Protein and Lipid Structural Transitions in Cytochrome *c* Oxidase–Dimyristoylphosphatidylcholine Reconstitutions†

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ABSTRACT: The thermotropic behavior of the mitochondrial enzyme cytochrome *c* oxidase (EC 1.9.3.1) reconstituted in dimyristoylphosphatidylcholine (DMPC) vesicles has been studied by using high-sensitivity differential scanning calorimetry and fluoresence spectroscopy. The incorporation of cytochrome *c* oxidase into the phospholipid bilayer perturbs the thermodynamic parameters associated with the lipid phase transition in a manner analogous to other integral membrane proteins: it reduces the enthalpy change, lowers the transition temperature, and reduces the cooperative behavior of the phospholipid molecules. Analysis of the dependence of the enthalpy change on the protein:lipid molar ratio indicates that cytochrome *c* oxidase prevents 99 ± 5 lipid molecules from participating in the main gel–liquid-crystalline transition. These phospholipid molecules presumably remain in the same physical state below and above the transition temperature of the bulk lipid, thus providing a more or less constant microenvironment to the protein molecule. The effect of the phospholipid bilayer matrix on the thermodynamic stability of the cytochrome *c* oxidase complex was examined by high-sensitivity differential scanning calorimetry. Detergent (Tween 80)-solubilized cytochrome *c* oxidase undergoes a complex, irreversible thermal denaturation process centered at 56 °C and characterized by an enthalpy change of 550 ± 50 kcal/mol of enzyme complex. Reconstitution of the cytochrome *c* oxidase complex into DMPC vesicles shifts the transition temperature upward to 63 °C, indicating that the phospholipid bilayer moiety stabilizes the native conformation of the enzyme. The lipid bilayer environment contributes ~10 kcal/mol to the free energy of stabilization of the enzyme complex. The thermal unfolding of cytochrome *c* oxidase is not a two-state process. Deconvolution analysis of the heat capacity function indicates that the overall curve is composed of at least four sequential melting steps. The calorimetric experiments have been complemented with thermal gel electrophoresis experiments directed to identify the enzyme subunits in the main melting steps. According to these experiments, the first melting step, for the membrane-reconstituted enzyme, at 52 °C, involves primarily subunit III. This step is followed by two closely spaced melting steps at 61 and 64 °C involving the bulk of the enzyme complex. According to the thermal gel analysis experiments, subunits I, II, IV, VII, and VIII melt between 60 and 65 °C. The melting of subunits V and VIb could not be detected by gel electrophoresis, and their melting temperatures could not be assigned.

The interaction of intrinsic membrane proteins with the lipid components of the membrane has been the subject of considerable interest in the past few years. Numerous studies have shown that this interaction is mostly of a local nature, involving primarily the protein molecule itself and the phospholipid molecules immediately adjacent to the protein (Chapman et al., 1979; Marsh et al., 1982; Freire et al., 1983). The properties of these lipid molecules, sometimes referred to as boundary lipid, differ in several respects from those of the bulk lipid (Jost et al., 1973; Kang et al., 1979). Many studies using differential scanning calorimetry have shown that the insertion of an intrinsic membrane protein into a phospholipid bilayer membrane results in a reduced enthalpy change for the phospholipid gel–liquid-crystalline transition (Alonso et al., 1982; Freire et al., 1983). This decreased enthalpy is consistent with a molecular picture in which the phospholipid molecules immediately adjacent to the protein are withdrawn from the phase transition, therefore remaining in an energetically equivalent configuration below and above the transition temperature of the bulk lipid. Studies using fluorescent lipid probes suggest that these molecules adopt an intermediate conformation between the gel and liquid-crystalline state (Kimelman et al., 1979; Freire et al., 1983).

Whereas numerous articles have appeared regarding the perturbation of lipid behavior by protein molecules, very little is known regarding the opposite effect, i.e., the perturbation of protein conformation by lipid molecules and/or the nature of protein conformational changes within the lipid bilayer matrix. In this paper, we also address this question using the mitochrondrial enzyme cytochrome *c* oxidase (EC 1.9.3.1) reconstituted into dimyristoylphosphatidylcholine vesicles as a model system to study the thermodynamics of the protein–lipid interaction. Cytochrome *c* oxidase is the terminal enzyme of the mitochrondrial electron-transport chain catalyzing the transfer of electrons from cytochrome *c* to molecular oxygen. Also, it has been demonstrated that cytochrome *c* oxidase exhibits proton-pumping ability and this activity is dependent on the presence of subunit III in the enzyme complex (Wikstrom et al., 1981; Penttila, 1983). Even though the enzyme requires a hydrophobic environment to function properly, no specific lipid requirements have been demonstrated except for a tight association with cardiolipin (Fry & Green, 1980). The activity of the enzyme does depend on the fluidity of the bilayer or degree of acyl chain unsaturation and appears to be insensitive to variations in phospholipid head group (Vik & Capaldi, 1977).

The studies in this paper address both the effects of cytochrome *c* oxidase on the thermotropic behavior of the phospholipid molecules and also the conformational stability of the

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protein within the bilayer matrix. The use of high-sensitivity differential scanning calorimetry in conjunction with fluorescence and absorption spectroscopy, as well as thermal gel electrophoresis analysis, has allowed us to identify and characterize these interactions from a thermodynamic and structural point of view.

Experimental Procedures

Materials. Dimyristoylphosphatidylcholine (DMPC) was obtained from Avanti Biochemicals (Birmingham, AL) and used without further purification. Sodium cholate and sodium deoxycholate were purchased from Sigma (St. Louis, MO) and recrystallized 3 times in ethanol to remove bile salt contaminants as described by Wikstrom (1979). Horse heart cytochrome *c* type VI was obtained from Sigma (St. Louis). Sodium hydrosulfite (dithionite) and potassium ferricyanide from Fisher Scientific (Fair Lawn, NJ) were used as reductant and oxidant, respectively, in the spectrophotometric determination of isolated cytochrome oxidase heme *a* content and activity.

Cytochrome oxidase was isolated from bovine heart following the protocol of Capaldi & Hayashi (1972) with some modifications. An additional ammonium sulfate fractionation step was necessary to obtain a purified preparation of the enzyme. To the final supernatant obtained from the aforementioned method, a saturated, neutralized ammonium sulfate solution was added (8 mL/100 mL of supernatant) and centrifuged as described. The resulting pellet was resuspended in 0.1 M phosphate buffer, pH 7.4, and dialyzed against the same buffer to remove ammonium sulfate and residual detergents before use in the following experiments. The heme *a*:protein ratio ranged from 9 to 11 nmol/mg over several preparations of the purified cytochrome oxidase using an extinction coefficient of $\epsilon_{605nm}^{red-ox} = 13.5\ mM^{-1}\ cm^{-1}$.

