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The formate complex of the cytochrome *bo* quinol oxidase of *Escherichia coli* exhibits a 'g = 12' EPR feature analogous to that of 'slow' cytochrome oxidase

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The cytochrome *bo* quinol oxidase of *Escherichia coli* is homologous in sequence and in structure to cytochrome aa_3 type cytochrome oxidase in subunit I, which contains the catalytic core. The cytochrome *bo* enzyme forms a formate complex which exhibits 'g = 12' and 'g = 2.9' EPR signals at X band; similar signals have previously been observed only in association with the 'slow' and formate-ligated states of cytochrome oxidase. These signals arise from transitions within integral spin multiplets identified with the homologous heme-copper binuclear catalytic centers in both enzymes.

Quinol oxidase; Cytochrome *bo*; Formate complex; EPR; *E. coli*

1. INTRODUCTION

Many preparations of cytochrome oxidase contain a significant proportion of the slow or resting state, a form of the enzyme which reacts sluggishly with added ligands but which can be converted to a more reactive form by cycling with reducing equivalents [1–3]. Slow enzyme is characterized by a blue shifted Soret band (near 417 nm) at neutral and mildly acidic pH [2]. An unusual electron paramagnetic resonance spectrum with a feature near g = 12 at X band, apparently arising from an excited doublet of an integral spin system, is associated with the slow state [2].

Detergent-solubilized cytochrome oxidase can be converted quantitatively to the slow form by incubation at slightly acid pH. Formate binding by cytochrome oxidase produces a state which mimics the slow enzyme both in reactivity to added ligands and in spectroscopic features [4,5]. In particular, binding of formate can fully elicit the g = 12 feature from preparations in which the fast form otherwise predominates. Schoonover and Palmer [5] have recently demonstrated that formate complexes resembling slow oxidase can be formed in intact mitochondrial membranes; this demonstrates that formate binding can produce such states in even the most intact preparations.

Cytochrome *bo*, one of two ubiquinol oxidases from *Escherichia coli*, is closely related to cytochrome aa_3 type terminal oxidases. Considerable sequence homology exists [6] and both enzymes contain heme–copper binuclear catalytic sites [7,8]. The *E. coli* enzyme has been much less extensively studied than cytochrome aa_3. While no evidence for a slow form of any quinol oxidase exists, binding of formate to cytochrome *bo* produces optical changes and slows the binding of other ligands. Analogous changes in the EPR spectra have not been reported; it is of interest to determine whether the *E. coli* forms spectroscopically similar states.

2. MATERIALS AND METHODS

E. coli strain RG145, which contains elevated levels of the cytochrome *bo* complex but lacks the cytochrome *bd* complex, was grown in a rich buffered medium as described previously [9,10]. Membranes were prepared as described by Lemieux et al. [11]. Briefly, cells were passed through a French pressure cell generating high shear forces, and membranes were collected by ultracentrifugation. Membranes were washed once with 50 mM TES, pH 7.0, 2 mM EDTA, and were resuspended in the same buffer at approximately 40 mg protein/ml.

Cytochrome oxidase was prepared by the method of Yu [12] with minor modifications introduced to reduce the exposure to cholate and to slightly raise the pH during the purification. This resulted in a reduced proportion of slow enzyme in the preparation, although ~20% of the enzyme as prepared was still in the slow form by spectroscopic criteria [2]. Samples were prepared by incubating membranes or purified enzymes with formate at 50 mM or 20 mM, transferring to a quartz EPR sample tube and rapidly freezing in cold isopentane/cyclohexane (5:1 v/v). Electron spin resonance spectra were recorded using a Bruker ER300 spectrometer equipped with an Oxford Instruments cryostat cooled with flowing liquid helium.

Abbreviations: TES, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid; EDTA, ethylenediamine tetraacetic acid; EPR, electron paramagnetic resonance.

