CONSTRAINTS ON THE MECHANISM OF REDUCTION OF MOLECULAR OXYGEN BY CYTOCHROME OXIDASE UNDER COUPLED CONDITIONS

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1. Introduction

Mitochondrial respiration involves the reduction of both atoms of molecular oxygen to water. Substrates entering the respiratory chain before the antimycin A-sensitive site are able to support H_2O_2 generation [1], but free peroxide cannot be detected as an intermediate in the terminal, cytochrome oxidase, reaction [2], strongly suggesting that the oxygen molecule must remain bound to the cytochrome oxidase for at least the time period of four-electron transfer. In intact mitochondria and coupled submitochondrial vesicles, the transfer of two electrons across a coupling span has been shown to give rise to one coupling intermediate or quantum of energised state, corresponding to the transport of two monovalent cations or the eventual synthesis of one molecule of ATP. Thus, during the reduction of molecular oxygen, the passage of four electrons through cytochrome oxidase could give rise to two coupling intermediates. The free energy changes involved make it unlikely that two coupling intermediates could coexist in the same domain at the same time and therefore there is a kinetic as well as mechanistic problem concerning the way in which the obligatorily-coupled transfer of pairs of electrons across a coupling span in cytochrome oxidase is related to a four-electron transfer to oxygen. One possible solution to this problem, that also accounts functionally for the stoichiometric excess of oxidase over other fixed catalytic assemblies of the respiratory chain, is that cytochrome oxidases act cooperatively in reducing molecular oxygen under coupling conditions. Two complexes, both containing two molecules of heme protein and two atoms of copper, would be engaged in oxygen reduction, each complex being involved in a two-electron transfer reaction. The life-time of any bound peroxide would not then necessarily be restricted by the lifetime or rate of discharge of the coupling intermediate, whatever form the latter may take. Each oxidase complex of a pair engaged in the reduction of a single molecule of oxygen could be associated with a separate coupling domain.

To test for such cooperativity, we have titrated respiration with cyanide under coupled and uncoupled conditions. If oxidases functioned as pairs in the reduction of molecular oxygen, then it would be expected that for a given amount of bound cyanide, a greater degree of inhibition of respiration would be observed than if each cytochrome oxidase unit were acting independently.

2. Materials and methods

Sonic vesicles were prepared from beef heart mitochondria according to Löw and Vallin [3] using

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a suspending medium of 250 mM sucrose, 10 mM Tris–sulphate buffer (pH 7.6), 15 mM magnesium chloride, 1 mM sodium succinate and 1 mM ATP. The final pellet was suspended in 0.25 M sucrose. Spectral data were obtained at room temp., unless otherwise indicated, using a Perkin–Elmer dual wavelength spectrophotometer. A Clark oxygen electrode was used for respiration measurements. Cytochrome oxidase concentration was determined using $\Delta E_{605-630\ nm}$ (dithionite reduced minus oxidised) = 28 $mM^{-1}\ cm^{-1}$ [4] and protein concentration obtained according to the method of Gornall et al. [5]. Potassium cyanide solutions were prepared fresh daily. Other experimental details are given in the figure legends.

3. Results and discussion

3.1. *Degree of coupling*

Table 1 shows that the preparation of submitochondrial vesicles retained the ability to reduce NAD^+ under conditions of ATP-dependent reverse electron flow, with succinate as substrate. This reaction was sensitive to both dinitrophenol and oligomycin. A further indication of the retention of energy-coupling capability in the preparation was the sensitivity of respiration to dinitrophenol in the presence of oligomycin (table 1). Stimulation by uncoupler of oligomycin inhibited respiration was found to be 1.6-fold.

Table 1
Energy dependent properties of the preparation of submitochondrial vesicles.

	Control	+DNP	+Oligomycin
Reverse electron flow (nmol NAD^+ reduced/min/mg protein	65 ± 5	0	0
Respiration (in presence of oligomycin) (n atoms O_2/min/mg protein)	21 ± 2	33 ± 3	–

NADH formation was monitored at 340 nm at 30°C using a reaction mixture of 250 mM sucrose, 50 mM Tris–sulphate buffer (pH 8.0), 6 mM magnesium chloride, 5 mM sodium succinate, 1 mM NAD^+, 1 mM potassium cyanide and 2 mM ATP at a protein concentration of approx. 0.1 mg/ml. DNP and oligomycin were added at final concentrations of 74 nmol/mg protein and 30 nmol/mg protein, respectively. Oxygen uptake was measured at room temp. in a reaction mixture of 125 mM sucrose, 0.5 mM EDTA, 2.5 mM Tris–chloride buffer (pH 7.4) 3 mM sodium succinate and 1.5 nmol oligomycin/mg protein at a protein concentration of approx. 5 mg/ml.

