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Differential Detergent Solubility Investigation of Thermally Induced Transitions in Cytochrome *c* Oxidase†

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ABSTRACT: The thermal denaturation of membrane-reconstituted cytochrome *c* oxidase (EC 1.9.3.1) occurs at ~63 °C as determined by high-sensitivity differential scanning calorimetry. The heat capacity profile associated with this process is characterized by the presence of two well-defined peaks, indicating that all the enzyme subunits do not have the same thermal stability. This thermal denaturation of the enzyme complex is coupled to a change in its solubility properties. This change in solubility allows separation of the native and denatured protein fractions by detergent solubilization followed by centrifugation under conditions in which only the native fraction is solubilized. Using this principle, it has been possible to study the denaturation of membrane-reconstituted cytochrome *c* oxidase and quantitatively identify the protein subunits undergoing thermal denaturation using computer-assisted gel electrophoresis analysis. This technique allows calculation of single-subunit thermal denaturation profiles within the intact enzyme complex, and as such, it can be used to obtain transition temperatures, molecular populations, and van't Hoff enthalpy changes for individual protein subunits, thus complementing results obtained by high-sensitivity differential scanning calorimetry.

A wide variety of physical techniques, including differential scanning calorimetry, NMR, and optical methods, have been used to study the thermal unfolding of proteins. Most of these studies have examined the temperature-dependent denaturation of small globular hydrophilic proteins and to a lesser extent complex multidomain hydrophilic proteins (Privalov, 1979, 1982). Until now, however, very little information is available on the thermal denaturation of heterologous multisubunit proteins or integral membrane proteins. In a previous paper, we have studied by high-sensitivity differential scanning calorimetry the melting process of the mitochondrial enzyme cytochrome *c* oxidase reconstituted into phospholipid bilayer vesicles (Rigell et al., 1985). It was shown that the denaturation process was accompanied by changes in the solubility of individual subunits of the enzyme complex and that the native subunits could be separated from denatured subunits by differential detergent solubilization following thermal denaturation. In this paper, we describe a further development in the application of differential solubility thermal gel analysis to the study of protein melting in membranes. In this paper, it is shown that differential solubility thermal gel analysis can be used in a quantitative fashion to measure the population of individual protein conformational states as a function of temperature. These individual subunit melting profiles can be used to calculate apparent thermodynamic parameters for individual subunits in complex systems. This method offers a natural complement to differential scanning calorimetry studies and particularly to the resolution of complex peaks into individual components.

Cytochrome *c* oxidase is the terminal member of the electron-transfer chain of the inner mitochondrial membrane, and it catalyzes the transfer of electrons from cytochrome *c* to molecular oxygen while simultaneously contributing to the proton gradient across the inner membrane. Bovine cytochrome *c* oxidase is a multisubunit integral membrane protein containing 13 subunits (Kadenbach et al., 1983), the 3 largest of which are encoded on the mitochondrial genome whereas the remaining subunits are nuclear gene products (Schwal et al., 1972; Sebald et al., 1972).

In a previous report, we presented a calorimetric characterization of the thermal unfolding of cytochrome *c* oxidase. Those experiments were complemented by gel electrophoresis of the protein in which the thermal denaturation of the subunits of the enzyme was followed by examining thermally induced solubility changes of the enzyme complex in a detergent-containing medium (Rigell et al., 1985). In this paper, we present the technique of differential solubility thermal gel analysis as a means of measuring the denaturation of individual subunits of the multisubunit enzyme cytochrome *c* oxidase. This technique when applied to the membrane-reconstituted enzyme allows a positive identification of the thermal denaturation peaks observed by differential scanning calorimetry as well as quantitative determination of transition tempera-

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tures, population of molecules in the native and denatured states, and van't Hoff enthalpies of denaturation for individual subunits of the enzyme.

Experimental Procedures

Materials. Dimyristoylphosphatidylcholine (DMPC) was obtained from Avanti Biochemicals (Birmingham, AL) and was used without further purification. Tween 80 was purchased from Fisher Scientific (Fair Lawn, NJ). Acrylamide, *N,N'*-methylenebis(acrylamide), and sodium cholate were obtained from Sigma (St. Louis, MO). Sodium cholate was recrystallized 3 times in ethanol to remove bile salt contaminants as described by Wikstrom (1979).

