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Spectroscopic Evidehce for an Oxygen Bridge between the Iron Atom of Cytochrome a_3 and Cu_B in the Class C Group of Compounds Formed in the Reaction of Mixed Valence State Cytochrome Oxidase with Oxygen

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Cytochrome oxidase (E.C.1.9.3.1) catalyses the reduction of dioxygen (O_2) to water in all higher organisms, animals and plants. The minimum functioning of cytochrome oxidase is thought to consist of two A type haems, cytochromes *a* and a_3, differing only in the nature of their axial ligands, and two copper atoms, Cu_A and Cu_B [1]. Cytochrome a_3 and Cu_B are antiferromagnetically coupled with $-J \gtrsim 200$ cm^{-1} which implies a maximum separation between the iron atom of cytochrome a_3 and Cu_B of 5 Å [2]. Cytochrome *a* and Cu_A are magnetically isolated [2].

Much interest in recent years has focused on the low temperature reactions of cytochrome oxidase with O_2 [3–12] in which seven intermediates have been discovered, four in the reaction of fully reduced ($Cu_A^+a^{2+}\cdot Cu_B^+a_3^{2+}$) and three in the reaction of mixed valence state ($Cu_A^{2+}a^{3+}\cdot Cu_B^+a_3^{2+}$) cytochrome oxidase with O_2 [10–12]. Of particular interest with regard to the configuration of the Cu_Ba_3 unit is whether O_2 acts as a bridging ligand between Cu_B and the iron atom of cytochrome a_3 in any of these intermediates, or simply remains bound to the iron atom of cytochrome a_3 as an end-on ligand. This is of importance because (1) to date there are no known examples in which a terminal non-bridging O_2 is reduced to the oxidation level of water [13], and (2) the demonstration of an O_2 bridge between the iron atom of cytochrome a_3 and Cu_B would make the possibility of the imidazole bridge model [2, 14–16] of the Cu_Ba_3 unit highly unlikely. In the present paper we present spectroscopic evidence for an O_2 bridge between the iron atom of cytochrome a_3 and Cu_B in the class C group of compounds formed in the reaction of mixed valence state cytochrome oxidase with oxygen at low temperatures.

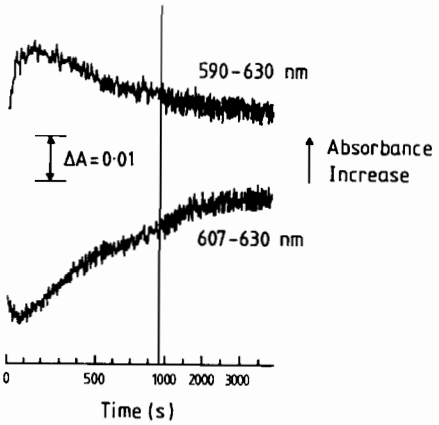

Fig. 1. Kinetics of the reaction of membrane bound mixed valence state cytochrome oxidase with O_2 at 173 K in intact potato mitochondria.

The kinetics were recorded using the CD66 rapid wavelength scanning optical spectrophotometer [20, 22]. Spectra were recorded at a rate of 100 per second with a spectra resolution of 0.567 nm per point. The kinetic traces represent the progress of the reaction at two selected wavelength pairs. Each point in the progress curve is obtained from a CATted spectrum in which 400 transient spectra have been averaged. The mixed valence state cytochrome oxidase-CO complex was prepared as described previously [3, 5, 10, 12] and the reaction with O_2 was initiated by photolysis of the CO complex with a *ca.* 99% saturating flash from a 500 J xenon flash lamp (pulse width 1ms). The first spectrum recorded after flash photolysis was unliganded mixed valence state cytochrome oxidase and was used as the reference spectrum in computing difference spectra. CO did not recombine to any detectable extent in the presence of the high concentration of O_2 employed here (1 m*M*) as demonstrated by control experiments in which repeated flashes over the course of the reaction with O_2 only produced about 1% further photolysis of the CO complex, the intermediates in the reaction with O_2 not being susceptible to photolysis at the flash intensity used [3–12]. The experimental conditions were: 13 mg/ml potato mitochondria [23] containing *ca.* 10 μ*M* cytochrome oxidase, 5 mm succinate, 20 m*M* sodium phosphate buffer pH 7.4 30% (v/v) ethylene glycol, 5 m*M* $K_3Fe(CN)_6$, 0.6 m*M* CO and 1 m*M* O_2. (Note the change of time scale at 870 s).

