Conformations of Cytochrome Oxidase: Thermodynamic Evaluation of the Interconversion of the 418- and 428-nm Forms†

Jack A. Kornblatt*,‡ and Gaston Hui Bon Hoa

ABSTRACT: Oxidized cytochrome *c* oxidase exists in two reasonably well-defined conformations, a high-spin conformation with maximal absorption at 418 nm and a low-spin conformation with maximal absorption at 428 nm. The equilibrium between these two conformations has been studied as a function of pH, pressure, and temperature. pH effects the equilibrium between the two conformations, the maximum fraction of the 418-nm form being found at about pH 6.8. Increasing pressure displaced the equilibrium toward the 428-nm form; the molar volume changes found are independent of pH but strongly dependent on temperature. Increasing temperature over the range −20 to 25 °C displaces the equilibrium toward the 428-nm form; the van't Hoff plots that result show a discontinuity at about 10 °C. Above 10 °C, ΔH is relatively constant as a function of pH; below 10 °C, ΔH is strongly pH dependent. ΔG, ΔH, ΔS, and ΔV have been evaluated for the equilibrium.

Cytochrome *c* oxidase (EC 1.9.3.1) is the terminal electron carrier of the mitochondrial electron transport chain and is associated with site III of oxidative phosphorylation. The mechanism by which oxidative energy is converted into useable ATP appears to involve the generation of a proton gradient during the transfer of electrons from reduced cytochrome *c* to oxygen (Mitchell, 1979). At least a portion of the proton gradient that results from catalysis is due to an active proton pump which transfers protons from the inner to the outer mitochondrial space (Wikstrom & Saari, 1977; Wikstrom, 1977; Casey et al., 1979; Wikstrom & Krab, 1979). The detailed nature of the pump is unknown, but several groups have suggested conformational changes during the catalytic cycle as being a possible cause of the pump (Wikstrom, 1975a; Wikstrom & Saari, 1976; Wikstrom & Krab, 1979; Krab & Wikstrom, 1979; Kornblatt, 1980a,b).

Cytochrome *c* oxidase is known to exist in several different but interrelated conformations (Wriggleworth & Nicholls, 1978; Kornblatt et al., 1975; Kornblatt, 1977, 1980a). The oxidized protein—with which we are concerned here—can exist as either the resting, high-spin (Tweedle et al., 1978) form (λ_{max} 418 nm) or the so-called "oxygenated", low-spin (Lanne et al., 1979; Nicholls, 1979) form (λ_{max} 428 nm); additionally, several other conformations have been recognized but have not been as thoroughly studied as the 418- and 428-nm forms.

In this report we attempt to evaluate the magnitude of the thermodynamic parameters associated with the interconversion of the 418- and 428-nm forms. Our approach has been to study the effect of high pressure on the 418 nm ⇌ 428 nm equilibrium and subsequently to study the effect of temperature on this reaction. The combined approach has allowed the evaluation of ΔG, ΔH, ΔS, ΔE, and ΔV for the reaction.

Materials and Methods

The preparation of the beef heart cytochrome oxidase has been described (Yonetani, 1966; Kornblatt et al., 1973). The final step in the purification entails passing the oxidase over Sepharose 6B-CL in order to eliminate trace protein contaminants, cholate and phospholipid. The buffer used for the final column in the purification differed from that used in the past; it contained 10 mM potassium phosphate, 100 mM potassium chloride, and 0.5% (v/v) Tween 80, pH 7.0.

In experiments in which the effects of temperature were studied, the oxidase was used directly in the phosphate-containing buffer, phosphate having a very small $\Delta pH/\Delta C$ (Bates, 1954). In experiments in which the effects of pressure were studied, Tris-HCl[1] or Bistris-HCl was added to a final concentration of 0.08 M. Tris was used for the pH range 8.9–7.1 and Bistris for the pH range 7.3–5.8. The two buffers show very little pH variation as a function of pressure (Neuman et al., 1973; J. A. Kornblatt, unpublished observations). All reagents other than the oxidase were purchased commercially and were of the highest purity available.

pH was determined by using Tritrimax system equipped with a Radiometer combination electrode. The electrode was calibrated at pH 9.22, 7.0, and 4.0 at the temperature of the experiment for all experiments except those at subzero temperatures; for the latter, pHs were estimated by using the data of Bates (1954).

Spectra were recorded on a Cary 219 UV–vis spectrophotometer especially modified for high-pressure, low-temperature studies (Hui Bon Hoa et al., 1982). Base lines were stored in memory and automatically subtracted from the oxidase spectra. Over a period of 5 days, the base-line shape varied by less than 0.0005*A*. Typically, base lines were recorded on day one while the oxidase was purified on the column. The same oxidase was then used on days two, three, and four. Base-line shape did not vary as a function of temperature, pH, or pressure; pressure, however, caused considerable shifts in the base-line position. Accordingly, all spectra were zeroed at 705 nm, a wavelength where both the oxidase and the buffer show minimal absorption. Oxidase concentrations in the cuvette (light path approximately 0.5 cm) were approximately 10 μM; the relatively high concentration helped to minimize errors resulting from shifts in the base line when the system was pressurized.

