STEADY STATE REDOX LEVELS IN CYTOCHROME OXIDASE: RELEVANCE FOR IN VIVO NEAR INFRARED SPECTROSCOPY (NIRS)

Chris E. Cooper[1], Martyn A. Sharpe[2], Maria G. Mason[1] and Peter Nicholls[1]

Abstract: In the visible/NIR (600 – 900 nm) three different redox centres are potentially detectable *in vivo* in mitochondrial cytochrome *c* oxidase: haem *a* (605nm), the binuclear haem a_3/Cu_B centre (655 nm) and Cu_A (830 nm). In this paper we report changes in the steady state reduction of these centres following increases in the rate of electron entry into the purified enzyme complex under conditions of saturating oxygen tension. As turnover is increased all three centres becomes progressively reduced. Analysis of the steady states indicated that all three centres remained in apparent equilibrium with cytochrome *c* throughout the titration. The calculated redox potentials of Cu_A (+224 mV) and haem *a* (+267 mV) were consistent with previous equilibrium data. The 655 nm band was also found to be oxygen and flux sensitive. It may be a useful additional *in vivo* detectable chromophore. However, it titrated with an apparent redox potential of +230mV, far from its equilibrium value (+400 mV). The implications of these results for the interpretation of non invasive measurements of mitochondrial function are discussed.

1. INTRODUCTION

There has been renewed interest recently in using optical techniques to study the mitochondrial cytochrome oxidase (CCO) redox state *in vivo*, both to address basic science[1, 2] and clinical[3, 4] questions. In most cases changes in redox state have been interpreted as due to changes in intracellular pO_2[4]. However, there are a number of other parameters that can change the redox state of CCO. CCO reacts with ferrocytochrome *c*

[1] Department of Biological Sciences, University of Essex, Wivenhoe Park, Colchester Essex, CO4 3SQ, UK ccooper@essex.ac.uk
[2] Biochemistry and Molecular Biology Department, Michigan State University, East Lansing, MI 48824-1319, USA

P. Liss et al. (eds.), *Oxygen Transport to Tissue XXX*, DOI 10.1007/978-0-387-85998-9_19,

and oxygen to produce water, ferricytochrome *c* and a proton motive force ($\Delta \bar{\mu}_{H+}$) across the inner mitochondrial membrane. In general flux through CCO can be modelled as a Michaelis-Menton function of its substrates (oxygen, ferrocytochrome *c*), modulated by product inhibition (from ferricytochrome *c* and $\Delta \bar{\mu}_{H+}$). Thus, in addition to oxygen, changes in the redox state of cytochrome *c* and Δp will contribute to any observed *in vivo* changes in redox state. Our "grand aim" is to develop a model of CCO function to aid the interpretation of *in vivo* mitochondrial spectroscopy. The interpretation of mitochondrial $\Delta \bar{\mu}_{H+}$ effects *in vivo* is complicated by the fact that $\Delta \bar{\mu}_{H+}$ can act at three parts of the chain (complexes I, II and III), making it impossible to *a priori* predict the effects at one site[5]. Similarly it is a non trivial, and controversial, process to measure redox states accurately at low oxygen tension[6, 7]. Therefore the initial, more tractable, aim of our project outlined here is to understand the relationship of CCO redox centres to changes in the cytochrome *c* redox state at saturating oxygen and zero $\Delta \bar{\mu}_{H+}$.

2. METHODS

Bovine heart CCO was purified according to the method of Kuboyama *et al.* [8] with Tween-80 substituting for Emasol. Maximum turnover (pH 7.4) was approximately 380 $e^{-}aa_3^{-1}$ s^{-1}. In a cuvette 10 μM CCO was incubated at 30 °C. with 10 μM horse heart cytochrome c and 100 nM bovine liver catalase, in 1 ml of 25 mM K^{+}-HEPES, 0.1% lauryl maltoside, pH 7.8. Sodium ascorbate (40 mM) was added to the cuvette, to initiate turnover of the enzyme; the solution was then allowed to go anaerobic. Following anaerobiosis hydrogen peroxide (H_2O_2) was added to the cuvette (final concentration 0.5 - 2 mM). The catalase rapidly decomposed the H_2O_2 into oxygen in the incubation medium, thus re-initiating enzyme turnover. Following the second anaerobiosis, the cuvette was re-oxygenated with H_2O_2 and a second steady state was produced. No difference was observed between the two steady states.

