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THERMODYNAMIC CHARACTERIZATION OF LIPID INTERACTIONS AND CONFORMATIONAL STABILITY OF MEMBRANE PROTEINS

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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ABSTRACT

The interaction of two membrane proteins, cytochrome b_5 and cytochrome c oxidase with phosphatidylcholine lipid bilayers has been investigated using high-sensitivity scanning calorimetry and fluorescence spectroscopy. In both cases, the incorporation of each of these integral membrane proteins into large single lamellar phospholipid vesicles causes a reduction in the enthalpy associated with the lipid phase transition, a lowering of the transition temperature and a broadening of the lipid melting profile. The dependence of the enthalpy change on the protein:lipid molar ratio indicates that cytochrome b_5 prevents 14 ± 1 lipids from undergoing the gel to liquid crystalline transition and cytochrome c oxidase prevents 99 ± 5 lipid molecules from undergoing the phase transition. Steady state fluorescence spectroscopy of the reconstituted membrane proteins indicates that integral membrane protein acts as a barrier to the lateral mobility of the lipid as probed by pyrene decanoic acid, and increases the apparent order of the phospholipid above the phase transition temperature as determined by diphenylhexatriene fluorescence anistropy measurements.

The thermal denaturation of cytochrome c oxidase was investigated using high sensitivity differential scanning calorimetry and differential solubility thermal gel analysis. The thermal denaturation of cytochrome oxidase is characterized by an enthalpy change of 550 ± 50 Kcal/mole enzyme complex. This irreversible transition occurs at 63°C with the membrane reconstituted enzyme as opposed to

56°C with the detergent-solubilized enzyme, indicating the lipid bilayer has a stabilizing influence on the enzyme complex. The denaturation of the enzyme complex is not a two state process but is composed of four melting steps. Differential solubility thermal gel analysis has permitted the quantitative identification of the contribution of individual subunits of the enzyme complex to the melting steps of the complex heat capacity profile. This gel analysis technique provides an ideal complement to the study of complex membrane protein systems by high sensitivity differential scanning calorimetry.

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INTRODUCTION

Biological membranes are crucial to the very existence of life. Membranes serve as highly selective permeability barriers which define the limits of each cell and in eukaryotic cells define the organelles within each cell. Biological membranes carry out a wide number of functions including ligand/receptor responses, generation of electrical or chemical signals, active or passive transport and energy conversion processes such as photosynthesis and oxidative phosphorylation. Therefore, contributions to the understanding of the structure and function of biological membranes are important to further the description of life at the molecular level.

Protein-Lipid Interactions

It is generally accepted that biological membranes consist of a heterogeneous fluid lipid bilayer which also contains associated protein. Membrane proteins range from being loosely attached to the surface of the membrane to being imbedded into the hydrophobic portion of the bilayer. These proteins are referred to as peripheral and integral membrane proteins, respectively. Peripheral membrane proteins are held to the exposed hydrophilic portion of the bilayer primarily by ionic interactions and may be removed from the membrane by changing the pH or ionic strength of the medium. Integral membrane proteins, however, are held by hydrophobic

interactions to the membrane and are usually only removed by detergent solubilization, chaotropic agents or solvent extraction. Since lipid and membrane protein are the main constituents of biological membranes, the interaction of these components is of fundamental interest.

As with other biochemical investigations of complex systems, biological membranes are often studied by isolating individual components of the membrane and reconstituting the constituents in a carefully defined manner so as to reduce the number of variables which might affect the results of the investigation. Model systems of purified membrane protein and liposomes of defined lipid composition have proven to be useful in the application of biophysical techniques to study the interaction of protein and lipid (Anderson, 1978). Among these techniques, high sensitivity differential scanning calorimetry and fluorescence spectroscopy have been especially useful in examining these interactions (Melchior & Stein, 1976; Mabrey & Sturtevant, 1978; Freire et al., 1983; and Shinktzky and Barenholz, 1978).

