Direct Calorimetric Analysis of the Enzymatic Activity of Yeast Cytochrome *c* Oxidase†

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ABSTRACT: The kinetic and thermodynamic parameters associated with the enzymatic reaction of yeast cytochrome *c* oxidase with its biological substrate, ferrocytochrome *c*, have been measured by using a titration microcalorimeter to monitor directly the rate of heat production or absorption as a function of time. This technique has allowed determination of both the energetics and the kinetics of the reaction under a variety of conditions within a single experiment. Experiments performed in buffer systems of varying ionization enthalpies allow determination of the net number of protons absorbed or released during the course of the reaction. For cytochrome *c* oxidase the intrinsic enthalpy of reaction was determined to be −16.5 kcal/mol with one (0.96) proton consumed for each ferrocytochrome *c* molecule oxidized. Activity measurements at salt concentrations ranging from 0 to 200 mM KCl in the presence of 10 mM potassium phosphate, pH 7.40, and 0.5 mM EDTA display a biphasic dependence of the electron transferase activity upon ionic strength with a peak activity observed near 50 mM KCl. The ionic strength dependence was similar for both detergent-solubilized and membrane-reconstituted cytochrome *c* oxidase. Despite the large ionic strength dependence of the kinetic parameters, the enthalpy measured for the reaction was found to be independent of ionic strength. Additional experiments involving direct transfer of the enzyme from low to high salt conditions produced negligible enthalpy changes that remained constant within experimental error throughout the salt concentrations studied (0–200 mM KCl). These results indicate that the salt effect on the enzyme activity is of entropic origin and further suggest the absence of a major conformational change in the enzyme due to changes in ionic strength. High-sensitivity differential scanning calorimetric experiments performed at very low ionic strength show a calorimetric transition profile characterized by two well-defined peaks centered at 50.5 and 60 °C. At high ionic strength, the high-temperature peak shifts to lower temperatures (57.4 °C), giving rise to a single but slightly asymetric transition profile. No shift in the transition temperature was observed for the low-temperature peak under the experimental conditions studied. These results suggest that variation of ionic strength primarily affects the structural stability of the protein subunits giving rise to the main transition peak, primarily subunits I and II.

Cytochrome *c* oxidase (ferrocytochrome *c*:O_2 oxidoreductase; EC 1.9.3.1) is an integral membrane protein found in the inner mitochondrial membrane. As the terminal member of the mitochondrial respiratory chain, it is repsonsible for the catalytic oxidation of cytochrome *c* and the reduction of molecular oxygen to water. For each electron transferred from cytochrome *c*, one proton is used in the reduction of oxygen while a second proton is translocated vectorially from the matrix across the inner membrane (Casey & Azzi, 1983; Wikström, 1984; Thelen et al., 1985). Part of the energy generated from these redox reactions is stored in the form of a proton electrochemical potential across the inner mitochondrial membrane (later utilized to drive adenosine triphosphate synthesis).

Previous steady-state measurements of the electron transfer activity of cytochrome *c* oxidase have relied on the use of either the spectrophotometric methods of Smith and Conrad (1956) or the polarographic method of Ferguson-Miller (1976) to monitor the extent of reaction completion as a function of ferrocytochrome *c* concentration or the consumption of O_2, respectively. Experiments presented in this paper show that by utilizing current microcalorimetric technology it is possible to study the kinetics of this enzymatic reaction by directly measuring the rate of heat production or absorption during the course of reaction. Advantages in using heat flow as an observable in measuring enzyme kinetics include its general applicability to any reaction evolving or absorbing heat, its ease of use for systems that would otherwise require tedious linkage schemes for determination of reaction progress, and in the case of redox systems such as cytochrome oxidase the method is also capable of determining the net proton flux for the reaction. Above all, microcalorimetric methods are able to report the energetics of the reaction, including the intrinsic reaction enthalpy. In this paper, high-sensitivity microcalorimetric techniques have been used to probe the origin of the ionic strength dependence of the enzyme activity of cytochrome *c* oxidase purified from bakers yeast.

It has been known for some time that the electron transfer activity of cytochrome *c* oxidase from beef heart has a peculiar behavior as a function of ionic strength (Wilms et al., 1981). As the ionic strength is increased from a value near zero to ~200 mM, the enzymatic activity first increases to a maximal value (~40 mM of monovalent cations) and then it monotonically decreases until reaching negligible levels. We have found that, despite the differences in subunit composition, the yeast enzyme exhibits a similar behavior. For the yeast enzyme, the activity increases with ionic strength until it reaches a maximum at about 50 mM. Beyond this point, the activity decreases monotonically as the ionic strength increases. Recently, several hypotheses have been advanced to explain this phenomenon. Kossekova et al. (1989) proposed that ionic strength affects the porportion of enzyme in monomeric and dimeric forms. Michel and Bosshard (1989) and Brzezinski and Malmström (1986), on the other hand, have postulated

