Pressure-Induced Effects on Cytochrome Oxidase: The Aerobic Steady State†

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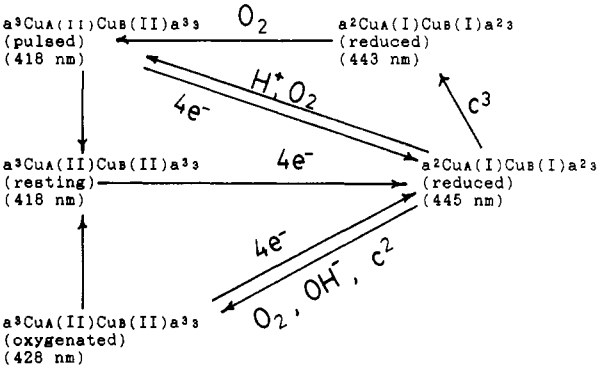
ABSTRACT: If cytochrome *c* oxidase is subjected to pressure during the aerobic steady state, large spectral changes are apparent. These seem to be associated with the inhibition of electron transport within the oxidase. The volume change for the transition is about 80 mL/mol. When the oxidase in the aerobic steady state, with porphyrin cytochrome *c* (the iron-free derivative of cytochrome *c*) bound to it, is subjected to pressure, the porphyrin derivative is released. This results from a change in the dissociation constant of the complex. Whereas the dissociation constant during turnover is about 1.25×10^{-8} M, during pressure-induced inhibition the dissociation constant appears to be about an order of magnitude greater. It appears as though the binding site of the inhibited, partially reduced enzyme more closely resembles that of the fully reduced enzyme than that of the enzyme during the aerobic steady state.

Except under conditions of severe oxygen deprivation, mitochondria normally respire; they are in one of several different aerobic steady states (Chance & Williams, 1955). In terms of the cytochrome *c* oxidase of these mitochondria, this means that it is continuously accepting electrons from the reductant, cytochrome *c*, and passing the electrons to oxygen. There is either fast or slow turnover of the enzyme in response to physiological need; the greater the demand on the system for ATP, the faster is the turnover of the electron-transport proteins (Chance & Williams, 1955). The overall rate of cytochrome oxidase activity is a function of several factors (Wilson et al., 1977; Wilson & Erecinska, 1979): the pH, the steady-state levels of reduced and oxidized cytochrome *c*, the membrane potential, and the ionic strength of the cytosolic compartment. In addition to electron-transport activity, the oxidase also acts as a proton pump (Wikstrom, 1977). The latter function is thought to result from conformational changes arising during the catalytic cycle of the protein [reviewed in Wikstrom et al. (1981)]. In some way, it is thought, the electron-transport function of the protein must be coupled to conformational changes and thereby to proton pumping. One possibility for coupling the multiple regulators, substrates, conformations, and functions is shown in Scheme I. So long as oxygen is present and electrons (4 e^-) are entering the cycle, the oxidase is turning over; it is in the aerobic steady state.

Different pathways are possible; these are associated with different conformations of the protein. The regulators of pH (H^+, OH^-), cytochrome *c*(2+) and cytochrome *c*(3+) determine whether the oxidase follows a pathway involving the protein conformations called "pulsed"[1] and reduced or, alternatively, "oxygenated" and reduced. Both "pulsed" and "oxygenated" forms are completely oxidized conformers of the oxidase. Their absorption maxima occur at 418 and 428 nm, respectively, and they can be identified on this basis or from their visible absorption spectra. The third totally oxidized form of the protein, the resting form, is the thermodynamically most stable form. Both oxygenated and pulsed proteins yield the resting form on standing. Two totally reduced forms absorbing at 445 and 443 nm do not have alternative names. Without doubt, partially oxidized and reduced forms also take part in the catalytic cycle of the oxidase, but these have been omitted for the sake of brevity.

Scheme I: A Catalytic and Conformational Cycle for the Cytochrome Oxidase [Modified from Kornblatt (1980a)][a]



[a] The different conformers of the oxidase are easily recognized. The resting oxidase has its absorption maxima at 418 and 600 nm while the pulsed has maxima at 418 and 605 nm; the oxygenated absorbs at 428 and 603 nm. All three species, resting, pulsed, and oxygenated conformers, are completely oxidized forms of the protein; they are distinguished on the basis of their spectra. The reduced forms are those absorbing at 445 and 443 nm. The nature of the reductants (e^-) and oxidants (O_2) is not critical. *Cytochrome c is the natural electron donor, but it also fulfills the role of conformational effector by interacting with the reduced oxidase.* Under conditions of rapid enzymatic turnover, the reduced oxidase (or the partially reduced oxidases) encounters oxidized *c* prior to itself becoming oxidized; because of the signals it receives from the oxidized cytochrome *c*, the oxidase follows the upper conformational pathway involving the pulsed and reduced forms. During slow turnover the reduced enzyme encounters reduced *c* prior to itself becoming oxidized. In the presence of cytochrome c^2 the oxidase follows a conformational pathway involving the oxygenated and reduced forms. As a consequence of receiving these conformational signals from cytochrome *c* as well as conformational signals arising from pH, the conformational cycle becomes linked to the catalytic cycle.

