Biochem. J. (1983) **215**, 57–66
Printed in Great Britain

Low-spin ferric forms of cytochrome a_3 in mixed-ligand and partially reduced cyanide-bound derivatives of cytochrome *c* oxidase

Bruce C. HILL,*‡ Tom BRITTAIN,*§ David G. EGLINTON,† Paul M. A. GADSBY,† Colin GREENWOOD,* Peter NICHOLLS,*‖ Jim PETERSON,† Andrew J. THOMSON† and Tai Chin WOON*¶
**School of Biological Sciences and †School of Chemical Sciences, University of East Anglia, Norwich NR4 7TJ, U.K.*

(*Received 22 March 1983/Accepted 23 May 1983*)

1. Optical-absorption-, e.p.r.- and m.c.d. (magnetic-circular-dichroism)-spectroscopic measurements were made on liganded derivatives of oxidized and partially reduced cytochrome *c* oxidase. 2. When NO was added to oxidized cyanide-bound cytochrome *c* oxidase, no changes occurred in the optical-absorption difference spectrum. In contrast, NO induced reduction of cytochrome a_3 and formation of the nitrosylferrohaem species when the oxidized resting enzyme was the starting material. 3. E.p.r. spectroscopy of the NO-treated oxidized cyanide-bound enzyme revealed the presence of a low-spin haem signal at $g = 3.40$, whereas the $g = 3.02$ and $g = 2.0$ signals of the oxidized enzyme remained unchanged. Both haem groups in this species are e.p.r.-detectable simultaneously. Examination of an identical sample by m.c.d. spectroscopy in the near-i.r. region identified two distinct low-spin species at 1565 and 1785 nm. 4. Irradiation with white light of the NO-treated cyanide-bound sample at 10 K resulted in the disappearance of the $g = 3.40$ e.p.r. signal and the m.c.d. signal at 1785 nm, whereas a band at 1950 nm increased in intensity. When the photolysed sample was warmed to 50 K and held in the dark for 15 min, the original spectrum returned. 5. Magnetization studies of the 1785 nm m.c.d. band support the assignment of this signal to the same metal centre that gives rise to the $g = 3.40$ e.p.r. signal. 6. The effect of NO on the oxidized cyanide-bound enzyme was compared with that obtained when the oxidized cyanide-bound species was taken to the partially reduced state. Cytochrome a_3 is e.p.r.-detectable with a *g*-value of 3.58 [Johnson, Eglinton, Gooding, Greenwood & Thomson (1981) *Biochem. J.* **193**, 699–708]. Its near-i.r. m.c.d. spectrum shifts from 1950 nm in the oxidized cyanide-bound enzyme to 1545 nm on addition of reductant. 7. A scheme is advanced for the structure of the cytochrome a_3–Cu_B site that allows for cyanide binding to Fe_{a_3} and NO binding to Cu_B. Cyanide is the bridging ligand in the ferromagnetically coupled cytochrome a_3–Cu_B pair of oxidized cyanide-bound cytochrome *c* oxidase. The bridged structure and the magnetic interaction are broken when the enzyme is partially reduced. However, when NO binds to Cu_B the cyanide bridge remains intact, but now the odd spins of NO and Cu_B are magnetically coupled.

Abbreviation used: m.c.d., magnetic circular dichroism.

‡ To whom correspondence should be addressed.
§ Present address: Department of Biochemistry, University of Auckland, Auckland, New Zealand.
‖ Present address: Department of Biological Sciences, Brock University, St. Catharines, Ontario L2S 3A1, Canada.
¶ Present address: Department of Chemistry, Memorial University, St. John's, Newfoundland A1B 3X7, Canada.

Cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) catalyses the transfer of electrons from cytochrome *c* to oxygen. It contains four redox-active metal centres: two haem A groups and two copper atoms (Malmström, 1979). E.p.r. spectroscopy of the isolated resting oxidized enzyme unambiguously identifies two of the four centres. These are one low-spin haem with *g*-values of 3.02, 2.21 and 1.45, assigned to cytochrome *a*, and one cupric copper with *g*-values

near 2.0, assigned to Cu_A (also called Cu_a or Cu_D) (Aasa *et al.*, 1976). The other two centres, cytochrome a_3 and Cu_B (also known as Cu_{a_3} or Cu_U), form a magnetically coupled pair (Van Gelder & Beinert, 1969). This binuclear pair may give rise to the unusual low-field e.p.r. signal seen in samples of the resting enzyme at an effective *g*-value of 12 (Greenaway *et al.*, 1977; Brudvig *et al.*, 1981; Hagen, 1982).

In the absence of exogenous ligands, cytochrome a_3 is probably high-spin (Thomson *et al.*, 1976; Babcock *et al.*, 1976). Addition of cyanide results in the formation of a low-spin complex of the ligand with cytochrome a_3 (Van Buuren *et al.*, 1972; Thomson *et al.*, 1981). The reaction of cyanide with the oxidized resting species is complete only after an incubation period of longer than 24h. No new e.p.r.-detectable species arise when the oxidized enzyme binds cyanide, but the low-field signal at $g = 12$ is lost (Brudvig *et al.*, 1981). M.c.d. spectroscopy in the near-i.r. region identifies a new low-spin ferric haem band when the oxidized enzyme binds cyanide (Thomson *et al.*, 1982). Its wavelength position and magnetization properties make it unique among the low-spin haem types examined thus far by this technique (Eglinton *et al.*, 1980; Rawlings *et al.*, 1977). We have attributed these unusual features to the coupling of cytochrome a_3 to Cu_B and have suggested that cyanide is bound as a bridging ligand between the metal atoms (Thomson *et al.*, 1982).

NO forms a complex with reduced cytochrome a_3, giving rise to a ferronitrosylhaem e.p.r. signal from cytochrome a_3^{2+}–NO$^{\bullet}$ (Blokzijl-Homan & Van Gelder, 1971). Stevens *et al.* (1979) produced a high-spin signal from cytochrome a_3 by adding NO to oxidized resting cytochrome *c* oxidase. These workers suggested that NO was binding to Cu_B, breaking its magnetic interaction with Fe_{a_3} and rendering the latter e.p.r.-detectable. Boelens *et al.* (1982) demonstrated that this species was photosensitive. It had a photoaction spectrum of a Cu^{2+}–NO species with a broad band in the visible region at 640nm. In addition, Brudvig *et al.* (1981) demonstrated that incubation of the oxidized cyanide-bound enzyme with NO produced an e.p.r. signal at '$g = 3.5$'. These workers equated this signal with that seen from cytochrome a_3 (at $g = 3.58$) when the oxidized cyanide-bound enzyme is reduced with dithionite (Johnson *et al.*, 1981).