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the existence of two conformational states of the enzyme. Other authors have postulated the existence of two binding sites, each with a different ionic strength binding dependence [see Cooper (1990) for a recent review]. In this paper, we address this issue from a thermodynamic standpoint in order to develop a set of energetic constraints to any proposed mechanism. Our calorimetric results indicate that the effect of ionic strength on the electron transfer activity of the enzyme is of entropic origin, with very small enthalpic effects. The magnitude of these changes in conjunction with the observed ionic strength dependence of the structural stability of the enzyme, as measured by high-sensitivity differential scanning calorimetry, are consistent with a rearrangement of subunit interactions and further suggest the absence of a major conformational change in the enzyme.

Experimental Procedures

Materials. Cytochrome *c* type VI from horse heart and sodium cholate were obtained from Sigma (St. Louis, MO). Protein grade Tween-80 was purchased from Calbiochem (La Jolla, CA). Dimyristoylphosphatidylcholine (DMPC) was obtained from Avanti Biochemicals (Birmingham, AL) and used without further purification. Cytochrome *c* oxidase was purified from bakers yeast (Gist Brocades, Oldbridge, NJ) essentially following the procedures of George-Nascimento and Poyton (1981) and Power et al. (1984) and adapted for use in our laboratory as described previously by Morin et al. (1989). In brief, this method consists of mechanical cell lysis performed with a Dynomill type KDL bead mill (W.A. Bachofen Machinenfabrick, Basel, Switzerland), detergent extraction of hydrophobic proteins from submitochondrial particles with cholic acid, and iterative ammonium sulfate fractionation followed by hydrophobic interaction chromatography over octyl-Sepharose. Typical batches of enzyme purified in this manner had a heme/protein ratio of 8–9.5 nmol (mg of protein)$^{-1}$ by the method of Mason et al. (1973) and a specific activity of 8.5×10^2 (min^{-1} mg^{-1}) under the assay conditions described below. These values are similar to those previously reported in the litrature (Power et al., 1984).

Membrane Reconstitution. When required, cytochrome *c* oxidase was reconstituted into phospholipid vesicles by the cholate detergent dialysis method previously described (Morin et al., 1989). The dialysis medium used for all reconstitutions performed in this paper was 10 mM potassium phosphate buffer, pH 7.40, and 1 mM EDTA.

Spectroscopic Measurement of Enzymatic Activity. The kinetics of oxidation of ferrocytochrome *c* by yeast cytochrome *c* oxidase were determined by a method similar to that described by Smith and Conrad (1956). The extent of reaction progress was followed spectroscopically by monitoring the absorbance change of the substrate at 550 nm. Data were collected digitally with a Hewlett Packard 8452 diode array spectrophotometer equipped with a thermostatically controlled cuvette holder. The cuvette contained a solution consisting of 33 μM ferrocytochrome *c*, 0.5% Tween-80, 0.5 mM EDTA, and 10 mM of the appropriate buffer, pH 7.40. The final volume for each reaction was 1.39 mL. Ferrocytochrome *c* was prepared by reduction of the resting protein with solid sodium dithionite according to the method of Rigell et al. (1985). All assays were performed with solutions of cytochrome *c* >95% in the reduced form. The reaction was initiated by addition of 20 μL of cytochrome *c* oxidase solution, diluted to an appropriate concentration in the same buffer (typically 0.5–1.0 μM). Enzymatic rates were determined by nonlinear least-squares analysis of the exponential decay in the concentration of ferrocytochrome *c* followed by monitoring the A_{550} as a function of time. In this paper, the rates are expressed as the specific first-order rate constant [min^{-1} (mg of cytochrome oxidase^{-1})] under the specified assay conditions.

Reaction Calorimetry. Reaction enthalpies and kinetic rate constants were obtained from thermograms collected with an Omega titration calorimeter (Microcal Inc., Northampton, MA) equipped with a Keithley model 181 nV meter for signal amplification and a microcomputer for process control. The design and functional principles of this instrument have been described previously by Wiseman et al. (1989). Reaction conditions were identical with those used in the spectrophotometric assay described above in a final volume of 1.388 mL, the volume of the instrument's reaction cell, and were initiated by injecting 20 μL of enzyme solution into the sample cell filled with substrate solution. The temperature during each calorimetric assay was held constant throughout each experiment, and ranged from 26 to 26.5 °C across experiments. Under the conditions of the experiments in this paper, heats of dilution and mixing were less than 0.5% of the total heat measured for the enzymatic reaction. In order to increase the sampling rate and to control the signal-averaging processes, our own data collection software was used. For this purpose, a second microcomputer system equipped with a Data Translation 2801 A/D converter was connected to the amplified output of the calorimeter. Thermograms collected in this manner were corrected for the response time of the instrument according to the method described previously by Mayorga and Freire (1987) and Randzio and Suurkuusk (1980) using a first-order approximation for the correction and an instrumental response time constant of 16 s. In order to further minimize the effects of thermal lag, experiments were designed such that the ratio of enzyme to substrate in the calorimetric cell resulted in reaction completion times at least one order of magnitude greater than the instrumental response time (i.e., >160 s). Enzymatic rates were determined from these corrected profiles by using nonlinear least-squares analysis of the exponential decay of the heat flux as a function of time. For kinetics analysis, only data collected after enzyme injection and mixing were complete were used in the nonlinear least-squares analysis. For each experiment, the enthalpy of reaction was measured by integrating the area under the calorimetric traces as described before (Schön & Freire, 1989).

