The Cytochrome c Oxidase of Paracoccus denitrificans Pumps Protons in a Reconstituted System*

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The purified two-subunit cytochrome c oxidase of Paracoccus denitrificans was reconstituted into phospholipid vesicles having a high internal buffering capacity and exhibiting a respiratory control index greater than 6.6. With these proteoliposomes, pH changes of the suspending medium were monitored in response to reductant pulses in the presence of valinomycin and potassium. When reduced cytochrome c was added to allow for a limited number of turnovers (2–12), a net acidification of the extravesicular space could be observed. This apparent proton ejection by the vesicles was abolished by inhibition of the oxidase with azide, by bypassing the oxidase with ferricyanide, or by preventing charge compensation by omitting valinomycin. Addition of uncoupler led to an alkalization, rather than an acidification, of the extravesicular space in response to reduced cytochrome c. We thus conclude that cytochrome c oxidase of P. denitrificans is a proton pump. Under the conditions described here, an apparent stoichiometry of 0.6 proton ejected/electron was obtained by extrapolation to zero turnovers.

In the last 2 years, bacterial cytochrome c oxidases (EC 1.9.3.1) containing hemes a and a3 and copper atoms have been purified from various microorganisms, such as the thermophilic bacterium PS3, Thiobacillus novellus, Thermus thermophilus, and Paracoccus denitrificans. The most striking difference between these prokaryotic enzymes and eukaryotic cytochrome c oxidase is the much simpler subunit composition of the bacterial enzymes (see Ref. 1 for review). Thus, purified cytochrome c oxidase of P. denitrificans appears to consist of only two polypeptide subunits of Mr = 45,000 and 28,000 (2). In contrast, eukaryotic cytochrome c oxidase is a highly complex enzyme, consisting of at least seven polypeptide subunits (3).

Both the prokaryotic and the eukaryotic cytochrome c oxidases serve as the terminal members of the electron transport chain, transferring electrons from reduced cytochrome c to oxygen. According to Mitchell (4), the oxidase forms the last electron-carrying arm of the respiratory chain loops and functions in the generation of a proton gradient by catalyzing the consumption of 1 matrix proton/electron for the formation of water. As a result of experiments on the mammalian enzyme, Wikstrom has proposed that cytochrome c oxidase, in addition, translocates 1 matrix proton/electron across the membrane, thus operating as a redox-linked proton pump (5, 6). This proton pumping activity of eukaryotic cytochrome c oxidase has been demonstrated with intact mitochondria as well as with the purified enzyme, reconstituted into phospholipid vesicles (5–13). The question arose at this point whether the much simpler prokaryotic cytochrome c oxidase is also capable of acting as a proton pump coupled to electron transport. Here, we report proton pumping by the purified two-subunit cytochrome c oxidase of P. denitrificans, reconstituted into phospholipid vesicles.

Materials and Methods

Reconstitution of Cytochrome c Oxidase into Liposomes—Cytochrome c oxidase of P. denitrificans was isolated as previously described (2) and further purified by affinity chromatography on a cytochrome c column (14). The enzyme (8.5 mg/ml, 50 mM KPi, pH 7.3, traces of Triton X-100 and cholate) contained 26.5 nmol of heme a/mg of protein, using an extinction coefficient of 11.7 cm-1 mM-1 (2) for reduced minus oxidized heme a at the wavelength couple 550-630 nm. The enzyme was devoid of other spectrally detectable cytochromes and contained less than 1 phospholipid molecule/aa.1

To reconstitute cytochrome c oxidase into liposomes, 12.5 mg of soybean phospholipids (Asolectin, Associated Concentrates, Woodside, NY) in ether were dried to a film in a tube and subsequently suspended in 0.5 ml of 200 mM choline-Hepes2, 30 mM KCl, 0.1 mM K-EDTA, 50 mM choline cholate, pH 7.3, by sonication to clarity at 0 °C under nitrogen. To this, 4 nmol of enzyme were added and the mixture was dialyzed against 200 ml of the same buffer without cholate, for 8–8 h at 0 °C, followed by dialysis against 200 ml of 100 mM choline chloride, 30 mM KCl, 0.1 mM K-EDTA, 1 mM K-Hepes, pH 7.1, for 7.5–9 additional h at 0 °C in 1-inch dialysis tubing (Union Carbide) with rapid stirring. The respiratory control index of the reconstituted vesicles, measured as described in Ref. 2, was at least 6.6 at 15 °C.

