FUNCTIONAL STUDIES ON CROSSLINKED BOVINE CYTOCHROME *c* OXIDASE

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1. Introduction

Cytochrome oxidase (EC 1.9.3.1.) acts as the terminal electron acceptor in the mitochondrial respiratory chain and catalyzes the reduction of molecular oxygen to water. It is comprised of four redox sites, two heme *a* groups associated with the cytochromes *a* and a_3 and two copper atoms distinguishable on both functional and spectral grounds (reviews [1,2]). It is known from SDS gel electrophoresis that cytochrome oxidases consist of at least six and possibly eight polypeptide chains [3–5]. The relationships between the functional properties of the redox sites and the polypeptide subunit structure of the enzyme are, however, largely unexplored.

Recent functional studies on isolated cytochrome oxidase have suggested that the oxidized enzyme, as prepared, is of secondary catalytic importance but rather acts as a pool of 'resting' enzyme from which an activated species, termed 'pulsed' oxidase, may be generated in response to a flux of electrons [6]. This activation process takes place upon the enzyme complex undergoing one cycle of full reduction and reaction with molecular oxygen and also goes part way to explain known discrepancies between the enzyme-turnover number as measured by transient and steady-state methods.

In this paper we report findings that indicate that activation of the oxidase can be accomplished not only for the isolated enzyme but also when it is imbedded in the membrane of the submitochondrial particles thus indicating a possible in vivo importance for this activation. Also to investigate the role played by conformational rearrangement between subunits in this activation process we have undertaken crosslinking experiments with purified enzyme in an attempt to impair the conformational flexibility of the oxidase assembly.

2. Materials and methods

Cytochrome oxidase and Keilin-Hartree particles were prepared as described [7].

Crosslinking was carried out by the method [8] using dimethyl suberimidate. Solutions, 25 μM, of either fully oxidized or dithionite reduced cytochrome oxidase were reacted at room temperature with dimethyl suberimidate for 3 h. The reaction was stopped by the addition of ammonium chloride to a final concentration of 0.1 M and the reaction mixture was then dialyzed against 0.1 M potassium phosphate buffer, pH 7.4, containing 1% Tween 80 overnight. All samples were in the fully oxidized state after

dialysis, as judged by their spectra. As a control a 25 μM solution of oxidized oxidase was treated in an identical manner except that suberimidate was omitted from the reaction mixture.

The concentration of cytochrome oxidase in terms of heme was determined using $\epsilon = 21\,000\ M^{-1}cm^{-1}$ at 605 nm for the fully reduced enzyme [7]. The oxidase concentrations are, however, expressed in terms of a two heme (aa_3) complex. SDS gel electrophoresis was performed according to [12].

3. Results and discussion

3.1. *SDS gels*

Figure 1 shows SDS gels of cytochrome oxidase which have been crosslinked in either the fully oxidized or the fully reduced state and compares these with uncrosslinked enzyme. The uncrosslinked enzyme exhibits a multibanded pattern in agreement with [3–5] and indicates the presence of eight polypeptide chains with approx. mol. wt range 51 000–16 000, together with some further higher molecular weight components. This pattern may be contrasted with that of the crosslinked enzyme.

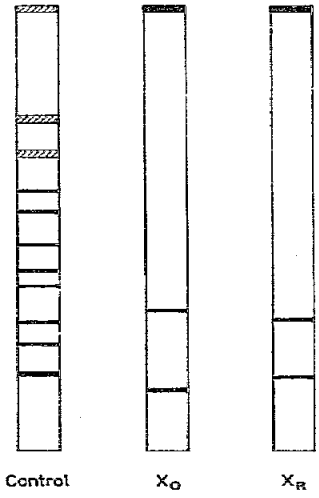

Fig.1. Polyacrylamide gel–SDS electrophoresis performed according to the method [12] and in the presence of the proteinase inhibitor. The major band app. mol. wts are: Control, 50 700, 44 700, 35 500, 30 200, 27 900, 22 400, 19 500 and 16 200; X_O, 24 000 and 14 500: X_R, 22 900 and 15 800.

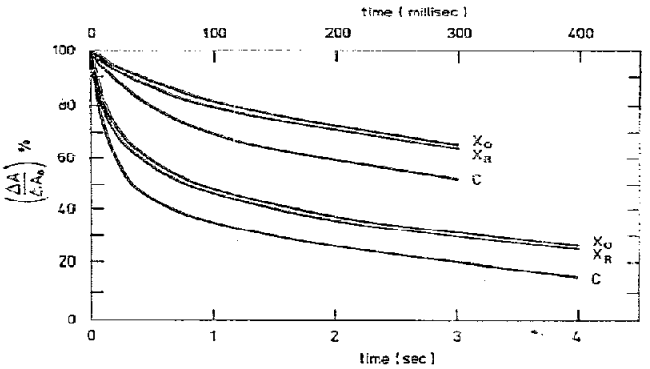

Fig.2. Electron transfer between horse ferrocytochrome *c* and bovine cytochrome oxidase in the uncrosslinked and crosslinked condition. Cytochrome oxidase concentration 1.3 μM; ferrocytochrome *c* 2 μM. C, X_O and X_R refer to the control, oxidase crosslinked in the oxidised condition and reduced condition, respectively. The upper time scale refers to the top three traces and the lower scale to the bottom three traces. Monitoring wavelength 550 nm. Potassium phosphate buffer 0.1 M, pH 7.4, containing 1% Tween 80. Temp. 20°C.