Differential Scanning Calorimetry. Scanning calorimetric experiments were performed with a Microcal MC2 differential scanning calorimeter (DSC) interfaced to a microcomputer equipped with a Data Translation DT-2801 A/D converter board for instrument control and automatic data collection (Myers et al., 1986). Scans of membrane-reconstituted enzyme were performed at a scanning rate of 45 °C/h. Protein concentrations on the order of 3 mg/mL were used in these experiments.

Protein Concentration Determinations. Concentrations of cytochrome *c* oxidase were determined by the Pierce BCA assay method with bovine serum albumin as a standard. Cytochrome *c* concentrations were determined spectrophotometrically with a millimolar extinction coefficient of 29.5 cm^{-1} for the fully reduced protein species. The extent of reduction of the ferrocytochrome *c* solutions used in the enzymatic experiments was measured by comparing the absorbances at 550 nm with that of identical samples fully reduced by the addition of dithionite and fully oxidized by the addition of potassium ferricyanide.

Results

Calorimetric Determination of Enzymatic Reaction. Figure 1 shows a typical calorimetric trace (heat flow versus time)

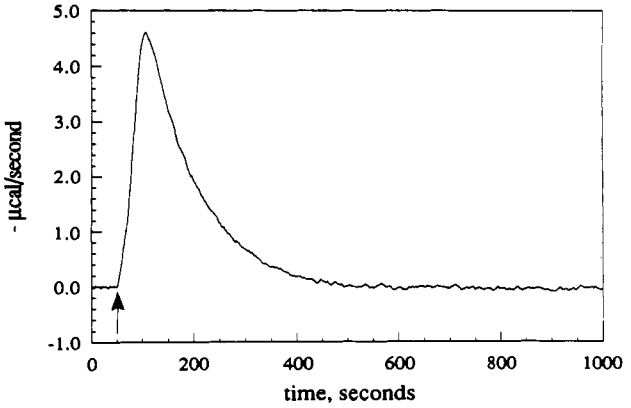

FIGURE 1: Typical calorimetric trace (heat flow versus time) obtained after addition of yeast cytochrome *c* oxidase (20 μL) to the calorimetric cell (1.388 mL) containing ferrocytochrome *c* (33 μM). The enzyme is added at the time indicated by the arrow. The total area under the curve divided by the amount of cytochrome *c* oxidized in the cell is equal to the enthalpy change (ΔH) for the reaction, while the time decay of the rate of heat flow depends on the kinetics of the reaction. This experiment was performed in 10 mM phosphate, pH 7.4, 0.5 mM EDTA, and 0.5% Tween-80 at 26 °C.

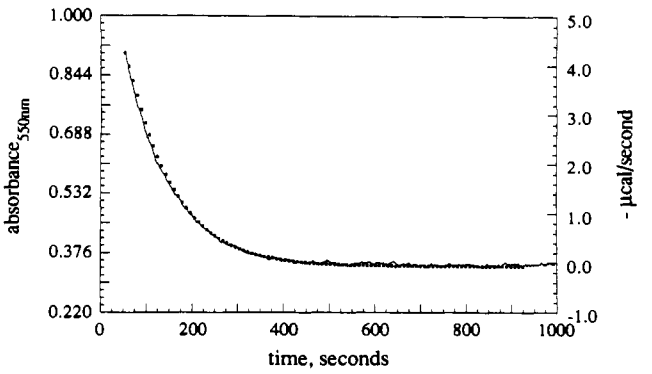

FIGURE 2: Comparison of the kinetic data obtained using the standard spectrophotometric assay (dots) and the calorimetric assay (solid lines). The two experiments were performed with aliquots of the same solutions at exactly the same concentrations as indicated in Figure 1. Calorimetrically, the kinetic parameters are measured from the rate of decay of heat flow after correction for the instrumental time response.

