Ser-82 mutant and Zn-substituted cytochrome *c* peroxidase prior to publication.

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Intrinsic Uncoupling in Proton-Pumping Cytochrome *c* Oxidase: pH Dependence of Cytochrome *c* Oxidation in Coupled and Uncoupled Phospholipid Vesicles†

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ABSTRACT: The pH dependence of the transient aerobic kinetics of cytochromes *c* and *a* has been investigated with cytochrome oxidase reconstituted in phospholipid vesicles in the absence and presence of an uncoupler and an ionophore. The cytochrome *a* reduction level immediately after the burst phase was 60-80% and was not significantly changed by the addition of uncoupler and/or ionophore. The coupled rate of ferrocytochrome *c* oxidation increases linearly with decreasing pH in the range 8.4-5.4. The increase in rate on uncoupling becomes less with decreasing pH and low cytochrome *c* concentration, being almost zero at pH 5.4. The coupled rate is increased by a lowering of the outside pH when the inside pH is constant. Varying the inside pH with a constant outside pH of 7.4 has little effect on the rate. It is suggested that the electrochemical potential has two separate effects on the coupled rate: the pH gradient mainly slows down the intramolecular electron transfer, but the membrane potential also lowers the second-order rate constant for the reaction with cytochrome *c*. The results are interpreted in terms of a model in which protonation of an acid-base group with a pK_a of 6.4 from the inside increases the catalytic constant. Protonation from the outside, on the other hand, leads to an intrinsic uncoupling, because the protonated enzyme in the output state can return to the input state. This has no adverse physiological effect, since it becomes significant only at pH values well below 7.

Cytochrome *c* oxidase, the terminal enzyme in cellular respiration, is a redox-linked proton pump (Wikström et al., 1981). There are certain basic principles governing the operation of such pumps, as emphasized in several recent reviews (Malmström, 1985; Blair et al., 1986; Krab & Wikström, 1987). An analysis of the kinetic properties of the enzyme within the framework of these principles had led to the formulation of a detailed reaction cycle, which describes the coupling between the catalytic electron-transfer reaction and the proton translocation (Malmström, 1987; Brzezinski & Malmström, 1987; Thörnström et al., 1988). According to this model the enzyme exists in two conformations, E_1 and E_2. Cytochrome *c* donates electrons to the primary acceptors, cytochrome *a* and Cu_A, in the E_1 conformation. The intramolecular electron transfer from the primary acceptors to the binuclear cytochrome a_3–Cu_B site, and the subsequent reaction

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with dioxygen, on the other hand, can only take place in the E_2 state. In addition to requiring the reduction of both cytochrome *a* and Cu_A, the transition from E_1 to E_2 needs protonation from the inside of the membrane of a group with a low pK_a. The pK_a remains the same in the two states, in accordance with the so-called transition-state mechanism (Malmström, 1985).

The mechanism just described has received support from studies of the pH dependence of the steady-state kinetic parameters (Thörnström et al., 1984) and of the transient kinetic behavior of the redox centers (Brzezinski & Malmström, 1987; Thörnström et al., 1988) under conditions in which the enzyme is completely uncoupled. In this paper we describe kinetic experiments with the enzyme reconstituted into phospholipid vesicles. The pH dependence of the rate of cytochrome *c* oxidation under coupled and uncoupled conditions has been measured. The experiments have been performed with the initial pH the same inside and outside as well as with constant inside and varying outside pH or with constant outside and varying inside pH. An analysis of the results suggests that the oxidation rate is increased by protonation from the inside of a group with a pK_a of 6.4. When the outside pH is lower than 7.4, the difference between the coupled and uncoupled rates decreases, and it becomes close to zero at pH 5.4. This can be explained if it is assumed that the protonated enzyme in the E_2 state can return to E_1 without prior dissociation of the proton. This would lead to an intrinsic uncoupling of the pump ("slip"). Such intrinsic uncoupling of mitochondrial proton pumps has been proposed earlier (Zoratti et al., 1986, Pietrobon et al., 1986) but has not previously been demonstrated specifically for cytochrome oxidase.