Results of these studies on peripheral membrane proteins such as the matrix protein of the vesicular stomatitis virus (Weiner et al., 1983) and cholera toxin (Goins & Freire, 1985) indicate that these proteins have specific interactions with lipid head groups and can lead to phase separation of lipid to which they are specifically bound. However, for lipid systems which exhibit a phase transition, the presence of peripheral membrane protein

has no effect on the enthalpy change associated with the lipid transition which results from cooperative conformational changes of the acyl chains within the lipid bilayer.

On the other hand, the reconstitution of purified integral membrane proteins into liposomes results in a pronounced decrease in the enthalpy associated with the lipid phase transition (Alonso et al., 1982; Chapman et al., 1979; and Curatolo et al., 1977). This effect has been interpreted as resulting from the inability of lipids adjacent to the integral membrane protein to undergo the gel to liquid crystalline phase transition. Calculation of the number of lipids perturbed by each integral membrane protein may be made from the observed enthalpy change as a function of the protein to lipid ratio (Correa-Freire et al., 1979; Freire et al., 1983; Rigell et al., 1985). These studies have indicated that the effect is only local and that the perturbation does not extend beyond one or two layers of lipid around the protein.

Other physical techniques such as Electron Spin Resonance Spectroscopy (ESR) and Nuclear Magnetic Resonance Spectroscopy (NMR) have been used to study the lipid affected by integral membrane proteins (for review see Devaux and Seigneuret, 1985). The affected lipid is referred to as the boundary lipid or the lipid annulus of the membrane protein. As in the case of high sensitivity differential scanning calorimetry, results obtained for glycophorin, $(Na^+ + K^+)$ - ATPase, rhodopsin and cytochrome oxidase indicate that the effect of integral membrane protein on surrounding lipid is a local effect

(Marsh et al., 1982; Jost et al., 1973; Von Zoelen et al., 1978). It must be noted that ESR and NMR measure events occurring on different time scales whereas high sensitivity differential scanning calorimetry measures the time independent equilibrium characteristics of the protein-lipid interaction.

Steady state and time-resolved fluorescence techniques have also been used to study the lipid dynamics within membranes and these techniques have been used to investigate the effect of incorporation of integral membrane proteins into phospholipid bilayers (Shinktzky & Barenholz, 1978; Ikegami et al., 1982). Fluorescence anistropy measurements of lipophilic probes such as diphenylhexatriene have provided information concerning the local fluidity of the lipid environment and the use of excimer forming probes such as derivatives of pyrene has provided information concerning the lateral mobility of lipid within membranes (Galla & Sackmann, 1974; Shinktzky & Barenholz, 1978).

In this thesis, the interactions of two integral membrane proteins, cytochrome b_5 and cytochrome c oxidase with phosphatidylcholine lipid bilayers is investigated using high sensitivity differential scanning calorimetry and steady-state fluorescence spectroscopy.

Conformational Stability of Membrane Protein

Whereas there is much information concerning the influence of protein on the lipid physical state, little is known about what

role the lipid environment plays in maintaining protein structure. It is known that some membrane bound enzymes exhibit different degrees of activity depending on the lipid environment suggesting that the lipid around the membrane protein is important (Ochoa et al., 1983; Kramer & Klingelberg, 1980; Mardershoot et al., 1978).

High sensitivity differential scanning calorimetry has not only been a useful tool for examining the interaction of proteins with lipids but also has provided information concerning the thermal unfolding of proteins. Previously, the application of differential scanning calorimetry has been limited to the study of the thermal denaturation of water soluble proteins. The study of small globular proteins such as ribonuclease, cytochrome c, lysozyme and α-chymotrypsin demonstrated that the unfolding of these water soluble globular proteins is essentially a two state phenomenon, i.e., only two thermodynamically stable states exist at any point during the denaturation (Privalov, 1979). This determination was possible because differential scanning calorimetry provides a measure of both the real enthalpy change for the denaturation as well as a measure of the Van't Hoff enthalpy change from the same set of experimental data.