It is apparent that suberimidate has linked together some of the polypeptide chains and only two lower molecular weight components (approx. mol. wt 23 000, 15 000) are now evident, the remainder of the enzyme failing to enter the gel.

3.2. *Functional studies*

Figure 2 shows typical progress curves of the electron transfer reaction between ferrocytochrome *c* and cytochrome oxidase at low protein concentrations. In agreement with [9] we observe a fast process (second-order rate approx. $3 \times 10^6\ M^{-1}\ s^{-1}$) corresponding to the oxidation of cytochrome *c* and a simultaneous reduction of cytochrome *a*. When monitored at a wavelength of 550 nm this reaction is followed by a slower oxidation of cytochrome *c* which by reference to the photochemical experiments [10] appears to be rate-limited by the internal transfer of electrons from cytochrome *a* to a_3. It is clear that the crosslinked enzyme behaves identically to the untreated enzyme with regard to the rate at which electrons enter the enzyme from cytochrome *c*. This similarity in the functional properties of the crosslinked and uncrosslinked oxidase is also seen in their reactions

with carbon monoxide. Table 1 compares the rates of combination of CO with dithionite reduced enzyme as measured by stopped-flow and photolytic methods. These rates are the same in all cases and are in close agreement with the values for the combination rate constant of CO with reduced cytochrome oxidase [11].

The fast electron entry into the oxidase molecule and the unimpaired ligand binding properties of crosslinked enzyme suggests that the cytochrome *c* interaction site, cytochrome *a*, and the accessibility and binding properties of the heme associated with cytochrome a_3 are unaffected by the crosslinking of the constituent polypeptide chains of the oxidase complex.

In fig.3 we report progress curves monitored at

Table 1
Reaction of reduced uncrosslinked and crosslinked oxidase with carbon monoxide

Protein	Protein (μM)	CO (μM)	rate (a e) (s^{-1})	Method
Control	4.4	250	15.00	Flow
Control	4.4	250	13.53	Flash
X_O	5.8	250	15.37	Flow
X_O	5.8	250	14.5	Flash
X_R	5.5	250	15.56	Flow
X_R	5.5	250	15.08	Flash

