Measurement of cytochrome oxidase and mitochondrial energetics by near-infrared spectroscopy

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SUMMARY

Cytochrome oxidase is the terminal electron acceptor of the mitochondrial respiratory chain. It is responsible for the vast majority of oxygen consumption in the body and essential for the efficient generation of cellular ATP. The enzyme contains four redox active metal centres; one of these, the binuclear CuA centre, has a strong absorbance in the near-infrared that enables it to be detectable in vivo by near-infrared spectroscopy. However, the fact that the concentration of this centre is less than 10% of that of haemoglobin means that its detection is not a trivial matter.

Unlike the case with deoxymyoglobin and oxyhaemoglobin, concentration changes of the total cytochrome oxidase protein occur very slowly (over days) and are therefore not easily detectable by near-infrared spectroscopy. However, the copper centre rapidly accepts and donates an electron, and can thus change its redox state quickly; this redox change is detectable by near-infrared spectroscopy. Many factors can affect the CuA redox state in vivo (Cooper et al. 1994), but the most significant is likely to be the molecular oxygen concentration (at low oxygen tensions, electrons build up on CuA as reduction of oxygen by the enzyme starts to limit the steady-state rate of electron transfer).

The factors underlying haemoglobin oxygenation, deoxygenation and blood volume changes are, in general, well understood by the clinicians and physiologists who perform near-infrared spectroscopy measurements. In contrast, the factors that control the steady-state redox level of CuA in cytochrome oxidase are still a matter of active debate, even amongst biochemists studying the isolated enzyme and mitochondria. Coupled with the difficulties of accurate in vivo measurements it is perhaps not surprising that the field of cytochrome oxidase near-infrared spectroscopy has a somewhat chequered past. Too often papers have been written with insufficient information to enable the measurements to be repeated and few attempts have been made to test the algorithms in vivo.

In recent years a number of research groups and commercial spectrometer manufacturers have made a concerted attempt to not only say how they are attempting to measure cytochrome oxidase by near-infrared spectroscopy but also to demonstrate that they are really doing so. We applaud these attempts, which in general fall into three areas: first, modelling of data can be performed to determine what problems are likely to derail cytochrome oxidase detection algorithms (Matcher et al. 1995); secondly, haemoglobin concentration changes can be made by deoxygenation (using saline or artificial blood substitutes) in animals (Tamura 1993) or patients (Skov & Greisen 1994); and thirdly, the cytochrome oxidase redox state can be fixed by the use of mitochondrial inhibitors and then attempts made to cause spurious cytochrome changes by dramatically varying haemoglobin oxygenation, haemoglobin concentration and light scattering (Cooper et al. 1997).

We have previously written reviews covering the difficulties of measuring the cytochrome oxidase near-infrared spectroscopy signal in vivo (Cooper et al. 1997) and the factors affecting the oxidation state of cytochrome oxidase CuA (Cooper et al. 1994). In this article we would like to strike a somewhat more optimistic note—we will stress the usefulness this measurement may have in the clinical environment, as well as describing conditions under which we can have confidence that we are measuring real changes in the CuA redox state.

1. ALGORITHMS FOR CYTOCHROME OXIDASE DETERMINATION AND THEIR VALIDATION

We have recently performed tests of the various methods for measuring the cytochrome oxidase redox state (Matcher et al. 1995). Algorithms for measuring CuA redox state changes in vivo rely on converting optical density changes into the three chromophore concentration changes—deoxymyoglobin (Hb), oxyhaemoglobin (HbO2) and CuA (the other major tissue chromophore in this region, water, is presumed to maintain a constant concentration). Although the purified spectra of these compounds are well characterized, problems arise in allowing for tissue-specific effects, such as the wavelength dependence of the
optical path length (Essenpreis et al. 1993) and possible changes in light scattering. Three algorithms, those developed at University College London (UCL) (Wray et al. 1988), Keele University (Wickramasinghe et al. 1990) and that used in the commercial Critikon NIRS 2000 are based on similar assumptions and would be expected to yield similar results (Matcher et al. 1995). They assume that no scattering changes occur and that the wavelength dependence of path length is constant throughout the measurement procedure. A derivation of the UCL algorithm that can theoretically correct for variations in the latter property has also been published (Cope et al. 1991). However, the data presented in this paper are based on the basic version of this technique with multiwavelength detection (UCLn as designated by Matcher et al. 1995).

