Zinc cytochrome *c* fluorescence as a probe for conformational changes in cytochrome *c* oxidase

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Zinc cytochrome *c* forms tight 1:1 complexes with a variety of derivatives of cytochrome *c* oxidase. On complex-formation the fluorescence of zinc cytochrome *c* is diminished. Titrations of zinc cytochrome *c* with cytochrome *c* oxidase, followed through the fluorescence emission of the former, have yielded both binding constants ($K \sim 7 \times 10^6 \text{ M}^{-1}$ for the fully oxidized and $2 \times 10^7 \text{ M}^{-1}$ for the fully reduced enzyme) and distance information. Comparison of steady-state measurements obtained by absorbance and fluorescence spectroscopy in the presence and in the absence of cyanide show that it is the reduction of cytochrome *a* and/or Cu_A that triggers a conformational change: this increases the zinc cytochrome *c* to acceptor (most probably cytochrome *a* itself) distance by some 0.5 nm. Ligand binding to the fully oxidized or fully reduced enzyme leaves the extent of fluorescence quenching unchanged, whereas binding of cyanide to the half-reduced enzyme ($a^{2+}\text{Cu}_A^+\text{Cu}_B^{2+}\text{CN}^-a_3^{3+}$) enhances fluorescence emission relative to that for the fully reduced enzyme, implying further relative movement of donor and acceptor.

INTRODUCTION

Cytochrome *c* oxidase is the terminal electron acceptor of the mitochondrial electron-transfer chain. It catalyses the reduction of molecular O_2 to water, transferring electrons from cytochrome *c*. The study of this enzyme has increased greatly over recent years in recognition of its central importance in cellular respiration, and we now possess detailed information concerning both its structure and its functional properties (see, e.g., refs. [1–4]). These studies have also brought to light the complexity of the enzyme's mechanism and the subtlety with which it performs its function, and we are still some considerable way from understanding the full details of its mechanism. Both the electron-transfer and O_2 -activation steps are incompletely understood, and the proton-pumping function of the enzyme, although now apparently well established [5–8], is comprehended in broad outline only. It is now certain that conformational changes do accompany the enzyme's catalytic cycle and may constitute part of the mechanism of proton pumping [9–11]. However, as yet we do not have much information concerning the conformational dynamics of the enzyme, and our knowledge of the nature and extent of conformational changes is rudimentary.

An approach that may be useful for studying these conformational changes is offered by the technique of fluorescence-resonance-energy transfer. This technique has been employed by Dockter *et al.* [12] to map distances between bound fluorescent probes and the metal centres in yeast cytochrome *c* oxidase. Their approach depended on measuring the fluorescence lifetimes of fluorescent probes either when bound to a calibrant protein bovine serum albumin or to cytochrome *c* oxidase. This latter partially 'quenches' the fluorescence and leads to a shorter fluorescence lifetime. It is considered that this 'quenching' results from resonance-energy transfer from the donor fluorescent probe to an acceptor haem group, which offers efficient de-excitation

pathways. The efficiency of the energy transfer depends on the proximity and the relative orientation of the donor and acceptor.

Our aim in the present work has been to investigate the nature and extent of conformational change that accompany changes in the enzyme's redox and ligation state. The approach has been similar to that used by others [12–16], but, although we have been able to make estimates of the absolute magnitude of donor-acceptor distance, our emphasis has been to establish the size of distance changes caused by electron transfer or ligand binding. Of equal importance has been to determine which of the centres in cytochrome *c* oxidase triggers the conformation change when it itself is reduced and/or binds ligands.

To achieve some of these objectives we have employed zinc cytochrome *c* as a fluorescent probe, to monitor through its fluorescence emission such conformational changes in the enzyme. This probe is useful as it forms (at low ionic strength) a tight 1:1 complex with the enzyme, binding at the same site at which the enzyme's natural substrate, cytochrome *c*, binds [17].

A preliminary report of this work has been presented at a meeting of the Biochemical Society [18].

MATERIALS AND METHODS

Preparation of cytochrome *c* oxidase

Cytochrome *c* oxidase (EC 1.9.3.1, ferrocycytochrome *c*:oxygen oxidoreductase) was prepared from bovine heart by the method of Yonetani [19]. The enzyme was further purified by gel filtration on a Sepharose 4B column (35 cm $\times$ 1.5 cm). The eluting buffer was either 0.1 M-sodium phosphate buffer, pH 7.4, containing 1% Tween 80 or 25 mM-Tris/acetate buffer, pH 7.8, containing 0.1% lauryl maltoside. Finally the oxidase was stored in small ampoules at -20°C until needed. All cytochrome *c* oxidase preparations used had an

$A_{444}^{\text{red.}}/A_{424}^{\text{red.}}$ of at least 2.2:1. Cytochrome *c* oxidase concentrations (total haem) were calculated by using $\epsilon_{605} = 21\,000\text{ M}^{-1}\cdot\text{cm}^{-1}$ for the reduced protein. To convert into concentration in functional units the concentration in total haem was divided by 2.

Monomeric (subunit-III-depleted) cytochrome *c* oxidase was prepared as described by Malatesta *et al.* [20]. The enzyme was stored at -20°C until needed.

Preparation and characterization of porphyrin cytochrome *c* and zinc cytochrome *c*

Anhydrous HF was produced by the action of H_2SO_4 on NaF. Both reagents were rigorously freed of water. H_2SO_4 was dried by the addition of anhydrous Na_2SO_4 . NaF was dried in a vacuum oven and stored over P_2O_5 in a desiccator. H_2SO_4 (22 ml) was added to NaF (7 g) in a Teflon apparatus in a fume hood. Distillation and collection of anhydrous HF was as described by Simons [21]. The HF so obtained was employed to prepare, with some minor modifications, porphyrin (iron-free) cytochrome *c* by the method of Vanderkooi & Erecińska [17]. Zinc cytochrome *c* was prepared from porphyrin cytochrome *c* by the method of Flatmark & Robinson [22], as modified by Dickinson & Chien [23], and Vanderkooi *et al.* [24].

The absorption coefficient of zinc cytochrome *c* was determined by the direct method of weighing. Portions of known volume (in triplicate) of aqueous solutions containing zinc cytochrome *c* (buffer- and salt-free) and with known absorbance at 422 nm in a 1 cm-pathlength cell were dried to constant weight. From this weight the concentration of the original solution was calculated by using an M_r value of 12394 for zinc cytochrome *c*.

The quantum yield of fluorescence of porphyrin cytochrome *c* and zinc cytochrome *c* were determined by the method of Parker & Rees [25], with the observed fluorescence being corrected for the variation of lamp output with wavelength. The standard used was quinine sulphate dissolved (1 mg/l) in distilled water; this compound has a quantum yield of 0.55 [26]. The protein solutions had absorbances 0.2–0.3 and were corrected for inner-filtering by using the equation:

$$I_c = (I_0 + I_d) \times \text{anti-ln}(A_1 + A_2/2)$$

I_c is the corrected fluorescence intensity, I_0 is the uncorrected fluorescence intensity, I_d is a factor that corrects for dilution, and A_1 and A_2 are the absorbances at the excitation and emission wavelengths respectively.