Cytochrome oxidase activity was measured spectrophotometrically with a Beckman DB UV–vis spectrophotometer according to the method of Smith (1955). When assaying the purified nonreconstituted enzyme, the medium was made 0.5% Tween 80. Cytochrome *c* was reduced by addition of a small amount of sodium hydrosulfite. Excess reductant was removed by using a rapid gel filtration–centrifugation technique (Tuszinski et al., 1980). Typical activities were in agreement with other reports, ranging from 8 to 11 μmol of cytochrome *c* oxidized min^{-1} (mg of protein)$^{-1}$ (Capaldi & Hayashi, 1972).

Protein concentrations were determined by the method of Lowry (Lowry et al., 1951), and phosphate analyses were performed by a modification of the Bartlett method as described by Marinetti (1962). The average amount of phospholipid phosphate associated with the purified oxidase was 0.14 μmol of phospholipid phosphate/mg of protein. Thin-layer chromatography according to the method of Robinson and Capaldi indicated that cardiolipin, phosphatidylcholine, and phosphatidylethanolamine were present as was reported for this preparation (Robinson & Capaldi, 1977).

Cytochrome Oxidase Reconstitution. Cytochrome oxidase was reconstituted with DMPC to form large unilamellar vesicles by using detergent dialysis. Typically, 5 mg of DMPC in chloroform was dried under a stream of N_2 and desiccated overnight. To the dried lipid was added 200 μL of 40 mM KH_2PO_4 and 40 mM *N*-[tris(hydroxymethyl)methyl]glycine (Tricine), pH 7.5 (buffer A), containing 1.5% cholate which was then vortexed to solubilize the DMPC. Cytochrome oxidase solution was added to obtain the desired protein to lipid ratio, and the solution was diluted to 1.0 mL with buffer A. This suspension was sonicated at 4 °C for 5 min by using a Laboratory Supplies Co. bath sonicator. Following sonication, the samples were dialyzed for 4 h against buffer A and then against 10 mM Tricine, pH 7.5, for 24 h. To determine the amount of residual cholate present after dialysis, [^{3}H]cholate was used as a tracer. Less than 4 mol of cholate/mol of cytochrome oxidase was present after 24 h of dialysis. The presence of residual cholate was also monitored by differential scanning calorimetry of pure DMPC reconstitutions as a function of dialysis time. Up to 20 h of dialysis, there was a shift in the T_m of the endotherms from 22.5 to 23.2 °C. Beyond this time, there was no further shift in the T_m of the transitions, indicating that the removal of cholate by dialysis was complete at this point with a cholate:lipid ratio of 1:200 as determined by the radioactive tracer. Evidence for incorporation was obtained from the comigration of protein and lipid as demonstrated by sucrose density gradient centrifugation of reconstituted cytochrome oxidase compared to protein and lipid control experiments. The diameter of the reconstituted cytochrome oxidase–DMPC vesicles, determined by negative-stain electron microscopy, ranged from 750 to 2000 Å. Cytochrome oxidase activity of the reconstituted samples was measured as described without the addition of Tween 80 to the sample and fell within the range of 2.5–4 μmol min^{-1} (mg of protein)$^{-1}$.

Cytochrome oxidase was also reconstituted with DMPC for high-temperature differential scanning calorimetry and temperature-dependent subunit composition analysis by gel electrophoresis using the following method. Lipid was dried under nitrogen and desiccated to remove solvent; 0.1 M PO_4, pH 7.4, buffer was added, and the lipid was suspended by vortexing and mild sonication in a bath sonicator. To this suspension was added cytochrome oxidase (20 mg/mL) to obtain the desired protein to lipid ratio. The sample was then vortexed and mildly sonicated to obtain a homogeneous suspension before calorimetry and electrophoresis.

Differential Scanning Calorimetry. Calorimetric experiments were performed with a Microcal MC1 differential scanning calorimeter. The sensitivity and precision of the basic calorimetric unit have been improved by the use of two Keithley amplifiers connected to the heat capacity and temperature outputs of the calorimeter and interfaced to an IBM PC microcomputer using a Data Translation (DT-805) A/D conversion board for automated data collection and analysis. With pure lipid dispersions, concentrations lower than 0.5 mg/mL can be used with a total sample volume of 0.7 mL. For the cytochrome oxidase reconstitutions by cholate dialysis, lipid concentrations of 5 mg/mL were used. All the calorimetric scans were performed at a scanning rate of 15 °C/h, except when noted otherwise.

Sodium Dodecyl Sulfate (SDS)–Polyacrylamide Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis was performed essentially by the method of Kadenbach et al. 1983. A Bio-Rad Protean vertical slab gel apparatus (16 × 18 × 0.15 cm) was used to run the gels. An 18.75% acrylamide separation gel [32:1 acrylamide–*N*,*N*′-methylenebis(acrylamide)] with an 8.3% acrylamide stacking gel was used with the buffers described in the aforementioned method. Electrophoresis was run for 1–2 h at 80 V (constant) and then for 9 h at 200 V (constant). Gels were stained by incubation in 0.2% Coomassie blue in 5:4:1 methanol–water–acetic acid at 45 °C with shaking for 30 min. Destaining was accomplished by incubation overnight at room temperature in a 8:1:1 water–methanol–acetic acid solution. Electrophoresis of the purified cytochrome oxidase exhibited 10 bands, including the 7 bands reported by Downer et al. (1976) with three other bands corresponding to VIa, VIc, and VIII according to the no-

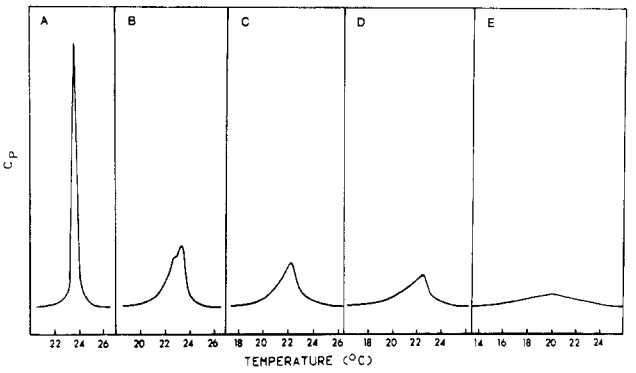

FIGURE 1: Excess heat capacity function of cytochrome *c* oxidase–DMPC reconstitutions at different protein:lipid molar ratios: (A) P:L = 0; (B) P:L = 1:1200; (C) P:L = 1:800; (D) P:L = 1:400; (E) P:L = 1:200. All the scans were run at a scanning rate of 15 °C/h in 10 mM Tricine, pH 7.5. Sample concentrations in the calorimeter cell were 5 mg of DMPC/mL.

menclature of Kadenbach et al. (1983).