In the present work we have examined the effect of NO addition to oxidized cyanide-bound cytochrome *c* oxidase by optical, e.p.r. and m.c.d. spectroscopies. These experiments distinguish the low-spin species produced by NO treatment of oxidized cyanide-bound enzyme from that obtained with the partially reduced cyanide-bound species. This mixed-ligand species was found to be photosensitive. We present a scheme in which cyanide binds to both cytochrome a_3 and Cu_B, acting as a bridge, and NO also binds to Cu_B.

Experimental

^2H_2O was obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K. Ethanediol, P_2O_5, KH_2PO_4, KCN, $Na_2S_2O_4$ and NO were from BDH Chemicals, Poole, Dorset, U.K. Tween-80 (polyoxyethylene sorbitan mono-oleate) was from Sigma Chemical Co., Poole, Dorset, U.K. Na_2HPO_4 was from Fisons Scientific Apparatus, Loughborough, Leics., U.K. Deuterated ethanediol was prepared by dilution of ethanediol into an equal volume of ^2H_2O. Excess of ^2H_2O was removed by standing the mixture over P_2O_5 for a month.

Cytochrome *c* oxidase was prepared from bovine heart muscle by a modified version of the method of Yonetani (1960) as described by Thomson *et al.* (1981). The preparation of deuterated samples for near-i.r. measurements was as outlined by Eglinton *et al.* (1980). The cyanide derivative of oxidized enzyme was prepared by incubation of the oxidized resting material with a 20-fold excess of KCN for 3 days at 4°C. The mixed-ligand complex was formed by incubating the cyanide-bound form of the oxidized enzyme under an atmosphere of NO for 24h at 20°C. The addition of NO was made anaerobically. This was accomplished by five cycles of evacuation and flushing with O_2-free N_2. After the final round of evacuation NO was dispensed from a cylinder to a pressure of 1 atm.

The dithionite titration of the oxidized cyanide-bound enzyme was performed as outlined by Johnson *et al.* (1981). A 25mM solution of $Na_2S_2O_4$ was prepared in N_2-equilibrated buffer (0.2M-sodium phosphate buffer, pH 7.4). The stock cytochrome aa_3 solution was taken to various stages of partial reduction by the addition of $Na_2S_2O_4$. Portions of the stock enzyme solution were removed with a gas-tight syringe, diluted with O_2-free ethanediol and distributed to anaerobic N_2-flushed cells for absorbance, e.p.r. and m.c.d. measurements.

E.p.r. spectra were recorded on a Brucker ER-200D spectrometer equipped with an Oxford Instruments ESR-900 flow cryostat and D.T.C.-2 temperature controller. The instrument was calibrated with reference to 1,1-diphenyl-2-picrylhydrazyl.

The dichrograph used for m.c.d. measurements was constructed in this laboratory and is described by Eglinton *et al.* (1980). An SM4 superconducting solenoid (Oxford Instruments) was used to provide fields up to 5T. The temperature of the sample was regulated by a heater on the sample block controlled by a D.T.C.-2 temperature controller. The temperature was measured by two

carbon/glass resistors, one mounted above and one below the sample.

Absorbance measurements were recorded on a Pye–Unicam SP. 8-200 spectrophotometer.

Results

Fig. 1 compares optical difference spectra obtained by treating the resting and cyanide-bound forms of oxidized cytochrome *c* oxidase with NO. When the oxidized resting enzyme is exposed to an atmosphere of NO at 20°C, positive bands appear at 430, 555 and 595 nm, with a trough at 415 nm (Fig. 1*a*). This reaction comes to completion after a period of about 12 h. The absorbance features listed above are indicative of the formation of the ferrocytochrome a_3-NO complex (Boelens *et al.*, 1982). Lack of absorbance at 445 and 605 nm shows that cytochrome *a* remains oxidized. This is confirmed by the e.p.r. spectrum of this species, which has a signal at $g = 3.02$ (not shown). This spectrum also shows the signals due to oxidized Cu_A, and in addition a strong signal at $g = 1.98$ assigned to excess NO. Although cytochrome a_3–NO is present, no hyperfine structure associated with the presence of NO is observed. This may be due to a second NO molecule bound to Cu_B, as suggested by Brudvig *et al.* (1980) for the fully reduced enzyme in the presence of excess of NO. This result demonstrates the ability of NO to act as both a reductant and a ligand to oxidized resting cytochrome *c* oxidase. In this respect NO resembles CO in the ability to reduce and bind to cytochrome a_3 while cytochrome *a* remains oxidized (Greenwood *et al.*, 1974). This species may be described as the mixed-valence NO-bound enzyme, and by analogy with the mixed-valence CO-bound species (Wilson & Miyata, 1977) the oxidation states of the metal atoms are as shown below:

$$a^{3+}\ a_3^{2+}\text{–NO}$$
$$Cu_A^{2+}\ Cu_B^{+}\text{–(NO)} \qquad (1)$$

In contrast, when the cyanide-bound oxidized enzyme is the starting material NO induces very little change in the u.v.–visible-absorption difference spectrum (see Fig. 1*b*). There are small bands at 445 and 602 nm that appear after about a 16 h incubation under an atmosphere of NO at 20°C. These changes probably result from a slight reduction, about 10%, of cytochrome *a*. Cyanide binding has blocked the ability of NO to reduce the enzyme, implying that the reduction reaction occurs at the cytochrome a_3–Cu_B site, as suggested by Brudvig *et al.* (1980). However, when a sample of oxidized cyanide-bound enzyme, treated in a manner identical with that indicated for Fig. 1(*b*), was examined by e.p.r. spectroscopy, a signal was seen at $g = 3.40$ along with the signals of the oxidized enzyme (see Fig. 2). The $g = 3.40$ signal is taken to be the g_z component of a low-spin haem species. Integration of this signal by the method of De Vries & Albracht (1979) for single isolated absorption peaks gives a value of 1.06 spins per cytochrome *a*. The $g = 3.02$ signal of the oxidized resting enzyme, which gives 0.9–1.1 haem groups per functional unit (Johnson *et al.*, 1981), was used as the standard. The cytochrome *a* signal of the NO-treated oxidized cyanide-bound oxidase was 90% of that of the resting enzyme. Therefore in this species both cytochromes *a* and a_3 are virtually fully and simultaneously e.p.r.-detectable. Complete formation of this species is obtained only when the starting material is fully cyanide-bound and the NO incubation is allowed to continue for about 20 h at 20°C. In the experiments reported by Chan and co-workers (Brudvig *et al.*, 1981) the cyanide-bound enzyme reacts rapidly with NO ($t_{\frac{1}{2}} < 100$ s) to give an e.p.r.

