THE OXIDATION–REDUCTION STATE OF CYTOCHROME OXIDASE IN FREEZE TRAPPED GERBIL BRAINS

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1. Introduction

The steady state level of reduction of cytochrome oxidase in purified mitochondrial preparations is usually extremely low [1,2] (<5%), the enzyme becoming significantly more reduced only when the oxygen tension of the medium falls below 1 mm Hg [3–5]. However, studies of the cat cerebral cortex suggest that in the aerobic steady state in vivo cytochrome oxidase may be up to 85% reduced [6,7]. The haem proteins associated with oxygen delivery and consumption, haemoglobin and mitochondrial cytochromes, can be conveniently monitored by their specific absorbance in the 500–650 nm spectral region using dual-wavelength spectroscopy either in the conventional transmission mode [1,2,8] or by reflectance [6,7,9,10]. Here, scanning dual-wavelength reflectance spectroscopy was employed in order to resolve the overlapping absorbance bands of haemoglobin and mitochondrial cytochromes in freeze-trapped gerbil brains. In these preparations the steady state level of cytochrome oxidase remains constant over a wide range of inspired oxygen concentrations; the enzyme exhibiting significant reduction only when the fraction of oxygen in the inspired gasses (FiO_2) fell below 0.05.

2. Experimental

Spectra were recorded with a Johnson Foundation Dual Wavelength Scanning spectrophotometer [11] under microprocessor control, which can store up to four different spectra. The output of the microprocessor to an $X-Y$ plotter represents the difference between any two of the stored spectra; absolute spectra can be obtained by subtraction of an indifferent 'baseline' spectrum such as of water. Reflectance measurements were made with a bifurcate fibre optic; one branch led from the output of the light chopper to the common terminal of the fibre, the second branch conducted reflected light from the common terminal back to the photomultiplier. White styrofoam was used as the 'baseline' for absolute reflectance spectra. Adult gerbils (70 g) were maintained on pentobarbital anaesthesia (30 mg/kg) and were allowed to breathe normally. Gases from a mixer were provided to the anaesthetised animals through a mask; 60 s after starting inhalation of a defined gas mixture the gerbil brain was freeze trapped using the funnel freezing procedure introduced by Kerr [12]. A plastic funnel with a 10 mm opening was fitted into a skin incision that exposed the skull, freezing was initiated by pouring liquid nitrogen into the funnel taking care that no nitrogen mixed with the gases delivered through the mask. Spontaneous breathing ceased after ~60 s of funnel freezing whereupon the whole gerbil was immersed in liquid nitrogen. For reflectance spectroscopy the skull of the frozen animal was removed by mechanical milling; both the milling and recording of spectra were performed while the animal was immersed in liquid nitrogen.

3. Results

Fig.1 illustrates typical reflectance spectra recorded from gerbils funnel frozen during the inspiration of

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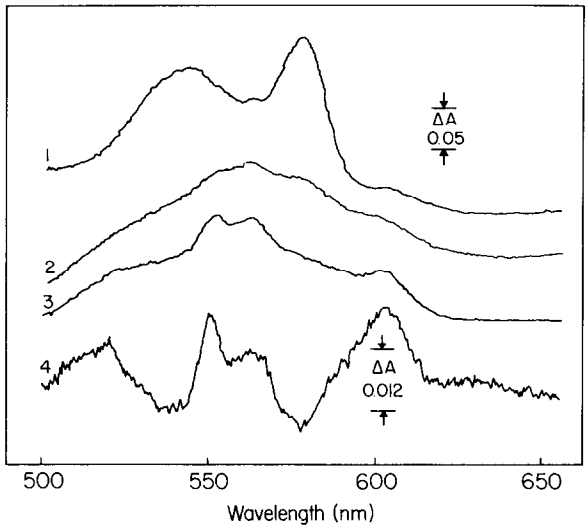

Fig.1. Reflectance spectra of freeze trapped gerbil cerebral cortex. Gerbil brains were funnel frozen 60 s after starting inspiration of: (1) 95% O_2, 5% CO_2; (2) 5% O_2, 90% N_2, 5% CO_2; (3) 95% N_2, 5% CO_2. Reflectance spectra 1–3 were recorded at 77 K from the right cortex using white styrofoam as the baseline and 630 nm as the reference wavelength. The difference reflectance spectrum (4) represents spectrum (3) minus spectrum (2).

