Volume 319, number 1,2, 151–154 FEBS 12233 March 1993

Distinct forms of the haem *o*-Cu binuclear site of oxidised cytochrome *bo* from *Escherichia coli*

Evidence from optical and EPR spectroscopy

Nicholas J. Watmough[a,b], Myles R. Cheesman[a], Robert B. Gennis[c], Colin Greenwood[b] and Andrew J. Thomson[a]

Centre for Metalloprotein Spectroscopy and Biology, School of Chemical Sciences[a] and School of Biological Sciences[b], University of East Anglia, Norwich NR4 7TJ, UK and *[c]Department of Biochemistry, University of Illinois, Urbana, IL 61801, USA*

Received 4 February 1993

Oxidised, formate-bound and fluoride-bound forms of *E. coli* cytochrome *bo* give rise to an electronic absorption band near 630 nm, diagnostic of high-spin ferric haem *o*, whose position is sensitive to the nature of the bound anion. In all three forms, haem *o* remains spin-coupled to Cu_B(II), resulting in distinct broad X-band EPR signals. Those of formate-bound cytochrome *bo* are similar to the signals seen in slow cytochrome aa_3 but cannot be induced by incubation at acid pH suggesting that the endogenous carboxylate believed to be important in slow cytochrome aa_3 is not present in cytochrome *bo*. The oxidised form gives rise to novel EPR signals at g = 3.74 and g = 3.08 which have not been detected in cytochrome aa_3 and may arise from a weak magnetic coupling between high-spin haem *o*, S = 5/2, and Cu_B(II), S = 1/2.

Cytochrome *bo*; Cytochrome c oxidase; EPR

1. INTRODUCTION

Cytochrome *bo*, one of two terminal ubiquinol oxidases of *E. coli*, consists of four subunits of which I contains the three redox-active metal sites; one low-spin protohaem IX group liganded by two histidine residues and one haem *o* magnetically coupled to a copper ion, Cu_B, at the site of oxygen reduction [1–3]. There is considerable sequence homology between this subunit and subunit I of mitochondrial cytochrome aa_3 [4], which also contains one low-spin haem and a haem-copper site. It remains to be discovered how functionally similar the binuclear oxygen reduction site is in cytochrome *bo* and cytochrome aa_3. This paper examines some aspects of this comparison for the oxidised form of the two enzymes.

Oxidised mitochondrial cytochrome aa_3 can exist as a mixture of conformers called fast and slow because the rate of reaction with exogenous anionic ligands, such as F^-, CN^-, $HCOO^-$, varies [5–9]. When isolated under mildly alkaline conditions a fast conformer predominates. A slow form dominates in preparations exposed to low pH and is characterised by a blue-shifted Soret maximum and broad X-band EPR signals centred close to g = 12 and g = 2.95 [6–8,10–12]. Conversion of fast to slow forms may be effected either by lowering the pH of the enzyme or by the addition of exogenous formate [6,13–16].

In the fast form of cytochrome aa_3 the magnetic coupling of Cu_B(II) and ferric haem a_3 results in a state which is EPR silent [17]. The magnetic state of haem a_3 within this coupled site has been deduced from susceptibility studies [18–20] and Mössbauer spectroscopy [21–22] to be high-spin ferric anti-ferromagnetically coupled to CuB(II) yielding a spin = 2 state. In the slow form, the coupling between ferric haem a_3 and Cu_B(II) also leads to an S = 2 state [23], but magnetic coupling and zero-field-splitting parameters are quantitatively different to those of the fast enzyme and result in the observation of the g = 12 and g = 2.95 signals [18–20,22].

Results are presented here which indicate that the binuclear centre of cytochrome *bo* can also exist in several different forms some of which are similar to those already described for cytochrome aa_3.

