EPR spectroscopy of *Escherichia coli* cytochrome *bo* which lacks Cu_B

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Abstract The spectroscopic and ligand-binding properties of a copper-deficient cytochrome bo_3, a member of the haem–copper superfamily of terminal oxidases, are reported and contrasted with those of the native enzyme. The enzyme lacks the copper atom (Cu_B) which is normally an integral part of the catalytic site. The consequences of loss of the Cu_B are the loss of antiferromagnetic coupling to the high-spin haem and an inability to form any of the integer-spin derivatives of the enzyme. Low-spin compounds of the normally high-spin haem are still formed with appropriate ligands, although these are modified.

Key words: Quinol oxidase; *Escherichia coli*; Copper depletion; Ligand binding; Electron paramagnetic resonance; Cytochrome aa_3

1. Introduction

Cytochrome bo_3 is a respiratory terminal oxidase which catalyses the reduction of oxygen to water by quinol and conserves part of the energy available from this process in the form of a transmembrane proton-electro-chemical potential via the transmembrane translocation of protons. The enzyme is closely related to the cytochrome *c* oxidases (cytochrome aa_3) with which it forms the 'haem–copper terminal oxidase' superfamily. Cytochrome bo_3 and cytochrome *c* oxidases are alike in all aspects of structure and function except for the replacement of the cytochrome *c*-binding site and attendant Cu_A centre with a quinol-binding site in subunit II. The catalytic core of these enzymes comprise a five-coordinate (high-spin) haem and a copper atom (Cu_B), which in the unligated oxidised form of the enzyme are antiferromagnetically coupled. It is at this site that oxygen and most inhibitors bind.

The binding of ligands to native cytochromes bo_3 and aa_3 has been investigated by both optical and EPR spectroscopy [for cytochrome bo_3 see [2–4]]. Ligand-binding studies are a useful method by which the catalytic centres of oxidases may be probed and have been extensively applied to these enzymes. Generally, ligands bind at the binuclear centre and fall into two classes: those which force the high-spin haem low-spin, making it six-coordinate, and those in which the ligand complex remains high-spin, which often gives rise to integer-spin complexes. This situation is complicated by different redox states of the centres and because some ligands can bind to either the Cu_B or the haem. Thus a range of ligands bind to the binuclear centre in these oxidases, and the nature of this binding can be used diagnostically.

It has been established that reactions at the binuclear centre of haem–copper oxidases which involve the uptake of negative charge (anion binding or reduction of the centre) also require the uptake of compensating positive charge via protonation [5–7] which has led to proposals for protonmotive mechanisms based on these findings [8]. Cu_B has been suggested as a possible site for protonation. The role of Cu_B has been investigated using cytochrome bo_3 that lacks this centre, obtained by growing *E. coli* under conditions of copper limitation [9–12]. The aim of the current work is to extend these observations by investigating the effect of lack of Cu_B on the electron paramagnetic resonance spectroscopy of the cytochrome *bo*–ligand complexes.

2. Materials and methods

2.1. Bacterial growth and membrane preparation

E. coli strain RG145, a gift from Prof. R. Gennis, University of Illinois, was used in this study. This strain does not express the second terminal oxidase, cytochrome *bd* and as grown has an approximately 5-fold amplified expression of cytochrome bo_3 [13]. Bacteria were grown under conditions described previously [14] except when copper availability was to be restricted. In these growths, copper supplementation was omitted and the growth medium was treated with 1 mM sodium ascorbate and 1–2 μM 2,2′-bicinchinonic acid (4,4′-dicarboxy 2,2′-biquinoline, BCA), which is a chelator of Cu(I) [15,16] for 1 h at 35°C prior to autoclaving. Membranes were prepared from the cells obtained as described in [14].

The cytochrome bo_3 content of the *E. coli* membranes thus prepared were determined using the CO-binding spectra of fully reduced membranes, in order to facilitate comparison of the EPR spectra. These are almost identical for Cu(+) and Cu(−) membranes [12]. The mean of $\Delta A_{536-556nm}$ and $\Delta A_{570-590nm}$ was used in this determination, for which the extinction coefficient was taken to be approximately 7 $mM^{-1}\,cm^{-1}$ [12]. Additionally, after ligand binding, samples were reduced with dithionite (10 mM, 1 min) and a dithionite-reduced *minus* air-oxidised (redox) spectrum generated, for which a value for $\Delta A_{560-580nm}$ could be obtained. For a given membrane preparation, there is a constant relationship between the CO-binding and redox spectra, hence the enzyme concentration in individual samples could be determined.