One algorithm, that published by Tamura and co-workers, attempts to correct for scattering changes by using a fourth reference wavelength (750 nm) where it is stated that there is no absorbance change due to cytochrome oxidase redox state changes (Hazeki et al. 1987). As can be seen from some of the problems described later this is a laudable aim, and we certainly hope that in future algorithms will be able to correct for scattering changes. The problem we have is with the statements these authors make that below 780 nm there are no absorbance change attributable to cytochrome oxidase. This is not true for the purified enzyme (Cooper et al. 1994), nor for in vivo near-infrared spectroscopy (NIRS) measurements by other groups (Wray et al. 1988; Cope 1991; Miyake et al. 1991). The clearest in vivo data showing this is that from Chance’s group in the fluorocarbon-perfused haemoglobin-free rat brain (Miyake et al. 1991). The lack of an isosbestic point in this region is as expected because no fewer than three chromophores contribute significantly to the mitochondrial absorption spectrum between 700 and 780 nm. As well as the Cu$_\alpha$ centre of cytochrome oxidase, there are the long wavelength tails of the significant absorption due both to the 695 nm band of the cytochrome $\epsilon$ iron–methionine charge-transfer band and the 655 nm band of the high-spin haem $a_2$-Cu$_\alpha$ centre of cytochrome oxidase. It is possible to simulate the absorbance changes seen by the group of Chance (Miyake et al. 1991) and the UCL group (Cope 1991) using the purified components, whereas it is not easy to see how it is possible to do this with the spectra published by Tamura and co-workers. In this case we feel the onus is on the latter group to explain the physical basis behind their unusual spectra. Nevertheless, this criticism notwithstanding, we found that, in general, cytochrome changes observed by the algorithms of the Tamura and the UCL group showed similar trends both in modelling studies and when comparing the in vivo data.

The latter is not true for the algorithm published by Piantadosi (1993), which we found, in most circumstances, had identical time courses for HbO$_2$ decline and Cu$_\alpha$ reduction (Matcher et al. 1995). It has recently been suggested that the published algorithm may have been miscalculated (Macnab & Gagnon 1996). Although we await clarification by the original authors on this issue, it should be noted that they consistently find a much closer in vivo correlation between haemoglobin desaturation and cytochrome oxidase reduction than other groups (Hampson et al. 1990; Piantadosi 1993) and the resolution of this discrepancy is likely to go deeper than a simple miscalculation in a table. Their paper (Piantadosi 1993) shows two spectra (figures 2 and 3) both claiming to be in vivo Cu$_\alpha$ oxidized minus reduced difference spectra. One has a large $\Delta$OD at 770 nm and a peak at 810 nm; the other has no $\Delta$OD at 770 nm and a peak at 830 nm (along with the interesting oxygen-dependent signal at 860 nm that is only detected by this group). The UCL and Chance groups in vivo spectra split the difference between these two spectra showing a significant $\Delta$OD at 770 nm and a peak at 830 nm (Wray et al. 1988; Cope 1991; Miyake et al. 1991). It is therefore not as easy as some have suggested (Macnab & Gagnon 1996) to reconstruct the Piantadosi algorithm from the raw data in this paper. In their defence it should be stated that this group have performed internal validations of the sort that we recommend to determine that, in their hands, there is no obvious ‘cross-talk’ between their HbO$_2$ and Cu$_\alpha$ signals (Piantadosi 1993). Nevertheless readers should be aware that, even more so than in the case with the Tamura algorithm, using the published algorithms from this paper will yield vastly different results to those obtained by using the Keele, Critikon or UCL algorithms described above, i.e. with the Piantadosi algorithm, reduction of Cu$_\alpha$ will occur at a much higher PO$_2$.