2. MATERIALS AND METHODS

Cytochrome *bo* was isolated from RG145, a previously described strain of *E. coli* which overexpresses cytochrome *bo* [24]. For spectroscopic experiments all protein samples were exchanged into 50 mM HEPES, 0.2% (w/v) octyl-β-D-glucopyranoside pH = 7.5. Enzyme concentrations were determined optically using e_{406} = 183 $mM^{-1}cm^{-1}$ [25] and verified by integration of the g = 2.98 EPR feature of haem

Correspondence address: M.R. Cheesman, School of Chemical Sciences, University of East Anglia, Norwich NR4 7TJ, UK. Fax: (44) (603) 259 396.

Published by Elsevier Science Publishers B.V.

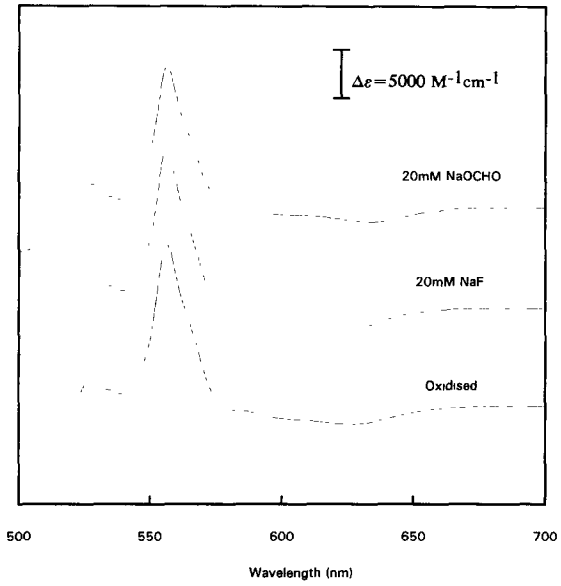

Fig. 1. Reduced minus oxidised difference electronic absortion spectra for unliganded, fluoride-bound and formate-bound forms of oxidised cytochrome *bo*. 15 μM cytochrome *bo* in 50 mM HEPES/0.2% OGP pH 7.5 was incubated at room temperature for two hours either in the absence of ligands or in presence of sodium formate (20 mM) or sodium fluoride (20 mM). Spectra were recorded before and after addition of excess sodium dithionite.

b against copper(II) EDTA standards using the method of Aasa and Vängärd [26].

Sodium fluoride was purchased from Aldrich. HEPES, sodium formate, and octyl-β-D-glucopyranoside were purchased from Sigma. The formate and fluoride derivatives of cytochrome *bo* were prepared by addition of 1 M solutions of their sodium salts to give a final ligand concentration of 20 mM. Binding was monitored by electronic absorption spectroscopy. When no further optical changes were observed, samples were frozen for EPR

Absorption spectra were recorded on a Hitachi U3200 or an Aminco DW2000C spectrophotometer. Wavelengths are quoted to within 0.5 nm. EPR spectra were recorded on an X-band ER-200D spectrometer (Bruker Spectrospin) interfaced to an ESP1600 computer and fitted with a liquid helium flow cryostat (ESR-9; Oxford Instruments).

3. RESULTS

Oxidised cytochrome *bo* when isolated by previously described methods [25] has a Soret band at 406.5 nm, maxima in the a,b region at 529 nm, 558 nm and a broad feature at 630 nm. Porphyrin *o* has UV-visible spectroscopic properties comparable to those of protoporphyrin [27] and thus the band near 630 nm is indicative of the presence of high-spin ferric haem *o* [28].

If oxidised cytochrome *bo* is incubated with 20 mM sodium formate or 20 mM sodium fluoride, the Soret absorption maximum undergoes a small blue-shift to 406.0 nm or 405.0 nm, respectively (not shown). Changes in the band near 630 nm also occur. These are illustrated in Fig. 1, which shows the reduced-oxidised optical difference spectra for oxidised cytochrome *bo* and the two derivatives. A distinct trough represents the disappearance of the band near 630 nm. Reduction of all three forms results in identical absolute absorbance spectra. The position of the trough is therefore a reflection of differences in the '630 nm' band in the three oxidised forms. It occurs at 625 nm for the oxidised enzyme but is shifted to 634 nm and 618 nm for the formate and fluoride derivatives respectively.