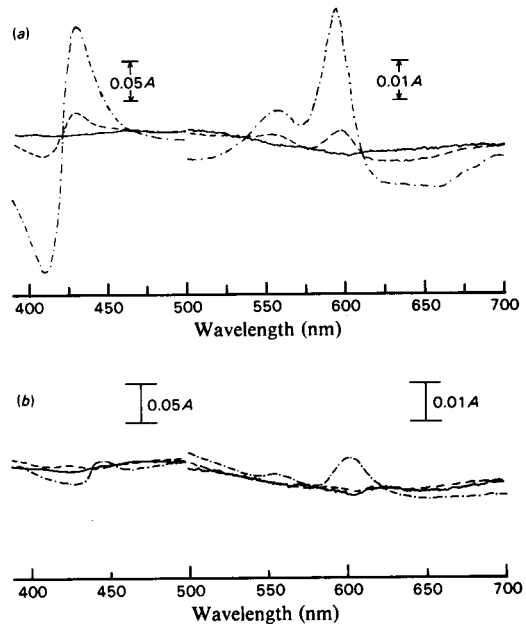

Fig. 1. *Effect of NO treatment on the absorption difference spectrum of oxidized cytochrome c oxidase*
The enzyme concentration was 3.55 μM and the pathlength 1 cm. (*a*) Spectra were with the oxidized resting enzyme as reference: ——, oxidized versus oxidized baseline; – – – –, immediately after NO addition; –·–·–, final NO spectrum after a 16 h incubation at 20°C. (*b*) The oxidized cyanide-bound enzyme (1 mM-KCN) was the reference for: ——, oxidized cyanide-bound versus oxidized cyanide-bound baseline; – – – –, immediately after NO addition; –·–·–, final NO spectrum after a 20 h incubation at 20°C.

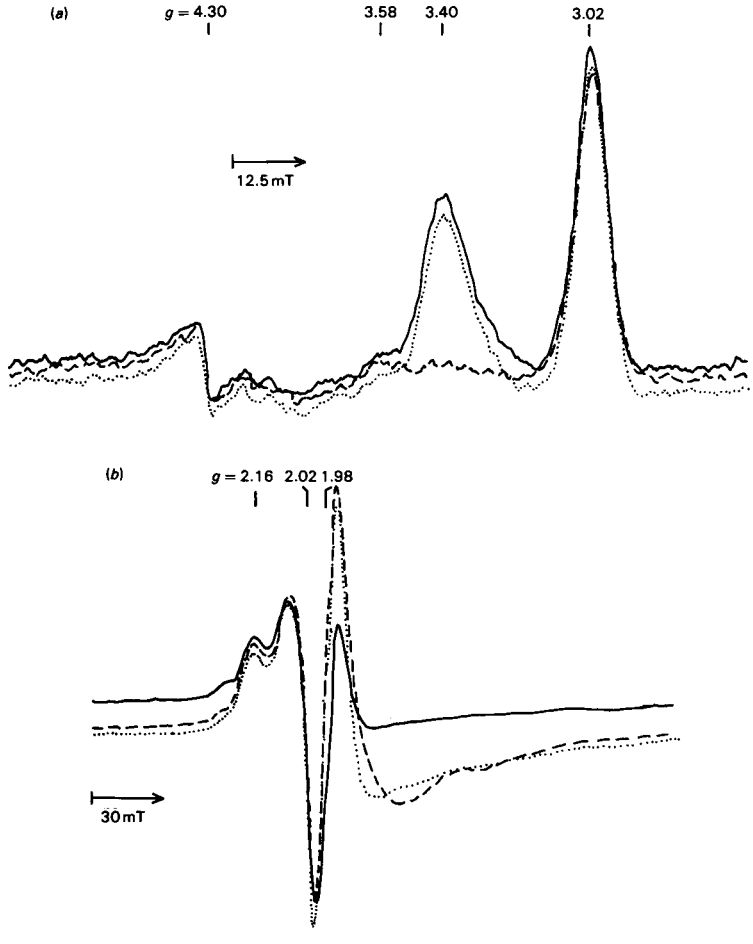

Fig. 2. *E.p.r. spectra of NO-treated oxidized cyanide-bound cytochrome c oxidase*
The enzyme concentration was 365 μM, with 5 mM-KCN. The cyanide-bound enzyme was incubated for 16 h under 1 atm. of NO at 20°C. The spectra are designated as follows: ——, NO-treated oxidized cyanide-bound; – – – –, then photolysed with white light at 10 K for 15 min; · · · · · ·, then warmed to 55 K in the dark for 10 min. (*a*) The $g = 3$ region; (*b*) the $g = 2$ region. The spectrometer settings were: 2 mW microwave power, 0.63 mT modulation amplitude, microwave frequency of 9.42 GHz at a temperature of 10 K.

signal at $g = 3.5$. This contrasts with the results obtained with our preparation. Brudvig *et al.* (1981) examined a number of different cytochrome *c* oxidase preparations and observed that the amount of enzyme able to react rapidly with NO was highly preparation-dependent.

Fig. 2 also shows the $g = 2$ region of the spectrum. The copper signals of the oxidized resting enzyme are evident, but are obscured by a sharp signal at $g = 1.98$. Stevens *et al.* (1979) have reported this signal previously and assigned it to excess, 'matrix-bound', NO. In contrast with these workers, we do not see this signal in the absence of the oxidase. Moreover, this excess-NO signal was not observed in similar studies of nitrate reductase from *Pseudomonas aeruginosa* performed in this laboratory (Johnson *et al.*, 1980). Therefore we suggest that this signal may represent the binding of NO to another site on cytochrome *c* oxidase. Perhaps this represents the occupation by NO of the gas pocket thought to be present near the cytochrome a_3–Cu_B pair (Harmon & Sharrock, 1978; Boelens *et al.*, 1982).