Preparation of cytochrome *c* oxidase derivatives

Ascorbate (0.5 M) was prepared in 25 mM-Tris/acetate buffer, pH 7.8, containing 0.1% lauryl maltoside. This buffer was used for the preparation of all cytochrome *c* oxidase species. TMPD (0.1 M) was prepared in distilled water.

Cytochrome *c* oxidase as isolated was in its fully oxidized state. Fully reduced cytochrome *c* oxidase was prepared by the reduction of the resting fully oxidized enzyme with 100 mM-ascorbate and 2 mM-TMPD. As a control to check any effects of ascorbate, titrations were performed with resting fully oxidized oxidase that had been incubated with 100 mM-ascorbate in the absence of TMPD (i.e. no reduction). The CO-cytochrome *c* oxidase complex was produced by bubbling CO through the fully reduced enzyme. Pulsed cytochrome *c* oxidase was generated during the experiments by titrating aerated

zinc cytochrome *c* with the fully reduced enzyme. The cyanide derivative of cytochrome *c* oxidase was prepared by the method of Van Buuren *et al.* [27]. Cytochrome *c* oxidase was incubated with a 5-fold excess of KCN for 18 h at 4°C in 25 mM-Tris/acetate buffer, pH 7.8, containing 0.1% lauryl maltoside. Unbound cyanide was removed by gel filtration on a Sephadex G-25 column (15 cm $\times$ 1 cm) equilibrated with the same buffer. The resulting cyanide-bound oxidase was in its fully oxidized form. Half-reduced CN^- -cytochrome *c* oxidase was obtained by reduction with 5 mM-ascorbate and 0.1 mM-TMPD. This species, $\alpha^{2+}\alpha^{3+}\text{-CN}^-$, was completely stable over the time course of titration experiments, as judged by absorbance spectroscopy.

Monomeric cytochrome *c* oxidase prepared as indicated above was in its resting fully oxidized state. The fully reduced enzyme was obtained by the addition of ascorbate (100 mM) and TMPD (2 mM).

Titration

Zinc cytochrome *c* was titrated at two different ionic strengths (0.01 and 0.03) with the several different forms of free and ligand-bound cytochrome *c* oxidase, prepared as described above.

Titration at I 0.010. Zinc cytochrome *c* was dissolved in 25 mM-Tris/acetate buffer, pH 7.8, containing 0.1% lauryl maltoside to give a $0.7\text{ }\mu\text{M}$ solution, and 2.5 ml of this was placed in a sealed cuvette. Tris/acetate buffer (200 μl) was now added, and the fluorescence intensity of the resulting solution was set at 100%. Portions of cytochrome *c* oxidase (15 μl , $75\text{ }\mu\text{M}_{\text{TH}}$) were titrated in and the fluorescence intensity was recorded after each addition. The fully reduced and CO-cytochrome *c* oxidase complex could not be studied under these conditions as addition of dithionite significantly increased the ionic strength.

For the vast majority of work the excitation wavelength was 432 nm and the emission wavelength was 590 nm. Other excitation wavelengths employed were 422 nm and 545 nm, and other emission wavelengths were 585, 595, 605, 615, 623 and 640 nm.

Titration at I 0.030. Zinc cytochrome *c* ($0.7\text{ }\mu\text{M}$) was prepared as above in degassed buffer. $\text{Na}_2\text{S}_2\text{O}_4$ (98 mM) was prepared in degassed 25 mM-Tris/acetate buffer, pH 8.8, containing 0.1% lauryl maltoside. The final pH was 7.8 ± 0.2 . Na_2SO_4 (98 mM) was prepared in the same Tris/acetate buffer, but pH 7.8.

Zinc cytochrome *c* (2.5 ml) was placed in sealed evacuated cuvettes, and $\text{Na}_2\text{S}_2\text{O}_4$ (200 μl) and Tris/acetate buffer (200 μl) were added. The fluorescence intensity of each solution was set at 100%, and 15 μl portions of either fully reduced cytochrome *c* oxidase or CO-cytochrome *c* oxidase were now titrated in. The role of the $\text{Na}_2\text{S}_2\text{O}_4$ was to maintain reduction through the course of the titration.

To ensure comparability between these experiments and titrations with the other species of cytochrome *c* oxidase (the fully oxidized resting form, the pulsed form, the oxidized cyanide complex and the half-reduced cyanide complex), these latter were performed with Na_2SO_4 (7 mM) replacing $\text{Na}_2\text{S}_2\text{O}_4$ (7 mM).

In all the experiments (i.e. at I 0.010 and I 0.030) the redox state of the enzyme during titration was regularly monitored by absorbance spectroscopy against a zinc

cytochrome *c* reference. The spectrum and hence the oxidation state of a given species of oxidase could thus be checked at any stage of the titration.

Control titrations

To control for the effects of inner-filtering each titration (whether at I 0.010 or at I 0.030) was repeated in the presence of 0.3 M-NaCl to abolish complex-formation between zinc cytochrome *c* and cytochrome *c* oxidase. This was achieved by replacing the 200 μ l addition of buffer with 200 μ l of 5 M-NaCl.

Zinc cytochrome *c*-cytochrome *c* oxidase complex studies by Sephadex G-100 filtration

Zinc cytochrome *c*-cytochrome *c* oxidase complexes were studied for the fully oxidized resting form, the pulsed form and the fully reduced form of the enzyme. The oxidized cyanide derivative and the half-reduced cyanide derivative were also studied. These forms of the enzyme were prepared as described above.

Zinc cytochrome *c* (47 μ M) was prepared in 25 mM-Tris/acetate buffer, pH 7.8, containing 1% Tween 80, and 200 μ l of this solution was mixed at room temperature with the different forms of cytochrome *c* oxidase (35 μ l, 350 μ M total haem). After gentle mixing for 5 min, each mixture was passed down a Sephadex G-100 column (35 cm $\times$ 1 cm) that had been previously equilibrated with the same Tris/acetate buffer. The column was run at 0.3 ml/min, and 0.8 ml fractions were collected. For the resting form, the pulsed form and the two forms of cyanide complexes, the elution buffer also contained Na₂S₂O₄ (2 mM). For the fully reduced enzyme, the buffer was degassed and contained 2 mM-Na₂S₂O₄, i.e. the column was run under anaerobic conditions. In this latter case, the top of the column was sealed with a Subaseal, through which the oxidase was applied. All experiments were performed in the dark at 4 °C, and spectra of the eluted complexes were recorded for the fully oxidized and the dithionite-reduced states. The composition of the complexes was determined from the fully reduced spectra taking absorption coefficients of 243 000 M⁻¹·cm⁻¹ and 5300 M⁻¹·cm⁻¹ for zinc cytochrome *c* and 44 000 M⁻¹·cm⁻¹ and 105 000 M⁻¹·cm⁻¹ for cytochrome *c* oxidase at 422 nm and 444 nm respectively [24,28].