Since the copper-deficient membranes produced by growth in the presence of BCA contain a proportion of Cu(+) Cyt. *bo*, it was essen-

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Abbreviations: Cu(+) Cyt. *bo*, cytochrome *bo* which contains Cu_B; Cu(−) Cyt. *bo*, cytochrome bo_3 lacking Cu_B; Cu(+) membranes, membranes derived from *E. coli* grown with a copper supplement, containing only Cu(+) Cyt. *bo*; Cu(−) membranes, membranes derived from *E. coli* grown without a copper supplement and in the presence of a Cu(I) chelator, containing a mixture of Cu(−) Cyt. *bo* and Cu(+) Cyt. *bo*); BCA, 2,2′-bicinchinonic acid (4,4′-dicarboxy 2,2′-biquinoline); EPR, electron paramagnetic resonance; Ox, unligated (oxidised) membranes; CN, cyanide-ligated membranes; Su, sulfide-ligated membranes; Az, azide-ligated membranes; Flu, fluoride-ligated membranes; For, formate-ligated membranes

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tial to determine the relative proportions of Cu(+) and Cu(−) Cyt. *bo*. This was determined from the cyanide-binding spectra of fully reduced membranes, since cyanide does not bind to fully reduced Cu(−) Cyt. *bo* [9,12]. The mean of $\Delta A_{528-548nm}$ and $\Delta A_{560-576nm}$ was used in this determination and related to individual samples using the dithionite-reduced *minus* air-oxidised (redox) spectrum. Table 1 summarises the measurements and calculations used to determine the degree of copper deficiency and relative cytochrome bo_3 concentrations. These values were then used to correct the EPR spectra of Cu(−) membranes for the contribution of residual Cu(+) Cyt. *bo* by subtraction of appropriately scaled Cu(+) membrane spectra.

2.2. Ligand binding

Membranes were diluted to 20–30 mg membrane protein/ml in 50 mM MOPS, pH 7.0, and the appropriate ligand was added from a fresh buffered stock solution to a concentration of 10 mM. Potassium cyanide, sodium sulfide, sodium azide, sodium fluoride and sodium formate were used as ligands. After incubation on ice for 30 min (cyanide, sulfide, azide) or 18 h (fluoride, formate), to allow the ligand complex to fully form, 250 μl aliquots of the treated membranes were removed, placed in EPR tubes, frozen and stored under liquid nitrogen.

2.3. EPR spectroscopy

EPR spectra were obtained using a Bruker ER200D spectrometer interfaced to a ESP3220 computer (Bruker Analytische Messtechnik GmBH, Silberstreifen, W-7512, Rheinstetten 4, Germany) equipped with a variable temperature cryostat and liquid helium transfer line (Oxford Instruments, Osney Mead, Oxford, UK). Spectrometer conditions are described in the figure legends.

3. Results

In this paper we report the consequences of phenotypically removing Cu_B on the spectroscopy and ligand-binding of the catalytic site of cytochrome bo_3. Ligand-induced changes in both the optical and EPR spectra of copper-replete cytochrome *bo* have been reported [2]. Comparison of the EPR spectra of Cu(+) and Cu(−) membranes in Figs. 1 and 2 shows that substantial changes occur when copper is removed from cytochrome bo_3 and the effects of certain ligands are altered. Fig. 1 compares the EPR spectra of the ligand compounds of copper-replete (Cu(+), Fig. 1a) and copper-depleted (Cu(−), Fig. 1b) cytochrome bo_3 in the region of the $g = 6$ resonance arising from ferric high-spin haem. It should be noted that the copper-replete spectra (Fig. 1a) are expanded 2-fold on the ordinate relative to the copper-depleted spectra (Fig. 1b) for clarity. Fig. 2 compares the EPR spectra in the low-spin region, from around $g = 4$ to $g = 2$.

3.1. Unligated cytochrome bo_3

A comparison of the EPR spectra of copper-replete and copper-depleted oxidised cytochrome bo_3 in the absence of exogenous ligands (Fig. 1a,b, top) shows that copper depletion results in a large increase in the intensity of the $g = 5.9$ resonance; this is expected because of the loss of the antiferromagnetic coupling between haem *o* and Cu_B^{2+}. In the low-spin spectral region (Fig. 2a), the signal from a putative integer-spin ($S = 2$) complex at $g' = 3.5$ is lost on copper depletion, whereas the $g_z = 3$ and $g_y = 2.2$ resonances from low-spin ferric haem remain. These results confirm that the presence of Cu_B is necessary to elicit both the integer-spin signals in the oxidase and the antiferromagnetic coupled state of the binuclear site.