In addition, Fig. 2 shows the effect on the e.p.r. spectrum, measured at 10 K, of irradiation of the sample at 10 K by white light for 15 min. The signal at $g = 3.40$ is lost, whereas those at $g = 3.02$ and $g = 2.0$ are unchanged. At 10 K this photolysis event

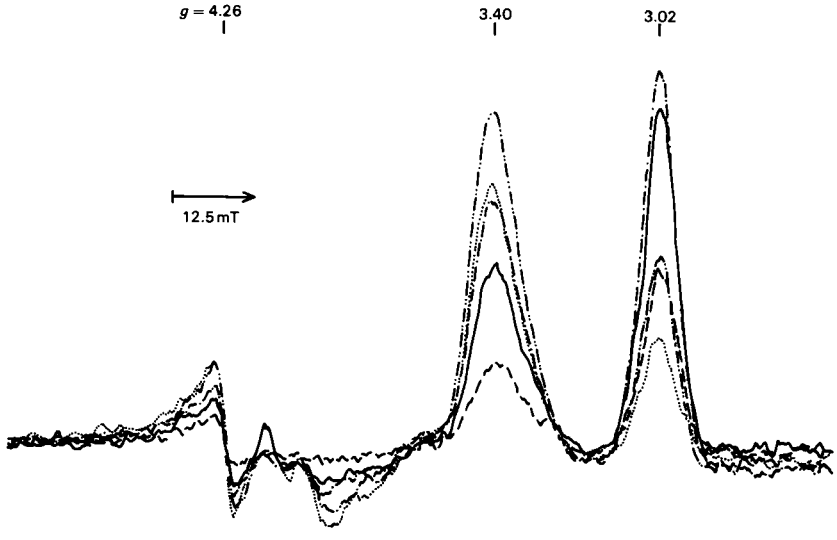

Fig. 3. *Temperature-dependence of g = 3.40 and g = 3.02 e.p.r. signals of oxidized cyanide-bound cytochrome c oxidase*
The sample and spectrometer settings were the same as those given in the legend to Fig. 2. The temperatures employed are shown as follows: ——, 10K; – – – –, 20K; –·–·–, 7.2K; –··–··–, 4.2K; ·····, 3.2K.

is irreversible. But when the sample is warmed to 50K in the dark for 10min and then re-cooled to 10K full re-formation of the e.p.r. signal at $g = 3.40$ is obtained, showing that recombination has occurred. This behaviour resembles photolysis of both carbonmonoxy and nitrosyl derivatives of ferrocytochrome a_3, which are also irreversibly photolysed at 10K and recombine only at higher temperatures (Chance *et al.*, 1965; Yoshida *et al.*, 1980). However, in those complexes there are accompanying changes in the visible-absorption spectrum indicative of ligation to a reduced haem centre.

In Fig. 3 the temperature-dependence of the $g = 3.02$ and $g = 3.40$ signals is illustrated. The signals vary differently with changing temperature at constant microwave power. The $g = 3.40$ signal increases as the temperature is lowered from 10K to 7K and then to 4K, but then decreases at 3.2K. The $g = 3.02$ signal also increases in intensity when the temperature is lowered from 10K to 7K, but then decreases going to 4.2K and 3.2K. Thus the latter appears to be saturating more readily than the former. This behaviour supports the suggestion that the two signals arise from two different centres as opposed to being an equilibrium mixture of the same centre in two states. Neither of these signals shows any shape changes as the temperature is varied. This behaviour is in contrast with the $g = 3.58$ signal of partially reduced cyanide-bound oxidase, which becomes more asymmetric as the temperature is lowered (Johnson *et al.*, 1981).

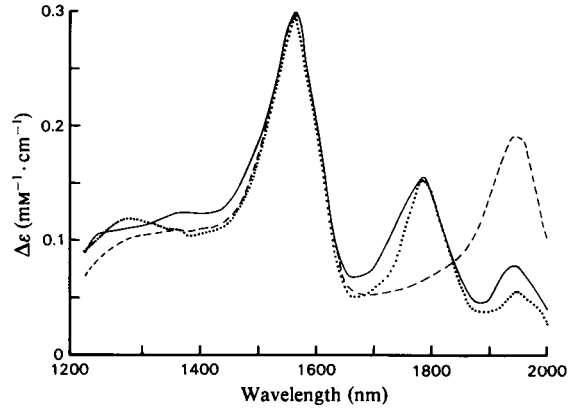

Fig. 4. *Near-i.r. m.c.d. spectrum of NO-treated oxidized cyanide-bound cytochrome c oxidase*
The enzyme concentration was 100μM, with 5mM-KCN and 1mM-NO in an equal mixture of buffer and ethylene glycol. The spectra are designated: ——, the NO-treated oxidized cyanide-bound species; – – – –, the photolysis product obtained by irradiation of the above derivative at 4.2K for 10min; ·····, the recombined species obtained by warming to 80K for 10min. The field was 4.9T and the temperature 4.2K throughout.

M.c.d. spectroscopy in the near-i.r. region gives a bandshape, comprised of a peak and accompanying shoulder to lower wavelength, that is diagnostic of low-spin haem groups (Stephens *et al.*, 1974;