Fluorimetric and spectrophotometric steady-state studies

Fluorimetric. Sodium ascorbate (0.5 M) and TMPD (0.1 M) solutions were prepared as described above, and 3 vol. of ascorbate was mixed with 1 vol. of TMPD. Zinc cytochrome *c* (0.7 μ M) was prepared in degassed buffer, and 2.7 ml of this solution was placed in an evacuated cuvette. The fluorescence intensity of the zinc cytochrome *c* was set at 100%, and a single portion of resting fully oxidized cytochrome *c* oxidase (50 μ l, 75 μ M total haem) was added. The new fluorescence intensity was recorded. At zero time ascorbate/TMPD was added to a final concentration of 18 mM-ascorbate and 1.2 mM-TMPD, and fluorescence intensity changes were observed.

The experiment was repeated, but before the addition of ascorbate/TMPD KCN to a final concentration of 5 μ M was added to the zinc cytochrome *c* solution.

Spectrophotometric. The experiments described in the preceding paragraphs were repeated spectrophotometrically at two wavelengths, 444 nm and 604 nm. In

these experiments the absorbance was recorded as a function of time. The reference cuvette contained zinc cytochrome *c* (0.7 μ M).

RESULTS

Properties of porphyrin cytochrome *c* and zinc cytochrome *c*

Porphyrin cytochrome *c* and zinc cytochrome *c* were prepared as described by Flatmark & Robinson [22] and by Vanderkooi *et al.* [24]. The absorbance and fluorescence spectra were determined and found to be in good agreement with those reported elsewhere.

Both modified cytochromes, when in free solution, were found to be light-sensitive. This sensitivity was more marked for porphyrin cytochrome *c*, the zinc derivative being much less affected by light and indeed hardly at all when in complex with cytochrome *c* oxidase. In addition, the fluorescence spectrum of porphyrin cytochrome *c* showed time-dependent changes in the presence of Na₂S₂O₄, whereas that of the zinc protein did not. The relative stability of zinc cytochrome *c* towards light and reducing agents made this the more appropriate fluorescent probe for the majority of the experiments.

The absorption coefficient of zinc cytochrome *c* was determined (see the Materials and methods section) as $\epsilon_{423} = 243\,000\text{ M}^{-1}\cdot\text{cm}^{-1}$, in excellent agreement with Vanderkooi *et al.* [24]. Like porphyrin cytochrome *c* [17], zinc cytochrome *c* was found to compete with native cytochrome *c* in its interactions with cytochrome *c* oxidase.

The values we obtained for the quantum yield of porphyrin cytochrome *c* are somewhat smaller than those reported by Dockter *et al.* [12], who used 8-anilino-naphthalene-1-sulphonate as a standard. We also were able to obtain higher, but varied, values when 1 mM-quinine bisulphate in 0.1 M-H₂SO₄ was employed as the standard. The reason for this variation is that quinine bisulphate does not strictly obey the Beer-Lambert Law, its absorption coefficient increasing on dilution (probably owing to dissociation of complexes). The absorbance of 1 mM-quinine bisulphate exceeds 1, and consequently dilutions are necessary for accurate fluorescence measurements. The results reported in Table 1 were obtained with quinine sulphate (1 mg/l) as the standard [26]. Dilutions are unnecessary when using this standard.

The quantum yield of fluorescence of zinc cytochrome *c* was determined under a number of solvent regimes, and the values obtained are given in Table 1.

Complexes of zinc cytochrome *c* and cytochrome *c* oxidase

A prerequisite for using zinc cytochrome *c* as a fluorescent probe to monitor state-dependent conformational changes in cytochrome *c* oxidase is that the probe and the enzyme form tight complexes irrespective of the enzyme derivative used. In order to determine that such complexes form, the two proteins were incubated together for 5 min and after incubation any complex was separated from free zinc cytochrome *c* by passage through a Sephadex G-100 column equilibrated with the same buffer either aerobically or anaerobically in the presence of dithionite (see the Materials and methods section).

The spectra of the separated complexes were recorded in the oxidized and dithionite-reduced states, and the

Table 1. Quantum yields of zinc cytochrome *c* and porphyrin cytochrome *c*

The quantum yields of porphyrin cytochrome *c* and zinc cytochrome *c* were determined (a) over a range of pH and ionic strengths and (b) in the presence of up to a 2-fold excess of bovine serum albumin (BSA). See the Materials and methods section for details.

Protein	$10^3 \times$ Quantum yield
Porphyrin cytochrome <i>c</i> , pH 6–8, ± 0.1 – 0.3 M-NaCl	1.3
Porphyrin cytochrome <i>c</i> + BSA, ratio 1:2	1.3
Zinc cytochrome <i>c</i> , pH 6–8, ± 0.1 – 0.3 M-NaCl	0.97
Zinc cytochrome <i>c</i> + BSA, ratio 1:2	0.97

concentrations of the components were calculated by assuming that addition of dithionite changed the absorption spectrum of only the oxidase, and using the absorption coefficients given in the Materials and methods section. The stoichiometries of the complexes so calculated were 0.8 mol of zinc cytochrome *c*/mol of oxidase functional unit at I 0.010 and 0.7 mol of zinc cytochrome *c*/mol of oxidase functional unit at I 0.016. These values were independent of the enzyme derivative used and were close to the values of 0.9 and 0.8 mol of ferricytochrome *c*/mol of oxidase functional unit found for the complex between native cytochrome *c* (iron-containing) and oxidase under the same conditions.

Although the stoichiometries found are non-integral and less than 1:1, we conclude that this is due to some dissociation of a tight 1:1 electrostatic complex of the two proteins on passage through the Sephadex column. A low flow rate for gel filtration was deliberately chosen in order to produce tight bands and good separation on elution, and hence some dissociation of complexes with even very low dissociation rates could be expected. We conclude that zinc cytochrome *c* binds with similar stoichiometry and association constant to oxidase as the native ferri protein and that the binding constant between zinc cytochrome *c* and cytochrome *c* oxidase does not vary greatly with the valence state of the metal centres in the latter.

At high ionic strength (i.e. 0.3 M-NaCl) no tight complexes form between the two proteins, and, in addition, complexes formed at low ionic strength dissociate rapidly on a column equilibrated and eluted at high ionic strength.

As a control for subsequent fluorescence experiments complex-formation between zinc cytochrome *c* and bovine serum albumin was also examined. Bovine serum albumin (2.4 mM) was first labelled with tetranitromethane (750 mM) to aid visual detection in the column and calculation of stoichiometry. Excess tetranitromethane was removed by gel filtration, and then the labelled albumin (0.5 mM) was incubated with zinc cytochrome *c* (4 mM).

A complex was separated by gel filtration at low ionic strength from the excess free cytochrome *c*, and the elution volume was noted.

