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Cytochrome *c* oxidase: the presumptive channel holds at least four water molecules

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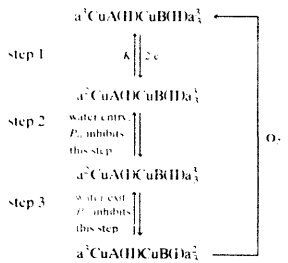
The catalytic cycle of cytochrome oxidase requires that different intermediates be differentially hydrated. The movement of water, into and out of the enzyme, is essential for but is not directly linked to electron transfer. For this reason it is felt that the water cycle represents an aspect of the energy transduction process. We show that a minimum of four waters can be trapped in the oxidase during arrested turnover.

Cytochrome *c* oxidase, a protein of the mitochondrial membrane, transfers electrons from cytochrome *c* to oxygen. This is chemiosmotically coupled to the pumping of protons across the membrane, thereby generating an electrochemical proton gradient. In the purified membrane-free state, the oxidase can still turnover but it can not store energy; there is no membrane. Do the pumping reactions of the oxidase occur when the soluble, membrane free enzyme turns over? The data relating to this question are equivocal [1–5].

We previously showed that the oxidase itself has some characteristics of an osmotically active system, the exterior of the protein acting as a semipermeable membrane [6] vis à vis the interior space. Experiments using hydrostatic pressure and osmotic pressure indicated that there is a water channel in the cytochrome *c* oxidase and that there are steps in the catalytic mechanism which involve entry or exit of water (see Scheme 1) [6]. We have inferred that a minimum of 10 water molecules must pass through the oxidase during each turnover. These water molecules enter at step 2 of the abbreviated catalytic cycle. A minimum of five waters are thought to exit at step 3 and all must have exited before the cycle can begin once more. If the water in the channel is sequestered from the bulk water and if it can enter or exit only at specific steps in the catalytic cycle, two predictions follow: (1) altering the activity of water should affect catalysis [7], and (2) it should be possible to trap solvent in the channel. We describe here experiments that confirm both predictions.

The use of osmolytes to alter the activity of water and the use of osmotic pressure to perturb the structures of biologically important structures has been described [8–11]. In figure 1 we show that the reaction catalyzed by the oxidase is inhibited by high concentrations of glycerol; ln k, the rate constant of the enzymatic reaction, scales as a linear function of the osmotic pressure of the solution (Fig. 1, top). For this experiment $\Delta V^{\ddagger}$, the activation volume, is 25 ml/mol; it is a complex function of several pressure sensitive terms in the rate equation [12,13]. Although we cannot correlate this $\Delta V^{\ddagger}$ with a single physical step in the enzymatic mechanism, it is clear from comparing the results in hydrogen containing solutions (Fig. 1, top) with those in deuterium containing solutions (Fig. 1, bottom) that osmotic pressure is probing steps that include the making and breaking of bonds in which hydrogen is involved [14]. $\Delta V^{\ddagger}$ is smaller in H_2O than in D_2O (25 vs. 56 ml/mol). This causes the kinetic



Scheme 1. An abbreviated catalytic cycle for cytochrome oxidase ($a^3CuA(II)CuB(II)a_3^3$) showing the steps at which water movement is thought to occur. P_h is hydrostatic pressure, P_o is osmotic pressure.

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isotope effect to increase with increasing osmotic pressure, from 1.51 at 1 bar to 1.95 at 200 bar. The implication of these data is that hydrogen bond making and breaking becomes increasingly difficult as the osmotic pressure is increased.

We sought a direct method to test whether water was implicated in the catalytic mechanism. If there is a channel and if it is closed during the resting state, then it might be possible to "trap" ^3H_2O in this compartment. The major problem associated with this type of quest is to distinguish specific trapping from non-specific binding or exchange. The basic outline of the experiment follows (see Table I):

1. Non-specific exchange. The resting protein was incubated with radioactive water for 5 minutes, bound to a Q-Sepharose column, and washed for 60 min with buffer containing no ^3H_2O. During this wash, ^3H exchanged out of the protein. The protein was then eluted with high salt and the radioactivity in the oxidase containing fractions was determined. This sample gave a baseline value for nonspecific radioactive uptake.

