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# Volume Changes Associated with Cytochrome *c* Oxidase–Porphyrin Cytochrome *c* Equilibrium†

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ABSTRACT: The binding of a fluorescent derivative of cytochrome *c* to cytochrome *c* oxidase has been studied by use of pressure to perturb the equilibrium. $\Delta V^\circ$ for the reaction

$$\text{oxidase–porphyrin cytochrome } c \overset{K_d}{\rightleftharpoons} \text{oxidase} + \text{porphyrin cytochrome } c$$

was small and favored dissociation of the complex. Pressure-induced dissociation is to be expected if the major forces governing the equilibrium are electrostatic in nature. The dependence of log $K_d$ on pressure is not linear but biphasic; high pressures lead to a decrease in $K_d$ and association of the reactants. The latter fact indicates that the net compressibility of the complexes is greater than that of the free reactants, an unexpected result.

Cytochrome *c* oxidase (EC 1.9.3.1) catalyzes electron transfer from cytochrome *c* to oxygen. The oxidase is a mitochondrial protein, the role of which is to couple electron transport to the production of ATP. This is accomplished through the intermediate establishment of an electrochemical gradient across the mitochondrial membrane. There appears little doubt that the gradient is set up, at least in part, by the active pumping by the oxidase of protons from the mitochondrial matrix space to the cytosolic space (Wikstrom, 1977). The oxidase is an intrinsic membrane protein; it is large ($M_r$ 200000) and contains at least seven subunits, two hemes, and two coppers. The structure of the oxidase has been admirably reviewed recently by Capaldi et al. (1983).

The interaction of cytochrome *c* oxidase with cytochrome *c* is similar to the interactions that cytochrome *c* shows with its other protein acceptors. A summary of the available information indicates that the interacting face of cytochrome *c* viewed from any of its acceptor molecules (cytochrome *c* reductase, mammalian sulfite oxidase, and cytochrome *c* peroxidase, as well as cytochrome *c* oxidase) consists of a positively charged surface with the center of the charge located near phenylalanine-82; Koppenol & Margoliash (1982) have postulated that the driving force for the orientation of the cytochrome *c* with respect to its partners is the large dipole moment that is associated with the sum total of positive and negative charge on the cytochrome *c*. The acceptor molecules have been postulated to have a complementary surface as part of their interaction domains. In support of this statement, all of the acceptor molecules show similar ionic strength dependencies (Wainio et al., 1960; Davies et al., 1964; Nicholls, 1964), similar interactions with modified cytochromes *c* or the cytochromes *c* of different species (Errede & Kamen, 1978; Davis et al., 1972; Brautigan et al., 1978; Ferguson-Miller et al., 1978; Smith et al., 1980, 1981), similar reaction rate constants (Errede & Kamen, 1978; Smith et al., 1981), and similar tendencies to form tight binding complexes (Yonetani & Ray, 1965; Nicholls, 1964; Mochan & Nicholls, 1972; Yu et al., 1975; Ferguson-Miller et al., 1976). In the case of the peroxidase, where a high-resolution three-dimensional structure is known, a negatively charged surface (Poulos & Kraut, 1980), complimentary to the positively charged surface of the cytochrone *c* (Swanson et al., 1977), has been located. In the case of the oxidase, it is likely that some of the negative charge on the oxidase comes from the cardiolipin that is normally associated with the protein (Erecinska et al., 1980).

One consequence of the binding of cytochrome *c* by the oxidase is that spaces between them are eliminated:

$$n\text{H}_2\text{O} + \text{enzyme–cytochrome } c \rightleftharpoons \text{enzyme–}p\text{H}_2\text{O} + \text{cytochrome } c\text{–}m\text{H}_2\text{O} \quad (1)$$

The total volume occupied by the reactants[1] of eq 1 may be greater or less than that of the products; $\Delta V^\circ$ for the reaction may be either positive or negative but is unlikely to be zero. As such, the equilibrium of (1) can be perturbed by pressure. From a study of the pressure dependence, one can deduce how

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[1] Reactants or products, as used here, must be taken to mean the sum of all the interacting species on either side of eq 1. This includes interactions of the oxidase and porphyrin cytochrome *c* with solvent, detergent, and other components of the buffer.