The experiment was repeated with unlabelled bovine serum albumin (tetranitromethane partially quenches zinc cytochrome *c* fluorescence), and the fluorescence properties of zinc cytochrome *c* in association with

Fig. 1. Fluorimetric titration of zinc cytochrome *c* with cytochrome *c* oxidase

Zinc cytochrome *c* ($0.7 \mu\text{M}$) was titrated with cytochrome *c* oxidase ($38 \mu\text{M}$ functional units). (a) The variation of fluorescence intensity occurring on the sequential addition of $15 \mu\text{l}$ portions of cytochrome *c* oxidase (at the times indicated) is shown. The lower series of traces represents the loss of fluorescence intensity that results from both complex-formation and inner-filtering. The upper traces show the loss of fluorescence intensity occurring when the titration is performed in the presence of 0.3 M-NaCl, which abolishes complex-formation. (b) The fluorescence emission spectrum of zinc cytochrome *c* shown at the beginning and end of each titration: —, in the presence and in the absence of 0.3 M-NaCl (no oxidase); ---, in the presence of cytochrome *c* oxidase ($1.2 \mu\text{M}$); ----, in the presence of cytochrome *c* oxidase ($1.2 \mu\text{M}$) and 0.3 M-NaCl. (c) Fluorescence-intensity changes due to complex-formation only, obtained by subtracting the upper traces from the lower traces in (a). F_0 is the initial fluorescence intensity and was arbitrarily set at 100%. F is the fluorescence intensity relative to F_0 . The buffer was 25 mM-Tris/acetate buffer, pH 7.8, containing 0.1% lauryl maltoside. Excitation was at 432 nm and the emission wavelength was 590 nm. The temperature was 25 °C.

Fig. 2. Fluorimetric titration of zinc cytochrome *c*

(a) Titration of zinc cytochrome *c* ($0.7 \mu\text{M}$) with cytochrome *c* oxidase at approx. I 0.010. $\circ$, Fully oxidized 'resting'; $\blacktriangle$, half-reduced cyanide-bound ($a^{2+}a_3^{3+}-\text{CN}^-$). F_0 is the initial fluorescence intensity and was arbitrarily set at 100%. F is the fluorescence intensity relative to F_0 . The buffer was 25 mM-Tris/acetate buffer, pH 7.8, containing 0.1% lauryl maltoside. (b) Titration of zinc cytochrome *c* ($0.7 \mu\text{M}$) with cytochrome *c* oxidase at approx. I 0.030. $\square$, Fully oxidized 'resting'; $\circ$, fully oxidized 'pulsed'; $\triangle$, fully reduced; $\bullet$, fully reduced CO adduct; $\blacksquare$, half-reduced cyanide-bound ($a^{2+}a_3^{3+}-\text{CN}^-$). The buffer was 25 mM-Tris/acetate buffer, pH 7.8, containing 0.1% lauryl maltoside. For the reduced oxidase the buffer also contained the reducing agent $\text{Na}_2\text{S}_2\text{O}_4$ (7 mM); for all of the other species the buffer contained Na_2SO_4 (7 mM). The excitation wavelength was 432 nm and the emission was monitored at 590 nm. The temperature was 25 °C.

bovine serum albumin are reported below (see Table 1).

Fluorescence titrations

In order to monitor the extent to which the fluorescence of zinc cytochrome *c* was quenched by complex-formation with cytochrome *c* oxidase, fluorescence titration experiments were carried out. A typical experiment is shown in Fig. 1. Fig. 1(b) shows the fluorescence emission spectra of zinc cytochrome *c* at high and low ionic strength and in the absence and in the presence of cytochrome *c* oxidase. It is seen, in agreement with the data given in Table 1, that the fluorescence emission of zinc cytochrome *c* is independent of ionic strength in the absence of the oxidase. On addition of cytochrome *c* oxidase, however, a loss of fluorescence intensity is observed. At high ionic strength, where column chromatography indicates that no complexes are present, the loss of intensity is due to inner-filtering caused by the addition of an absorbing species. At low ionic strength the fluorescence intensity loss is significantly greater, and thus we conclude is due to specific complex-formation under these conditions, as indicated by the gel-filtration experiments.

Figs. 1(a) and 1(b) also show the fluorescence intensity at 590 nm, the peak of the emission spectra, as a function of cytochrome *c* oxidase concentration. The difference between the titrations conducted at low and high ionic strength reports the increasing concentration of the complex on serial addition of the oxidase. This difference is plotted as a function of cytochrome *c* oxidase concentration in Fig. 1(c).

Fig. 2 shows the results of a number of such titrations with different forms of cytochrome *c* oxidase and at two ionic strengths. Fig. 2(a) reports the experiments conducted at I 0.010 (i.e. in 25 mM-Tris/acetate buffer, pH 7.8). Under these conditions it was difficult to obtain

results with the reduced protein, as addition of $\text{Na}_2\text{S}_2\text{O}_4$ significantly altered the ionic strength. To obtain comparable data for the reduced protein we therefore performed experiments in the presence of 7 mM- $\text{Na}_2\text{S}_2\text{O}_4$ and these are compared with titrations of the other cytochrome *c* oxidase species in buffer to which 7 mM- Na_2SO_4 had been added to equalize ionic strength (at approx. I 0.030). These titrations are shown in Fig. 2(b). Examination of computer fitting of these titration curves for the resting oxidized form, the pulsed form and the oxidized cyanide complex determined at approx. I 0.030 show that these conform to a simple equilibrium formulation of the form:

The value of K is $\sim 7 \times 10^6 \text{ M}^{-1}$. This value is in reasonable agreement with binding constants obtained under similar conditions by other methods. Thus the binding constant for the formation of the 1:1 complex of zinc cytochrome *c* oxidase does not appear to vary between these enzyme species. Similar analysis of the titration with the fully reduced enzyme indicated that this also conformed to eqn. (1) but in this case with a somewhat higher value of $K = 3.5 \times 10^7 \text{ M}^{-1}$. What is also clear is that the extent of quenching is strongly dependent on the redox state of the oxidase species used. Reduction of the enzyme decreases quenching considerably (by 14%), in agreement with a recent report by Kornblatt & Luu [14], who found a 13% decrease in quenching. Ligand binding to cytochrome *a*, in either the fully oxidized or the fully reduced enzyme, had no effect.

At the lowest ionic strength (I 0.010) the overall results are largely unchanged, all the oxidized species again behaving identically but leading to somewhat higher quenching than at I 0.030. The half-reduced cyanide complex form also appears to quench more than at

Fig. 3. Fluorimetric titration of zinc cytochrome *c* with monomeric cytochrome *c* oxidase

Titration of zinc cytochrome *c* ($0.7 \mu\text{M}$) with monomeric (subunit III-depleted) cytochrome *c* oxidase. ○, Resting fully oxidized; □, fully reduced. F_0 is the initial fluorescence intensity and was arbitrarily set at 100%. F is the observed fluorescence intensity relative to F_0 . The buffer was 25 mM-Tris/acetate buffer, pH 7.8, containing 0.1% lauryl maltoside. For the reduced enzyme the buffer also contained $\text{Na}_2\text{S}_2\text{O}_4$ (7 mM); this was replaced with Na_2SO_4 (7 mM) for the oxidized enzyme.

the higher ionic strength, but the difference between the oxidized and half-reduced cyanide complex forms remains the same for the two conditions. The shapes of the curves at I 0.010 do not conform exactly, however, to the simplest formulation given in eqn. (1). Examination of these curves suggests that some additional cytochrome *c* binding (1.2 mol of zinc cytochrome *c*/mol of oxidase functional unit) occurs to each oxidase molecule at these very low ionic strengths.