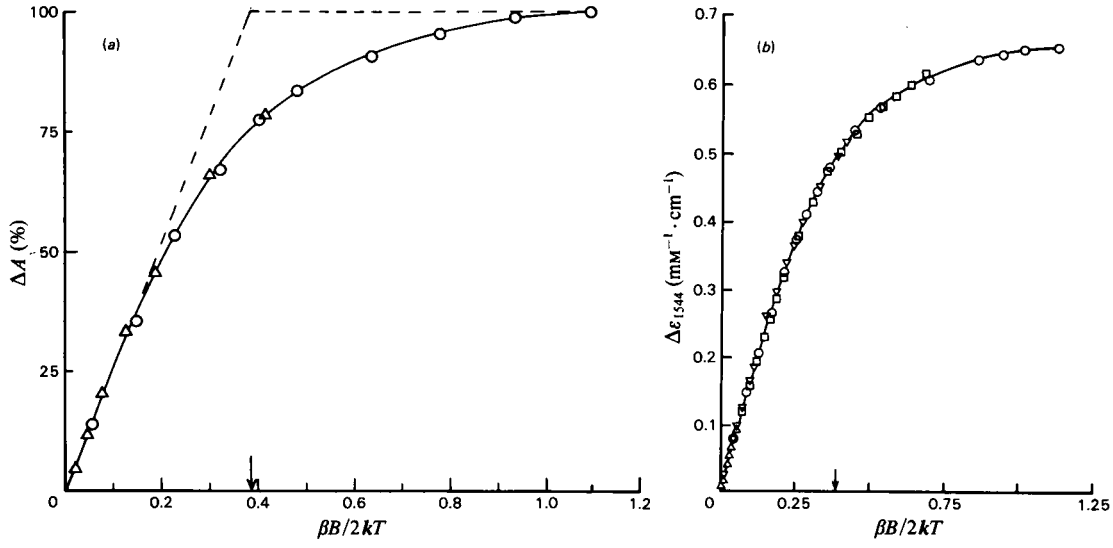

Fig. 5. *M.c.d. magnetization curves for cytochrome a_3 measured in the near-i.r. region*
(*a*) The NO-treated oxidized cyanide-bound species. The sample was the same as that described in the legend to Fig. 4. The signal intensity was measured at field values of 0.25, 0.66, 1.01, 1.44, 1.81, 2.16, 2.87, 3.56, 4.25 and 4.94 T at temperatures of (○) 1.5 K and (△) 4.2 K. (*b*) The partially reduced cyanide-bound species. The sample is the same as that described in the legend to Fig. 6 after the final dithionite addition. The m.c.d. intensity was recorded at fields of 0.21, 0.39, 0.56, 0.75, 0.92, 1.08, 1.24, 1.40, 1.56, 2.31, 3.03, 3.38, 3.74, 4.09, 4.80 and 5.15 T and at temperatures of (○) 1.5 K, (□) 2.0 K, (▽) 4.2 K and (△) 18 K.

Rawlings *et al.*, 1977; Eglinton *et al.*, 1980). These signals arise from porphyrin-to-iron charge-transfer transitions and are therefore sensitive in spectral position to the axial ligand field of the Fe(III). When a sample showing the $g=3.40$ and $g=3.02$ signals was examined by m.c.d. spectroscopy in the near-i.r. region, three peaks typical of low-spin haem signals could be seen (see Fig. 4). The peak at 1565 nm is present in the oxidized enzyme and has previously been assigned to oxidized cytochrome *a* (Eglinton *et al.*, 1980; Johnson *et al.*, 1981). The peak at 1950 nm arises from the low-spin cytochrome a_3 centre coupled to Cu_B in the oxidized cyanide-bound enzyme (Thomson *et al.*, 1982). The cytochrome a_3 centre is e.p.r.-silent in this species. In the NO-treated sample this signal was present to 20% that seen in the oxidized cyanide-bound enzyme that has not been exposed to NO. This probably results from some loss of NO during transfer to the m.c.d. cell due to the low affinity of this site for NO (Stevens *et al.*, 1979). NO treatment produces the other low-spin band of this sample at 1785 nm. When this sample was exposed to light the 1785 nm band disappeared whereas that at 1950 nm increased (Fig. 4). The band at 1565 nm was virtually unaffected by this treatment. Thus the photolysed product has spectroscopic qualities indistinguishable from those of the oxidized cyanide-bound enzyme. This result is strong evidence that the species giving rise to the $g=3.40$ signal is the same as the centre having a near-i.r. m.c.d. band at 1785 nm. This is confirmed by the following experiment: when the photolysed m.c.d. sample was warmed to 80 K in the dark for 10 min and then re-cooled to 4.2 K, the 1785 nm m.c.d. band had re-appeared and the 1940 nm band had collapsed. This shows that thermal recombination of NO had occurred.

M.c.d. spectra of chromophores with paramagnetic ground states exhibit signals whose intensities are dependent on temperature and magnetic field (Thomson & Johnson, 1980). Thomson & Johnson. (1980) have shown that the dependence of intensity of an m.c.d. signal on field and temperature may be used to define the ground-state magnetic properties of a paramagnet, and thereby aid in assigning m.c.d. bands to particular centres. We have used this technique to distinguish the low-spin bands of cytochromes *a* and a_3 in the oxidized cyanide-bound enzyme (Thomson *et al.*, 1982). In Fig. 5(*a*) the magnetization plot of the new band at 1785 nm is shown. The smooth form of the curve with all the points on a single line implies a simple doublet ground state (Thomson & Johnson, 1980). At low values of $\beta B/2kT$ (β is the Bohr magneton, B is the magnetic field strength, k is the Boltzmann constant and T the absolute temperature) the signal intensity increases in a linear manner. At high values of

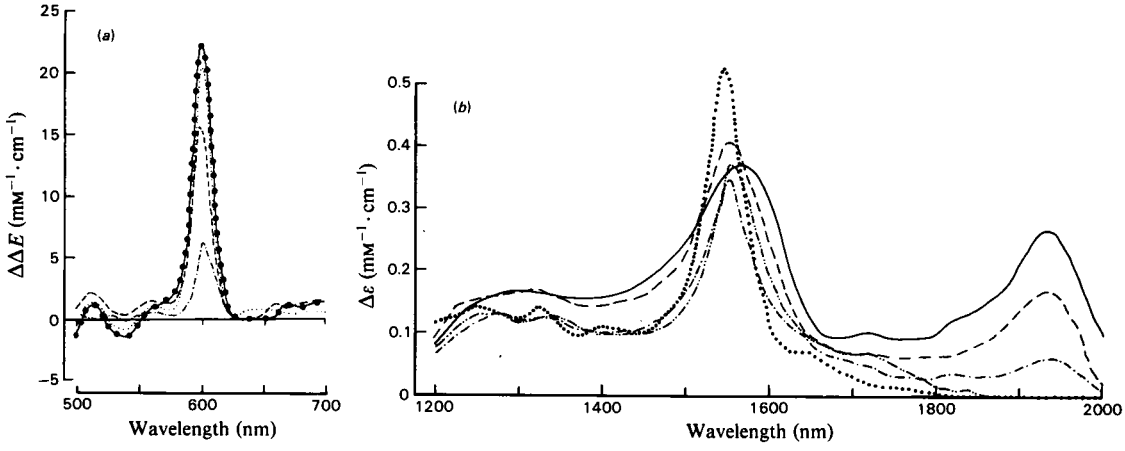

Fig. 6. *Dithionite titration of oxidized cyanide-bound cytochrome c oxidase*

(*a*) Visible-absorption difference spectra monitored at room temperature. The *y*-axis is a double-difference extinction coefficient ($\Delta\Delta E$) comprising the difference between the partially reduced and the oxidized oxidation states and the reference wavelength 620nm. The enzyme concentration was 95μM, with 5mM-KCN in an equal mixture of buffer and ethanediol. Dithionite concentrations were: ----, 120μM; –·–·–, 200μM; –··–··–, 400μM; ·····, 680μM. (*b*) Near-i.r. m.c.d. spectra of the same samples as above in (*a*). The temperature was 4.2K and the field strength 5.1T.

