Reactivity of photoreduced cytochrome aa_3 complexes with molecular oxygen

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Cytochrome *c* oxidase (ox heart cytochrome aa_3) is reduced on illumination in the presence of a photocatalyst system containing deazaflavin and EDTA. The photo-reduced enzyme reacts with oxygen at neutral pH to give a form of ferric enzyme, whereas a corresponding sample partially reduced by light in the absence of any photocatalyst reacts with oxygen to give an oxyferri species ('oxygenated' enzyme). Reduction by the photocatalyst system at an alkaline pH value (9.0) also gives rise to fully reduced oxidase (both haem groups ferrous). At these pH values the immediate product after oxygen addition is a species with a 605–606 nm absorption band, not identical with ferrous cytochrome *a*, but capable of oxidizing added cytochrome *c*. This intermediate, which is unstable at neutral pH, may be analogous to the 'compound B' obtained by Chance and co-workers [Chance, Saronio & Leigh (1975) *J. Biol. Chem.* **250**, 9226–9237; Chance, Saronio & Leigh (1979) *Biochem. J.* **177**, 931–941] at low temperatures.

The pathway by which oxygen is reduced to water by cytochrome *c* oxidase is still uncertain. Even the stable product obtained when the fully reduced enzyme is oxidized sometimes appears as a high-spin (ferric) form and at other times as a low-spin (oxyferri) form (Wharton & Gibson, 1968; Lemberg & Mansley, 1966). The latter species may be of more than one kind (Orii & King, 1976), only one of which may correspond to the original 'oxygenated' form (Sekuzu *et al.*, 1959), whereas others are claimed as the reactive intermediate in catalysis (Antonini *et al.*, 1977; Shaw *et al.*, 1979; Brunori *et al.*, 1979). When oxygen reacts with the reduced enzyme (ferrocycytochrome a_3) at low temperatures, the initial complex formed seems to be a true oxygenated (oxyferro) species, called 'compound A' by Chance *et al.* (1975). The fate of compound A depends on the redox state of cytochrome *a* (the oxidase component that does not directly interact with molecular oxygen). If cytochrome *a* is initially reduced, 'compound A' decays to 'compound B', an intermediate in which at least one of the two haem groups is oxidized, and that contains oxidizing equivalents in addition to those attributable to the ferric and cupric components present (Chance, 1980). If cytochrome *a* is initially oxidized, that is, if the oxidase starts out in a 'mixed-valence' state (Greenwood *et al.*, 1974), then 'compound A' decays to 'compound C', an intermediate (Chance & Leigh, 1977) in which one of the haem groups,

probably that of cytochrome a_3 (Chance *et al.*, 1979; Nicholls, 1979) is reduced and that is capable of its own oxidative activity, in particular the oxidation of CO to CO₂ (Nicholls, 1980).

One of the problems in monitoring the reaction of oxygen with the reduced enzyme is the elimination of side reactions induced or catalysed by excess reductant in the system. Even when the reduced enzyme is formed anaerobically by stoichiometric quantities of reductant (Wever *et al.*, 1977), or electrochemically (Anderson *et al.*, 1976), catalytic steps involving electron exchange may occur on admission of air. At the suggestion of Dr. Vincent Massey we have therefore employed a photoreduction method using a deazaflavin derivative in the presence of excess EDTA, under vacuum, as the reducing system. This system when illuminated becomes a powerful reductant, capable of reducing the usually inert catalase as well as other haems and flavoenzymes (Massey & Hemmerich, 1978). In the dark, reduction ceases, and presumably little or no reducing substances remain.

As described below, we have been able to reduce the enzyme fully by light in the presence of the deazaflavin system, and partially by light in the absence of photocatalyst. Some modulation of the reduction pattern is possible at high pH or in the presence of carbon monoxide, and the results obtained are interpreted in terms of the occurrence of at least one intermediate in oxygen reduction

between the ferrous cytochrome a_3 -oxygen complex ('oxyferro' species) and the oxidized ('oxyferri') form. The chemical nature of this type of intermediate remains uncertain (cf. Nicholls, 1979, 1980).

