Studies of the Reaction of Cytochrome *c* Oxidase with Oxygen at Low Temperature*

BO KARLSSON,[a] LARS-ERIK ANDRÉASSON,[a] ROLAND AASA,[a] BO G. MALMSTRÖM [a] and G. MARIUS CLORE [b]

[a] Department of Biochemistry and Biophysics, Chalmers Institute of Technology, University of Göteborg, S-412 96 Göteborg, Sweden and
[b] Department of Biochemistry, University College London, Gower Street, London WC1E 6BT, U.K.

Cytochrome oxidase (ferrocytochrome c:O_2 oxidoreductase, E.C. 1.9.3.1.) is the terminal oxidase in the respiratory chain in higher organisms. The minimum functional unit consists of two A type haems, cytochrome *a* and a_3, and two copper atoms, Cu_A and Cu_B. Cytochrome *a* and Cu_A are detectable by EPR in the oxidized enzyme whereas cytochrome a_3 and Cu_B are antiferromagnetically coupled [1] and hence not EPR detectable. Recent low-temperature kinetic studies by optical methods have indicated the appearance of several intermediates in the reaction of fully reduced ($a^{2+}Cu_A^{+}$ Cu_B^{+} a_3^{2+}) and of partially reduced mixed valence state ($a^{3+}Cu_A^{2+}$ Cu_B^{+} a_3^{2+}), membrane bound cytochrome oxidase with O_2. The nature of these intermediates is still practically unknown and assignments of the valence state of the metal centres have been based largely on optical data.[2]

The purpose of this investigation was to characterize further these intermediates by optical and EPR methods using pure solubilized enzyme to gain a deeper understanding of the mechanism of O_2 reduction by cytochrome oxidase.

Experimental. Samples for studying the reaction of fully reduced and mixed valence cytochrome oxidase with O_2 were prepared essentially as described by Clore and Chance [3] in 50 mM sodium phosphate buffer, pH 7.4, with 0.3 mM NADH and 1.6 μM phenazine-methosulfate as reductant, using an enzyme concentration of 10 μM and 100 μM for the optical and EPR experiments, respectively. The properties of the enzyme preparation were as described earlier.[4] Photodissociation of the CO complexes was carried out at 77 K using 10 J, 3 μs light flashes from a xenon flash lamp (Model 610 B, Photochemical Research Associates Inc.,

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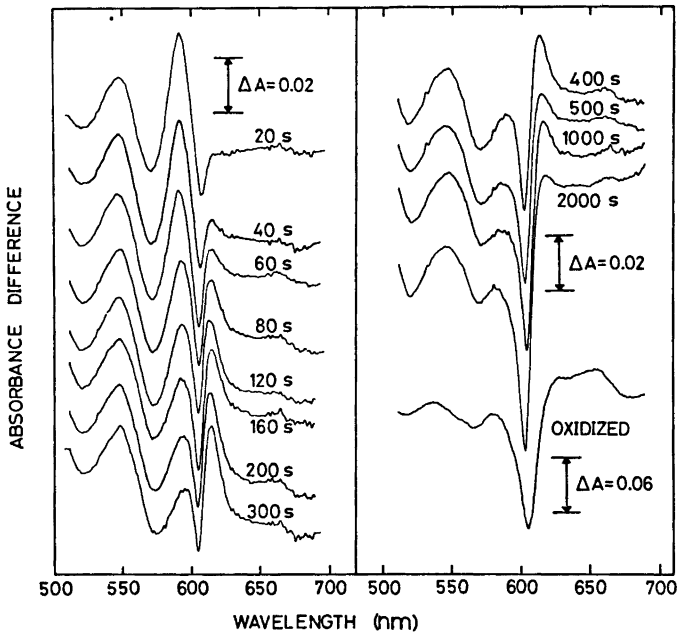

Fig. 1. Optical difference spectra (*vs.* fully reduced cytochrome oxidase) recorded during the reaction of fully reduced cytochrome oxidase with O_2 at 173 K. A difference spectrum of fully oxidized minus fully reduced enzyme is also shown for comparison.