For some time we have been engaged in determining the effects of pressure on the equilibria existing between the various forms (Kornblatt & Hui Bon Hoa, 1982; Kornblatt

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[1] Different conformations of the cytochrome oxidase are referred to throughout this work. They are the *resting enzyme* (that form which is obtained on isolation of the protein or after long periods of standing), the *pulsed enzyme* (Antonini et al., 1977; Brunori et al., 1979), the *oxygenated enzyme* (Okunuki et al., 1958; Orri & King, 1976; Kumar et al., 1984), and the *reduced, 445 nm, enzyme* and the *reduced, 443 nm, enzyme* (Kornblatt, 1980a).

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et al., 1984; Kornblatt & Luu, 1986). Our goal is to identify the equilibria that involve large conformational changes as well as large volume changes in the hope that these can be correlated with steps in energy transduction in the oxidase. Since cytochrome *c* plays a role in determining steady-state ratios of the conformations during the catalytic cycle (Kornblatt, 1977), we have looked also at the effects of pressure on the cytochrome *c*/cytochrome *c* oxidase interactions. To date, most of our analysis has revolved around the resting oxidase and the oxygenated form. We expand it here to include the reduced and pulsed and integrate the data with those of the resting and oxygenated.

Materials and Methods

The preparation of cytochrome *c* oxidase (Yonetani, 1966; Kornblatt et al., 1973) and porphyrin cytochrome *c* has been completely described (Robinson & Kamen, 1968; Kornblatt et al., 1984).

Absorbance changes and spectra during pressure experiments were recorded on either on Aminco DW-2 or Cary 219 spectrophotometer interfaced to the pressure bomb (Hui Bon Hoa et al., 1982). Fluorescence changes under pressure were monitored with an apparatus constructed in the laboratory (Hui Bon Hoa et al., 1982; Kornblatt et al., 1984).

The stopped-flow experiments under pressure were performed on an apparatus previously described (Heremans et al., 1980, 1982). The design of this apparatus is such that it uses Plexiglass windows with pressure seals; it cannot be operated above 1.5 kbar (1 bar = 0.1 MPA). One syringe contained 43 μM cytochrome *c* plus 5 mM ascorbate. The second syringe contained 6 μM cytochrome *c* oxidase (12 μM heme *a*). The apparatus was assembled and brought to the desired pressure; 5 min were allowed for temperature equilibration prior to starting the measurements. Cytochrome *c* oxidation was followed at 550 nm with a 10-nm bandwidth; the latter necessitated using an experimentally determined extincton coefficient which included corrections for cytochrome oxidase.

The buffer used throughout the experiments was 10 mM [bis(2-hydoxyethyl)amino]tris(hydroxymethyl)methane (Bistris)–1% Tween 80, pH 7. The ionic strength was adjusted with KCl; the reductant concentrations are as reported below.

The reduced oxidase and the aerobic steady state were generated in the same manner. Oxidase was mixed with 0.1 mM TMPD (*N,N,N′,N′*-tetramethyl-1,4-phenylenediamine) plus 1–5 mM ascorbate and allowed to go anaerobic for the reduced experiments. For the aerobic steady state experiments, TMPD and ascorbate were added at the same concentrations, and the enzyme underwent several turnovers before measurements were started; these experiments were terminated prior to the consumption of all the oxygen. All experiments are representative of at least three repetitions, and all reported volume changes were determined by regression analysis; correlation coefficients were better than 0.999.

Results

Effects of Pressure on the Reduced Oxidase and on the Aerobic Steady State. The reduced oxidase showed no change when subjected to pressure (data not shown). This indicates that the molar volume change for the 445 nm/443 nm equilibrium (Kornblatt, 1980a) is either quite small or positive and that the latter conformer cannot be seen by this technique.

When we subjected the oxidase which was in the aerobic steady state (Kornblatt & Luu, 1986) to pressure, we were surprised to find that there were large changes in the oxidase spectrum; representative traces are shown in Figure 1. In the

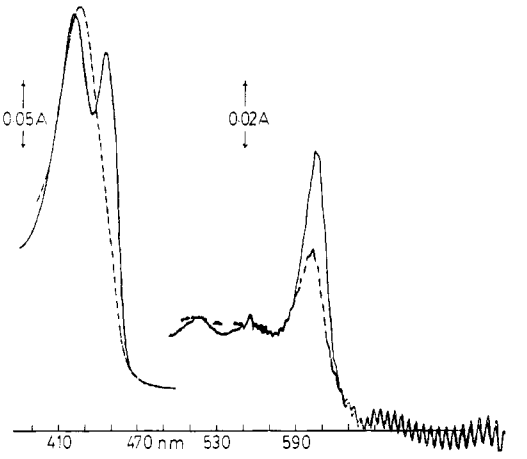


FIGURE 1: Effects of pressure on the aerobic steady state of cytochrome oxidase. Heme *a* (9.3 μM) was treated with 2 mM ascorbate plus 200 μM TMPD at pH 7, 4 °C. The spectrum before pressure, dashed trace, is characteristic of the oxygenated oxidase. The spectrum after application of pressure (2600 bar) is characteristic of the partially reduced oxidase (solid trace). Upon a return to 1 bar, the spectrum reverts immediately to the dashed trace.