obtained after the injection of catalytic quantities of cytochrome *c* oxidase into the calorimetric cell preloaded and equilibrated with a solution of ferrocytochrome *c*. The concentration of ferrocytochrome c in the calorimeter cell (total volume = 1.388 mL) was 33 μM. At the indicated time, 20 μL of cytochrome *c* oxidase was added to the calorimeter cell in order to initiate the reaction. Throughout this process the rate of heat absorption or release was monitored continuously. As shown in Figure 1, the the enzymatically catalyzed oxidation of ferrocytochrome *c* is characterized by the release of heat, as expected for an exothermic process. The heat flow reaches a maximum soon after the addition of enzyme to the calorimetric cell and then decays to the baseline level as the substrate is depleted. Such traces contain information concerning two characteristics of the enzymatic reaction. First, the area under the curve is equal to the total heat produced by the reaction under the specific conditions employed. The enthalpy change for the reaction is obtained by dividing the amount of heat produced by the amount of cytochrome *c* oxidized. Second, the shape of these curves is defined by the kinetic behavior of the reaction. Specifically, the heat flow (dQ/dt) is directly proportional to the rate with which the reaction product is formed and can be expressed in terms of the enzymatic rate equation

$$dQ/dt = \Delta HV(d[P]/dt) \quad (1)$$

In the preceding equation, ΔH is the enthalpy change for the reaction, V is the volume of the calorimetric cell, and [P] is the concentration of product. The oxidation of cytochrome *c* by cytochrome *c* oxidase under the conditions of the experiments described in this paper has been described in terms of first-order rate equations (Smith & Conrad, 1956; Minnaert, 1961). If such is the case, the heat flow measured by the calorimeter is given by

$$dQ/dt = \Delta HVk[C_o] \exp(-kt) \quad (2)$$

where k is the rate constant and $[C_o]$ is the initial concentration of substrate. Equation 2 indicates that the heat flow decays exponentially and that the calorimetric data can be used to determine the kinetics of the reaction. Figure 2 represents the time decay of the heat flow, after correction for the instrumental response time as described by Mayorga and Freire (1987). Analysis of the collected data indicates that it obeys monoexponential kinetics under the experimental conditions employed. In order to examine the accuracy of the kinetic parameters determined from calorimetric traces, tandem experiments were performed calorimetrically and spectrophotometrically using aliquots of the same enzyme and substrate solutions. The experiments were performed with identical volumes of reagents in both the titration calorimeter and the spectrophotometer. Figure 2 also shows the data obtained spectrophotometrically. As can be seen in the figure, the decay in the heat flow with respect to time and the decay observed in the spectral absorbance with respect to time are in good agreement with one another. The rate constants obtained from the calorimetric and spectral determinations shown in Figure 2 were found to be within 3% of each other [7.48 and 7.28 $\times 10^2$ (min^{-1} mg^{-1}), respectively]. For the entire set of experiments presented in this paper, the differences observed between the two methods were typically within 5% and never greater than 13%.

As mentioned above, the determination of the reaction enthalpy requires knowledge of the amount of cytochrome *c* oxidized. Under each condition studied, two methods were employed to check the completion of the reaction performed in the calorimeter cell. First, spectroscopic measurements were performed on the reaction mixture removed from the calorimeter immediately after the heat flow had returned to baseline. Second, in other experiments a second injection of enzyme into the calorimetric cell was made after the heat flow from the first injection had returned to baseline. Both methods showed that all ferrocytochrome *c* in the calorimeter cell was fully oxidized upon return of the signal to baseline level. Under these conditions the calorimetric enthalpies were calculated by dividing the total heat produced during the reaction by the amount of reduced cytochrome *c* originally in the reaction cell prior to the addition of enzyme. In phosphate buffer at pH 7.4, the apparent enthalpy for the oxidation of cytochrome *c* was -15.5 ± 0.2 kcal/mol.

Calorimetric Determination of Intrinsic Reaction Enthalpy and Net Proton Flux. The apparent enthalpy change of any reaction that involves the release or absorption of protons is sensitive to the enthalpy of ionization, ΔH_b, of the buffer used in the reaction medium. Figure 3 displays a series of calorimetric traces collected for the enzymatic reaction of cytochrome *c* oxidase with ferrocytochrome *c*. Each of the four panels shown in the figure were obtained under identical conditions except for the buffer component present in the reaction medium. The four buffers used in these experiments