The X-band EPR spectra of these three forms of cytochrome *bo* are shown in Fig. 2. The features at g = 2.98, 2.26 and 1.50 persist in all forms of cytochrome *bo* discussed in this work and are due to low-spin ferric heme *b* [2].

In addition to these signals, the EPR spectrum of oxidised cytochrome *bo* shows unusual broad features at g = 3.74 and g = 3.08 which are less easily power saturated than those of heme *b*. These dominate the spectrum at temperatures of ≤ 10K and powers in excess of 100 mW. This property is typical of signals aris-

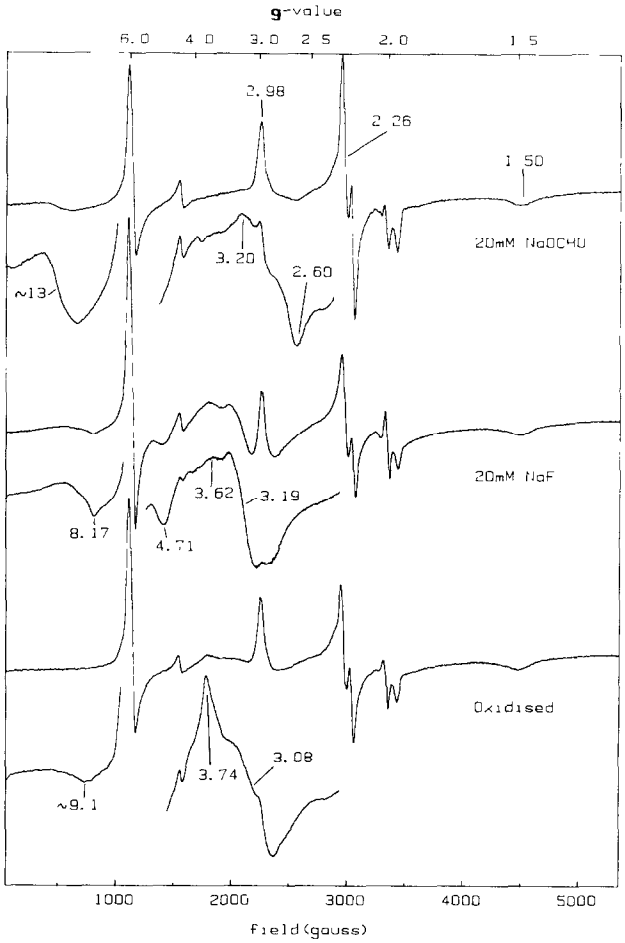

Fig. 2 X-band EPR spectra of oxidised, fluoride-bound and formate-bound forms of cytochrome *bo* at 10K using 2.03 mW microwave power. Shown below each is the spectrum of the same sample recorded at 5K using 203 mW microwave power. Operating conditions: modulation amplitude, 10G; microwave frequency, 9.39 GHz. Concentrations of cytochrome *bo* were 165 μM (oxidised), 190 μM (NaF) and 227 μM (NaOCHO).

ing from integral-spin (non-Kramers) systems. No change in the form of these signals nor the corresponding absorption spectrum is observed on changing the pH of the buffer from 7.5 to 6.5. These broad signals and the '630 nm' band are abolished by addition of sodium cyanide, hydrogen peroxide or reductants such as sodium dithionite, sodium ascorbate or NADH (not shown). When reduced cytochrome *bo* is reoxidised by stirring in air, the Soret maximum is again at 406.5 nm and both the 630 nm band and the broad EPR signals return.

The X-band EPR spectrum of formate-bound cytochrome *bo* shows a broad derivative feature at g = 13 and the features labelled g = 2.60 and g = 3.20 which appear to be the extrema of another derivative signal centred near g = 2.8 and underlying the g_z = 2.98 signal of the low-spin heme *b*.