figure the dashed trace represents the oxidase at pH 7 and 1 bar during turnover. On the basis of the absorption values we calculate that the extinction coefficient of the α peak is 11.5 mM^{-1} and that of the Soret is 65 mM^{-1}. The peak positions are 602 and 427 nm, respectively. The coefficients and peak positions are characteristic of the "oxygenated" form of the enzyme (Okunuki et al., 1958; Orii & King, 1976). Subjecting the same sample to pressures greater than 1.7 kbar caused the spectrum to completely convert to a form resembling that of the partially reduced enzyme (solid trace). Both the α region and the Soret indicate that a block in electron transfer has been introduced between the two iron centers. It appears from the spectrum that cyt *a* is almost completely reduced while cyt a_3 is completely oxidized. This conclusion is based on the calculated extinction coefficient of the pressurized form in the α region, which is 18 mM^{-1}, and the fact that the Soret peak splits; the peak appearing at 442 nm is weak compared to that remaining at 421 nm. The α region coefficient and the split Soret peak agree reasonably well with Vanneste's (1966) original computation of the *a* and a_3 contribution to the total spectrum.

We have used the extinction coefficients of the oxygenated (Okunuki et al., 1958; Orii & King, 1976) and the partially reduced forms (Vanneste, 1966) to calculate their concentrations during the steady state. The concentrations were then used to calculate "equilibrium" constants (K_{app}) at each pressure.[2] Figure 2 shows that ln K_{app} and pressure are linearly related. The data were fit to

$$\partial(\ln K_{app})/\partial P = -\Delta V/RT$$

ΔV for the process is extremely large, −76 mL/mol.

[2] We realize that the treatment of steady-state concentrations as though they were equilibrium concentrations is not totally justified. Accordingly, our "equilibrium" constants are reported as K_{app}. The application of steady-state data to thermodynamics is treated in Dixon and Webb (1979). The volume changes that we report here are not standard molar volume changes ($\Delta V°$). They are, rather, quantities representing the pressure sensitivity of the reactions

$$(1,2)e^- + a^3a^3_3 \rightarrow a^2a^3_3$$

$$a^2a^3_3 \rightarrow a^3a^2_3$$

$$a^3a^2_3 + O_2 \rightarrow a^3a^3_3 + H_2O$$

The volume changes are valid quantities for the reaction conditions found here but do not relate to standard states.

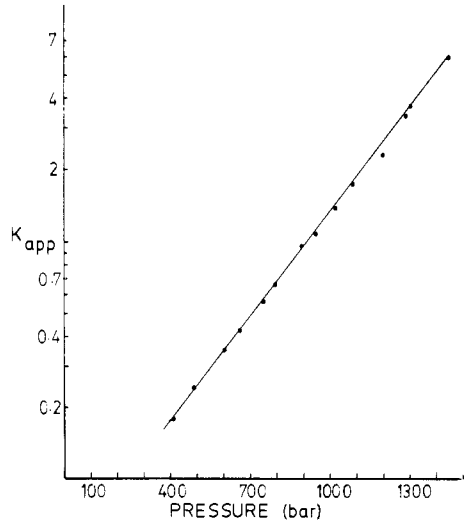


FIGURE 2: Effects of pressure on the aerobic steady state. The relative concentratons of the oxygenated and partially reduced forms were evaluated as indicated under Materials and Methods. ln K (partially reduced/oxygenated) and pressure are linearly related. ΔV evaluates to 76 mL mol^{-1}. The value of K_{app} shown was independent of the direction (high or low) from which the final pressure was attained; the process was completely and immediately reversible.

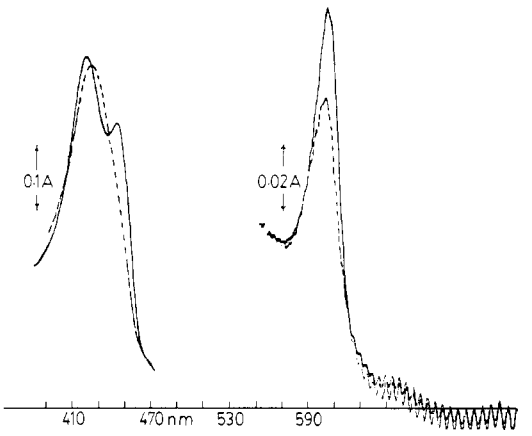


FIGURE 3: Effects of pressure on the pulsed oxidase during the aerobic steady state. Heme a (19 μM) was treated with 2 mM ascorbate plus 200 μM TMPD at pH 6, 5 °C. Prior to pressure the oxidase is pulsed (dashed trace). After pressure is applied (2500 bar) the oxidase is partially reduced (solid trace).