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well the reactants fit one another, whether reactants and products are particularly deformable, and whether, on interaction, other compensatory changes occur in the noninterface region.

The discussion that follows deals with the effects of pressure on the equilibrium of eq 1. The binding is monitored by energy-transfer quenching where the acceptor molecule is the oxidase and the donor molecule is the fluorescent derivative of cytochrome *c*, porphyrin *c*.[2]

## Materials and Methods

Cytochrome *c* oxidase was prepared by the modification of the Yonetani (1966) procedure routinely used in this laboratory (Kornblatt et al., 1973). Following column chromatography, the enzyme was precipitated with 29% saturated ammonium sulfate (the oxidase precipitates, but the vast majority of the Tween 80 stays in solution until 33%). The pelleted oxidase was taken up in a small volume of buffer and dialyzed extensively against 10 mM Bistris, 1 mM EDTA, and 1% Tween 80, pH 7. The spectral properties of this preparation did not differ from those of the original; solutions were optically clear and contained no detectable *b* and *c* type cytochromes. The aggregation state of the oxidase did differ under conditions of high and low salt; this will be dealt with under Results.

Porphyrin *c* was prepared from horse heart cytochrome *c* (Sigma type III) essentially as described by Robinson & Kamen (1968). The characteristics of the preparation are described under Results.

The buffer used throughout the study was 10 mM Bistris, 1 mM EDTA, and 1% Tween 80, pH 7.0. Bistris was chosen as the buffer since its p$K$ does not change appreciably with pressure (J. A. Kornblatt, unpublished results). KCl was added, in varying amounts, to the buffer; in all cases, this caused an increase in pH but never by more than 0.2 pH unit; we made no attempt to correct for this small change.

The pressure apparatus used in the study has been described (Hui Bon Hoa et al., 1982). Pressure was developed and transmitted by using pentane in the press and in the bomb. For absorbance measurements under pressure, the apparatus was interfaced to a Cary 219 spectrophotometer. For fluorescence measurements, the apparatus was interfaced to a fluorometer assembled in the laboratory. In essence, the fluorometer consisted of a xenon light source, a Balzer no. 1 filter (maximum transmission at about 400 nm), the bomb containing the sample, a Balzer no. 6 emission filter (maximum transmission at about 620 nm), and two photomultipliers (PM). The first PM was used to monitor lamp output; the second, red sensitive, to monitor the output form the sample. The bomb and sample were maintained at 16 °C throughout the experiments.

Fluorescence spectra and polarization of fluorescence were obtained either on a Perkin-Elmer MPF 44B fluorometer or on a SLM 4800 equipped with a SMC 210 monochrometer. Fluorescent lifetimes by the phase-modulation technique were determined on the latter instrument.

Dissociation constants at 1 bar were evaluated by ultrafiltration of oxidase–porphyrin *c* mixtures through Amicon PM30 membranes (procedure from Amicon Publication No. 459); free porphyrin *c* passes through the membrane while the oxidase and the oxidase–porphyrin *c* complex do not. One milliliter of filtrate was collected from a total volume of 10 mL in the diaflow cell. Since the equilibrium between oxidase and porphyrin *c* is dynamic and since removal of 1 mL of solution results in concentrating the solutes that do not pass the filter, the act of measurement introduces an error into the determination of $K_d$. We estimate this error to be a maximum of ±10%. From the value of $K_d$ at 1 bar, we related fluorescence intensities to the concentrations of free and bound porphyrin *c*, at higher pressures. Concentrations were corrected for the change in the density of water at different pressures (Grindley & Lind, 1971).

$\Delta V°$ and compressibilities ($\beta$) were evaluated from eq 2

$$\frac{\partial(\ln K_d)}{\partial P} = \frac{\Delta V° + \beta V_p P}{RT} \quad (2)$$

(Gekko & Noguchi, 1979; Paladini & Weber, 1981) by computer fitting to the integrated form

$$\ln K = \frac{\Delta V° P}{RT} + \frac{\beta V_p P^2}{2RT} \quad (3)$$

where $\Delta V°$ is the standard molar volume change at 1 bar, $\beta$ is the average compressibility of the system, and $V_p$ is the average volume of the reactants and products.