The EPR spectrum of the fluoride derivative shows the appearance between 0 and 3000G of complicated broad signals with power saturation characteristics comparable to those of the g = 3.74 and g = 13 signals of the oxidised and formate-bound forms of the enzyme. The significant features at g = 8.17, 4.71, 3.62 and 3.19 are analogous to those observed when fluoride is added to cytochrome aa_3 [7,9].

4. DISCUSSION

Using EPR and UV-visible absorbance spectroscopy it can be shown that the binuclear site of cytochrome *bo* can exist in three distinct electronic states which appear to be counterparts of forms of the binuclear site in cytochrome aa_3.

The formate-induced form of cytochrome *bo* shows the most direct correlation, with slow cytochrome aa_3. The EPR signals from slow cytochrome aa_3 at g = 12 and g = 2.95 correspond to features at g = 13 and g = 3.20 in the spectrum of cytochrome *bo* with formate. The EPR features observed here are similar to those seen near g = 12 and g = 2.9 when cytochrome *bo*-containing membranes were treated with formate [29]. In contrast to the behaviour of cytochrome oxidase, these signals are not observed in our native preparations of cytochrome *bo*. Neither have we been able to induce them by prolonged incubation of the enzyme at pH = 6.5.

Although the formate derivatives of the binuclear sites of the two enzymes are similar by EPR spectroscopy, cytochrome *bo* appears unable to adopt the g = 13 configuration without exogenous formate ion, suggesting that the endogenous carboxylate believed to be capable of effecting the equivalent transition in cytochrome aa_3 is absent in cytochrome *bo*. There are 14 carboxylate residues in the primary amino acid sequence of cytochrome aa_3 subunit I which are not conserved in cytochrome *bo* [4].

Cooper and Salerno [12] proposed that the g = 12 and g = 2.95 signals could arise from the highest doublet and lowest pair of states respectively of a spin = 2 multiplet with zero-field-splitting parameters of D = 1.0 cm^{-1} and E/D = 0.28. These values of D and E are slight modifications of those originally proposed by Hagen [23] on the evidence of the g = 12 signal alone. The resonance position of such signals is extremely sensitive to small changes in these parameters. Using the same spin-Hamiltonian with D = 1.0 cm^{-1}, changes in E/D of the order of 0.01 would be sufficient to alter the resonance fields to those observed for formate-treated cytochrome *bo*.

No form of cytochrome aa_3 is known to give rise to EPR signals comparable to those we have observed at g = 3.74 for oxidised cytochrome *bo*. The Soret maximum at 406.5 nm and the presence of the 630 nm band indicate that the ferric haem *o* is in the high-spin form. Since the EPR signals are not those of an isolated high-spin ferric haem, it can be assumed that they arise from the binuclear site in which the haem *o* is spin coupled to the copper(II) ion. In the limit of strong anti-ferromagnetic coupling, an S = 2 quintet results. If the axial-zero-field splitting parameter D is greater than the Zeeman energy and the rhombic parameter E is close to zero, the doublets $m_s = \pm 2$ and ± 1 have g-values of ≥ 8 and ≥ 4, respectively. However, at low values of E the the transition intensity in perpendicular mode EPR will be weak. The g = 3.74 signal is therefore unlikely to arise from such transitions. As discussed above, the g = 2.95 EPR signal of slow cytochrome aa_3 may arise from a transition between the lowest two components of an S = 2 quintet with a positive D-value [12]. The g = 3.74 signal too may have such an origin. However, both cases suffer from the criticism that a D-value of + 1 cm^{-1} is significantly smaller than that expected to arise from high-spin ferric heme. For example, metmyoglobin-fluoride has a D-value of $\approx 7 cm^{-1}$ [30]. As discussed by Salerno and Cooper [12], an alternative explanation may involve a spin coupling between high-spin ferric haem *o* and Cu(II) of magnitude $J << D$. This can result in states sufficiently close in energy to permit EPR transitions while maintaining a larger local D-value at the haem. We are at present studying the g = 3.74 signal at Q-band and in parallel mode EPR spectroscopy in order to investigate this possibility.