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London, Ontario). The reaction with O_2 was initiated by warming the photolyzed samples in a liquid N_2-cooled isopentane bath for a given time and stopped by cooling the EPR tubes in liquid N_2. This procedure was repeated and optical and EPR spectra were recorded after each warming and cooling cycle. Optical spectra were recorded at 77 K in EPR tubes using a dual wavelength spectrophotometer (Johnson Research Foundation DBS-2) with provisions for storing the spectrum of a reference substance digitally. EPR spectra at 9 GHz were recorded at 77 K with a Varian E-3 spectrometer and at temperatures between 5 and 80 K in a Varian E-9 instrument.

Results and discussion. Fig. 1 shows optical difference (*vs* fully reduced enzyme) spectra recorded during the reaction of fully reduced cytochrome oxidase with O_2 at 173 K. Three optically distinct species may be distinguished, represented by the 20, 200 and 2000 s spectra, respectively.

The only EPR signals detected during the course of the reaction are those attributed to low-spin cytochrome a^{3+} and Cu_A^{2+}. The positions and lineshapes of these signals are very similar to those in fully oxidized oxidase. At the end of the reaction at 173 K, Cu_A is 90 % oxidized whereas only 40 % of cytochrome *a* is in the oxidized state (Table 1).

Table 1. Intensities of the EPR detectable species during the reaction of fully reduced cytochrome oxidase with O_2 at 173 K.

Time s	Low-spin cytochrome a^{3+} mol/mol oxidase	Cu_A^{2+} mol/mol oxidase
0	0	0
20	0.05	0.05
60	0.16	0.19
120	0.30	0.45
200	0.36	0.61
400	0.40	0.69
1000	0.42	0.80
2000	0.42	0.87

The reaction of mixed valence state cytochrome oxidase with O_2 at 173 K to 193 K confirm the existence of three intermediates [3] (I_M, II_M, III_M). From the EPR data it is evident that cytochrome *a* and Cu_A remain in their low-spin Fe^{3+} and Cu^{2+} states, respectively,

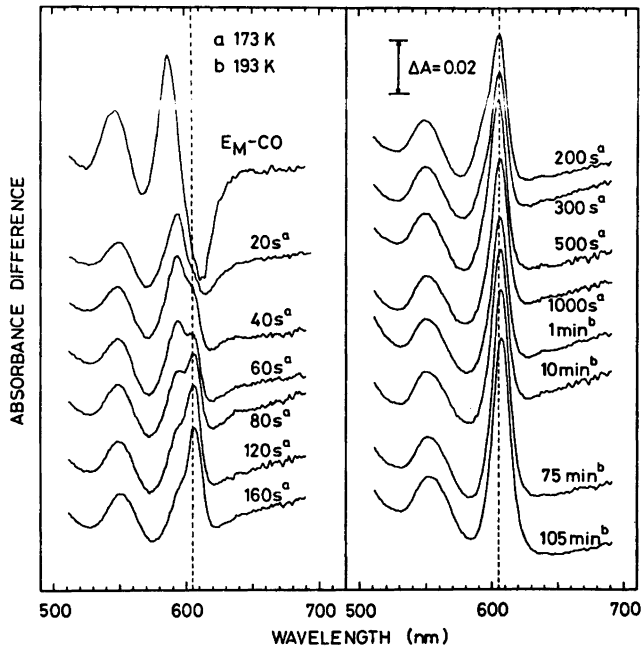

Fig. 2. Optical difference spectra (*vs.* mixed valence state cytochrome oxidase) recorded during the reaction of mixed valence state cytochrome oxidase with O_2 at 173 and 193 K. A difference spectrum of mixed valence state cytochrome oxidase – CO complex is shown for comparison. The spectra at 193 K were obtained after the 1 000 s spectrum at 173 K.

