fraction of site bound by ligand, and care must be taken in the analysis of the data.

Registry No. DNase I, 9003-98-9; d(TAGCGCTA), 115710-85-5; actinomycin D, 50-76-0.

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Thermal Stability of Membrane-Reconstituted Yeast Cytochrome *c* Oxidase†

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Received July 10, 1989; Revised Manuscript Received September 7, 1989

ABSTRACT: The thermal dependence of the structural stability of membrane-reconstituted yeast cytochrome *c* oxidase has been studied by using different techniques including high-sensitivity differential scanning calorimetry, differential detergent solubility thermal gel analysis, and enzyme activity measurements. For these studies, the enzyme has been reconstituted into dimyristoylphosphatidylcholine (DMPC) and dielaidoylphosphatidylcholine (DEPC) vesicles using detergent dialysis. The phospholipid moiety affects the stability of the enzyme as judged by the dependence of the denaturation temperature on the lipid composition of the bilayer. The enzyme is more stable when reconstituted with the 18-carbon, unsaturated phospholipid (DEPC) than with the 14-carbon saturated phospholipid (DMPC). In addition, the shapes of the calorimetric transition profiles are different in the two lipid systems, indicating that not all of the subunits are affected equally by the lipid moiety. The overall enthalpy change for the enzyme denaturation is essentially the same for the two lipid reconstitutions (405 kcal/mol of protein for the DMPC and 425 kcal/mol for the DEPC-reconstituted enzyme). In both systems, the van't Hoff to calorimetric enthalpy ratios are less than 0.2, indicating that the unfolding of the enzyme cannot be represented as a two-state process. Differential detergent solubility experiments have allowed us to determine individual subunit thermal denaturation profiles. These experiments indicate that the major contributors to the main transition peak observed calorimetrically are subunits I and II and that the transition temperature of subunit III is the most affected by the phospholipid moiety. Experiments performed at different scanning rates indicate that the thermal denaturation of the enzyme is a kinetically controlled process characterized by activation energies on the order of 40 kcal/mol. These studies have allowed us to quantitatively model the thermal denaturation mechanism of the enzyme.

The formation of functionally active integral membrane protein assemblies involves membrane insertion, folding, and subunit association of the constituent polypeptide units. The molecular details and energetics of those processes are still not completely understood. Cytochrome *c* oxidase is the terminal enzyme of the respiratory chain, catalyzing the transfer of electrons from cytochrome *c* to molecular oxygen while simultaneously serving as a proton pump. Cytochrome *c* oxidase is a multisubunit enzyme composed of mitochondrially synthesized subunits and subunits imported from the cytoplasm. In most species, the three largest subunits (I, II, III) are synthesized in the mitochondria and the remaining subunits

† Supported by grants from the National Institues of Health (GM 37911 and RR 04328).

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0006-2960/90/0429-781$02.50/0

in the cytoplasm (Sebald et al., 1972; Mason & Schatz 1973). The number of imported subunits appears to vary among different species, amounting to 10 for the beef heart enzyme and to 4–6 for the yeast enzyme (Kadenbach et al., 1983; Power et al., 1984). Even though some aspects of the targeting and import process of the cytoplasmic subunits are known, little is understood about the mechanism of folding and assembly of the various subunits. The experiments presented in this paper are directed to evaluate the magnitude of the forces that stabilize the membrane structure of the yeast enzyme.

For these experiments, the yeast enzyme has been purified and reconstituted into phospholipid vesicles in active form. These preparations have been used to measure the temperature dependence of the structural stability and electron-transfer activity of the enzyme. High-sensitivity differential scanning calorimetry in conjunction with differential solubility thermal gel analysis of the membrane-reconstituted enzyme has been used to characterize the energetics of unfolding and to identify the subunits involved at each stage of the overall process. These experiments indicate that the overall stability of the enzyme is affected by the lipid moiety and that the thermal denaturation of the enzyme complex is a kinetically controlled process. From the point of view of the unfolding mechanism, the enzyme appears to be composed of a minimum of three cooperative elements. These elements include subunits I and II, subunit III, and subunits IV and VI as their major constituents. The thermal behavior of the subunits forming those cooperative elements appears to be coupled to one another, suggesting the existence of stronger interactions within than between cooperative elements.

Experimental Procedures

Materials. Dimyristoylphosphatidylcholine (DMPC) and dielaidoylphosphatidylcholine (DEPC) were obtained from Avanti Biochemicals (Birmingham, AL) and used without further purification. Octyl-Sepharose was purchased from Pharmacia. Enzyme-grade Tween-20 was purchased from Fisher Scientific (Fair Lawn, NJ) and Tween-80 from Calbiochem (La Jolla, CA). Cytochrome *c* type VI from horse heart, reduced Triton X-100, acrylamide, *N,N'*-methylenebis(acrylamide), and 98% sodium cholate were obtained from Sigma (St. Louis, MO). Sigma 98% sodium cholate was recrystallized repeatedly in ethanol to remove bile salt contaminants as described previously by Wikstrom (1979). Fresh brewers yeast was obtained from Anhauser Busch (St. Louis, MO).

Protein Isolation and Purification. Cytochrome *c* oxidase was purified from *Saccharomyces cerevisiae* essentially following the methods of George-Nascimento and Poyton (1981) and Power et al. (1984). The three-part procedure consisted of cell rupture, cholate extraction/ammonium sulfate fractionation, and octyl-Sepharose chromatography.

