Observation of the Resting and Pulsed States of Cytochrome *c* Oxidase in Electrode-Supported Lipid Bilayer Membranes

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Abstract: Cytochrome *c* oxidase immobilized in lipid bilayer membranes on gold electrodes mediates electron transfer between reduced cytochrome *c* in solution and the electrode. Under flow conditions, fixed potential amperometry of anaerobic solutions of reduced cytochrome *c* shows changes in the rate of enzyme mediated electron transfer from cytochrome *c* to oxidase modified electrodes. These data are consistent with literature that describes both activation of the resting oxidase state to the pulsed oxidase state and subsequent decay of the pulsed oxidase state to the resting oxidase state.

Introduction

Cytochrome *c* oxidase is immobilized in electrode-supported lipid bilayer membranes by using a method[1,2] developed from initial work by Cullison et al.[3] Deoxycholate dialysis[4] is used to form a lipid bilayer membrane containing cytochrome *c* oxidase on gold electrodes[2,3] that was initially covered with 1.6 monolayers of electrodeposited silver. The lipid bilayer membrane is anchored to the silver surface with a submonolayer of octadecyl mercaptan that also becomes part of the bilayer.[1−3] The immobilized oxidase enzyme is able to directly transfer electrons with the electrode and to mediate electron transfer between solution resident cytochrome *c*, its native redox partner, and the electrode.[2,3]

Mammalian cytochrome *c* oxidase, the terminal enzyme of oxidative phosphorylation,[5−9] is located in the inner mitochondrial membrane where it pumps protons against a concentration gradient from the matrix to the cytosol to support the production of ATP.[10] Beef heart cytochrome *c* oxidase has 13 subunits (molecular weight ca. 204 000)[5] and contains four redox active sites,[11] a binuclear Cu site (Cu_a), heme a, and a heme a_3-Cu_b binuclear site where oxygen is reduced to water.[7]

The conformational state of enzymes is known to affect their activities.[12−14] Cytochrome *c* oxidase exists in two distinct conformations that have been called the resting and pulsed states.[7,15] The pulsed oxidase state catalyzes the reduction of oxygen by cytochrome *c* faster than does the resting oxidase state (2−10 times faster depending on the experimental conditions).[7,16] This increased catalytic activity has been attributed to faster intramolecular electron transfer to heme a_3-Cu_b[7,16,17] resulting from complete reduction of the enzyme followed by complete oxidation.[15] The pulsed and resting states have been proposed as the stable conformations of the fully reduced and oxidized states, respectively.[18,19] During reactions in which the oxidase is continuously reducing oxygen to water, however, the oxidase can exist in a mixed valence state[7] and its distribution between the resting and pulsed states may depend on the electron flux through the system[15] (i.e., the resting state is activated to the pulsed state during reduction). Interconversion between the resting and pulsed states is a reversible process,[7] but the equilibrium constant for the transition is not known.[20] The transition from the resting state to the pulsed state has been shown for detergent solubilized oxidase,[15] oxidase imbedded in the membrane of submitochondrial particles,[21] and oxidase inserted into liposomal vesicles.[22]

The model of this oxidase modified electrode has the enzyme positioned in the supported lipid bilayer with its larger hydro-

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philic end, containing the cytochrome *c* binding site,[7] exposed at the surface of the bilayer membrane and protruding into solution.[2] The submonolayer of octadecyl mercaptan, which is chemisorbed to the electrode through sulfur−silver bonds, stabilizes the lipid bilayer at this interface by allowing hydrophobic interactions between the thiol alkane tails and the lipid hydrocarbon tails. The regions of the silver surface that are not reacted with octadecyl mercaptan accommodate the smaller hydrophilic end of the oxidase as well as water and polar lipid headgroups. The proposed sequence of electron transfer is from cytochrome *c* in solution to Cu_a (the cytochrome *c* binding site of the oxidase) and from Cu_a to heme a_3-Cu_b (the oxygen binding site of the oxidase) or from Cu_a to heme a and then to heme a_3-Cu_b.[7] Enzyme oxidation likely results from electron transfer from heme a_3-Cu_b to the electrode.