Titration with monomeric cytochrome *c* oxidase

Cytochrome *c* oxidase as isolated exists in solution as a stable dimer consisting of two functional monomeric units, each containing its complement of four metal

centres [1]. These monomeric units are thought to combine to form a cleft between them into which cytochrome *c* fits. In this position cytochrome *c* is in close proximity to both monomeric units. It is important to know if the quenching that we observe on binding zinc cytochrome *c* to the oxidase is due to the proximity of (quenching) metal centres in the functional unit to which the zinc cytochrome *c* is directly binding (via the positive lysine residues surrounding the haem crevice) or by metal centres in the other functional unit. If this latter were to be the case, then changes in monomer–monomer interactions might well lead to changes in quenching. To examine any role of such monomer–monomer interaction, we carried out fluorescence titration experiments with monomeric cytochrome *c* oxidase prepared by the procedure of Malatesta *et al.* [20], in which subunit III is removed from the enzyme.

Fig. 3 reports the results of such a titration. The extent of quenching and the dependence on the oxidase concentration are very similar to those that we reported in Fig. 2 for the dimeric enzyme. We conclude therefore that quenching of zinc cytochrome *c* is by centres in the oxidase functional unit to which it is bound.

Absorbance and fluorescence change during turnover

In order to identify which of the two haem groups in cytochrome *c* oxidase plays the major role in quenching fluorescence of bound zinc cytochrome *c*, experiments were undertaken to monitor simultaneously changes in absorbance and fluorescence spectra. Fig. 4 shows the results. At 444 nm, where both cytochrome *a* and cytochrome *a*₃ make comparable contributions to the difference spectrum between the fully oxidized and the fully reduced enzyme, and in the absence of cyanide a steady state is achieved on addition of TMPD and ascorbate. This steady state collapses (after exhaustion of O_2 dissolved in the buffer, approx. 5 min under these conditions) leading to the full reduction of the enzyme.

Fig. 4. Absorbance and fluorescence changes during steady-state turnover of cytochrome *c* oxidase in complex with zinc cytochrome *c* in the presence and in the absence of cyanide

(a) The absorbance changes, as a percentage of the total expected change from fully oxidized to fully reduced cytochrome *c* oxidase, monitored at 444 nm. At zero time, TMPD and ascorbate were added to initiate the steady state. Absorbances were measured against a zinc cytochrome *c* reference. (b) Similar absorbance measurements at 605 nm (—) and normalized fluorescence changes (----) monitored in a parallel experiment. The cytochrome *c* oxidase concentration was $0.5 \mu\text{M}$, and that of zinc cytochrome *c* $0.7 \mu\text{M}$. The TMPD and ascorbate concentrations were 1.2 mM and 18 mM respectively. The cyanide concentration was $5 \mu\text{M}$. The buffer used was degassed 25 mM-Tris/acetate buffer, pH 7.8, containing 0.3% lauryl maltoside, and the temperature was 25°C .

In the presence of cyanide initial reduction also occurs, leading to a steady state that is very much diminished in lifetime and finishes after about $\frac{1}{2}$ min. This behaviour is to be expected, as cyanide is a potent inhibitor of the enzyme under turnover, blocking oxidation of cytochrome a_3 , which is trapped, together with Cu_B , in the oxidized state. Reduction of the enzyme in the presence of cyanide yields therefore a species in which only cytochrome *a* (and Cu_A) are reduced.

Experiments monitoring absorbance at 605 nm showed the same phenomenon of a shortened steady state in the presence of cyanide, but the magnitude of the absorbance changes in the presence and in the absence of cyanide are similar, as at this wavelength cytochrome *a* makes the dominant contribution (at least 80 %).

Fig. 4(b) also shows the intensity changes in the fluorescence of zinc cytochrome *c*. As expected, these show an increase in fluorescence intensity on reduction of the enzyme and, consistent with the results in Fig. 2, this increase is somewhat larger in the presence of cyanide. The normalized fluorescence intensity changes shown in Fig. 4(b) are clearly not greatly affected by cyanide, even though this ligand prevents cytochrome a_3 reduction. We conclude therefore that it is the reduction of cytochrome *a* that leads to the major changes in fluorescence intensity, and thus in the oxidized enzyme it is cytochrome *a* that is the quenching centre.

Difference spectroscopy

Two dual-compartment cuvettes were filled with zinc cytochrome *c* in one compartment and cytochrome *c* oxidase in the other. After a baseline was obtained by running the identical cuvettes against each other the contents of the compartments of one cuvette were mixed. If on mixing no interaction between solution components takes place, then no spectral change should occur. (The concentration of each component is halved, but the path-length doubled in the mixed cuvette.) We found, however, that small spectral changes do occur on binding zinc cytochrome *c* to the oxidase. These amount to an approx. 2% decrease in absorbance in the Soret region.

DISCUSSION

There are a number of ways to account for the quenching of zinc cytochrome *c* in the presence of cytochrome *c* oxidase. The most obvious and trivial is that of inner-filtering, i.e. absorption of the exciting light and the emitted fluorescence by the presence of a chromophore. This possibility we discount on the grounds that control experiments at high ionic strength show less quenching than those in which complexes form while leaving the total absorbance practically unchanged (Fig. 1). In addition, measurements taken at a number of wavelengths (not shown) yield the same overall result even though the effects of inner-filtering should be wavelength-dependent.

Another possibility is that the fluorescence quantum yield of zinc cytochrome *c* decreases on binding to the oxidase because the structure of the zinc protein changes. This might be considered a possibility because the structure of the zinc derivative is likely to be somewhat more flexible than the native protein, since quadrivalent zinc cannot stabilize the cytochrome *c* crevice so

effectively as sexavalent iron, which co-ordinates not only the porphyrin but also histidine-18 and methionine-80 residues in the protein. We consider this explanation for the decrease in fluorescence intensity to be unlikely. In the first instance the quantum yield of zinc cytochrome *c* was found to be independent of a wide range of solvent conditions, such as pH and ionic strength, that might be expected to modify the protein structure. We did note, however, a very small (approx. 2%) change in the absorption spectrum of the zinc cytochrome *c*-oxidase complex when compared with that of the separated components. This change amounted to a small decrease of the absorption coefficient of the proteins in the Soret region. A similar though somewhat smaller decrease was observed when zinc cytochrome *c* was complexed with bovine serum albumin. The fact that bovine serum albumin, like cytochrome *c* oxidase, perturbed the absorbance spectrum of zinc cytochrome *c* but had no effect on its fluorescence demonstrates that binding as such does not alter (lower) ϕ .