Cytochrome *c* oxidase was isolated from bovine heart as described before (Rigell et al., 1985). Briefly, this method follows the procedure described by Capaldi and Hayashi (1972). The cytochrome *c* oxidase pellet was repurified by dialyzing away ammonium sulfate and cholate against 0.1 M PO_4 buffer, pH 7.4, overnight, redissolving the protein in cholate, and refractionating with a saturated neutralized ammonium sulfate solution as described in the aforementioned procedure. The heme *a*/protein ratio and activity measurements of the preparations used in this study fell within the ranges reported previously for purified cytochrome *c* oxidase: 9–11 nmol/mg of protein for the heme *a*/protein ratio and 8–11 μmol of cytochrome *c* oxidized min^{-1} (mg of protein)$^{-1}$ for the activity measurement.

Protein concentrations were determined by the method of Lowry (Lowry et al., 1951).

Sodium Dodecyl Sulfate (SDS)–Polyacrylamide Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis was performed by using the method of Kadenbach et al. (1983). A Bio-Rad protein 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 (same ratio) was used with the buffers described in the protocol. Electrophoresis was run for 2 h at 80 V (constant) and then for 10 h at 200 V (constant). Gels were prefixed in a 50% methanol/10% acetic acid solution for 3 h at ambient temperature and stained in a 0.2% Coomassie blue/(5/4/1) methanol/water/acetic acid solution at 45 °C with shaking for 1 h. Destaining was accomplished by incubation overnight at room temperature in an 8/1/1 water/methanol/acetic acid solution.

Cytochrome c Oxidase Reconstitution. Cytochrome *c* oxidase was reconstituted into large unilamellar DMPC vesicles using a detergent dialysis technique (Rigell et al., 1985). Five milligrams of DMPC in chloroform was dried under a stream of nitrogen and desiccated overnight to remove residual solvent. The dried lipid was suspended in 10 mM *N*-[tris(hydroxymethyl)methyl]glycine (Tricine), pH 7.5, containing enough cholate such that the final concentration of cholate in the solution was 1.5% w/v. Before addition of protein, the lipid was vortexed and sonicated for 30 s in a bath sonicator (Laboratory Supplies, Hicksville, NY). Protein was then added to obtain a protein/lipid molar ratio of 1/400 assuming a molecular weight of 165 000 for cytochrome *c* oxidase (Deatherage et al., 1982). This solution was gently bath sonicated for 10 s at 4 °C before dialysis against 4 L of 10 mM Tricine, pH 7.5, for 24 h at 4 °C.

Thermal Denaturation of Cytochrome c Oxidase. A separate aliquot of reconstituted cytochrome *c* oxidase was prepared for each temperature point of the thermal denaturation experiments. Each aliquot contained 150 μL of the cytochrome *c* oxidase/DMPC reconstitution (containing 5 mg/mL DMPC, P/L = 1/400). The samples were placed in an RTE-8 Neslab refrigerated circulating water bath equipped with the Neslab temperature programmer ETP-3. The temperature of the samples was monitored by using a thermistor connected to a Hewlett Packard 3467A logging multimeter. The thermistor was placed adjacent to the sample test tubes in a similar test tube containing 150 μL of 10 mM Tricine, pH 7.5. Thermal denaturation was performed by scanning the temperature upward at a heating rate of 20 °C/h. At the desired temperatures, the samples were immediately placed on ice. Ten minutes later, 100 μL of 1.25% Tween 80 in 10 mM Tricine, pH 7.5, was added to make the resultant solution 0.5% Tween 80. At this point, 20 μL of a 0.25 mg/mL carbonic anhydrase stock solution was added as an internal concentration standard. All samples were centrifuged in an IEC HN-SII centrifuge for 90 s at 1400 rpm to remove condensate from the walls of the test tubes. Each sample was sonicated for 30 s in a bath-type sonicator, recentrifuged at 1400 rpm, and transferred to airfuge tubes. Samples were centrifuged in a Beckman airfuge at 178000*g* for 10 min to pellet insoluble protein. Following this last centrifugation, 150 μL of each supernatant was diluted to 300 μL with electrophoresis sample buffer [625 mM tris(hydroxymethyl)aminomethane (Tris) base, 20% glycerol, and 4% sodium dodecyl sulfate, pH 6.8]. Samples were then made 1% β-mercaptoethanol to improve resolution of the subunits. Calibration gels were run in order to ensure that the optical densities of the bands, as measured by our laser densitometer, were linearly dependent on protein concentration for the concentration ranges used in these experiments.