Water soluble proteins which do not represent simple two state transitions have also been studied using high sensitivity differential scanning calorimetry (Privalov, 1982). Freire and Biltonen (1978) demonstrated that the calorimetric data may be used to obtain the partition function for the system, and that the partition function may be used to obtain the thermodynamic parameters associated with

individual melting steps contributing to a non-two-state transition without any a priori assumptions. This deconvolution technique has been successfully applied in this and other laboratories to several water soluble proteins which do not represent simple two-state denaturation transitions and the melting steps obtained from deconvolution of the calorimetric data has been assigned to the unfolding of specific domains of these proteins (Tsalkova & Privalov, 1985; for review see Privalov et al., 1981).

Whereas there have been numerous calorimetric investigations of the thermal unfolding of many types of water soluble proteins, until recently, no study has been performed on the thermal denaturation of a membrane protein. In this thesis, calorimetry has been used to examine the thermal unfolding of the multisubunit integral membrane protein, cytochrome c oxidase and determine the effect of the hydrophobic environment on the thermal stability of the enzyme complex. Deconvolution analysis of the calorimetric data for the enzyme denaturation results in several melting steps. The protein subunits contributing to each step have been identified using a new technique, differential solubility thermal gel analysis. It is also shown that this technique can be used to obtain apparent enthalpies and melting temperatures for the denaturation of individual subunits of the membrane protein, thus contributing to the development of a quantitative characterization of the thermal unfolding of multisubunit membrane protein complexes.

INTRODUCTION

REFERENCES

REFERENCES

Alonso, A., Restall, C. J., Turner, M., Gomez-Fernandez, J. C., Goni, F. M., & Chapman, D. (1982) Biochim. Biophys. Acta 689, 283-289.

Anderson, H. C. (1978) Ann. Rev. Biochem. 47, 359-383.

Chapman, D., Gomez-Fernandez, J. C. & Goni, F. M. (1979) FEBS Lett. 98, 211-228.

Correa-Freire, M. C., Freire, E., Barneholz, Y., Biltonen, R. L., & Thompson, T. E. (1979) Biochemistry 18, 442-445.

Curatolo, W., Sakura, J. D., Small, D. M., & Shipley, G. G. (1977) Biochemistry 16, 2313-2319.

Devaux, P. F., & Seigneuret, M. (1985) Biochim. Biophys. Acta 822, 63-125.

Freire, E., & Biltonen, R. L. (1978) Biopolymers 17, 463-479.

Freire, E., Markello, T., Rigell, C., & Holloway, P. W. (1983) Biochemistry 22, 1675-1680.

Galla, H. J., & Sackmann, E. (1974) Biochim. Biophys. Acta 339, 103-115.

Goins, B., & Frieire, E. (1985) Biochemistry 24, 1791-1797.

Ikegami, A., Kinosita, K., Kouyama, T., & Kauato, S. (1982) in Structure, Dynamics and Biogenesis of Biomembranes (Sato, R., & Ohnishi, S., Ed.) pp 1-32, Plenum Press, New York.

Jost, P. C., Griffith, O. H., Capaldi, R. A., & Vanderkooi, G. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 480-484.

Kramer, R., & Klingelberg, M. (1980) FEBS Lett. 119, 257-260.

Mabrey, S., & Sturterant, J. M. (1978) in Methods in Membrane Biology vol. 9 (Korn, E. D., Ed.) pp 237-274, Plenum Press, New York.

Mardershoot, J. G., Roelofsen, B., & De Gler, J. (1978) Biochim. Biophys. Acta 508, 478-485.

Marsh, D., Watts, A., Pates, R. D., Uhl, R., Knowles, P. F., & Esmann, M. (1982) Biophys. J. 37, 265-271.

Melchior, D. L., & Steim, J. M. (1976) Ann. Rev. Biophys. Bioeng. 5, 205-238.

Ochoa, E. L. M., Dalziel, A. W., & McNamee, M. G. (1983) Biochim. Biophys. Acta 727, 151-162.

Privalov, P. L. (1979) Adv. Prot. Chem. 33, 167-241.

Privalov, P. L. (1982) Adv. Prot. Chem. 35, 1-104.

Privalov, P. L., Mateo, P. L., Khechinashvili, N. N., Stepanov, V. M., & Revina, L. P. (1981) J. Mol. Biol. 152, 445-464.