Fixed potential amperometry under sample flow conditions[23] with a wall-jet configuration was used to monitor the oxidation of anaerobic solutions of reduced cytochrome *c* at the oxidase modified electrodes. Reported here are kinetic measurements of oxidase mediated electron transfer from solution resident reduced cytochrome *c* to the electrode. The current responses show a kinetic transition involving an increase in the rate of cytochrome *c* oxidase mediated electron transfer from reduced cytochrome *c* in solution to the electrode. Lowering the concentration of reduced cytochrome *c* in the solution that is reacting with the oxidase results in a kinetic transition with a decrease in the rate of oxidase mediated electron transfer. This behavior is discussed in terms of a transition between the resting and pulsed oxidase states of cytochrome *c* oxidase in the electrode-supported lipid bilayer membrane.

Experimental Section

The instrumentation,[2] cell design,[2] preparation of the electrodes, and experimental conditions have been reported.[1−3] The current responses shown in this paper involve flowing buffer past the oxidase modified electrodes to obtain a baseline current and then changing the flow to buffer containing reduced cytochrome *c*. For the experiments shown in Figure 4 the flow is changed one additional time to a lower cytochrome *c* concentration. For anaerobic experiments, buffer and reduced cytochrome *c* solutions were purged with pre-pure nitrogen (BOC Gases) for at least 5 min and then pulled into a glass syringe through a stainless steel needle. The syringe was then quickly connected to stainless steel tubing (Cole-Parmer), which carried the sample to the cell. The cell was kept in a dry bag (Tools for Scientists, I^2R) under a positive nitrogen pressure. The water used in all the experiments was deionized and then further purified with a Milli RO-4/Milli-Q system (Millipore Corp.) to exhibit a resistivity of 17−18 MΩ·cm upon delivery. The buffer used was sodium phosphate (0.1 M, ACS reagent grade, pH 7.4). Horse heart cytochrome *c* (Sigma Chemical Co., 98%) was purified, reduced, and desalted as described earlier.[2] Bovine cytochrome *c* oxidase was isolated from fresh beef hearts essentially following the procedure published by Soulimane and Buse.[24] The amperometric experiments were conducted at an applied potential of 472 mV vs NHE.

Electron-transfer rates given in Table 1 were calculated assuming a 50% coverage of oxidase on the electrode (electrode area is 0.2 cm^2).[2] The diameter of the oxidase[11] is about 80 Å giving 2×10^{11} molecules of oxidase on each electrode. Electron-transfer rates were calculated by using the Final Plateau currents in Table 1 to determine the rate of electron flow. The rate of electron flow divided by the number of oxidase molecules on the electrode gives the turnover rates shown in Table 1.

Results and Discussion

The current measured for the oxidation of reduced cytochrome *c* at oxidase modified electrodes under anaerobic conditions depends on the history of the electrode. All oxidase modified electrodes exhibit a biphasic current response (i.e., a kinetic transition) upon introduction of anaerobic solutions of reduced cytochrome *c* at the appropriate concentrations (vide infra) after the electrode is held under stagnant buffer for at least ca. 15 h at an applied potential of 472 mV vs NHE. Voltammetry shows that the enzyme is oxidized at this potential[2,3] and, as mentioned

