Titration and steady-state behaviour of the 830 nm chromophore in cytochrome *c* oxidase

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Titration of cyanide-incubated cytochrome *c* oxidase (ox heart cytochrome *aa*₃) with ferrocycytochrome *c* or with *NNN'**N'*-tetramethyl-*p*-phenylenediamine initially introduces two reducing equivalents per mol of cytochrome *aa*₃. The first equivalent reduces the cytochrome *a* haem iron; the second reducing equivalent is not associated with reduction of the 830 nm chromophores (e.p.r.-detectable copper) but is probably required for reduction of the e.p.r.-undetectable copper. Excess reductant introduces a third reducing equivalent into the cyanide complex of cytochrome *aa*₃. During steady-state respiration in the presence of cytochrome *c* and ascorbate, the 830 nm chromophore is almost completely oxidized. It is reduced more slowly than cytochrome *a* on anaerobiosis. In the presence of formate or azide, some reduction at 830 nm can be seen in the steady state; in an oxygen-pulsed system, a decrease in steady-state reduction of cytochromes *c* and *a* is associated with an increased reduction of the 830 nm species. In the formate-inhibited system the reduction of *a*₃ on anaerobiosis shows a lag phase, the duration of which corresponds to the time taken for the 830 nm species to be reduced. It is concluded that the e.p.r.-undetectable copper (Cu_U) is reduced early in the reaction sequence, whereas the detectable copper (Cu_D) is reduced late. The latter species is probably that responsible for reduction of the cytochrome *a*₃ haem. The magnetic association between undetectable copper and the *a*₃ haem may not imply capability for electron transfer, which occurs more readily between cytochrome *a*₃ and the 830 nm species.

Cytochrome *c* oxidase, the terminal enzyme of the eukaryotic respiratory chain, contains four redox centres, two haems (cytochromes *a* and *a*₃) and two copper atoms (see Erecinska & Wilson, 1978). The two cytochrome components are characterized by their different absorbances in the Soret and visible regions of the spectrum; and a single absorption band at 830 nm is seen, possibly characteristic of a copper centre or centres. In the 'resting' ferric enzyme, one haem and one copper centre are e.p.r.-detectable, whereas the other haem and copper are undetectable, indicating a probable magnetic interaction (van Gelder & Beinert, 1969). The e.p.r.-detectable haem is essentially identical with the component identified spectrophotometrically as ferric cytochrome *a* (Hartzell & Beinert, 1974). The e.p.r.-detectable copper, with a signal at *g* = 2 in the cupric state, is closely related to the 830 nm

chromophore (Beinert *et al.*, 1980). The final and the 830 nm band appear and disappear concertedly on oxidoreduction of the native enzyme (Babcock *et al.*, 1978), its CO-inhibited form (Wever *et al.*, 1977) and its cyanide complexes (Yong & King, 1972). Contributions from the e.p.r.-undetectable copper to the 830 nm band, suggested by Powers *et al.* (1979), must be quantitatively very small (Beinert *et al.*, 1980).

When the enzyme is reduced in the presence of cyanide, cytochrome *a* becomes ferrous and the 830 nm chromophore is bleached. At the same time, the cyanocycytochrome *a*₃ centre, which remains ferric, becomes e.p.r.-detectable, with an absorption at *g* = 3.58 (Shaw *et al.*, 1978). Yong & King (1972) found that two oxidizing equivalents were taken up in the oxidation of 1 mol of the partially reduced cyanide-inhibited enzyme (cytochrome *a*²⁺*a*₃³⁺HCN). Similarly, Nicholls & Hildebrandt (1978a) claimed that the reduction of the cyanocycytochrome *aa*₃ complex involved only the cyto-

Abbreviation used: TMPD, *NNN'**N'*-tetramethyl-*p*-phenylenediamine.