## Results

*Properties of Porphyrin c.* Porphyrin *c* used in this study was prepared as described by Robinson & Kamen (1968) as modified by Vanderkooi & Erecinska (1975). The derivative is prepared by exposing the cytochrome *c* to anhydrous HF. If the sample absorbs water at this time, denaturation can occur. The porphyrin *c* used here was judged by the following criteria to be similar to that used in other studies: (1) The absorption and emission spectra of the derivative were identical with published spectra. There was no trace of either native cytochrome *c* nor of low molecular weight degradation products of the porphyrin or the protein in the preparation. (2) At least 80% of the porphyrin *c* comigrated with cytochrome *c* on Sephadex G-75 chromatography. The protein absorbance of the derivative comigrated with the porphyrin absorbance. A small amount (less than 20%) of the preparation migrated with a higher molecular weight than the cytochrome *c* but was still associated with porphyrin. (3) The molecular size of the derivative was judged by polarization of fluorescence to be approximately the same as that of preparations used in other work. Our experimentally determined polarization values measured at 620 nm (excitation 505 nm) were 0.14 in buffer and 0.30 in 90% glycerol; the lifetime of the probe was estimated by the phase-modulation technique to be 8.5–12 ns. Use of the Perrin equation leads to rotational correlation times of 7.7–11 ns. The latter values would correspond to a perfect sphere, nonhydrated, with a $M_r$ of 17 000–25 000 (Cantor & Schimmel, 1980). The polarization values that we have obtained are the same as those found by Dixit et al. (1982); our lifetime values are higher than the values reported in Vanderkooi et al. (1976) but lower than those reported in their later work (Dixit et al. (1982).

The effects of pressure on the absorption spectrum of the porphyrin *c* are shown in Figure 1. Pressure, up to 4 kbar, has no effect on the absorbance. While the acid and alkaline transitions of the cytochrome *c* are large (Ogunmola et al., 1977), on the order of 50 mL mol$^{-1}$, the native protein shows little or no change in spectral properties at pressures up to 8 kbar (Ogunmola et al., 1977).

In the Tween-containing buffer used here, pressure had no effect on porphyrin *c* fluorescence intensities at pressures up to 4 kbar. When Tween is omitted from the buffer, there is as much as 20% quenching at pressures between 1 and 3 kbar.

[2] Abbreviations: porphyrin *c*, porphyrin cytochrome *c*; Bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid.

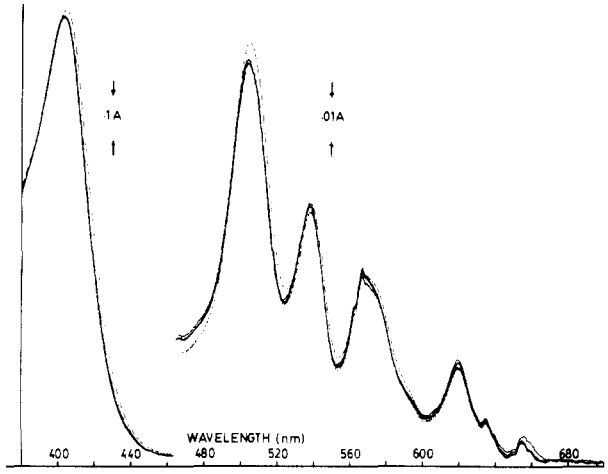


FIGURE 1: Pressure has little effect on the absorption spectrum of porphyrin *c*. Porphyrin *c*, 20 μM in Bistris, EDTA, and Tween 80 buffer, pH 7.0, containing 50 mM KCl, was subjected to pressures between 1 bar and 1.5 kbar in 500-bar steps. The spectra show no significant differences at the different pressures. Lack of coincidence in the spectra is the result of solvent compression as the pressure was increased. Porphyrin *c*, in the same buffer, was also treated with pressures to 4 kbar (spectra not shown), and the spectra, after correction for solvent compression, did not differ from the 1-bar spectrum. The spectrum at pressures of 2–4 kbar was stable for at least 20 h.

