the dehydrogenase in anaerobically grown cells is soluble and is lost during ETP preparation (Kistler & Lin, 1972). Glycollate, glutamate, malate, and β-hydroxybutyrate showed negligible oxidase activities.

EPR spectra of the reduced ETP are shown in Fig. 1(a, b). The spectra in Fig. 1(a) are of dithionite-reduced particles and taken at different temperatures, whilst the spectra in Fig. 1(b) are of ETP allowed to go anaerobic in the presence of substrate (z-glycerophosphate in the case shown, but other substrates gave similar results); the gain used in the latter case was twice that used in Fig. 1(a). In both cases, at relatively high temperatures (150 K), a ferredoxin is observed with apparently rhombic symmetry and gₛ at 2.037, gₓ centred at 1.937 and gₓ at 1.923; this centre is as fully reduced by substrate as by dithionite (note different gains). For convenience, this centre will be termed centre 1, pending a more accurate description of its role. As the
Fig. 1. EPR spectra of reduced ETP at different temperatures. The numbers by each spectrum refer to the temperature (K). The numbers at the bottom of the figure are g-values and, where appropriate, are linked to indicate resonances belonging to a single centre. Membranes were reduced with (a) dithionite or (b) by adding 10 mM-a-glycerophosphate for 5 min. The receiver gain was \(2.5 \times 10^4\) in (a) and \(5 \times 10^4\) in (b). Protein concentration was 52 mg ml\(^{-1}\). In both (a) and (b), the microwave frequency was 9.47 GHz, microwave power 24 mW, modulation frequency 100 kHz, and modulation intensity 1 mT.

As the temperature was lowered, the \(g_x\) and \(g_z\) absorbance of centre I were increasingly obscured by other signals. The \(g_x\) absorbance decreased as the temperature was lowered to 30 K, below which temperature it was also obscured by other signals. This decrease in signal height on lowering the temperature is due to a phenomenon called saturation. On lowering the temperature, the rate at which the photon-excited state can relax back to the ground state decreases and there comes a point at which the relaxation rate cannot keep up with the excitation rate (the rate of photon absorption), so as a consequence the latter has to decrease. This saturation phenomenon is thus dependent on the intrinsic relaxation rate of the species under investigation, the temperature, and the photon flux density (microwave power). As a result of this process, each centre will have its own temperature profile, with the signal height increasing with decreasing temperature to a temperature below which the signal height decreases.

On lowering the temperature from 150 K, a second species was increasingly observed at 100 K and 50 K, overlapping with centre I. This centre appears to have slight rhombic distortion with \(g_x\) centred at 1.915 and \(g_z\) at 2.022. These resonances increased in amplitude as the temperature was lowered further: in the case of the dithionite-reduced sample (Fig. 1a), saturation did not occur until temperatures below 10 K were obtained, whereas in the substrate-reduced sample, saturation was observed below 30 K. The component that is not reduced by
substrate but is reduced by dithionite has a relatively rapid relaxation rate (i.e. is most prominent at very low temperatures) which also enables the component to be distinguished in the redox titrations (see later). The centres described thus far will be referred to as IIa, b, c on the basis of resolution into three components by redox titration (IIa having the highest mid-point potential and IIc the lowest).

Below 30 K, two further sets of signals were observed. The resonances are paired on the basis of their similar temperature profiles and redox behaviour (see later) and will be referred to as ferrodoxin centres 3 (g = 2.045, 1.89) and 4 (g = 2.07, 1.875).

In addition to these ferrodoxin-type iron–sulphur centres, signals in the g = 1.98 to 1.96 region were observed. These signals were relatively larger in the substrate-reduced sample. The signals in this region are due to at least two species with different relaxation times and redox behaviour (Figs 1 and 2). These signals and their possible implications are discussed later.

Oxidation–reduction titrations of EPR signals

The results of a redox titration are shown in Fig. 2. Samples were examined at different temperatures to aid the resolution of components. At 150 K, a single ferrodoxin-like component
is seen, centre I (Fig 2a). The curve drawn for this component is a theoretical \( n = 1 \) curve and this gave the best statistical fit (Table 1). Also seen at 150 K is a \( g = 1.978 \) signal, titrating in a bell-shaped manner; the mid-points of the two half-reactions are \(-20 \text{ mV} \) and \(-270 \text{ mV} \), each with an \( n \)-value of 1. This signal, observed at relatively high temperatures, has a different temperature profile and different mid-point potentials to a \( g = 1.978 \) signal which was observed at low temperatures (see below). The signal seen at 150 K is referred to as the ‘slow’ \( g = 1.978 \) signal as its temperature dependence shows that it has the slower relaxation rate of the two \( 1.978 \) absorbing centres.

When the redox titration samples were observed at 25 K a different profile was observed (Fig. 2b): the \( g = 1.978 \) signal was less prominent (in Fig. 2 the signal units are arbitrary) and titrated differently than at 150 K. Centre I could not be distinguished. At 25 K, ferredoxins with \( g_s = 1.915 \) (not shown in Fig. 2), \( g_s = 1.927 \) and \( g_s = 2.02 \) (Fig. 1) were observed titrating as multiple centres (centres IIa, b, c). These ferredoxins were resolved better at 12.5 K (Fig. 2c).