Laser Densitometry and Data Acquisition. Densitometry of the destained gels was performed by using an LKB 2202 Ultroscan laser densitometer interfaced to an IBM PC equipped with a Data Translation DT-2805 A/D converter board. The resolution of the laser densitometer is better than 50 μm, and about 1000 data points were collected for every lane of the gel, each corresponding to one temperature point of the thermal denaturation. Each lane was scanned 3 times, each time, with a different horizontal positioning of the laser beam. The output of the laser densitometer was not electronically filtered prior to computer digitization; while filtering reduces high-frequency noise, it also distorts the shape of the peaks, precluding a precise quantitative analysis. Subsequent data manipulation to obtain normalized peak intensities and areas was performed by using software developed in this laboratory.

Results

Temperature-Induced Change in Cytochrome c Oxidase Subunit Solubility. Cytochrome *c* oxidase is an integral membrane protein, and as such, solubilization of the protein complex requires the addition of detergent. The thermal denaturation of cytochrome *c* oxidase reconstituted into DMPC vesicles results in changes in the solubility of the enzyme complex. The addition of detergent to the reconstituted enzyme in the native, nondenatured, form results in the solubilization of all of the subunits. However, when detergent is added to the reconstituted enzyme following thermal denaturation, most of the subunits of cytochrome oxidase can no longer be solubilized and therefore can be removed from solution by centrifugation. This phenomenon has allowed us to examine the denaturation of individual subunits in the enzyme complex by observing the change in subunit solubility as a function of temperature (Rigell et al., 1985). A similar approach has been used before by Lysko et al. (1981) to measure protein aggregation in red blood cells.

The protein solubility changes examined in these studies are induced by temperature and are associated to the thermal

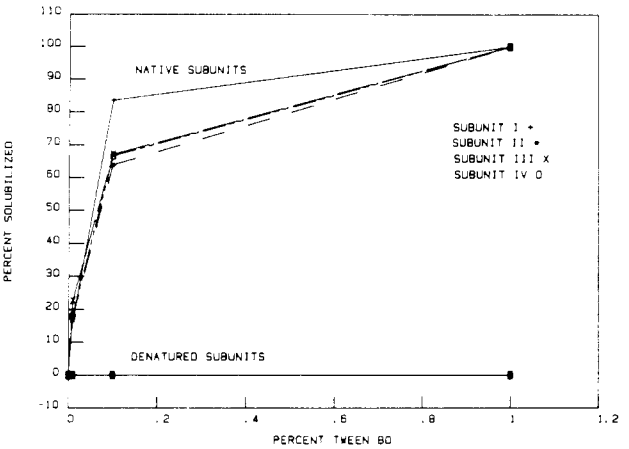

FIGURE 1: Solubilization of individual subunits of membrane-reconstituted cytochrome *c* oxidase by Tween 80 as determined by SDS–polyacrylamide gel electrophoresis for subunit I (+), subunit II (*), subunit III (×), and subunit IV (○). The reconstituted enzyme was incubated either at 25 °C (native) or at 95 °C (denatured).

denaturation of the enzyme as evidenced by the coincidence of the transition temperatures observed by solubility changes with those obtained by differential scanning calorimetry, fluorescence and absorbance spectroscopy, and enzymatic analysis (Rigell et al., 1985). It must be noted that a solubility change alone would not be a sufficient condition to infer thermal denaturation since solubility changes can be induced by many other processes including local conformational changes. In this case, the magnitude of the enthalpy change measured by differential scanning calorimetry (600 kcal/mol of protein) indicates that the solubility changes are coupled to the thermal denaturation of the enzyme complex.