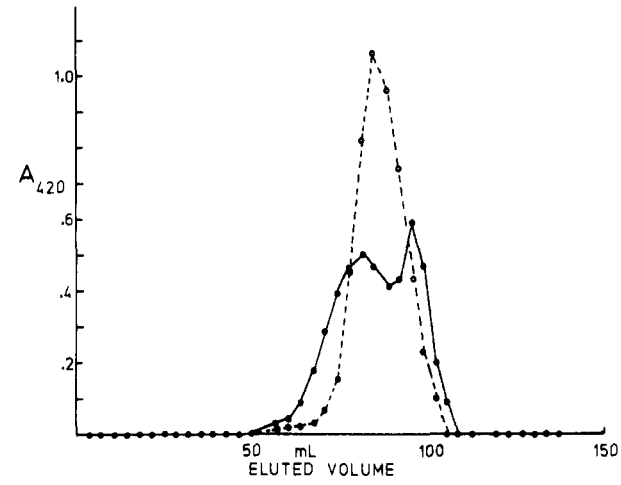


FIGURE 2: Aggregation state of cytochrome oxidase probably changes as a function of KCl concentration. Samples of cytochrome oxidase (120 nmol) were dialyzed against either Bistris, EDTA, and Tween 80 buffer, pH 7.0, or the same buffer containing 50 mM KCl. The samples were applied to a 135-mL Sepharose CL-6B column that had been equilibrated against the same buffer, and the eluate was monitored for cytochrome oxidase. (Open circles, dashed line) Buffer with 50 mM KCl; (closed circles, solid line) no added KCl.

Table I: Dissociation Constants of Porphyrin *c*–Cytochrome *c* Oxidase Equilibrium as a Function of KCl Concentration[a]

| [KCl] (mM) | $K_d$ (×10⁶) | [KCl] (mM) | $K_d$ (×10⁶) |
|---|---|---|---|
| 0 | 0.13 | 40 | 2.06 |
| 10 | 0.24 | 50 | 4.06 |
| 20 | 0.57 | 60 | 6.41 |
| 30 | 1.14 | 80 | 9.54 |

[a] A total of 10 mL of Bistris, EDTA, and Tween 80 buffer, pH 7.0, containing 10 μM cytochrome *c* oxidase and 8 μM porphyrin *c* was filtered through an Amicon PM30 membrane. One milliliter of filtrate was collected and the porphyrin *c* concentration determined. The filtrate was recombined with the oxidase–porphyrin *c* solution and the total solution adjusted to contain 10 mM KCl. The filtration and determination of porphyrin *c* were repeated; the combined solution was subsequently adjusted to the KCl concentrations shown in the table. After each addition of KCl, the filtration and porphyrin *c* determination were repeated. The temperature was maintained at 16 °C throughout the procedure.

The salts used in the experiments had no effect on the emission either at 1 bar or at higher pressure.

The fluorescence of the porphyrin *c* solutions was proportional to concentration at concentrations up to 10 μM. Above this value we began to see a diminution in fluorescence.

*Properties of Cytochrome c Oxidase.* The behavior of the oxidase under pressure has been described (Kornblatt & Hui Bon Hoa, 1982). The protein undergoes a reversible high-spin/low-spin transition when subjected to pressure, the $\Delta V^\circ$ of which varies between 4 and 8 mL mol$^{-1}$ depending on the temperature. Solutions of the oxidase showed no fluorescence under any of the conditions used here.

Salt was used to adjust the porphyrin *c*–oxidase dissociation constant. It has recently been claimed that KCl effects the aggregation state of the oxidase in lauryl maltoside solutions but not in Triton X100 solutions (Nalecz et al., 1983). We report here that the aggregation state of the oxidase in Tween-containing buffers is also effected by KCl. Figure 2 shows the elution profiles of the oxidase from Sepharose CL-6B columns; the conditions are that of 50 mM KCl and no added KCl. In the first case, the protein elutes as a single, symmetrical peak, which is consistent with previous studies (Kornblatt et al., 1975; Love et al., 1970; Cabral & Love, 1972). In the low-salt condition the oxidase has clearly split into two components, both of which have oxidase properties. The elution profiles, which are exceptionally repeatable, are puzzling. They are not consistent with a simple monomer–dimer interaction since the leading peak in the low-salt case elutes before the peak in the high-salt case; close inspection of the profiles in the Nalecz et al. (1983) paper show that they witnessed a similar phenomenon. The low-salt profile is also not consistent with a rapid equilibrium between forms since we would then expect to see a single peak, perhaps somewhat broader than the high-salt peak, that eluted later than the high-salt peak. We currently have no explanation for the splitting of the two peaks; it may very well be that Nalecz and co-workers are correct in their interpretation, which has been supported by their kinetic data, but that will have to be established by methods other than sieving chromatography, which itself is very sensitive to salt effects. Regardless of the true nature of the low salt induced retardation of the oxidase on the columns, we do not think that it plays a role in the pressure-induced binding phenomena that we will describe.