$\beta B/2kT$, corresponding to low temperatures and high values of magnetic field, the signal intensity approaches a limiting value. When the slope of the initial linear portion of the curve is extrapolated to intersect the asymptotic limit a value, I, on the $\beta B/2kT$ scale is obtained. Values of I expected may be obtained from ground-state g-factors observed by e.p.r. spectroscopy (Thomson & Johnson, 1980). The value of the intercept of the species giving an m.c.d. band at 1785nm is 0.39. This compares well with the other low-spin haem groups (see Table 1), and supports our assignment of this band to the same chromophore giving the low-spin ferric $g = 3.40$ e.p.r. signal.

When dithionite is added to oxidized cyanide-bound cytochrome *c* oxidase, all the metal centres except cytochrome a_3 are reduced (Johnson *et al.*, 1981). Fig. 6(*a*) shows the effect of a reductive titration on the α-band of the absorption spectrum of the oxidized cyanide-bound enzyme. The α-band absorbance increases continuously as the reductant concentration is increased. Absorbance in this region is dominated by cytochrome *a*, but there are contributions from other centres. At 1950nm in the near-i.r. m.c.d. spectrum (Fig. 6*b*) the intensity decreases as reduction proceeds, whereas at 1565nm the band sharpens and shifts to 1545nm. The 1950nm m.c.d. band has completely disappeared when the absorbance change of the visible band is only 80% complete. The final near-i.r. spectrum has a single low-spin band at 1545nm arising from the e.p.r.-detectable cytochrome a_3–CN^- species. The magnetization curve of the m.c.d. band at 1545nm from the partially reduced cyanide-bound enzyme is shown in Fig. 5(*b*). Its smooth form indicates a simple doublet magnetic ground state. The intercept value, I (see Table 1), is typical of a low-spin haem species. A value of g_z may be estimated from the m.c.d. data and the g-value anisotropy, and it agrees well with the value estimated from experimental e.p.r. g-values. These results distinguish the two e.p.r.- and near-i.r.-m.c.d.-detectable states obtained by adding NO or reductant to the oxidized cyanide-bound enzyme.

Discussion

The results presented in the preceding section enable a comparison to be drawn between the e.p.r. and near-i.r. m.c.d. spectra of three derivatives of cytochrome *c* oxidase, all of which are bound by CN^- ion. They are the partially reduced, the oxidized and the NO-bound enzyme. The electronic properties of cytochrome a_3^{3+} in these species are summarized in Table 1. Also included for completeness are the properties of cytochrome *a* in the same three derivatives. Cytochrome a_3^{3+} can be detected as the low-spin ferric haem by near-i.r. m.c.d. spectroscopy in all three species, but by e.p.r. spectroscopy only in the partially reduced and NO-bound forms of the enzyme. The structural assignments of the binuclear metal site, a_3–Cu_B, in these derivatives are shown in Fig. 7 and identified

Table 1. *E.p.r. and near-i.r. m.c.d. properties of cytochromes a and a_3 in various derivatives of cytochrome c oxidase*
For experimental details see the text.

Derivatives of cytochrome *c* oxidase	Metal components	Spin	E.p.r. *g*-values	Peak of near-i.r. m.c.d. band (nm)	Intercept value of m.c.d. magnetization plot	Reference
Resting, oxidized	a^{3+}	$S=\frac{1}{2}$	3.02, 2.21, 1.45	1565	0.41	Thomson *et al.* (1982)
Oxidized, cyanide	a^{3+}	$S=\frac{1}{2}$	3.02, 2.21, 1.45	1565	0.40	Johnson *et al.* (1981)
	$a_3{}^{3+}$–CN–$Cu_B{}^{2+}$	$S=1$	Silent	1950	0.29	Thomson *et al.* (1982)
Oxidized, cyanide plus NO	a^{3+}	$S=\frac{1}{2}$	3.02, 2,21, 1.45	1565	0.40	Present work
	$a_3{}^{3+}$–CN–$Cu_B{}^{2+}$–NO	$S=\frac{1}{2}$	3.40*	1785	0.39	Present work
Partially reduced, cyanide	$a_3{}^{3+}$–CN	$S=\frac{1}{2}$	3.58, 1.56, 1.0	1545	0.39	Johnson *et al.* (1981); present work
Metmyoglobin cyanide		$S=\frac{1}{2}$	3.45, 1.89, 0.93	1595	0.40	Eglinton *et al.* (1983)

* Only one component of the e.p.r. spectrum detectable owing to overlap with signals from other centres.

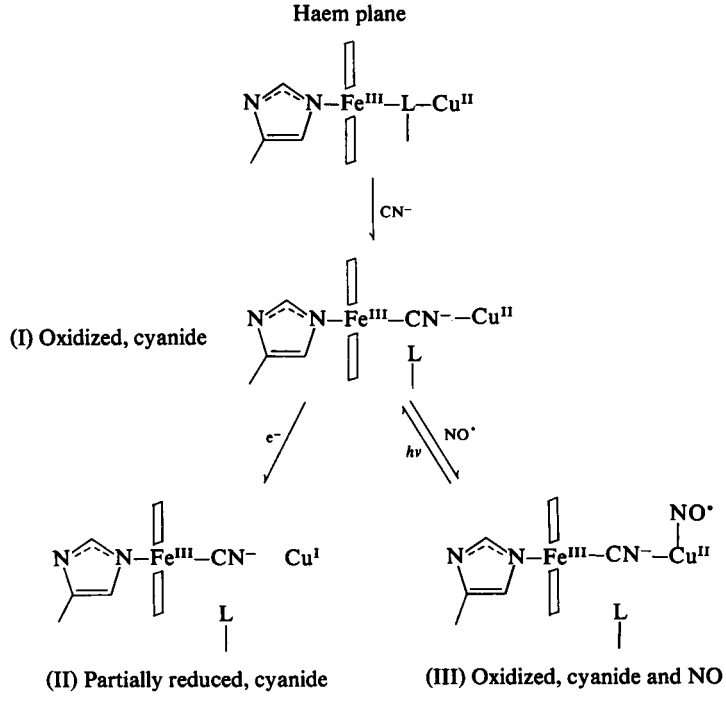

Fig. 7. *Scheme depicting the binding of cyanide and NO to the binuclear cytochrome a_3–Cu_B pair*
Only the Fe of cytochrome a_3 and Cu_B are shown. L refers to the bridging ligand of the resting enzyme. Other ligands to Cu_B are not shown.

by numerals I–III. The detailed arguments leading to these structural formulae are as follows.