The same electrophoretic technique was used for thermal gel analysis experiments designed to examine the effect of temperature on the subunit structure of the enzyme. Cytochrome *c* oxidase samples, either dissolved in 0.1 M PO_4, pH 7.4, containing 0.1% Tween 80 or reconstituted in phospholipid vesicles, were placed in the calorimeter and scanned to the desired temperature. The scans were terminated at the desired temperature and the samples removed. Immediately after removal from the calorimeter, the membrane-reconstituted cytochrome oxidase samples were made 0.1% Tween 80 and sonicated briefly (few seconds) in a bath sonicator. Each sample was centrifuged in a Fisher Model 235A microcentrifuge at 15000*g* for 5 min to sediment insoluble protein. The resulting supernatant was removed and examined for subunit composition by electrophoresis as described. A somewhat similar approach has been used before to study the aggregation of membrane proteins in red blood cells (Lysko et al., 1981).

Fluorescence Spectroscopy. Steady-state fluorescence depolarization experiments were performed by using a Perkin-Elmer LS-5 spectrofluorometer equipped with 3M 105ML glass polarizers in the excitation and emission beams. The temperature of the cuvette was controlled with a Neslab RTE-8 refrigerated bath circulator and the temperature monitored within ±0.1 °C with a Keithley digital thermometer. 1,6-Diphenyl-1,3,5-hexatriene (DPH) (Molecular Probes, Junction City, OR) was dissolved in acetonitrile and added to the vesicle suspensions at a ratio of 1 probe per 500 phospholipid molecules. All samples were incubated for 1 h at 24 °C prior to the experiments to ensure complete equilibration of the fluorescent probe. The total lipid concentration for these experiments was 0.2 mg/mL. The excitation wavelength was set at 360 nm, and the emission intensity was measured at 430 nm parallel and perpendicular to the plane of excitation. Anisotropy was calculated as described in a previous communication (Freire et al., 1983).

Spectroscopic Measurements. Absorption spectra of cytochrome oxidase used in the calculation of heme *a* to protein ratios and in determining the effect of temperature on the protein conformational state were measured by using an Aminco-Bowman DW-2 spectrophotometer equipped with a thermostated cuvette holder.

RESULTS

Cytochrome c Oxidase Perturbation of the Phospholipid Main Transition. Figure 1 shows the excess heat capacity function associated with the main transition of dimyristoyl-

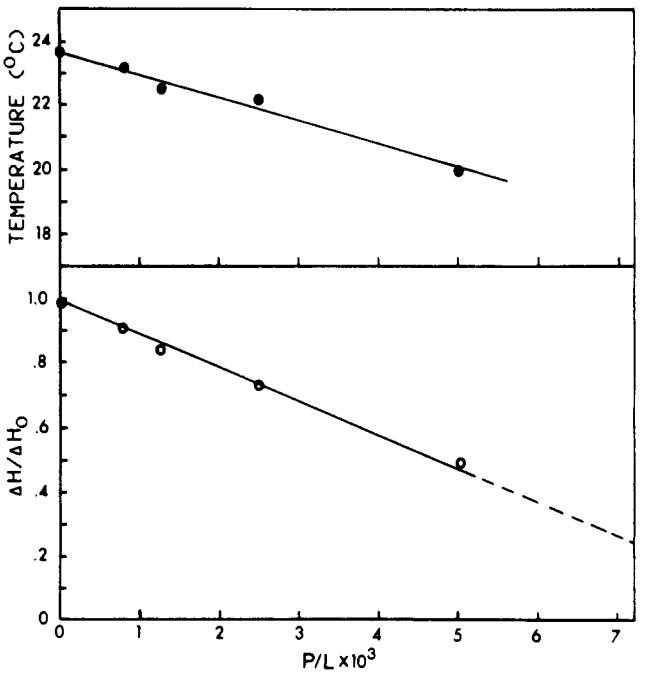

FIGURE 2: Dependence of thermodynamic parameters for the DMPC main gel–liquid-crystalline transition on the protein:lipid molar ratio.

phosphatidylcholine reconstituted with different amounts of cytochrome *c* oxidase. All the samples in this sequence of scans were passed through the lipid phase transition temperature immediately before the calorimetric experiments. Samples treated in this way yielded highly reproducible calorimetric scans; i.e., repeated scans of the same sample gave identical calorimetric traces, indicating that the membrane preparations were in an equilibrium configuration. Samples kept at 4 °C and scanned without having been at or above the phospholipid main transition temperature gave rise to a metastable peak centered at 17 °C. This metastable peak disappears after passage of the sample through the main lipid transition and most likely arises from the presence of extended bilayer sheets in samples that have never been annealed by passage through the phase transition. In this respect, Lentz et al. (1985) have previously observed that cold reconstitution of the calcium pump protein of sarcoplasmic reticulum results in extended bilayer sheets and that these sheets close into vesicular structures upon passage of the sample through the lipid phase transition. As shown in the figure, the incorporation of cytochrome *c* oxidase has a pronounced effect on the thermotropic behavior of the phospholipid molecules. At protein:phospholipid molar ratios as low as 1:1200, cytochrome *c* oxidase induces a significant broadening of the heat capacity function. The half-height width ($\Delta T_{1/2}$) of the pure phospholipid transition is 0.4 °C, whereas at a protein:phospholipid ratio of 1:1200 it becomes 1.5 °C and at a protein:phospholipid ratio of 1:200 it is more than 4 °C. This broadening of the phospholipid phase transition indicates that cytochrome *c* oxidase greatly disrupts the cooperative behavior of the phospholipid molecules. In the absence of protein, the cooperative unit size is 300 molecules, and at a protein:lipid ratio of 1:200, it is only 50 phospholipid molecules. Parallel to these changes in cooperative behavior, the incorporation of the protein shifts the transition temperature to lower temperatures and reduces the magnitude of the enthalpy change (ΔH) associated with the phospholipid phase transition. These results are summarized in Figure 2. The decrease in ΔH upon increasing the protein:lipid ratio indicates that the protein molecules are preventing some of the phospholipid molecules

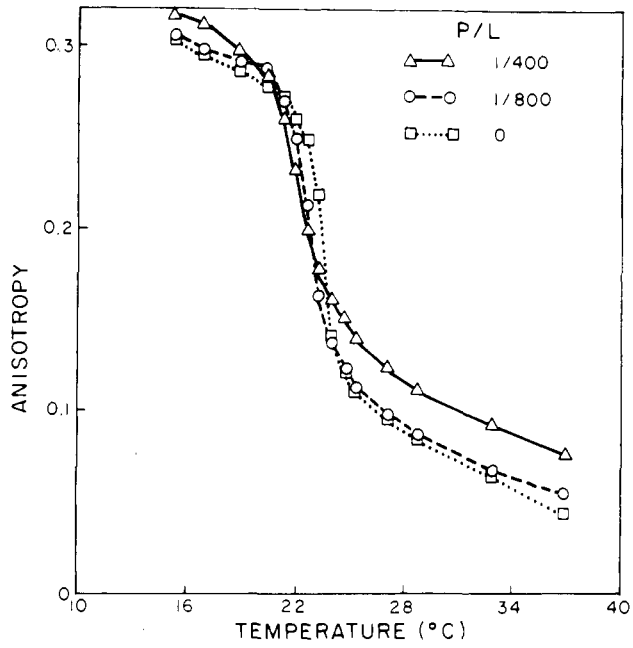

FIGURE 3: Steady-state fluorescence anisotropy of DPH as a function of temperature for pure DMPC vesicles (□) and for DMPC vesicles containing cytochrome *c* oxidase at molar ratios of 1:800 (○) and 1:400 (△).