chrome *a* and e.p.r.-detectable (830 nm) copper. Titration with either cytochrome *c* or TMPD showed that two reducing equivalents are initially needed per cytochrome *a* reduced. But this conclusion has recently been challenged (Johnson *et al.*, 1981); the latter workers report that the cyanide-inhibited partially reduced enzyme contains at least three reducing equivalents, indicating reduction of both copper atoms as well as one haem group (cytochrome *a*). Nicholls *et al.* (1972) had also claimed that the species formed by oxygenation of the species $a^{2+}a_3^{2+}\text{HCN}$ was identical with that produced by reduction of the form $a^{3+}a_3^{3+}\text{HCN}$. If the claims of Nicholls *et al.* (1972) be true, then any revision of the assigned redox state for the partially reduced species will entail a corresponding revision of the redox state assigned to the partially oxidized form.

We have therefore re-examined the titration behaviour of the oxidase in the presence of cyanide, by using higher concentrations of enzyme and monitoring the 830 nm band presumed to be due to e.p.r.-detectable cupric copper; we have also carried out experiments with uninhibited and partially inhibited systems to analyse the steady-state behaviour of this chromophore. And we have recalculated some of the titration results previously reported in the literature (Wever *et al.*, 1977; Babcock *et al.*, 1978; Schroedl & Hartzell, 1977a) in order to compare the behaviour of the two copper atoms in the enzyme under different conditions.

We conclude that the criticisms of Johnson *et al.* (1981) are indeed well-founded, and that excess reductant permits the introduction of three reducing equivalents into cyano-cytochrome aa_3 . This conclusion leads to some reassignment of roles for the two copper atoms in the functioning of cytochrome *c* oxidase.

Materials and methods

Ox heart cytochrome *c* oxidase (cytochrome aa_3) was prepared by the method of Kuboyama *et al.* (1972) and stored at -75°C , at a concentration of approx. $320\ \mu\text{M}$, in 100 mM-sodium phosphate buffer (pH 7.4)/0.25% Tween 80 solution as described by Nicholls & Hildebrandt (1978b). Cytochrome *c* (horse heart, type VI), sodium ascorbate, NaN_3 , sodium formate and $\text{NNN}'\text{N}'$ -tetramethyl-*p*-phenylenediamine (TMPD) as dihydrochloride were from Sigma Chemical Co., St. Louis, MO, U.S.A. KCN (AnalaR) was from BDH, Toronto, Canada, and CO was a Linde Speciality Gas from Union Carbide Co., Toronto, Canada.

The cytochrome $aa_3\text{-CN}^-$ complex was formed by incubating $20\ \mu\text{M}$ -cytochrome aa_3 with 2.1 mM-KCN for 3 days in 67 mM-sodium phosphate buffer (pH 7.4)/0.015% Tween 80 at 4°C . Tween 80 was

not added to the experimental reaction medium, and therefore for experiments with $3\ \mu\text{M}$ -cytochrome aa_3 its final concentration was 0.002%, whereas for experiments with $20\ \mu\text{M}$ -cytochrome aa_3 its final concentration was 0.015%. Cytochrome *c* was reduced with a small portion of dithionite and then passed through a Sephadex G-25 column to remove excess dithionite and oxidation products.

The aerobic reduction of the cytochrome aa_3 -cyanide complex was monitored spectrophotometrically as described by Nicholls & Hildebrandt (1978a) with standard 1 cm cuvettes containing approx. 3 ml of cyano-cytochrome aa_3 solution. Additions were made either of portions of reduced cytochrome *c* (c^{2+}) or of portions of TMPD in the presence or absence of oxidized cytochrome *c* (c^{3+}). The subsequent distribution of reducing equivalents was monitored for cytochrome *a*, cytochrome *c* and the 830 nm chromophore. The wavelengths and absorption coefficients employed were: cytochrome *a* at 605–630 nm ($\Delta\epsilon_{\text{mM}} = 22.7$) or 605–619 nm ($\Delta\epsilon_{\text{mM}} = 18.8$), cytochrome *c* at 550–540 nm ($\Delta\epsilon_{\text{mM}} = 19.8$, reduced; $\Delta\epsilon_{\text{mM}} = 2.0$, oxidized) and the e.p.r.-detectable copper, Cu_D , at 830–740 nm ($\Delta\epsilon_{\text{mM}} = 1.0$). Higher ($20\ \mu\text{M}$) concentrations of cytochrome aa_3 were used for the experiments at the latter wavelengths. Approximate redox potentials for cytochromes *a* and *c* were calculated by using the Nernst equation.