Cell rupture was carried out in batches ranging in size from 2 to 10 lb. of yeast. For large batches, mechanical lysis of a 50% yeast slurry (wet packed cell weight) in lysis buffer (50 mM potassium phosphate, 1 mM EDTA, 0.9% KCl, and 0.5 mM PMSF, pH 8.4) was achieved by using a Dynomill-type KDL bead mill (W. A. Bachofen Machinenfabrick, Basel, Switzerland) with a 10 m/s tip speed and a feed rate of 10 L/h. Small batches of 2–3 lb. were lysed on a Beadbeater (Biospec Products, Bartlesville, OK). During lysis, the temperature was kept below 7 or 8 °C by use of a large-volume circulating water bath in the case of the KDL and by ice/salt baths and pulsed operation in the case of the Beadbeater. In either sequence, the homogenate was centrifuged in a Beckman JA-10 rotor at $3200g_{max}$ for 30 min to remove unbroken cells and debris. The supernatant was decanted and then pelleted for 1 h at $30000g_{max}$ in a JA-14 rotor. The soft pellet was resuspended with a glass–Teflon homogenizer in 75 mL of lysis buffer per pound of yeast processed. This suspension was then pelleted in a JA-14 rotor as before and the pellet resuspended to approximately 40 mg of protein/mL prior to freezing at −70 °C.

When needed, the mitochondria-rich suspensions were thawed slowly and then sonicated in 60-mL aliquots using a bath sonicator (Laboratory Supplies, Hicksville, NY) for 45 s. The suspension was then centrifuged in a Beckman 45Ti rotor at $119000g_{max}$ for 90 min, and the pellet was resuspended in lysis buffer to a final concentration of 20 mg/mL. To this suspension of submitochondrial particles (SMP) was added 15 mL of 20% cholic acid (Sigma 98% grade recrystallized once, pH 8.3) for every gram of protein. Solid ammonium sulfate (184 g/L) was added slowly to this mixture, the pH was adjusted to 7.2 with KOH, and the solution stirred for 10–14 h.

After extraction, the mixture was centrifuged at $30000g_{max}$ in a JA-20 rotor for 20 min. To the supernatant was added 184 g/L solid ammonium sulfate and the suspension centrifuged again at $30000g_{max}$ for 20 min. The resulting pellet was resuspended in 15 mL of 0.25 M sucrose, 0.01 M Tris-HCl, and 0.5% sodium cholate, pH 7.4, per gram of SMP protein. In this buffer, the suspension was fractionated twice again, this time with saturated, neutralized ammonium sulfate at 30% and 46%. The center fraction was retained through centrifugation at $35000g_{max}$ for 15 min in a JA-20 rotor, and the greenish pellet was resuspended in 5 mL of 0.05 M MOPS, 1 mM EDTA, and 2% cholate, pH 7.5 (ME–2% cholate), per gram of SMP protein. Prior to chromatography, the solution was clarified via ultracentrifugation at 40 000 rpm in a Beckman 45Ti rotor.

The final purification of cytochrome *c* oxidase was accomplished chromatographically using an octyl-Sepharose column (15 mL of resin/g of SMP protein) equilibrated in ME–2% cholate. Once loaded, the column was rinsed with 5 volumes of ME–2% cholate and then 5 volumes of ME–2% cholate containing 15% ammonium sulfate. Loosely bound proteins were removed with 5 volumes of ME–2% Tween 20/15% ammonium sulfate. Cytochrome *c* oxidase was eluted in a concentrated band with ME–3% Triton X-100 (reduced). The excess Triton in the eluted fraction was exchanged for cholate by layering the eluate over a small pad (3–4 mL) of ME–1.0% cholate/50% sucrose, in the bottom of an ultracentrifuge tube which was then spun at $185000g_{max}$ in a 45Ti rotor for 13–16 h. The dark green layer was removed, and the enzyme was separated from the sugar by passing it over a Bio-Rad P-6 column equilibrated and run in ME–0.5% cholate. When necessary, the enzyme was concentrated by ultracentrifugation at $200000g_{max}$ in a 70Ti rotor for 12 h, and the pellet was resuspended by gentle overnight swirling on a blood rotator. The specific activity [K (min^{-1} mg^{-1} × 10^{-2})] of the purified enzyme used in this study fell in the range of 10–11.5 under assay conditions of 50 mM potassium phosphate, pH 6.65, 30 μM cytochrome *c*, and 0.5% Tween-80. The heme *a*:protein ratio ranged from 8 to 9.5 nmol mg^{-1} using the method of Mason et al. (1973). These values are similar to those obtained by other investigators (Powers et al., 1984; Mason et al., 1973). All protein concentrations were determined by using the Pierce BCA assay. Bovine serum albumin was used as a standard.

Membrane Reconstitution. Cytochrome *c* oxidase was reconstituted into phospholipid vesicles of DMPC or DEPC by use of a detergent dialysis technique (Rigell et al., 1985). Lipid

dissolved in chloroform was dried under a stream of nitrogen and then lyophilized overnight to remove any residual chloroform. The dried lipid was dissolved in the appropriate buffer (10 mM Tricine, pH 7.5, or 50 mM potassium phosphate, pH 7.5) containing enough cholate (recrystallized 3 times) to obtain a final concentration of 1.5% after addition of the desired amount of protein/cholate suspension. The lipid/detergent mixture was vortexed vigorously at a temperature above the lipid transition temperature, sonicated in a Laboratory Supplies Co. bath sonicator, and allowed to equilibrate at 4 °C. At this point, the protein/cholate solution was added to the lipid/cholate mixture, typically to a concentration of 5 mg/mL protein. The resulting protein/lipid/detergent mixture was bath-sonicated for 5 s and then allowed to remain at 4 °C for 30 min prior to dialysis against either 10 mM Tricine, pH 7.5, or 50 mM potassium phosphate, pH 7.5. Dialysis was performed by using three buffer changes of 4 L each over a period of 24–28 h at 4 °C.

Differential Scanning Calorimetry. 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). All calorimetric scans of membrane-reconstituted enzyme were performed at a scanning rate of 45 °C/h. Protein concentrations of 2–3 mg/mL were used in these experiments.