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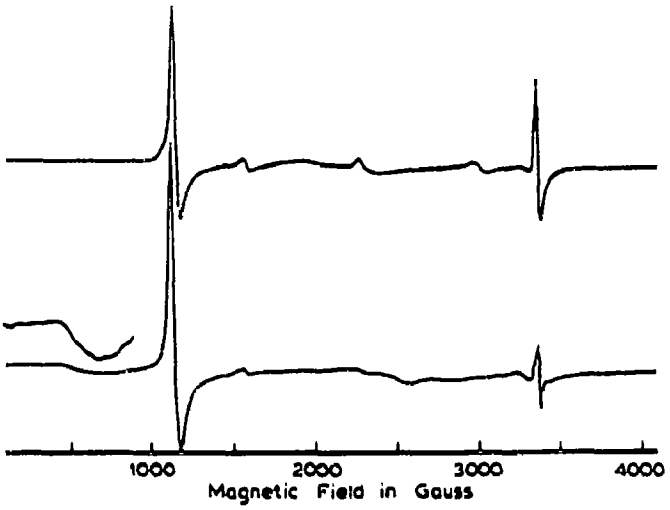

Fig. 1. EPR spectrum of membranes from *E. coli* strain RG145, amplified in cytochrome *bo*. A. As isolated with no additions. B. Incubated with 20 mM formate; inset, $g' = 12$ region with gain increased by a factor of 4. EPR conditions were: temperature, 13K; microwave power, 20 mW; microwave frequency, 9.44 GHz; modulation amplitude, 20 G; field sweep, 0.4 mT; center field position, 0.21 mT; time constant, 163.84 ms; digital conversion interval, 327.68 ms; resolution, 1024 field points; sweep time, 335.54 s. 1 G = 10^{-4} tesla (T).

3. RESULTS

Membranes of strain RG145, prepared as described above, were highly amplified in cytochrome *bo* and contained no cytochrome *bd*. The electron paramagnetic resonance spectrum of the membranes as isolated is shown in Fig. 1; signals from the low spin ferric heme are visible at $g = 2.98$ and $g = 2.24$. A signal at $g = 6$ arises from the high spin heme site. The relatively small size of the $g = 6$ signal is due to the tight coupling between heme and copper in the binuclear center; when both metals are oxidized, the coupling leads to an EPR silent integral spin state. The $g = 6$ signals observed represent a minority population in which the copper is either in the cuprous state or absent. These signals are greatly increased at intermediate potentials, reaching a maximum at about 300 mV.

Fig. 1B shows the effects of formate addition on the EPR spectrum of cytochrome *bo*. An additional signal at $g = 12$ is visible. For purposes of comparison, the EPR spectrum of cytochrome aa_3 oxidase in the presence of 20 mM formate is shown in Fig. 2. An almost identical signal at $g = 12$ is evident. In both Fig. 1B and Fig. 2 a broad signal at $g = 2.9$ is seen which is overlapped with the g_x feature of the low spin heme near $g = 3$. This has been attributed to the same state of the enzyme which gives rise to the $g = 12$ signal in cytochrome oxidase [13]. It may represent transitions between two other states in the integral spin multiplet which gives rise to the $g = 12$ signal.

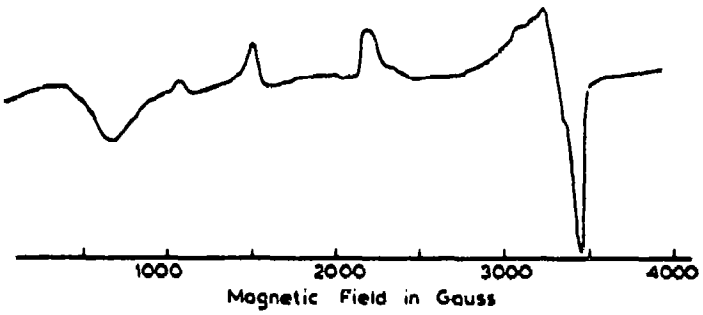

Fig. 2. EPR spectra of cytochrome aa_3 terminal oxidase after incubation with 20 mM formate. EPR conditions were as described in Fig. 1.

4. DISCUSSION

The $g = 12$ signal in slow and formate ligated cytochrome oxidase arises from an excited doublet of an integral spin system. Several models for such a system have been proposed. The integral spin system may represent $S = 2$ ferryl heme [14,15], but a coupled system involving antiferromagnetically coupled $S = 5/2$ ferric heme to $S = 1/2$ cupric copper to produce an $S = 2$ state has also been proposed [16,17]. It has been recently pointed out [18] that the $g = 2.9$ feature could arise from a different transition within the same integral spin multiplet, but that some proposals (e.g. [16]) could not explain both transitions.