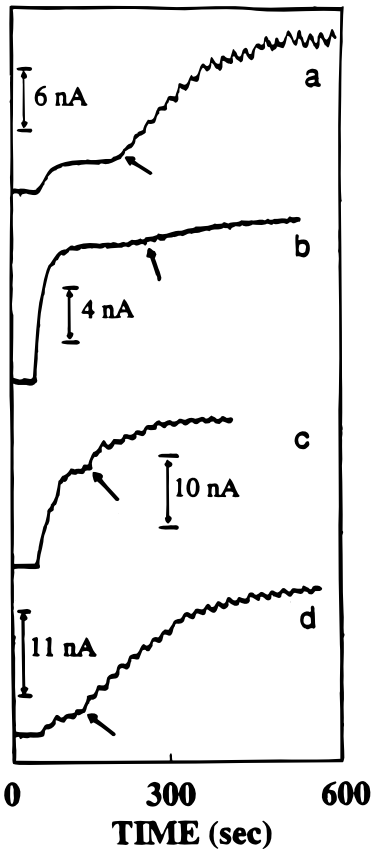

Figure 1. Anaerobic amperometric analysis of reduced cytochrome *c* at four oxidase modified electrodes (a, b, c, d). The electrode area is 0.2 cm^2, the cytochrome *c* concentration is 10 μM, the flow rate is 0.5 mL/min, and the applied potential is 472 mV vs NHE.

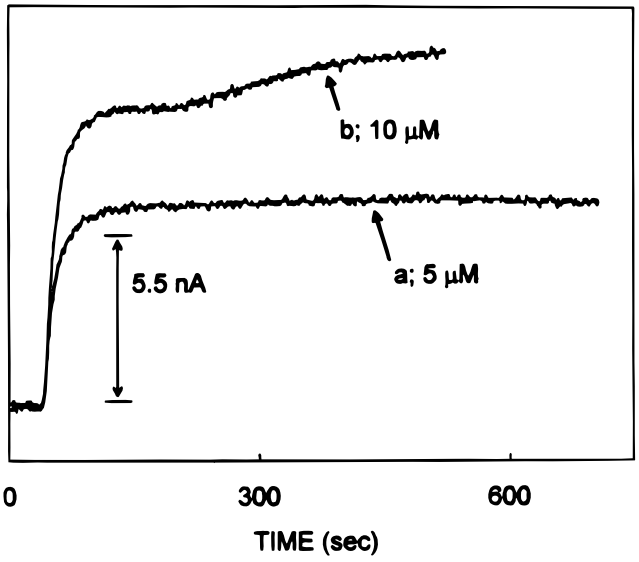

Figure 2. Anaerobic amperometric analysis of reduced cytochrome *c* at an oxidase modified electrode: (a) 5 μM cytochrome *c* and (b) 10 μM cytochrome *c*. Part a is also shown in Figure 1b. Other conditions are the same as in Figure 1.

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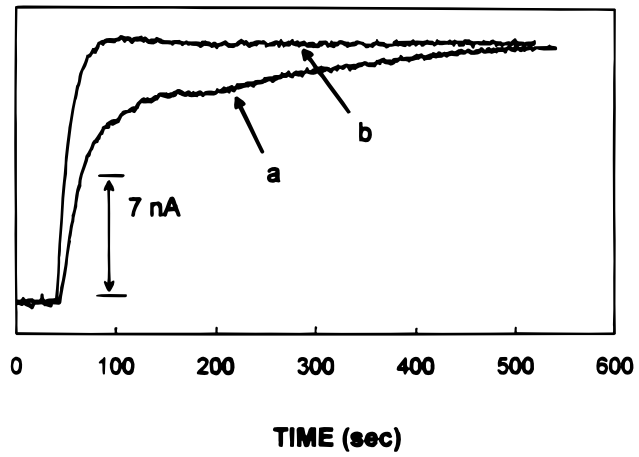

Figure 3. Anaerobic amperometric analysis of reduced cytochrome *c* at an oxidase modified electrode: (a) first 10 μM cytochrome *c* flow and (b) second 10 μM cytochrome *c* flow. The electrode is the same as for Figures 1 and 2. Other conditions are the same as in Figure 1.