Sodium Dodecyl Sulfate (SDS)–Polyacrylamide Gel Electrophoresis. An 18 × 16 cm 15% acrylamide separation gel [32:1 acrylamide:*N*,*N*′-methylenebis(acrylamide) ratio] with a 5% acrylamide stacking gel was used. Electrophoresis was carried out at 80 V (constant) for 1–2 h and then at 150 V for 12 h. Gels were stained in 5:4:1 methanol/water/acetic acid containing 0.2% Coomassie blue-2R. Destaining took place overnight in 1:8:1 methanol/water/acetic acid.

Differential Detergent Solubility Thermal Gel Analysis. These experiments were performed as described previously (Rigell & Freire, 1987). A separate 75-μL aliquot of membrane-reconstituted protein was placed in an Eppendorf microfuge tube for each temperature point studied. The entire battery of microfuge tubes was immersed in a circulating water bath controlled by a Neslab ETP-3 temperature programmer (Portsmouth, NH). At this point, the temperature was increased at the desired linear rate and was monitored by using a thermistor immersed in an identical tube containing buffer and placed adjacent to those containing the enzyme samples. At each temperature point studied, a tube was removed from the bath and immediately placed on ice. Ten minutes later, 50 μL of buffer containing 1.25% Tween-80 was added to yield a final concentration of 0.5% Tween-80.

Prior to electrophoresis, samples were centrifuged briefly in a microcentrifuge to collect any condensate from the test tube walls and then sonicated for 30 s in a bath sonicator. Ten microliters of bovine albumin solution (0.5 mg/mL) was added as an internal concentration standard. The samples were then briefly centrifuged again, transferred to Beckman airfuge tubes, and centrifuged in a Beckman airfuge ultracentrifuge at 178000g_{max} for 10 min. An aliquot of 100 μL of supernatant was removed and combined with 100 μL of electrophoresis sample buffer containing 2% BME. Fifty microliters of the resulting samples was loaded on each lane of the polyacrylamide gel.

Cytochrome c Oxidase Activity Measurements. The electron-transfer activity of the protein was measured according to the method described previously (Rigell et al., 1985).

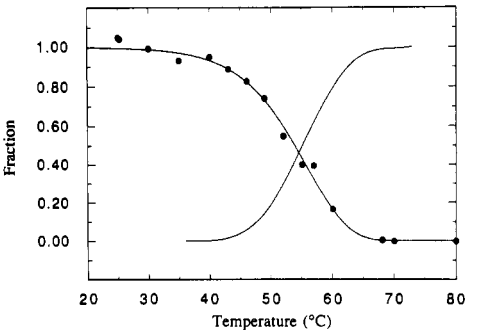

FIGURE 1: Residual activity of DMPC-reconstituted yeast cytochrome *c* oxidase following temperature scanning up to the indicated temperature (closed circles) and fractional degree of enzyme denaturation determined by integration of the excess heat capacity function obtained by high-sensitivity differential scanning calorimetry. In both cases, the scanning rate was 45 °C/h. The activity measurements were made at 25 °C.

Spectroscopic measurements were made at 25 °C in 50 mM potassium phosphate using a Perkin Elmer Lambda-4B spectrophotometer equipped with a Model C550-0555 thermoelectric cell holder. Enzymatic rates were obtained by nonlinear least-squares analysis of the exponential decay of the absorbance at 550 nm as a function of time. When assaying the nonreconstituted enzyme, the medium was made 0.5% in Tween-80. Activity measurements of the membrane-reconstituted enzymes were made in the absence of detergent.

The dependence of activity upon the temperature scan rate was measured by incubating a battery of samples placed in Eppendorf tubes in a temperature scanning water bath configured as described above. Samples were removed from the bath at selected temperatures and immediately placed on ice. Measurement of each sample's electron-transfer activity was then made in a random order.

The dependence of activity upon varied exposure times to a fixed temperature was carried out in a similar manner. Sample aliquots of 120 μL were placed in Eppendorf tubes and immersed in the bath for the desired length of time, and then immediately placed on ice.

Laser Densitometry. Polyacrylamide gels were digitized by using an LKB 2202 Ultrascan laser densitometer interfaced to a microcomputer equipped with a Data Translation DT-2805 A/D converter. Each lane was scanned 5 times, using a different horizontal positioning of the laser beam across the lane; the resulting intensities were averaged, and then multiplied by the lane width. Analysis of the data was performed with software developed in this laboratory as described below.

RESULTS

The electron-transfer activity at 25 °C of membrane-reconstituted yeast cytochrome *c* oxidase, that has been scanned upward in temperature at a rate of 45 °C/h, is shown in Figure 1. As indicated in the figure, the activity profile exhibits a characteristic sigmoidal dependence with an inflection point centered at 54 °C. Since all the activity measurements are performed at 25 °C, the observed decrease in activity is, in each case, proportional to the amount of enzyme that has been irreversibly inactivated by scanning the sample up to the indicated temperature. The origin of the inactivation is the thermal denaturation of the enzyme as indicated by the observation that the enzyme inactivation profile coincides with the thermal denaturation profile obtained by high-sensitivity differential scanning calorimetry under identical conditions, as shown in the same figure and also in Figure 2.

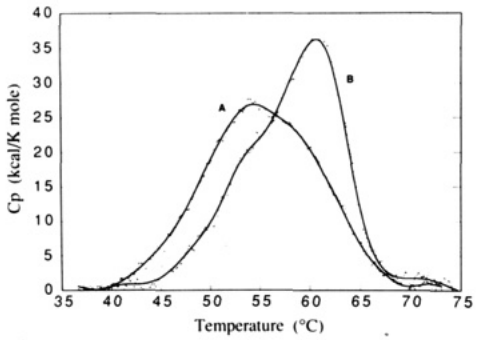

FIGURE 2: Excess heat capacity function associated with the thermal denaturation of DMPC- (curve A) and DEPC- (curve B) reconstituted yeast cytochrome *c* oxidase. Both experiments were performed at a scanning rate of 45 °C/h and at a protein:lipid molar ratio of 1:500.