2. Trapping. The oxidase was allowed to cycle catalytically for 5 min in the presence of radioactive water. Turnover was stopped. The enzyme was then absorbed, washed and eluted as in Expt. 1. This sample gave a value for how much radioactivity was taken up both non-specifically and by the presumptive channel.

3. Trapping plus catalytic washout. The oxidase was allowed to cycle catalytically with radioactive water present. The reaction was stopped; the protein was absorbed and washed as in Expt. 2. Reductants were then added so that the Q-Sepharose bound enzyme could undergo further turnover in the presence of non-radioactive water. Any radioactive water trapped during the first period of turnover should be replaced by non-radioactive water during the second. The radioactivity washed out of the protein and that eluted with the protein were determined. These two values should represent the water that was trapped within the oxidase during turnover and that non-specifically taken up by the protein. The two numbers that come from Expt. 3 should sum to the total in Expt. 2. In addition, the non-specific labelling of Expt. 1 should be the same as that seen in Expt. 3.

The data from the three experiments are shown in Table I. They indicate that the oxidase contains at least two types of sites that will exchange with radioactive water. The first is that which exchanges independently of turnover [15]. The second and more interesting of the two sites is that associated with turnover. When catalysis begins, the site becomes accessible, and when

TABLE I

Water trapping by cytochrome oxidase during turnover

In experiments of type 1, 0.2 ml of oxidase (170 μM aa_3, purified and dialysed as described in the legend to Fig. 1) was diluted into 0.2 ml ^3H_2O (100 μCi). The mixture was incubated for 5.0 min and then diluted into 15 ml of 1.5 mM EDTA, 1.5 mM Tris, 1% Tween 80 (pH 7.0). The diluted material was applied to a 10 ml Q-Sepharose column and allowed to absorb to the resin. The column was washed with three 10 ml aliquots of the diluting buffer over a period of 60 min. The oxidase was finally eluted from the column with the same diluting buffer containing 0.5 M KCl. The oxidase eluted as a single sharp band; the radioactive counts in the oxidase-containing samples were 4-times the counts contained in the adjacent, non-oxidase-containing, samples. In experiments of type 2, 0.2 ml of oxidase (170 μM aa_3, purified and dialysed as described in the legend to Fig. 1) was diluted into 0.2 ml ^3H_2O (100 μCi) containing 2.5 mM ascorbate, 0.25 mM TMPD and 9 μM cytochrome *c*. The ascorbate was exhausted after less than 3 min, but the incubation was allowed to continue for the full 300 s. It was then diluted into 15 ml of 1.5 mM EDTA, 1.5 mM Tris, 1% Tween 80 (pH 7.0) and processed exactly as in Expt. 1. Once again, the oxidase eluted as a sharp band and the counting precision was high. Experiments of type 3 were identical to type 2 through the binding of protein to Q-Sepharose and the 3 × 10 ml buffer wash. The column was then washed with 20 ml of the same buffer containing 0.5 mM ascorbate, 0.1 mM TMPD and 3.6 μM cytochrome *c*. This latter solution allowed the oxidase to cycle on the column but did not elute the oxidase. The column was washed with another 10 ml aliquot of buffer before the final elution with 0.5 M KCl in buffer. The counts in the samples containing 'turnover wash' (ascorbate, TMPD, cytochrome *c*) were only 1.5-times those in the adjacent samples ml; the precision and accuracy of the tritiated water contained in these fractions was low. The oxidase, as in the other experiments, eluted as a single sharp band; the counting accuracy was again high. The entire series of experiments was performed twice; portions of the series – expt. 1 vs. expt. 2, expt. 2 vs. expt. 3, expt. 3 alone – were performed two or three times in order to establish trends. The numbers in the table represent the means and absolute limits of all the determinations.