Equilibrium coefficients for the reaction 418-nm oxidase ("resting", "high spin") ⇌ 428-nm oxidase ("oxygenated", "low

† From the Institut de Biologie Physico-Chimique-Service de Biospectroscopie, 75005 Paris, France. *Received May 17, 1982.* This research was supported by research grants from the Délégation Générale à la Researche Scientifique et Technique (Contract 81.E.0418) and the Institut National de la Santé et de la Researche Médicale (Unité 128) and by a travel grant from NSERC (Canada).

‡ Permanent address: Department of Biology, Concordia University, Montréal, P.Q., Canada H3G 1M8.

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; Bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid.

0006-2960/82/0421-5439$01.25/0

spin") were calculated on the basis of the following millimolar extinction coefficients (mM^{-1} cm^{-1}): 418-nm form, $E_{418} = 70$, $E_{428}^{418} = 58.4$; 428-nm form, $E_{418}^{428} = 57.6$, $E_{428} = 66.8$; $E_{424.5}$ (isosbestic point) = 64.3. All extinction coefficients refer to the concentration of heme *a* and all oxidase concentrations in the text are heme *a* concentrations. The concentration of active oxidase (aa_3) is half the value of that listed in the text or in the legends to the figures.

The extinction coefficients were evaluated with freshly prepared enzyme contained in 10 mM Tris, 10 mM EDTA, 100 mM KCl, and 0.5% Tween 80, pH 6.8, at 20 °C. Extinction coefficients and peak shapes of the individual forms were assumed to be invariant over the temperature and pressure range used in the study.

The enzyme in the Tris, EDTA, KCl, and Tween buffer shows an absorption maximum at 417.5 nm and was assumed to be the 100% pure 418-nm form. The assumption is obviously incorrect; enzyme at pH 6.8, contained in the above buffer, is a mixture of 418-nm and 428-nm forms but contains minimal 428 nm. Extinction coefficients for the 428-nm form were determined by treating the 418-nm form with 50 mM ascorbate; after complete reduction of the anaerobic system, oxygen was admitted to the cuvette and the spectrum of the 428-nm form recorded. The resulting spectrum is stable for 24 h so long as ascorbate and oxygen are present (Kornblatt et al., 1975). The ascorbate-generated, 428-nm spectrum was assumed to be 100% pure 428-nm form. As with the 418-nm spectrum, the assumption is not completely valid; the ascorbate-generated spectrum is predominantly the 428-nm form but does contain a small, unknown percentage of the 418-nm form.

The equilibrium coefficients were calculated by using the relationship $K_{e(T,P)}$ = (428-nm form)(418-nm form)$^{-1}$ or

$$K_{e(T,P)} = \frac{A_{428}(E_{428}E_{418} - E_{428}^{418}E_{418}^{428}) - E_{428}^{418}(A_{418}E_{428} - A_{428}E_{418}^{428})}{E_{428}(A_{418}E_{428} - A_{428}E_{418}^{428})}$$

Recoveries of enzyme were calculated by using the data of Grindley & Lind (1971) for pressure correction and the isosbestic point for total oxidase in order to ensure that denaturation was not occurring. Denaturation, as exhibited by less than 95 ± 5% total oxidase recovery, only occurred at temperatures above 30 °C and pressures greater than 3.5 kbars. At subzero temperatures, pressures greater than 3.5 kbars could be employed without any sign of denaturation, as evidenced either by poor spectral recovery or by irreversible processes occurring.

Results

Figure 1 shows the absorption spectra of the high-spin (418-nm) and low-spin (428-nm) forms of the oxidized cytochrome *c* oxidase. The spectra of Figure 1 were used to calculate the extinction coefficients listed under Materials and Methods. An extinction coefficient of 70 mM^{-1} cm^{-1} for the 418-nm form was assumed on the basis of the data of Yonetani (1966); all other extinction coefficients were expressed relative to this value. Peak positions for the two forms of the oxidized protein were 417.5 and 428.5 nm; the isobestic point occurred at 424.5 nm. The peak positions are as low as has been reported for the 418-nm conformer and as high as has been reported for the 428-nm conformer, leading us to have a relatively high degree of confidence that we are looking at the "almost pure" spectra of the two forms. That the 418-nm form is not pure may be easily seen from the shoulder which appears in the spectrum in the region of 428 nm. The shoulder, which

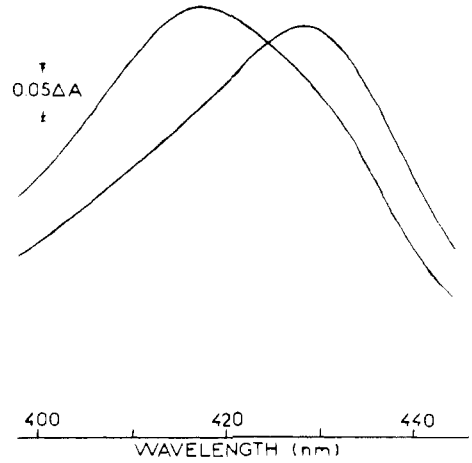

FIGURE 1: Absorption spectrum of the high- and low-spin forms of the oxidized oxidase. 5.3 μM cytochrome oxidase before and after treatment with 50 mM ascorbate. The resting (high-spin) form exhibits a λ_{max} of 417.5 nm; the ascorbate-treated, oxygenated (low-spin) form has a λ_{max} at 428.5 nm. Noteworthy is the shoulder in the high-spin spectrum, indicating that it is the sum of predominantly high-spin protein with some low-spin contamination. Judged by its symmetry, the low-spin form is almost spin pure.