The relatively small (1–2 cm^{-1}) values of the zero field splitting parameter D needed to account for the signals has been presented as evidence for the ferryl heme model [14]. While it is true that ferric heme D values are typically about five times this large and that *nonheme* ferryl iron complexes used as models have D values of the correct magnitude, it is worth pointing out that nonheme high-spin *ferric* D values are also often small. The large D values of high spin ferrihemes reflect the geometry imposed by the tetrapyrrole. Recent evidence suggests that in the cytochrome oxidase formate complex heme a_3 is high spin ferric and that the ground state is an $S = 2$ quintet [17]; no existing model accounts for all the data satisfactorily.

Transitions between the excited state doublets are likely to be first order forbidden, at least with the microwave magnetic field component in its conventional orientation (perpendicular to the Zeeman field). The $g = 12$ signals in both oxidases are very broad and less intense than the $g = 6$, $g = 3$ and $g = 2.24$ signals of the ferric hemes. In addition, it is necessary to use lower temperatures and/or higher microwave power to obtain reasonable signal to noise ratios for the $g = 12$ species at enzyme concentrations usually used to study EPR signals from the ferric heme species. It is probable that this is the reason that this signal has not been previously reported in the cytochrome *bo* system.

Formate ligation does not generally produce '$g = 12$' states in heme model systems; high-spin ferric heme is

often observed. The observation of this unusual spectroscopic feature almost unaltered in the cytochrome *bo* system is further evidence for close structural homology between the two oxidase systems. The sequence homology and structural similarity between the oxidases, particularly within subunit I, preserves enough common detail at the atomic level within the binuclear catalytic site to allow the formation of such otherwise unique complexes.

Superior ability to manipulate the cytochrome *bo* system should aid in our ability to choose between different models for unusual states such as the formate ligated and resting enzymes, and to obtain as a result added insight into the structure of the catalytic core of both the cytochrome aa_3 and cytochrome *bo* systems. Advantages include relatively easy genetic manipulation, ability to obtain copper depleted but heme sufficient enzyme, and ready isotopic enrichment.

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REFERENCES

[1] Antonini, E., Brunori, M., Colosimo, A., Greenwood, C. and Wilson, M. (1981) Proc. Natl. Acad. Sci. USA 78, 7115–7119.
[2] Baker, G.M. and Palmer, G. (1987) J. Biol. Chem. 262, 595–604.
[3] Young, L.J. and Palmer, G. (1985) J. Biol. Chem. 261, 13031–13033.
[4] Moody, A.J., Cooper, C.E. and Rich, P.R. (1991) Biochim. Biophys. Acta 1059, 189–207.
[5] Schoonover, J.R. and Palmer, G. (1991) Biochemistry 30, 7541–7550.
[6] Chepuri, V., Lemieux, L., Hill, J., Alben, J.O. and Gennis, R.B. (1990) Biochim. Biophys. Acta 1018, 124–127.
[7] Salerno, J.C., Bolgiano, B. and Ingledew, W.J. (1989) FEBS Lett. 247, 101–107.
[8] Salerno, J.C., Bolgiano, B., Poole, R.K., Gennis, R.B. and Ingledew, W.J. (1990) J. Biol. Chem. 265, 4364–4368.
[9] Au, D.C.-T. and Gennis, R.B. (1987) J. Bacteriol. 161, 123–127.
[10] Minghetti, K.C., Goswitz, V.C., Gabriel, N.E., Hill, J.J., Barassi, C.A., Chan, S.I. and Gennis, R.B., submitted to Biochemistry.
[11] Lemieux, L.J., Calhoun, M.W., Thomas, J.W., Ingledew, W.J. and Gennis, R.B. (1992) J. Biol. Chem. 267, 2105–2113.
[12] Yu, C.A., Yu, L. and King, T.E. (1975) J. Biol. Chem. 250, 1383–1392.
[13] Cooper, C.E., Moody, A.J., Rich, P.R., Wrigglesworth, J.M. and Ioannidis, N. (1991) Biochem. Soc. Trans. 19, 259S.
[14] Hagen, W.R. (1982) Biochim. Biophys. Acta 708, 82–98.
[15] Dunham, W.R., Sands, R.H., Shaw, R.W. and Beinert, H. (1983) Biochim. Biophys. Acta 748, 73–85.
[16] Brudvig, G.W., Stevens, T.H., Morse, R.H. and Chan, S.I. (1981) Biochemistry 20, 3912–3921.
[17] Barnes, Z.K., Babcock, G.T. and Dye, J.L. (1991) Biochemistry 30, 7597–7603.
[18] Cooper, C.E. and Salerno, J.C. (1992) J. Biol. Chem. 267, 280–285.