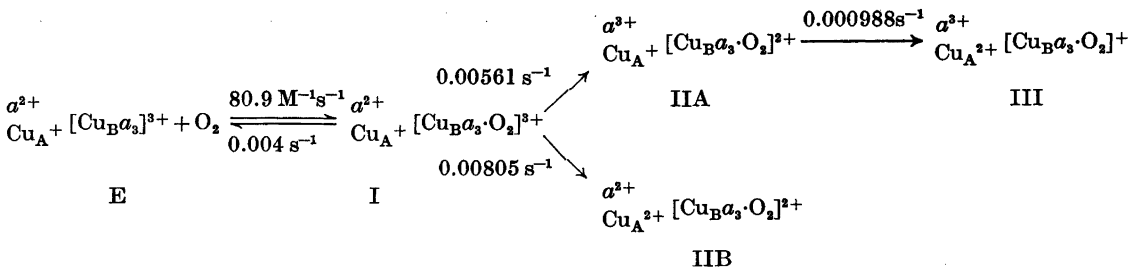

Fig. 3. Scheme for the reaction of fully reduced cytochrome oxidase with O_2 at 173 K. The optimized values for the rate constants are indicated in the figure.

throughout the reaction. The concentration of low-spin haem remains the same in the intermediates, but significant differences in linewidths and positions of the *g* 3 "absorption" peak are seen during the reaction. No evidence of new EPR signals has been found in the reaction of mixed valence state cytochrome oxidase with O_2 in conflict with the report by Chance *et al.*[5] Optical difference spectra (*vs.* photolyzed mixed valence state enzyme) of the intermediates are shown Fig. 2. The difference spectrum of intermediate I_M (20 s) is qualitatively similar to that of the CO complex but with significant differences in the positions of the peaks and troughs in the α and β regions. Intermediate II_M (peak at 605 nm) appears to be fairly stable at 173 K (up to 1000 s). On warming the sample to 193 K, II_M is slowly converted to intermediate III_M (characterized by a shift of the a peak to 607 nm and a gradual increase in intensity of this peak). In neither of the intermediates is the 655 nm band of the fully oxidized oxidase observed.

An analysis of the kinetics of the reaction of fully reduced oxidase with O_2 based on changes in the optical absorption differences (590 − 630, 604 − 630 and 614 − 630 nm) and changes in the EPR intensities has been made. (For a description of the numerical techniques used, definitions of statistical parameters and the statistical evaluation, see Ref. 3.) A simple scheme which accounts for both the optical and EPR data with an overall standard deviation within the standard error of the data (2.5 %, given by the weighted mean of the standard error of the individual progress curves [3]), a random distribution of residuals and a good determination [3] of the optimized parameters (*i.e.* rate constants and optical contributions) is a four-intermediate branching scheme where the second optical species is actually a mixture of two intermediates (Fig. 3). Three-intermediate schemes fail to fit the data (overall standard deviation substantially larger than 2.5 %). On the other hand, improving the fit by increasing the number of intermediates (*i.e.* making the model more complex), results in a very poor determination of the optimized parameters.

Cytochrome a_3 and Cu_B constitute a coupled binuclear metal center where O_2 is known to react with cytochrome a_3. Neither the optical nor the EPR spectra provide any direct information on the valence states of cytochrome a_3 or Cu_B in the intermediates, and it may therefore be convenient to consider cytochrome a_3, Cu_B and O_2 as a single unit $[Cu_B\ a_3\ O_2]$ where only the total charge can be specified. Formation of intermediate I of the fully reduced enzyme involves no change in the charge of this unit but comparisons of the absorption spectrum with those of model haem-O_2 compounds indicate electron transfer within the unit.[6] With the above consideration the structure is probably best represented by $[Cu_B^{1+\delta_1}\ a_3^{2+\delta_1}\ O_2^{-(\delta_1+\delta_2)}]$, where $(\delta_1 + \delta_2) \approx 1$. The conversion of intermediate I into the other intermediates involves successive one-electron transfer from cytochrome a^{2+} and Cu_A^+ to the $[Cu_B\ a_3\ O_2]$ unit possibly with the formation of H_2O. Formally intermediate III, $Cu_A^{2+}\ a^{3+}$-$[Cu_B\ a_3\ O_2]^+$, has the same oxidation state as fully oxidized oxidase and two molecules of H_2O. It may be noted, however, that the absorption band at 655 nm, ascribed to antiferromagnetic coupling between Cu_B and cytochrome a_3 in the fully oxidized enzyme,[7] is absent during the reaction of fully oxidized enzyme with O_2. This shows that intermediate III is not identical with the oxidized enzyme and indicates that reduction of oxygen is incomplete at this stage.