Materials and methods

Ox heart cytochrome c oxidase (cytochrome aa_3) was prepared as described by Kuboyama *et al.* (1972) and stored at -75°C as described by Nicholls & Hildebrandt (1978). Cytochrome c (type VI from horse heart) and EDTA (disodium salt) were from Sigma Co. (St. Louis, MO., U.S.A.). The deazaflavin photocatalyst 3,10-dimethyl-5-deaza-isoalloxazine (compound I below) was the gift of Dr. V. Massey, Department of Biochemistry, University of Michigan, Ann Arbor, MI, U.S.A. Gases (N_2 , CO) were from Linde (Union Carbide, Toronto, Ont., Canada).

(I)

Irradiation of cytochrome aa_3 was normally carried out in Thunberg tubes with attached cuvettes of optical glass, with the enzyme dissolved in 50 mM-sodium phosphate/0.25% Tween-80, pH 7.4, or 50 mM-sodium pyrophosphate/0.25% Tween-80, pH 9.0, in the presence or absence of 2–3 μM -3,10-dimethyl-5-deaza-isoalloxazine and 10–12 mM-EDTA, for a period of 5–30 min. The Thunberg tube was immersed in a 25°C water bath with its optical face 5 cm from the projector lens of a Kodak projector with 150 W quartz/iodine lamp. Control experiments showed that catalase reduction (Massey & Hemmerich, 1978) could be brought about under these conditions. Enzyme photoreduced at pH 7.4 ($\pm$ deazaflavin) shows no change in activity when subsequently assayed polarographically. Enzyme photoreduced at alkaline pH is also catalytically active when assayed at neutral pH, provided that it is not left for prolonged periods at the higher pH.

Photoreduction of the enzyme at high light intensities in the absence of photocatalysts has also previously been shown by Salmeen *et al.* (1978) and Adar & Yonetani (1978).

Subsequent spectroscopic studies were performed at room temperature (30°C) with an Aminco DW-2 spectrophotometer.

Results

Fig. 1(a) shows the absolute spectra of cytochrome c oxidase in the oxidized ('resting') state and after 12 min photoreduction, catalysed by deazaflavin. Both cytochromes a and a_3 are in the ferrous state, and, after admission of air, an oxidized form of the enzyme is restored. The peak shifts of the final product compared with the resting enzyme are, however, smaller than those obtained by aerating the dithionite-reduced enzyme (see, e.g., Fig. 4 of Nicholls *et al.*, 1976). When the corresponding experiment is carried out with no photocatalyst present (Fig. 1b), the reduction achieved (after 60 min irradiation under N_2) is largely of cytochrome a , with a smaller proportion of reduced cytochrome a_3 (i.e. a 'mixed-valence' state). Admission of air oxidizes both species, and the product ($\dots$, Fig. 1b) is now 'oxyferricytochrome aa_3 ', closely similar to the product of oxygenating the dithionite-reduced enzyme. In the absence of light, appreciable reduction only occurs in the presence of CO (Greenwood *et al.*, 1974; Nicholls, 1980), which can itself act as a weak source of reducing equivalents.

Although a powerful reducing system (Massey & Hemmerich, 1978), the deazaflavin/EDTA mixture, unlike the dithionite/Methyl Viologen mixture (Nicholls *et al.*, 1980), cannot reduce cytochrome a_3 . Photocatalysed reduction of the oxidized enzyme-cyanide complex gave rise to the half-reduced species, cytochrome $a^{2+}a_3^{3+}\text{HCN}$, which cannot react with added oxygen (cf. Nicholls *et al.*, 1972).