Figure 1 shows the detergent (Tween 80) concentration dependence of the degree of solubilization of the four largest subunits of cytochrome *c* oxidase in both the native and denatured states. In the absence of detergent, the membrane-reconstituted enzyme, in the native state (25 °C), can be removed entirely from solution by centrifugation at 178000*g* for 10 min. SDS–polyacrylamide gel electrophoresis of the resulting supernatant exhibits no subunit bands after being stained with Coomassie blue. Addition of as little as 0.01% Tween 80 results in a measurable degree of solubilization for each of the subunits (~20% of maximum). For the concentration of protein and lipid used in these experiments ([DMPC] = 5 mg/mL, [cytochrome oxidase] = 3 mg/mL), addition of between 0.1% and 1.0% Tween 80 results in maximal solubilization of the enzyme complex. However, if the reconstituted enzyme is first denatured by incubation at 95 °C for 15 min, none of these subunits can be solubilized at any of the concentrations of Tween 80 tested. Incubation of the reconstituted enzyme at intermediate temperatures and subsequent addition of detergent result in the selective solubilization of only those subunits remaining in the native state.

In our original paper (Rigell et al., 1985), detergent was added as soon as the sample reached the desired final temperature without cooling the samples to a reference temperature. Since the thermal denaturation of cytochrome *c* oxidase is totally irreversible, similar results are obtained if the samples are immediately cooled to a reference temperature. In this paper, we have used his second method in order to avoid any possible interference from the temperature dependence of the detergent properties.

Under the conditions of these experiments, detergent concentrations ranging between 0.19 and 1% selectively solubilize only the native components of the enzyme. This is true for

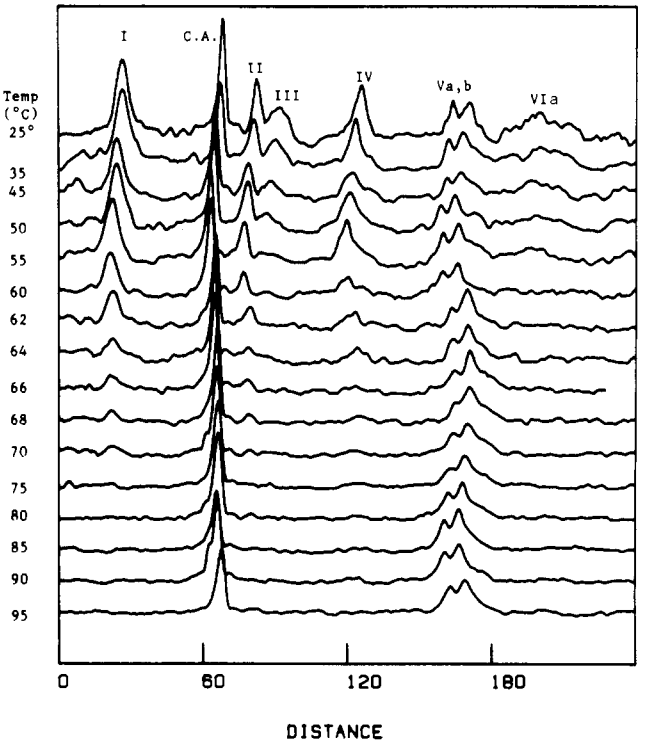

FIGURE 2: Laser densitometer tracing of the differential solubility thermal gel analysis of cytochrome *c* oxidase reconstituted into DMPC vesicles. Carbonic anhydrase (C.A.) was added as an internal concentration standard after detergent solubilization as explained in the text.

all of the subunits except Va and Vb which can be solubilized under all conditions studied in this work. Apparently, the thermal denaturation of these two subunits is not accompanied by a detergent solubility change. These denaturation-induced detergent solubility changes constitute the basis for the technique of differential solubility thermal gel analysis, that is, the use of an appropriate detergent concentration to separate the native subunits from the denatured subunits of the enzyme complex.