3.2. *Respiration and cyanide binding*

Fig. 1 shows the percentage change in the steady state reduction level of cytochrome *c* when cyanide was added to submitochondrial vesicles in the presence and absence of oligomycin. The steady state reduction levels were measured at (a) high cytochrome aa_3 concentration so that, in effect, all the cyanide was bound [6], and (b) at low rates of electron flow (succinate in the presence of malonate) to prevent the concentration and rate of reoxidation of cytochrome *c* from exerting a rate limiting effect on respiration. Little change in reduction level was observed until the molar ratio of cyanide to cytochrome aa_3 exceeded 1.0. Thus, for each molecule of cyanide bound, only one cytochrome oxidase unit was inhibited in its reaction with oxygen. Under cooperative conditions, the molar ratio of cyanide to oxidase for the degree of inhibition corresponding to the break in the steady state reduction curve for cytochrome *c* would be almost 0.6 if the oxidase units were acting in pairs and less than this for any higher degree of cooperativity, assuming one binding site for cyanide per cytochrome oxidase [7]. Since no significant difference is observed in the presence and absence of oligomycin (similar results were also found in DNP treated vesicles), it can be concluded that cooperativity between oxidases does not occur, even under conditions of imposed respiratory control and that each cytochrome oxidase unit acts independently to reduce molecular oxygen.

The results also exclude the possibility of sequential reduction of molecular oxygen by a pair of oxidases as suggested by Green and Ji [8]. Sequential reduction of the oxygen molecule, first to peroxide and then to water, by two cytochrome oxidase complexes in a "supermolecular" assembly without the liberation of free peroxide would give a break point in the titration curve at a cyanide/oxidase ratio of less than 0.6.

3.3. *Respiratory control and the mechanism of oxygen reduction*

The independence of cytochrome oxidase units in reducing molecular oxygen under conditions of respiratory control places certain energy dependent restraints on the kinetics of the catalytic mechanism.

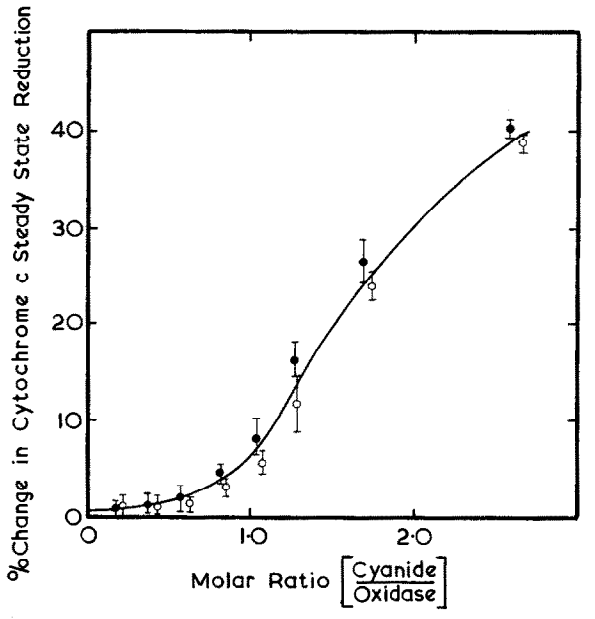

Fig. 1. Titration of steady state reduction level of cytochrome *c* with cyanide in submitochondrial vesicles in the presence (○) and absence (●) of oligomycin (approx. 1.5 nmol/mg protein). The reaction was followed by measuring 551 nm absorbance on addition of 3 mM sodium succinate and 1.5 mM sodium malonate to a suspension of submitochondrial vesicles at approx. 50 mg protein/ml in 125 mM sucrose, 0.5 mM EDTA and 2.5 mM Tris–chloride buffer (pH 7.4). After anaerobiosis, cyanide was added and oxygen was introduced and the steady-state reduction level was monitored. After the mixture had gone anaerobic again, more cyanide and oxygen were added and the new steady-state reduction level was recorded.

If it is in fact true that no free peroxide is produced during the cytochrome oxidase reaction, then any intermediate at the level of peroxide formed under energised conditions must remain bound until discharge of the energised state occurs, since a further two-electron transfer would be slowed in the high energy state. Alternatively, it could be postulated that the affinity of oxidase for oxygen is decreased under energised conditions both in the partially reduced and fully reduced states. Only after discharge of the energised state would oxidation of a fully reduced complex by molecular oxygen take place (with the generation of an energised fully-oxidised complex). The kinetics of peroxide formation and reduction would then be independent of respiratory control.

This latter alternative is supported by recent evidence on ligand binding affinities in cytochrome a_3 under energised and non-energised conditions. Wohlrab and Ogunmola [9] have found that the presence of ATP decreases the affinity of ferrous cytochrome a_3 for carbon monoxide by a factor of two and Erecinska et al. [10] report a decrease in cyanide affinity to both the oxidised and reduced forms of cytochrome oxidase under energised conditions. Under steady state conditions, the apparent K_m for oxygen uptake of coupled mitochondria has been measured by Degn and Wohlrab [11] as 0.5 μM whilst the apparent K_m for the uncoupled state was found to be less than 0.05 μM.

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