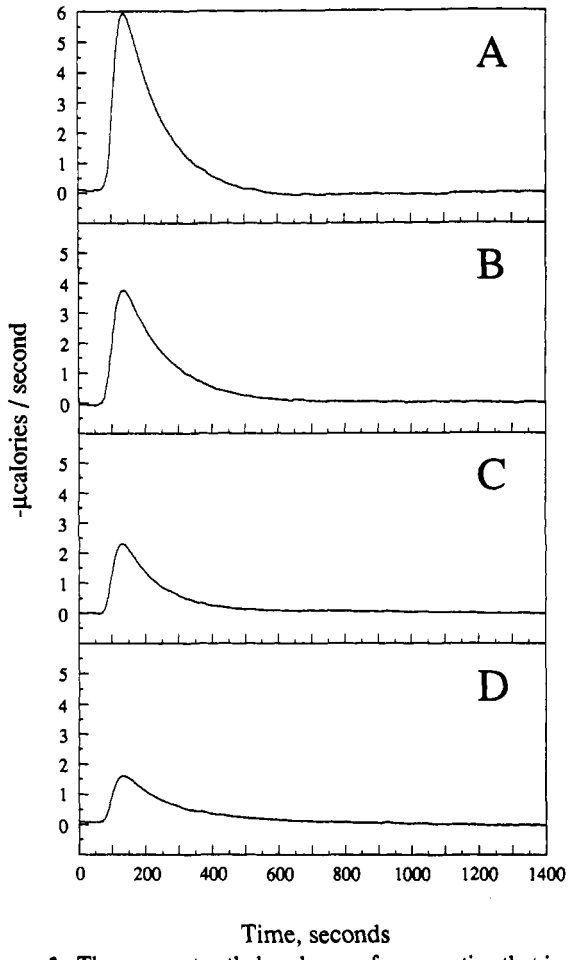

FIGURE 3: The apparent enthalpy change of any reaction that involves the release or absorption of protons is sensitive to the enthalpy of ionization, ΔH_b, of the buffer used in the reaction media. The traces correspond to the cytochrome *c* oxidase reaction performed in (A) phosphate, ΔH_b = 1.22 kcal/mol; (B) MOPS, ΔH_b = 5.29 kcal/mol; (C) Tricine, ΔH_b = 7.76 kcal/mol; and (D) Tris, ΔH_b = 11.51 kcal/mol.

have different enthalpies of ionization at 25 °C (1.22, 5.29, 7.76, and 11.51 kcal/mol for phosphate, MOPS, Tricine, and Tris, respectively) and have been previously measured to great precision (H. Fukada and K. Takahashi, personal communication). The data from these experiments indicate that while the enzymatic rates for the reaction remain essentially unchanged in the various buffer systems, the reaction enthalpy per mole of substrate consumed differs by as much as a factor of 3 depending on the buffer in which the experiments are performed. These differences in observed enthalpy are due to the ionization of buffer molecules in solution during the course of the reaction. As the reaction progresses, a net consumption of protons from the solution occurs and is compensated by the ionization of a similar number of buffer molecules. Since the buffers have different ionization enthalpies, the apparent reaction enthalpies exhibit a buffer dependence. The apparent reaction enthalpy observed per turnover of substrate to product (ΔH_{app}) includes both the intrinsic enthalpy of the enzymatic reaction plus the enthalpy of ionization (ΔH_b) for each proton released or absorbed by the buffer system. This process is represented by

$$\Delta H_{app} = \Delta H_{rxn} + n\Delta H_b \quad (3)$$

where n represents the number of protons released by the buffer and ΔH_{rxn} represent the intrinsic enthalpy change for the reaction. The apparent enthalpy changes obtained from

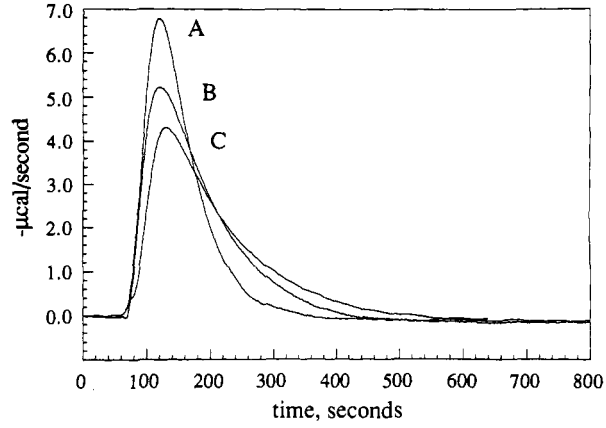

FIGURE 4: Typical reaction thermograms obtained at different ionic strengths. From top to bottom the curves correspond to (A) 50, (B) 0, and (C) 150 mM KCl. All the experiments shown were performed in 10 mM phosphate, pH 7.4, 0.5 mM EDTA, and 0.5% Tween-80. The observed enthalpy changes were −15.2, −15.6, and −15.7 kcal/mol of cytochrome *c*, respectively.