The oxygenated form of the oxidase is found during slow turnover when cytochrome c is not present. During rapid turnover or in the presence of cyt c, the pulsed form of the enzyme is found (Antonini et al., 1977; Brunori et al., 1979). Which of the two forms predominates during the aerobic steady state is also a function of the pH. Low pH favors pulsed; high pH favors oxygenated. In any turnover experiment it is likely that a mixture of forms is present; nonetheless, the overall spectrum is governed by the major (oxygenated or pulsed) species. In Figure 1 we showed that the oxygenated oxidase converted to a partially reduced form on application of pressure. We repeated this experiment using the pulsed enzyme at pH 6.0. The oxidase at pH 6.0 was monitored during the aerobic steady state and then subjected to pressure (Figure 3). Prior to pressure, the extinction coefficient in the α region was 13.8; that in the Soret was 70. Peak positions were at 605 and 421 nm, respectively. These values are characteristic of the pulsed form (Brunori et al., 1979). Subjecting the sample to 2.5 kbar (Figure 3) caused it to convert to a partially reduced form similar to that seen in Figure 1. Once more the α region extinction coefficient was 18, and the Soret peak split. It should be noted that the contributions of the Soret peaks in the pressure experiment of Figure 3 are not the same as those of Figure 1. The difference is due to the change in the reduced Soret peak as the pH changes. We earlier showed that this peak diminishes as the pH is lowered; the pK for the change is about 6.5 (Kornblatt, 1980). In all essential aspects, the results of the two experiments are the same. Pressure appears to introduce a block in electron transfer somewhere between the two iron centers. We have not quantitated the volume change of Figure 3; for technical reasons, the data at high enzyme concentration and pH 6 are difficult to attain. We note however that all spectral changes at this pH are complete by 1.7 kbar; in this respect, pulsed and oxygenated behave identically.

Pressure Induces a Block in Electron Transport Prior to the Cu_B–Cyt a_3 Couple. To this point, we have considered the oxidase only from the point of view of its heme. The functional unit of the protein contains two hemes and two coppers [see Wikstrom et al. (1981) for a review]. Additionally, one magnesium and one zinc (Einarsdottir & Caughey, 1985) as well as a third copper (Steffens et al., 1987; Bombelka et al. 1986) seem to be reproducibly found with the oxidase. While the role of the two hemes (a and a_3) and the first two coppers (Cu_A and Cu_B) are reasonably well-defined, those of the third copper, the magnesium, and the zinc are unknown. Cyt a is considered to the immediate electron acceptor from cyt c(2+) and is considered to be in rapid equilibrium with Cu_A (Antalis & Palmer, 1982); the two are frequently thought of as forming a reactive couple, the cyt a–Cu_A center. Similarly, Cu_B and cyt a_3 are thought of as forming a reactive pair, the Cu_B–cyt a_3 center. Electron transfer from the cyt a–Cu_A center to the Cu_B–cyt a_3 center may be effected in one or two electron steps [reviewed in Hill et al. (1986)]. The reduced Cu_B–cyt a_3 center is the reductant of O_2.

We have used two approaches to locate the pressure-induced block, viz., stopped flow under pressure and quantitation of the 830-nm, Cu_A, band during the aerobic steady state under pressure. In the first approach we used the stopped-flow device to count the number of electrons transferred from cytochrome c to the oxidase when, after the separate solutions were brought to pressure, they were rapidly mixed. A "burst", similar to that seen with chymotrypsin (Hartley & Kilby, 1954), followed by a steady-state consumption of cytochrome c results. Extrapolation of the steady-state rate back to zero time yields the total number of electrons rapidly transfered to the oxidase on the time scale of the experiment. For example, at 1 bar (Figure 4A) all of the available electrons (seven) in cytochrome c are transferred to the oxidase and then to oxygen. At 1200 bar about 1.5 electrons from cytochrome c are transferred to the oxidase (Figure 4B). In a series of four experiments, at pressures greater than 1000 bar, we found an average value of 1.35 ± 0.2.

Ideally, these experiments would have been carried out at pressure greater than 2 kbar where the block in electron transfer was complete. The apparatus did not allow us to work above 1.5 kbar.

In the second approach to determining the position of the block, we monitored the 830-nm, Cu_A, band in the resting enzyme, during the aerobic "steady state" (two turnovers), and after subjecting the latter to 2.5 kbar. The experiment is difficult to carry out properly since the low extinction coefficient [$\Delta\epsilon$ (oxidized – reduced) = 1.2 mM^{-1} cm^{-1} (Griffiths & Wharton, 1961; Muijsers et al., 1971)] and short path length of the pressure cuvette necessitate high concentrations of oxidase. While the Cary spectrophotometer is easily able to resolve differences in absorbance of 0.01, the presence of