The excess heat capacity curves obtained by high-sensitivity differential scanning calorimetry for the thermal denaturation of the DMPC and DEPC membrane-reconstituted enzyme are shown in Figure 2. In DMPC, the peak maximum for the protein denaturation transition is centered at 54 °C whereas in DEPC the transition is shifted upward to 60.5 °C. In both lipid systems, the bilayer is in the fluid phase during the protein transition. The DEPC gel–fluid phase transition is centered at 13 °C and the DMPC transition at 24 °C. These results indicate that the lipid moiety affects the stability of the enzyme and that the protein complex is more stable when reconstituted with the longer, monounsaturated phospholipid (DEPC, C18:1) than with the shorter, saturated phospholipid (DMPC, C14:0). Similar conclusions have been obtained before for the human erythrocyte band 3 protein (Maneri & Low, 1988). Also, membrane protein functional parameters have been observed to depend on phospholipid acyl chain length (Caffrey & Feigenson, 1981; Lewis & Engelman, 1983). It should also be noted that, in addition to the shift in transition temperature, the shape of the calorimetric curves is also affected. In the case of the DEPC reconstitution, the transition profile is sharper and asymmetric toward the low-temperature side of the transition. This change in shape suggests that the lipid environment preferentially interacts with some of the enzyme subunits, presumably those in direct contact with the phospholipid moiety, and that this effect is not completely transduced to the remaining subunits. The total transition enthalpy is essentially the same, within experimental error, for the two systems (ΔH = 405 kcal/mol for the enzyme in DMPC and 425 kcal/mol for the enzyme in DEPC). The van't Hoff enthalpy is larger for the DEPC reconstitution (ΔH_{vh} = 75 kcal/mol) than for the DMPC reconstitution (ΔH_{vh} = 58 kcal/mol), reflecting the fact that the transition occurs over a more narrow temperature range in DEPC. For the beef heart enzyme, we have previously measured a calorimetric transition enthalpy of 550 kcal/mol and a van't Hoff enthalpy of 115 kcal/mol (Rigell et al., 1985; Rigell & Freire, 1987). These values are of similar magnitude if the larger molecular weight of the beef heart enzyme is taken into consideration. It should also be noted that on a weight basis the denaturation enthalpy for these two membrane proteins, as well as other membrane proteins that have been measured calorimetrically (Brouillette et al., 1987; Cladera et al., 1988), is smaller in magnitude than those found for water-soluble proteins (Privalov, 1979). Since the denaturation enthalpy is made up of contributions arising from the melting of the protein secondary structure as well as the exposure of previously buried hydrophobic groups to the solvent, this effect could reflect a smaller degree of secondary structure in membrane proteins, the nonexposure of hydrophobic residues to water after protein denaturation in the case of intrinsic membrane proteins, or both.

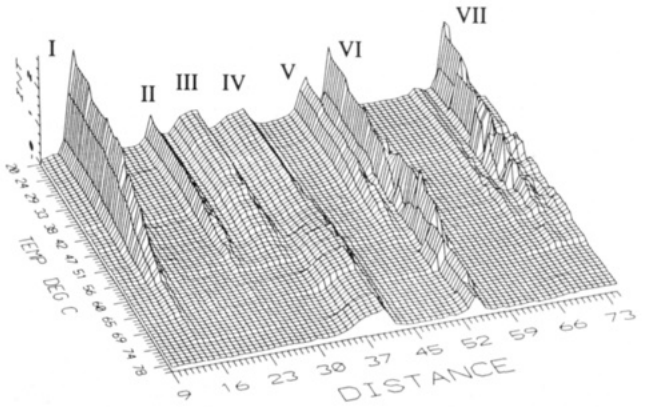

FIGURE 3: Results of differential detergent solubility thermal gel analysis. Shown in the figure is the digitized image of a SDS gel corresponding to the thermal denaturation of DMPC-reconstituted yeast cytochrome *c* oxidase at a scanning rate of 45 °C/h. The Roman numerals indicate the location of the various enzyme subunits in the gel.

Differential Detergent Solubility Thermal Gel Analysis. Differential scanning calorimetry reports the overall thermal denaturation of the enzyme complex. Since yeast cytochrome *c* oxidase is a multisubunit protein, the observed calorimetric heat capacity profiles represent the superposition of several denaturation events corresponding to the various subunits of the enzyme. The fact that the van't Hoff to calorimetric enthalpy ratios ($\Delta H_{vh}/\Delta H$) for both the DMPC- and DEPC-reconstituted enzyme are significantly less than 1 (0.14 for the DMPC- and 0.18 for the DEPC-reconstituted enzyme, respectively) also supports the notion that the overall denaturation process cannot be represented as a two-state transition (Biltonen & Freire, 1978; Rigell & Freire, 1987). A complete characterization of unfolding profiles of this type often requires the use of complementary techniques directed to identify and dissect the contributions of individual subunits to the entire process.