We propose the following criteria for authors claiming that they are measuring changes in the Cu$_\alpha$ redox state: (i) the algorithm used for deconvolution should, if possible, be explainable in terms of the known optical properties of the biological tissue and chromophores measured; (ii) there should be no large change in optical path length during the measurement, or if there is the algorithm should account for this; (iii) if possible the fitting technique should contain significantly more wavelengths than chromophores and the residuals of the fit should be random; (iv) the absorbance change observed should be reasonable for the amount of cytochrome oxidase in the tissue (when known); and (v) Cu$_\alpha$ redox state variations should not be a simple function of haemoglobin oxygenation or concentration changes, i.e. there should be limited ‘cross-talk’ in the system.

Our algorithm for cytochrome oxidase detection (Wray et al. 1988; Matcher et al. 1995) was designed for the adult rat brain in transmittance mode. We were concerned about transferring it to the neonatal brain where there are clear differences in the optical geometry of the measurement, the tissue studied (amount of skull) and the total cytochrome oxidase concentration (lower in the neonate). We tested our algorithm by determining that the cytochrome oxidase ‘oxidized minus reduced’ NIR spectrum in the perfuorocarbon-perfused piglet brain was identical to that of the adult rat (Cooper et al. 1997) and that the wavelength dependence of the optical path length in the blooed brain were also identical (point (i) above). Changes in the optical path length during short-term
hypoxia or alterations in arterial CO$_2$ were measured by the water path length and found to be small (point (ii)), and the three chromophores were a good fit to the optical density changes (point (iii)). The reduction of Cu$_A$ upon anoxia in the bloodig piglet was consistent with in vitro measurements of total cytochrome oxidase content (point (iv)). Finally we tested point (v) by causing large changes in haemoglobin concentration and oxygenation under conditions (following a 5 mg kg$^{-1}$ cyanide bolus i.v.) where the cytochrome oxidase redox state should not change and, as in the rat (Cooper et al. 1997), found no significant ‘cross-talk’ in the system. We were thus confident that the algorithm, in this case, could be transferred across the species and developmental age barrier, an important finding, as point (v), the most critical one, is difficult to determine in humans. Despite this ‘success’ it is important to remember that the points listed above may not hold for all the measurements in a particular system (see later for a case where some, but not all, of the observed ‘cytochrome oxidase NIRS signal’ can be attributed to a Cu$_A$ redox state change).

2. OXYGEN DEPENDENCE OF THE Cu$_A$ NIRS SIGNAL

As stated previously there has been controversy over how readily the Cu$_A$ centre becomes reduced following a drop in oxygen tension. With the problems of measuring this signal in the blooded brain, perhaps this is not surprising. More worrying is that two groups have recently presented diametrically opposing views as to the ease of reducibility of Cu$_A$ in the blood-free brain. In one case it is claimed that the Cu$_A$ is extremely difficult to reduce and remains oxidized even after significant haem a reduction (Hoshi et al. 1993; Inagaki & Tamura 1993). Another group, which had more conventional views (Ferrari et al. 1996) now suggests that Cu$_A$ reduction occurs very early in the hypoxic process (Stingele et al. 1996). In the purified enzyme we have repeated and agree with previous work (Nicholls & Chanady 1982) showing that Cu$_A$ occurs late in the hypoxic process and simultaneously with haem a reduction. One possibility for the discrepancy between these groups might be that in one case rapid anoxia is initiated and in the other slow hypoxia; the latter has been suggested to induce a change in the nature of the cytochrome oxidase molecule (Chandel et al. 1995). However, our own findings show identical kinetics of haem a and Cu$_A$ reduction in the bloodless brain during rapid anoxia (Cope 1991) and those of the Chance group show a similar result for slow-graded hypoxia (Miyake et al. 1991). Whilst the resolution of this controversy awaits further developments we are happy to sit in the middle of the debate with in vitro characteristics of the enzyme.

3. CYTOCHROME OXIDASE NIRS—WHY BOTHER?

(a) Tissue dysoxia

It is easy to measure concentration changes in HbO$_2$ and Hb and, as shown above, it is difficult to do the same for cytochrome oxidase. It is also likely that in the not-too-distant future absolute quantitation of haemoglobin oxygenation will be available. Bioenergetics are interested in the characteristics and behaviour of cytochrome oxidase in vivo in its own right, but the clinician is likely to require more convincing in order to feel the need to struggle through the literature of mitochondrial redox-state measurements.