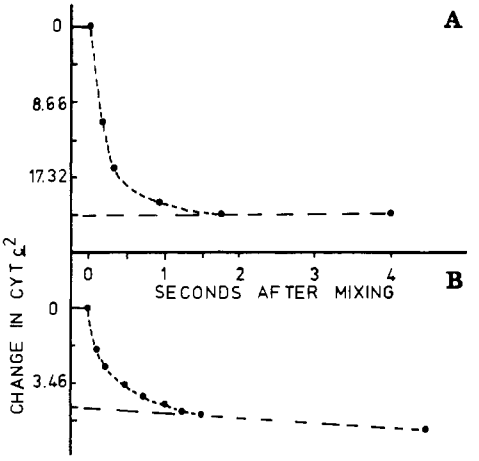


FIGURE 4: Electron transfer from cytochrome c^2 to cytochrome oxidase as a function of pressure. In both (A) and (B) the stopped-flow syringes contained 43 μM c(2+) (syringe 1) and 6 μM aa_3 (syringe 2); the final concentration of reactants in the mixing chamber was half the above values. Oxygen was saturating in both syringes; T = 25 °C. (A) Mixing at 1 bar. There is rapid and complete electron transfer from the c(2+) to aa_3 to O_2. (B) Mixing at 1200 bar. There is rapid transfer of 1.5 electrons from c(2+) to aa_3 followed by slow transfer to O_2. In both (A) and (B) the samples went anaerobic after all the O_2 was consumed. This resulted from the presence of 2.5 mM ascorbate (final concentration) in the chamber. The half-time for ascorbate reduction of the c^3 (22 μM c^3, 2.5 mM ascorbate) was about 2 min. Rereduction of the c, oxidized during the first 4 s, was considered to be negligible.

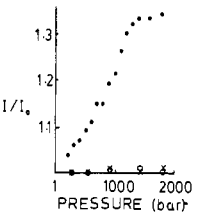


FIGURE 5: Effects of pressure on the binding of porphyrin cytochrome c to the reduced oxidase and to the oxidase in the aerobic steady state. Three experiments are presented; all three were performed at pH 7, 5 °C, 0.1 mM TMPD, and 1 mM ascorbate. The initial conditions were such that association or dissociation could be observed. In the two reduced oxidase experiments, the initial K_d's were 2.7×10^{-7} M (×) and 6.3×10^{-5} M (○) resulting in 9% or 56% free porphyrin. In the aerobic steady state experiment the K_d was 15 nm, and 6% of the porphyrin was free. The concentrations of oxidase and porphyrin were not the same in the two types of experiments. The increase in fluorescence yield (I/I_0) on applying pressure to the aerobic steady state is probably the result of partial release of bound porphyrin cytochrome c as well as lower quenching of the porphyrin that stays bound. See Results.

the pressure bomb introduces interference patterns into the spectrum which are most apparent at wavelengths greater than 700 nm; these patterns decrease the sensitivity of the apparatus such that concentrations above 100 μM oxidase are required. We found that in the presence of an electron source (17 mM ascorbate) at 2.5 kbar the α-band indicated that cyt a was 80% reduced and the 830-nm band showed that 60% of the Cu_A signal was still Cu(II). This indicates that at the time we made our first measurement at 2.5 kbar, 7 min after mixing, about 1.2 electrons were stocked in the inhibited oxidase. This number is far less precise than that determined in the stopped-flow experiment; it could represent either one or two electrons but probably not three or four.

Effects of Pressure on the Interaction between Porphyrin Cytochrome c and the Oxidase: Reduced and Aerobic Steady State. Cytochrome c is the natural electron donor for cytochrome oxidase; cytochrome c itself has no optical signals that monitor only binding to the oxidase. The iron-free derivative of cytochrome c, porphyrin cytochrome c, is fluorescent, and the fluorescence is quenched on binding to the oxidase. Porphyrin cytochrome c, while not a perfect analogue, has been used to model the binding of cytochrome c to the oxidase. The reduced oxidase was mixed with porphyrin cytochrome c under conditions of concentration and ionic strength such that either association or dissociation of the complex could be observed. The state of association was inferred from the fluorescence yield of the porphyrin cytochrome c. Bound porphyrin has a yield of 72% compared to that of the free porphyrin. Subjecting the complex to pressure showed that there was neither association nor dissociation [Figure 5 (×, ○)]. As is the case with the b_5/P-450 couple (Fisher et al., 1986), the resting oxidase (Kornblatt et al., 1984), and cytochrome c peroxidase (Kornblatt et al., 1986), the porphyrin derivative and the reduced protein show a small or no volume change on interacting.

The steeply ascending curve of Figure 5 indicates that the oxidase which at 1 bar was in the aerobic steady state is behaving differently. In this experiment the oxidase and the porphyrin are mixed with TMPD and ascorbate at 1 bar; pressure is applied, and the data are taken prior to the onset of anaerobic conditions; with the exception of oxygen, the conditions of this experiment are similar to those of the low ionic strength curve of the reduced experiment. In the presence of porphyrin cytochrome c the form of the oxidase present at 1 bar is pulsed; this contrasts with the situation in Figure 1 where the oxygenated oxidase was present and corresponds instead to the situation in Figure 3. The concentrations of porphyrin c and oxidase as well as the ionic strength were set such that the initial equilibrium vastly favored the complex over the free components; the initial dissociation constant was 15 nM (Kornblatt & Luu, 1986). Two important facts emerge from the figure. (1) Pressure causes an increase of 32% in the fluorescence of the mixture. (2) There are no additional pressure effects after about 1700 bar.