The first step in the reaction of the mixed valence state cytochrome oxidase with O_2 must be very similar to that of the fully reduced enzyme. No electron transfer to the $[Cu_B\ a_3\ O_2]$ unit is possible since cytochrome *a* and Cu_A are oxidized initially. The formation of intermediates II_M and III_M must be limited to electron redistribution within the $[Cu_B\ a_3\ O_2]$ unit. The absence of new EPR signals is easily explained by the fact that the $[Cu_B\ a_3\ O_2]$ unit always contains an even number of unpaired electrons.

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Regulation of Intermediary Phosphorylation of K^+-ATPase from Pig Gastric Mucosa by Sodium Ions *, **

MAGNUS LJUNGSTRÖM,[a]
BJÖRN WALLMARK [b] and SVEN MÅRDH [a]

[a] Inst. of Medical and Physiological Chemistry, Biomedical Centre, Uppsala University, Box 575, S-751 23 Uppsala, Sweden and
[b] AB Hässle, Fack, S-431 20 Mölndal 1, Sweden

Vesicles from the microsomal fraction of gastric mucosa hydrolyze ATP with a concomittant K^+-dependent uptake of H^+.[1] Broken membranes derived from these vesicles contain a K^+-stimulated ATPase which is believed to constitute an integral part of the proton pump. In the presence of Mg^{2+} and ATP a phosphorylated form of the ATPase appears.[2] The extent of phosphorylation is reduced by K^+. In a recent report evidence was presented that the phosphoenzyme is an intermediate in the hydrolysis of ATP.[3] It was found also that Na^+ inhibited the K^+-stimulated hydrolysis of ATP. This study shows that already low concentrations of Na^+ effectively reduce the rate of formation of the phosphoenzyme intermediate.

Experimental. The Tris-salt of ATP was prepared as described previously.[4] [γ-^{32}P]ATP was a product of New England Nuclear. K^+-ATPase was prepared from the gastric mucosa of pig stomachs (fraction GII, Ref. 5). The ATPase activity was about 6 μmol (mg protein)$^{-1}$ h^{-1} at 21 °C in the presence of 5 μM ATP, 2 mM $MgCl_2$ and 10 mM KCl in 40 mM Tris-HCl buffer, pH 7.4. Phosphorylation experiments were carried out at 20 – 22 °C at 5 μM ATP by means of a rapid-mixing apparatus.[4] Maximal amount of phosphoenzyme was obtained by phosphorylation of the enzyme in this apparatus, or by calculation of the upper limit to which the experimental values extrapolated in a time-dependent study. Both methods gave maximally about 1.5 nmol per mg protein. Curve fitting of experimental data points was performed by the method of least squares on a Wang 600 calculator assuming first-order or pseudo first-order kinetics. The correlation coefficient was 0.997 or better in all experiments.

Results and discussion. In order to investigate further interactions of Na^+ with the K^+-ATPase, the rate of formation of the phosphoenzyme intermediate was studied at various

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** The abbreviations used are: K^+-ATPase, potassium-stimulated ATP phosphohydrolase; (Na^+,K^+)-ATPase, sodium plus potassium ion transport ATP phosphohydrolase.

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