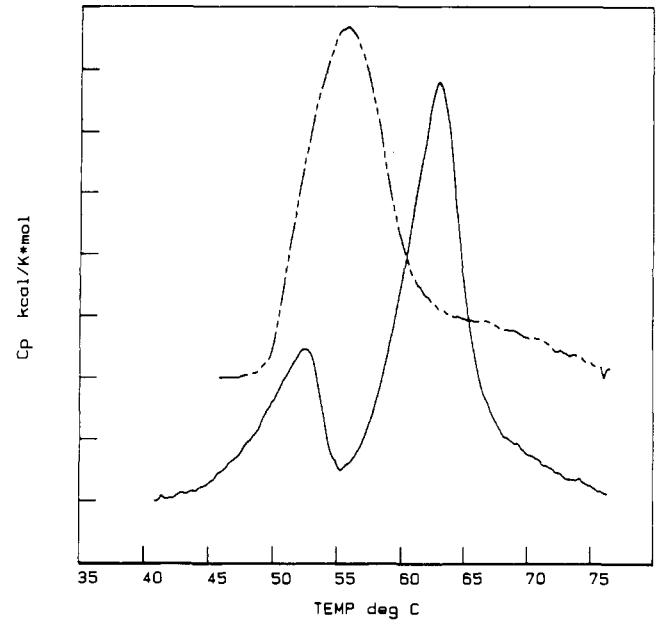

FIGURE 4: Excess heat capacity function for cytochrome oxidase solubilized in 0.1% Tween 80 and 0.1 M PO_4, pH 7.4 (dashed line), and reconstituted in DMPC vesicles at a protein:lipid ratio of 1:130 (solid line). The scanning rate for this experiment was 41.9 °C/h.

from undergoing the lipid phase transition. The dependence of ΔH on the protein:lipid molar ratio can be analyzed in terms of the equation (Correa-Freire et al., 1979; Freire et al., 1983)

$$\Delta H/\Delta H_0 = 1 - N_a(P/L)$$

where ΔH_0 is the enthalpy change in the absence of protein and N_a is the mean number of lipid molecules prevented from participating in the gel–liquid-crystalline transition per protein molecule. As shown in Figure 2, a linear least-squares analysis of the data indicates that each cytochrome *c* oxidase molecule prevents 99 ± 5 phospholipids from participating in the phase transition.

The thermotropic behavior of the reconstituted vesicles was also examined by steady-state fluorescence anistropy measurements using DPH. A typical sequence of experiments is shown in Figure 3. In agreement with the calorimetric results, the fluorescence results also report a downward shift in the transition temperature and a broadening of the transition upon increasing the protein:phospholipid ratio. Most significant, however, is the observation that the fluorescence probe reports increasing anisotropy values above T_m with increasing protein:phospholipid ratios. These results are in agreement with those found by Kinosita et al. (1981) and indicate that the incorporation of cytochrome *c* oxidase increases the apparent order of the lipid bilayer above T_m, suggesting that the phospholipid molecules perturbed by the protein are in a more ordered configuration than the bulk fluid lipid. These results are also in agreement with ESR results using spin-label probes (Jost et al., 1973; Marsh et al. 1982) which indicate that, in the fluid phase, a fraction of the phospholipid molecules is partially immobilized by the presence of the protein.

Thermal Unfolding of Cytochrome c Oxidase. The thermal unfolding of cytochrome *c* oxidase was also studied by using high-senstivity differential scanning calorimetry in both detergent-solubilized and membrane-reconstituted samples. As shown in Figure 4, the detergent-solubilized (Tween 80) enzyme undergoes a broad unfolding transition centered at 56 °C. The overall unfolding transition is irreversible and characterized by a ΔH of 550 kcal/mol of enzyme assuming a molecular weight of 165 000 as reported by Deatherage et al. (1982a). The ratio of the calorimetric to the van't Hoff enthalpy (ΔH:ΔH_{VH}) was calculated according to the formula (Privalov & Khechinashvilli, 1974; Biltonen & Freire, 1978)

$$\Delta H/\Delta H_{VH} = \Delta H^2/C_{p,max}4RT_m^2$$

where ΔH is the calorimetric enthalpy (area under the heat capacity curve), $C_{p,max}$ is the maximum in the heat capacity function, and T_m is the transition temperature. The ratio ΔH:ΔH_{VH} is equal to 1 if the transition is of the two-state type and greater than 1 if more than two states are involved in the melting process. For the detergent-solubilized enzyme, the ratio ΔH:ΔH_{VH} is 5.7, indicating that the unfolding reaction is not a two-state process and that most likely involves the separate melting of its various subunits. It must be noted that the definition of two state or multistate above is related to the degree of cooperative interactions between subunits and only indirectly to the transition temperatures of each subunit; i.e., it is possible for all the subunits to have exactly the same transition temperature and for the transition to be multistate according to the $\Delta H/\Delta H_{VH}$ criteria. If the transition temperatures are different, then the overall transition is necessarily multistate (Biltonen & Freire, 1978).

Reconstitution of the cytochrome *c* oxidase molecule into DMPC vesicles or vesicles prepared with a phospholipid composition similar to that of the inner mitochondrial membrane (40% egg PC, 35% egg PE, and 25% cardiolipin; Krebs et al., 1979) resulted in an upward shift of 7 °C in the temperature of the maximum in the heat capacity function. The total ΔH for the transition remained the same within experimental error; however, the shape of the heat capacity function was not identical with the one obtained with the detergent-solubilized enzyme. In fact, the heat capacity function of the lipid-reconstituted enzyme was characterized by two well-defined peaks, a small peak centered at 52 °C and a larger peak centered at 63 °C. The smaller peak at 52 °C should not be confused with the peak of the detergent-solubilized enzyme at 56 °C. The 52 °C peak is absent in samples containing a protein:lipid molar ratio smaller than 1:50; it appears as a shoulder at a protein:lipid ratio of 1:50 and be-

Table I: Deconvolution of the Heat Capacity Function of Cytochrome *c* Oxidase[a]

	T_{m1}	ΔH_1	T_{m2}	ΔH_2	T_{m3}	ΔH_3	T_{m4}	ΔH_4
detergent solubilized	53.6	180	56.9	171	59.9	105	68.3	82
membrane reconstituted	51.7	139	60.7	160	63.5	195	68.4	89

[a] Temperatures are in degrees centigrade. Enthalpies are in kilocalories per mole of enzyme. The thermodynamic parameters in this table generate a heat capacity function with standard deviations of 1.0 and 1.6 kcal K^{-1} mol^{-1} for detergent-solubilized and membrane-reconstituted samples, respectively.

comes a distinct peak at protein:lipid molar ratios larger than 1:100.