The most likely cause of quenching therefore, and one that has been invoked by others, is the proximity of a metal centre in cytochrome *c* oxidase. The fluorescence energy of zinc cytochrome *c* is transferred by a non-radiative resonance process to the acceptor, here a nearby metal site, where de-excitation occurs by a non-radiative process. This site is shown to be in the same functional unit to which the zinc cytochrome *c* is bound by experiments in which monomeric cytochrome *c* oxidase substitutes for the dimer that is generally prepared. The dimeric form offers the possibility of zinc cytochrome *c* being bound to one monomer while being partially quenched by an acceptor in the partner monomer.

The nature of the acceptor is unlikely to be an amino acid residue, such as an aromatic species, within the enzyme, as these components have a practically zero value of the overlap integral, *J*, an important parameter defining resonance-energy-transfer efficiency (see below). On similar grounds we have also discounted the copper atoms. However, it is possible that weak copper absorption bands may lie beneath and be obscured by the α -bands of the haem groups, and these weak bands could in principle contribute to the observed quenching. In the absence of quantitative knowledge with regard to such bands in the visible region, we have ignored the copper atoms and have taken the likely quenching site(s) as one or both of the haem residues.

Our steady-state measurements (Fig. 4) show that changes in the valence state of cytochrome *a* leads to the major changes in measured fluorescence intensity of bound zinc cytochrome *c*, indicating that it is this haem that is the acceptor site. This conclusion is strengthened by the observation that ligand binding to cytochrome a_3 , in either the fully oxidized or the fully reduced enzyme, has no effect on the fluorescence emission. Electron transfer between native cytochrome *c* and cytochrome *a* is known to be very rapid ($k \sim 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$ at $I \rightarrow 0$), suggesting that these centres are in close proximity, at least much closer than cytochrome *c* is to cytochrome a_3 . It is, however, possible, if unlikely, that cytochrome a_3 is the quenching centre and reduction of cytochrome *a* (and/or Cu_A) triggers a conformational change (see below) that removes cytochrome a_3 from the vicinity of zinc cytochrome *c*. However, in the absence of definitive proof, we favour the simpler and more natural

hypothesis that cytochrome *a* itself is the centre that quenches the fluorescence of zinc cytochrome *c*.

Given that resonance-energy transfer between zinc cytochrome *c* and cytochrome *a* may largely account for our results, we must now enquire as to the reasons for the decreased fluorescence quenching observed when cytochrome *a* is reduced. Here again there are at least three possible explanations. A decrease in binding constant between the two proteins on reduction of cytochrome *a*, i.e. some dissociation of the complex, we discount on the basis of the computer fit of the titration data, which if anything suggests a tighter complex when cytochrome *a* is reduced. The results of our column-chromatography studies also suggest no such dissociation. Similarly changes in overlap integral cannot explain our findings. Indeed, reduction of cytochrome *a* leads to an increase of the overlap between its absorption spectrum and the fluorescence emission spectrum of zinc cytochrome *c*, and would be expected to increase resonance-energy transfer efficiency and hence to increase the magnitude of fluorescence quenching. The opposite in fact occurs: reduction of cytochrome *a* decreases the fluorescence quenching (Fig. 2). Kornblatt & Luu [14] also observed similar changes in fluorescence intensity on reduction of the enzyme.

The remaining possibility left, therefore, is that reduction of cytochrome *a* and/or Cu_A, with which it is in rapid electron-transfer equilibrium, alters the relative position of the donor and acceptor, i.e. a conformational change accompanies valence changes of cytochrome *a* and/or Cu_A. Conformational changes occurring as a consequence of reduction have also been reported by King *et al.* [29] on the basis of c.d. studies. At this stage we cannot distinguish whether reduction of cytochrome *a* or Cu_A or both is responsible for triggering this conformational change.

The c.d. results obtained by King *et al.* [29] show that on reduction the orientation of the haem groups within cytochrome *c* oxidase change with respect to the protein backbone. Calculations based on Förster's [30] theory for resonance-energy transfer (eqns. 2–4) show that such re-orientation of haem *a* can account for only a small fraction of our observed fluorescence changes. The reason for this is that both the donor and the acceptor are porphyrins and thus possess doubly degenerate symmetry, and consequently, K^2 , the orientation parameter of Förster's equation, can never approach zero, and has been shown by Vanderkooi & Erecińska [17] to have a value of between 1 and 2. The value is 2 when the two haem groups are in the same plane.

Since re-orientation of haem *a* can only account for a small fraction of observed changes in resonance-energy transfer, it seems that an increase in the cytochrome *c*–haem *a* distance forms the major part of the conformational change that accompanies reduction of cytochrome *a*.

Förster's theory of resonance-energy transfer is generally expressed in the following equations:

$$E = \frac{R_0^6}{R_0^6 + R^6} \quad (2)$$

where R is the distance between the donor and the acceptor, R_0 is the distance at which the energy-transfer efficiency (E) is 50%, and E is the efficiency of non-radiative energy transfer from the fluorescent donor to the fluorescent acceptor and is experimentally determined

by either one of the following two methods: (a) by measuring the decreased quantum yield of the fluorescent donor by steady-state fluorescence measurement as we have done here, or (b) by measuring the decreased fluorescence lifetime of the excited donor by nanosecond fluorimetry. R_0 is calculated from eqn. (3):

$$R_0 = 9.7 \times 10^2 (n^{-4} K^2 Q_D J)^{\frac{1}{6}} \text{ nm} \quad (3)$$

where n is the refractive index of the medium (measured here as 1.33), Q_D the quantum yield of the donor in the absence of energy transfer (see Table 1), K^2 is an orientation factor of the particular dipole–dipole system, and J is the 'spectral overlap integral', which expresses the degree of overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor, calculated from eqn. (4) (see Campbell & Dwek [31]):

$$J = \frac{\sum_{\lambda_i} F_D(\lambda_i) \epsilon_A(\lambda_i) \lambda_i^4 \delta \lambda}{\sum_{\lambda_i} F_D(\lambda_i) \delta \lambda} \quad (4)$$

where $F_D(\lambda_i)$ is the uncorrected fluorescence intensity at wavelength λ_i , and $\epsilon_A(\lambda_i)$ is the molar absorption coefficient of the acceptor at λ_i .

We have used these equations together with our experimental data to compute the distance (R) between the fluorescence donor, here zinc cytochrome *c*, and the acceptor, cytochrome *a*. The results of these calculations are given in Table 2.

The value of R for the resting fully oxidized enzyme (~ 2.4) is in good agreement with values reported by Dockter *et al.* [12], if somewhat smaller than the values given by Vanderkooi *et al.* [13] of 4.4 nm. The most important feature of Table 2 is not, however, the absolute values of R , which are open to a number of criticisms (see, e.g., Dale and co-workers [32–34]), but the fact that R changes by approx. 0.7 nm on reduction of cytochrome *a*. This donor–acceptor distance (R) is, of course, sensitive to the numerical values of the parameters J and K^2 chosen; however, the value of ΔR , the change in distance on reduction, is less so, and even most unfavourable values still yield ΔR values close to 0.5 nm.

