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The oxygen reactive species of cytochrome-*c*-oxidase: an alternative view

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In a recent review article Babcok and Wikström (Nature, 1992, 356, 301–309) proposed that the species of cytochrome-*c*-oxidase which binds molecular oxygen during turnover is the so-called mixed valence enzyme, in which the binuclear center cytochrome a_3-Cu_B is reduced, while the cytochrome a/Cu_A sites are oxidized. This proposal is based on earlier work (Morgan and Wikström, Biochemistry 1991, 30, 948–958) in which it was found that the steady-state reduction levels of cytochrome *c* and cytochrome *a* in respiring rat liver mitochondria (sustained by ascorbate and TMPD) are quite different, the latter being much more oxidized than the former; evaluation of the steady-state reduction levels demanded a large correction due to the optical contribution of oxidized $TMPD^+$ which overlaps with the cytochromes. We report below that application of transient spectroscopy and SVD analysis to respiring rat heart myocytes, under conditions in which the contribution of $TMPD^+$ is very small or absent, allows to show that the steady-state reduction levels of cytochrome *c* and cytochrome *a* are comparable at all times accessible to measurement in the rapid-scanning stopped-flow spectrophotometer. Our conclusion, in agreement with previous results, is that mixed valence cytochrome-*c*-oxidase as defined above is not the prevailing oxygen binding species of cytochrome-*c*-oxidase, unless electron donation to cytochrome *c* becomes rate limiting.

Cytochrome oxidase; Electron transfer; Mitochondria; Kinetics

1. INTRODUCTION

The reaction between oxygen and cytochrome-*c*-oxidase, the crucial enzyme of cell respiration, has been reviewed with deep insight by Babcock and Wikström [1] who payed particular attention to the oxygen intermediates and their role in the mechanism of proton pumping. One point to which they draw attention is the oxidation state of the enzyme which reacts with oxygen during aerobic turnover in mitochondria; this, they state, may be either the fully reduced (a^{2+} CuA^+ /a_3^{2+} CuB^+) or the so-called mixed valence (a^{3+} CuA^{2+}/a_3^{2+} CuB^+) enzyme in which only the binuclear center (where oxygen binds) is reduced. This is an important question because the efficiency of proton pumping by cytochrome-*c*-oxidase may be crucially linked to the nature of the oxygen reacting species; as stated in [1],'under steady-state turnover conditions heme *a* (which we call cytochrome *a*) is largely oxidized and oxygen reacts for the most part with the enzyme in what is effectively a mixed valence state', based on the experimental data of Morgan and Wikström [2]. Babcock and Wikström [1] somewhat cavalierly discount previous results [3–5] indicating (i) that the rate-limiting step is the internal electron transfer process leading to reduction of the binuclear center (or a conformational change of the enzyme which rate-limits eT, see [6]), and (ii) that the mixed valence state (as defined above) is not the oxygen reactive species. We dissent from their conclusion, and present below the experimental evidence to support our viewpoint.

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Abbreviations: TMPD, *N,N,N',N'*-tetramethyl *para*-phenylenediamine; eT, electron transfer; SVD, singular value decomposition; RCR, respiratory control ratio.

2. BACKGROUND

In a highly simplified form, electron transfer (eT) from cytochrome *c* to oxygen may be depicted schematically as follows:

$$c \overset{k_1}{\longleftrightarrow} (a/CuA) \overset{k_2}{\longleftrightarrow} (a_3/CuB) \overset{k_3}{\longrightarrow} O_2$$

where: k_1 represents the second order rate constant for eT from reduced cytochrome *c* to oxidized cytochrome *a*/*CuA* (which are in very rapid internal equilibrium, τ^{-1}=1–2 ×10^4 s^{-1}, refs. [1] and [7]); k_2 is the intramolecular rate constant for the eT step(s) leading to reduction of the binuclear center, cytochrome a_3/*CuB*, a prerequisite for the binding of oxygen and CO at this site; and k_3 groups together all the reactions of binding and eT

leading (via several intermediate states) to complete reduction of oxygen to water. Because the slowest rate constant for the latter process measured in classical flow-flash experiments [8,9] is very fast (0.5– 1× 10^3 s^{-1}) compared to turnover, it is immediately apparent that if electron donation from cytochrome *c* (k_1) is slow the oxygen reactive species will indeed be the mixed valence state of the enzyme as concluded by Babcock and Wikström [1]; on the other hand if the internal eT is rate-limiting, as we believe, then the matter demands more careful consideration, as we shall discuss below.