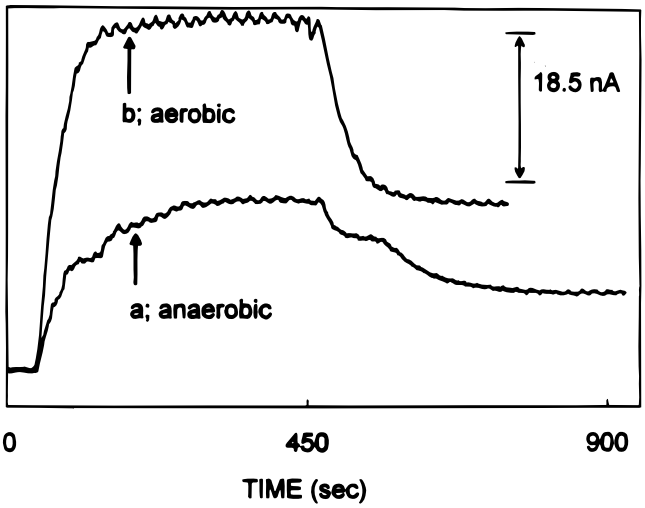

Figure 4. Anaerobic amperometric analysis of reduced cytochrome *c* at an oxidase modified electrode: (a) sequential anaerobic flows of first 10 μM cytochrome *c* and second 5 μM cytochrome *c* and (b) sequential aerobic flows of first 10 μM cytochrome *c* and second 5 μM cytochrome *c*. The first current response shown in (a) is also shown in Figure 1c. The cytochrome *c* concentration was changed from 10 μM to 5 μM at 466ss. Other conditions are the same as in Figure 1.

Table 1. Summary of Anaerobic FIA Data at Oxidase Modified Electrodes

electrode	start of transition (s)	final plateau (s)	final plateau (nA)	electron transfer rate[a] (s^{-1})
a	190	>500	14	0.4
b	200	>500	12	0.4
c	150	>350	21	0.7
d	140	>600	19	0.6

[a] Electron transfer rate is oxidase molecules reduced in electrons/s (see Experimental Section).

above, the resting state is believed to be favored when the enzyme is oxidized.[18,19] The biphasic current response is attributed to the transition of cytochrome *c* oxidase from its resting state to its pulsed state. Oxygen is not required for transition from the resting to pulsed state.[17,18,20,25,26] Figure 1 shows current responses observed at four different oxidase modified electrodes upon introduction of anaerobic 10 μM reduced cytochrome *c*. Every oxidase modified electrode studied under these conditions (8 electrodes) gave a current response that was, in general, similar to one of the responses shown in Figure 1. Table 1 summarizes the time and current profiles of the responses. Antonini et al.[16] have reported that resting state activities should be observed when the turnover rate is below 4 s^{-1}, about a factor of 10 larger than values reported here. In agreement with Antonini's original work,[15] at increased turnover rates in the presence of 50 μM reduced cytochrome *c*, the kinetic transition occurs during the initial current increase. Temporary initial current plateaus are not observed but kinetic transitions are apparent (the responses are biphasic).

For some oxidase modified electrodes no transition was observed at cytochrome *c* concentrations of 5 μM, even after 10 min. Figure 2a shows an anaerobic 5 μM cytochrome *c* current response and no transition is apparent. Figure 2b shows the subsequent anaerobic 10 μM cytochrome *c* response, and a transition is apparent. This suggests a minimum turnover rate is necessary to induce the pulsed state, as proposed by Antonini et al.[15] The result shown in Figure 2a was not limited by mass transfer of cytochrome *c* to the immobilized oxidase. Control experiments using the same cytochrome *c* concentration at promoter modified gold electrodes showed larger currents.[2]

The transition from the resting to pulsed oxidase state may be influenced by the valence state of the oxidase. The oxidase is likely in a more reduced valence state under faster electron-transfer conditions as the rate-limiting step of oxidase mediated electron transfer from cytochrome *c* in solution to the electrode is believed to be intramolecular electron transfer through the oxidase[7] (from Cu_a to heme a_3-Cu_b), i.e., more Cu_a is reduced under faster reaction conditions. As discussed above, the resting oxidase state is favored when the enzyme is fully oxidized and the pulsed oxidase state is favored when the enzyme is fully reduced.[18,19] Thus, the pulsed state is favored under faster reaction conditions, i.e., when increased current is observed.