Bovine heart cytochrome c oxidase, isolated as described (15); 15 mg/ml in 10 mM KPi, 0.1% Tween-80, pH 7.4, 11.8 nmol of heme a/mg was reconstituted by the same procedure. The respiratory control index was at least 4 at 15 °C.

Reductant Pulse Experiments—Unless stated otherwise, reconstituted cytochrome c oxidase was incubated with 2 μg of valinomycin in a total volume of 1 ml of 100 mM choline chloride, 30 mM KCl, 0.1 mM K-EDTA, 0.5 mM K-Hepes, pH 7.1, in a thermostated glass vessel equipped with magnetic stirring and a glass pH electrode (Philips CA/02). The response time of the electrode was reduced by stirring it in 1% HF for 1 min. The electrode signal was amplified with a pH meter (Philips PW 9409) and fed into a strip chart recorder (Perkin-Elmer, Model 56). Cytochrome c (horse heart, type III, Sigma) was reduced just before use with dichromite, followed by chromatography on Sephadex G-25 (Pharmacia) in the same buffer used in the reductant pulse experiments. The concentration of reduced cytochrome c (cytochrome c2+) was determined spectrophotometrically E (650 nm, reduced-oxidized) = 19.2 cm-1 mM-1 (16).

For simultaneous measurement of cytochrome c2+ oxidation and pH, a 3-ml thermostated glass cuvette was fitted with the pH electrode and magnetic stirring in an 8-wavelength spectrophotometer (MB3, Johnson Foundation). Reduced cytochrome c was monitored as absorbance difference of 550–540 nm and the signal, together with the pH signal, was fed into a 2-channel strip chart recorder (Perkin-Elmer, Model 56). The reaction conditions were those described above, except that the total volume was 2 ml. Due to the higher respiratory control index of vesicles containing Paracoccus cyto-

1 B. Ludwig, unpublished.
2 The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; cytochrome c2+, reduced cytochrome c.
Proton Ejection in Response to Reductant Pulses—When cytochrome c oxidase of *P. denitrificans*, reconstituted into phospholipid vesicles, was supplied with a pulse of reduced cytochrome c, acidification of the extravesicular space was observed (Fig. 1, trace B). Eight turnovers of the enzyme (8 nmol of reduced cytochrome c added/0.25 nmol of aa3) led to the appearance of protons in the suspending medium with a stoichiometry of 0.35 H+/e-. The relatively slow decay of this acidification was greatly accelerated by the addition of uncoupler, which allows protons to freely permeate the lipid membrane. The resulting net alkalization of the system exhibited a stoichiometry of -1 H+/e-, as expected for the consumption of 1 proton/electron for the formation of water inside the vesicles. This stoichiometry was accurately maintained for several consecutive reductant pulses added to the uncoupled system (Fig. 1, trace B), thus providing evidence that no protons, other than those required for the formation of water, were generated or released in the system. If a very small amount of uncoupler (6 mg of carbonyl cyanide m-chlorophenylhydrazone/mg of lipids) was added to the vesicles before the experiment, nearly the same initial acidification following the addition of cytochrome c2+ could be observed, but was followed by a rapid alkalization of the expected final stoichiometry of -1 H+/e- (not shown). We conclude that the initial acidification of the extravesicular space in response to reduced cytochrome c represents proton pumping by the bacterial heart cytochrome c oxidase. Trace A in Fig. 1 shows, for comparison, a similar experiment conducted with vesicles containing beef heart cytochrome c oxidase. Eight turnovers produced an acidification of 0.7 H+/e- for the beef heart enzyme, it has repeatedly been shown that protons are pumped at an initial stoichiometry of about 1 H+/e- (5-13).