What is the cause of the increase in fluorescence on pressurizing mixtures of cytochrome oxidase and porphyrin cytochrome c during the aerobic steady state? Are the complexes completely dissociated by pressure? Pulsed oxidase quenches porphyrin cytochrome c fluorescence by 36%. Complete dissociation of the porphyrin from the oxidase should result, therefore, in an increase in I/I_0 from 1.0 at 1 bar to 1.56 at pressure. We find a ratio of only 1.32, and importantly, this is a plateau value; additional pressure has no effect. We conclude that the oxidase and the porphyrin are probably not completely dissociating as a result of the poor fit between the components. Had such been the case, the fluorescence yield would have continued to increase to a value of 1.56 with increasing pressure. Alternatively, is the equilibrium between the porphyrin cytochrome c and the oxidase shifting as a result of conversion to the partially reduced form? Two points argue that this is indeed the case. (1) If we take 1.0 as the fluorescence yield of the completely bound porphyrin and 1.32 as the fluorescence yield of the porphyrin in the final state, we can calculate the relative "equilibrium constant" of the intermediate stages during the pressure experiment. A plot of the logarithm of these relative values versus the pressure yields a value for the volume change associated with the process of −81 mL/mL, a value similar to that found for inhibition of electron transport (Figure 2). (2) The dissociation constant for the reduced oxidase at this ionic strength is 270 nM (Kornblatt & Luu, 1986). At the concentrations of oxidase and porphyrin used in the experiment, this would result in 29% of the porphyrin being unbound, 71% being bound. In

the initial state, prior to pressure (dissociation constant of 15 nM), virtually all the porphyrin is bound. Taking into account the fact that the partially reduced enzyme would probably quench the fluorescence of the bound porphyrin by only 28%, not the 36% (Kornblatt & Luu, 1986) of the pulsed, we calculate that the difference between the original and final states in terms of fluorescence would be a factor of 1.25. This number, involving as it does many assumptions, is quite close to the value of 1.32 that we observe. We consider it likely that the effect of pressure is to act on the steady-state enzyme converting it into the partially reduced form; this species has binding characteristics more closely related to those of the reduced form than it does to those of the pulsed. Dissociation of a portion of the porphyrin cytochrome *c* results from the change in the binding constants of the two forms.

DISCUSSION

In the work presented here, we have shown that cytochrome *c* oxidase—in a complex medium in which electron transfer is occurring at a slow rate—undergoes a large volume change when the system is subjected to pressure. We would like to ascribe a physical significance to this volume change. It would appear as though the large ΔV_{app} is not the result of pressure effects on the detergent or on the reductants. All our studies on the resting oxidase contain the same components (without reductants) as the study carried out here; with the resting oxidase we see only very small volume changes on pressurizing the system. Similarly the reductants are probably not contributing significantly to the volume changes during the aerobic steady state. The studies on the reduced oxidase act as controls for the steady state. In both instances the same reductants are present at almost the same concentrations. Were there a large volume change associated with the equilibria involving ascorbate and TMPD, we might expect to see some effect on the reduced protein. Certainly there may be pressure effects that result in alteration of the driving force of the reductants (Heremans et al., 1982); in the steady state these would either increase electron flow or decrease it but would not cause cytochrome *a* to go completely reduced at 1700 bar while leaving a_3 oxidized. Not only does this latter go oxidized, but it stays oxidized; the mixtures do not go anaerobic.

An additional point that argues against effects on the reductants is the fact that our pressure stopped-flow experiments indicate that cytochrome *c* is competent as an electron donor but will pass only 1.3–1.5 electrons to the pressurized oxidase. Cytochrome *c* is relatively insensitive to pressure. No gross conformational changes occur below 8 kbar (Ogunmula et al., 1977), nor are there differences between the partial specific volumes of the reduced and oxidized cytochrome *c* (Eden et al., 1982). There is a large difference in the compressibilities of the reduced and oxidized forms. Eden et al. have concluded that this difference represents the greater molecular motion of the side chains of the ferricytochrome *c* relative to those of ferrocytochrome *c*. The difference in compressibilities of the ferri- and ferrocytochrome *c* is such that the midpoint potential of the protein would change by about 27 mV at 1 kbar, a rather small change in driving force. The major effect of pressure on the aerobic steady state, we conclude, is on the oxidase, not on the reductants.

Does the large ΔV represent a change in conformation of the oxidase? We feel that this postulate probably accounts for at least a part of the volume change. Our reasons for so thinking are as follows. It is well established that the rate of electron transfer from cytochrome *c* to cytochrome *a* to cytochrome a_3 to O_2 can vary substantially depending on reaction conditions (Peterson & Cox, 1980; Jones et al., 1983; Hill & Greenwood, 1984). Bickar et al. (1986) have shown that the rate of internal electron transfer is a function of the reductant used. The rate is fast when cyt *c* or its redox-competent derivatives are used and slow when other reductants are used. Bickar et al. reason that the rate enhancement is the result of altering the redox potential of the a_3 portion of the oxidase; this occurs on conversion of the resting enzyme to the pulsed.[3] Scholes and Malmstrom (1986) have studied the two-electron transfer in the oxidase and have inferred that partial reduction (a–Cu_A) leads to a large conformational change. Subsequently, Fabian et al. (1987) showed that intramolecular electron transfer from cyt *a* to the Cu_B–a_3 center is rapid only if both Cu_A and cyt *a* are reduced. The addition of one electron to the oxidase functional unit results in its being shared between the cyt *a* and Cu_A (Antalis & Palmer, 1982). Fabian et al. (1987) feel that the two-electron reduction of the oxidase is necessary to trigger the conformational change which results in rapid transfer to the Cu_B–a_3 center.