This weak-coupling scheme has been suggested as an explanation of the Mössbauer properties of the analogous binuclear site of cytochrome $\mathbf{c_1}aa_3$ from *Thermus thermophilus* [31]. Interestingly, this oxidase also exhibits broad intense EPR signals between g = 3 and g = 4, assigned to cytochrome $\mathbf{c_1}$. But it is not clear that the intensity is sufficient to account for only one low-spin ferric haem [32]. It should be noted that the cytochrome $\mathbf{c_1aa_3}$ Mössbauer results could also be rationalised on the basis of a strong coupling scheme [31] comparable to those used to explain Mössbauer and magnetic susceptibility data for fast cytochrome aa_3 [18–22]. There-

Table I

A comparison of oxidised forms of the binuclear site in cytochrome aa_3 and cytochrome *bo*

Cytochrome aa_3	Cytochrome *bo*
fast	oxidised
λ_{Soret} = 424 nm	λ_{Soret} = 406.5 nm
EPR: silent	EPR: g = 3.74, 3.08
slow	formate-bound
λ_{Soret} = 417 nm	λ_{Soret} = 406.0 nm
EPR: g = 12, 2.95	EPR: g = 13, 3.20/2.60
fluoride-bound	fluoride-bound
λ_{Soret} = 416 nm	λ_{Soret} = 405.0 nm
EPR: g = 8.5, 5, 3.20	EPR: g = 8.17, 4.71, 3.19

fore it cannot be excluded that oxidised cytochrome *bo* represents a form of the binuclear site analogous to that of fast cytochrome aa_3. We also note however that a species, transiently observed within milliseconds of reoxidation of reduced cytochrome oxidase, exhibits a derivative EPR signal near g = 5 [33]. Until such a pulsed form of cytochrome *bo* can be identified, it is possible that the g = 3.7 EPR signal represents a stable equivalent of the g = 5 species.

The broad EPR signals arising from the fluoride derivative of cytochrome *bo* are comparable to a set at g = 8.5, 5 and 3.2 which were observed following addition of fluoride to cytochrome aa_3 [7,9]. Rapid fluoride binding was reported to be a property of the redox-cycled form of cytochrome aa_3 [9]. Similarly, the oxidised (406.5 nm) form of cytochrome *bo* binds fluoride rapidly and is the form obtained after redox-cycling of the enzyme.

The binuclear site of cytochrome *bo* exists in three distinct forms which involve high-spin ferric haem *o* spin-coupled to Cu_B(II). The EPR signatures of these forms link them to known states of the binuclear site of cytochrome aa_3. These comparisons are summarised in Table I. The differing properties of O-type haem as compared to A-type haem allow the presence of high-spin ferric haem to be easily recognised using optical spectroscopy. The '630 nm' band is sensitive to the binding of different high-spin ligands but is abolished, along with the broad EPR signals, by the addition of cyanide, peroxide or dithionite which results in either a spin or oxidation state change at haem *o*.

Acknowledgements. Support of this work by the SERC Molecular Recognition Initiative and TRAMPS initiative is gratefully acknowledged.