Three centre II components were resolved by redox titration, having \( E_m,\gamma \) values of IIa \(-50 \text{ mV} \), IIb \(-244 \text{ mV} \) and IIc \(-340 \text{ mV} \); best fits to the points were obtained with theoretical curves having \( n \)-values of 1. A comparison of the plots at 25 K and 12.5 K revealed that the lower potential components had more rapid relaxation rates than the higher potential component.

These observations correlate with the spectra in Fig. 1 to indicate that it is the lowest potential component which is not substrate-reducible. The \( g = 1.89 \) (centre III) and \( g = 1.875 \) (centre IV) components (Fig. 2c) titrated with \( E_m,\gamma \) values of \(-150 \text{ mV} \) and \(-250 \text{ mV} \), respectively (both these centres being reducible by substrate). The ‘fast’ (low temperature) \( g = 1.978 \) signal also gave a ‘bell-shaped’ titration curve, but with mid-points at approximately \(+50 \text{ mV} \) and \(-100 \text{ mV} \).

When the samples were observed at 6 K (Fig. 2d), the \( g = 1.89 \) and 1.875 signals gave a slightly different redox titration profile with apparent \( E_m,\gamma \) values of approximately \(-290 \text{ mV} \).

It is, however, impossible to say whether these differences are due to spectral distortion caused by overlapping (or interacting) components or are new species (centres V and VI?).

### The EPR signals of oxidized ETP

Shown in Fig. 3 are EPR spectra, taken at different temperatures, of oxidized ETP. A prominent peak occurs at \( g = 2.023 \) to 2.017 and troughs from \( g = 2.011 \) to 1.976. The peak at \( g = 2.023 \) appears to pair with the trough at 2.011, giving a high potential iron–sulphur protein (HiPIP)-like signal similar to that of mitochondrial succinate dehydrogenase (Ohnishi et al., 1976a). The designation ‘HiPIP-like’ is based on (1) the centre being paramagnetic in the oxidized state and (2) its spectral similarity to the HiPIP signal of succinate dehydrogenase.

This centre is most clearly discerned in spectra taken above 15 K (Fig. 3) where the broad trough at \( g = 1.976 \) and the overlapping peak at \( g = 2.017 \) are no longer so prominent. The temperature-dependencies of these peaks and troughs confirm the presence of at least two distinct centres, one of which has a peak at \( g = 2.017 \) and a trough at \( g = 2.011 \), and the other with a peak at \( g = 2.017 \) and a trough at about \( g = 1.976 \); the exact position of this latter trough changes with temperature. The temperature-dependencies in Fig. 3 are complicated by the fact that the \( g = 2.017 \) and \( g = 2.073 \) signals extensively overlap. These signals titrate potentiometrically with mid-point potentials of approximately \(+120 \text{ mV} \) (results not shown).

#### Redox poise of EPR-detectable components in the steady state

The redox poise of the centres during steady state oxidation of NADH, succinate or lactate by oxygen was studied. In all cases, the ‘slow’ \( g = 1.978 \) signal was detected, indicating some partial reduction of this component as the centre is only observed at an intermediate redox state.

The amplitude of the \( g = 1.978 \) signal was similar in the samples oxidizing NADH or lactate, but in the presence of succinate the signal approximately doubled in amplitude, reaching almost maximum amplitude for this centre. As the \( g = 1.978 \) signal is due to an intermediate redox state, it cannot be said whether the increased amplitude during succinate oxidation is due to greater oxidation or reduction than with other substrates.
During NADH oxidation, only centre I of the ferredoxins was extensively reduced (Fig. 4a, b). The temperature profile of the \(g = 1.94\) signal (Fig. 4a) is typical of a single homogeneous component. This is confirmed in Fig. 4b where a microwave power profile over a saturating range shows that a single component contributes to the \(g = 1.94\) signal. Analysis of the redox poise of the ferredoxins during succinate and lactate oxidation is more complex, as more than one centre was reduced (Fig. 4a). In the case of membranes oxidizing lactate, the profile indicates partial reduction of centre I (approximately 50%) and some reduction of a second centre with a more rapid relaxation state (probably centre IIa, see Figs 1 and 2). During the steady state oxidation of succinate, at least three different \(g = 1.94\) ferredoxins are partially reduced; by comparisons of line-shapes and temperature dependencies these are centre I and probably centres IIa and b. Although these samples were freeze-trapped and then stored in liquid \(N_2\) until used, it is possible that intracomplex electron transfer could continue to occur under these conditions.