3.2. Cyanide-ligated ferric cytochrome bo_3

In the copper-replete enzyme, cyanide is known to bind to the high-spin haem, producing a low-spin adduct with a g_z at 3.2 when the Cu_B is cuprous [2], the cupric form is EPR-silent. This is apparent in Figs. 1 and 2b. In Fig. 1 the high-spin ferric haem *o* signal is lost on cyanide addition in both Cu(+) and Cu(−) Cyt. *bo*. As expected, the low-spin haem *b* features at $g_z = 3$ and $g_y = 2.2$ are unchanged. However, the cyanide-ligated low-spin haem *o* is slightly different in the two forms of the enzyme, having a greater rhombic distortion in the absence of copper (Fig. 2b). The low-spin ferric haem *o*–cyanide complex gives rise to a low-spin resonance at $g = 3.2$ [1] in Cu(+) Cyt. *bo* (Fig. 2b, top) and in the Cu(−) enzyme the resonance shifts to $g = 3.3$ (Fig. 2b, bottom). Thus cyanide binds to haem *o* in the presence and absence of copper at the catalytic site, but with a minor change in site geometry.

3.3. Azide-ligated cytochrome bo_3

The spectroscopic consequence of azide ligation by the copper-replete enzyme depends on the redox state; in the fully oxidised enzyme an integer-spin compound is formed, which develops into a low-spin haem ($g_z = 2.8$, Fig. 2c) compound as the Cu_B becomes reduced [2]. Addition of azide to the copper-depleted enzyme alters the EPR spectrum around the $g = 6.0$ high-spin haem resonance in Cu(−) membranes to a more rhombic line-shape (Fig. 1b). This does not appear to be due to a temperature-dependent equilibrium between axial and rhombic forms as the overall lineshape of the signal is not temperature dependent (not shown). Azide binding to the copper-depleted enzyme also results in a low-spin compound, the g_z of which is shifted to $g = 2.9$ and is of lower intensity, with additional resonances at $g = 3.6$ and $g = 3.2$ (Fig. 2c, bottom). The azide-induced low-spin compound in the copper-replete enzyme has a g_z of $g = 2.8$ (Fig. 2c, top). These results show that azide binding to copper-depleted enzyme results in an equilibrium mixture of rhombic high-spin and low-spin forms, with a heterogeneity in the latter and confirm that integer-spin compounds cannot be formed in the absence of

Table 1
Copper deficiency level and concentrations of cytochrome bo_3 in *E. coli* membrane preparations used for EPR

Preparation of membranes	Measurements ($n = 2$)		Ratio	Fraction of Cu(+) Cyt.bo	[Cytochrome *bo*] μM
	B/A (Cyanide-binding/reduced *minus* oxidised)[a]	C/A (CO-binding/reduced *minus* oxidised)[b]	(B/A)/(C/A) → (B/C)	Relative to Cu(+) Membranes	(based on CO-binding)
Cu(+) Membranes	0.323	0.262	1.233	1	16.1
Cu(−) Membranes	0.088	0.296	0.297	0.241	10.2

[a] $((\Delta A_{528-548nm} + \Delta A_{560-576nm}) \times 0.5)/\Delta A_{560-580nm(redox)}$.
[b] $((\Delta A_{536-556nm} + \Delta_{A570-590nm}) \times 0.5)/\Delta A_{560-580nm(redox)}$.