The aerobic steady-state behaviour of cytochrome *a* (605–630 nm), cytochrome a_3 (445–470 nm), cytochrome *c* (550–540 nm) and the 830 nm chromophore (830–740 nm) was monitored essentially as described by Yonetani (1960) and by Nicholls (1976a,b). An aerobic ($235\ \mu\text{M-O}_2$) solution containing cytochrome aa_3 in the absence or in the presence of cytochrome *c* was treated with reductant (ascorbate plus various amounts of TMPD) and the absorbance changes monitored until anaerobiosis supervened. After anaerobiosis a brief pulse of air mixed into the open cuvette induced a new steady state. Similar experiments were carried out in the presence of the terminal inhibitors formate (Nicholls, 1976a) and azide. All spectral studies were carried out at 30°C with an Aminco DW-2 instrument either in the double-wavelength or in the split-beam mode; the '>600 nm' filter provided was used in the 680–860 nm region. For further experimental details, see the Figure legends.

Results

Fig. 1 illustrates the results of titrating cyano-cytochrome aa_3 as described by Nicholls & Hildebrandt (1978a), including measurements at 830 nm as well as in the visible region. With ferrocytochrome *c* as titrant (Fig. 1a) the 830 nm region is obscured by absorption increases produced by

Fig. 1. Titrations of the cytochrome *aa*₃ complex

(a) Titration with ferrocytochrome *c*. Cytochrome *aa*₃ (20 μM) was incubated for 96 h with 2 mM-KCN in 67 mM-sodium phosphate buffer, pH 7.4, at 4°C. Titration was carried out in a 3 ml cuvette at 30°C with portions of reduced cytochrome *c* solution (see the Materials and methods section). Δ, Changes at 830–740 nm corrected for ferricytochrome *c* ($\Delta\epsilon_{\text{nm}} = 0.37$), Cu_D^+ ; ●, changes at 605–630 nm (cytochrome *a*²⁺); ○, changes at 550–540 nm (cytochrome *c*²⁺), using correction factor for ferricytochrome *c*; □, sum of $[c^{2+}] + 2[a^{2+}]$; 1, line of equality (μ -equiv. found = μ -equiv. added); 2, expected asymptote for *c*²⁺ if only 2 equiv. are taken up by the enzyme; 3, expected asymptote for *c*²⁺ if only 3 equiv. are taken up by the enzyme. (b) Titration with TMPD. Conditions were as in (a). Δ, Changes at 830–740 nm (Cu_D^+); ●, changes at 605–619 nm (*a*²⁺); □, sum of $2[a^{2+}] + [\text{Cu}_D^+]$; ■, changes at 630–650 nm (TMPD⁺). (c) Titration with TMPD in the presence of cytochrome *c*. Conditions were as in (a). Δ, Changes at 830–740 nm (Cu_D^+); ●, changes at 605–619 nm (*a*²⁺); ○, changes at 550–581 nm (*c*²⁺); □, sum of $[c^{2+}] + 2[a^{2+}] + [\text{Cu}_D^+]$; ■, changes at 630–650 nm (TMPD⁺).

ferricytochrome *c*. It is clear, however, that very little absorbance is bleached during the early part of the titration, although the ratio of *c* oxidized to *a* reduced is close to 2.0, as previously reported. Cytochrome *a* titrates as a single species (one electron acceptor) with a redox potential some 30 mV more positive than cytochrome *c* under these conditions (cf. Erecinska & Wilson, 1978). Calculations from the data in Fig. 1(a) confirm this, and indicate that the extra reducing equivalent is taken up at a redox potential close to that of cytochrome *a*.