Materials and Methods

Reagents. All reagents used were of analytical grade or further purified. FCCP[1] was obtained from Aldrich Chemical Co. and valinomycin from Boehringer. Asolectin (Sigma type II) was purified by acetone–ether fractionation as described by Kagawa and Racker (1971) but with the use of 1 mM dithiothreitol as antioxidant. Cholate was recrystallized from 70% ethanol.

Cytochrome *c* was obtained from horse hearts according to the procedure described by Brautigan et al. (1978) and then further purified by ion-exchange chromatography. Stock solutions of approximately 3 mM reduced cytochrome *c* were prepared by addition of dithionite to 15 mM ferricytochrome *c* in 0.5 M Hepes, pH 7.4, followed by gel filtration on a Sephadex G-25 column to remove excess dithionite. The ferrocytochrome *c* thus obtained was typically 97% reduced.

Beef heart cytochrome *c* oxidase was isolated essentially according to the method of Van Buuren (1972).

Reconstitution. To achieve reproducible respiratory control ratios, generally in the range of 4–6, a sucrose gradient ultracentrifugation of the enzyme was carried out prior to reconstitution (Finel & Wikström, 1986). Gradients of 5–30% sucrose (w/v) in 0.5 M potassium phosphate buffer, pH 7.4, containing 2% cholate were prepared in Quickseal centrifuge tubes. The enzyme was incubated, at a concentration of 0.2 mM, in 10 mM sulfobetaine 12 and 0.45 M KCl for 4 h on ice. This treatment of the enzyme favors dimerization leading to better reconstitution (Finel & Wikström, 1986). Samples (0.5 mL) of the incubated enzyme were applied to the gradients, and the tubes were centrifuged for 15 h at 40000 rpm in a Beckman Ti-75 rotor. The band containing dimerized cytochrome oxidase was collected manually and concentrated. The enzyme thus obtained was either used directly for reconstitution or stored at −80 °C until used.

Reconstitution of the enzyme into phospholipid vesicles was done according to the cholate–dialysis method described by Casey (1986), but with some changes in the buffers used. The second dialysis buffer consisted of 10 mM Hepes, 20 mM K_2SO_4, and 70 mM sucrose, pH 7.4. The third dialysis buffer used was 0.1 mM Hepes, 22 mM K_2SO_4, and 75 mM sucrose, adjusted to pH 7.4 just before use. A fourth dialysis step of 15 h was included with the same buffer as for the third dialysis step. After dialysis the vesicles were centrifuged (25000*g*, 30 min) to remove larger particles.

Respiratory control ratios were measured as described by Nilsson et al. (1988). Ratios in the range of 4–6 were generally obtained, but lower ratios were always found in the stopped-flow experiments (see Results).

Stopped-Flow Experiments. The stopped-flow apparatus used has been previously described (Andréasson et al., 1972). The buffers used in all experiments were as follows: for pH 5.4 and 6.4, 50 mM Mes; for pH 7.4 and 8.4, 50 mM Hepes. All buffers contained 0.167 M K_2SO_4 to give a constant ionic strength of 0.5 M at all pH values. The reconstituted oxidase vesicles were diluted to 2 μM oxidase with the same buffer as for the last dialysis step, and the pH was adjusted to that desired with KOH or H_2SO_4.

Ferrocytochrome *c* was diluted in buffer of the desired pH to give a concentration after mixing in the stopped-flow apparatus of 16 or 40 μM. A small amount of ascorbate was present in the solution to keep cytochrome *c* reduced. The concentration of cytochrome *c* oxidase after mixing was 1 μM. The concentrations of both the enzyme and ferrocytochrome *c* were checked after dilution.

Three types of experiment were carried out: (1) The cytochrome oxidase containing vesicles were mixed with cytochrome *c* in buffer at the same pH as that of the vesicles. (2) The vesicle suspension was adjusted to pH 7.4 (pH in) and mixed with cytochrome *c* at different pH values (pH out). (3) The pH of the vesicles was varied and mixed with cytochrome *c* in buffer at pH 7.4. In all three cases the experiments were done with the coupled, partially coupled (10 μM FCCP or 5 μM valinomycin present), or fully uncoupled (10 μM FCCP + 5 μM valinomycin present) oxidase.