For example, we have calculated the value of the spectral overlap integral J under the assumption that both cytochromes *a* and *a*₃ make equal contribution to the oxidized spectrum, whereas cytochrome *a* makes the greater contribution to the reduced spectrum (80% of the oxidized-minus-reduced difference spectrum [35]). Alternatively, J may be calculated by assuming that cytochrome *a* makes an equal contribution to both oxidized and reduced spectra. These different assumptions give different values for R (see Table 2), but the value of ΔR still remains close to 0.7 nm. Similarly, large variations (up to 50%) in the allowed values of Q makes relatively small changes to ΔR (approx. 10%). Choosing a different value for K^2 (between 1 and 2) and recalculating the R values again makes little difference to ΔR (Table 2). However, allowing K^2 to vary from derivative to derivative (say between the fully oxidized and the fully reduced enzyme) does effect ΔR and can decrease this value from 0.7 nm to 0.5 nm (Table 2). Given that some re-orientation of haem groups may occur [29], we concluded that a realistic value for ΔR may be around 0.5 nm.

Although our values of R (fully oxidized) are similar to

Table 2. Resonance-energy-transfer parameters and calculated (resulting) distances

The parameters are as defined in eqns. (2)–(4) (Discussion section). The maximum efficiencies (*E*) of resonance-energy transfer shown are from the computer fit of the data presented in Fig. 2(b). The values of the overlap integral, *J*, have been calculated on the assumption that cytochrome *a* makes an equal contribution to both the oxidized and the reduced absorbance spectra of cytochrome *c* oxidase. The asterisked (*) *J* values are calculated on the assumption that both cytochrome *a* and *a*₃ make equal contributions to the oxidized spectrum, but cytochrome *a* contributes far more to the spectrum of the fully reduced enzyme [35] (also see the Discussion section). Both assumptions yield ΔR (oxidized–reduced) = 0.8 nm for $K^2 = 2$. The dagger (†) denotes measurements at very low ionic strength as shown in Fig. 2(a). The refraction index, μ , was 1.33.

Cytochrome <i>c</i> oxidase derivative	Maximum <i>E</i> (%)	$10^{13} \times J$ (cm ³ ·M ⁻¹)	K^2	$10 \times R^0$ (nm)	$10 \times R$ (nm)
Resting fully oxidized	20.5	0.92	2	19.2	24.1
	20.5	0.92	1.5	18.3	23.0
	20.5	0.46*	2	17.1	21.4
Fully reduced	6.2	1.21	2	20.1	31.6
	6.2	1.21	1.5	19.1	30.1
	6.2	0.68*	2	18.3	28.8
Pulsed fully oxidized	20.5	0.98	2	19.4	24.3
	20.5	0.98	1.5	18.5	23.2
Fully reduced CO complex	6.2	1.24	2	20.2	31.8
	6.2	1.24	1.5	19.2	30.3
Oxidized cyanide complex	20.5	1.06	2	19.7	24.7
Half-reduced cyanide complex(†)	8.5	1.21	2	20.1	29.9

those reported by others, ΔR is not. Vanderkooi & Erecińska [17] in fact report no significant change in *R* on reduction. However, in their original experiments no quenching of porphyrin fluorescence was observed on binding to cytochrome *c* oxidase, and thus it would be impossible to see a decrease in quenching on reduction. In a later paper, Vanderkooi *et al.* [13] did observe fluorescence quenching when cytochrome *c* oxidase was bound to either tin or zinc cytochrome *c*. In these experiments they examined the fully oxidized enzyme using tin cytochrome *c* and the fully reduced enzyme using zinc cytochrome *c*, and therefore were unable to place any significance on the different extent of fluorescence quenching observed.

In a more recent paper Kornblatt & Luu [14] have presented data on porphyrin cytochrome *c* fluorescence quenching by both reduced and oxidized cytochrome *c* oxidase. In many respects our data and theirs are similar. For example, we both observe a stoichiometry of approx. 1 mol of cytochrome *c* (porphyrin or zinc) bound/mol of oxidase functional unit, and a decrease in quenching (some 14%) on reduction of the enzyme. However, the overall extent of quenching reported by Kornblatt & Luu [14] is almost twice that shown in Fig. 2(b). This discrepancy may be due to the fact that these authors did not correct the titration of porphyrin cytochrome *c* with cytochrome *c* oxidase for inner-filtering by subtracting the quenching effects observed at high ionic strength from those at low ionic strength, but relied on solutions having a low absorbance (below 0.05) at the emission wavelength. The absorbance at the excitation wavelength, however, in solutions of approx. 8 μ M in each of ferricytochrome *c*, cytochrome *c* oxidase and porphyrin cytochrome *c* (as used in some of their experiments) will attenuate the excitation beam.

Kornblatt & Luu [14] also reported binding constants

between porphyrin cytochrome *c* and cytochrome *c* oxidase in its oxidized and reduced states. Our values agree well with theirs at comparable ionic strength.

Our finding that reduction of cytochrome *a* and/or Cu_A triggers a conformational change in the enzyme, reflected by an increase in the cytochrome *c*–cytochrome *a* distance, is in keeping with a number of other lines of evidence suggesting that valence changes in one or other of these metal centres leads to structural changes in the molecule. Cyanide binding to cytochrome *a*₃, a process that is exceedingly slow in the fully oxidized enzyme, becomes rapid once electrons have entered into cytochrome *a* [36,37]. This apparent ‘opening’ of the ligand-binding site only when electrons have become available must have significance to the mechanism by which O₂ is bound and stabilized as a peroxide species within the enzyme. Blair *et al.* [38] have found that the standard entropy of reduction of cytochrome *a* when compared to that of other low-spin cytochromes is larger and more negative (ΔS^0 approx. 50 kJ·mol⁻¹·K⁻¹), indicating a significant conformational change within cytochrome *c* oxidase when cytochrome *a* is reduced. In addition, this conformational change has also been considered to form an important mobile element of the proton pump, by which cytochrome *c* oxidase transfers one proton from the interior to the exterior of the mitochondrion for each electron passed to O₂. Independent data from a number of groups have shown that this transfer is tied to redox changes in either cytochrome *a* or copper A.

It is also possible that conformational changes lead to the apparent discrepancy between the redox potential of cytochrome *a*, measured either statically (340 mV in the fully oxidized enzyme) or kinetically (~280 mV) by monitoring electron entry and distribution in the millisecond time range. This difference may reflect conformational changes in the enzyme on cytochrome *a* reduction

occurring over a period of time that is short compared with the time taken for static measurement but longer than the electron-transfer reactions.