appears in virtually all published spectra of the oxidase at temperatures above 0 °C, indicates that the enzyme is a mixture of both high- and low-spin forms. Since the peak position of the oxidase occurs at the low value of 417.5 nm, we have assumed, for the purpose of calculation, that the high-spin spectrum of Figure 1 represents that of the pure protein in the pure high-spin form; the assumption is not valid but has allowed us to approximate the thermodynamic values desired. The spectrum of the low-spin form shown in Figure 1 exhibits no shoulder and has been assumed to represent that of the low-spin pure protein; the assumption is not strictly valid but has allowed us to proceed. Brudvig et al. (1981) have shown that at least one EPR detectable intermediate forms upon reoxidation of the reduced enzyme, that the intermediate has the same Soret spectrum as the classical oxygenated enzyme, and that the intermediate decays to the oxygenated compound with a half-time of 100 s. Since the half-time for the generation of the 428-nm form is about 20 min (Kornblatt et al., 1975), we anticipate that the classical spectrum is probably composed of 10% intermediate and 90% classical oxygenated form.

That the high-spin/low-spin equilibrium can be displaced by pH and exogenously supplied ligands has been shown qualitatively in previous work (Nicholls & Hildebrandt, 1978; Maurel et al., 1978; Lanne et al., 1979; Yamamoto & Orii, 1973; Cabral & Love, 1974; van Gelder et al., 1977; Nicholls et al., 1976; Hartzell & Beinert, 1976; Wikstrom, 1975b; Kornblatt, 1980b). pH can alter the equilibrium coefficient by a factor of 3 as can be seen in Figure 2. The data shown were taken in the presence and absence of 50 μM CaCl. When similar titrations were performed in EDTA-containing buffers, the general shape of the curve was the same but the minimum value of K_e obtained was 0.09 as opposed to 0.18 in the absence of EDTA. At the extremities of the titration curve, there was no difference between curves with or without EDTA.

Qualitatively, the spectra which lead to the equilibrium coefficients of Figure 2 show the following progression. When the titrations are started with the enzyme at pH 5, increasing pH causes a small progressive blue shift in the Soret peak up to about pH 7. Further increases in pH are accompanied by a red shift and a diminution in the Soret maximum. It is probable that at least two groups contribute to the formation

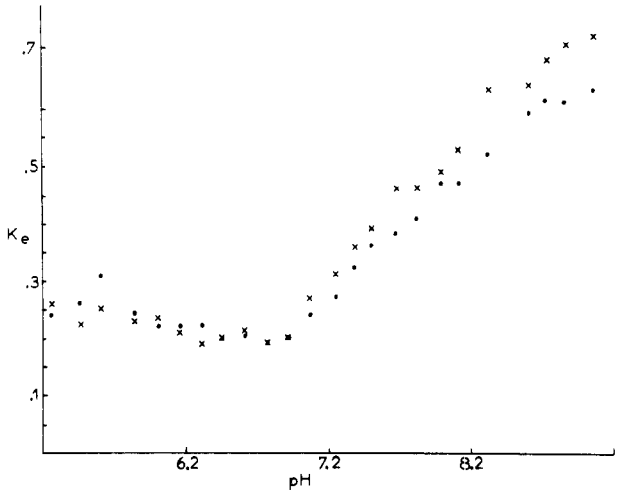

FIGURE 2: Effect of pH on the high-spin/low-spin equilibrium of the cytochrome *c* oxidase. The equilibrium constants were determined on enzyme contained in the phosphate buffer listed under Materials and Methods (···); $CaCl_2$ was added to a final concentration of 50 μM (×).

of the 418-nm form; one group must be protonated and the other deprotonated for the 418-nm form to predominate at equilibrium. Other pH-sensitive groups contribute to instability of the enzyme. The pH response of the oxidized protein below 5.4 and above 9.0 (in the phosphate-containing buffer used here) is complicated by the occurrence of other phenomena which, frequently, are not reversible.

It is necessary to postulate that one is looking at a single equilibrium process over the entire pH range, i.e., that on both sides of the pH optimum of Figure 2 the same 428-nm form exists. Kinetic studies have shown that the 428-nm enzyme is probably an obligate intermediate in many catalytic cycles of the oxidase, that it can be formed at both high and low pH, and that it decays to the equilibrium position more rapidly at low pH (Kornblatt, 1980b) than at high pH. In the equilibrium condition, which concerns us here, it would appear that if other forms of the oxidase are present, they are spectrally indistinguishable from the high- and low-spin forms of Figure 1. The single equilibrium assumption is reasonable in view of the fact that total spectral recoveries of the oxidase are invariant over a K_e range of 0.095–0.7.