In order to examine the contributions of individual subunits to the overall melting profile, the thermal denaturation of the membrane-reconstituted yeast cytochrome *c* oxidase was also studied by the technique of differential detergent solubility thermal gel analysis. Previously, we have used this technique to study the thermal denaturation of beef heart cytochrome *c* oxidase (Rigell et al., 1985; Rigell & Freire, 1987). This technique takes advantage of changes in protein solubility that are coupled to the thermal denaturation in order to separate subunits in the denatured state from those in the native state. For these experiments, the enzyme was thermally scanned under conditions similar to those used in the calorimetric scans, as described under Experimental Procedures. At each temperature value to be recorded, an aliquot was removed from the water bath, immediately placed on ice, and then exposed to a detergent concentration sufficient to solubilize the native but not the denatured subunits (Rigell & Freire, 1987). Nonsolubilized materials were removed by centrifugation, and the samples were analyzed by SDS gel electrophoresis. Figure 3 shows the digitized image of a gel corresponding to the thermal denaturation of DMPC-reconstituted yeast cytochrome *c* oxidase. As shown in the figure, all of the subunits, except subunits IV and VI, exhibit a clearly distinguishable temperature-induced transition. At low temperatures, the intensity of the bands is maximal and the banding pattern similar to gel patterns previously reported in the literature for native yeast cytochrome *c* oxidase (Power et al., 1984). The seven bands in the digitized picture of the gel are characterized

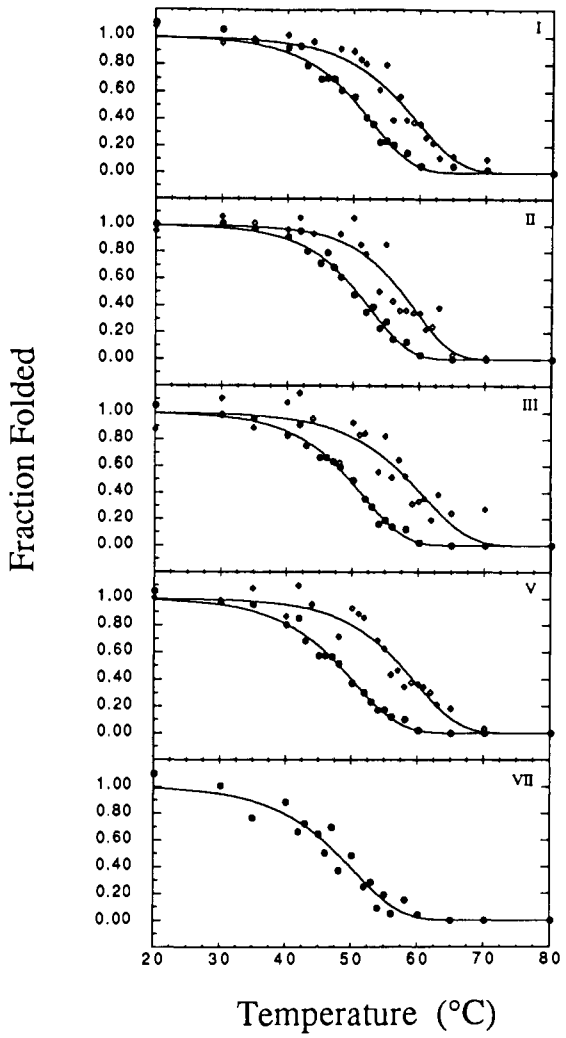

FIGURE 4: Individual subunit temperature denaturation profiles obtained from differential detergent solubility thermal gel analysis experiments. Melting profiles are obtained by integration of the peak intensities (areas under the peaks) for enzyme subunits as a function of temperature. DMPC-reconstituted enzyme (closed circles); DEPC-reconstituted enzyme (open diamonds). Both sets of data were obtained at 45 °C/h.

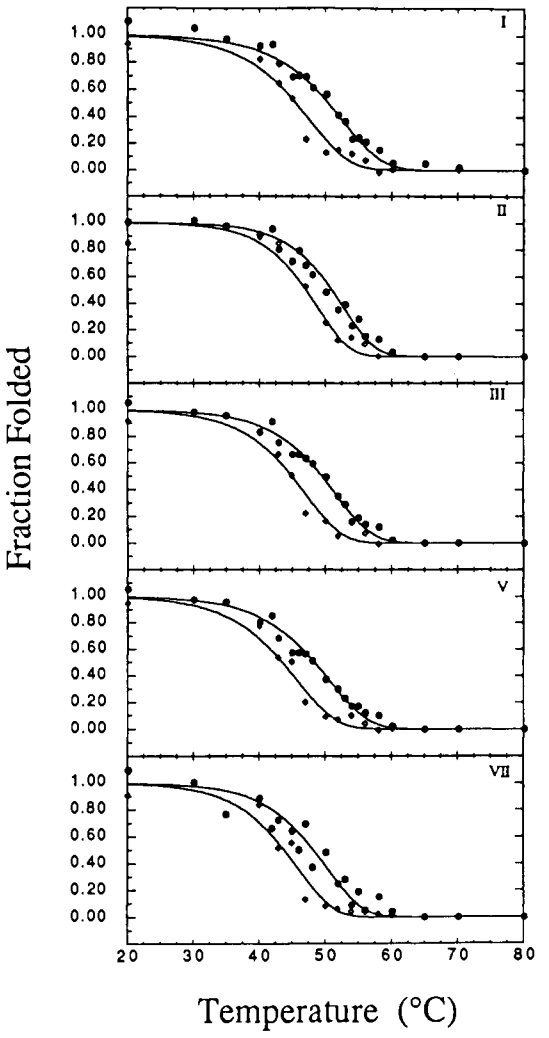

FIGURE 5: Individual subunit temperature denaturation profiles obtained from differential detergent solubility thermal gel analysis experiments with DMPC-reconstituted yeast cytochrome *c* oxidase at scanning rates of 20 °C/h (open diamonds) and 45 °C/h (closed circles).

by apparent molecular weights of 44 000 for subunit I, 28 500 for subunit II, 21 500 for subunit III, 16 500 for subunit IV, 11 700 for subunit V, 9700 for subunit VI, and 4000 for subunits VIIa/b. As the temperature is increased and the transition temperature approached, the bands progressively lose intensity until they disappear at approximately 70 °C, in agreement with the differential scanning calorimetric data. Subunit IV and, to a somewhat lesser extent, subunit VI remain soluble at all temperatures, precluding the use of this technique to study their denaturation. Additional experiments indicate that these two subunits are water soluble even after prolonged boiling and that their thermal denaturation cannot be studied by differential solubility experiments. This observation agrees with previous observations (Poyton & Schatz, 1975) and with their primarily hydrophilic character deduced from the known amino acid sequence of these two subunits (Gregor & Tsugita, 1982; Maarse et al., 1984).

