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Rapid proton release during flash-induced oxidation of cytochrome *c* oxidase

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Protolytic reactions during the reaction between fully reduced cytochrome oxidase and oxygen have been investigated using the flow-flash methodology. Enzyme reconstituted into phospholipid vesicles has been used and pH changes in the extravesicular medium have been detected with the pH indicator Phenol red. The results show the release of protons to the medium with a rate of $9 \cdot 10^2$ s^{-1} during reoxidation. The amplitude of the absorption change corresponds to at least one proton per functional unit of cytochrome oxidase.

Cytochrome oxidase; Vesicle; Flow-flash; Proton pumping

1. INTRODUCTION

Cytochrome oxidase catalyses the transfer of electrons from cytochrome *c* to molecular oxygen coupled with the translocation of protons across the mitochondrial membrane.

Several of the electron transfers involved in the overall reaction have been studied with the flow-flash methodology pioneered by Gibson and Greenwood [1]. This method utilises flash photolysis to displace carbon monoxide bound to the fully reduced enzyme. If oxygen is present, this initiates reoxidation. The approach has led to considerable insight into the electron transfers that occur during the reoxidation reaction (see [2] for a review). Some features of these rapid reactions as well as other kinetic and equilibrium properties can be related to the function of cytochrome oxidase as a redox-linked proton pump [3]. The elucidation of the mechanism of proton pumping does, however, also require a detailed picture of the proton transfers involved in the overall reaction.

In the present work, we have used the flow-flash approach to study protolysis reactions during reoxidation of the fully reduced enzyme. We have used cytochrome oxidase reconstituted into phospholipid vesicles to allow the observation of protonation events at the outside of the membrane only, and the reactions have been monitored optically with a pH indicator. Our results show that at least one proton is released rapidly ($9 \cdot 10^2$ s^{-1}) into the extravesicular medium when the carbon monoxide complex of the fully reduced enzyme is photolysed in the presence of oxygen.

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Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazinesulfonic acid

2. MATERIALS AND METHODS

2.1. *Materials*

All reagents used were of analytical grade. Carbon monoxide (99.94%) was from Alfax and asolectin (type II) was obtained from Sigma and further purified as in [4]. Cytochrome oxidase was isolated from bovine hearts by the method of Van Buuren [5], and further purified by sucrose gradient ultracentrifugation as described in [4]. The purified enzyme was reconstituted into phospholipid vesicles by cholate dialysis using the protocol of Maison-Peteri and Malmström [4]. The last dialysis buffer was 0.1 mM Hepes, 22 mM K_2SO_4, 75 mM sucrose, adjusted to pH 7.5 just before use. Respiratory control was measured as in [4], and respiratory control ratios were found to be routinely above 4.

2.2. *Flow-flash kinetics*

An aliquot of the vesicles (typically ca. 10 ml, containing ca. 6 μM of cytochrome oxidase) was supplemented with 40 μM valinomycin and 10 μM hexaammineruthenium (III) chloride. The pH was adjusted to 7.5 (under N_2 flush to prevent CO_2 uptake) and the sample was transferred to a Thunberg cuvette equipped with a septum port. Enough Phenol red to make a 40 μM solution when mixed with the vesicles was added to the sidearm. The cuvette was then repeatedly evacuated and flushed with N_2 that had been passed over an oxygen-removing catalyst (Anoxy-Cil). Degassed ascorbate was added with a syringe (final concentration ca 0.5 mM), and the atmosphere in the cuvette was changed to CO. An optical spectrum was taken to check the formation of the fully reduced CO complex. The absorbance change at 605–630 nm corresponded to the reduction of about 70% of the cytochrome oxidase, suggesting that this fraction was available for reduction by ascorbate/hexaammineruthenium on the outside of the vesicles. Then the Phenol red in the sidearm was added and the sample was transferred to the storage syringe of the flow-flash apparatus. The other syringe contained 50 mM choline chloride, 50 mM KCl and 40 μM Phenol red, adjusted to pH 7.5 and saturated with ox-

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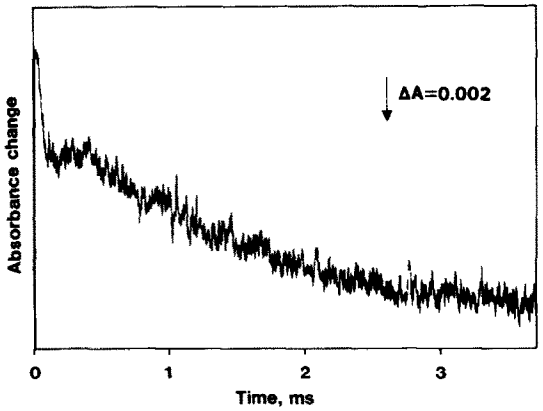

Fig.1. Absorption changes at 605 nm following flash photolysis of the fully reduced vesicular cytochrome oxidase-CO complex in the presence of O_2. The concentrations of enzyme and oxygen after mixing were about 1 μM and 1 mM, respectively. The trace is the average of 15 transients.