Pressure is an additional environmental perturbant capable of displacing the high-spin/low-spin equilibrium of the oxidase. Figure 3 shows the pressure sensitivity of the oxidase at pH 7, 15 °C, in Bistris-containing buffer; the spectra are representative of the changes that occur over the pH range 6–9 at temperatures between −20 and 25 °C. When the pressure on the enzyme is started at 1 bar, the peak position of the Soret band shifts from 418 to 420.5 nm at 3000 bars. The continuous increase in peak height seen in the figure is an artifact of the manipulation; when corrections are applied for solvent contraction—about 10% at 3000 bars—one finds that the peak height remains approximately constant.

The changes shown in Figure 3 are readily reversible up to pressures of 3500 bars and probably beyond. The stippled trace, superimposed on the lowest curve, is the spectrum that is found at the end of the experiment after the enzyme had gone through a pressurizing cycle (3500 bars) and then returned to 1 bar. There is no detectable difference between the spectrum taken before pressurizing and that taken after. The exception to the previous sentence occurred at the extremes of pH at elevated temperatures; when irreversible changes were found in either pressure change or temperature

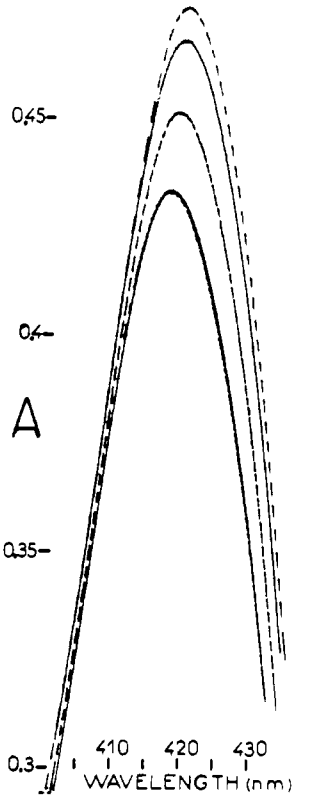

FIGURE 3: Effect of pressure on the spectrum of the oxidized cytochrome *c* oxidase. Approximately 12 μM oxidase contained in 80 mM Bistris, 10 mM phosphate, 10 mM KCl, and 0.5% Tween 80, pH 7.0. Lowest solid trace, 1 bar, λ_{max} 418 mm; lowest dashed trace, 1 kbar, λ_{max} 419 mm; solid trace, 2 kbars, λ_{max} 420 nm; dashed trace, 3 kbars, λ_{max} 420.5 nm; stippled trace superimposed on lowest trace, 1 bar after 10-min exposures to 1, 2, and 3 kbars. The superposition indicates that the equilibrium at pH 7, 14 °C, is completely reversible.

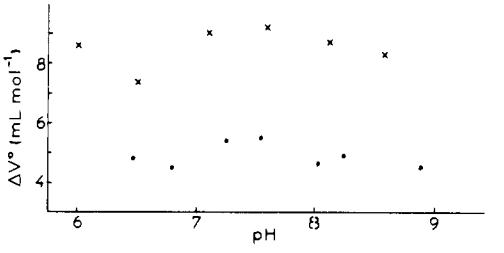

FIGURE 4: pH does not influence ΔV^0 over the range 6–8.5. Spectra such as those of Figure 3 were evaluated to yield ΔV^0 at the pHs shown. ΔV^0 was invariant as a function of pH but doubled as the temperature was raised from 274 (···) to 298 K (×).

change experiments, the manipulations were stopped and the data rejected.

In the pressure, temperature, and pH range where perturbation does not irreversibly alter the oxidase, equilibrium coefficients were calculated for each of a series of pressure changes occurring at constant temperature and pH. Since

$$\left(\frac{\partial \ln K_e}{\partial P}\right)_T = \frac{-\Delta V^0}{RT}$$

a plot of ln K_e vs. P yields ΔV^0, the molar volume changes associated with the 418-nm/428-nm equilibrium.

Figure 4 shows data compiled for two sets of temperatures over the pH range 6–9. Each ΔV^0 was evaluated from spectra such as those of Figure 3 by using at least eight pressures equally spaced between 1 and 3500 bars. In the plots of ln K_e vs. P, no systematic deviation from linearity was found. Volume changes associated with the 418-nm/428-nm equi-