the calorimetric thermograms shown in Figure 3 were −15.3 ± 0.8, −11.47 ± 0.3, −9.16 ± 0.3 and −5.38 ± 0.82 kcal/mol for the experiments performed in phosphate, MOPS, Tricine, and Tris buffers, respectively. As predicted by eq 3, a plot of these values versus the enthalpy of ionization of the buffers yields a straight line with a correlation coefficient of 0.9998. The slope is equal to the number of protons released by the buffer and the *x*-axis intercept is equal to the intrinsic enthalpy for the reaction. A linear regression of the data indicates a net release of one (0.964) buffer proton per cytochrome *c* oxidized, in agreement with literature values (Wikström, 1977; Wikström et al., 1981), and an intrinsic reaction enthalpy of −16.5 kcal/mol of ferrocytochrome *c*. Until now, the intrinsic enthalpy for the enzymatic oxidation of ferrocytochrome *c* has not been measured directly even though indirect methods have yielded values of −15 kcal/mol at pH 7.4 (Watt & Sturtevant, 1969). The Gibbs free energy for the oxidation of ferrocytochrome *c* has been determined to be on the order of −11.5 kcal/mol of electrons transferred at 25 °C (Wikström, 1981). With the enthalpy change measured here, the entropy change for the reaction can be calculated to be −16.8 cal/K mol at 25 °C.

Effects of Ionic Strength. Figure 4 displays cytochrome *c* oxidase activity thermograms obtained at different ionic strengths. As can be seen in the Figure, the rate of heat evolution varies with the concentration of KCl present in the medium while the reaction enthalpy, the area under each curve, remains constant. In particular, it should be noted that the rate obtained at 50 mM KCl is faster than the rates observed at lower or higher KCl concentrations. Figure 5 (panel A) displays the calculated rate constants (expressed as specific activities) determined both calorimetrically and spectrophotometrically for a total of nine salt concentrations. The specific activity of the enzyme was found to increase with increasing KCl concentration until approximately 50 mM. As the KCl concentration is raised further, the specific activity decreases monotonically until it reaches a value of 0.75×10^2 min^{-1} mg^{-1} at 200 mM KCl. Similar ionic strength dependencies were observed for detergent-solubilized cytochrome oxidase and for the protein when reconstituted into DMPC vesicles. Previously, a similar dependence of oxidase activity upon ionic strength was reported for cytochrome *c* oxidase purified from other sources (Davies et al., 1964; Wilms et al., 1981; Bolli et al., 1985; Reimann et al., 1988). Figure 5 (panel B) displays the reaction enthalpies obtained for the cytochrome *c* oxidase

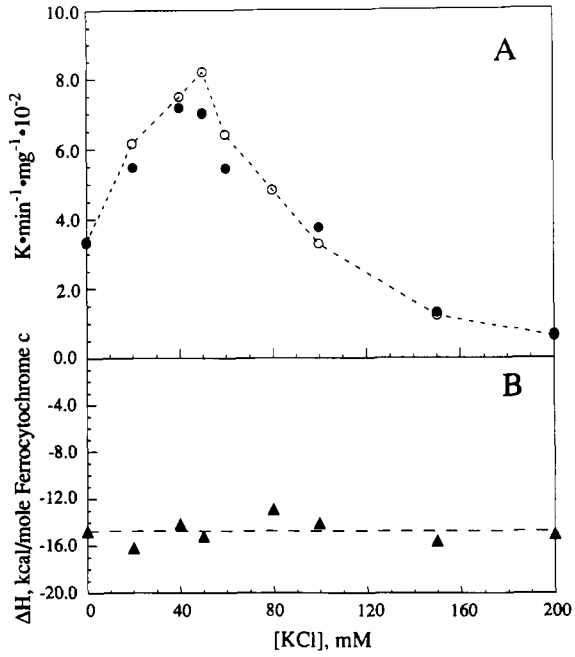

FIGURE 5: (A) Ionic strength dependence of the electron transfer activity as measured calorimetrically (closed circles) and spectrophotometrically (open circles) in the presence of 10 mM potassium phosphate and 0.5 mM EDTA, pH 7.40. (B) The enthalpy change associated with the reaction is independent of ionic strength within the experimental error.

reaction at different ionic strengths. In these experiments it can be seen that, within experimental error, the observed reaction enthalpy remains constant over the range of salt concentrations tested. This lack of ionic strength effect on the reaction enthalpy should be contrasted with the large ionic strength dependence observed for the reaction rate and further indicates that the ionic strength effect is of an entropic origin.