**Conclusions**

The characteristics of the centres resolved are compiled in Table 1. The picture is complex, perhaps not surprisingly so, as these membranes contain hydrogenase, formate dehydrogenase, fumarate reductase and succinate dehydrogenase. In other electron transport chains examples are known of these dehydrogenases which contain EPR-detectable iron–sulphur centres (Yoch & Carithers, 1979). There is no indication that the almost ubiquitous Rieske iron–sulphur centre is present in these membranes; this may correlate with the absence of a cytochrome \(bc\) (or \(bf\)) complex in *E. coli* with which the Rieske centre is associated in other systems. Three of the iron–
Fig. 4. An analysis by temperature and microwave power profiles of the ferredoxins reduced under aerobic steady state conditions. (a) Temperature dependence of the 'g = 1.94' signal of the samples freeze-trapped in the aerobic steady state during oxidation of NADH (●), succinate (△) or lactate (○). (b) Power saturation profile at 12 K of the single 'g = 1.94' signal observed during steady state oxidation of NADH. EPR conditions were as for Fig. 1. Protein concentration was 52 mg ml⁻¹. Substrate concentrations were: lactate, 10 mM; succinate, 10 mM; and NADH, 6 mM. A small quantity of hydrogen peroxide was added to prolong aerobiosis long enough to freeze-trap the samples.

Table 1. Characteristics of resolved iron–sulphur centres

<table>
<thead>
<tr>
<th>Centre*</th>
<th>g-value</th>
<th>$E_{m,\gamma}$ (mV)</th>
<th>n-value</th>
<th>Optimum temperature (K)</th>
<th>Possible source</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>2-037</td>
<td>1.937 – 1.923</td>
<td>20</td>
<td>150</td>
<td>Succinate dehydrogenase</td>
</tr>
<tr>
<td>IIa</td>
<td>2-022</td>
<td>1.927</td>
<td>1</td>
<td>30</td>
<td>Succinate dehydrogenase†</td>
</tr>
<tr>
<td>IIb</td>
<td>2-022</td>
<td>1.927</td>
<td>1</td>
<td>10</td>
<td>Succinate dehydrogenase</td>
</tr>
<tr>
<td>IIc</td>
<td>2-022</td>
<td>1.927</td>
<td>1</td>
<td>10</td>
<td>Succinate dehydrogenase</td>
</tr>
<tr>
<td>III</td>
<td>2-045</td>
<td>1.89</td>
<td>1</td>
<td>12</td>
<td>Succinate dehydrogenase</td>
</tr>
<tr>
<td>IV</td>
<td>2-07</td>
<td>1.875</td>
<td>1</td>
<td>12</td>
<td>Succinate dehydrogenase</td>
</tr>
<tr>
<td>V?</td>
<td>1.89</td>
<td>–290</td>
<td>1</td>
<td>6</td>
<td>Succinate dehydrogenase</td>
</tr>
<tr>
<td>VI?</td>
<td>1.875</td>
<td>290</td>
<td>1</td>
<td>6</td>
<td>Succinate dehydrogenase</td>
</tr>
<tr>
<td>HiPIPb</td>
<td>2-037</td>
<td>2-017</td>
<td>120</td>
<td>20</td>
<td>Succinate dehydrogenase</td>
</tr>
<tr>
<td>HiPf</td>
<td>2-017</td>
<td>1-976</td>
<td>120</td>
<td>9</td>
<td>Formate dehydrogenase Mo (V)</td>
</tr>
<tr>
<td>‘Slow’ 1-978</td>
<td>1-978</td>
<td>20</td>
<td>1</td>
<td>100</td>
<td>Formate dehydrogenase Mo (V)</td>
</tr>
<tr>
<td>‘Fast’ 1-978</td>
<td>1-978</td>
<td>–50</td>
<td>&lt;1</td>
<td>9</td>
<td>Formate dehydrogenase Mo (V)</td>
</tr>
</tbody>
</table>

---, n-value not adequately resolved.
* Centres I–VI are paramagnetic in the reduced form and are referred to as ferredoxin-like. The name HiPIP is used only to indicate that the centre is paramagnetic and therefore EPR-detectable in its oxidized form.
† Not substrate-reducible.

The sulphur centres detected can be tentatively assigned to the succinate dehydrogenase on the basis of similarities between their properties and those described for succinate dehydrogenase centres from a variety of sources (e.g. Ohnishi et al., 1976b, Ingledew & Prince, 1977); these centres are IIa, IIc and the HiPIPb centre (see Table 1).
The source of the $g = 1.978$ signals is most probably molybdenum in its pentavalent state, because of similarities between the redox behaviour of the centre giving rise to the signal, and its $g$-value, to the behaviour reported for other molybdenum centres (Barber et al., 1977; Bray, 1975). Furthermore, growth experiments show the dependence of the signal on molybdenum in the growth medium (W. J. Ingledew, unpublished results). Molybdenum is known to be a constituent of two $E. coli$ oxido-reductases: the nitrate reductase (absent in membranes of glycerol fumarate-grown cells) and formate dehydrogenase (present in membranes of glycerol fumarate-grown cells) (Haddock & Mandrand-Berthelot, 1982; Boxer et al., 1982). Consequently, it is possible that the signals observed come specifically from the molybdenum centre of formate dehydrogenase. This requires confirmation.

The complex picture represented by Table 1 should clarify as more becomes known about the individual dehydrogenases. Some of these dehydrogenases have been cloned and amplified (Cole & Guest, 1982); this will assist in future studies, both in the resolution of the redox centres and in specifically determining their sidedness and orientation in the membrane.

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