At least two uses for this signal are apparent. Firstly as many (Bashford et al. 1980; Hazeki et al. 1987; Ferrari et al. 1995; Matcher et al. 1995), but not all (Hampson et al. 1990; Jobis-Vandervliet 1991; Stingele et al. 1996) groups contend, it requires low oxygen tensions before cytochrome oxidase Cu$_A$ is reduced, whereas haemoglobin deoxygenates much more readily. This is because cytochrome oxidase has both a low $K_m$ (Michaelis constant) for oxygen and is sensitive to mitochondrial, not tissue or cytosolic PO$_2$. Therefore cytochrome oxidase may be a useful marker of actual, rather than impending, tissue dysoxia. Two recent clinical studies stand out in this regard. One (Nollett et al. 1995) measured a number of parameters, including NIRS, during cardiac surgery and found that the only parameter correlating with subsequent neurological dysfunction was the reduction of cytochrome oxidase Cu$_A$. Another study on neonates (McCormick et al. 1993) showed that the standard treatment of patent ductus arteriosus, indomethacin, caused a significant unwanted side-effect, namely a drop in the Cu$_A$ redox state. Alternative treatments with ibuprofen appear not to have this problem (Patel et al. 1995) and this, to our knowledge, is likely to become the first example of clinical practice being altered, at least in part due to a cytochrome oxidase NIRS measurement.

Although the Cu$_A$ reduction was the best marker for mitochondrial dysoxia in these cases there were also significant haemodynamic changes and decreased haemoglobin oxygenation. Therefore one might argue that as improved measurements of absolute haemoglobin oxygenation become available, measurements of cytochrome oxidase NIRS will become less useful. It would be interesting to find instances where changes in the cytochrome oxidase NIRS signal correlate with energy failure, but haemoglobin oxygenation does not decrease significantly. We believe that such a situation arises in neonatal hypoxia–ischaemia.

(b) Neonatal hypoxia–ischaemia

Magnetic resonance spectroscopy of the newborn human infant, following birth asphyxia, reveals little change in brain energy metabolites on the first day of life (Hope et al. 1984; Azzopardi et al. 1989). However, NIRS reveals haemodynamic changes, including an increased cerebral blood flow (CBF) and cerebral blood volume (CBV), and a much-reduced CBV.
response to changes in $P_{\text{a}CO_2}$ (CBVR) ($P_{\text{a}}$ is arterial CO$_2$ tension). By day 2 many infants have impaired energy metabolism, resulting in a reduced cerebral PCR/P$_i$ ratio (where PCR stands for phosphocreatine) (Hope et al. 1984; Azzopardi et al. 1989), the level of which is predictive of the subsequent neurological outcome (Azzopardi et al. 1989; Roth et al. 1992).

We have modelled the nuclear magnetic resonance (NMR) changes in a piglet model of hypoxia–ischaemia (Cady et al. 1993, 1994; Lorek et al. 1994; Mehmet et al. 1994; Thoreson et al. 1995) and shown that the primary cerebral energy failure in the insult provokes the same secondary (delayed) energy failure observed in the human. Most significantly lactate is also seen to increase in the secondary phase (Cady et al. 1993), strongly suggesting that an impairment in mitochondrial energy metabolism is directly responsible for the secondary fall in PCR and ATP levels. We have shown that there is an increase in apoptosis during secondary energy failure (Mehmet et al. 1994) and that mild hypothermia both prevents this rise in apoptosis (Edwards et al. 1995) and is cerebroprotective in this model (Thoreson et al. 1995).

Our current working hypothesis for the mechanism of this delayed energy failure is that there is an induction of nitric oxide synthase as occurs in adult models of hypoxia–ischaemia (Iadecola et al. 1995). Nitric oxide (NO) is now a well-established intercellular messenger, produced from L-arginine and oxygen by nitric oxide synthase(s) (Knowles & Moncada 1994). However, NO is also cytotoxic and has been implicated in neurotoxicity (Lipton et al. 1993; Iadecola et al. 1994). In the brain NO is responsible for the maintenance of cerebrovascular tone (Iadecola et al. 1994) and probably mediates the increased CBF and CBV following hypercapnia (Iadecola et al. 1994).