Differential Scanning Calorimetry. In order to test for any possible effects of ionic strength on the structural stability of the enzyme, the thermal stability of the protein was measured by high-sensitivity differential scanning calorimetry. The excess heat capacity curves obtained by differential scanning calorimetry for the thermal denaturation of the DMPC membrane reconstituted enzyme at low and high ionic strengths are shown in Figure 6. As shown in the figure, at low ionic strength the unfolding profile is characterized by two well-resolved peaks. These peaks are centered at 50.7 ± 0.2 °C and 59.9 ± 0.2 °C and contain approximately 43 ± 4% and 57 ± 4% of the total enthalpy, respectively. The differential scanning calorimetry data represent the average of three independent calorimetric scans under each condition. At high ionic strength (150 mM KCl), the high-temperature peak is shifted downward 2.5 °C and becomes slightly broader while the low-temperature peak remains at the same position and unchanged. Under these conditions, the calorimetric profile consists of a single albeit highly asymmetric peak as shown in Figure 6. Within the experimental error, the enthalpy change for the thermal unfolding was independent of ionic strength. Deconvolution of the calorimetric profiles obtained at high and low ionic strengths indicates that the relative intensities of the two peaks remain approximately the same within the experimental error. These results indicate that the ionic strength effect on the differential scanning calorimetry data most likely reflects a change in the stability of the subunits that comprise the high-temperature peak since this is the peak affected by ionic strength. Previously, the high-temperature peak has been associated with the thermal denaturation of the

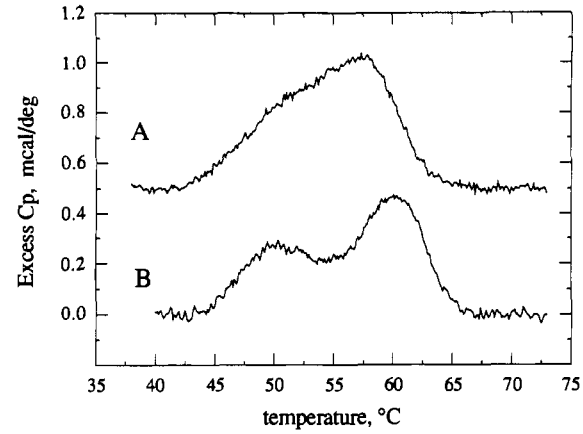

FIGURE 6: Excess heat capacity function versus temperature for DMPC-reconstituted yeast cytochrome *c* oxidase in the presence of 150 mM KCl (curve A) and in the absence of additional KCl (curve B). In both experiments, the buffer used was 10 mM potassium phosphate, pH 7.40. The scans were performed at a rate of 45 °C/h.

major subunits I and II for both the beef and yeast enzymes (Rigell & Freire, 1987; Morin et al., 1989). For the beef enzyme, the low-temperature peak has been shown unambiguously to reflect the thermal denaturation of subunit III. It is noteworthy that the transition temperature of the low-temperature peak changes less than 0.2 °C, suggesting a low degree of correlation between the low-temperature and high-temperature peaks.

Isothermal Titration Calorimetry. In order to test directly the effect of ionic strength on the enzyme conformation, the heat effects associated with the transfer of the enzyme from low to high ionic strength were measured calorimetrically at constant temperature. For these experiments, enzyme aliquots equilibrated at low ionic strengths were injected into the calorimeter reaction cell containing high ionic strength buffer. Control experiments were performed by injecting the enzyme into the same buffer (heat of dilution of the enzyme) and by injecting the low ionic strength buffer into the high ionic strength buffer. In all experiments only negligible heats of reaction were observed within the experimental salt concentration range considered (0–200 mM KCl, 10 mM potassium phosphate). The small size of the evolved heats (<5 kcal/mol) and the lack of dependence upon ionic strength were consistent with the absence of any enthalpic change induced by ionic strength. Enthalpic effects are usually associated with conformational rearrangements involving changes in secondary structure. The absence of significant enthalpic changes upon transferring the enzyme from low to high ionic strengths, in the differential scanning calorimetric profiles at low and high ionic strengths and in the reaction enthalpy at low and high ionic strengths, indicates that the ionic strength effect on the enzyme is of an entropic nature and that it does not involve a major structural change in the enzyme. Most likely, ionic strength affects the stability and interactions between subunits I and II, primarily responsible for the main transition peak observed by differential scanning calorimetry.

Discussion

The idea of measuring reaction kinetics using calorimetry is very old [see Sturtevant (1937) and Randzio and Suurkuusk (1980)]; however, the technologies to accomplish this in very dilute macromolecular solutions have been developed only recently. In this paper, high-sensitivity isothermal reaction calorimetry has been used to assesss simultaneously the kinetics and energetics of the enzymatic reaction between yeast cytochrome *c* oxidase and its biological substrate cytochrome

c. While calorimetry is unique in its ability to measure the reaction enthalpy, this paper demonstrates that enzyme kinetic parameters can be measured with a precision similar to that of the standard spectroscopic method. It must be noted that, in general, the calorimetric assay does not require the sample to be optically clear or to exhibit any optical changes associated with the reaction, thus providing a very general enzymatic assay procedure.

The calorimetric measurements presented here indicate that the intrinsic enthalpy for the enzymatic oxidation of cytochrome c is -16.5 ± 0.2 kcal/mol and independent of the ionic strength. The reaction consumes one proton (0.96) for every ferrocytochrome c molecule oxidized as determined by experiments performed in buffers with different enthalpies of ionization. The measured thermodynamic parameters for the reaction were independent of ionic strength.