The ratio of the calorimetric to the van't Hoff enthalpy for the low-temperature peak was 1.17, indicating that this peak approaches a two-state peak. The high-temperature peak, on the other hand, is characterized by a ΔH:ΔH_{VH} ratio of 2.5, clearly indicating that this peak involves more than two states.

The thermal unfolding of cytochrome *c* oxidase is also characterized by changes in the absorption and fluorescence emission spectra of the protein. The fluorescence emission spectrum (λ_{ex} 280 nm) is shifted to longer wavelengths, suggesting exposure of tryptophan residues to a more polar environment. The absorption spectrum also undergoes changes in the 55–70 °C temperature range as indicated in Figure 5. Similar spectral changes have been reported before (Kornblatt & Hui Bon Hoa, 1982) as a function of pH.

From a thermodynamic point of view, the incorporation of the cytochrome *c* oxidase molecule into a lipid bilayer has a 2-fold effect: (1) an overall stabilization of the enzyme complex amounting to an additional ~10 kcal/mol in the free energy of stabilization as indicated by the upward shift in the transition temperature; and (2) a modification in the magnitude and/or nature of the intersubunit interactions, reflected in the different shapes of the heat capacity profiles of the solubilized and membrane-reconstituted enzymes.

Deconvolution of the Heat Capacity Function of Cytochrome c Oxidase. It has been previously demonstrated (Freire & Biltonen, 1978) that the heat capacity function associated with the thermal unfolding of a macromolecule can be used to experimentally determine the partition function for the unfolding reaction and that this partition function can be used to estimate the number of steps in a sequential melting process as well as the thermodynamic parameters associated with each step. This deconvolution technique has been successfully applied to globular proteins as well as multidomain proteins (Freire & Biltonen, 1978; Biltonen & Freire, 1978; Privalov, 1982).

The heat capacity profiles of both detergent-solubilized and membrane-reconstituted cytochrome *c* oxidase were deconvoluted by using a two-pass deconvolution technique as described in the Appendix. The resulting deconvolution parameters were optimized by nonlinear least-squares analysis. The results of the deconvolution analysis are shown in Table I. Figures 6 and 7 show the deconvoluted curves as well as the theoretical curves obtained with the thermodynamic parameters in Table I. For the detergent-solubilized enzyme, the standard deviation between the theoretical and experimental curves is 1.0 kcal K^{-1} mol^{-1} and for the membrane-reconstituted sample, the standard deviation is 1.6 kcal K^{-1} mol^{-1}. In all cases, the thermal unfolding of cytochrome *c* oxidase was characterized by at least four melting steps. Attempts to use only three transitions yielded standard deviations for the fit larger than 4 kcal K^{-1} mol^{-1}, indicating that four transitions is the minimum number of transitions required to fit the data. Since the oxidase molecule has 7–13 subunits (Downer et al., 1976; Kadenbach et al., 1983), these melting steps, or at least some of them, must involve the simultaneous melting of more than one subunit. As shown in Table I, the

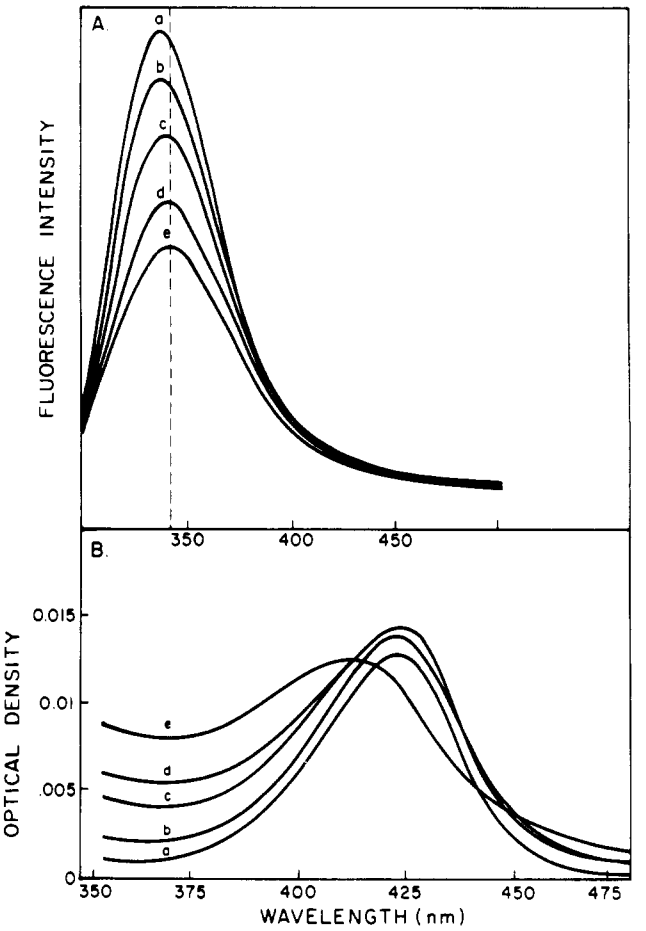

FIGURE 5: Steady-state tryptophan fluorescence spectra (A) and heme absorption spectra (B) vs. temperature for cytochrome *c* oxidase reconstituted into DMPC vesicles. The fluorescence spectra were measured at the following temperatures: (a) 36, (b) 43, (c) 51, (d) 61, and (e) 66 °C. The heme absorption spectra were measured at (a) 17, (b) 21, (c) 37.5, (d) 55.5, and (e) 72.2 °C.

enthalpies of two of the melting steps (steps 2 and 4) appear to be unchanged in both the detergent-solubilized and membrane-reconstituted enzymes, suggesting that they probably involve the same subunits in both samples. This is not the case for the first and third melting steps. The enthalpy of the first melting step is significantly larger in the detergent-solubilized enzyme whereas that of the third melting step is larger than in the membrane-reconstituted sample. It appears that some of the protein subunits that melt in the first step in the detergent-solubilized enzyme are greatly stabilized by the incorporation of the enzyme into the lipid bilayer and melt with the bulk of the enzyme in the third melting step. These results indicate that while membrane reconstitution induces an overall stabilization of the enzyme complex the magnitude of the stabilizing effect is not the same for all subunits. For example, the second melting step is only shifted upward 3 °C, and the fourth melting step remains unchanged with regard to both enthalpy and transition temperature. Thus, the most dramatic change induced by membrane reconstitution is the stabilization of some of the lower melting components of the enzyme