When TMPD is used, the disappearance of reductant may be monitored by the appearance of the oxidation product, Wurster's Blue (TMPD⁺) (Fig. 1b), and both cytochrome *a* and the 830 nm chromophore are seen to be reduced. Cytochrome *a* titrates as a one-electron acceptor with a calculated redox potential some 45 mV more positive than that of the TMPD/TMPD⁺ couple. The 830 nm chromophore, however, hardly changes until more than 20 μ-equiv. of TMPD/litre have been added and the cytochrome *a* is nearly 50% reduced. The reduction then proceeds 'sigmoidally', suggesting that it depends upon the prior reduction of some other acceptor (see the Discussion section). As in the titration with cytochrome *c*, however, the initial reduction of the cytochrome *a* iron centre from the

ferric to the ferrous state is accompanied by the disappearance of a second reducing equivalent.

Fig. 1(c) shows these responses in a system containing equal amounts of ferricytochrome *c* and cytochrome *c* oxidase. The initial reduction of cytochromes *a* and *c* is accompanied by the appearance of extra TMPD⁺. We attribute this (see the Discussion section) to the reduction of a species approximately equal in concentration and apparent redox potential to the cytochrome *a*. At the same time the 830 nm chromophore is hardly reduced, indicating an initial potential at least 40 mV more negative than that of cytochrome *c*. However, towards the end of the titration some bleaching of the 830 nm absorbance occurs, as in Fig. 1(b).

The unexpected titration behaviour of the 830 nm chromophore prompted examination of its steady-state behaviour. Four types of steady-state system were examined: (i) the ascorbate-*c-aa*₃-O₂ system; (ii) the ascorbate-TMPD-*c-aa*₃-O₂ system commencing with 'resting' ferric enzyme; (iii) the ascorbate-TMPD-*c-aa*₃-O₂ system commencing with reduced enzyme; and (iv) the ascorbate-TMPD-*aa*₃-O₂ system, in which TMPD acts directly as a weak substrate (Kimmelberg & Nicholls, 1969).

Fig. 2 summarizes the results obtained in the first

Fig. 2. Steady-state reduction of cytochromes *c* and *a* as a function of ascorbate concentration and flux

(a) Percentage reduction plotted against ascorbate added. Cytochrome *aa*₃ (4.0 μM) plus 4.0 μM -cytochrome *c* in 67 mM-sodium phosphate buffer, pH 7.4 (30°C) were treated with ascorbate at the indicated concentrations and the percentage reduction monitored in the aerobic steady state. Δ , Changes at 550–540 nm (cytochrome *c*); $\circ$, changes at 605–630 nm (cytochrome *a*); $\square$, changes at 445–470 nm (cytochromes *a* and *a*₃). (b) Respiration rate plotted against percentage reduction. Experiments were as in (a) and the average rate of respiration was calculated by dividing four times the oxygen concentration in solution ($4 \times 235 \mu\text{M}$) by the time taken to anaerobiosis, to give results in $\mu\text{equiv.} \cdot \text{s}^{-1} \cdot \text{litre}^{-1}$. Δ , Changes at 550–540 nm (cytochrome *c*); $\circ$, changes at 605–630 nm (cytochrome *a*).

of these systems. Fig. 2(a) shows that cytochrome *a* is markedly more reduced than cytochrome *c* (cf. Yonetani, 1960), but the relationship is not simple. Although the rate of respiration is proportional to the amount of ferrocytochrome *c* present, it is not proportional to the amount of reduced cytochrome *a*

Fig. 3. Plot of the flux ratio, Γ , for the cytochrome *c*/cytochrome *a* system against the apparent respiration rate constant