Rigell, C. W., de Saussure, C., & Freire, E. (1985). Biochemistry 24, 5638-5646.

Shinitzky, M., & Barenholz, Y. (1978) Biochim. Biophys. Acta 515, 367-394.

Van Zoelen, E. J. J., Van Dijck, P. W. M., De Kruijff, B., Verkleij, A. J., & Van Deenen, L. L. M. (1978) Biochim. Biophys. Acta 514, 9-24.

Weiner, J. R., Wagner, R. R., & Freire, E. (1983) Biochemistry 22, 6117-6123.

PART 1

CALORIMETRIC AND FLUORESCENCE CHARACTERIZATION OF INTERACTIONS BETWEEN CYTOCHROME b_5 AND PHOSPHATIDYLCHOLINE BILAYERS

The arrangement of membrane proteins within the lipid bilayer matrix as well as the interactions between protein and lipid components of biological membranes has been the subject of considerable interest in recent years. From those studies, it became evident that the incorporation of membrane proteins into lipid bilayer membranes had a perturbing effect on the structural state of the surrounding lipid and that this perturbation might have profound effects on the properties and behavior of the membrane. The availability of purified membrane proteins that can be reconstituted into well-characterized lipid vesicles has permitted the use of a variety of physical techniques directed to probe the nature of the protein-lipid interaction (Curatolo et al., 1977; Chapman et al., 1979; Marsh et al., 1982). In this study, we present the results of a calorimetric and fluorescence investigation of the interactions of cytochrome b_5 with single lamellar vesicles prepared from dimyristoylphosphatidylcholine and dipalmitoylphosphatidylcholine.

Cytochrome b_5 is an intrinsic membrane protein with a molecular weight of 16700 (Spatz & Strittmatter, 1971); it consists of two independent domains, a hydrophilic region that bears the heme group and a functionally distinct hydrophobic domain that anchors the molecule to the membrane (Visser et al., 1975; Spatz & Strittmatter, 1971; Strittmatter et al., 1972). Cytochrome b_5 is mainly found

in the endoplasmic reticulum of liver cells (Jarasch et al., 1979) and can be isolated by detergent extraction. The isolated lipid-free protein binds rapidly and completely preformed phosphatidylcholine vesicles and other membranes (Strittmatter et al., 1972; Sullivan & Holloway, 1973; Roseman et al., 1978; Leto & Holloway, 1979). It has also been documented (Roseman et al., 1977; Enoch et al., 1977; Leto et al., 1980) that cytochrome b_5 exchanges rapidly between phosphatidylcholine vesicles and other membrane systems. This intermembrane exchange of an intrinsic membrane protein may prove to be of importance in a variety of biological processes including the assembly of biomembranes within the cell.

The studies presented in this part describe the effects of cytochrome b_5 on the thermotropic behavior of the lipid bilayer and evaluate the nature and magnitude of the lipid perturbation exerted by the protein. This investigation has been accomplished by combining high-sensitivity scanning calorimetry with fluorescence measurements that measure the rate of excimer formation of the pyrene probe.

Materials and Methods

Dimyristoylphosphatidylcholine and dipalmitoylphosphatidylcholine were obtained from Avanti Biochemicals (Birmingham, AL) and used without further purification. Rabbit liver cytochrome b_5 was prepared by the method originally described by Ozols (1974) and modified by Leto (1980). Pyrenedecanoic acid was obtained from Molecular Probes

(Plano, TX) and used without further purification. Stock solutions of pyrenedecanoic acid were prepared with tetrahydrofurane (THF) or acetonitrile as solvent and stored under nitrogen, in the dark at 4°C.

All the vesicle preparations used for these experiments were fused unilamellar vesicles prepared essentially as described by Schullery et al. (1980). DMPC or DPPC was first dried from a chloroform solution and lyophilized overnight. The dried lipid was suspended in 50 mM KCl containing 0.02% sodium azide to give a concentration of 70 mM. The lipid suspensions were sonicated and centrifuged above the lipid phase transition temperature and then incubated at 4°C for one week as described by Wong et al. (1982). This low-temperature incubation triggers a spontaneous fusion process and produces a homogeneous population of single lamellar vesicles of ~700-Å diameter (Wong et al., 1982).