The reaction of reduced cytochrome *c* with the reconstituted oxidase was followed at 550 and 605 nm over a period of 10 s.

The difference absorbance coefficients (reduced − oxidized) used for the determination of the concentrations and the degree of reduction were 21.1 mM^{-1} cm^{-1} at 550 nm for cytochrome *c* and 24 mM^{-1} cm^{-1} at 605 nm for cytochrome *c* oxidase, at which wavelength 80% of the absorbance change is attributed to cytochrome *a*. In the determination of the absorbance change for cytochrome *a*, corrections were made for the contribution of cytochrome *c* at 605 nm.

Results

An example of our primary stopped-flow traces at 550 and 605 nm is shown in Figure 1 for pH 7.4. Complete oxidation of cytochrome *c* was always much faster than that of cytochrome *a*; full oxidation of the latter took at least several hours. First-order plots of the 550-nm data (insert) were used to estimate the initial rate of cytochrome *c* oxidation according to Smith and Conrad (1956). The molecular activity was then calculated by multiplying the first-order rate constant with the initial concentration of reduced cytochrome *c* and dividing

[1] Abbreviations: FCCP, carbonyl cyanide 4-(trifluoromethoxy)-phenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

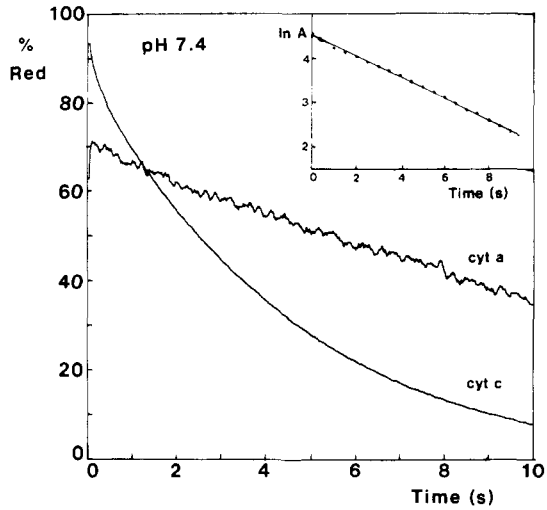

FIGURE 1: Time course of the degree of reduction of cytochrome *c* (550 nm) and cytochrome *a* (605 nm) with cytochrome oxidase reconstituted into phospholipid vesicles. Both the inside and the outside pH were 7.4 initially. The concentrations after mixing were 1 μM oxidase, 16 μM cytochrome *c*, and 137 μM O_2.

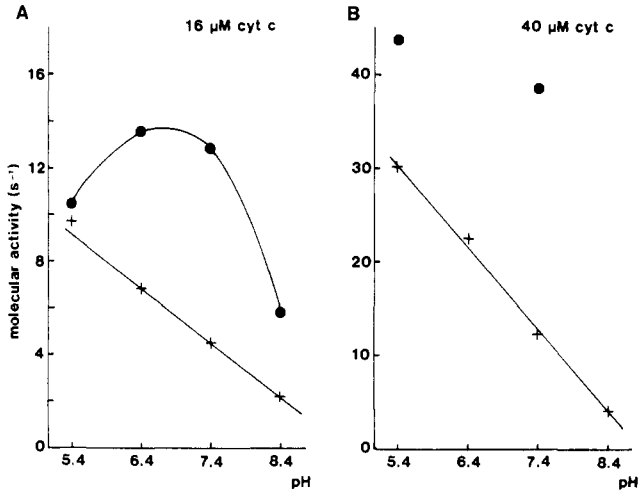

FIGURE 2: Effect of pH on the molecular activity of cytochrome oxidase in coupled (+) and uncoupled (●) vesicles with 16 (A) or 40 μM (B) cytochrome *c*. Other concentrations were as in Figure 1. The inside and the outside pH were the same initially.

by the enzyme concentration. The logarithmic plots (insert) were always linear immediately after the burst except at pH 5.4, where there was an initial increase in the value of the negative slope to a constant value after about 2 s in the experiments both with coupled and uncoupled oxidase. The molecular activities measured were rather low, because the K_m values are quite high at the ionic strength used and because only 70% of the oxidase molecules were correctly oriented in the vesicles (Thörnström et al., 1984).