Potassium phosphate, 0.1 M, pH 7.4, 1% Tween-80. Temp. 20°C

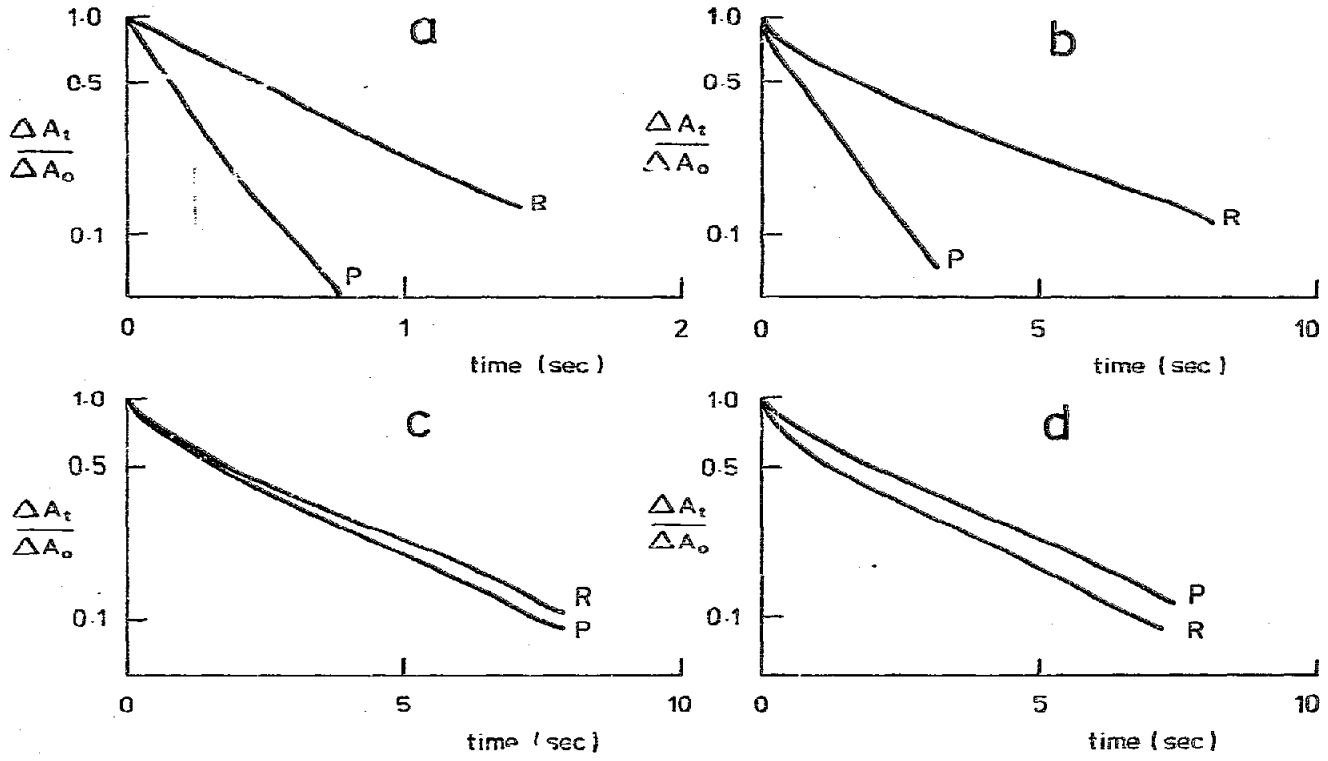

Fig.3. Oxidation of horse ferrocytochrome *c* by membrane bound, isolated and crosslinked bovine cytochrome oxidase. (a) Keilin-Hartree particles, oxidase concentration 2.1 μM, cytochrome *c*, 10 μM. (b) Isolated oxidase 1.3 μM, cytochrome *c*, 6.25 μM, (c) Isolated oxidase crosslinked while in the oxidised state 1.3 μM, cytochrome *c*, 6.25 μM. (d) Isolated oxidase crosslinked while in the reduced state 1.3 μM, cytochrome *c*, 6.25 μM. R and P denote experiments with the 'resting' or 'pulsed' enzyme (see text) respectively. Experiments were performed in 0.1 M potassium phosphate buffer, pH 7.4, which, for those experiments using the isolated enzyme, also contained 1% Tween 80. Oxygen concentration 135 μM, sodium ascorbate 1 mM. Monitoring wavelength 550_{nm}. Temp. 20°C.

550 nm for the reaction between ferrocytochrome *c* and either isolated cytochrome oxidase, crosslinked and uncrosslinked, or Keilin-Hartree particles. The concentrations are chosen so that each molecule of cytochrome oxidase will turn over approximately once on average, that is the ferrocytochrome *c* concentration is approximately equal to the concentration of redox sites in the oxidase or 4-fold greater than the total oxidase complex concentration.

This reaction has been studied in two ways. Firstly, by mixing an aerobic solution of oxidized 'resting' cytochrome oxidase with ascorbate-reduced cytochrome *c* and secondly, by reacting a mixture of ascorbate-reduced cytochrome *c* and cytochrome oxidase with an oxygen-containing buffer. In the first method we observe the oxidation of ferrocytochrome *c* by cytochrome oxidase which has been in the oxidized or 'resting' state for a long period of time while in the second method we see the action of freshly-oxidized oxidase or 'pulsed' oxidase, which is produced within the deadtime of the stopped-flow apparatus by the oxidation of oxidase by molecular oxygen.

As reported for uncrosslinked oxidase [6] the two methods show clearly different rates as illustrated in fig.3b and table 2, indicating that the rate of internal electron transfer within the pulsed enzyme is approx. 3-fold greater than in the resting enzyme. The rate of reaction of the crosslinked enzyme, however, is the same by both methods and the same as the rate exhibited by the uncrosslinked, resting enzyme (see table 2). We may conclude from this experiment that whereas the crosslinked enzyme is able to oxidize ferrocytochrome *c* as rapidly as uncrosslinked resting enzyme it is unable to become activated and display the enhanced rates that are characteristic of the untreated enzyme. The differences between the crosslinked and uncrosslinked enzymes reside in the fact that the subunits are linked together in the former case and, presumably, this interferes with the conformational flexibility of the molecules. The activation process would therefore seem to rely on the oxidase molecule entering in its activated state a conformation different from either the fully-oxidized resting enzyme or the fully-reduced enzyme. This activated state corresponds to the condition of the enzyme when it is turning over, as a cycle of reduction and oxidation in a steady-state experiment should produce the activated enzyme in an analogous fashion to the way described above.

Table 2
Rate of the slow phase of oxidation of ferrocytochrome *c* by membrane-bound, isolated and crosslinked bovine cytochrome oxidase

Protein	Rate (s^{-1})	
	'Resting'	'Pulsed'
Oxidase in particles	1.25	3.95
Control	0.26	0.74
X_O	0.25	0.28
X_R	0.33	0.24

Values taken from plots in fig.3

In order to ascertain whether the activation process is an artifact generated by the isolation procedure we have conducted some experiments on Keilin-Hartree particles. In fig.3 the resting and pulsed particles are compared in their rate of oxidation of ferrocytochrome *c*. It is clear that the activation process occurs leading to a 3-fold enhancement of the rate. The fact that this enhancement occurs in the membrane-bound enzyme suggests that it is physiologically relevant and that the activation process may have an important role in the control of the respiratory chain as discussed [6]. We also see (table 2, fig.3) that under comparable conditions the membrane-bound oxidase oxidises ferrocytochrome *c* considerably faster (~ 5-fold) than the isolated enzyme. This is in agreement with the known activation of cytochrome oxidase by lipids [13].

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