Difference spectroscopy may be used to probe the reaction sequences more closely. The control experiment shown in Fig. 2 gives the difference spectra (oxidized 'resting' enzyme in the reference cuvette) for the reduction and reoxidation processes in Fig. 1(a). Light induces full reduction in Soret (Fig. 2a) and visible regions (Fig. 2b) and reoxygenation leaves only a small proportion of the low-spin 'oxyferri' form. If the photoreduced enzyme is treated with CO, the resulting oxidation is slower, detectable amounts of reduced species (ferrocycytochrome a) occur as intermediates and the final product (results not shown) contains a larger amount of the 'oxyferri' form than in the absence of CO (Fig. 2).

In the absence of photocatalyst (cf. Fig. 1b) and in the presence of CO, a partially reduced species containing both $a^{3+}a_3^{2+}\text{CO}$ and $a^{2+}a_3^{3+}$ forms is produced (Fig. 3). On standing this becomes the mixed-valence species (Greenwood *et al.*, 1974), cytochrome $a^{3+}a_3^{2+}\text{CO}$. Admission of air now gives rise to the 607 nm species (Nicholls, 1979; Chance & Leigh, 1977) with characteristic α - and β -bands in the visible region. The appearance of this compound thus depends on the initial presence of cytochrome a in the ferric state.

Fig. 1. Photoreduction of cytochrome c oxidase: absolute spectra

(a) In the presence of the deazaflavin catalyst. Cytochrome aa_3 ($3.53\mu\text{M}$) was dissolved in 50 mM-sodium phosphate/0.25% Tween-80, pH 7.4, 30°C and irradiated under N_2 in the presence of 11.5 mM-EDTA and $2.6\mu\text{M}$ -3,10-dimethyl-5-deazaalloxazine for 12 min (see the Materials and methods section), to give the fully reduced species (----). Air was then admitted to the Thunberg cuvette and the spectrum retraced (....). The continuous line (—) shows the spectrum of the initial fully oxidized form. (b) In the absence of the photocatalyst. Cytochrome aa_3 ($3.53\mu\text{M}$) was dissolved in 50 mM-sodium phosphate/0.25% Tween-80, pH 7.4, 30°C (—) and irradiated under N_2 in absence of any photocatalyst for 1 h to give the partially reduced species (----). Addition of air to the Thunberg cuvette gave the oxygenated spectrum (....).

The rates of some of the processes involved can be slowed by carrying out the reactions at a more alkaline pH value (Kornblatt, 1977). Experiments were performed in pyrophosphate buffer, pH 9, for

comparison with results at neutral pH. Fig. 4 shows the products obtained by chemical reduction. At high pH, reduction of cytochrome a by dithionite is fast, but reduction of cytochrome a_3 is slow.

Fig. 2. *Difference spectra of photoreduced enzyme before and after oxygenation*

(a) Soret region; (b) visible region. Cytochrome aa_3 ($3.5\mu\text{M}$) was irradiated in the presence of EDTA and 3,10-dimethyl-5-deazaalloxazine as described in the Materials and methods section. A similar enzyme sample was placed in an aerobic reference cuvette, to give the baseline (—). The fully reduced spectrum after irradiation (----) is immediately abolished on admission of air (-.-.-) and little change occurs thereafter (.....).

Fig. 3. *Difference spectra of enzyme in the presence of CO, and in the absence of photocatalyst, before and after oxygenation*

Irradiation of $3.53\mu\text{M}$ -cytochrome aa_3 under CO for 10 min gave the partially reduced spectrum (----), which was transformed into the mixed-valence species (.....) on overnight incubation at 4°C . Subsequent admission of air gave the 607 nm complex (-.-.-). The reference cuvette contained oxidized enzyme in aerobic buffer (50 mM-sodium phosphate, pH 7.4, plus 0.25% Tween-80) at 30°C .