The ratio $[a^{2+}][c^{3+}]/[a^{3+}][c^{2+}]$, or ' Γ ', is plotted against $v/[c^{2+}][a^{3+}]$ for experiments such as those in Fig. 2, in the absence and presence of TMPD. Cytochrome *aa*₃ (4 μM) plus 4 μM -cytochrome *c* in 67 mM-sodium phosphate buffer, pH 7.4 at 30°C, were incubated aerobically with various amounts of ascorbate and TMPD. Velocity is measured in $\mu\text{equiv.} \cdot \text{s}^{-1} \cdot \text{litre}^{-1}$. Δ , Ascorbate alone (5–40 mM); $\circ$, 5 mM-ascorbate plus 20–60 μM -TMPD, starting from 'resting' enzyme; $\bullet$, 5 mM-ascorbate plus 20–60 μM -TMPD, starting from fully reduced enzyme and then 'pulsing' with air.

(Fig. 2b). The ratio $[a^{2+}][c^{3+}]/[a^{3+}][c^{2+}]$, or Γ (Nicholls, 1976b), is a function of respiratory flux, as shown in Fig. 3, which includes data from each of the first three types of steady-state system. In the absence of cytochrome *c* (system iv), the respiration rate is directly proportional to TMPD concentration, with a rate constant (eqn. 1) equal to the rate constant for reduction of cytochrome *a* by TMPD in the stopped-flow system (Kimmelberg & Nicholls, 1969).

$$v = 10^4 \times [aa_3] \times [\text{TMPD}] \quad (1)$$

At high TMPD concentrations, slight (<20%) and equal reductions are seen at 605–630 nm and 830–740 nm, presumably due to rather inactive enzyme molecules, in which all species except cytochrome *a*₃ are reduced (results not shown). In the presence of cytochrome *c* (systems ii and iii), reduction of the 830 nm chromophore cannot be detected with the present spectrophotometric technique in steady-state systems, even when cytochrome *a* is as much as 40% reduced (Fig. 4). The TMPD-induced steady state is characterized by a slight increase in absorbance measured at 830–740 nm. The reduced cytochrome *a* is not accompanied by an equivalent amount of reduced 'e.p.r.-detectable copper' (see the Discussion section).

Reduction of the 830 nm component can be secured under steady-state conditions in the presence of a terminal inhibitor. Fig. 5 shows experiments carried out in the presence of azide, whereas

Fig. 4. Steady-state reduction of cytochromes *a* and *c*, and the 830 nm chromophore, in a complete *c-aa₃* system. Cytochrome *aa₃* (20 μM) plus 20 μM-cytochrome *c* were incubated aerobically in 67 mM-sodium phosphate, pH 7.4 at 30°C. Steady-state reduction was induced by the addition of 5 mM-sodium ascorbate and the indicated amounts of TMPD. Percentage reduction at three pairs of wavelengths is plotted for the 'resting' and 'pulsed' oxidase systems against the TMPD concentrations. Δ, ▲, Reduction at 550–540 nm (cytochrome *c*); ○, ●, reduction at 605–630 nm (cytochrome *a*); □, ■, steady-state changes at 830–740 nm; open symbols, reaction was started from 'resting' ferric enzyme; closed symbols, reaction was started from reduced enzyme by 'pulsing' with air (cf. Fig. 3).

Fig. 5. Steady-state reductions of the cytochrome *c*-cytochrome *aa₃* system in the presence of NaN_3 . Cytochrome *aa₃* (20 μM) plus 20 μM-cytochrome *c* were incubated aerobically in 67 mM-sodium phosphate, pH 7.4 at 30°C, in the presence of 0.8 mM- NaN_3 . Steady-state reduction was induced by the addition of 5 mM-sodium ascorbate and various quantities of TMPD as indicated, Δ, ▲. Steady-state reduction at 550–540 nm; ○, ●, steady-state reduction at 605–630 nm; □, ■, (upper scale), reduction at 830–740 nm; open symbols, reaction was started from 'resting' ferric enzyme; closed symbols, reaction was started from reduced enzyme by 'pulsing' with air (cf. Fig. 3).