NO inhibits mitochondrial respiration (Stadler et al. 1991; Bolanos et al. 1994; Brown & Cooper 1994; Cleeter et al. 1994). It has generally been assumed that this is due to inhibition of mitochondrial iron–sulphur enzymes (Henry et al. 1991). However, iron–sulphur enzymes are very resistant to NO (Vanin et al. 1992), although they are sensitive to peroxynitrite (Heiss et al. 1994; Pantopoulous et al. 1994). We have recently shown that physiological levels of NO reversibly inhibit cytochrome oxidase activity by competing for oxygen at the active site (Brown & Cooper 1994). This is clearly the primary mechanism for NO inhibition of cellular respiration. Long-term damage may be caused by the increase in superoxide ions generated as cytochrome oxidase is inhibited, reacting with NO to generate peroxynitrite (Castro et al. 1994; Cleeter et al. 1994; Hausladen & Fridovich 1994). A similar sequence of events has recently been proposed (Cleeter et al. 1994) to explain the link between mitochondrial impairment and long-term neurological disorders, e.g. Huntington’s disease (Jenkins et al. 1993) and Parkinson’s disease (Hantraye et al. 1996).

It is therefore possible that a rise in cerebral NO production, subsequent to the hypoxic–ischaemic insult, is implicated in the haemodynamic changes and/or the secondary energy failure observed in the neonate. In this picture NO generated secondary to the insult would inhibit mitochondrial respiration initially at cytochrome oxidase; generation of peroxynitrite would then cause long-term mitochondrial damage and secondary energy failure primarily via an increase in apoptotic cell death. The increased levels of NO immediately subsequent to the insult would cause the observed increases in CBF, CBV and the absent CBVR (this response would already be saturated with NO). Hypothermia is cerebroprotective in our piglet model; intriguingly hyperthermia has recently been shown to stimulate NO production in vivo (Hall et al. 1994). Also, the close links recently shown between nitric oxide, mitochondrial damage and apoptosis are also consistent with our model (Shimaoka et al. 1995; Liu et al. 1996).

How is this model testable? A strong prediction is that, in contrast to the indomethacin and cardiac surgery situations described above, energy failure should be accompanied by an increase in haemoglobin oxygenation. As we (Brown & Cooper 1994) have shown, the highly effective inhibition of cytochrome oxidase by nitric oxide in vitro takes place at the oxygen reduction site of cytochrome oxidase, the haem $a_{2}$–Cu$_{h}$ centre. NO inhibition therefore causes an increase in the redox state of the electron donor prior to this site, haem $a$, despite adequate oxygenation of the medium. Cu$_{h}$ would also be expected to become more reduced. Our prediction is therefore that in neonatal hypoxia–ischaemia, in the primary insult haemoglobin oxygenation will fall and Cu$_{h}$ will become reduced. However, in the delayed energy failure haemoglobin oxygenation will not fall (indeed HbO$_2$ should increase as cerebral blood volume and flow rise) and yet cytochrome oxidase Cu$_{h}$ will become more reduced. This latter event should correlate with other measures of energy failure. Results consistent with part of this hypothesis have come from studies involving ischaemic insults on the foetal sheep brain. A delayed rise in cerebral blood volume was detected, along with an increase in Cu$_{h}$ reduction (Marks et al. 1996a). The latter correlated with histological damage and a decline in electrocortical activity. The addition of a nitric oxide synthase inhibitor decreased the amplitude of the rise in blood volume, although the histological damage was worse (Marks et al. 1996b). Unfortunately the effect of NO synthase inhibition on the Cu$_{h}$ signal during secondary energy failure was not reported. Measurements of cytochrome oxidase redox state have been attempted in the neonatal human following hypoxia–ischaemia (Van Bel et al. 1993)—Cu$_{h}$ reduction increased somewhat earlier than would be predicted from the animal models and, in contrast to other clinical finding, no delayed rise in cerebral blood volume was observed (Wyatt 1993).