In a very recent study Copeland et al. (1987) showed that the reduction of Cu_A is accompanied by a large change in intrinsic tryptophan fluorescence. The approximately 50 tryptophans of the oxidase appear to be buried in the resting enzyme and show a composite emission maximum of 328 nm (Hill et al., 1987). On reduction of all the redox centers the emission shifts to 345 nm indicating that the majority of the tryptophans are now in a more polar environment (Copeland et al., 1987). The data suggest that reduction of Cu_A is probably sufficient to effect this change and that it occurs independently of events occurring at cytochrome *a*. Copeland et al. conclude that the change in fluorescence is probably the result of a large conformational change induced by reduction of Cu_A; their conclusions are in agreement with those of Fabian et al. (1987); Alleyne and Wilson (1987) have also found evidence for a conformational change associated with cyt *a* or Cu_A reduction.

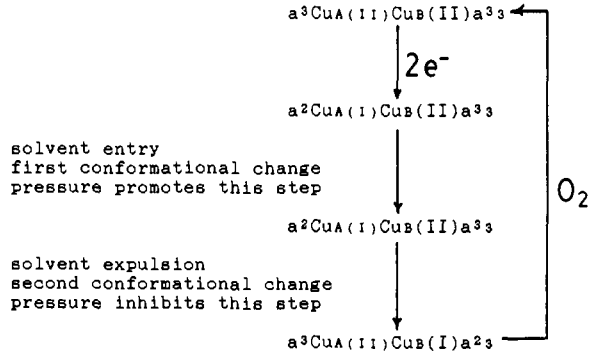
The data we present here complement those of the groups cited above. The most attractive, but not the only, interpretation of the combined data is that that reduction of cyt *a* or both cyt *a* and CuA triggers a conformational change which modulates the redox potential of Cu_A (Fabian et al., 1987; Copeland et al., 1987). The value of the potential, we suggest, is "tunable"; it depends on the average environment of Cu_A. During the initial one- or two-electron reduction, the potential of Cu_A is adjusted via (1) conformational changes and (2) solvent movement.[4]

The fully oxidized oxidase has a partial specific volume greater than that of one of the partially reduced forms. The tryptophans of the fully oxidized protein are shielded from solvent (Hill et al., 1986). The volume of the protein/solvent on reduction of cyt *a* or a–Cu_A is smaller than that of both the fully oxidized protein and the protein after electron transfer to the Cu_B–a_3 center. In this globally smaller form, solvent has access to the interior of the protein; this is reflected in the altered fluorescence of the tryptophans (Copeland et al., 1987). The volume difference between the partially reduced protein $a^2Cu_A^{I}Cu_B^{II}a^3{}_3$ with its exposed tryptophans and the intermediate $a^3Cu_A^{II}Cu_B^{I}a^2{}_3$ is about 80 mL mol^{-1} and possibly reflects solvent that would be expelled in the absence of pressure. Solvent expulsion from the ordered environment of

[3] Our results on the formation of the pulsed enzyme are not completely consistant with those of Bickar et al. In our hands, porphyrin cytochrome *c* promotes the transition to the pulsed enzyme (Kornblatt & Luu, 1986) whereas they find that the metal-free derivative does not promote the conversion.

[4] The term solvent is used here to signify water, detergent, and the salts that contribute to the buffer.

Scheme II: Electron Transfer, Conformational Changes, and Solvent Movement Associated with the Oxidation and Reduction of the Cytochrome a–Cu_A Couple[a]



[a] The scheme is based on, and attempts to account for, the following: (1) In any cycle in which a conformational change is postulated, at least one more additional conformational change must occur in the cycle in order to restore the original form of the molecule. (2) A two-electron reduction of the fully oxidized oxidase ($a^3Cu_A^{II}$ to $a^2Cu_A^{I}$) is necessary to "activate" electron transfer to the $Cu_B^{II}a^3_3$ (Fabian et al., 1987). This activation step is associated with a conformational change from the original form of the fully oxidized enzyme. Activation here would be associated with the first conformational change shown. (3) Reduction of Cu_A is associated with changed fluorescence of the majority of the oxidase's tryptophans (Copelane et al., 1987). Following reduction of the Cu_A the tryptophans appear to be in a more polar environment then they previously experienced. This could result from a global shift in the peptide backbone of the oxidase or a large-scale entry of solvent into a hydrophobic crevice or both. Pressure would promote formation of this complex with its ordered solvent. (4) The overall conformation of the oxidase, as monitored by tryptophan fluorescence, must be restored on electron transfer from the a–Cu_A center to the Cu–a_3 center. This statement is based on Copeland's Table I which shows that all species containing $a^2Cu_A(I)$ have the tryptophans in the exposed or polar condition whereas species of the type $a^3Cu_A^{II}Cu_B^{I}a^2_3$ (the a–Cu_A center oxidized, the Cu_B–a_3 center reduced) have the tryptophans once again in a nonpolar environment. Accordingly, pressure would act to prevent solvent transfer from the ordered to the disordered environment and thereby prevent electron transfer from the reduced a–Cu_A to the oxidized Cu_B–a_3. Pressure would be modulating the Cu_A driving force. (5) Subsequent steps, electron transfer to oxygen and restoration of the original oxidation state of the oxidase, do not appear to be associated with large conformational changes; they have at least not been detected. (6) The question must be asked, Why propose what appears to be a futile cycle in which solvent moves first in one direction and then in the other? It is currently felt by Wikstrom, Malmstom, Chan, and their respective coworkers (Wikstrom & Casey, 1985; Fabian et al., 1987; Copeland et al., 1987) that the primary step in energy transduction, proton pumping by the oxidase, is associated with electron transfer to and from the Cu_A center or perhaps the a–Cu_A center. The futile cycle proposed here could of course be nothing other than just that, a futile cycle; such things are known in biochemistry. It may as well be another manifestation of the proton pump in which binding and release of a proton by the oxidase during the catalytic cycle is associated with solvent movement.