Fig. 6 summarizes the corresponding results obtained with formate in the system. The steady-state response of the 830 nm chromophore is opposite to that of cytochrome *a* when experiments commencing with 'resting' and with 'reduced' enzyme are compared. With increased flux, cytochrome *a* is less reduced, but the 830 nm species is more reduced. One of the effects of 'pulsing' the reduced oxidase may thus be pinpointed as lying between cytochrome *a* and the 830 nm species, which gives rise to a 'cross-over' point under these conditions.

When the formate-inhibited system becomes anaerobic (Fig. 7), because the release of the inhibitor from cytochrome *a₃* (Fig. 7b) is slower than that of azide (Fig. 7a), clearly biphasic reduction steps are seen at 605–630 nm; similar changes (results not shown) were observed at 445–455 nm.

The first step represents the reduction of the residual cytochrome *a*, in near equilibrium with cytochrome *c* but at a more positive standard redox potential. The second step, also observed by Brittain *et al.* (1977), represents the reduction of cytochrome *a₃*, and is limited by the rate of formate dissociation at about 0.03 s^{-1} . But in between a plateau is seen, indicating that the component responsible for the reduction of cytochrome *a₃* has itself not yet been reduced. It will be argued below that this component is in fact the 830 nm chromophore, i.e., the 'detectable' copper in the enzyme (see the Discussion section).

Discussion

Chance & Leigh (1977) and Powers *et al.* (1979) have suggested that the 830 nm chromophore contains components from both e.p.r.-detectable and

Fig. 6. Steady-state reductions of the cytochrome *c*-cytochrome *aa*₃ system in the presence of sodium formate. Conditions were the same as those in Fig. 5 with the exception that 0.1 M-sodium formate was present instead of NaN₃. Δ , Steady-state reduction at 550–540 nm; $\circ$, steady-state reduction at 605–630 nm; $\square$, steady-state reduction at 830–740 nm. The arrow indicates the maximum 'rapid' reduction at 605–630 nm obtained on anaerobiosis (i.e. the apparent contribution due to cytochrome *a*). N.B. A 'pulsed' system is not possible in the presence of formate, owing to the slow rebinding of the ligand.

-undetectable copper atoms, although this conclusion has been rejected by Beinert *et al.* (1980). The present results are more consistent with the latter viewpoint. The extra reducing equivalent taken up by the enzyme in the initial phase of the titrations is not associated with any 830 nm change. The absorption band at 830 nm measured under the present conditions was fully developed in both resting and cyanide-blocked enzyme, and completely abolished in fully reduced and cyanide-inhibited reduced enzyme. Mixed-valence species of the other type, in which cytochrome *a*₃ is reduced and cytochrome *a* oxidized, show a slight diminution of the 830 nm absorbance in difference spectra associated with some haem contribution to the slope of the baseline (cf. B. C. Hill & C. Greenwood, personal communication). It is now clear that the copper atom initially reduced in the titration of the cyanide-cytochrome *aa*₃ complex with reductant is not this 830 nm (e.p.r.-detectable) copper, but the other (e.p.r.-undetectable) copper. We have analysed the anaerobic titrations reported by Schroedl & Hartzell (1977a), Wever *et al.* (1977), Babcock *et al.* (1978) and Johnson *et al.* (1981) and find their

results consistent with this proposal. In the presence or absence of a terminal inhibitor, the e.p.r.-undetectable acceptor is the first to be reduced (Wever *et al.*, 1977; Babcock *et al.*, 1978) and the last to be oxidized when starting from fully reduced enzyme (Schroedl & Hartzell, 1977a,b,c).

In the presence of CO, this acceptor and the *a*₃ haem are reduced concurrently (Wever *et al.*, 1977); the last two electrons go to cytochrome *a* and the 830 nm copper. Conversely, on oxidation with ferricyanide, the latter two species are the first to disappear (Schroedl & Hartzell, 1977a). Both in the presence and in the absence of CO, the 830 nm copper titrates 'anomalously', first behaving as a more negative redox centre than cytochrome *a*, but later titrating before cytochrome *a* as if it had a more positive redox potential. Such sigmoidal titration curves were also seen in the present experiments (Fig. 1c) and in the dithionite titrations of Johnson *et al.* (1981).