The degree of reduction of cytochrome *a* following the burst was always in the range of 60–80% at all pH values for both concentrations of cytochrome *c*. The reduction levels did not change significantly on the addition of uncouplers, despite the fact that the rate of cytochrome *c* oxidation increased considerably, particularly at high pH and cytochrome *c* concentration.

The pH dependence of the molecular activity under coupled and uncoupled conditions with the same pH inside and outside the vesicles is shown in Figure 2 for two different concentrations of cytochrome *c*. The data for the partially uncoupled enzyme are not shown, but the rates were always lower than for the fully uncoupled enzyme and higher than for the coupled

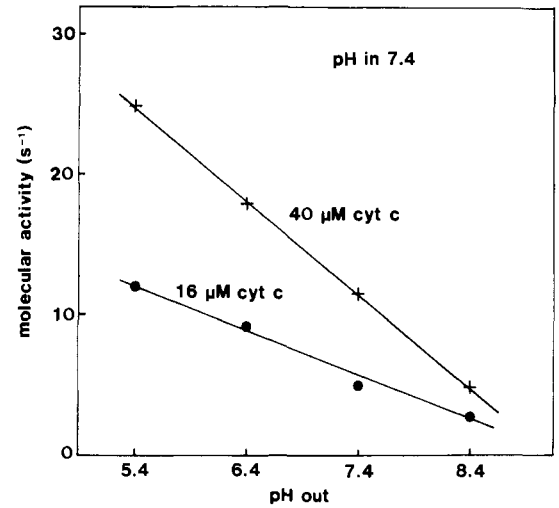

FIGURE 3: Effect of outside pH, with inside pH 7.4, on the molecular activity of cytochrome oxidase in coupled vesicles. Other conditions were as in Figure 2.

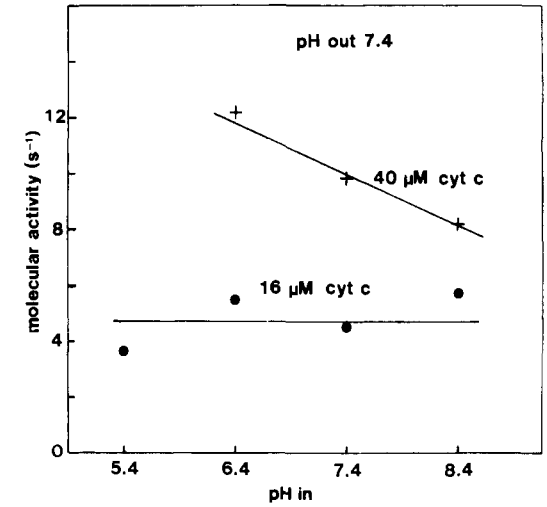

FIGURE 4: Effect of inside pH, with outside pH 7.4, on the molecular activity of cytochrome oxidase in coupled vesicles. Other conditions were as in Figure 2.

enzyme. In this as well as in the following figures, each point for 16 μM cytochrome *c* represents the mean of four to five experiments, whereas the points for 40 μM cytochrome *c* are estimated from two experiments only.

Figure 3 shows the effect of outside pH on the molecular activities with coupled oxidase and two different concentrations of cytochrome *c*, when the inside pH is always 7.4. If one plots the ratio between the activity at a given pH and that at pH 7.4, straight lines with slopes of approximately −0.4 are obtained. Data (not shown) from experiments with a constant inside pH of 6.4 result in the same slope.

The dependence of the molecular activity of the coupled oxidase on the inside pH with the outside pH constant at 7.4 for two concentrations of cytochrome *c* is shown in Figure 4.