Absorbance changes between 520 and 800 nm were monitored using a Beckman DU 7400 diode array spectrophotometer. Redox changes of cytochrome c were monitored at 550-540 nm. Redox changes of haem *a* were monitored at 605-630 nm, assuming a 14% contribution at this wave pair of haem a_3 in the reduced enzyme and no contribution of haem a_3 in the steady state[9]. Absorbance changes in the near infrared were recorded using a Aminco DW2 spectrophotometer under identical assay conditions; the Cu_A redox state was measured using the 780-830 nm wavepair. 100% oxidation of all chromophores was asssumed in the absence of added reductants and 100% reduction was assumed upon anaerobiosis. Repeating the experiment at increasing concentrations of the reductant TMPD (N,N,N',N' -tetramethyl-p-phenylenediamine) resulted in an increase in the cytochrome *c* redox state and a consequent increase in enzyme turnover. The latter was measured by using the time taken for the sample to go anaerobic (independent measurements with an oxygen electrode demonstrated that under these conditions the rate of oxygen consumption is essentially linear with respect to the complete time of the assay, due to the low, submicromolar, K_m of the enzyme for oxygen under these conditions).

3. RESULTS

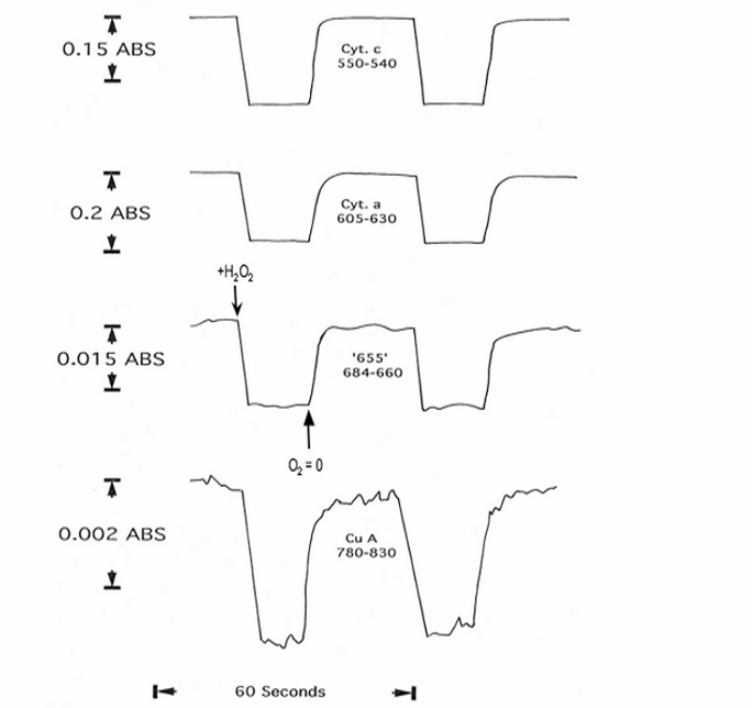

Figure 1. Absorbance changes during repeated cycles of oxygenation/deoxygenation (illustrative additions of peroxide and effects of anaerobiosis indicated)

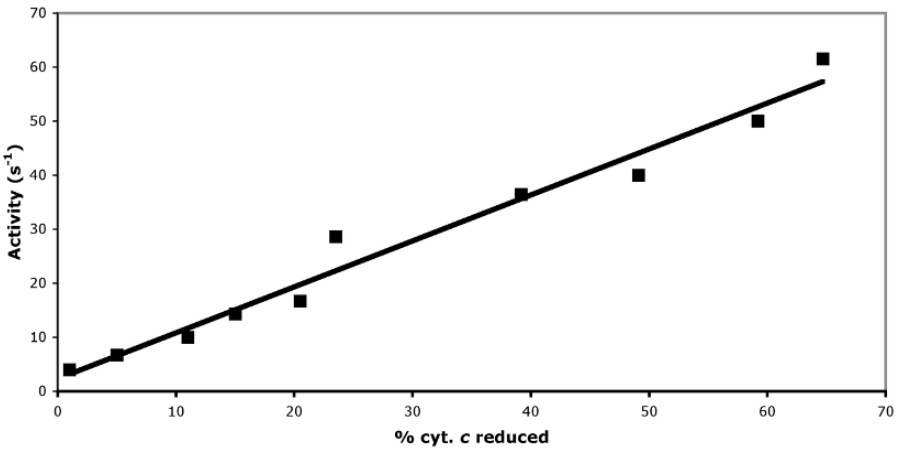

Figure 2. Variation of enzyme activity (electrons s^{-1}) with increasing fraction of reduced cytochrome *c*