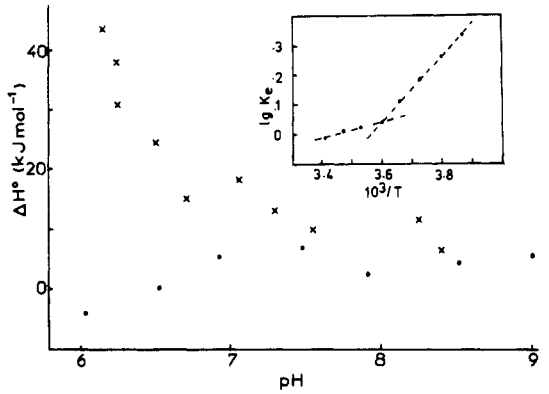

FIGURE 5: Effect of pH on the enthalpy of the 418-nm/428-nm equilibrium. Data were evaluated for the temperature ranges 10–25 (···) and −20 to 5 °C (×). Data for the high temperature range were collected at 1 bar and for the low temperature range at 2 kbars. When selected points for the high-temperature range were evaluated at 2 kbars, the enthalpies for the two ranges were still different from one another as can be seen in the inset of the figure. The inset curve represents a van't Hoff plot in which data were collected at 2 kbars at a working pH of 7.05. ΔH for the low-temperature range is 18.2 kJ mol^{-1}; for the high range it is about 5 kJ mol^{-1}.

librium were quite reproducible; measurements made over a period of 3 days yielded ΔVs within 5% of one another. The data of Figure 4 show that ΔV^0 is virtually independent of pH over the range pH 6–9. The data for either individual temperature series reinforce the assumption that we are dealing with a single equilibrium over the pH range shown and that if other forms of the oxidase are present, they share the spectral characteristics and volume changes of the 418- and 428-nm forms at that temperature. That ΔV for the two temperature series is not the same is disturbing and will be dealt with later.

The volume changes indicated in Figure 4 are small, between 4 and 8 mL per mol, but are of the same magnitude as is found with other heme proteins (Ogunmola et al., 1977; Ogunmola, 1980). The molar volume of the oxidase is approximately 10^5 mL (Fuller et al., 1979); a volume change of 8 mL represents only 10^{-2}% of the total. It should be emphasized that the volume change viewed here is a global quantity and sums both positive and negative changes occurring during the 418-nm/428-nm transition. ΔV indicates, in an unequivocal way, the magnitude of difference but does not indicate whether it is taking place in one or several regions of the protein. Moreover, ΔV does not indicate whether the changes are associated with the protein itself, i.e., the amino acid residues, with the hydration shell of the protein, or, importantly in this case, with the detergent "shell" of the protein. The utility of ΔV is that it allows us to view the overall change associated with the process. The small ΔV associated with the equilibrium is rather surprising. Previous work showed that the sedimentation coefficients of the two forms differed by 2.5%, the 428-nm form being the more compact (Kornblatt et al., 1975). Were ΔV reflecting changes only in the protein moiety of the oxidase, one would expect ΔV to be considerably greater than that found here.

The data of Figure 4 were compiled at only two temperatures, 25 and 1 °C. The variation of K_e as a function of temperature is required in order to achieve a complete thermodynamic description of the 418-nm/428-nm equilibrium. Preliminary van't Hoff plots showed that ΔH was not constant over the temperature range 0–25 °C and that a definite discontinuity occurred at about 10 °C (Figure 5). Accordingly, van't Hoff plots were determined for the ranges 10–25 and −20 to 5 °C. The results of such plots, as a function of pH, are shown in Figure 5.

For the upper temperature range, data were not collected above 25 °C since strict reversibility was not found above that temperature. Accordingly, the enthalpy values shown for the upper range were based, in most cases, on only four data points and carry a relatively large probable error (±15%).

For the lower temperature range, data were collected by lowering the temperature of the cuvette to 5 °C, pressurizing the system to 2 kbars, and then lowering the temperature in steps of 5 °C. Data could easily be collected at temperatures as low as −20 °C, *in the absence of cryoprotectants*; at 2 kbars the freezing point of water in this cuvette is about −30 °C (Hui Bon Hoa et al., 1982). ΔH for the lower temperature range was based on a minimum of five points; probable errors are considerably smaller than for the upper range.

For the upper temperature range, ΔH values are small and appear to be almost independent of pH. For the lower range, the data are quite different. Above pH 7 ΔH is small and more or less constant. Below pH 7 ΔH increases in value as the pH is lowered. The data would indicate that we are viewing the effects of a temperature-dependent pK on the enzyme, one which has a pK at elevated temperature of about 6 or less such that it tends to be completely deprotonated over the majority of the pH range. At the lower temperatures, the pK appears to be shifted toward the basic range such that the group, in the pH range below 6.5, becomes protonated. Such temperature-dependent behavior might be expected for a weakly basic group such as an amine (Bates, 1954) or imidazole (Datta & Grzybowski, 1966). Disturbing in this interpretation is the fact that plots of ln K_e vs. $1/T$ do not show a continuously varying slope between −20 and 25 °C as might be expected for a temperature-sensitive pK acting independently. Rather the plots show a discontinuity at about 10 °C with straight lines above and below this temperature. Such behavior would be more likely to occur if we were dealing with a concerted change in protein structure, induced by temperature and reflected in the altered pK.