Fig. 4. *Dithionite-induced reduction and reoxidation of cytochrome c oxidase at alkaline pH: difference spectra* Cytochrome *aa*₃ (3.67 μ M) was dissolved in 50 mM-sodium pyrophosphate buffer, final pH 9.0, plus 0.25% Tween-80 at 30°C. A few milligrams of sodium dithionite added to an aerobic cuvette produced an initial reduction (.....), which gave way to full reduction after 30 min (---, final spectrum); the sample was then re-oxidized by addition of air (----). In a parallel experiment, ferricyanide oxidized all the components absorbing at 605 nm (—). The reference cuvette contained oxidized enzyme at pH 7.4, and the 'oxidized-minus-oxidized' trace (——) therefore reflects the slight high- to low-spin shift in the oxidase on raising the pH.

Intermediate half-reduced spectra (....., Fig. 4) are seen before final reduction (---). Ferricyanide oxidizes all the haem components (—). Oxygen admission to a similar reduced sample (----) produces an initial intermediate differing perhaps either in spin state or in redox state from the resting oxidized form. A similar intermediate is produced on addition of oxygen to the photoreduced enzyme at alkaline pH (Fig. 5). It is spectroscopically identical neither with ferrocytochrome *a* nor with the 607 nm compound; in the difference spectrum a trough at 412 nm is accompanied by a peak at 439 nm, and in the visible region by a peak at 605.5 nm, slightly to the 'red' side of the fully-reduced-enzyme spectrum.

The same intermediate is formed on causing the alkaline photoreduced CO complex to react with oxygen (Fig. 6). The complex is only slowly destroyed by ferricyanide (Fig. 6a), but the absorption bands are immediately removed on addition of sufficient HCl to restore a pH of 7.0–7.5 (results not shown). It is also decomposed on addition of

stoichiometric ($\sim 4 \mu$ M) amounts of ferrocytochrome *c* from the side-arm of the Thunberg cuvette, after the system has been re-evacuated (Fig. 6b). This resembles the behaviour reported by Chance (1980) for 'compound B' produced by the reaction of oxygen with reduced enzyme at low temperatures, and seems to be inconsistent with the idea that the intermediate is only a partially reduced form of the enzyme in which ferrocytochrome *a* is sluggishly reactive.

Discussion

The dispute between Lemberg and Gibson (see, e.g., Lemberg, 1966) as to the immediate product of the reaction between reduced oxidase and oxygen was not resolved even by the careful study of Gilmour *et al.* (1969), who found mixtures of 'ferric' and 'oxyferri' enzyme in the reaction products, even in supposedly kinetically homogeneous membrane preparations. More recently, Antonini and his co-

Fig. 5. Photoreduction of cytochrome *c* oxidase at an alkaline pH and the reoxidation of reduced enzyme by O_2 : difference spectra

Cytochrome aa_3 was dissolved in sodium pyrophosphate buffer, pH 9.0, with 0.25% Tween-80 at 30°C as in Fig. 4. Photoreduction was carried out under N_2 with 11.5 mM-EDTA plus 2.6 μ M-3,10-dimethyl-5-deazaalloxazine by using a 10 min irradiation period (see the Materials and methods section). The resulting partially reduced compound is shown by ----. Admission of O_2 to the Thunberg cuvette resulted in the partial reoxidation shown by The reference cuvette contained oxidized (resting) enzyme at pH 7.4 as in Fig. 4.

workers (Antonini *et al.*, 1977; Brunori *et al.*, 1979) have identified a third ferric species ('pulsed' oxidase) as a reaction intermediate with a spectrum intermediate between the resting ferric and oxyferri forms. Greenwood *et al.* (1974) distinguished between the products of oxygenation of mixed-valence enzyme and the products of oxygenation of fully reduced enzyme; the former appeared largely oxyferri (low-spin, or 'oxygenated'), whereas the latter were essentially ferric (Wharton & Gibson, 1968) or

spectral transitions of ferric cytochrome a_3 was associated with the reduction of cytochrome *a* and involved a haem-haem interaction perhaps the same as that involved in the formation of the e.p.r. signals characteristic of the ferric cytochrome a_3 centre present in mixed-valence enzyme (Hartzell & Beinert, 1976). This finding might be used to link two catalytic cycles (eqns. 1 and 2) undergone by the fully reduced (eqn. 1) and mixed-valence (eqn. 2) enzymes respectively:

perhaps 'pulsed' ferric enzyme (Brunori *et al.*, 1979).