When cyanide is added to the resting enzyme it binds in a slow kinetically complex process, replacing any bridging ligand, L, present. This reaction supplied some of the first evidence that the isolated oxidized enzyme exists as a mixture of conformational states with different reactivities towards cyanide (Van Buuren *et al.*, 1972). In the cyanide-bound state the cytochrome a_3–Cu_B site appears closer to homogeneity than in the resting enzyme, as the e.p.r. signals at $g=12$ and $g=6$ have disappeared (Brudvig *et al.*, 1981). Furthermore, the site can be made e.p.r.-detectable by addition of NO, and over 90% of one spin can be recovered by integration. We have demonstrated

that in the cyanide-bound oxidized state of the enzyme cytochrome a_3 is e.p.r.-silent but detectable as low-spin ferric haem with an m.c.d. band at 1946nm with magnetic properties indicative of a ferromagnetic interaction with Cu_B (Thomson *et al.*, 1981, 1982). The effective *g*-values obtained from analysis of the m.c.d. magnetization curves strongly suggest that the bridged binuclear couple, $Fe_{a_3}^{III}$–CN–Cu_B^{II}, is linear (A. J. Thomson, C. Greenwood, D. G. Eglinton & B. C. Hill, unpublished work).

When NO is added to the oxidized cyanide-bound enzyme, cytochrome a_3 becomes e.p.r.-detectable in a complex that is photosensitive. This species cannot involve direct binding of NO to the Fe^{III} ion of cytochrome a_3, for the following two reasons. First, the reaction is not accompanied by any change in the haem visible-absorption spectrum. Secondly, binding of NO to a ferric haem renders it e.p.r.-silent (Johnson *et al.*, 1980), presumably by the addition of an extra electron to the *d*-shell of the low-spin Fe^{III} ion. In the present case the haem is rendered e.p.r.-detectable by the addition of NO. However, the near-i.r. charge-transfer band of cytochrome a_3 is shifted substantially from 1946nm to 1785nm by ligation of NO. Thus the binding of NO is sensed by the Fe^{III} ion of cytochrome a_3. Therefore we propose (structure III in Fig. 7) that NO binds to Cu_B^{2+} without disrupting the cyanide bridge between haem and copper.

Photolytic removal of NO at 4.2K (or 1.5K) leads to the re-formation of the cyanide-bound enzyme, I, with an m.c.d. peak at 1946nm and loss of the haem e.p.r. signal at $g = 3.40$. Since the amount of ligand re-organization that can take place at 4.2K is minimal, we conclude that the cyanide bridge remains intact in structure III. Addition of NO to Cu_B^{2+} leads to a coupling of the spins of the two and a resultant spin $S = 0$. Therefore complex III has overall an odd number of electron spins and is e.p.r.-detectable. The e.p.r. signal is haem-like, since structure III can be regarded as a magnetically coupled species with $S = \frac{1}{2}$ on the haem moiety and $S = 0$ on the Cu^{II} ion. This is expected to give rise to a low-spin ferric haem e.p.r. spectrum only modified by the change to the bridging group. There is a precedent for such a coupling induced by a bridging ligand between a haem group and another metal centre with spin $S = 0$. This is the case of *Escherichia coli* sulphite reductase, in which a $[4Fe–4S]^{2+}$ cluster with spin $S = 0$ is coupled to a high-spin ferric haem, a sirohaem (Christner *et al.*, 1981). Since the spin on the coupled cluster is zero, the e.p.r. signals are those typical of high-spin ferric haem. Note, however, that structure III is spectroscopically distinct from the partially reduced species, II, formed by addition of an electron to the Cu_B of the binuclear metal pair. The *g*-values are different and the positions of the near-i.r. m.c.d. peaks are dissimilar (Table 1).

We have previously argued (Thomson *et al.*, 1982) that the effect of binding the oxidized ion, Cu_B^{2+}, to the N end of the CN^- ion causes the porphyrin-to-Fe^{III}-ion charge-transfer band to be shifted from 1545nm to 1950nm. It seems quite reasonable then that the addition of the ligand NO to Cu_B^{2+} should decrease the oxidizing power of the latter, causing the charge-transfer band to lie at an intermediate wavelength of 1785nm, as in structure III.

The near-i.r. spectrum of structure II, the partially reduced cyanide-bound derivative, is strikingly similar to that of the cyanide-bound form of metmyoglobin (Thomson *et al.*, 1982; Eglinton *et al.*, 1983). The wavelengths of the peaks are similar, and both spectra develop additional fine structure. This result leads us to speculate that cytochrome a_3^{3+} has the same ligands as metmyoglobin cyanide, and therefore that the endogenous ligand is an imidazole N atom of a histidine residue. This has also been shown to be the case in the reduced NO-bound species of cytochrome *c* oxidase (Blokzijl-Homan & Van Gelder, 1971). The wavelength maxima of the near-i.r. m.c.d. bands as well as the g_z resonance values in the e.p.r. spectra distinguish the low-spin forms of cytochrome a_3^{3+} in structures II and III. This distinction was left unclear by the assignment of a g_z value of 3.50 to both these forms of cytochrome a_3^{3+} by Brudvig *et al.* (1981). It seems most probable that in complex II Cu_B^+ does not bind to the CN^- ion and that the cyanide bridge is broken.

In structure II both cytochrome *a* and Cu_A are reduced (Johnson *et al.*, 1981), whereas in both structures I and III these two redox centres are oxidized. Hence there are two possible mechanisms for the breaking of the cyanide bridge on going from structure I to structure III. One is the reduction of Cu_B, and the other is the reduction of either or both of cytochrome *a* and Cu_A. This is an important question to answer, since it may have a functional significance. It could be that oxygen is bound to reduced cytochrome a_3 only in the initial step of its reaction with cytochrome *c* oxidase, but becomes bridged between a_3 and Cu_B in a linear fashion analogous to the binding of CN^- to the oxidized enzyme either when Cu_B or when cytochrome *a* or Cu_A becomes oxidized. If so, this would suggest a mechanism for the tight binding of the intermediates of oxygen reduction as a prerequisite for the breaking of the O–O bond.