	Additions and conditions	Water bound to oxidase as exchanged ^3H or as trapped ^3H_2O
Expt. 1	oxidase + ^3H_2O	3.8 ± 1.0
Expt. 2	oxidase + ^3H_2O + ascorbate, TMPD and cytochrome *c*; turnover followed by complete oxidation of the reductants	7 ± 1.5
Expt. 3	oxidase + ^3H_2O + ascorbate, TMPD and cytochrome *c*; turnover followed by complete oxidation of the reductants; water washed out during 20 ml of turnover wash	4.2 ± 2
	Protein eluate	4.2 ± 1

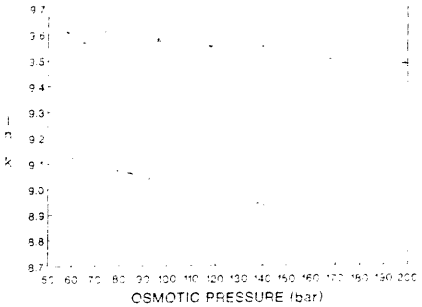

Fig. 1. Cytochrome oxidase activity is a direct function of water activity. 546 nmol (heme *a*) of cytochrome oxidase was purified as previously described [18], following the final column it was precipitated with 31% satd. ammonium sulfate and centrifuged for 30 min at 14000 rpm. The pellet was dissolved in 1 ml of 10 mM EDTA, 10 mM Tris, 2% Tween 80 (pH 7.0) and dialysed against two changes of the same buffer. The resultant protein solution was optically clear at a concentration of 70 μM oxidase (140 μM heme *a*). Activities were measured in a Clark type oxygen electrode thermostated at 37°C. The final solutions in the electrode contained 10 mM EDTA, 6.6 mM Tris, 0.1% Tween 80, 2 mM ascorbate, 0.2 mM TMPD (*N,N,N',N'*-tetramethylphenylenediamine), 40 μM cytochrome *c* and 34 nM cytochrome *c* oxidase. In the absence of glycerol, the measured pH of the solution was 6.5 in protium buffers and 6.7 in deuterium buffers (pD of the latter was 7.1). The concentration of glycerol in each protium solution was determined by picnometry. The accuracy of the measurements was better than one part in 10^3. The values were verified when possible by monitoring the freezing point depression of the solutions. In the deuterium-containing solutions, deuterated glycerol (100%, MSD Isotopes) replaced glycerol and all solutions were prepared in deuterated water. The actual concentration, the osmolality, of the solutions was determined by freezing point depression taking the molal freezing point depression of D_2O as 2.009 deg·mol [19] and the freezing point of D_2O as 3.82 [20]. $\Delta H^{\ddagger}$ was evaluated from $(\partial \ln k / \partial \Pi) = -\Delta V^{\ddagger} / RT$ where Π is osmotic pressure. $\Delta V^{\ddagger}$ is a linear function of osmotic pressure only over a small range. $\Delta V^{\ddagger}$ is also dependent on a hidden viscosity term which can be ignored below relative viscosities of 5, which is the case here.

it stops, the contents are sequestered from the bulk of the solvent. Reinstating turnover allows the contents of the site to once again equilibrate with the bulk of the solvent.

Four waters (Table I, 4.2 ± 2) are associated with the sequestered site of cytochrome *c* oxidase; they have the characteristics that we would have predicted for waters located in the presumptive channel of the protein. Rousseau and co-workers [16] have shown that there are at least two water molecules that are associated with the heme of the oxidase. This water is present both during turnover and during the resting state. We previously thought [6] that Rousseau's water and ours could not be the same. The water discussed here gains entrance during turnover and is trapped in the resting state. Accordingly, it is quite possible that of the four waters discussed in this work, two could be the same as seen by Sassaroli et al. [16]. It is interesting to note that Papadopoulos et al. [17], working with a different proton pump, that of the purple membrane, have evidence for four waters tightly bound in the region of the retinal chromophore. They, just as we, feel that there is reason to believe that the waters are part of the proton pumping mechanism. In our case, the channel is open only during catalysis and its opening and closing appears to be obligatory for electron transport to occur. It is for this reason that we feel that the four waters may be tightly associated with the proton channel of the oxidase. If this turns out to be correct, we have identified one of the few auxiliary characteristics of a proton pump of the mitochondrion.

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