Determination of Denaturation Parameters for Individual Subunits of Cytochrome c Oxidase. Figure 2 shows a family of laser densitometer profiles obtained from an SDS–polyacrylamide gel of the detergent-soluble subunits of the reconstituted enzyme complex following incubation up to the indicated temperatures. Each densitometer trace in this figure contains only the contributions from the subunits which retain solubility in 0.5% Tween 80/10 mM Tricine, pH 7.5, after heating to the indicated temperature. The additional band labeled C.A. corresponds to carbonic anhydrase which is used as an internal concentration standard. The reason for using carbonic anhydrase is to compensate for concentration errors arising from sample handling steps necessary to run the gels. As explained under Experimental Procedures, carbonic anhydrase is only added after the thermal denaturation has been completed and the samples have been detergent solubilized. Carbonic anhydrase migrates to a position between subunits I and II on the SDS–polyacrylamide gels, facilitating peak area determinations.

For each lane of the gel (each temperature point), the area under each peak was divided by the area of the carbonic anhydrase peak in order to obtain the normalized areas which were used for the calculation of the transition parameters:

$$\text{area(normalized)} = \frac{\text{area(observed)}}{\text{area(carbonic anhydrase)}} \quad (1)$$

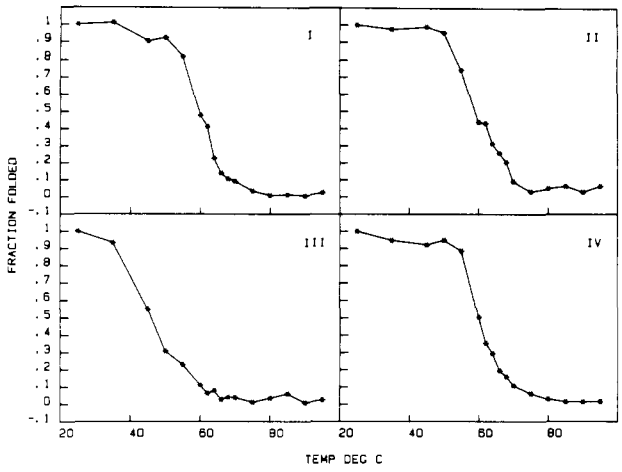

FIGURE 3: Fraction of molecules in the native state as a function of temperature (degrees centigrade) for each of the four largest subunits of cytochrome *c* oxidase as determined from the peak areas obtained by laser densitometry analysis of gel electrophoresis bands.

The densitometer tracings in Figure 2 represent the unnormalized, unfiltered raw data for the denaturation of cytochrome *c* oxidase. Over the temperature range examined, it is evident that there is a decrease in all the cytochrome *c* oxidase subunit peak areas with the exception of subunits Va and Vb which remain detergent soluble even after incubation of the enzyme complex at 95 °C for 15 min.

The areas obtained at 25 °C were used to represent the subunit populations in the completely native state. The fraction of each subunit remaining in the native state at higher temperatures (F_n) was obtained by dividing the area for each subunit peak at the indicated temperature over the area of the corresponding peak at 25 °C as follows:

$$F_n(T) = \frac{A(T)}{A(25\ °C)} \tag{2}$$

where $A(T)$ and $A(25\ °C)$ represent the normalized areas at temperature T and 25 °C, respectively. Plots of the fraction of native subunit as a function of temperature are shown in Figure 3 for the four largest subunits of the enzyme complex. As can be observed in the figure, each curve has the typical sigmoidal shape characteristic of a thermally induced transition. The melting temperature, T_m, of each subunit can be defined as the temperature at which the fraction in the native state is 0.5. Subunits I, II, and IV have melting temperatures (T_m's) ranging between 60 and 63 °C; however, subunit III is rendered insoluble at a much lower temperature (T_m = 47 °C) than the other subunits shown in this figure. Also, there is little temperature dependence of the individual subunit solubility for subunits I, II, and IV below the onset of the unfolding transition, indicating that these experiments are sensitive to detergent solubility changes induced by protein denaturation. Using the data shown in Figure 3, it is possible to calculate the ratio of the population of molecules in the denatured state to that of the population in the native state:

$$K_{app}(T) = \frac{1 - F_n(T)}{F_n(T)} = \frac{F_d(T)}{F_n(T)} \tag{3}$$

This ratio of populations is defined in analogy to the reversible situation, and in the limit of thermodynamic equilibrium, it becomes equal to the equilibrium constant. Because of the nature of the technique (i.e., direct measurement of subunit populations), this is a true ratio of populations even if the transition under study is irreversible. This should be contrasted with the most common situation in which molecular