Oxygen may be trapped in channels within the oxidase enzyme[5] and may not be removed during nitrogen purging. The oxidase enzyme reacts with molecular oxygen fast[7,15,16,27] and residual oxygen may cause the differences in the current responses between the electrodes shown in Figure 1. Any residual oxygen may be reduced during electron transfer, causing the valence state of the oxidase to be more oxidized (favoring the resting oxidase state).

The current plateau variations between electrodes shown in Figure 1 could also be due to differences in the rates of electron transfer from the oxidase to the electrodes. This change in electron-transfer rate could affect the transition from the resting to pulsed state because faster electron transfer from the oxidase to the electrode will favor the oxidized (resting) state of the oxidase.[16]

Another possibility concerning the current plateau variations between electrodes shown in Figure 1 is that the individual oxidase molecules are in different states (i.e., the enzyme population consists of oxidase in the resting and pulsed state).[16] The various current magnitudes of the final plateaus shown in Figure 1 may be due to differences in the degree of activation to the pulsed state (i.e., incomplete activation to the pulsed state for the smaller current responses). The current of the initial plateau may similarly depend on the distribution of the oxidase between the resting and pulsed states before the apparent transition begins, as self-activation has been reported earlier.[16]

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Anaerobic experiments with 10 μM cytochrome *c*, conducted a few minutes after the first anaerobic 10 μM experiment, show that the oxidase population (depending on the electrode under study) remains largely in the pulsed state. Figure 3a shows the first anaerobic 10 μM cytochrome *c* current response at an oxidase modified electrode, and Figure 3b shows a subsequent anaerobic 10 μM cytochrome *c* current response. The first experiment shows transition from the resting state to the pulsed state and the second experiment shows only pulsed state activity, i.e., the current plateau shown in Figure 3b is about equal to the final plateau shown in Figure 3a. The reason for the slight decrease in current after the plateau shown in Figure 3b is not known. Apparently all of the oxidase activated remained in the pulsed state during the time between experiments. However, most of the oxidase modified electrodes show that part of the oxidase population decays back to the resting state between experiments conducted a few minutes apart. The oxidized pulsed state has been reported to decay to the resting state with a half-time in the order of minutes.[16,18]

The rate of electron transfer through oxidase dictates the distribution of the oxidase between its resting and pulsed states. As discussed above, this is due to the dependence of the electron-transfer rate on the valence state of oxidase. Figure 4 shows experiments with buffer alone flowing over the oxidase modified electrode to establish a baseline current. Then the solution is changed to contain 10 μM reduced cytochrome *c* in the same buffer. Once a constant current is reached with the 10 μM reduced cytochrome *c* solution the solution is changed again to contain 5 μM reduced cytochrome *c* in buffer. Figure 4 shows sequential current responses for the introduction of 10 μM reduced cytochrome *c* solution followed by 5 μM reduced cytochrome *c* solution under anaerobic (a) and aerobic (b) conditions. The anaerobic current response at 5 μM cytochrome *c* (Figure 4a, starting at 466 s) is biphasic, indicating that a decrease in oxidase reduction rate results in decay of the oxidase from its pulsed to resting state. Again, the decrease in electron-transfer rate, upon changing from 10 μM cytochrome *c* to 5 μM cytochrome *c*, results in a more oxidized valence state of the enzyme, which favors the resting conformation.