The data in Figure 3 can be used to calculate thermal denaturation profiles for individual subunits within the enzyme complex. This is shown in Figure 4 for the DMPC- and DEPC-reconstituted systems. As in the case of the calorimetric data, the differential solubility thermal gel analysis also indicates a higher melting temperature for the DEPC-reconstituted enzyme. For the DMPC-reconstituted enzyme, the transitions of subunits I and II are centered at 51 °C, subunit III at 49 °C, and the smaller subunits V and VII at 48 °C. This pattern of unfolding agrees with the broad excess heat capacity envelope observed by differential scanning calorimetry. For the DEPC-reconstituted enzyme, the transition temperatures are shifted upward 7 °C on the average; however, the shift is larger for subunit III which under these conditions melts together with subunits I and II at 58 °C. The transition of subunit V is centered at 56.5 °C. The transition of the smallest subunits (VIIa/b) could not be measured in the presence of DEPC due to optical interference from this lipid in the gel at R_f values near 1. These results indicate that subunit III is the most affected by the phospholipid environment, suggesting the existence of different stabilizing interactions between the phospholipid molecules and the enzyme subunits. van't Hoff analysis of the data obtained from the two phospholipid systems indicates an additional 8 kcal/mol to the overall apparent free energy of stabilization for the DEPC-reconstituted enzyme.

Kinetics of Irreversible Denaturation. The thermal unfolding of yeast cytochrome *c* oxidase is irreversible judging from the absence of a transition upon rescanning of the samples. The thermal denaturation of the enzyme is a kinetically controlled process as demonstrated by experiments performed at different scanning rates. Figure 5 shows the results of

Table I: Energetics of Thermal Denaturation of Membrane-Reconstituted Yeast Cytochrome *c* Oxidase

lipid	subunit	ΔH^* (kcal/mol)	ΔH_{vh} (kcal/mol)	θ (K)
DMPC	I	37.5 ± 2.5	55.2 ± 3.7	355.68
	II	38.7 ± 3.1	56.9 ± 4.6	354.45
	III	35.4 ± 4.4	52.1 ± 6.5	356.65
	V	32.6 ± 3.8	48.0 ± 5.6	359.35
	VII	31.8 ± 3.3	46.8 ± 4.9	360.55
DEPC	I	42.9 ± 3.1	63.1 ± 4.6	358.95
	II	42.8 ± 2.7	63.0 ± 4.0	359.85
	III	39.9 ± 3.7	58.7 ± 5.4	362.25
	V	36.2 ± 3.6	53.3 ± 5.9	365.45
	VII	ND		

differential detergent solubility thermal gel analysis experiments performed at scanning rates of 20 and 45 °C/h. As expected for kinetically controlled processes, the melting curves are shifted to higher temperatures at faster scanning rates. This phenomenon is not unique to membrane proteins and has been observed for water-soluble proteins as well. In fact, more than 3 decades ago, Lumry and Eyring (1954) proposed the following model to account for the irreversible unfolding behavior of many proteins:

$$F \underset{k_2}{\overset{k_1}{\rightleftharpoons}} U \xrightarrow{k_3} D \quad (1)$$

According to this model, the equilibrium between the folded and unfolded states of the protein is followed by an irreversible step. The origin of the irreversible step is not unique to all proteins and may originate in several ways including protein precipitation, chemical modifications at high temperatures, autolysis in the case of proteolytic enzymes, etc. Recently, Sanchez-Ruiz et al. (1988a,b) have extensively discussed the validity of the above model and its application to obtain quantitative information for the irreversible denaturation of thermolysin and procarboxypeptidase A. These authors derived several linearized expressions to obtain kinetic parameters from experiments performed at different scanning rates. An alternative way of analyzing the data is by nonlinear least-squares analysis using the numerically solved expression for the population of molecules in the native state. In this way, it is possible to fit directly the observed denaturation profiles versus the independent variable, minimizing potential numerical problems arising from double-logarithmic or inverse temperature dependence plots. For the model in eq 1 under conditions in which $k_3 \gg k_2$, the fraction of molecules in the native state is given by

$$F_0(T) = \exp\left[-(1/\alpha)\int_0^T A \exp(-\Delta H^*/RT)\, dT\right] \quad (2)$$

where ΔH^* is the activation energy, A the preexponential factor, and α the scanning rate. This equation was used to fit the data for each subunit in Figure 5 as well as the data in Figure 4 and the enzyme activity data in Figure 1. In all cases, the solid lines passing through the experimental points correspond to the theoretical curve obtained with eq 2 and the best-fitting parameters summarized in Table I.