ΔH is relatively constant as a function of pH at pH above 7.5; nonetheless, for the two temperature ranges (at the same pressure) ΔH at pH 7.5 is not the same. Low temperature range data were obtained at 2 kbars. When $P\Delta V$ is subtracted from the low temperature range data to yield ΔE, the values for the two ranges are closer but still distinct. Similarly, for a high- and low-temperature range, ΔV values were found to be constant as a function of pH but different as a function of temperature. The coupled ΔH and ΔV data lead us to think that we would probably find that there were two volume changes associated with the proposed equilibrium. We anticipated that if we determined ΔV as a function of T we would find a stable, high-temperature ΔV followed by a discontinuity in the plot followed by a stable low temperature ΔV. Our expectation was that a two-state model—such as we have employed throughout this work—would probably not fit the data and that other states, having similar spectral characteristics, would have to be invoked.

Figure 6 shows the variation of ΔV with temperature over the range 25 to −20 °C. The data were all compiled at a working pH of approximately 7.5 where ΔV does not vary with pH. To a first approximation the data show an almost linear relationship between T and ΔV at temperatures above −10 °C. Below −10 °C the data deviate from the relationship, and a new phenomenon appears to take place. Interestingly, the spectra still show only reversible changes with pressure as the temperature is lowered.

We have summarized the thermodynamic data in Table I; the data shown have been calculated for 25 °C, 1 bar only.

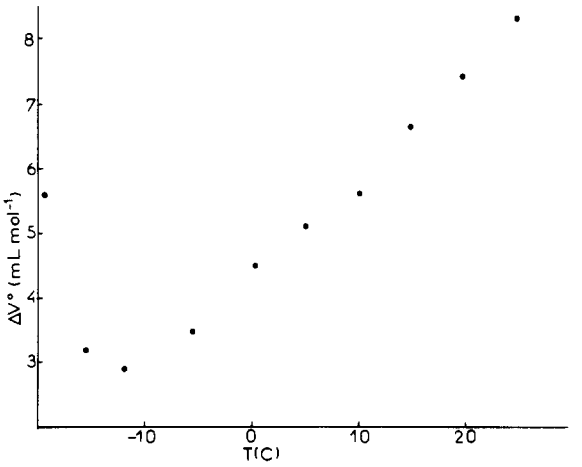

FIGURE 6: Effect of temperature on ΔV^0 at pH 7.5. Data above 0 °C were taken between 1 bar and 3 kbars. Below zero, the enzyme was brought to 0.5 (−5.5 °C), 1 (−12 °C), 1.5 (−15.5 °C), and 2 kbars (−19.5 °C) before the temperature of the sample was lowered to the value indicated. Data were then collected between the initial pressure and 3.9 kbars.

Table I: Thermodynamic Values for the 418-nm/428-nm Equilibrium at 298 K and 1 bar

pH	$\Delta G°$ (kJ mol^{-1})	$\Delta H°$ (kJ mol^{-1})	$\Delta S°$ (eu)	$\Delta V°$ (mL mol^{-1})
6.0	3.6	−4	−25.5	8.5
6.5	3.9	2	−6.4	8.5
7.0	3.6	4	0.12	8.5
7.5	2.4	4	4.8	8.5
8.0	1.7	4	6.9	8.5
8.5	1.3	4	8.0	8.5
9.0	0.9	4	9.3	8.5

It can be easily seen that the values for ΔG for the 418-nm/428-nm equilibrium are small and that the values decrease on both sides of a maximum occurring in the vicinity of pH 6.8. ΔH (which is approximately equal to ΔE at 1 bar) is approximately constant above pH 7 and decreases below. ΔS, reflecting the difference between ΔG and ΔH, shows a continual increase in value with increasing pH. ΔV for the equilibrium is constant over the pH range shown.

Discussion

To the best of our knowledge, the work presented here represents the first attempt to quantify the thermodynamic parameters associated with the 418-nm/428-nm equilibrium. We have made several assumptions in our approach to the project.

Our first assumption was that the ϵ's for the pure 418- and 428-nm forms could be derived from their spectra. The assumption is valid as a first approximation only. The "pure", 418-nm spectrum (Figure 1) shows obvious 428-nm contamination; the extent to which the 428-nm compound contributes to the observed spectrum is unknown but may represent as much as 10% based on estimates of the acidic and basic pKs of Figure 2. There is no clear evidence for 418-nm contamination of the "pure" 428-nm compound. The method by which the 428-nm spectrum was generated—continuous slow reduction of the oxidase followed by rapid reoxidation—favors the steady-state accumulation of the 428-nm compound. Nonetheless, like the 418-nm form, the assumption of spectral purity is a first approximation only.

Our second assumption was that in the overall equilibium $A \rightleftharpoons B \rightleftharpoons 418\text{ nm} \rightleftharpoons N \rightleftharpoons O \rightleftharpoons 428\text{ nm} \rightleftharpoons Y \rightleftharpoons Z$, the only forms present in significant concentrations were the 418- and 428-nm forms. The assumption is reinforced by the fact that good spectral recoveries of the oxidase were found as temperature and pressure were varied and that both volume and enthalpy measurements yielded plots in which there was no systematic deviation from linearity. Were other forms of the oxidase present, either as intermediates or as side products, one would expect significant deviations from linearity if their extinction coefficients, volumes, or enthalpies differed.