The rate-limiting step of electron transfer from cytochrome *c* in solution to the electrode through oxidase is not known. However, the kinetic transitions shown here likely reflect faster intramolecular electron transfer to heme a_3-Cu_b upon activation to the pulsed state.[16,17] As reported earlier, electron transfer from the oxidase to the electrode is believed to occur from either heme a or heme a_3-Cu_b.[2,3] Structural changes near heme a and heme a_3-Cu_b occur upon transition from the resting to pulsed oxidase state,[7] causing the reduction potentials of both heme a[22] and heme a_3[20] to be higher in the pulsed state. However, the possibility that the rate-limiting step is electron transfer from the oxidase to the electrode cannot be ruled out. The conformational change associated with the transition from the resting to pulsed oxidase state[7] may cause a change in orientation of the enzyme relative to the electrode surface and/or a change in distance between the oxidase and the electrode, both of which could result in faster electron transfer from the oxidase to the electrode. Irrespective of the rate-limiting step (electron transfer to the electrode or to heme a_3-Cu_b), faster electron transfer will result in the oxidase being in a more reduced valence state.

The data indicate that, under anaerobic conditions, the activity of the oxidase (both the resting and the pulsed state) depends on the concentration of cytochrome *c* that is reacting with it. Under anaerobic conditions the final plateau current for the 10 μM experiment (pulsed state activity at 10 μM) compared with the initial plateau current for the 5 μM experiment (pulsed state activity at 5 μM) indicates that the pulsed state activity is higher for 5 μM reduced cytochrome *c* relative to 10 μM reduced cytochrome *c* (see Figure 4a prior to and just after the cytochrome *c* concentration is reduced from 10 μM to 5 μM at 466 s). Constant pulsed state activity would result in the current for 5 μM reduced cytochrome *c* being half that of the final current plateau at 10 μM (pulsed state activity at 10 μM), i.e., a linear relationship between the pulsed state current and the cytochrome *c* concentration. It follows that the current of the final plateau at 5 μM anaerobic cytochrome *c* (resting state activity at 5 μM after decay from the pulsed state) should be less than half that of the final plateau at 10 μM anaerobic cytochrome *c* (pulsed state activity at 10 μM). For the experiment shown in Figure 4a, the current of the final plateau at 5 μM (resting state at 5 μM) is about half that of the final plateau at 10 μM (pulsed state at 10 μM). These data indicate that both the resting and pulsed state activities are increased under lower anaerobic cytochrome *c* concentrations. This trend is also apparent in the data shown in Figure 2; the current of the plateau for the 5 μM response (resting state activity at 5 μM) is more than half that of the initial plateau for the 10 μM response (resting state activity at 10 μM). A possible explanation for the lower activities of both the resting and pulsed oxidase states under anaerobic conditions at 10 μM reduced cytochrome *c* relative to 5 μM reduced cytochrome *c* is an increase in binding of reduced cytochrome *c* to the oxidase at higher concentrations.

For the aerobic experiments no transitions are observed. In Figure 4b the current of the plateau at 5 μM is about half that observed at 10 μM (i.e., the oxidase activity is constant). The aerobic current plateaus are about two times larger than the final anaerobic current plateaus. Intramolecular electron transfer to heme a_3-Cu_b may be faster when partially reduced oxygen intermediates are present (but before significant accumulation).[7] The presence of oxygen at the heme a_3-Cu_b site is required for proton pumping activity.[28] Nonfaradaic current resulting from protons being pumped[10] away from the electrode by the oxidase enzyme could contribute to the increased current responses observed under aerobic conditions.

Conclusions

The current responses of anaerobic reduced cytochrome *c* reacting at the oxidase modified electrodes show kinetic transitions that are consistent with interconversion between the resting and pulsed states of cytochrome *c* oxidase. The distribution of oxidase between its resting and pulsed states is governed by the concentration of reduced cytochrome *c* that reacts with the immobilized oxidase. Experiments aimed at probing the dependencies of this behavior on the structure of the reduced cytochrome *c* delivering the electrons (e.g., cytochrome *c* from different species) and on the oxygen concentration are underway.

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