When the evidence from e.p.r. and m.c.d. measurements (Babcock *et al.*, 1978; Carithers & Palmer, 1981) is also taken into account, the following conclusions can be drawn.

(i) The $g = 2$ e.p.r. signal and the 830 nm band always titrate together, and nearly always show a 'lag' while some other components are reduced.

(ii) The 'undetectable' component (the reduction of which can be estimated by difference) is always reduced first, albeit in parallel with *a*₃ in the presence of CO.

(iii) In the absence of ligands, cytochromes *a* and *a*₃ are reduced together, the reduction of *a* usually slightly preceding that of *a*₃.

(iv) The disappearance of the $g = 3$ (low-spin iron) signal parallels or slightly precedes the reduction of cytochrome *a* both in the presence and absence of cyanide.

(v) $g = 6$ (high-spin iron) signals appear in the absence of ligands as cytochrome *a* and 'undetectable' copper are reduced, and disappear on reduction of cytochrome *a*₃.

(vi) Lastly, the appearance of the $g = 3.6$ (low-spin iron cyanide) signal is associated with the reduction of cytochromes *a* and undetectable copper.

Both in the presence and absence of ligands, the e.p.r.-undetectable copper is apparently reduced early, which releases the interaction with the *a*₃ centre. The inhibitor-dependent low-spin iron signals are evidently due to the ferric cytochrome *a*₃ centre in the presence of cuprous e.p.r.-undetectable copper. The $g = 6$ signals seen in the absence of ligands may similarly be attributed to a cytochrome *a*₃ Fe³⁺Cu⁺ centre. However, there are at least two kinds of $g = 6$ signal, an axial and a rhombic type, and the ratio between the two forms is not constant (Hartzell & Beinert, 1976; Beinert & Shaw, 1977). It

Fig. 7. Transition to anaerobiosis in the presence of terminal inhibitors

(a) Transitions in the presence of 800 μ M-azide. Experimental conditions were identical with those in Fig. 5. Changes are seen on the transition from aerobic steady state to anaerobiosis in a system containing 20 μ M-cytochrome *c* and 20 μ M-cytochrome *aa₃*. a, changes at 605–630 nm; b, changes at 830–740 nm; c, changes at 550–540 nm; (i) in the presence of 5 mM-ascorbate (asc.) alone; (ii) in the presence of 5 mM-ascorbate plus 60 μ M-TMPD. (b) Transitions in the presence of 0.1 M-formate. Experimental conditions were identical with those in Fig. 6, and the technique was as described in (a), except that 0.1 M-sodium formate was present instead of 0.8 mM- NaN_3 . a, changes at 605–630 nm; b, changes at 830–740 nm; c, changes at 550–540 nm; (i) in the presence of 5 mM-ascorbate alone; (ii) in the presence of 5 mM-ascorbate plus 60 μ M-TMPD.

is also possible to prepare samples of enzyme by reoxidation in which an appreciable proportion of the *aa₃* complexes present show both high-spin ($g = 6$) and low-spin ($g = 3, 1.5$) signals in amounts indicating that both haems are paramagnetically detectable (Beinert & Shaw, 1977). Under such conditions e.p.r.-undetectable copper must be less readily oxidized than cytochrome *a*.