Proton ejection by *Paracoccus* (and beef heart) oxidase is critically dependent on the presence of sufficient concentrations of valinomycin and potassium. If valinomycin is omitted, a large transmembrane potential, interior negative, is rapidly generated by the flow of electrons from cytochrome c2+ on the outside, to O2 in the inside of the vesicles, and proton pumping is suppressed (Fig. 1, trace D). If the cytochrome c oxidase is 90% inhibited with 1 mM sodium azide, only a very small acidification (10% of that observed in the absence of sodium azide) is seen (Fig. 1, trace E). This argues against the generation of protons by a process not related to the transport of electrons from cytochrome c to oxygen, such as the binding of cytochrome c to the vesicles. No significant pH change takes place if cytochrome c2+ is added to vesicles containing 30 μM ferricyanide in the suspending medium (Fig. 1, trace F). Under these conditions, cytochrome c oxidase is efficiently bypassed and electrons are transferred directly from cytochrome c2+ to ferricyanide. Addition of uncoupler shows that no alkalization of the vesicle interior has taken place. If the oxidation of cytochrome c2+ *per se* were to generate protons, an acidification should be observed in this experiment.

The Stoichiometry of Proton Ejection at Zero Turnovers—When the number of turnovers of the enzyme is increased from 2 to 12 by adding increasing amounts of reduced cytochrome c in the experiments of Fig. 1, A and B, the observed acidification of the extravesicular space increases in absolute terms, but the observed H+/e- stoichiometries decrease. In Fig. 2, the H+/e- ratios for beef heart and *Paracoccus* cytochrome c oxidase are plotted as a function of the number of turnovers. The highest values measured were 0.9 H+/e- at 4 turnovers of beef heart oxidase and 0.57 H+/e- at 2 turnovers of the *Paracoccus* enzyme. With increasing numbers of turn-

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**Fig. 1.** pH responses in reductant pulse experiments. A, to vesicles containing beef heart cytochrome c oxidase (0.25 nmol of aa3) in the presence of 2 μg of valinomycin, the additions indicated were made. B and C, to vesicles containing *Paracoccus* cytochrome c oxidase (0.25 nmol of aa3) in the presence of 2 μg of valinomycin, the indicated additions were made. D, as B, but valinomycin was omitted. E, as B, but in the presence of 1 mM NaNs. F, as B, but in the presence of 30 μM ferricyanide. The *numbers* are nanomoles added and correspond also to microliters added. CCCP, carbonyl cyanide m-chlorophenylhydrazene, 0.1 μg added as a 100-μg/ml solution in ethanol; c2+, reduced cytochrome c. An upward deflection of the trace corresponds to a decrease in pH.

**Fig. 2.** Stoichiometries of proton ejection for beef heart and *Paracoccus* cytochrome c oxidase vesicles as a function of the number of enzyme turnovers. Measurements of proton ejection were made as in Fig. 1, A and B, respectively. The extent of the initial acidification upon addition of various amounts of reduced cytochrome c was quantitated, based on a calibration with HCl before the start of the reaction. One turnover is defined as the oxidation of 4 nmol of cytochrome c2+ by 1 nmol of aa3, and the stoichiometry of proton ejection is expressed as nanomoles of protons appearing in the extravesicular space/nmol of cytochrome c2+ added (H+/e-). ⋄, beef heart cytochrome c oxidase; ⋄, *Paracoccus* cytochrome c oxidase.
Proton Pumping by Paracoccus Cytochrome c Oxidase

Protein pumping by Paracoccus cytochrome c oxidase was not significantly affected by raising the lipid-to-protein ratio for the reconstitution by factors of 2 or 4 (not shown). Measurements at pH 6.8 yielded approximately the same stoichiometry of proton ejection as that found at pH 7.1, whereas at pH 7.4 and 7.7, the stoichiometries were reduced by about 0.2 H+/e- (data not shown). This could be due to the reduced reaction rate of the oxidase at increased pH (80% of that at pH 7.1) and/or the reduced buffering capacity of the Hepes-buffered intravesicular space at higher pH.

Proton Pumping and Oxidation of Cytochrome c2+ Show the Same Time Course—An important test of the validity of the proposed proton pumping linked to the oxidation of added cytochrome c2+ by Paracoccus oxidase is the demonstration that these processes occur at the same rate. Fig. 3 depicts the results of an experiment in which the oxidation of cytochrome c2+ and the external changes in pH were monitored simultaneously. Although the relative slowness of the pH electrode (90% response in less than 2 s) does not permit precise estimates of initial rates, it can be seen that cytochrome c2+ oxidation and proton ejection follow approximately the same time course.