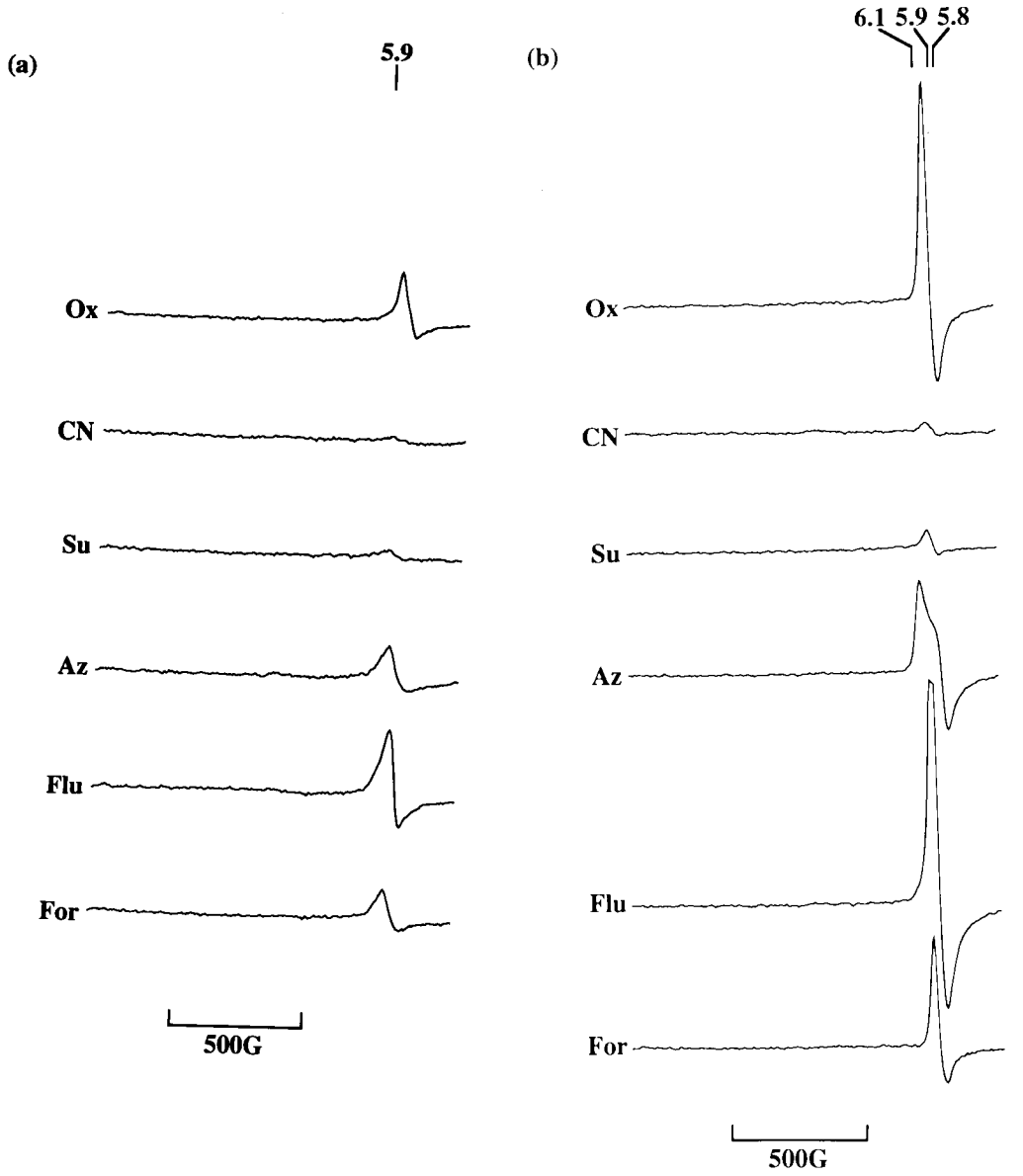

Fig. 1. EPR spectra of the ligand compounds of cytochrome bo_3 in the low field region. a: Copper-replete membranes. b: Copper-depleted membranes. Note that (a) is expanded 2-fold on the ordinate relative to (b). EPR spectrometer conditions were: modulation amplitude 4 G, modulation frequency 100 kHz, microwave frequency 9.45 GHz. Spectra were recorded at a temperature of 7 K and microwave power of 100 mW.

Cu_B. Azide-treated membranes can also display integer-spin resonances at approx. $g' = 10$, these are also lost in the Cu(−) membranes (not shown).

3.4. *Fluoride-treated cytochrome bo_3*

Fluoride is known to form an integer-spin complex with copper-replete cytochrome bo_3 [2]. Fluoride-treated membranes display a prominent integer-spin derived set of resonances at $g' = 3.7$ to $g' = 3.35$, which are lost in the Cu(−) membranes (Fig. 2d). No additional low-spin compounds are formed on fluoride ligation in either form of the enzyme. However, small distortions in the $g = 6$ region of the spectrum of copper-depleted membranes (Fig. 1b, comparing Ox spectrum with Flu spectrum) suggest that fluoride may still bind to Cu(−) Cyt. *bo*. Fluoride-treated membranes also show integer-spin derived resonances at around $g' = 10$ [2], which are observed at very low temperatures and high microwave power. These signals are not seen in the Cu(−) membranes (not shown).