It is useful to compare changes in the cytochrome oxidase NIRS signal with other non-invasive measurements of brain energy function. Correlations have previously been shown with cortical impedance (Marks et al. 1996a), [ATP] (Tsujii et al. 1996) and EEG & Tamura 1993). We therefore used our neonatal piglet model (Lorek et al. 1994) to perform simultaneous NIRS and magnetic resonance spec-
Figure 1. The effect of hypoxia–ischaemia on the haemodynamic and energetic properties of the neonatal brain. Simultaneous NIRS and MRS measurements were made on the neonatal piglet head. At time zero the carotid artery was occluded and $F_{iO_2}$ reduced to 11%. Changes in Hb, HbO$_2$ and the cytochrome oxidase Cu$_A$ redox state were calculated as described previously (UCLn algorithm). NTP (nucleotide triphosphate) was measured by MRS as a fraction of the total exchangeable phosphate pool (EPP), i.e. $[\text{NTP}] = ([P_i] + [\text{PCr}] + [\text{NTP}])$ (Lorek et al. 1994). Cytochrome oxidase Cu$_A$ redox state changes are displayed on a $\times 10$ expanded scale.

Figure 1 shows that as expected in the initial hypoxic–ischaemic period there is an initial drop in HbO$_2$ and rise in Hb, accompanied by a small fall in the total cerebral haemoglobin concentration (CHC). Consistent with our previous findings (Matcher et al. 1995), subsequent to the fall in HbO$_2$ there is an increase in Cu$_A$ reduction. The latter has the same time course as the fall in cerebral [ATP]. Upon reversal of the hypoxia–ischaemia the NIRS and MRS parameters return to close to baseline values. Note that in all the observed figures the Cu$_A$ signal is shown on a $\times 10$ expanded scale with respect to the haemoglobin signals.

What happens during delayed energy failure (figure 2)? In this case there is a clear rise in HbO$_2$ and CHC, consistent with previous findings from the neonatal human (Wyatt 1993). However, there is a significant fall in the ‘cytochrome oxidase NIRS signal’ that, again, occurs over a similar time scale as the fall in [ATP]. An excellent correlation of the cytochrome oxidase NIRS signal with cerebral [ATP], although interestingly not with PCr/P$_i$, is maintained throughout the period of delayed energy failure (figure 3).

One should always beware of results that appear to agree too readily with a cherished hypothesis. In this case, although the data appear on the surface to be exactly as expected if nitric oxide is inhibiting cytochrome oxidase in vivo, problems lurk in the details. In effect the results are too good. Our algorithm, when coupled to the continuous optical path-length measurements, enables us to measure quantitative redox-state changes. The redox-state change in cytochrome oxidase measured in the delayed energy failure of up to 8 µM is over double that observed in the primary insult. We have used the method developed by Brown to measure the total amount of cytochrome oxidase in the neonatal pig brain homogenates (Brown et al. 1991)—in no case have we found a value higher than 4 µM and we are therefore sceptical of redox-state changes larger than this value. We have independent evidence that the optical properties of the brain are changing during the energy failure—there is an increase in the mean optical path length and the residuals of the multilinear least-squares fits of the pure chromophores to the optical density changes become less random (this path length variation could be interesting in its own right as it might be due to a change in cell volume, consistent with our simultaneous measurements of changes in the cerebral water diffusion rate by magnetic resonance...
Figure 2. Delayed energy failure in the neonatal brain as monitored by NIRS and MRS. Simultaneous NIRS (Matcher et al. 1995) and MRS (Lorek et al. 1994) measurements were made on the neonatal piglet for 48 hours following the hypoxic–ischaemic insult described in figure 1. Cytochrome oxidase Cu₄ redox state changes are displayed on a ×10 expanded scale. Abbreviations as per figure 1.

Figure 3. Correlation of energy failure measured by MRS and NIRS. From the data of figure 2, correlations were drawn between the cytochrome oxidase Cu₄ redox state, PCr/P_i and NTP/EPP (as defined in figure 1).
biochemical process being monitored—references to the Cu₃ redox state would be used when we were clear that the signal observed was shown to be due to redox-state changes in this centre by the methods described previously.

4. CONCLUSIONS

Large changes in near-infrared spectroscopy measurements of ‘cytochrome oxidase’ correlate with energy failure in a wide variety of systems. In some cases, but not all, these can be directly attributed to redox-state changes of the Cu₃ centre in the enzyme.

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REFERENCES