different gas mixtures. In order to maintain high cerebral blood flow and a reasonably constant cerebral blood volume the animals were maintained moderately hypercapnic by providing 5% CO_2 in all the gas mixtures. At this level of inspired CO_2 arterial P_{CO_2} is ~60 mm Hg and intracellular pH ~6.98 [13]. When the animal inhaled 95% oxygen (spectrum 1, fig.1) the absolute reflectance spectrum of the frozen cortex is essentially that of oxyhaemoglobin with A_{578} and A_{543} maxima together with a smaller component absorbing at 605 nm. When the FiO_2 was reduced to 0.05 the absolute spectrum (spectrum 2, fig.1) is predominantly deoxyhaemoglobin with an A_{564} max although a shoulder due to oxyhaemoglobin remains at 578 nm. The component at 605 nm remains at a level similar to that found when the FiO_2 was 0.95 and there are indications of additional bands at 522 and 550 nm. If the FiO_2 is reduced to zero (spectrum 3, fig.1) the amplitude of the 605 nm band increases and the band at 550 nm is clearly resolved. The assignment of the absorption bands is relatively straightforward. The bands at 578 and 543 nm arise from oxyhaemoglobin, that at 563 nm from deoxyhaemoglobin, that at 550 nm from ferrocytochrome *c* and that at 605 nm from ferrocytochrome aa_3 in cytochrome oxidase. Furthermore, the changes in absorbance ascribed to the mitochondrial cytochromes do not occur until most of the cerebral haemoglobin is deoxygenated. Thus a reduced minus oxidised spectrum of cortical mitochondria, largely free of contributions from the deoxy minus oxy haemoglobin difference spectrum, is obtained when the absolute reflectance spectrum of an animal funnel frozen at an FiO_2 of 0.05 is subtracted from that of an animal with an FiO_2 of 0 (spectrum 4, fig.1). The reduced minus oxidised difference spectrum of cortical mitochondria in situ does not differ significantly from the spectrum obtained from purified mitochondrial preparations [1,2].

Fig.2 presents the amplitudes of the A_{605} band as a function of FiO_2. Clearly there is little reduction of cytochrome aa_3 until FiO_2 falls below 0.05. The behaviour of the mitochondrial pyridine nucleotide fluorescence from rat brain in vivo as a function of FiO_2 [3,4] is included in fig.2 for comparison with the cytochrome oxidase signal. Clearly both indicators exhibit very similar sensitivities to inspired oxygen. Indeed, the mitochondrial pyridine nucleotide and flavoprotein fluorescence signals from the freeze

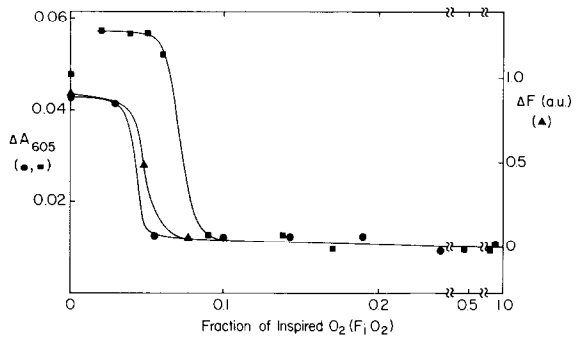

Fig.2. Mitochondrial signals from cerebral cortex. The A_{605} of cytochrome oxidase was measured from reflectance spectra from freeze trapped gerbil cerebral cortex (see fig.1). The amplitude of the A_{605} was taken as the vertical distance, at 605 nm, from the spectrum to a straight line connecting the minima in the spectrum at either side of this wavelength. Gerbils were funnel frozen 60 s after starting to inspire gas mixtures that contained either 0% CO (●) or 4% CO (■) and O_2, N_2 and 5% CO_2 to give fractions of inspired oxygen (FiO_2) as indicated. The data for pyridine nucleotide fluorescence (▲) from rat cerebral cortex was taken from [2]. Fluorescence at 460 nm was excited at 366 nm and recorded from the exposed cortex of anaesthetised animals as FiO_2 was varied.

trapped brains employed here show an identical dependence on FiO_2 as the cytochrome signal (data not shown).

If 4% carbon monoxide is present in the inhaled gases cytochrome aa_3 becomes reduced at higher FiO_2 values, around 0.07. Carbon monoxide has multiple effects on oxygen consumption by the brain because it both diminishes the oxygen supply by its interaction with haemoglobin [13] and it is a competitive inhibitor of cytochrome oxidase [14]. The combination of these effects of carbon monoxide decreases the apparent affinity of cytochrome oxidase for oxygen by a factor of 2 (fig.2). However, the shape and the end points (oxidised and reduced) of the oxygen titrations of cytochrome oxidase are little affected by the presence of 4% carbon monoxide.

4. Discussion

The observation that cytochrome aa_3 in funnel-frozen, normoxic gerbil brains is <20% reduced contrasts with results obtained with in vivo reflectance spectroscopy of the cat cerebral cortex [6,7] which indicates that the cytochrome is up to 85% reduced under similar conditions. The oxidation–reduction state of cytochrome aa_3 varied [6,7] over a wide range of arterial oxygen and carbon dioxide partial pressures. In contrast, these results indicate that the concentration of ferrocytochrome aa_3 in gerbil brain does not change significantly with the FiO_2 until the steady state level of haemoglobin oxygenation falls to very low values (fig.1). 50% reduction of cytochrome aa_3 is found at FiO_2 values of ~0.04 during mild hypercapnia and 0.07 in the presence of 4% carbon monoxide (fig.2). Studies of purified cytochrome oxidase [15] indicate that both the oxidised and reduced forms of the enzyme exhibit A_{600} maxima. It is thus uncertain whether the band observed in this spectral region in normoxic brains (fig.1) arises from ferri or ferrocytochrome aa_3. In this case, the 20% ferrocytochrome aa_3 represents an upper limit and the true level of reduced cytochrome oxidase in normoxic brains may be much lower, as would be expected from the behaviour of this enzyme in isolated mitochondrial preparations [1,2] and from the observation of reduced pyridine nucleotide signals in cells and tissues [3,4].

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