REFERENCES

[1] Wikstrom, M., Krab, K. and Saraste, M. (1981) Cytochrome Oxidase, A Synthesis, Academic Press.
[2] Salerno, J.C., Bolgiano, B. and Ingledew, W.J (1989) FEBS Lett. 247, 101–105.
[3] Salerno, J.C., Bolgiano, B., Poole, R.K., Gennis, R.B. and Ingledew, W.J. (1990) J. Biol. Chem. 265, 4364–4368.
[4] Chepuri, V., Lemieux, L., Au, D.C. and Gennis, R.B. (1990) J Biol. Chem. 265, 11185–11192.
[5] Baker, G.M. and Palmer, G. (1987) Biochemistry 26, 3038–3044.
[6] Moody, A.J., Cooper, C.E. and Rich, P.R. (1991) Biochim. Biophys Acta 1059, 189–207.
[7] Brudwig, G.W., Stevens, T.H., Morse, R.H. and Chan, S I (1981) Biochemistry 20, 3912–3921.
[8] Palmer, G., Baker, G.M. and Noguchi, M. (1988) Chem Scripta 28A, 41–46.
[9] Young, L.J. (1988) Biochemistry 27, 5115–5121.
[10] Beinert, H. and Shaw, R.W. (1977) Biochim. Biophys. Acta 462, 121–130.
[11] Greenaway, F.T., Chan, S.H P. and Vincow, G. (1977) Biochim. Biophys. Acta 490, 62–68.
[12] Cooper, C.E. and Salerno, J.C. (1992) J. Biol. Chem. 267, 280–285.
[13] Schoonover, J.R. and Palmer, G. (1991) Biochemistry 30, 7541–7550
[14] Baker, G.M., Noguchi, M. and Palmer, G. (1987) J. Biol. Chem. 262, 595–604.
[15] Boelens, R. and Wever, R. (1979) Biochim. Biophys. Acta 547, 296–310.
[16] Keyhani, J. and Keyhani, E. (1980) Biochem. Biophys. Res. Commun. 92, 327–333.
[17] Van Gelder, B F. and Beinert, H. (1969) Biochim. Biophys. Acta 189, 1 24.
[18] Tweedle, M.F., Wilson, L.J., García-Iñguez, L., Babcock, G.T. and Palmer, G. (1978) J. Biol. Chem 253, 8065–8071
[19] Moss, T.H., Shapiro, E., King, T.E., Beinert, H and Hartzell, C. (1978) J. Biol. Chem. 253, 8072–8073.
[20] Day, E.D., Peterson, J., Schoonover, J.R. and Palmer, G. (1989) J Inorg. Biochem. 36, 266.
[21] Kent, T.A., Young, L.J., Palmer, G., Fee, J.A. and Münck, E. (1983) J. Biol. Chem. 258, 8543–8546.
[22] Barnes, Z.K., Babcock, G.T and Dye, J.L. (1991) Biochemistry 30, 7597–7603.
[23] Hagen, W.R. (1982) Biochim. Biophys. Acta 708, 82–98.
[24] Au, D.C. and Gennis, R.B. (1987) J. Bacteriol. 169, 3237–3242
[25] Cheesman, M.R., Watmough, N.J., Pires, C.A., Turner, R , Brittain, T , Gennis, R.B., Greenwood, C, and Thomson, A.J. (1993) Biochem. J 289, 709–718.
[26] Aasa, R. and Vänngärd, T (1975) J. Magn. Reson. 19, 308–315
[27] Wu, W., Chang, C K., Varotsis, C., Babcock, G.T., Puustinen, A. and Wikström, M. (1992) J. Am. Chem. Soc. 114, 1182–1187.
[28] Cheng, J.C., Osborne, G.A , Stephens, P.J and Eaton, W A. (1973) Nature 241, 193–194.
[29] Calhoun, M.W., Gennis, R.B. and Salerno, J.C. (1992) FEBS Lett 309, 127–129.
[30] Feher, G. (1970) Electron Paramagnetic Resonance with Applications to Selected Problems in Biology, Gordon and Beach, New York.
[31] Rusnack, F M., Münck, E., Nitsche, C.I., Zimmermann, B.H. and Fee, J.A. (1987) J. Biol. Chem. 262, 16328–16332.
[32] Fee, J.A., Choc, M.G., Findling, K.L., Lorence, R. and Yoshida, T (1980) Proc. Natl. Acad. Sci. USA 77, 147–151.
[33] Shaw, R.W , Hansen, R.E. and Beinert, H. (1978) J. Biol Chem. 253, 6637–6640