*Properties of Porphyrin c–Oxidase Interaction. (i) Quenching of Porphyrin c Fluorescence by Cytochrome c Oxidase.* The oxidase and cytochrome *c* have repeatedly been shown to form tight binding complexes at low ionic strength. The stoichiometry of the complexes has not been agreed upon; it would appear that all oxidase preparations contain at least one high-affinity site for cytochrome *c* per two hemes; the dissociation constant for this high-affinity site varies from between 10 and 100 nM at very low ionic strength to between 10 and 100 μM at high ionic strengths (Nicholls, 1964; Kimelberg & Nicholls, 1969; Errede et al., 1976; Errede & Kamen, 1978; Ferguson-Miller et al., 1976, 1978; Smith et al., 1979; Osheroff et al., 1980). It has also been repeatedly pointed out that the oxidase can have a low-affinity site for cytochrome *c* in which the dissociation constants are at least an order of magnitude greater than those of the high-affinity sites. In Figure 3 (closed circles) we show that the fluorescence of porphyrin *c*, in the presence of the oxidase, is quenched at low ionic strength and that the quenching is relieved as the ionic strength is raised, i.e., as the dissociation constant is raised.

In order to relate fluorescence to the dissociation constant of eq 1, we measured the extent of porphyrin *c* binding at

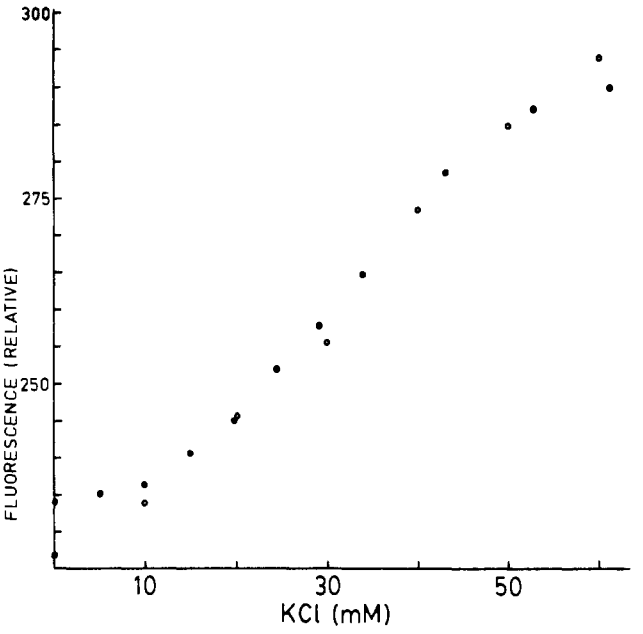


FIGURE 3: Fluorescence of porphyrin *c* is quenched on binding to cytrochrome oxidase. A 2.8 $\mu$M cytochrome oxidase aliquot in Bistris, EDTA, and Tween 80 buffer, pH 7.0, was titrated with KCl. The sample, in the bomb, was maintained at 16 °C. Closed circles represent the data points taken during the titration. Open circles represent the values that would have been obtained by assuming the dissociation coefficients of Table I and the fluorescence coefficients described in the text.