Cytochrome b_5 was incorporated into DMPC or DPPC fused unilamellar vesicles by incubating the desired amounts of protein and lipid as outlined by Leto et al. (1980). These membrane preparations were checked for degree and sidedness of protein incorporation and stability to heme loss. Aliquots containing 1 mg of lipid were layered on a Sephadex G-100 column (0.8 x 25 cm) and eluted with Pipes buffer, pH 8.1-1.0 mM EDTA. The 0.5-mL fractions were collected, and spectral scans were taken from 280 to 450 nm. The cytochrome b_5 concentrations were calculated from an extinction coefficient of 114000 M^{-1} cm^{-1} at 412 nm. A total of 50 µL of each fraction was

analyzed for lipid phosphorus by the method of Bartlett (1959). The incorporation of cytochrome b_5 was found to be ~100% into the outer monolayer with less than 1% loss of heme absorption over the course of all experiments performed.

The sideness of cytochrome b_5 was checked by placing an aliquot of the stock (final concentration 20 μg of lipid/mL) in a cuvette containing 3.7 μg of purified NADH-cytochrome b_5 reductase protein (water-soluble proteolytic fragment, M_r 40,000). The absorbtion spectrum was recorded, and the absorbance at 412 and 425 nm was measured. The cuvette solution was made 50 mM in NADH, and the spectrum was remeasured. Additional NADH had no effect, nor did NADH without reductase present. Finally, a few crystals of dithionite (a membrane-permeable reductant) were added to the cuvette, and no further reduction was observed (<2%), indicating that all the cytochrome b_5 was located in the outer layer of the vesicles.

The calorimetric experiments were performed with a Microcal MC1 differential scanning calorimeter at a scanning rate of 20°C/h. Lipid concentrations for the calorimetric experiments ranged between 5 and 10 mg/mL.

All temperature scanning fluorescence experiments were performed in a modified Aminco Bowman spectrofluorometer equipped with a photon counter and a thermostated cuvette holder connected to a Neslab RTE-8 refrigerated bath circulator equipped with a temperature programmer (ETP-3). The temperature of the sample

preparations was monitored during the experiments with a thermistor immersed inside the sample cell. Fluorescence intensities and temperatures were simultaneously recorded on a four-channel Hewlett-Packard 3467A logging multimeter.

Results

Scanning Calorimetry. Scanning calorimetric scans were performed on DMPC and DPPC large unilamellar vesicles containing different mole fractions of cytochrome b_5. These large unilamellar vesicles give rise to very sharp peaks in the calorimeter, similar to those observed with multilamellar liposomes. The gel-liquid-crystalline phase transition of these vesicles is characterized by a ΔH of 5.4 kcal/mol and a T_m of 24.1°C for DMPC and a ΔH of 8.6 kcal/mol and a T_m of 41.2°C for DPPC. The half-height widths ($\Delta T_{1/2}$) are 0.4 and 0.7°C for DPPC and DMPC, respectively. As shown in Figure 1-1, the incorporation of cytochrome b_5 into these vesicles causes a monotonic decrease in the enthalpy change (area under the heat capacity curve) for the gel-liquid-crystalline transition but only a negligible change in the phase transition temperature. It should also be mentioned that the half-height width of the transition increases only 0.1°C up to a protein/lipid molar ratio of 1/100, indicating that cytochrome b_5 does not largely disrupt the cooperative behavior of the lipid bilayer matrix. Only at protein/lipid ratios higher than 1/50 does the heat capacity function become skewed toward the low-temperature end of the transition, apparently due to the existence of two superimposed