With cytochrome-*c*-oxidase solubilized in detergent(s) or reconstituted into small unilamellar vesicles (displaying proton pumping, respiratory control and high enzyme activity), our kinetic experiments [3–5] show that the rate-determining step in turnover is the internal eT process. Given that the initial eT is bimolecular, and because under these conditions the concentration of cytochrome *c* can be increased 'ad libitum', this conclusion rests on a very firm basis. In contrast, Morgan and Wikström [2] concluded that in (rat liver) mitochondria the mixed valence enzyme is the oxygen-reacting species because during aerobic steady-state (sustained by ascorbate and *N,N,N′,N′*-tetramethyl *para*-phenylendiamine, TMPD) the reduction level cytochrome *c* is much greater than that of cytochrome *a*. This conclusion rests entirely on experiments (such as that shown in Fig. 2 of ref. [2]) where the reduction levels of cytochrome *a* and cytochrome *c* are estimated after subtraction of a very large contribution from oxidized $TMPD^+$ (incidentally, the concentration of ascorbate used in this and other experiments reported in [2] was not given). We challenge this result and therefore question a major conclusion of Morgan and Wikström [2], referred in the Nature Review article [1] as an established fact.

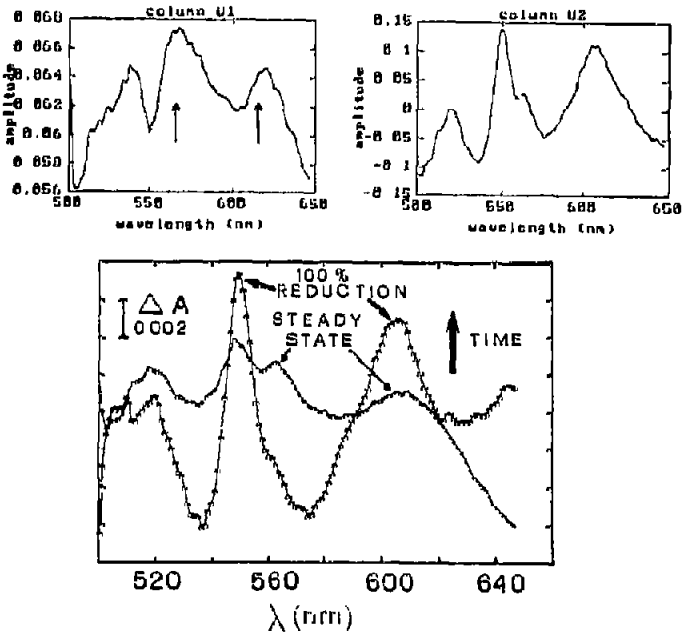

Fig. 1. Transient spectra of rat liver mitochondria in the visible region. 62 spectra were sequentially acquired (from 10 ms to 30 s after mixing) with a Tracor Northern TN6500 (Tracor, USA) photodiode array spectrometer adapted to a Gibson-Durrum stopped-flow apparatus. Aerobic rat liver mitochondria (15 mg/ml total protein), prepared according to [2], having a RCR of 3, suspended in a buffer containing 180 mM sucrose, 40 mM KCl, 1 mM EGTA, 3 mM Li/HEPES pH 7.1, and in the presence of 8 μM myothiazole and 10 μM rotenone, *were mixed* with 10 mM ascorbate and 0.4 mM TMPD, dissolved in the same buffer. Results are plotted as difference spectra; the reference spectrum being the aerobic suspension of rat liver mitochondria in the absence of ascorbate and TMPD. Top panels: SVD analysis of the difference spectra, showing U columns 1 and 2 with the following *S* values: 2.867, 0.21. The arrows in column U_1 indicate the wavelength of the maxima of the difference spectrum ($TMPD^+$ – TMPD), at 565 and 615 nm. Bottom panel. Difference spectra obtained after reconstruction from SVD columns 1 and 2. (–×–×–), spectrum during steady state; (–○–○–), spectrum after oxygen exhaustion and complete reduction.

3. EXPERIMENTAL RESULTS

Figs. 1 and 2 show some of the results obtained by transient optical spectroscopy and singular value decomposition (SVD) analysis on a suspension of rat liver mitochondria and rat heart myocytes. SVD analysis of the absorbance/time/wavelength matrix allows deconvolution of absorbancies into basis spectra and time courses [10,11]. In the experiment with mitochondria, carried out under conditions similar to those employed by Morgan and Wikström [2], the SVD analysis (Fig. 1, top panels), shows that the two basis spectra represented by columns U_1 and U_2 contain the significant information with the spectral contributions of $TMPD^+$ and the cytochromes; in particular, in column U_1, a small contribution of cytochrome *c* and a large contribution of $TMPD^+$ can be easily distinguished.