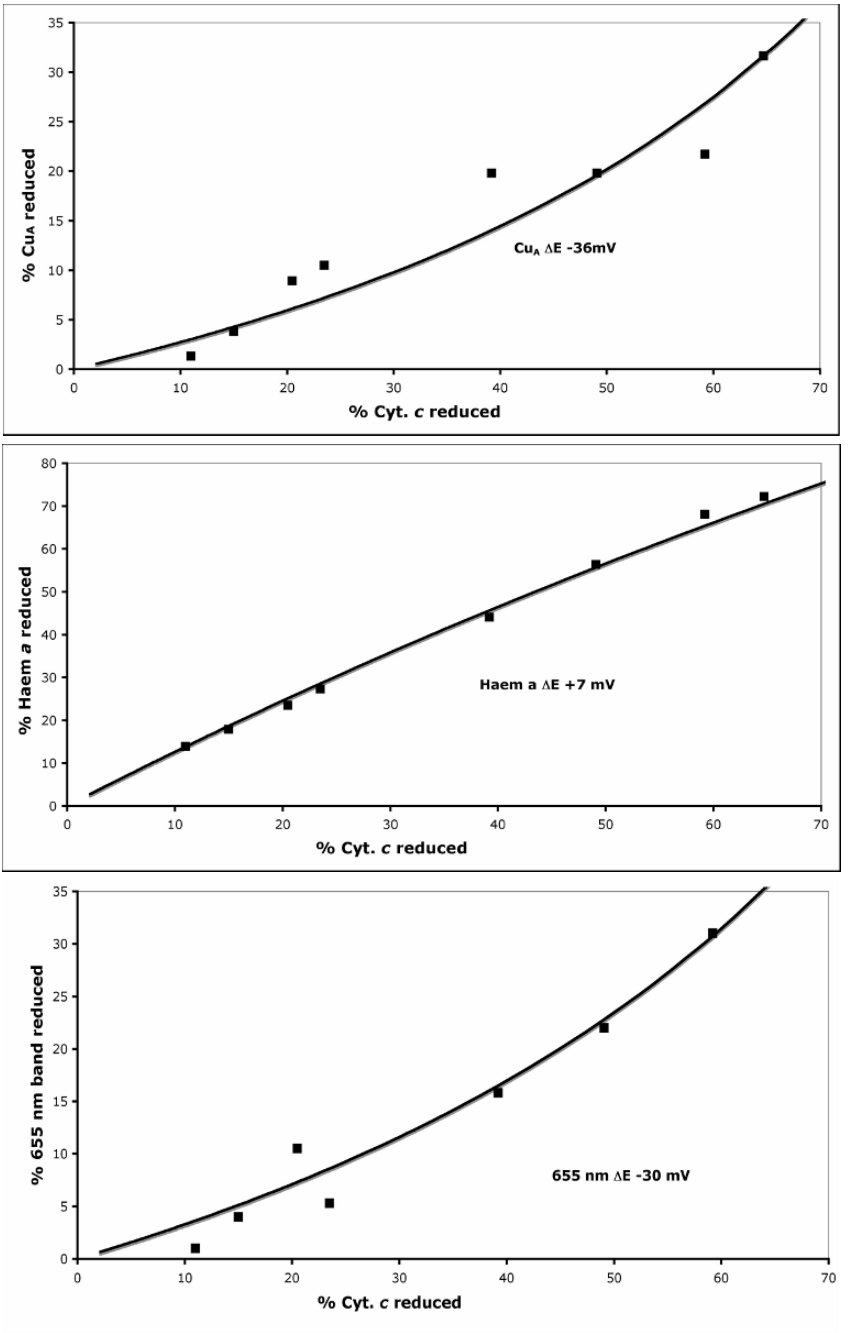

Figure 3. Relationship between cytochrome oxidase and cytochrome *c* redox states during turnover. (data fitted to Nernst equation, relative redox potential with respect to cytochrome *c* indicated)