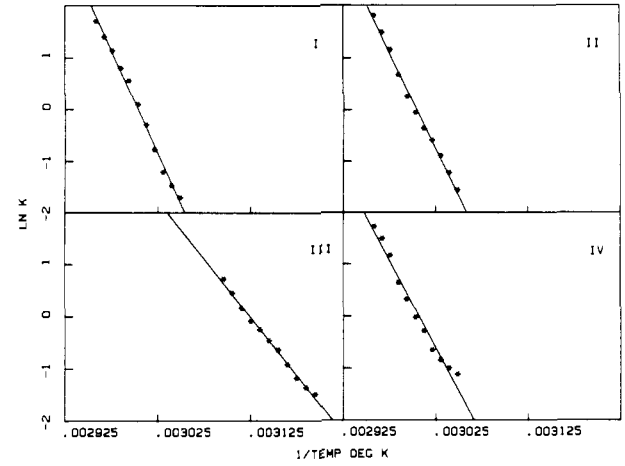

FIGURE 4: van't Hoff plots (ln K vs. $1/T$) for the thermal denaturation of the four largest subunits of cytochrome oxidase.

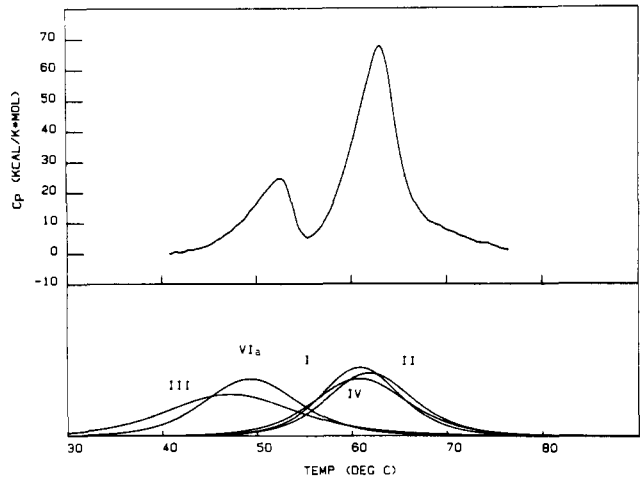

FIGURE 5: Heat capacity profile (top) and individual subunit differential melting profiles calculated from differential solubility gel analysis (bottom) for the denaturation of reconstituted cytochrome *c* oxidase.

populations are estimated from other parameters. In general, the ratio of populations defined by eq 3 has the meaning of an "apparent equilibrium constant", and as such, it can be used to estimate "apparent thermodynamic parameters". Of particular interest is the apparent or van't Hoff enthalpy change. This quantity is only equal to the true thermodynamic enthalpy if the transition under study is a reversible two-state process. Under other conditions (e.g., reversible multistate transitions or irreversible processes), the van't Hoff enthalpy is only a measure of the sharpness of a thermally induced transition rather than the heat effects associated with the process.

The van't Hoff plot of the calculated apparent equilibrium constants for each subunit in Figure 3 is presented in Figure 4. Data points for that van't Hoff analysis were obtained from the smoothed melting curve for each subunit at 1 °C intervals around the center of the transition. The linearity of these plots suggests that, even though the thermal denaturation of these subunits is irreversible, the calculated K_{app}'s also obey an exponential dependence on the inverse absolute temperature. Subunits I, II, and IV all exhibit similar slopes, indicating that the sharpness of the denaturation transition and therefore the van't Hoff enthalpy change is about the same for each of these subunits. This fact, along with the proximity of the melting temperatures for these subunits, suggest, that the denaturation of these subunits may be interdependent. In fact, the melting of these subunits corresponds to the main calorimetric peak (see Figure 5) and constitutes the melting of the bulk of the