the pocket into the bulk solution should be accompanied by a large positive change in volume (Schneider, 1972; Pande & Wishnia, 1986). Pressure acts to prevent this change from occurring. This proposal is summarized in Scheme II.

Transfer of metal ions from the protein interior to the solvent could also account for all or a portion of the volume change. The oxidase contains sequestered metals for which no role has been assigned. One Zn, Mg, and Cu have been found per functional unit (Bombelka et al., 1986; Einarsdottir & Caughey, 1985; Steffens et al., 1987) in addition to the two heme iron and two functional copper. If, on partial reduction, the sequestered metals were less tightly bound, pressure could drive them into the bulk solution; ΔV for the electrostriction of the Zn and Mg would be about 36 and 32 mL/mol, respectively (Hovey & Tremaine, 1985). Were this to be occurring, it would imply that the metals were required, in some undefined way, for subsequent reduction of the oxidase.

It seems probable, if one considers the data from the Goteburg group, the data from the Caltech group, and that presented here, that a conformational change is a likely result of cytochrome a or cyt a–Cu_A reduction. We have invoked solvent movement or metal ion electrostriction as a consequence of the conformational change because the size of ΔV is quite large, too large, we feel, to be the result of the movement of protein from one region in space to another. Solvent modulation of the redox potential of Cu_A is attractive (Moore et al., 1986).

The idea that solvent movements might be responsible for modulation of activity, spin state, redox potential, and other functions is not novel (Brown et al., 1983; Findsen et al., 1986). It is implied in the studies of Frauenfelder, Petsko, and Karplus (Frauenfelder et al., 1987; Gekko & Hasegawa, 1986) and has recently been explicitly defined by Douzou (1987). In the most well-studied case, solvent movement is probably responsible for at least a portion of the volume changes shown by cytochrome P-450 (CAM).

The latter has been subjected to pressure; volume changes of −30 mL/mol have been found for the high-spin/low-spin equilibrium (Hui Bon Hoa & Marden, 1982). These have been shown to be associated with extrusion of camphor from the heme pocket (Fisher et al., 1985). The crystal structures of the P-450 with (Poulos et al., 1985) and without (Poulos et al., 1986) camphor have recently been published; the data are extremely clear. With the exception of a short excursion by Phe-87 in the heme pocket, the entry of six to seven water molecules, and increased thermal motion of three sections of the protein, there are no major differences in the structure of the protein with and without camphor. It would seem, therefore, that solvent movement into the heme pocket combined with the extrusion of camphor account for the majority of the volume change associated with spin equilibrium. It may, of course, turn out that differences in solution characteristics of the P-450 do not find counterparts in the crystal structure; this seems to be the case in cytochrome c where there is ample evidence that the reduced and oxidized proteins are different in solution but no significant differences can be detected in their crystal structures (Salemme, 1977).

Until we have further, independent evidence, we prefer to exercise caution with the oxidase; the large ΔV that we find can be the result of a large conformational change, but it could also be the result of the movement of five to ten solvent molecules from a disordered to an ordered environment.

The dissociation of the porphyrin cytochrome c/pulsed oxidase complex that results from pressurizing the aerobic steady state is a consequence of the difference in dissociation constants of the pulsed form (very tight binding) and partially reduced form (weaker binding). The change however does not occur because the binding site is grossly deformed; ΔV for the change is virtually the same as that for the conversion of the aerobic steady state to the partially reduced, i.e., $\Delta\Delta V$ of binding is approximately zero. This implies that dissociation occurs because of cytochrome a or cyt a–Cu_A reduction and is communicated to the binding surface via subtle changes in the protein. Decreased binding affinity cannot be the result of simple electrostatic effects since the oxidase, on partial reduction, becomes more negative and this should support tighter binding of porphyrin cytochrome c.

What is quite clear is that Scheme I, presented at the beginning of this paper, no longer adequately describes our system. Between the pulsed/reduced pair and the oxygen-

ated/reduced pair must be introduced partially reduced species, at least two conformations for both the upper and lower pathways. Both sets of partially reduced conformers differ from the pulsed or oxygenated species by about the same amount. We conclude, therefore, that the pulsed and oxygenated conformers do not differ substantially in molar volume.

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