Table I summarizes the energetic parameters that best fit the differential solubility thermal gel analysis data. For the DMPC-reconstituted enzyme, the thermal denaturation of subunits I, II, and III is characterized by activation energies on the order of 37 kcal/mol, and that of subunits V and VII by somewhat smaller activation energies. For the DEPC-reconstituted enzyme, the activation energies are approximately 5 kcal/mol larger on the average. It should be emphasized that eq 2 correctly predicts the thermal stability behavior of the enzyme subunits at the scanning rates studied. Unfortunately, eq 2 does not have an exact closed-form solution; however, the transition midpoint for each of the subunits can be predicted as a function of the scanning rate by the approximate equation:

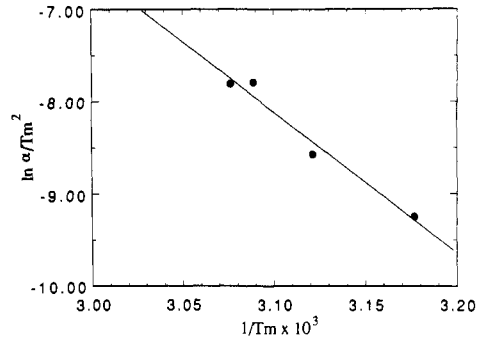

FIGURE 6: Linearized representation of the scanning rate (α) dependence of the midpoint of temperature inactivation (T_m) of DMPC-reconstituted yeast cytochrome *c* oxidase.

$$T_{m,i} = \theta_i + (\ln \alpha - 0.3665)\frac{R\theta_i^2}{\Delta H_i^*} \quad (3)$$

where $T_{m,i}$ is the temperature at which half of the ith subunit molecules have denatured at a scanning rate of α °C/s. The parameter θ has the meaning of a standard reference temperature and is equal to the transition temperature at a scanning rate of 1.4427 °C/s. The enzyme activity data in Figure 1 are also consistent with an activation energy of 39.3 kcal/mol in excellent agreement with the thermal denaturation data. The activation energies for the kinetic model and the van't Hoff enthalpies obtained from the maximum in the excess heat capacity function (Biltonen & Freire, 1978; Sanchez-Ruiz et al., 1988a) are related by the equation $\Delta H_{vh} = 4\Delta H^*/e$. This equation predicts a ΔH_{vh} of 57.8 kcal/mol for the DMPC calorimetric data, in excellent agreement with the experimental value obtained calorimetrically.

An alternative way of obtaining kinetic parameters is by analyzing the scanning rate dependence of the transition temperature. Transition temperatures obtained from additional activity measurements performed on samples scanned at different rates were also consistent with the behavior predicted by the kinetic model. A linearized plot of the scanning rate dependence of the transition temperature obtained from these activity measurements is shown in Figure 6. Analysis of these data as described by Sanchez-Ruiz et al. (1988a) yields an activation energy of 32 kcal/mol, in good agreement with the results obtained from a direct analysis of the thermal denaturation curves.

Discussion

The folding and assembly of multisubunit membrane proteins is a complex process of which very little is known. In the case of mitochondria, this process is additionally compounded by the fact that most of its constituent proteins are synthesized in the cytoplasm and then imported to their final compartment within the mitochondria. In eukaryotes, the three largest subunits of cytochrome *c* oxidase are synthesized within the mitochondria, and the remaining subunits are imported from the cytoplasm. Whether folding and assembly are fully concerted, partially concerted, or independent events is not known.

In order to provide a basic characterization of the structural stability of the enzyme and to investigate the magnitude of the interactions between its various subunits, thermal stability studies were performed under different conditions. As in the

case of the beef heart enzyme previously reported by us (Rigell et al., 1985; Rigell & Freire, 1987), the thermal denaturation of the yeast enzyme is characterized by three important features: (1) The bulk of the enzymes (subunits I and II) melt together and comprise the principal contributors to the main peak observed by differential scanning calorimetry. (2) Subunit III behaves somewhat independently and gives rise to a separate low-temperature peak in the beef heart enzyme and a shoulder in the low-temperature side of the yeast enzyme denaturation profile in the DMPC-reconstituted enzyme; additionally, experiments using two different phospholipid moieties suggest that the stability of subunit III can be affected differently than subunits I and II. (3) The cytoplasmically imported subunits IV and VI in the yeast enzyme and the homologous subunits Va and Vb in the beef heart enzyme [using the nomenclature of Kadenbach et al. (1983)] dissociate from the enzyme complex at the denaturation temperature and stay in aqueous solution after denaturation of the bulk of the enzyme. The thermal stability experiments, in both the beef heart and yeast enzyme, are consistent with the existence of hierarchical intersubunit interactions that define three primary behavioral structures. Apparently, subunits I and II, subunit III, and the imported subunits IV and VI in yeast or Va and Vb in beef heart define the main constituents of these structures. These findings are consistent with other observations in the literature. For example, subunit III in the beef heart enzyme can be selectively removed by either detergent solubilization at high pH (Saraste et al., 1981; Pentilla, 1983) or chymotryptic digestion (Capaldi et al. 1983), resulting in an enzyme that retains electron-transfer activity. Subunits IV and VI in the yeast enzyme can be dissociated from the rest of the enzyme by urea or GuHCl at concentrations below those required for denaturation of the bulk of the enzyme (Poyton & Schatz, 1975; D. Montgomery, unpublished results from this laboratory). Subunit Va in the beef enzyme 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 sequences of subunits Va and Vb in the beef heart enzyme indicates that they are largely water-soluble subunits (Tanaka et al., 1979) as is the case for subunits IV and VI in the yeast enzyme (Gregor & Tsugita, 1982; Maarse et al., 1984).

All the experiments presented in this paper for the yeast enzyme as well as those published previously for the beef heart enzyme indicate that the thermotropic behavior of subunits I and II cannot be uncoupled from each other under the conditions studied, suggesting that the interactions between these two subunits are stronger than those existing with or between other subunits. A plausible, albeit speculative, assembly mechanism consistent with the above observations is that the core of the enzyme is first formed by the association of subunits I and II and that the addition of subunit III, the imported subunits IV and VI in yeast (Va and Vb in beef heart), and the remaining minor imported subunits is only made after the enzyme core is formed. Additional experiments in this laboratory are also consistent with this mechanism and indicate that removal of subunit III does not affect the thermotropic behavior of the beef heart cytochrome *c* oxidase (C. Rigell, unpublished results) and that the dissociated subunits IV and VI from the yeast enzyme possess a tertiary structure and are able to undergo a folding/unfolding transition in the absence of the remaining subunits (D. Montgomery and N. Semo, unpublished results).