Table I: Physicochemical Parameters for Thermal Denaturation of Cytochrome *c* Oxidase Reconstituted into DMPC Vesicles

	calorimeter		differential detergent solubility		
transition	T_m (°C)	ΔH (kcal/mol)	T_m (°C)	ΔH_{vh} (kcal/mol)	subunit
1	51.7	139	47	50	III
			48	70	VIa
2	60.7	160	60.5	78	I
			60.5	70	IV
3	63.5	195	62	76	II
			60–65	ND	VII–VIII (?)
4	68.4	89	ND[a]	ND	ND

[a] Not determined.

enzyme complex. Subunit III, on the other hand, melts at a lower temperature and with a lower van't Hoff enthalpy, indicating that the unfolding of this subunit occurs independently of subunits I, II, and IV. The melting temperature of this subunit coincides with that of the low-temperature temperature peak in the calorimetric profile.

Individual Subunit Melting Profiles for Cytochrome c Oxidase. In a previous paper, we demonstrated that the thermal denaturation of cytochrome *c* oxidase reconstituted into a lipid membrane (DMPC vesicles) exhibits a complex melting profile as determined by high-sensitivity differential scanning calorimetry (Rigell et al., 1985). The transition associated with the denaturation of reconstituted cytochrome *c* oxidase consists of two well-resolved peaks: one smaller peak at 52 °C and the main peak at 63 °C. Analysis of the calorimetric data revealed that the low-temperature peak is characterized by a calorimetric to van't Hoff enthalpy ratio ($\Delta H/\Delta H_{vh}$) of 1.17 and that the high-temperature peak is characterized by a $\Delta H/\Delta H_{vh}$ ratio of 2.5, indicating that neither of these peaks represents two-state transitions. Deconvolution analysis of this melting profile indicated that at least four different melting steps take part in the thermal unfolding of the enzyme complex. Table I presents a comparison of the transition parameters obtained from the deconvolution of the calorimetric data and the parameters obtained from the differential detergent solubility gel analysis. Although the fractional melting profile data are not shown, subunit VIa (see Figure 2) melts at a temperature similar to that observed for subunit III, with a van't Hoff enthalpy of 70 kcal/mol. The melting of these two subunits contributes to the first melting step at 51.7 °C in the deconvoluted heat capacity profile. Subunits I and IV have melting temperatures corresponding to that of the second melting step whereas subunit II, which has a slightly higher melting temperature as measured by differential detergent solubility gel analysis, corresponds to the third melting step. The other low molecular weight subunits were not well resolved in the SDS–polyacrylamide gels for a quantitative analysis but were observed to denature with the bulk of the enzyme around 62 °C. These low molecular weight subunits presumably may also contribute to the third melting step. No subunits could be directly assigned to the fourth melting step. The unresolved low molecular weight subunits or those subunits which do not exhibit any thermally induced change in detergent solubility (Va and Vb) may contribute to this melting step.

Figure 5 (top panel) shows the observed heat capacity profile for the membrane-reconstituted enzyme and the individual subunit differential melting profiles generated from the parameters obtained from the gel analysis (lower panel). These curves were calculated by using the following representation of the unfolding process for each subunit:

$$\frac{\partial F_i}{\partial T} = \frac{K_{app_i}}{(1 + K_{app_i})^2}\frac{\Delta H_{vh,i}}{RT^2} = F_i(1 - F_i)\frac{\Delta H_{vh,i}}{RT^2} \quad (4)$$

where F_i is the fraction of subunit i remaining in the native state at temperature T and K_{app_i} is the apparent equilibrium constant evaluated at temperature T. The individual subunit differential melting profiles obtained by thermal gel analysis provide an independent way of positively identifying and locating contributions of individual subunits of the enzyme complex to the overall heat capacity curve. It must be noted, however, that differential solubility thermal gel analysis, like any other noncalorimetric technique, only provides values for the van't Hoff enthalpies of denaturation and as such it cannot be used to predict the magnitude of the heat capacity function or the enthalpy change unless the transition is of the two-state type.