Nicholls *et al.* (1976) and Nicholls & Hildebrandt (1978) suggested that at least one of the

where $a^{3+}a_3^{3+}$ (high-spin) and $a^{2+}a_3^{3+}$ (high-spin) indicate the presumed 'resting' or 'pulsed' forms of the enzyme and $a^{2+}a_3^{3+}$ (low-spin) and $a^{3+}a_3^{3+}$

Fig. 6. Photocatalysed reduction of cytochrome *c* oxidase at an alkaline pH in the presence of CO: difference spectra (a) Effects of oxygen and ferricyanide. Cytochrome *aa*₃ was photoreduced at pH 9.0 in a Thunberg cuvette under an atmosphere of CO (other conditions as in Figs. 4 and 5). The fully reduced spectrum is given by —. Admission of O₂ (air) to the cuvette immediately gave rise to the species indicated by ····, which slowly decayed to a partially oxidized state (----). The latter could only slowly be further oxidized by addition of ferricyanide (-·-·-). (b) Effect of ferrocytochrome *c* addition. Cytochrome *c* oxidase was photoreduced at pH 9.0 by 20 min irradiation under CO and in the presence of the photocatalyst as in Figs. 4 and 5. The fully reduced spectrum is given by —. Admission of O₂ as in Fig. 6(a) gave rise to the species indicated by the dashed spectrum (----). A few microlitres of ferrocytochrome *c* solution were then added from the side-arm to a final concentration of 4 μ M (a very slight excess over the molar concentration of oxidase present), resulting in the loss of the 605.5 nm species shown by ····. The reference cuvette contained oxidized (resting) enzyme at pH 7.4 as in Fig. 4.

(low-spin) are the presumed 'oxyferri' forms (Nicholls, 1979), and compounds B and C are the intermediate identified at low temperature by Chance & Leigh (1977) or the corresponding intermediates discussed here and by Nicholls (1979, 1980).

The stable form in the oxidized state, and the form produced by oxygen attack on the fully reduced enzyme, is a high-spin cytochrome $a^{3+}a_3^{3+}$, a ferric or perhaps 'pulsed' form. This form can be reduced to a half-reduced species (lower line, eqn. 1) that can either accept more electrons to become fully reduced enzyme (top line, eqn. 1), which then recycles via compound B, or (in absence of adequate reducing power, or when a_3^{3+} is blocked by an inhibitor) change to another half-reduced species, a low-spin form of $a^{2+}a_3^{3+}$, the 'oxyferri' state, via compound C. Only when compound C, the 607 nm intermediate (Fig. 3), is produced, does the low-spin state terminate the reaction sequence as a product of compound C reduction (Fig. 1). This occurs more readily in dithionite-initiated cycles (Lemberg & Mansley, 1966) because of the various mixed-valence and half-reduced forms that can intervene during oxygenation.

The relative importance of the cycle via fully reduced enzyme and compound B (eqn. 1) and the cycle via the mixed-valence species and compound C (eqn. 2) will be determined by the relative rates of reduction and spin-state change of the species $a^{2+}a_3^{3+}$ (high-spin), which will presumably be a function of the redox state of the catalytic system involved. We conclude that both fully reduced and mixed-valence enzyme are 'catalytically competent' to maintain a rapid rate of oxygen uptake and oxidation of cytochrome *c*. Whether there are bioenergetic differences between the two types of cycle, however, remains to be determined (cf. Nicholls & Wrigglesworth, 1980).

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