If the 830 nm component behaves as if magnetically independent of the other three components, why does it show a sigmoid titration curve? Babcock *et al.* (1978) make the ingenious suggestion that this species is equilibrating with a high-potential haem pair over the first half of the titration and a low-potential haem pair over the second half of the titration. Unfortunately this does

not account for the similar responses of the Cu_D component in the CO system, where the redox titrations of the two haem groups are quite separate (Wever *et al.*, 1977), or the cyanide system, in which only cytochrome *a* is titrated (Johnson *et al.*, 1981). Another possibility is that the 830 nm copper cannot be reduced until the other copper centre has been reduced. Tiesjema *et al.* (1973) reported that the two copper centres could act as a two-electron acceptor system under certain conditions; Carithers & Palmer (1981), however, find that the $g = 2$ signal behaves as if it originated from a simple one-electron centre, even when haem-haem interaction between the cytochromes is observed. These latter workers attribute titration anomalies to the occurrence of time-dependent transitions in the enzyme protein.

The kinetic (steady-state) relationship between cytochrome *a* and the two copper centres remains uncertain. Under steady-state conditions, cytochromes *a* and *c* are classically considered at (Yonetani, 1960), or close to (Minnaert, 1961), equilibrium. In such a system the flux can be represented by eqn. (2):

For this electron-transfer chain, we have:

$$v/[c^{2+}][a^{3+}] = k_{-2}(K_{\text{eq.}} - \Gamma) \quad (3)$$

where $\Gamma = [a^{2+}][c^{3+}]/[a^{3+}][c^{2+}]$ in the steady state (Nicholls, 1976b). A plot of the ratio Γ against $v/[c^{2+}][a^{3+}]$ as in Fig. 3 should give a straight line extrapolating to $K_{\text{eq.}}$ as $v/[c^{2+}][a^{3+}] \rightarrow 0$ and to k_{+2} as $\Gamma \rightarrow 0$. Fig. 3 shows that these extrapolations give values of $K_{\text{eq.}} = 4$ and $k_{+2} = 65 \text{ s}^{-1}$. When the reaction is started from reduced enzyme by admitting oxygen [generating the 'pulsed' enzyme of Antonini *et al.* (1977)] the rate also seems to be proportional to the percentage of reduced cytochrome *a*, with a similar rate constant. In the systems commencing with 'resting' enzyme, the percentage reduction of cytochrome *a* in the steady state is much higher, indicating that the effect of pulsing with oxygen is to increase the rate of electron transfer from cytochrome *a* to a subsequent component, perhaps the 830 nm chromophore (Fig. 5).

Upon anaerobiosis cytochrome a_3 is the last component to become reduced; the lag in the reduction of cytochrome a_3 upon anaerobiosis seems to be associated with the need to reduce the detectable copper (Fig. 7). Although not seen by Brittain *et al.* (1977) under slightly different conditions, this phenomenon has so far not yielded to 'trivial' explanations.

The 830 nm species is oxidized much more rapidly than cytochrome *a* in the flow-flash system (Gibson & Greenwood, 1965) and can be reduced by

ferrocycytochrome a_3 when the latter is formed by photolysis of the mixed-valence CO complex (Boelens & Wever, 1980, 1981). Cytochrome *c* is initially a poor reductant of cytochrome a_3 . Yet under steady-state conditions, when up to 100 molecules of O_2 are metabolized/s by each cytochrome aa_3 centre, cytochrome a_3 reduction must take less than 10 ms. Eqn. (4) summarizes the pattern involved:

where arrows indicate electron transfer and broken lines indicate 'allosteric' interactions between centres. According to eqn. (4) and contrary to Nicholls & Hildebrandt (1978a), the action of cyanide is to bind ferric cytochrome a_3 and to block its reduction by the e.p.r.-detectable copper centre (Cu_D). Cyanocycytochrome aa_3 in the presence of excess reductant therefore contains three reducing equivalents. A spectroscopically similar product (cytochrome $a^{2+}a_3^{3+}\text{HCN}$) is obtained by oxygenating the anaerobic cyanide complex, cytochrome $a^{2+}a_3^{2+}\text{HCN}$ (Nicholls *et al.*, 1972). If it too has the structure $a^{2+}\text{Cu}_D^+a_3^{3+}\text{HCNCu}_U^+$, then its formation involves a one-electron rather than a two-electron reduction of oxygen.

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