Certainly, other forms of the oxidized oxidase have been described. The original kinetic description of alternative forms by Orii & King (1972, 1976) has since been amplified by Palmer's group (Carithers & Palmer, 1981; Carter et al., 1981) as well as several other laboratories (Brudvig et al., 1981; Kitagawa & Orii, 1979; Yong & King, 1970; Shaw et al., 1978). To the extent that we are capable of recognizing these alternative forms, it would appear that they do not significantly contribute to the observed spectra. We have started a study on the kinetics of the 418-nm/428-nm change in response to pH and pressure; if intermediates or substates are involved in the establishment of the equilibrium our available techniques should allow us to see them.

Our third assumption was that temperature-induced and pressure-induced changes occurred on a time scale that was faster than that at which measurements were made. Spectra were recorded only after stable readings were achieved. At moderate temperatures (10–25 °C) and pH (6.5–7.5) stable values were obtained in the time required to change conditions. With decreasing temperatures and increasing pH, stability was only obtained after a long delay (5–45 min). Changes in equilibrium that required long waits in one direction frequently required long waits in the opposite direction.

The temperature- and pressure-induced changes of Figures 4 and 5 are represented as functions at pH. The latter has been known for some time to be a potent modulator of oxidase activity (Yonetani, 1961), spin state (Hartzell & Beinert, 1976), and spectrum (Kornblatt, 1980b). It was of interest to see whether ΔV and ΔH were sensitive to pH changes. That ΔV is more or less constant as a function of pH indicates that although K_e changes with pH, pressurizing the system leads to parallel changes in K_e at all investigated pH values. Similarly ΔH and ΔE are relatively insensitive to changes in pH except at low temperature in the pH range below 7. The large values of ΔH and ΔE in the low-temperature, low-pH range are indicative of a weakly basic group involved in the 418-nm/428-nm transition. Alternatively, the large change may reflect the reversible dissociation of one of the heme ligands; Stevens & Chan (1981) have discussed the possibility that the axial ligand of cytochrome a_3 is histidine.

Orii and his co-workers (Kawato et al., 1981; Yoshida et al., 1979) have found temperature-induced conformational transitions with soluble cytochrome oxidase. Using a covalently attached anilinonaphthyl fluorescent probe, the above authors found a thermally induced transition at about 20 °C which correlated well with a break in the enzymatically determined Arrhenius plot. Working in the temperature range −20 to 25 °C, we would not expect to see the effects of this transition if it was either very sharp or quite feeble. One notes that in their discussion, Kawato et al. (1981) commented that they were not able to detect substantial changes (greater than 3%) in the Soret absorption spectrum of the oxidase over the temperature range 4–35 °C. The results reported here are in agreement with their data. Below 4 °C we see spectral changes much greater than those which we see above 4 °C.

Figure 6 is of interest because it brings together the two variables central to this work, temperature and pressure. Molar volumes associated with the 418-nm/428-nm equilib-

rium are not constant but vary in an approximately linear fashion with temperature. Such a result, but in the opposite direction, has also been seen for the equilibrium between the high-spin and low-spin forms of cytochome P450 from *Pseudomonas putida* (Hui Bon Hoa & Marden, 1982). In neither case is there an adequate theoretical explanation for the data. For the cytochrome oxidase equilibrium several explanations are possible.

(1) At any given temperature there exist hydration shells associated with the two protein forms. Changing temperature changes the extent of hydration of one form more than it does of the second.

(2) At any given temperature there exist detergent shells associated with the two forms of the oxidase, the detergent shells being in equilibrium with free detergent and micellar detergent. Changing temperature changes the extent of detergent binding of one oxidase form more than another.

(3) At any given temperature, both forms of the oxidase display a degree of flexibility. Their net molar volumes are a reflection of both conformation and flexibility. Changing temperature changes the degree of flexibility of one form more than another with the result that there is an apparent change in ΔV.

(4) At any given temperature and pressure, the 418-nm/428-nm equilibrium monitors the equilibrium of a second reaction that is not spectrally visible. ΔV for the spectrally silent process is temperature sensitive, and the oxidase equilibrium reflects this sensitivity.

In summary, we have established the magnitude of the thermodynamic parameters associated with the 418-nm/428-nm equilibrium of the oxidized cytochrome oxidase. Several questions, however, remain unanswered. Among these is the question of intermediates in the reaction scheme. We have started a kinetic study of the pressure- and pH-induced changes; the study should allow us to state whether or how many intermediates exist between the two principle states. More difficult is the question of the reason for the linear relation between ΔV and T, the answer to which will have to await theoretical developments.

Acknowledgments

We express our gratitude to Professor P. Douzou for his continual interest in the work presented here and for his critical comments. J.A.K. thanks le Service de Biospectroscopie, U128 (INSERM) IBPC, for providing facilities during the sabbatical year and Concordia University for the sabbatical year.

References

Bates, R. G. (1954) in *Determination of pH, Theory and Practice*, Wiley, New York.

Brudvig, G. W., Stevens, T. H., Morse, R. H., & Chan, S. I. (1981) *Biochemistry 20*, 3912.

Cabral, F., & Love, B. (1974) *Biochemistry 13*, 2038.