The reconstructed difference spectrum of rat liver mitochondria at steady state (bottom panel of Fig. 1) is indeed very similar to that published by Morgan and Wikström [2] in Fig. 2, panel a. The broad band in the 600 nm region has a maximum well above that characteristic of cytochrome-*c*-oxidase (i.e. 605 nm [12]) and a band at 562 nm is observed, both features being related to the large spectral contribution of $TMPD^+$, which accumulates during the reaction and distorts the spectra of the cytochromes [2]. When oxygen is exhausted the reduction of $TMPD^+$ by excess ascorbate leads to a bleaching of its contribution to the difference spectrum (also in Fig. 1). It is self evident that an objective estimate of the steady-state reduction levels of cytochrome *c* and cytochrome *a* is difficult and subject to uncertainties, and the large corrections to be introduced may jeopardize the conclusions (especially if the absorbance of $TMPD^+$ is 'arbitrarely scaled to match' the observed steady-state spectrum, as stated by Morgan and Wikström in Fig. 2 of [2]).

On the other hand a similar experiment carried out

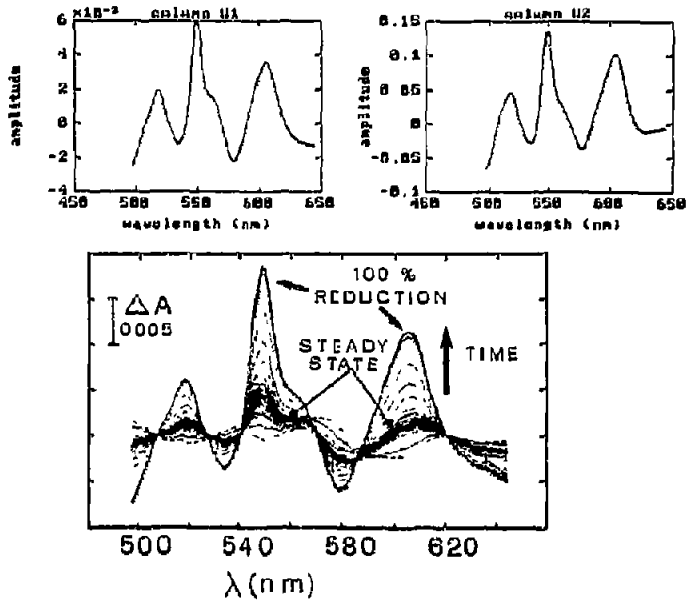

Fig. 2. Transient spectra of rat heart myocytes in the visible region 60 spectra were sequentially acquired (from 10 ms to 150 s after mixing) with the photodiode array spectrometer described in Fig. 1. Aerobic rat heart myocytes (10^6 cells/ml) in PBS Dulbecco's pH 7.2, 1 mM $CaCl_2$, 3% polyvinylpyrrolidone *were mixed* with ascorbate (10 mM) and TMPD (0.2 mM) dissolved in the same buffer. T = 20°C Myoglobin was 'frozen' as the carbon-monoxide derivative throughout (see ref. [13] for experimental details). Results are plotted as difference spectra, the reference spectrum being the aerobic suspension of rat heart myocytes in the absence of ascorbate and TMPD. Top panels. SVD analysis of the difference spectra, showing U columns 1 and 2 with the following *S* values. 17.10, 0.33. Bottom panel: Difference spectra obtained after reconstruction from SVD columns 1 and 2.

with rat heart myocytes (see [13] for experimental details) yields cleaner spectral-kinetic data. SVD analysis (Fig. 2, top panels) provides no indication for a significant spectral contribution of $TMPD^+$ in columns U_1 and U_2 which together account for > 98% of the total absorbance changes. The reconstructed difference spectra (Fig. 2, bottom panel) show that the observed maxima are correct (being 549 nm for cytochrome *c* and 606 nm for cytochrome *a*, with ±1 nm tolerance because of the grating, the band-width and the calibration of the photoarray); isosbestic points are seen throughout the time course. Therefore, no significant accumulation of $TMPD^+$ is detected, because we believe this is rapidly reduced in the suspension of intact myocytes. No spectral corrections are therefore necessary. As may be seen from Fig. 2, the steady state reduction levels of cytochrome *c* and cytochrome *a* under the conditions employed are very similar (approx. 25%). It may be pointed out that this corresponds however to a *minimum estimate* given that the reference base line spectrum employed in the analysis was that of a myocyte suspension in air; thus, it did not necessarily correspond to 100% oxidation.