The most unusual and in many ways the most interesting of the species formed in the reactions of cytochrome oxidase with O_2 are those belonging to class C which are characterised by an intense absorption band in the 606–610 nm region [3, 4, 9, 11]. EPR and optical studies in the visible and near infra-red regions have strongly suggested that the identity of this set of compounds is $Cu_A^{2+}a^{3+}\cdot Cu_B^{2+}a_3^{2+}\cdot O_2^-$ [5, 6, 10, 12, 17]. In cytochrome oxidase derived from beef heart mitochondria there are

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two such species, II_M and III_M* characterized by peaks at 606 and 610 nm respectively in the membrane bound form of oxidase, and by peaks at 605 and 607 nm respectively in the soluble form of the oxidase [10, 12]. The 605–610 nm band is approximately symmetrical, has a large extinction coefficient (~15–20 mM^{-1} cm^{-1}) and a narrow bandwidth (f.w.h.h.** = 350–550 cm^{-1}) [5, 10, 12]. These features are strongly suggestive of a charge transfer band. Although it has been suggested that the 605–610 nm absorption band is due to a σ_S (thiolate) → $d_{x^2-y^2}$ (Cu) charge transfer transition of a type 1 blue copper [17], such an assignment is highly unlikely for two reasons: (1) the extinction coefficient of the 605–610 nm band in species II_M and III_M is much larger than those seen in blue copper proteins (3.5–5 mM^{-1} cm^{-1}) [18], and (2) the bandwidth of the 605–610 nm band of species II_M and III_M is much narrower than those in blue copper proteins (f.w.h.h. = 3000–5000 cm^{-1}) [18]. The most likely assignment is that the 605–610 nm band is due to a charge transfer transition arising from interaction between Cu_B^{2+}, a_3^{2+} and O_2^- [5, 10, 12].

The reaction of membrane bound mixed valence state cytochrome oxidase with O_2 in intact potato mitochondria differs from that in intact beef heart mitochondria in that there is no evidence for the slow conversion of species II_M into III_M. The kinetic traces in Fig. 1 at 590–630 and 607–630 nm clearly show two distinct phases. The first phase is due to the formation of species I_M† (also known as compound A_2) [3, 4, 10, 12], and the second phase by a decrease in absorbance at 590–630 nm and an increase at 607–630 nm, due to the conversion of species I_M into species II_M.

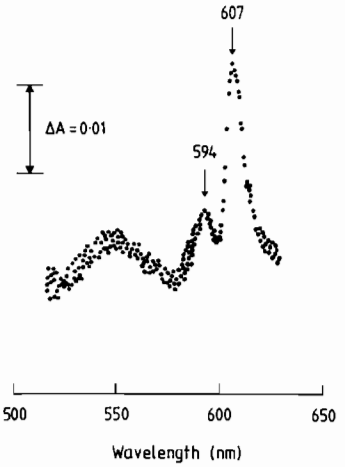

Fig. 2. Difference spectrum of species II_M (C_{607}^{594}) minus unliganded mixed valence state cytochrome oxidase obtained with membrane bound cytochrome oxidase in potato mitochondria at 173 K.

The difference spectrum was obtained by subtracting the spectrum obtained 1 h after the initiation of the reaction with O_2 at 173 K, at which time the reaction was complete, from the first spectrum obtained after photolysis of the CO complex, using 630 nm as the reference wavelength. Each spectrum was the average of 400 transient spectra recorded at a rate of 100 spectra per second with a spectral resolution of 0.576 nm per point as described in the legend to Fig. 1. Experimental conditions: as in Fig. 1.