Discussion

Differential detergent solubility thermal gel analysis appears to be an ideal technique to complement high-sensitivity differential scanning calorimetry in the study of the thermally induced denaturation of complex membrane proteins. First, it allows positive identification of the protein subunits undergoing thermal denaturation. In addition, it permits the calculation of physicochemical parameters associated with the thermal denaturation of individual subunits of a multisubunit membrane protein like cytochrome *c* oxidase. Additional studies in this laboratory using cholera toxin (B. Goins, unpublished results) and band III from erythrocyte membranes (C. Wortman, unpublished results) indicate that this technique can be applied to a wide range of systems, the prerequisite being that the native and denatured states of the membrane protein have different detergent solubilities. In these cases, differential solubility thermal gel analysis makes it possible to experimentally dissect complex multistate denaturation transitions observed by high-sensitivity differential scanning calorimetry into the contributions from the denaturation of individual proteins or protein subunits.

The thermal denaturation of cytochrome oxidase results in a change in the detergent solubility of the various subunits of the enzyme complex. The melting temperature for subunit III as determined by the gel analysis corresponds to the first melting step in the heat capacity curve measured by differential scanning calorimetry. Other researchers have reported that removal of the subunit III from the enzyme complex by detergent solubilization at high pH (Saraste et al., 1981; Pentilla, 1983) or by using chymotryptic digestion (Capaldi et al., 1983) results in an enzyme complex which retains electron-transfer activity but not the proton pumping capacity. This evidence has been used to assign the proton pumping activity of cytochrome *c* oxidase to the presence of subunit III in the enzyme complex (Casey et al., 1980; Prochaska et al., 1981). Sone and Nicholls (1984) observed that the proton pumping function of the reconstituted oxidase is thermally inactivated at a temperature of 43–45 °C while the enzyme still retains electron-transfer activity. Studies in our laboratory (data not shown) indicate that the electron-transfer activity of the enzyme becomes inactive at ~60 °C, a temperature corresponding to the denaturation temperature of the bulk of the enzyme complex. The results using differential detergent solubility thermal gel analysis and differential scanning calorimetry also indicate that it is the thermal unfolding of subunit III which accounts for the temperature-induced loss of proton pumping capacity.

Quantitative data from differential detergent solubility thermal gel analysis of reconstituted cytochrome *c* oxidase

indicate that subunits I and IV denature within 2 °C of subunit II and that all three subunits exhibit approximately the same van't Hoff enthalpy of denturation. Subunits Va and Vb remain in detergent solution even after the enzyme complex is boiled for more than 5 min. Since these subunits show no temperature-dependent change in solubility, no information could be obtained for the denaturation of these subunits using this technique. It must be noted that subunits Va and Vb have considerable hydrophilic character. Subunit Va has been shown to be largely exposed to the aqueous phase by its tryptic removal from the reconstituted enzyme complex (Zhang et al., 1984). Also, examination of the amino acid sequence of subunit Vb indicates that it is largely a water-soluble protein (Tanaka et al., 1979). Thus, it is not surprising that these subunits are insensitive to differential detergent solubility analysis.

The thermal denaturation profiles measured in these studies were independent of scanning rate for the range studied (20–60 °C/h), suggesting that the effects observed at any particular temperature correspond to the protein denaturation per se and not to postdenaturational events arising from the irreversible nature of these transitions. It must be noted that protein aggregation or precipitation usually gives rise to the appearance of exothermic peaks in the calorimetric melting profiles. In the case of cytochrome *c* oxidase, this situation has been observed with the detergent-solubilized enzyme but not with membrane-reconstituted samples (Semin et al., 1984; Rigell et al., 1985). Our overall transition temperature of 63 °C obtained by differential scanning calorimetry and differential detergent solubility is also in excellent agreement with the transition temperature of 64 °C obtained by Semin et al. (1984) using calorimetry and with the report by Knox and Tsong (1984) that cytochrome *c* oxidase activity is thermally inactivated at 63 °C. Since all these studies cover a wide range of concentrations, it can also be concluded that, under the conditions of these studies, the thermal denaturation of the enzyme is not significantly affected by protein aggregation.

The method of differential detergent solubility thermal gel analysis to complement high-sensitivity differential scanning calorimetry is currently being applied to other membrane protein systems in this laboratory. In conjunction, these techniques should prove useful in providing thermodynamic and structural information on biological membranes by identifying the constituents which contribute to the individual heat capacity peaks observed in complex melting transitions.

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