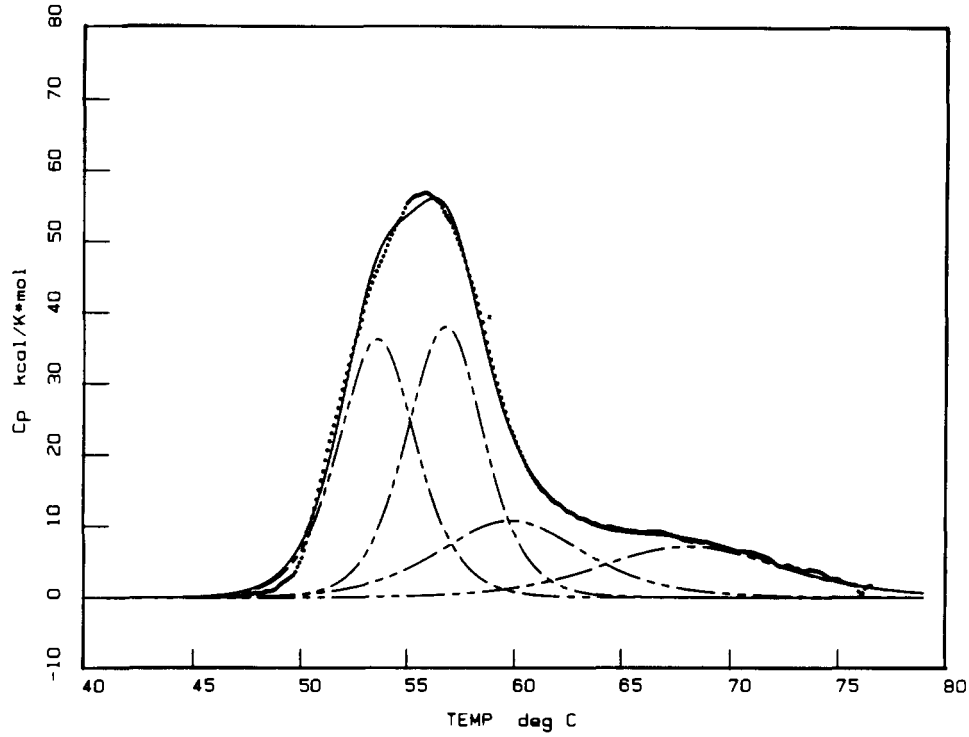

FIGURE 6: Deconvolution of the heat capacity function of detergent-solubilized cytochrome oxidase.

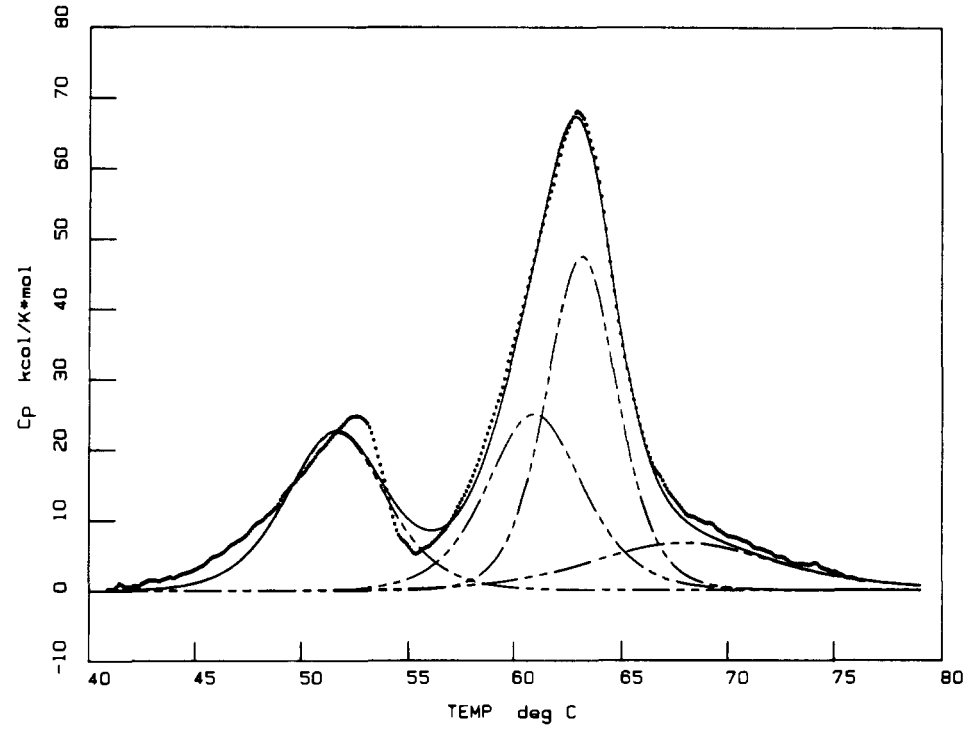

FIGURE 7: Deconvolution of the heat capacity function for membrane-reconstituted (DMPC) cytochrome *c* oxidase.

complex. The third melting step contributes the most (35% of the total enthalpy change) to the overall melting profile in the membrane-reconstituted enzyme whereas it only contributes 20% of the total enthalpy in the detergent-solubilized enzyme. In general, the bulk of the melting process occurs at a higher and narrower temperature interval in the membrane-reconstituted enzyme.

Subunit Contribution to the Denaturation of Cytochrome c Oxidase. The identification of the cytochrome *c* oxidase subunits undergoing thermal unfolding at any particular temperature was accomplished by thermal gel analysis as described under Experimental Procedures. Briefly, detergent-solubilized or membrane-reconstituted samples were scanned under conditions identical with those of the calorimetric experiments, removed at 5 °C intervals, detergent solubilized, and centrifuged to pellet any nonsoluble material. This technique allows separation of protein subunits on the basis of thermally induced solubility changes; thus, those cytochrome *c* oxidase subunits whose membrane solubility is lost upon denaturation will not be solubilized by detergent treatment and will be separated by centrifugation from those whose membrane solubility remains intact. Obviously, if the thermal

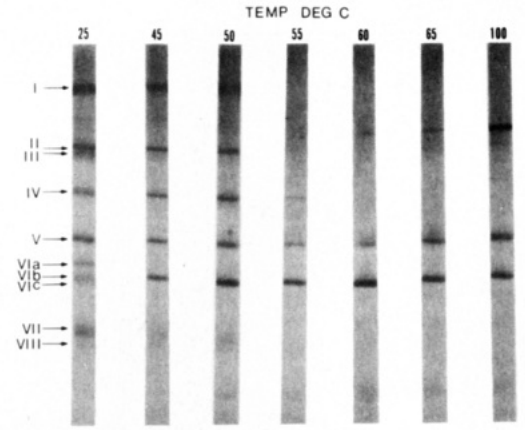

FIGURE 8: SDS–polyacrylamide gel of the detergent-soluble fraction following thermal denaturation of detergent-solubilized cytochrome *c* oxidase. Samples were heated to the indicated temperatures, and the postcentrifugation supernatant was run on an 18.75% polyacrylamide gel as described under Experimental Procedures.