The thermal denaturation of yeast cytochrome *c* oxidase is a kinetically restricted process as demonstrated by experiments performed at different scanning rates. At low temperatures, the rate constants are very small, and as such, the enzyme is stable in the native conformation for long periods of time. For example, at 25 °C, the rate constant is 6.5×10^{-5} s^{-1} for the DMPC-reconstituted enzyme and 9.2×10^{-6} s^{-1} for the DEPC-reconstituted enzyme. These rate constants are consistent with relaxation times of 4.5 and 30 h, respectively. As the temperature increases, the rate constants increase in magnitude as dictated by the positive activation energies summarized in Table I. So, for example, at 60 °C, the rate constants are 0.04 s^{-1} for the DMPC- and 0.013 s^{-1} for the DEPC-reconstituted enzyme, respectively. Under the conditions studied, the rate of irreversible thermal denaturation is faster for the DMPC- than for the DEPC-reconstituted enzyme, resulting in a reduced enzyme stability in the former lipid. The intriguing possibility arises that the stabilization of some membrane proteins might not be entirely a thermodynamic phenomenon but a process modulated by kinetic contraints.

Registry No. DMPC, 13699-48-4; DEPC, 52088-89-8; cytochrome *c* oxidase, 9001-16-5.

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Hairpin Structures in DNA Containing Arabinofuranosylcytosine. A Combination of Nuclear Magnetic Resonance and Molecular Dynamics†

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Received May 3, 1989; Revised Manuscript Received August 21, 1989

ABSTRACT: Nuclear magnetic resonance (NMR) and model-building studies were carried out on the hairpin form of the octamer d(CG^{a}CTAGCG) (^{a}C = arabinofuranosylcytosine), referred to as the TA compound. The nonexchangeable protons of the TA compound were assigned by means of nuclear Overhauser effect spectroscopy (NOESY) and correlated spectroscopy (COSY). From a detailed analysis of the coupling data and of the NOESY spectra the following conclusions are reached: (i) The hairpin consists of a stem of three Watson-Crick type base pairs, and the two remaining residues, T(4) and dA(5), participate in a loop. (ii) All sugar rings show conformational flexibility although a strong preference for the S-type (C2′-endo) conformer is observed. (iii) The thymine does not stack upon the 3′ side of the stem as expected, but swings into the minor groove. (This folding principle of the loop involves an unusual α^t conformer in residue T(4).) (iv) At the 5′-3′ loop-stem junction a stacking discontinuity occurs as a consequence of a sharp turn in that part of the backbone, caused by the unusual β^+ and γ^t torsion angles in residue dG(6). (v) The A base slides over the 5′ side of the stem to stack upon the ^{a}C(3) residue at the 3′ side of the stem in an antiparallel fashion. On the basis of J couplings and a set of approximate proton-proton distances from NOE cross peaks, a model for the hairpin was constructed. This model was then refined by using an iterative relaxation matrix approach (IRMA) in combination with restrained molecular dynamics calculations. The resulting final model satisfactorily explains all the distance constraints.

Hairpin structures of RNA and DNA play an important role in biological systems (Sinden & Pettijohn, 1984; Weaver & DePamphilis, 1984; Sheflin & Kowalski, 1985). Particularly, hairpin structures in the anticodon regions of tRNAs and in the control mechanisms of gene expression (Sinden & Pettijohn, 1984; Weaver & DePamphilis, 1984; Sheflin & Kowalski, 1985) are of biological significance. Most of our knowledge with regard to the structural and kinetic, as well as the thermodynamic, aspects of hairpin structures has been obtained by systematic investigations of small synthetic oligonucleotides both in the crystalline state and in solution.

Recently, our NMR[1] studies on the hairpin form of the mismatched octamer d(m^{5}CGm^{5}CGTGm^{5}CG) (m^{5}C = 5-methylcytidine) revealed that this hairpin consists of a loop of only two residues, which bridges the minor groove (Orbons et al., 1987a,b; Altona et al., 1988a,b). A combined electrophoretic and spectroscopic study reported by Xodo et al. (1988) also showed evidence for hairpin structures of DNA compounds in which the loop is limited by two residues. This discovery stands in contrast to earlier intimations in the literature (Haasnoot et al., 1984, 1986; Hilbers et al., 1985), where it was reported that in DNA a four- to five-membered loop leads to the most stable hairpin fragment. The miniloop reported by Orbons et al. (1987b) shows some salient features. The O5′-C5′-C4′-C3′ torsion angle γ of residue dG(6) at the 5′-3′ loop-stem junction takes up the trans (γ^t) rotamer instead of the usual gauche plus (γ^+) conformer. As a consequence, the attached phosphate swings into the minor groove and the phosphate-phosphate distance across the minor groove decreases from the normal value of ca. 18 to 13.3 Å, which

† This research was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organisation for Scientific Research (NWO). This is part 62 of the series "Nucleic Acid Constituents" from this laboratory; for part 61 see van den Hoogen et al. (1989).

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[1] Abbreviations: ^{a}C, arabinofuranosylcytosine; m^{5}C, 5-methylcytidine; DSS, sodium 4,4-dimethyl-4-silapentanesulfonate; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser effect spectroscopy; NOE, nuclear Overhauser effect; COSY, correlated spectroscopy; IRMA, iterative relaxation matrix approach; MD, molecular dynamics.

0006-2960/90/0429-788$02.50/0