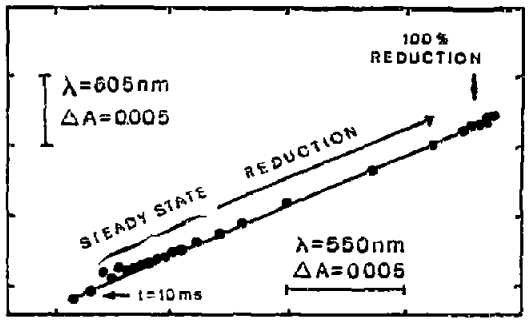

Fig. 3. Correlation between absorbance changes at 550 and 605 nm from the experiment carried out with rat heart myocytes. Absorption changes observed at 550 nm (mainly cytochrome *c* reduction) are plotted against absorption changes at 605 nm (mainly cytochrome *a* reduction) at times from 10 ms to 150 s; cytochrome a_3 is always > 95% oxidized at steady-state.

4. CONCLUSION

As shown above, the steady-state level of reduction of cytochrome *c* in mitochondria of intact myocytes is not higher than that of cytochrome *a*, and the two change together at all times from initial observation to complete reduction after 150 s, i.e. before, during and after steady state. This is clearly borne out by the linear correlation shown in Fig. 3; thus the rate of the internal eT (k_2) cannot be faster than electron donation from cytochrome c^{2+}(k_1). This conclusion is consistent with the notion that the 'concentration' of cytochrome *c* in the space limited by the inner and outer mitochondrial membranes is high (up to 0.5 mM, see [14]), and thus electron equilibration between cytochrome *c* and cytochrome-*c*-oxidase is expected to be fast. We suggest that in respiring mitochondria, using ascorbate and TMPD to sustain respiration, Morgan and Wikström [2] may have reached a similar conclusion without the large and difficult corrections for the optical contribution of $TMPD^+$.

It is well established since Gibson and Greenwood's [8] classical flow-flash experiments that oxidation of cytochrome *a* and of CuA (with eT to the binuclear center) is indeed very fast (k = 0.5–1 × 10^3 s^{-1}) in some states of the enzyme populated after oxygen binding. This is not surprising given that with partially reduced oxygen intermediates bound at the binuclear center the driving force is enormously increased and the coordination of the active site is changed compared to the oxidized enzyme. The significance of the structural and thermodynamic properties of the oxidase in controlling internal eT rates has been lucidly analyzed by Gray and Malmström [6] within the framework of Marcus theory. Rates of eT to oxygen intermediates have been recently confirmed by Nilsson [15] employing the P (peroxy) and F (ferryl) states (see [1]), and measuring eT from cytochrome a^{2+} rapidly reduced by a photochemical technique. However the rates of eT in these oxygen bound

states are not relevant, in our opinion, to understanding the process of internal eT from cytochrome *a*/*CuA* to the oxidized binuclear center, the final species following reduction of oxygen to water. It is the reduction of the binuclear center, a *prerequisite* for the binding of and eT to dioxygen, which we believe to be rate limiting, based (among other) on the demonstration that the steady-state reduction levels of cytochrome *c* and cytochrome *a* are comparable, not only in detergent solution and in artificial vesicles but also in intact myocytes (see above).

If therefore the internal eT is the rate-limiting step also in mitochondria, the proposition [1] that the mixed-valence state (a^{3+} CuA^{2+}/a_3^{2+} CuB^{+}) is, by-and-large, the oxygen reactive species [1] may still be valid, when (for example) the rate of reduction of cytochrome *c* in vivo or in vitro becomes limiting. However it was shown by Malatesta et al. [4] that both metals at the binuclear center must be reduced before CO can bind, and that eT to this site proceeds in two successive steps, each corresponding to one electron and both slower than electron entry from cytochrome *c*, with CuB^{2+} being reduced first. At sufficiently high cytochrome c^{2+} and CO concentrations (see [4] and [16]), the internal eT rate measured by the CO-trapping technique matches the turnover number of the enzyme in solution and in artificial uncoupled vesicles (COV) implying that, like CO, oxygen only binds once both metals at the binuclear centre are reduced, as agreed by Babcock and Wikström [1]. The consequence of this line of argument is that the mixed valence state with cytochrome *a* fully oxidized cannot be the primary oxygen reacting species, unless electron donation to cytochrome *c* becomes rate limiting; and thus the fully reduced enzyme, or a 3 electron-reduced state in which only cytochrome *a* or *CuA* is oxidized , is the species reacting with oxygen. As discussed by Babcock and Wikström [1] this conclusion is of great significance to the mechanism and the yield of the proton pump, and highlights the need for measuring proton translocation stoichiometries coupled to electron transfer in single turnover experiments.

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