3.5. *Sulfide- and formate-treated cytochrome bo_3*

Both sulfide and formate bind to oxidised cytochrome bo_3. The former produces a low-spin haem compound, the latter an integer-spin compound [2]. In the membranous systems used herein, both these ligands presented experimental problems; they caused extensive reduction of the enzyme, sulfide non-specifically and formate via the presence of formate dehydrogenase activity. Both ligands cause loss of intensity of the $g = 6.0$ signal from high-spin ferric haem in both Cu(+) and Cu(−) membranes. No low-spin ferric-haem or integer-spin signals were observed.

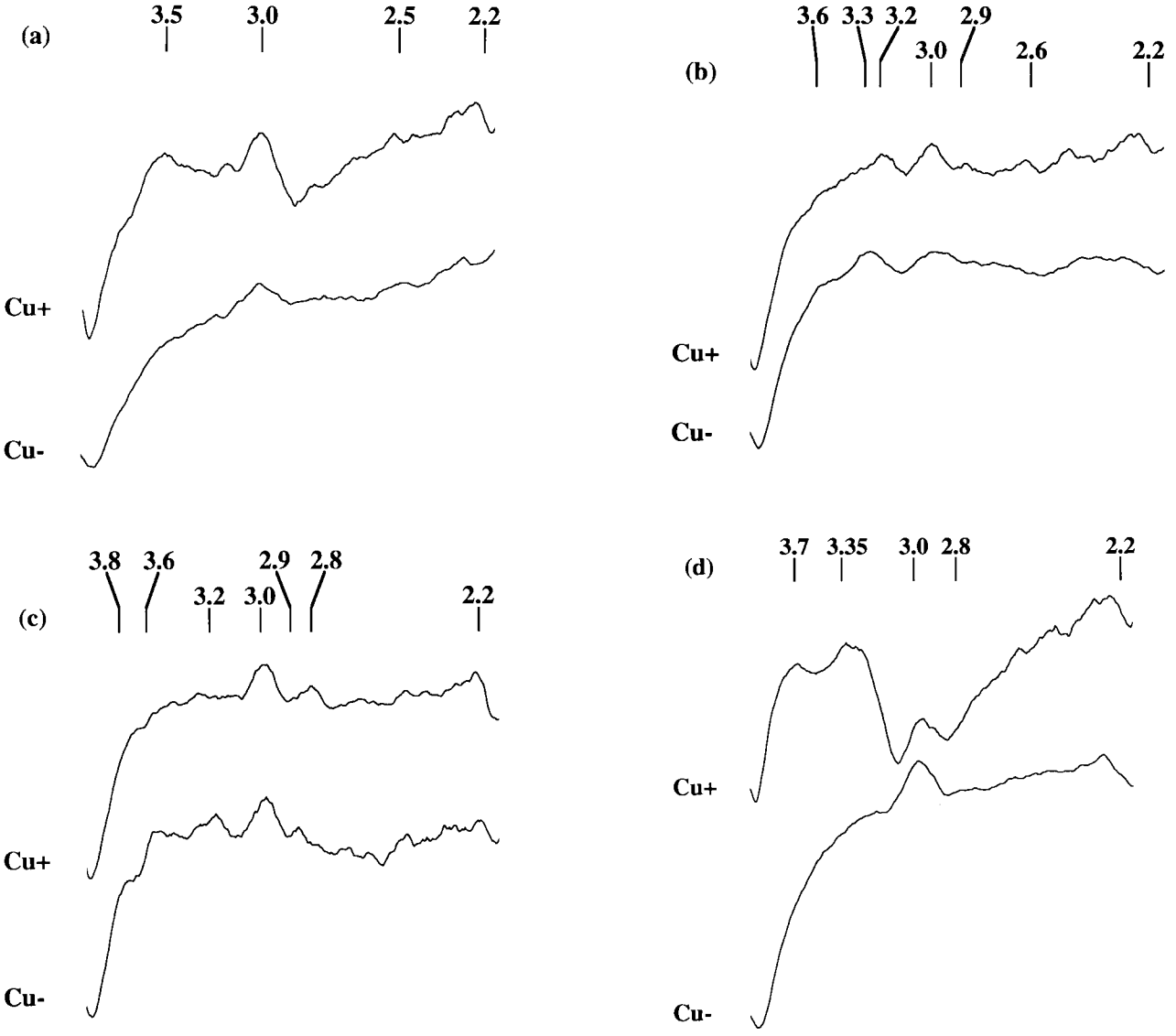

Fig. 2. EPR spectra of the ligand compounds of cytochrome bo_3 in the $g=4$ to $g=2$ region. a: Unligated (oxidised) membranes. b: Cyanide-ligated membranes. c: Azide-ligated membranes. d: Fluoride-ligated membranes. The spectra presented are accumulations of 10 scans, each with time constant and conversion time 40 ms. EPR spectrometer conditions were: modulation amplitude 4 G, frequency 9.45 GHz. Spectra of oxidised and fluoride-treated membranes were recorded at a temperature of 7 K and microwave power of 100 mW, spectra of cyanide- and azide-treated membranes were recorded at a temperature of 17 K and microwave power of 20 mW.

4. Discussion

Removal of Cu_B from cytochrome bo_3 gives rise to extensive changes in the properties of the high-spin haem, as expected from the structure of the binuclear centre. In addition to loss of oxidase activity [10], these include changes in the kinetics of CO binding and in both the kinetics and affinity of cyanide binding [12]. There is also a considerable decrease in the affinity of the oxidase for a range of anionic ligands which require co-binding of a proton [9]. These changes in ligand-binding properties are reflected by changes in the EPR spectra of the ligand complexes. The EPR spectra show that copper depletion of cytochrome bo_3 results in the loss of antiferromagnetic coupling due to disruption of the binuclear centre, loss of the ability to form integer-spin compounds and in the formation of altered low-spin ligand complexes of haem *o*. The absence of copper from the Cu_B (Cu^I/Cu^{II}) site may necessitate a replacement ligand. The most likely replacement species are proton(s), however the presence of a non-paramagnetic species such as Zn^{2+} cannot be ruled out. No spectroscopic evidence for replacement of Cu_B by paramagnetic species such as nickel or cobalt ions was observed.

In conclusion these data show that loss of the Cu_B gives rise to loss of antiferromagnetic coupling to the high-spin haem and to an inability to form any of the integer-spin derivatives of the enzyme. Low-spin compounds of the high-spin haem *o* are still formed with appropriate ligands, though these appear modified.

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References

[1] Puustinen, A., Morgan, J.E., Verkhovsky, M., Thomas, J.W., Gennis, R.B. and Wikström, M. (1992) Biochemistry 31, 10363–10369.
[2] Ingledew, W.J., Horrocks, J. and Salerno, J.C. (1993) Eur. J. Biochem. 212, 657–664.
[3] Watmough, N.J., Cheesman, M.R., Gennis, R.B., Greenwood, C. and Thomson, A.J. (1993) FEBS Lett. 319, (1-2) 151–154.
[4] Calhoun, M.W., Gennis, R.B. and Salerno, J.C. (1992) FEBS Lett. 309, (2) 127–129.
[5] Mitchell, R., Brown, S., Mitchell, P. and Rich, P.R. (1992) Biochim. Biophys. Acta 1100, 40–48.
[6] Rich, P.R., Brown, S., Moody, A.J. and Mitchell, J.R. (1993) Biol. Chem. Hoppe-Seyler 374, 823.
[7] Mitchell, R. and Rich, P.R. (1994) Biochim. Biophys. Acta 1186, 19–26.
[8] Rich, P.R. (1995) Aust. J. Plant Physiol. 22, 479–486.
[9] Rich, P.R., Meunier, B., Mitchell, R.M. and Moody, A.J. (1996) Biochim. Biophys. Acta 1275, 91–95.
[10] Salerno, J.C., Bolgiano, B., Poole, R.K., Gennis, R.B. and Ingledew, W.J. (1990) J. Biol. Chem. 265, 4364–4368.
[11] Ciccognani, D.T., Hughes, D.T. and Poole, R.K. (1992) FEMS Microbiol. Lett. 94, 1–6.
[12] Mitchell, R., Moody, A.J. and Rich, P.R. (1995) Biochemistry 34, 7576–7585.
[13] Au, D.C.-T. and Gennis, R.B. (1987) J. Bacteriol. 169, 3237–3242.
[14] Moody, A.J. and Rich, P.R. (1994) Eur. J. Biochem. 226, 731–737.
[15] Case, D.A. and Karplus, M. (1979) J. Mol. Biol. 132, 343–368.
[16] Brenner, A.J. and Harris, E.D. (1995) Anal. Biochem. 226, 80–84.