The effect of ionic strength on the specific activity of beef heart cytochrome c oxidase has been studied in the past (Davies et al., 1964; Wilms et al., 1981; Reimann et al., 1988; Koissekova et al., 1989). The experiments described in this paper demonstrate a similar behavior for the yeast enzyme. Even though the precise origin of the effect remains unclear, the present thermodynamic data provide precise energetic constraints to any proposed mechanism. First, we have found that the transfer of the enzyme from a low to a high ionic strength medium is not accompanied by a significant enthalpy change (<5 kcal/mol) and that the magnitude of this change does not vary with ionic strength. Second, differential scanning calorimetry indicates that upon increasing ionic strength the main transition peak shifts to lower temperatures and becomes somewhat broader. Since the main transition peak has been assigned to the major subunits I and II (Morin et al., 1990), the calorimetric data are consistent with an ionic strength effect mediated by subunits I and II. Judging from the scanning calorimetric results, these subunits are less stable at high salt concentrations and appear to exhibit diminished cooperative interactions.

Several hypothesis have been advanced to account for the ionic strength dependence of the enzymatic activity of cytochrome c oxidase. It has been postulated [see Cooper (1990) for a recent review] that cytochrome c oxidase has two binding sites for cytochrome c, one catalytic and the other regulatory. These sites presumably have different ionic strength binding dependencies. It has also been postulated that the ionic dependence of the activity is mediated by the ionic effects on the monomer–dimer equilibrium of the enzyme (Kossekova et al., 1989). Finally, a third hypothesis postulates the existence of two different conformations of the enzyme (Michel & Bossard, 1989). These authors dealt primarily with the salt dependence of the biphasic response of cytochrome c oxidase to the concentration of cytochrome c and found the data to be consistent with the existence of two conformations of the enzyme. Michel and Bosshard (1989) hypothesized that, during the enzymatic cycle, one of the conformations exists when the enzyme accepts the protons and the other one after it releases the protons. According to this hypothesis, high ionic strength favors the second conformation, resulting in decreasing enzymatic rate constants at increasing ionic strengths.

It is clear from the calorimetric data that ionic strength induces a change in the enzyme: either a conformational change, a change in the state of aggregation, or both. The data presented in this paper are consistent with the existence of an ionic strength induced conformational change in the enzyme; however, since the salt effect is predominantly entropic, the conformational change should not be associated with a major change in secondary structure and most likely involves a change in the functional coupling between subunits I and II. Hazzard et al. (1991) have recently pointed out that, in the complex between cytochrome c and cytochrome c oxidases, the majority of the observed spectroscopic changes are related to cytochrome c with minimal, if any, effects observed in the oxidase molecule. The same authors also pointed out that any conformational changes in the oxidase upon cytochrome c binding must be coupled to electron transfer to heme a. This view is consistent with the calorimetric results presented in this paper suggesting that the effect of ionic strength is most likely associated with a small conformational change that results in an altered functional coupling between subunits I and II. The absence of any significant enthalpy change associated with the transfer of the enzyme from a low to a high ionic strength medium is indicative of the absence of a major salt-dependent conformational change in the oxidase molecule. To get an approximate idea of the magnitude of this change, we may consider that the enthalpy change for the complete unfolding of the enzyme is on the order of 450 kcal/mol (Morin et al., 1990), whereas the ionic strength induced change is only ~5 kcal/mol; i.e. about 1% of the structure. The calorimetric data by itself cannot rule out the possibility that the above changes are triggered by a salt-induced change in the state of aggregation of the protein as a function of ionic strength. If this is the case, however, those changes in the state of aggregation must be linked to changes in the stability and interactions of the main enzyme subunits as indicated by the calorimetric experiments.

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Registry No. Cytochrome c oxidase, 9001-16-5; cytochrome c, 9007-43-6.

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CORRECTION

Modulation of Plasminogen Activation and Type IV Collagenase Activity by a Synthetic Peptide Derived from the Laminin A Chain, by Sharon Stack, Robert D. Gray, and Salvatore V. Pizzo*, Volume 30, Number 8, February 26, 1991, pages 2073–2077.

Page 2075. In column 1, the sentence beginning on line 16 should read as follows: In addition, treatment of a modified form of the peptide, in which Gln_7 was substituted by Glu, with *Staphylococcus aureus* V8 proteinase, which introduces a single cleavage in the peptide (verified by HPLC, data not shown), removed the stimulatory effect, providing further evidence that the intact peptide is necessary for stimulation of Pg activation. The stimulatory effect of the intact Glu_7-substituted peptide was identical with that of the original Gln-containing peptide (data not shown).