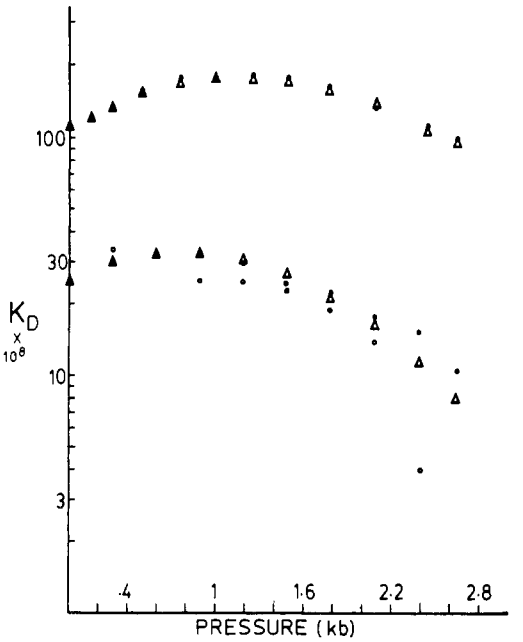


FIGURE 4: Effects of pressure on the porphyrin *c*–cytochrome oxidase equilibrium. Cytochrome oxidase–porphyrin *c* mixtures were placed in the bomb and subjected to pressure. The values shown (circles) were averages of ascending and descending cycles. All values were dependent only on the pressure at which the measurement was made and not on whether it was approached from a higher or lower pressure. (Upper curve) 2.6 $\mu$M porphyrin *c*–2.6 $\mu$M cytochrome oxidase in Bistris, EDTA, Tween 80, pH 7.0, and 30 mM KCl. (Lower curve) (Closed circles) 2.6 $\mu$M cytochrome oxidase–2.6 $\mu$M porphyrin *c*; (open circles) 2.6 $\mu$M cytochrome oxidase–1.3 $\mu$M porphyrin *c* in Bistris, EDTA, and Tween 80 with no added KCl. (Both curves, triangles) Data fit to eq 3.

several ionic strengths and calculated the resulting constants on the basis of the stoichiometry of eq 1. Table I shows how the dissociation constant varies as a function of KCl concentration.[3]

The relative fluorescence of any given mixture in Figure 3 is the result of the fluorescence of the free and bound porphyrin *c*. We have used the $K_d$'s at 20 mM and 40 mM to calculate the concentrations of free and bound porphyrin *c* in these two solutions; using these concentrations and a pair of simultaneous equations

$$F(\text{free})[\text{por } c] + F(\text{bound})[aa_3\text{–por } c] = \text{relative fluorescence} \quad (4)$$

we calculated the "fluorescence coefficients", $F$(free) and $F$(bound), for the experiment. The values obtained are 116 $\mu M^{-1}$ for the free porphyrin and 71.7 $\mu M^{-1}$ for the bound porphyrin. The numerical values of the two coefficients are valid only for the experiment of Figure 3, but the ratio of the two coefficients has general validity.

With the above fluorescence coefficients and the concentrations of free and bound porphyrin *c* at each of the different KCl concentrations of Table I, we calculated the anticipated fluorescence at each KCl concentration; the anticipated values are plotted in Figure 3 as open circles. The agreement between the expected and found values is reasonably good except at the two extremes where the values differ by 3% at the low KCl side and 1.3% at the 60 mM side.

*(ii) Effects of Pressure on Oxidase–Porphyrin c Equilibrium.* Oxidase and porphyrin *c* were mixed either in the absence of KCl or in the presence of 30 mM KCl; the dissociation constants at 1 bar for each salt concentration are listed in Table I. The resulting mixtures were submitted to pressures between 1 bar and 3 kbar. The fluorescence of the solutions as a function of pressure was biphasic and reversible; originally, the fluorescence increased with increasing pressure, but further increases in pressure resulted in a decrease in fluorescence. The fluorescence intensity at each pressure was used to calculate the displacement from the original equilibrium and thereby the new dissociation constant. The results of the plots of $K_d$ vs. pressure are shown in Figure 4. The biphasic behavior of the fluorescence is reflected in the biphasic behavior of $K_d$.

The upper curve shows data from a representative experiment at 30 mM KCl. The closed circles represent the experimental points. The lower curve represents data taken from two different experiments in which there was no KCl present. The two experiments were run 1 month apart; one (open circles) contained twice as much porphyrin *c* as oxidase; the other (closed circles), equivalent concentrations.