Carithers, R. P., & Palmer, G. (1981) *J. Biol. Chem. 256*, 7967.

Carter, K. R., Antalis, T. M., Palmer, G., Ferris, N. S., & Woodruff, W. H. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1652.

Casey, R. P., Chappell, J. B., & Assi, A. (1979) *Biochem. J. 182*, 149.

Datta, S. P., & Grzybowski, A. (1966) *J. Chem. Soc. B*, 136.

Fuller, S. D., Capaldi, R. A., & Henderson, R. (1979) *J. Mol. Biol. 134*, 305.

Grindley, T., & Lind, J. E. (1971) *J. Chem. Phys. 54*, 3983.

Hartzell, C. R., & Beinert, H. (1976) *Biochim. Biophys. Acta 423*, 323.

Hui Bon Hoa, G., & Marden, M. C. (1982) *Eur. J. Biochem.* (in press).

Hui Bon Hoa, G., Balny, C., Dahan, N., & Douzou, P. (1982) *Anal. Biochem. 120*, 125.

Kawato, S., Yoshida, S., Orii, Y., Ikegami, A., & Kinosita, K. (1981) *Biochim. Biophys. Acta 634*, 85.

Kitagawa, T., & Orii, Y. (1979) in *Cytochrome Oxidase* (King, T. E., et al., Eds.) Elsevier, Amsterdam.

Kornblatt, J. A. (1977) *Can. J. Biochem. 55*, 458.

Kornblatt, J. A. (1980a) *J. Biol. Chem. 255*, 7225.

Kornblatt, J. A. (1980b) *Can. J. Biochem. 58*, 840.

Kornblatt, J. A., Baraff, G. A., & Williams, G. R. (1973) *Can. J. Biochem. 51*, 1417–1427.

Kornblatt, J. A., Kells, D. I. C., & Williams, G. R. (1975) *Can. J. Biochem. 53*, 461.

Krab, K., & Wikstrom, M. K. F. (1979) *Biochim. Biophys. Acta 548*, 1.

Lanne, B., Malmstrom, B. G., & Vanngard, T. (1979) *Biochim. Biophys. Acta 545*, 205.

Maurel, P., Douzou, P., Waldman, J., & Yonetani, T. (1978) *Biochim. Biophys. Acta 525*, 314.

Mitchell, P. (1979) *Science (Washington, D.C.) 206*, 1148.

Neuman, R. C., Kauzmann, W., & Zipp, A. (1973) *J. Phys. Chem. 77*, 2687.

Nicholls, P. (1979) *Biochem. J. 183*, 519–529.

Nicholls, P., & Hildebrandt, V. (1978) *Biochem. J. 173*, 65.

Nicholls, P., Petersen, L. C., Miller, M., & Hansen, F. B. (1976) *Biochim. Biophys. Acta 449*, 188.

Ogunmola, G. N. (1980) *Biophys. Chem. 11*, 23.

Ogunmola, G. B., Zipp, A., Chen, F., & Kauzmann, W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1.

Orii, Y., & King, T. E. (1972) *FEBS Lett. 21*, 199.

Orii, Y., & King, T. E. (1976) *J. Biol. Chem. 251*, 7487.

Stevens, T. H., & Chan, S. I. (1981) *J. Biol. Chem. 256*, 1069.

Tweedle, M. F., Wilson, L. J., Garcia-Iñguez, L., Babcock, G. T., & Palmer, G. (1978) *J. Biol. Chem. 253*, 8065–8071.

Van Gelder, R. F., Van Rijn, J. L. M., Schilder, G. J. A., & Wilms, J. (1977) in *Structure & Function of Energy Transducing Membranes* (Van Dam, K., et al., Eds.) pp 61–68, Elsevier/North-Holland, Amsterdam.

Wikstrom, M. K. F. (1975a) *Electron Transfer Chains Oxid. Phosphorylation, Proc. Int. Symp.*, 97–103.

Wikstrom, M. K. F. (1975b) *Biochim. Biophys. Acta 408*, 170.

Wikstrom, M. K. F. (1977) *Nature (London) 266*, 271.

Wikstrom, M. K. F., & Saari, H. T. (1976) *Mol. Cell. Biochem. 11*, 17.

Wikstrom, M. K. F., & Saari, H. T. (1977) *Biochim. Biophys. Acta 462*, 347.

Wikstrom, M. K. F., & Krab, K. (1979) *Biochim. Biophys. Acta 549*, 177.

Wrigglesworth, J. M., & Nicholls, P. (1978) *FEBS Lett. 91*, 190.

Yamamoto, T., & Orii, Y. (1973) *J. Biochem. (Tokyo) 74*, 473.

Yonetani, T. (1961) *J. Biol. Chem. 236*, 1680.

Yonetani, T. (1966) *Biochem. Prep.*, 14–20.

Yong, F. C., & King, T. E. (1970) *Biochem. Biophys. Res. Commun. 40*, 1445.

Yoshida, S., Orii, Y., Kawato, S., & Ikegami, A. (1979) *J. Biochem. (Tokyo) 86*, 1443.