Figure 1 illustrates the changes in redox states during repeated cycles of respiration driven deoxygenation and peroxide/catalase–driven reoxygenation of the system. All centres respond essentially simultaneously, becoming partially reduced in the presence of the oxygen and fully reduced when the oxygen is used up. It is possible to set-up different steady states at different concentrations of reductant (TMPD). Increasing the TMPD concentration increases the steady state reduction of cytochrome *c* and hence the rate of enzyme turnover, which is linearly dependent on the cytochrome *c* redox state (Figure 2). It is possible to compare the steady state of cytochrome *c* to that of the optical chromophores in CCO. By fitting to the Nernst equation it can be seen whether an individual redox centre behaves as if it were in redox equilibrium with cytochrome *c* and, if so, what the relative redox potentials are (illustrated for the single electron transition between cytochrome *c* and Cu_A in Equation 1).

$$E_{CuA} - E_{cytc} = \frac{RT}{F}\left(\left(\ln\frac{cytc^{3+}}{cytc^{2+}}\right) - \left(\ln\frac{Cu_A{}^{2+}}{Cu_A{}^{+}}\right)\right) \qquad \text{Equation 1}$$

Figure 3 indicates that Cu_A, haem *a* and the 655 nm band all behave as if in apparent redox equilibrium with cytochrome *c*. Assuming a redox potential for cytochrome *c* in free solution of + 260 mV, this results in apparent potentials for the other centres of: Cu_A (+ 224 mV), haem *a* (+ 267 mV) and 655 nm band (+ 230mV).

4. DISCUSSION

These findings indicate that a variety of redox centres within mitochondrial CCO (Cu_A, haem *a*, haem a_3/Cu_B) are sensitive to changes in the concentration of the substrates ferrocytochrome *c* and oxygen. This is not surprising[10] although the sensitivity to substrate delivery reported here is rarely discussed when interpreting *in vivo* data. Although all signals appear in an apparent redox equilibrium with cytochrome *c*, the values for these "steady state" redox potentials vary.

In the case of Cu_A the potential measured (+230 mV) falls within the range of values (230 - 265 mV) calculated from true equilibrium experiments performed in the absence of oxygen or in the presence of an inhibitor of oxygen consumption[11-14]. For haem *a* the situation is more problematic. The finding that the haem *a* potential is slightly more positive than cytochrome *c* is consistent with other steady state[15] or pseudo steady-state experiments[16,17]. However, when haem a_3 is allowed to become reduced in an equilibrium titration there is a strong redox interaction between haem *a* and a_3[14] such that haem *a* no longer titrates as an n = 1 electron donor; its apparent redox potential can be as high as 360 mV. It is therefore difficult to make quantitative comparisons between the steady state and true equilibrium data for haem *a*. The 655 nm band is more problematic, both in spectral assignment and redox potential. It probably a charge transfer band of ferric high-spin haem a_3, which is modulated by the redox state of Cu_B. In equilibrium titrations it titrates at a potential of 400 mV. Clearly this is far removed from our value of +230 mV. Perhaps not surprisingly the signal that is part of the oxygen reduction site titrates far from equilibrium in a steady state when oxygen is being consumed.

What implications does this have for *in vivo* detection of CCO? Clearly these three centres that can all detect flux changes in the enzyme. However, the interpretation of changes in the haem *a* 605 and haem a_3 655 nm band is problematic, due to their complex redox and oxygen interactions. Furthermore these two centres are buried inside the enzyme and their response to $\Delta\ \mu_{H+}$ is unpredictable. In contrast the Cu_A centre is likely

to remain in equilibrium with cytochrome *c* under most conceivable conditions. It is physically close to the cytochrome *c* binding site, sensing a similar $\Delta \bar{\mu}_{H+}$[12]. Although more work is needed, particularly at low $[O_2]$ and in the presence of $\Delta \mu_{H+}$, it seems likely that Cu_A will prove to be a robust marker of the cytochrome *c* oxidation state *in vivo*.

5. ACKNOWLEDGEMENTS

We would like to thank BBSRC (D0609821/1) and EPSRC (EP/F006551/1) for financial support.