The NO-binding reaction with Cu_B occurs only very slowly with our enzyme preparation. In addition, our preparation did not produce a high-spin signal when exposed to NO in the oxidized resting form. Identical results were obtained by us

with a preparation of the oxidase made by the method of Kuboyama *et al.* (1972). This places these preparations along with that described by Yu *et al.* (1975), as examined by Brudvig *et al.* (1981), in a group that does not show rapid reactivity with NO, and distinct from the preparation described by Hartzell & Beinert (1974). This slow reactivity between NO and Cu_B suggests that a conformational change is necessary to expose a Cu_B ligand site and that the conversion rate between the conformers is low.

In structure III both cytochromes *a* and a_3 are completely e.p.r.-detectable simultaneously. This finding gives strong support to the neo-classical model of cytochrome oxidase, which requires distinct spectral properties for cytochromes *a* and a_3 (Wikström *et al.*, 1981). The ability of cytochrome a_3 and Cu_B to bind ligands simultaneously affords new possibilities for the concerted action of these two metals in the reduction of oxygen. In order to bind NO while still attached to CN^-, either Cu_B must lose a ligand or expand its co-ordination number. The latter seems the most likely to us, in view of the known readiness of Cu^{II} ions to accept co-ordination numbers between four and six.

B. C. H. acknowledges the Association of Commonwealth Universities for the award of a Commonwealth Postgraduate Scholarship. C. G. and A. J. T. thank the Science and Engineering Research Council and The Royal Society for grants in support of this work.

References

Aasa, R., Albracht, S. P. J., Falk, K. E., Lanne, B. & Vänngård, T. (1976) *Biochim. Biophys. Acta* **422**, 260–272

Babcock, G. T., Vickery, L. E. & Palmer, G. (1976) *J. Biol. Chem.* **251**, 7907–7919

Blokzijl-Homan, M. F. J. & Van Gelder, B. F. (1971) *Biochim. Biophys. Acta* **234**, 493–498

Boelens, R., Rademaker, H., Pel, R. & Wever, R. (1982) *Biochim. Biophys. Acta* **679**, 84–94

Brudvig, G. W., Stevens, T. H. & Chan, S. I. (1980) *Biochemistry* **19**, 5275–5285

Brudvig, G. W., Stevens, T. H., Morse, R. H. & Chan, S. I. (1981) *Biochemistry* **20**, 3912–3921

Chance, B., Schoener, B. & Yonetani, T. (1965) in *Oxidases and Related Redox Systems* (King, T. E., Mason, H. S. & Morrison, M., eds.), vol. 2, pp. 609–614, John Wiley and Sons, New York

Christner, J. A., Munck, E., Janick, P. A. & Siegel, L. M. (1981) *J. Biol. Chem.* **256**, 2098–2101

De Vries, S. & Albracht, S. P. J. (1979) *Biochim. Biophys. Acta* **549**, 334–340

Eglinton, D. G., Johnson, M. K., Thomson, A. J., Gooding, P. E. & Greenwood, C. (1980) *Biochem. J.* **191**, 319–331

Eglinton, D. G., Gadsby, P. M. A., Sievers, G., Peterson, J. & Thomson, A. J. (1983) *Biochim. Biophys. Acta* **742**, 648–658

Greenaway, F. T., Chan, S. H. P. & Vincow, G. (1977) *Biochim. Biophys. Acta* **490**, 62–78

Greenwood, C., Wilson, M. T. & Brunori, M. (1974) *Biochem. J.* **137**, 205–215

Hagen, W. R. (1982) *Biochim. Biophys. Acta* **708**, 82–98

Harmon, H. J. & Sharrock, M. (1978) *Biochim. Biophys. Acta* **503**, 56–66

Hartzell, C. R. & Beinert, H. (1974) *Biochim. Biophys. Acta* **368**, 318–338

Johnson, M. K., Thomson, A. J., Walsh, T. A., Barber, D. & Greenwood, C. (1980) *Biochem. J.* **189**, 285–294

Johnson, M. K., Eglinton, D. G., Gooding, P. E., Greenwood, C. & Thomson, A. J. (1981) *Biochem. J.* **193**, 699–708

Kuboyama, M., Yong, F. C. & King, T. E. (1972) *J. Biol. Chem.* **247**, 6375–6383

Malmström, B. G. (1979) *Biochim. Biophys. Acta* **549**, 281–303

Palmer, G., Babcock, G. T. & Vickery, L. E. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 2206–2210

Rawlings, J., Stephens, P. J., Nafie, L. A. & Kamen, M. D. (1977) *Biochemistry* **16**, 1725–1729

Stephens, P. J., Sutherland, J. C., Cheng, J. C. & Eaton, W. A. (1974) in *The Excited States of Biological Molecules* (Birks, J. B., ed.), pp. 434–442, John Wiley and Sons, New York

Stevens, T. H., Brudvig, G. W., Bocian, D. F. & Chan, S. I. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3320–3324

Thomson, A. J. & Johnson, M. K. (1980) *Biochem. J.* **191**, 411–420

Thomson, A. J., Brittain, T., Greenwood, C. & Springall, J. (1976) *FEBS Lett.* **67**, 94–98

Thomson, A. J., Johnson, M. K., Greenwood, C. & Gooding, P. E. (1981) *Biochem. J.* **193**, 687–697

Thomson, A. J., Eglinton, D. G., Hill, B. C. & Greenwood, C. (1982) *Biochem. J.* **207**, 167–170

Van Buuren, K. F. H., Nicholls, P. & Van Gelder, B. F. (1972) *Biochim. Biophys. Acta* **256**, 258–276

Van Gelder, B. F. & Beinert, H. (1969) *Biochim. Biophys. Acta* **189**, 1–24

Wikström, M., Krab, K. & Saraste, M. (1981) *Cytochrome Oxidase*, Academic Press, London

Wilson, D. F. & Miyata, Y. (1977) *Biochim. Biophys. Acta* **461**, 218–230

Yonetani, T. (1960) *J. Biol. Chem.* **235**, 845–852

Yoshida, S., Hori, H. & Orii, Y. (1980) *J. Biochem. (Tokyo)* **88**, 1623–1627

Yu, C., Yu, L. & King, T. E. (1975) *J. Biol. Chem.* **250**, 1383–1392