Discussion

The proposal that eukaryotic cytochrome c oxidase pumps protons coupled to electron transport from cytochrome c2+ to oxygen has now gained almost universal acceptance. In reconstituted systems, only the oxidase molecules inserted into the membrane in a “right-side-out” orientation can interact with cytochrome c2+, which is excluded from the vesicle interior. Consequently, the protons required for the formation of water will be drawn exclusively from the intravesicular space. It follows that, in vesicles impermeable to protons, the alkalinization escribed by the equation: 4 cytochrome c2+ + 4H+ + O2 = 4 cytochrome c3+ + 2 H2O, will not be apparent in the initial phase of the reaction. On the contrary, alkalinization of the extravesicular space takes place and has been attributed to the proton pumping activity of cytochrome c oxidase.

In our attempt to demonstrate proton pumping by cytochrome c oxidase of P. denitrificans, we have carried out reductant pulse experiments similar to those previously used to demonstrate proton pumping by mammalian cytochrome c oxidase (12, 13). This system only contains the natural substrates of the oxidase, namely reduced cytochrome c (used to start the reaction) and oxygen, and is not complicated by the presence of artificial electron donors or acceptors. Paracoccus cytochrome c oxidase, reconstituted as described here, shows an 80% right-side-out orientation (17). The number of turnovers in this work is based on the total amount of heme a3 present in the reaction and does not take into account that approximately 20% of the oxidase will be inoperative. However, H+/e- ratios obtained by extrapolation to zero turnovers will not be affected by this uncertainty.

A number of factors were of critical importance to the successful measurement of proton translocation by Paracoccus cytochrome c oxidase. First, the respiratory control index of the reconstituted oxidase had to be high (above 6 in the experiments reported here). Second, a high concentration of potassium in the medium was required for charge compensation via valinomycin to be fast. This prevents the build-up of a membrane potential, which would counteract proton ejection (the high K+-concentration in the medium precluded, in turn, the monitoring of charge movements with a potassium electrode). Third, to minimize the alkalinization of the intravesicular space and the concomitant nonspecific leakage of protons into the vesicles, the vesicle interior had to be very well buffered. Excess alkalinization of the intravesicular space was further avoided by allowing the enzyme to turn over only a few times.

Recently, Van Verseveld et al. (18) described measurements of proton extrusion by whole methanol-grown cells of P. denitrificans. Their experiments indicate that the cytochrome c oxidase of this organism pumps protons with a stoichiometry of 1 H+/e-. We have measured a maximal stoichiometry of 0.57 H+/e- at 2 enzyme turnovers and have obtained an extrapolated stoichiometry of 0.6 H+/e- at zero turnovers. The reliability of our measurements is demonstrated by the stoichiometry of proton pumping of 1.1 proton/electron that we have obtained under comparable conditions for beef heart oxidase, in close agreement with previous measurements on native and reconstituted systems (5–13). Studies on the mechanism of proton translocation will have to reveal whether there are actually fundamental differences in the function of the two enzymes. The fact that the pumping stoichiometry is
less than unity for the Paracoccus enzyme has no obvious explanation and is currently under investigation.

In the mammalian enzyme, subunit II is implicated in proton translocation, based on the following findings: (i) cytochrome c oxidase lacking subunit II does not pump protons (19), and (ii) dicyclohexylcarbodiimide inhibits proton translocation and covalently binds to subunit II (20, 21). The purified Paracoccus cytochrome c oxidase used here to demonstrate proton pumping consists of only two polypeptides of $M_r = 45,000$ and 28,000. Antibodies against subunit I of yeast cytochrome c oxidase cross-react with the small subunit of the bacterial enzyme and vice versa (1). The small subunit of Paracoccus oxidase therefore seems to be analogous to subunit I of the eukaryotic enzyme. A polypeptide corresponding to subunit II of eukaryotic cytochrome c oxidase is absent in this prokaryotic oxidase. Thus, a third polypeptide per se is not required for this enzyme to act as a proton pump. One might speculate that the structures involved in subunit II functions of the mammalian enzyme are integrated into subunit I of the oxidase of P. denitrificans. Future experiments will show whether the prokaryotic enzyme is also susceptible to inhibition and covalent labeling by dicyclohexylcarbodiimide.

We have thus shown, for the first time, proton pumping by a purified two-subunit cytochrome c oxidase, reconstituted into phospholipid vesicles. We suggest that the oxidase from P. denitrificans provides a simple model system to investigate molecular aspects of proton translocation in the terminal span of the respiratory chain.

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