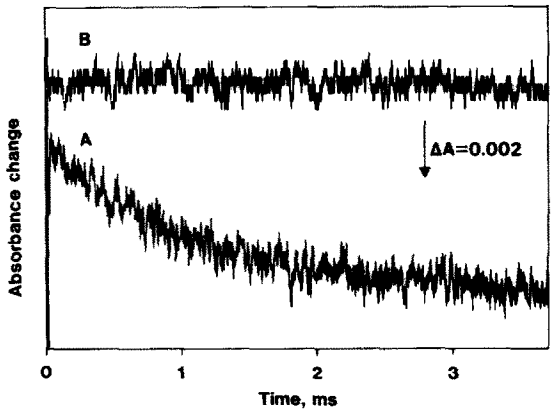

Fig.2. Absorption changes at 556 nm in the presence (A) or absence (B) of Phenol red following flash photolysis under the same conditions as in fig.1. Traces A and B are the averages of 9 and 19 transients, respectively.

ygen. In experiments where absorption changes in the enzyme were followed at 605 nm, Phenol red was omitted. Mixing, flash photolysis and data collection were as in [6], except that the time constant of the detection system was ca. 10 μs.

3. RESULTS

Fig.1 shows the absorption changes at 605 nm induced by flash photolysis of the fully reduced CO complex of vesicular cytochrome oxidase in the presence of oxygen. As observed in earlier work with the solubilised enzyme [2], three kinetic phases are resolved. Analysis of the curve gives rates constants of $2 \cdot 10^4$, $1 \cdot 10^4$, and $7 \cdot 10^2$ s^{-1}, in good agreement with those found earlier.

To monitor extravesicular pH changes associated with the reoxidation, absorption changes of the pH indicator Phenol red were followed at 556 nm. This is close to the absorption peak for the alkaline form of the indicator, whereas the contribution from the chromophores in the enzyme is minimal. Fig.2A shows the result obtained. When the experiment was repeated omitting the indicator, the result was the trace shown in fig.2B. In the presence of buffer (100 mM Hepes-K^+) and indicator, no absorption changes were observed (not shown). The downward deflection in trace A is thus very likely due to the release of protons during reoxidation of cytochrome oxidase. The rate constant obtained for the absorption decrease was $9 \cdot 10^2$ s^{-1}.

To relate the amplitude of the absorption change to the number of protons released, the exhaust from the flow cell was collected and adjusted to the initial pH. The changes in absorption brought about by additions of known amounts of HCl were then recorded in a spectrophotometer. This procedure gives a stoichiometry of about 1.2 protons released per enzyme molecule.

When the reaction was followed over a longer time scale, a large absorption decrease with a half-life of about 1 s was observed. This was also found with vesicles containing oxidised enzyme, or vesicles not containing cytochrome oxidase. No fast absorption changes were seen in these samples. This slow change is probably due to the release of protons from the lipids when the vesicles are diluted in the flow-flash apparatus as suggested by Sarti et al. [7].

In an attempt to investigate the effect of uncoupling on the fast acidification, we found that very high concentrations (>50 μM) of the uncoupler carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) were required to abolish the signal (not shown). However, in studies of uncoupler-catalysed proton equilibration in vesicle systems [8,9], half-lives of a few milliseconds have been found at comparable concentrations of uncouplers. Conclusions from experiments using uncouplers would require that proton equilibration across the lipid membrane is rapid compared to the process under study, and therefore more work seems necessary to define conditions for complete uncoupling on the sub-millisecond time scale.

4. DISCUSSION

The rapid absorption decrease seen in fig.2A shows that protons are released to the extravesicular space during reoxidation of the fully reduced enzyme. Since the cytochrome a_3-Cu_B, where the oxygen chemistry takes place, is connected with the vesicular lumen [10], the presently observed proton release is most likely the result of a partial reaction in the proton pumping cycle. A comparison of the rate constant for proton release, $9 \cdot 10^2$ s^{-1}, with k_{cat} measured under comparable conditions [11] shows that it is kineticaly competent to be a part of normal catalysis. The amplitude of the absorption decrease corresponds to the release of at least one proton per enzyme molecule, but this number should be considered tentative given the uncertainties of the calibration procedure. We have obtained values slightly

above one proton in several experiments, which indicates that the actual stoichiometry could be higher.

The overall stoichiometry of proton pumping by cytochrome oxidase is close to one H^+/e^-. Recent results by Wikström [12] do, however, suggest that it is only the electron transfers from cytochrome *c* to the peroxide and 3-electron intermediates that are coupled to proton translocation. In these steps, two protons per electron are translocated. If all proton release in the proton pumping catalytic cycle took place during the oxidative reaction, one would then expect to see four protons in the present experiment. Although our value for the number of released protons could be an underestimate, it seems unlikely that the actual value is as high as four. There are, however, two important differences between our experiment and those of Wikström that can account for the presently found release of less than four protons. First, we have monitored only the oxydative part of the catalytic cycle, whereas during catalysis, proton release might occur also on reduction. Second, the fully reduced enzyme may not be a catalytically relevant species, and consequently one of the electron transfers from the low-potential sites obtained in a flow-flash experiment may not be a part of normal turnover.

From its rate, the deprotonation seems to be associated with the slowest electron transfer step. It is, however, also possible that proton release is the result of a (comparatively) slow conformational change induced by a faster electron transfer step. In any case, it seems clear that deprotonation is brought about by the electron transfer from one of the low-potential sites to the oxygen-binding site.

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