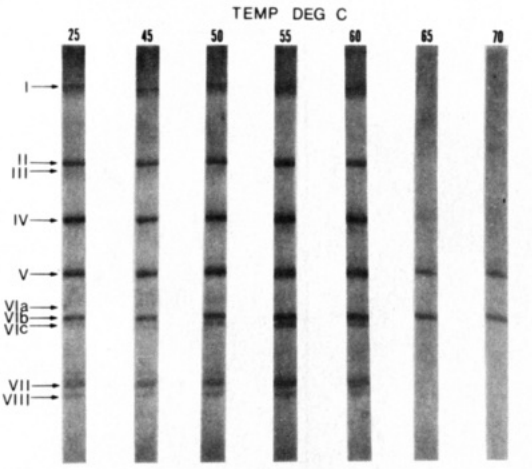

FIGURE 9: SDS–polyacrylamide gel of detergent-soluble subunits following thermal denaturation of cytochrome *c* oxidase reconstituted with DMPC.

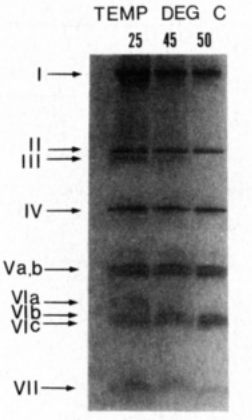

FIGURE 10: SDS–polyacrylamide gel of detergent-soluble subunits of cytochrome oxidase reconstituted with DMPC following incubation at 25, 45, and 50 °C. The samples were treated with 1% β-mercaptoethanol to improve resolution of subunit III.

denaturation is not associated with a solubility change, this technique will be insensitive to the process. Fortunately, in the case of cytochrome *c* oxidase (see below), only two of the subunits appear to be insensitive to thermal gel analysis studies.

The thermal gel analysis experiments were performed with detergent-solubilized and membrane-reconstituted samples. Figure 8 shows the resulting gel patterns for the detergent-solubilized samples (0.1% Tween 80). In all cases, the samples were heated under the same conditions up to the temperature indicated in the figure. At 25 °C, 10 bands are seen in the first lane of the gel. These bands correspond to subunits I, II, III, IV, V, VIa, VIb, VIc, VII, and VIII according to the nomenclature of Kadenbach et al. (1983). Subunit III, which does not stain well with Coomassie blue, is seen as a faint diffuse band immediately below the second band. The major change in the subunit composition of the gels occurs in the 50–60 °C temperature interval, in agreement with the calorimetric scans. In this temperature interval, the bulk of the enzyme complex (subunits I, II, and IV) undergoes thermal denaturation. Subunits V and VIb stayed soluble in the supernatant even after being heated at 100 °C for 5 min. This could result from no thermal denaturation of these subunits or from no thermally induced change in solubility upon denaturation. The high molecular weight band seen in the last three lanes is probably some soluble aggregation artifact of the denaturing enzyme complex at high temperatures.

A second series of samples containing cytochrome oxidase reconstituted with DMPC was subjected to the same procedure. Figure 9 is an SDS–polyacrylamide gel of the samples heated to 25, 45, 50, 55, 60, 65, and 70 °C. In this case, the major change in the gel occurs in the temperature interval between 60 and 65 °C. At this temperature, subunits I, II, VII, and VIII and most of subunit IV are no longer present in the soluble fraction. These changes in the gel correspond to the main transition peak observed in the calorimetric profile. As in the case of the detergent-solubilized enzyme, subunits V and VIb remain soluble even after incubation in boiling water for 5 min.

As shown in Figure 10, for a gel run in the presence of 1% v/v β-mercaptoethanol to improve subunit resolution (Kadenbach et al., 1983), subunit III is clearly present in the 25 °C temperature sample, becomes fainter at 45 °C, and is no longer discernible in the gel at 50 °C. This melting sequence for subunit III is in good agreement with the location of the first deconvoluted peak in the calorimetric results (See Figure 7) and indicates that subunit III is the first subunit to denature in the membrane-reconstituted system.

The detergent-solubilized enzyme and the DMPC-reconstituted enzyme exhibited marked differences in the subunit solubility experiments. The most important difference is the temperature at which the bulk of the enzyme denatures. According to the results of gel electrophoresis, the two largest subunits disappeared 5–15 °C higher in the DMPC-reconstituted system. Also, in the membrane-reconstituted system, the small molecular weight subunits VII and VIII denatured with the bulk of the enzyme at 60 °C, whereas in the detergent-solubilized enzyme, they appear to denature with subunit III at lower temperatures.

Discussion

The effect of cytochrome *c* oxidase on the thermodynamic behavior of DMPC vesicles resembles that observed with other integral membrane proteins: it produces a downward shift in the transition temperature, a broadening of the melting profile, and a reduction in the enthalpy change associated with the lipid- gel–liquid-crystalline transition. This reduction in enthalpy has been interpreted as arising from a decrease in the total number of phospholipid molecules which are able to undergo the gel–liquid-crystalline transition. Presumably, integral membrane proteins perturb their immediate lipid environment in such a way that the phospholipid molecules located in these regions of the bilayer are precluded from undergoing a cooperative phase transition, thus remaining in

more or less the same physical state below and above the transition of the bulk lipid. There are several lines of evidence, thermodynamic as well as spectroscopic, indicating that the perturbation induced by integral membrane proteins is of a local nature and that any overall effects arise from the superposition of local effects (Kimelman et al., 1979; Freire et al., 1983).

From the decrease in the enthalpy change associated with the phospholipid phase transition, we have estimated that each cytochrome oxidase molecule withdraws approximately 99 phospholipid molecules from the transition. This number is somewhat larger than the expected value of 50–60 calculated from the reported dimensions of the protein if only a single layer of lipid were perturbed by the protein; however, it is less than the number expected for two layers of lipid around the protein (Jost et al., 1973; Marsh et al., 1982; Deatherage et al., 1982b). Previously, calorimetric studies on different integral membrane proteins have reported perturbed lipid values ranging from one layer to as high as three to four layers of lipid (Freire et al., 1983; Hesketh et al., 1976; Curatolo et al., 1977). The lipid perturbation induced by cytochrome *c* oxidase appears to be of a local nature involving between one and two layers of phospholipid around the imbedded portion of the protein. Knowles et al. (1979) and Jost et al. (1973), using phospholipid spin-label probes, have estimated a boundary layer of motionally restricted lipid of ~50 lipid molecules. Conceivably, the difference in the number of perturbed lipids measured by ESR and DSC for cytochrome oxidase may be characteristic of the nature of the lipid perturbation as viewed by the two techniques. The motionally restricted phospholipids determined by ESR may not include all the lipid whose conformation is perturbed by the protein and are prevented from undergoing the phase transition as measured by DSC. In this respect, it should be noted that for glycophorin the reported number of perturbed lipid molecules was higher when estimated from calorimetric data than from ^{31}P NMR (Van Zoelen et al., 1978). One possible explanation for these apparent discrepancies is that calorimetry measures an equilibrium perturbation whereas ESR and NMR measure motional restrictions within the characteristic time scale of each technique. Nevertheless, the perturbation of the lipid moiety by cytochrome *c* oxidase is only of a local nature and does not extend beyond the first and maybe part of the second layer of lipid surrounding the enzyme.