Discussion

In all of our experiments the return of the absorbance at 605 nm to the level for the oxidized enzyme is much slower than the oxidation of the cytochrome *c*, as illustrated by the example in Figure 1. This behavior was first observed with the resting enzyme by Antonini et al. (1970) and has later been demonstrated also with pulsed or oxygenated oxidase (Fabian et al., 1987). To a small extent it can be ascribed to contributions of oxygen intermediates to the 605-nm absorbance

(Thörnström et al., 1988). The main part of the slow return is, however, most likely caused by an inability of the one-electron-reduced enzyme to react with dioxygen (Antonini et al., 1970). Thus, as reduced cytochrome *c* is consumed, one-electron-reduced oxidase molecules formed in the final stages of the reaction can only be reoxidized by first acquiring a second electron in a bimolecular electron-exchange reaction. This interpretation is strengthened by our observation that the reoxidation with reconstituted oxidase is much slower than that with the solubilized enzyme in detergent solution, as the frequency of collision between oxidase molecules must be very low, when they are incorporated into vesicles.

The nonlinearity in the plot of the logarithm of the absorbance at 550 nm against time which we observe at pH 5.4 is most likely due to a transition from the resting to the pulsed form of the oxidase, which then must be different at the other pH values. The reduction levels of cytochrome *a* in our experiments are similar to those found with resting oxidase in detergent solution, whereas the levels with the pulsed enzyme are lower because of the more rapid intramolecular electron transfer (Thörnström et al., 1988). Our molecular activities with the uncoupled vesicles are, on the other hand, close to those determined earlier for uncoupled pulsed oxidase in vesicles (Thörnström et al., 1984). Antonini et al. (1985) have reported that if the resting enzyme is kept for some hours at room temperature, the molecular activity approaches that of pulsed oxidase. Our vesicle preparation takes several days at 0 °C so it is possible that the enzyme has become pulsed (except at pH 5.4). The higher reduction levels could then be caused by an increase in k_1, the second-order rate constant for the reaction between cytochrome *c* and cytochrome *a*, due to an electrostatic effect from the negative membrane surface.

It may seem surprising that the reduction levels of cytochrome *a* do not change significantly on uncoupling, as the molecular activities increase considerably. This may be due to two opposing effects of $\Delta\tilde{\mu}_{H^+}$, the electrochemical potential gradient, on the reduction level. Removal of the chemical component (ΔpH) would tend to lower the level because of the increased rate of intramolecular electron transfer, whereas removal of the membrane potential ($\Delta\psi$) would increase the level because of an increase in k_1 when the outer membrane surface becomes more negative. Such separate effects of ΔpH on the intramolecular step and $\Delta\psi$ on the bimolecular step have been demonstrated by Gregory and Ferguson-Miller (1988a).

The catalytic constant (k_{cat}) of cytochrome oxidase increases continuously with decreasing pH, whereas k_{cat}/K_m remains essentially constant (Wilms et al., 1980; Thörnström et al., 1984; Gregory & Ferguson-Miller, 1988b). This has the effect that at low cytochrome *c* concentration the pH dependence of the molecular activity displays a maximum (Thörnström et al., 1988), as seen for the uncoupled activities with 16 μM cytochrome *c* in Figure 2A. The activity drops at the lowest pH, despite the highest k_{cat}, because the high K_m has the effect that the bimolecular step becomes largely rate limiting. This effect is less pronounced when the bimolecular rate is higher with 40 μM cytochrome *c* (Figure 2B), so that in this case the activity at pH 5.4 is higher than that at pH 7.4.

In view of what has just been said, it is surprising that the rate under coupled conditions increases linearly with the decreasing pH at both cytochrome *c* concentrations (Figure 2). This can be explained if lowering the pH results in an intrinsic uncoupling, as illustrated by the scheme in Figure 5. This is based on a reaction cycle formulated earlier (Thörnström et al., 1988), but steps that are not essential to the present argument have been combined. At physiological pH the proton