The titration data in Fig. 2 also show the distinctive behaviour of the half-reduced species in which cytochrome *a* has been reduced, and has therefore presumably triggered the restructuring discussed above, while cytochrome *a*₃ remains oxidized and ligated to cyanide. When this species is titrated into zinc cytochrome *c*, then little if any quenching of fluorescence was observed (at 1/0.03) within the limits of our experimental accuracy $\pm 2\%$. This is in spite of the fact that the oxidase in this form retains the ability to make a tight complex with zinc cytochrome *c*. If we are to maintain the position that cytochrome *a* is the major quenching centre, then we must suppose that in this derivative this site is yet further removed from cytochrome *c*. This is tenable, as cyanide bridges between the iron atom of ferricytochrome *a*₃ and Cu_B (II) and can change (from high to low) the spin state of the iron [39]. Configurational changes, resulting from cyanide binding, may be transmitted and amplified, leading to movement of the cytochrome *a* centre (cf. haemoglobin). This conjecture, if true, only applies when cytochrome *a* is itself reduced, as no such effect was observed on cyanide binding to the oxidized enzyme.

It is somewhat puzzling that no differences in quenching were observed between the resting and pulsed derivatives (thought to be conformational variants) of the oxidized enzyme. It may be that the pulsed enzyme relaxed rapidly back to the resting form on binding zinc cytochrome *c* [14]. Alternatively, the conformational changes between resting and pulsed forms may not affect the cytochrome *c* oxidase–cytochrome *c* distance, a possibility made more likely by the fact that the electron entry rate into cytochrome *a* from cytochrome *c* is similar (within a factor of 2-fold) for the two forms of the enzyme. A third possibility is that the same degree of fluorescence quenching would be observed if the cytochrome *c*–haem *a* distance was smaller by 0.1 nm provided that the haem orientation in the pulsed enzyme also changed. Whichever of these is the case must await more extensive investigations using probes bound specifically to the other sites on the enzyme.

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REFERENCES

- Wikström, M., Krab, K. & Saraste, M. (1981) *Cytochrome Oxidase, a Synthesis*, Academic Press, New York
- Brunori, M., Antonini, E. & Wilson, M. (1981) in *Metal Ions in Biological Systems*, vol. 13 (Sigel, H., ed.), pp. 187–221, Marcel Dekker, New York
- Azzi, A. (1980) *Biochim. Biophys. Acta* **594**, 231–252
- Brunori, M., Wikström, M., Greenwood, C. & Wilson, M. (1985) *J. Inorg. Biochem. Spec. Issue: Cytochrome c Oxidase, Structure Function and Physiological Role*, Elsevier, Amsterdam
- Wikström, M. (1977) *Nature (London)* **266**, 271–273
- Casey, R. P., Thelen, M. & Azzi, A. (1980) *J. Biol. Chem.* **255**, 3994–4000
- Soloz, M., Carafoli, E. & Ludwig, B. (1982) *J. Biol. Chem.* **257**, 1579–1582
- Sarti, P., Jones, M., Antonini, G., Malatesta, F., Colosimo, A., Wilson, M. & Brunori, M. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4876–4880
- Malmström, B. (1985) *Biochim. Biophys. Acta* **811**, 1–12
- Blair, D. F., Gelles, J. & Chan, S. (1986) *Biophys. J.* **50**, 713–733
- Brzezinsky, P. & Malust, B. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 4282–4286
- Dockter, M. E., Steinemann, A. & Schatz, G. (1978) *J. Biol. Chem.* **253**, 311–317
- Vanderkooi, J. M., Landesberg, R., Hayden, G. W. & Owen, C. S. (1977) *Eur. J. Biochem.* **81**, 339–347
- Kornblatt, J. A. & Luu, H. A. (1986) *Eur. J. Biochem.* **159**, 407–413
- Geren, L. M. & Millet, F. (1981) *J. Biol. Chem.* **256**, 10485–10489
- Miki, M. & Takayoshi, H. (1984) *Biochim. Biophys. Acta* **790**, 201–207
- Vanderkooi, J. M. & Erecińska, M. (1975) *Eur. J. Biochem.* **60**, 199–207
- Alleyne, T. A. & Wilson, M. T. (1987) *Biochem. Soc. Trans.* **15**, 524–525
- Yonetani, T. (1960) *J. Biol. Chem.* **235**, 845–852
- Malatesta, F., Georgevich, G. & Capaldi, R. A. (1983) in *Structure and Function of Membrane Proteins* (Quagliariello, E. & Palmieri, F., eds.), pp. 223–235, Elsevier, Amsterdam
- Simons, J. H. (1950) in *Fluorine Chemistry*, vol. 1 (Simons, J. H., ed.), p. 225, Academic Press, New York
- Flatmark, T. & Robinson, A. B. (1968) in *Structure and Function of Cytochromes* (Okunuki, K., Kamen, M. D. & Sekuzu, I., eds.), pp. 383–387, University Park Press, Baltimore
- Dickinson, L. C. & Chien, J. C. W. (1975) *Biochemistry* **14**, 3526–3534
- Vanderkooi, J. M., Adar, F. & Erecińska, M. (1976) *Eur. J. Biochem.* **64**, 381–387
- Parker, C. A. & Rees, W. T. (1960) *Analyst (London)* **85**, 587–600
- Drushel, H. V., Sommer, A. L. & Cox, R. C. (1963) *Anal. Chem.* **35**, 2166–2172
- Van Buuren, K. J., Zuurendonk, P. F., Van Gelder, B. F. & Muijsers, A. O. (1972) *Biochim. Biophys. Acta* **256**, 243–257
- Yonetani, T. (1961) *J. Biol. Chem.* **236**, 1680–1688
- King, T. E., Bailey, P. M. & Young, F. C. (1971) *Eur. J. Biochem.* **20**, 103–110
- Förster, Th. (1948) *Ann. Phys. (Leipzig)* **6**, 55–75
- Campbell, I. D. & Dwek, R. A. (1984) *Biological Spectroscopy*, pp. 91–126, Benjamin/Cummings Publishing Co., London
- Eisinger, J. & Dale, R. E. (1974) *J. Mol. Biol.* **84**, 643–647
- Dale, R. E. & Eisinger, J. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 271–273
- Dale, R. E., Eisinger, J. & Blumberg, W. E. (1979) *Biophys. J.* **26**, 161–194
- Wikström, M. K., Harmon, H. J. & Chance, B. (1976) *FEBS Lett.* **65**, 259–277
- Jones, M. G., Bickar, D., Wilson, M. T., Brunori, M., Colosimo, A. & Sarti, P. (1984) *Biochem. J.* **220**, 57–66
- Jensen, P., Wilson, M. T., Aasa, R. & Malmström, B. (1984) *Biochem. J.* **224**, 829–837
- Blair, D. F., Ellis, W. R., Wang, H., Jr., Gray, H. B. & Chan, S. I. (1986) *J. Biol. Chem.* **261**, 11524–11537
- Thompson, A. J., Greenwood, C., Gadsby, P. M., Peterson, J., Eglington, D. G., Hill, B. C. & Nicholls, P. (1985) *J. Inorg. Biochem.* **23**, 187–197