The experimental points were fit to eq 3 and plotted on Figure 4 as open triangles. The simulated data of the upper

[3] The data were fit to the limiting form of the Debye–Hückel equation, $\log K_a = \log (K_a + 1.02 Z_c Z_o \mu^{1/2})$, where $K_a$ is the association constant at zero ionic strength, $Z_c$ and $Z_o$ are the interaction charges on porphyrin *c* and the oxidase, and $\mu$ is the ionic strength. $K_d$ ($1/K_a$) was calculated to be 25 nM; the slope is equal to –9.3, and the correlation coefficient for the fit was –0.99. Under the solution conditions, zero KCl, $\mu$ = 5 mM. The results are in reasonable agreement with those of Veerman et al. (1983). Since the plot of $\log K_a$ vs. $\mu^{1/2}$ is a straight line, we infer that the vast majority of the porphyrin *c* was available for binding to the oxidase and that it all bound with a single affinity constant at any given ionic strength. This conclusion is reinforced by data to be presented in Figure 4, where it is clear that the same results are obtained at different ratios of porphyrin *c* to oxidase. Were we to have a substantial fraction of porphyrin *c* that was binding to the oxidase with a different affinity constant than the bulk of the prophyrin *c* or were we to have two (or more) populations of oxidase, each binding with different affinities, we would expect curvature in the Debye–Hückel plot and poor agreement between samples that had different ratios of oxidase and porphyrin *c*. Since we do not find such deviations, we conclude that the majority of the oxidase and porphyrin *c* behaves as though each was homogeneous.

curve could be fit to a maximum difference between the found and the anticipated values of 6%. In the case of the lower curve, the maximum difference was 19%. Changing either $\Delta V^\circ$ or $\beta V_p$ by 10% led to unacceptable fits in both cases; the differences between the found and simulated values climbed to above 20%; more important, systematic discrepancies were introduced. The values that we have obtained for the two experiments are 18 mL mol$^{-1}$ and −0.015 mL mol$^{-1}$ bar$^{-1}$ for $\Delta V^\circ$ and $\beta V_p$ for the upper curve, and 16 mL mol$^{-1}$ and −0.02 mL mol$^{-1}$ bar$^{-1}$ for the lower curve.

## Discussion

Much of the work reported here has been devoted to the controls required for the interpretation of the data on the pressure sensitivity of the porphyrin *c*–oxidase equilibrium. Even with the controls, there are several assumptions that underlie the conclusions, assumptions that have not been stated.

First is that the porphyrin *c*–cytochrome oxidase system is a good representation of the cytochrome *c*–cytochrome oxidase system. The dissociation constants that we have measured for the porphyrin derivative are approximately the same as those found for the native cytochrome *c*. $K_d$ determined for both prophyrin *c* and cytochrome *c* was 25 ± 5 nm;[3] both compounds showed the same variation of $K_d$ with salt. Moreover, Veerman et al. (1982) have shown that the porphyrin derivative, reduced by hydrated electrons, is capable of transferring electrons to the oxidase with a second-order rate constant about the same as that of cytochrome *c*. On the basis of these two facts we feel that the model of the porphyrin binding in the same manner as cytochrome *c* is at least valid to a first approximation.

Second among the assumptions is that the relative geometry of the closer heme and porphyrin does not change during the transition from low pressure to high pressure. Geometry here is taken to mean (1) orientation and (2) separation. Were the orientation to change, the $k^2$ term in the Forster (1951) equation would change; since this term is ultimately expressed as the sixth root, it should have almost no effect on our energy-transfer quenching. If the separation between the heme and the porphyrin were to change there would, of course, be a change in the efficiency of energy transfer from the porphyrin to the heme. For this altered efficiency to have an effect of more than a few percent on our derived fluorescence coefficients and the $K_d$'s that we calculate from them, the separation between the heme and porphyrin would have to change by more than 0.1 nm. As we show when we discuss the significance of $\beta V_p$, this is possible though unlikely.

The third assumption we have made is that the volume term $\Delta V^\circ$ represents the volume change associated with the equilibrium between chromophore and fluorophore and that it does not include a contribution from the volume changes associated with the spin changes/conformation changes of the oxidase. We know that the oxidase undergoes changes in conformation with increasing pressure but have not been able to determine, because the absorption bands overlap, whether the porphyrin *c*–oxidase combinations also undergo the same conformational changes when subjected to pressure. If they also occurred at pressure, the net effect would be to decrease the absolute value of $\Delta V^\circ$.

Our final assumption deals with the phenomenological constant $\beta V_p$. Since the plots of ln $K_d$ vs. pressure are clearly not linear, we have to incorporate a pressure-dependent term, $\beta V_p$, in the simulations. We think, although we have no measurements to justify it, that the pressure-dependent term probably represents the compressibility of the binding site. Weber & Drickamer (1983) point out that compressibility is likely to represent surface compressibility. Eden et al. (1982) feel that it may represent phenomena associated with the protein core. Since the surfaces of the individual reactants must be greater than that of the porphyrin–oxidase complex and if compressibility were acting only on surfaces, we would expect the pressure-dependent term to drive the equilibrium in the direction of dissociation, just the opposite of that which we find.