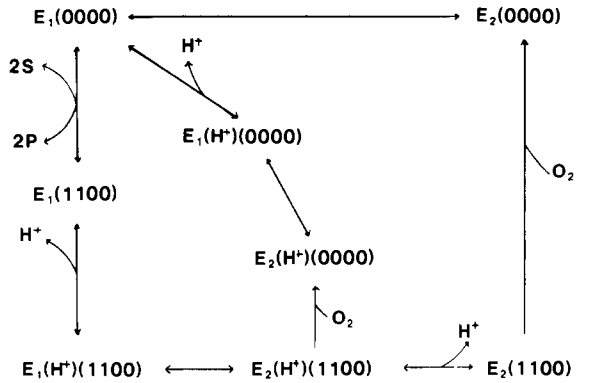

FIGURE 5: Simplified reaction scheme for cytochrome oxidase, modified from Thörnström et al. (1988). E_1 and E_2 represent two different conformations of the oxidase. The digits in parentheses designate the four redox centers in the order cytochrome *a*, Cu_A, Cu_B, and cytochrome a_3, 0 representing an oxidized center and 1 a reduced one. S is ferrocytochrome *c* and P is ferricytochrome *c*.

would dissociate from E_2 before the enzyme returns to the E_1 conformation. When the pH becomes close to or lower than the pK_a of the acid–base group, however, then the enzyme remains protonated in the E_2 state. If the protonated E_2 can also return to E_1, this would result in a proton leak.

The explanation just given is supported by a comparison with results from an earlier study of the pH dependence of the cytochrome oxidase kinetics (Thörnström et al., 1988). According to those, the pK_a of the group controlling the transition from E_1 to E_2 is 6.4. The data in Figure 2A show that the ratio between the uncoupled and the coupled rates is approximately 3 at pH 8.4 and 7.4 but becomes 1.5 at pH 6.4 and close to 1 at pH 5.4. This is in agreement with the fact that at pH 6.4 half of E_2 will be protonated, so that the leak rate is half of the maximum rate, and that at pH 5.4 the protonation is almost complete, so that all the enzyme leaks. With 40 μM cytochrome *c* the activity still increases on uncoupling at pH 5.4 (Figure 2B). This is probably due to the electron-transfer rate being larger than the leak rate, which thus should have a maximum value lower than 30 s^{-1}.

According to the reaction cycle in Figure 5, k_{cat} should be controlled by the inside pH, as it has been shown to be limited by the transition from E_1 to E_2 (Thörnström et al., 1988). In the experiments of Figure 3, the inside pH is constant, and hence so is k_{cat}. The finding that under these conditions the coupled rates are controlled by the outside pH again supports the idea of an intrinsic uncoupling. It has been mentioned that the ratios of the activities at the different pH values to the activity at a given pH give a straight line with a slope of approximately –0.4. This is expected in the pH range studied with a pK_a of 6.4, if the rate is proportional to the concentration of the protonated E_2 form. In terms of mole ratios, this concentration would increase from 0.5 at pH 6.4 to 0.91 at pH 5.4, i.e., but 0.41, and decrease to 0.09 at pH 7.4, i.e., by –0.41. It should, of course, go down to 0.01 at pH 8.4. The fact that the rate is still appreciable at this pH must be caused by extrinsic uncoupling due to proton leaks through the lipid bilayer, which thus must have a rate of at least 5 s^{-1}. Below pH 5.4, the molecular activity should become constant, but this pH range is not experimentally available because of enzyme instability.

When the inside pH is varied, k_{cat} should increase as the pH is lowered. The coupled rate is, however, not changed with 16 μM cytochrome *c* and increases only slightly at the higher concentration (Figure 4). This must be due to the fact that when the outside pH is 7.4, E_2 is always deprotonated, so that $\Delta\tilde{\mu}_{H^+}$ is efficiently built up. This is evidenced by the low

molecular activities compared to those in Figure 2. The coupled rate is decreased both by the increase in inside pH and by the effect of $\Delta\psi$ on k_1. In this way, the differential effect discussed earlier keeps the activity constant at low cytochrome *c* concentration but leads to a slight increase at the higher concentration.

Intrinsic uncoupling of a proton pump may seem to be physiologically disadvantageous. In mitochondria the outside pH is, however, expected to be rather constant at a pH above 7. Thus, with a pK_a of 6.4, the uncoupling rate will always be small under physiological conditions. Should the outside pH actually fall below 7, an intrinsic uncoupling may, in fact, be favorable in the case of cytochrome oxidase, as the scalar reaction will still go on with a concomitant consumption of protons from the inside (Wikström, 1988).

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