Figure 2 shows the difference spectrum of species II_M minus unliganded mixed valence state cytochrome oxidase obtained with membrane bound cytochrome oxidase in intact potato mitochondria 1 h after the initiation of the reaction of mixed valence state cytochrome oxidase with O_2 at 173 K, at which stage the reaction is complete and no further absorbance changes are seen (see Fig. 1). The striking feature of this difference spectrum is that the single intense absorption band in the α region seen for species II_M and III_M obtained with cytochrome oxidase from beef heart mitochondria, is split into a prominent peak at 607 nm and a side peak at 594 nm. For this reason we will refer to this species as C_{607}^{594}. Both the 607 and 594 nm peaks have narrow bandwidths (f.w.h.h. = 280 and 420 cm^{-1} respectively) which strongly suggests that they both represent charge transfer bands. The most likely explanation for the splitting of the α band in species C_{607}^{594} is that one of the bands is due to an O_2^- → Cu_B^{2+} charge transfer transition, and the other to an O_2^- → $Fe^{2+}(a_3)$ charge transfer transition, the two transitions differing in energy by ~4.3 kJ mol^{-1}. It should be noted that the assignment of O_2^- → Cu_B^{2+} and O_2^- → $Fe^{2+}(a_3)$ charge transfer transitions to bands in the 590–610 nm region is consistent with the known O_2^{2-} → Cu^{2+} charge transfer band at

*There is general agreement that species II_M is $Cu_A^{2+}a^{3+}$·$Cu_B^{2+}a_3^{2+}$·O_2^- (see ref. 12 for a detailed discussion). For species III_M, however, it has been suggested that O_2^- is reduced to O_2^{2-} with the concomitant oxidation of either a protein moiety or the porphyrin ring to a cation radical X^+ (see ref. 12). The alternative and equally probable possibility is that the red shift in the α band occurring on the conversion of species II_M into III_M is due to a conformation change in the vicinity of the $Cu_Ba_3O_2$ unit. In both cases the valence states of the metal centres in species III_M remain unchanged from those in species II_M.

**The bandwidth is defined at the full width at half height, f.w.h.h.

†The difference spectrum of species I_M minus unliganded mixed valence state cytochrome oxidase (not shown) obtained from membrane bound cytochrome oxidase in intact potato mitochondria is essentially identical to that obtained in intact beef heart mitochondria, the former being characterized by a peak at 591 nm and a trough at 602 nm, and the latter by a peak at 590 nm and a trough at 608 nm (ref. 10) in the α region.

570 nm in arthropod oxyhaemocyanin [19], and the known $O_2^{\delta-} \rightarrow Fe^{2+\delta}(a_3)$ $(1 \geqslant \delta > 0.5)$ charge transfer band around 590 nm in species I and I_M of cytochrome oxidase [9, 11, 12]. In species II_M and III_M obtained with cytochrome oxidase from beef heart mitochondria, the energy of the charge transfer band at 594 nm seen in species C_{607}^{594} is decreased by 3–5 kJ mol^{-1} as a result of 'accidental' degeneracy resulting in a red shift of 10–16 nm, so that the energy of both charge transfer bands is approximately the same and only a single absorption band is seen in the 605–610 nm region. This is further supported by the observation that the bandwidth of the 605–610 nm absorption band in species II_M and III_M (f.w.h.h. = 350–550 cm^{-1} [10, 12] obtained with cytochrome oxidase from beef heart mitochondria is approximately the same as that of the 594 nm band (f.w.h.h. = 420 cm^{-1}) but is significantly larger than that of the 607 nm band (f.w.h.h. = 280 cm^{-1}) of species C_{607}^{594}.

The presence of both O_2^- D Cu_B^{2+} and $O_2^- \rightarrow Fe^{2+}$ (a_3) charge transfer bands in the class C group of compounds implies that O_2^- acts as a bridging ligand between Cu_B and the iron atom of cytochrome a_3. The $Cu_B a_3 \cdot O_2$ would then best be represented in the class C group of compounds by the configuration

$$[Cu_B^{2+}\text{——}O\text{——}O\text{——}a_3^{2+}]$$

(i)

The presence of an O_2^- bridging ligand would easily account for the absence of an EPR signal attributable to Cu_B^{2+} in the class C group of compounds [12] as O_2^- and Cu_B^{2+} could undergo spin-spin coupling to form an exchange coupled complex of whole spin (S = 0 or 1) which would be undetectable by EPR. Configuration (i) is probably an extreme representation of the electron distribution within the $Cu_B a_3 \cdot O_2$ unit as electron delocalization is likely to occur [11, 12] so that a more realistic representation would be

$$[Cu_B^{1+\delta_1}\text{——}O\text{——}O\text{——}a_3^{2+\delta_2}]^{-(\delta_1+\delta_2)}$$

(ii)

where $(\delta_1 + \delta_2) \sim 1, \delta_1 > 0.5$ and $\delta_2 < 0.5$.

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