We have carried out preliminary pressure experiments on the cytochrome *c* peroxidase–porphyrin *c* equilibrium. The data are not yet refined but clearly indicate that the compressibility term in that equilibrium is approximately the same as that in the oxidase case. Since the two proteins, the oxidase and the peroxidase, are extremely different, it would be surprising if the $\beta V_p$ term represented something other than their common factor, the binding site for cytochrome *c* or porphyrin *c* itself.

If the binding site is undergoing compression, is it then likely that the fluorescence coefficients that we derived are valid only at 1 bar? If the site is being compressed, it is probable that the heme and porphyrin are coming closer together. The compressibility term has a value of about 20 mL mol$^{-1}$ kbar$^{-1}$. The interaction site of the peroxidase–cytochrome *c* couple, and by inference the oxidase–porphyrin *c* couple, may be estimated from the computer models of Poulos & Kraut (1980), or the data that has come from Margoliash's (Kippenol & Margoliash, 1982) group, to consist of a surface of approximately 1 nm$^2$. If the compression of the site were uniform, an increase in pressure of 1 kbar would result in a compression of about 20 mL mol$^{-1}$ kbar$^{-1}$ or about 0.03 nm molecule$^{-1}$. The calculation, while very approximate, leads us to conclude that, since the chromophore–fluorophore distance does not change by a significant amount on compression, the fluorescence coefficients that we have used are probably valid at higher pressures just as they are at 1 bar. In the worst case, $\beta V_p$ would represent a sum of two functions, a true compressibility of the binding site and the altered fluorescence coefficient arising from the compression; in the best case, the term $\beta V_p$ would represent only the overall compressibility of the site.

We have used the values obtained for the relative fluorescence coefficients in the presence and absence of enzyme to calculate the distance between the porphyrin and the heme of the oxidase (Forster, 1951; Dockter et al., 1978). We used the *J* value ($2.35 \times 10^{-14}$ cm$^3$ M$^{-1}$) determined by Dockter et al. (1978) for the spectral overlap between the oxidase and the porphyrin as well as their quantum yield for the porphyrin fluorescence (0.014); $k^2$ was taken to be 1. The *J* value was determined for the yeast oxidase, but the spectral properties of the yeast and beef heart enzymes are sufficiently similar that the results do not change appreciably with the latter. The value that we obtain for the distance between the porphyrin and the heme is about 2.8 nm, which compares favorably with the value of 2.3–2.6 nm obtained by Dockter et al. (1978) and 2.5–3.5 nm deterimined by Vanderkooi et al. (1977, 1978).

Taking into account all the data on the oxidase in its interaction with porphyrin *c*, it is clear that the data are internally consistent and consistent with the data of other workers. The internal agreement between our $\Delta V^\circ$ values obtained at the high and low salt concentrations indicates that the values of $\Delta V^\circ$ are probably a reasonable approximation of reality. $\beta V_p$, since it does not vary with salt concentration, is probably a correct numerical value, the interpretation of which is difficult. It most probably represents a sum of two terms, a compressibility term and a distance term. We think

that the compressibility term is associated with the oxidase–porphyrin *c* interaction site but have no evidence to reinforce the speculation.

By far, the most significant aspect of the work that we report here is that the volume change associated with the equilibrium between the cytochrome substrate and its cytochrome acceptor is—in contrast to most other enzyme–substrate combinations or most other interactions between macromolecules—extremely small (Morild, 1981; Heremans, 1982). While it must be true that the enzyme and porphyrin *c* (cytochrome *c*) lose water to the bulk phase as they approach one another, a positive volume change, there must be other negative volume changes taking place as the two come together such that the net change is as small as it is. In terms of fit, the oxidase and its substrate, cytochrome *c*/porphyrin *c*, are made for one another.

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**Registry No.** Cytochrome *c* oxidase, 9001-16-5; porphyrin *c*, 635-50-7; cytochrome *c*, 9007-43-6.

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