High-sensitivity differential scanning calorimetry of cytochrome *c* oxidase with either a detergent or a phospholipid environment revealed that the thermodynamic stability of the protein complex is sensitive to the enzyme environment, as indicated by the upward shift in transition temperature after membrane reconstitution. The stabilization effect is, however, not identical for all the enzyme subunits, judging from the different shapes of the transition profiles and the results of the deconvolution analysis. The presence of a phospholipid environment stabilizes the protein structure and appears to alter the subunit quaternary interactions. The shift of the heat capacity maximum of the DMPC-reconstituted system to a temperature 7 °C higher than that of the Tween 80 reconstituted enzyme was substantiated by SDS gel electrophoresis which also demonstrated a shift in the temperature of denaturation of the bulk of the enzyme. In neither case was the transition found to be two state, and both transitions were found to consist of four melting steps after deconvolution of the heat capacity functions. The fact that the thermal unfolding of cytochrome *c* oxidase is not a two-state process is not surprising considering the multisubunit composition of the enzyme. However, in each of the two hydrophobic environments tested, calorimetry and thermal gel analysis indicate that the bulk of the enzyme is denatured within a relatively small temperature range. This suggests that subunit interactions are an important factor in contributing to the overall stability of the enzyme complex. Judging from thermal gel analysis of the enzyme subunit composition, subunit III is the most easily removed from the enzyme complex and accounts for the first melting step in the phospholipid-reconstituted system. The observation that subunit III is the first subunit denatured corresponds to reports by Saraste and others, who have removed the third subunit by detergent solubilization at high pH (Saraste et al., 1981; Penttila, 1983). This removal of subunit III resulted in an enzyme complex deficient in proton-pumping ability but still capable of electron-transfer activity. The low molecular weight subunits VII and VIII appear to be greatly stabilized by the presence of phospholipid. According to the calorimetric results, the stabilization of these smaller subunits is consistent with the enthalpy changes observed in the first and third melting steps of the deconvoluted heat capacity functions of the detergent-solubilized and membrane-reconstituted enzymes. Comparing the two systems, the first melting step of the detergent-solubilized enzyme has a higher enthalpy than its DMPC-reconstituted counterpart, and the third melting step has a lower enthalpy than that in the phospholipid-reconstituted system.

Quaternary interactions of the subunits have been observed by other researchers. Corbley and Azzi have dissected the enzyme complex into smaller fractions by controlled lithium dodecyl sulfate denaturation. They isolated two fractions containing the first three subunits, indicating that these subunits are intimately associated in the native enzyme (Corbley & Azzi, 1984). Fry et al. (1978) obtained a fraction by solvent extraction in which the first three subunits were accompanied by subunit VII. Since subunit VII[1] was isolated with the first three subunits in one case but not the other, these observations correlate well with the denaturation of subunits VII and VIII together with I and II for the membrane-reconstituted enzyme. The denaturation of the two largest subunits corresponds to the two closely spaced melting steps at 61 and 64 °C of the deconvoluted heat capacity function of the phospholipid-reconstituted enzyme complex. As subunits V and VIb did not exhibit any noticeable change in solubility, the denaturation of these subunits, if any, could not be assigned to one of the melting steps.

In a study of the thermal inactivation of the mitochrondrial electron-transport chain, it was reported that cytochrome oxidase activity was thermally inactivated at 63 °C and calorimetry of submitochrondrial particles resulted in several irreversible transitions, one of which occurs at 62 °C (Knox & Tsong, 1984). This is in good agreement with the transition temperature observed with the phospholipid-reconstituted enzyme and that observed with cytochrome oxidase reconstituted in a membrane environment like that of the inner mitochondrial membrane. Thus, it appears that the thermodynamic behavior of cytochrome oxidase is dependent on the nature of the hydrophobic environment and appears to be the

[1] Subunit VII according to the nomenclature of Downer et al. (1976) has been shown by Kadenbach to consist of four polypeptides. In our gel analysis, two bands were resolved which we ascribed to the three bands of subunit VII (a, b, and c) and to subunit VIII according to Kadenbach's nomenclature (Kadenbach et al., 1983). Samples treated with 1% β-mercaptoethanol before electrophoresis exhibited a doublet for subunit V which corresponds to subunits Va and Vb using Kadenbach's nomenclature.

same in membrane-reconstituted samples as in submitochondrial particles.

Appendix

Two-Pass Deconvolution Analysis. The two-pass deconvolution analysis is essentially as described previously [see Freire & Biltonen (1978) for complete details] except for the following computational improvements:

(1) To minimize any possible errors arising from the finite choice of the initial temperature (T_0) for integration of the excess heat capacity function, each step in the deconvolution sequence is performed twice. The results of the first pass are used to estimate the excess heat capacity integral from 0 K to T_0. This value is then added to the experimental excess enthalpy function prior to the second pass.

(2) In the original paper (Freire & Biltonen, 1978), the enthalpy change for each transition step is calculated from the minimum in the function $\langle \Delta H_i \rangle / (1 - Q_i^{-1})$ where ΔH_i and Q_i are the excess enthalpy function and the transition partition function for the ith deconvolution step. While this equation is exact, we have found that it is experimentally more accurate to numerically calculate the temperature derivative of this function in order to obtain an excess heat capacity function, $\langle C_{p,i} \rangle$, corresponding to the ith deconvolution step. The enthalpy change for the ith transition is then estimated by the equation $\Delta H_i = C_{p,i-1} - C_{p,i}$. The advantage of this method is that the entire set of experimental data points is used to calculate the enthalpy values rather than the few values that determine the minimum of $\langle \Delta H_i \rangle / (1 - Q_i^{-1})$.

(3) The deconvolution parameters are optimized by a nonlinear least-squares analysis. The best set of parameters is defined as the one that minimizes the function $\sum_T [C_{p(T)} - C_{p'(T)}]^2$ where $C_{p(T)}$ and $C_{p'(T)}$ are the experimental and calculated values for the excess heat capacity function at temperature T, respectively.

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