6. REFERENCES

1. M. M. Tisdall, I. Tachtsidis, T. S. Leung, C. E. Elwell, and M. Smith, Near-infrared spectroscopic quantification of changes in the concentration of oxidized cytochrome c oxidase in the healthy human brain during hypoxemia, *J. Biomed. Opt.* **12**, 024002 (2007).
2. G. De Visscher, R. Springett, D. T. Delpy, J. Van Reempts, M. Borgers, and K. van Rossem, Nitric oxide does not inhibit cerebral cytochrome oxidase in vivo or in the reactive hyperemic phase after brief anoxia in the adult rat, *J. Cereb. Blood Flow. Metab.* **22**, 515-519. (2002).
3. I. Tachtsidis, M. Tisdall, T. S. Leung, C. E. Cooper, D. T. Delpy, M. Smith, and C. E. Elwell, Investigation of in vivo measurement of cerebral cytochrome-c-oxidase redox changes using near-infrared spectroscopy in patients with orthostatic hypotension, *Physiol. Meas.* **28**, 199-211 (2007).
4. Y. Kakihana, T. Kuniyoshi, S. Isowaki, K. Tobo, E. Nagata, N. Okayama, K. Kitahara, T. Moriyama, T. Omae, M. Kawakami, Y. Kanmura, and M. Tamura, Relationship between redox behavior of brain cytochrome oxidase and neurological prognosis, *Adv. Exp. Med. Biol.* **530**, 413-419 (2003).
5. M. Banaji, A generic model of electron transport in mitochondria, *J. Theor. Biol.* **243**, 501-516 (2006).
6. N. Oshino, T. Sugano, R. Oshino, and B. Chance, Mitochondrial function under hypoxic conditions: the steady states of cytochrome $a+a_3$ and their relation to mitochondrial energy state, *Biochim. Biophys. Acta* **368**, 298-310 (1974).
7. D. F. Wilson, M. Erecinska, C. Drown, and I. A. Silver, The oxygen dependence of cellular energy metabolism, *Arch. Biochem. Biophys.* **195**, 485-493 (1979).
8. M. Kuboyama, F. C. Yong, and T. E. King, Studies on cytochrome oxidase VIII. Preparation and some properties of cardiac cytochrome oxidase., *J. Biol. Chem.* **247**, 6375-6383 (1972).
9. P. Nicholls, The steady state behaviour of cytochrome c oxidase in proteoliposomes, *FEBS Lett.* **327**, 194-198 (1993).
10. C. E. Cooper, S. J. Matcher, J. S. Wyatt, M. Cope, G. C. Brown, E. M. Nemoto, and D. T. Delpy, Near infrared spectroscopy of the brain: relevance to cytochrome oxidase bioenergetics, *Biochem. Soc. Trans.* **22**, 974-980 (1994).
11. A. J. Moody and P. R. Rich, The effect of pH on redox titrations of haem a in cyanide-liganded cytochrome-c oxidase: experimental and modelling studies, *Biochim. Biophys. Acta* **1015**, 205-215 (1990).
12. P. R. Rich, I. C. West, and P. Mitchell, The location of Cu_A in mammalian cytochrome *c* oxidase, *FEBS Lett.* **233**, 25-30 (1988).
13. D. F. Wilson, M. Erecinska, and P. L. Dutton, Thermodynamic relationships in mitochondrial oxidative phosphorylation, *Annu. Rev. Biophys. Bioeng.* **3**, 203-230 (1974).
14. E. A. Gorbikova, K. Vuorilehto, M. Wikstrom, and M. I. Verkhovsky, Redox titration of all electron carriers of cytochrome c oxidase by Fourier transform infrared spectroscopy, *Biochemistry* **45**, 5641-5649 (2006).
15. P.-E. Thörnström, P. Brzezinski, P.-O. Fredriksson, and B. G. Malmström, Cytochrome *c* Oxidase as an Electron-Transort-Driven Proton Pump: pH Dependence of the Reduction Levels of the Redox Centres during Turnover, *Biochemistry* **27**, 5441-5447 (1988).
16. M. Brunori, A. Colosimo, G. Rainoni, M. T. Wilson, and E. Antonini, Functional intermediates of cytochrome oxidase. Role of "pulsed" oxidase in the pre-steady state and steady state reactions of the beef enzyme, *J. Biol. Chem.* **254**, 10769-10775 (1979).
17. I. Belevich, D. A. Bloch, N. Belevich, M. Wikstrom, and M. I. Verkhovsky, Exploring the proton pump mechanism of cytochrome c oxidase in real time, *Proc. Natl. Acad. Sci. U S A* **104**, 2685-2690 (2007).