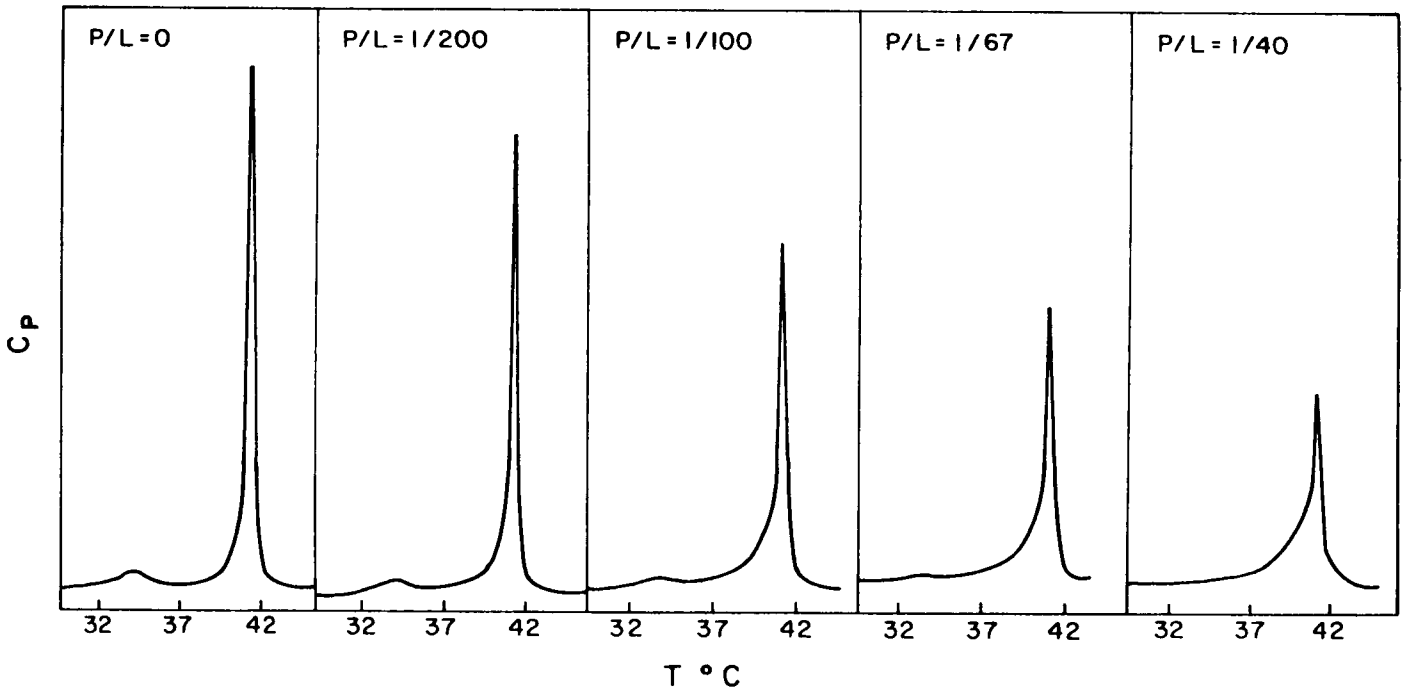


Figure 1-1. Excess heat capacity function vs. temperature for DPPC large single lamellar vesicles containing different concentrations of cytochrome b_5.

peaks, a sharp component having the transition characteristics of the pure lipid and a broader component centered at a somewhat lower temperature. As seen in Figure 1-1, the incorporation of cytochrome b_5 also affects the phospholipid pretransition; it gradually diminishes its amplitude until it is not longer detectable by the calorimeter at protein/lipid mole ratios greater than 1/50. The decrease in ΔH upon increasing the protein/lipid ratio is consistent with the model that the protein molecules are preventing some of the lipid molecules 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)

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

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 phase transition per protein molecule. As shown in Figure 1-2, a linear least-squares analysis of the data for both DMPC and DPPC indicates that each cytochrome b_5 molecule subtracts 14 ± 1 lipid molecules from the phase transition.

Cleavage of the hydrophilic domain of cytochrome b_5 by the addition of trypsin to the vesicle preparations did not affect the heat capacity profiles, indicating that the hydrophobic portion of the protein is responsible for the decrease in ΔH and that the hydrophilic domain interacts very little if at all with the membrane surface. This result is consistent with the proposed three-dimensional structure of cytochrome b_5 in which the hydrophilic

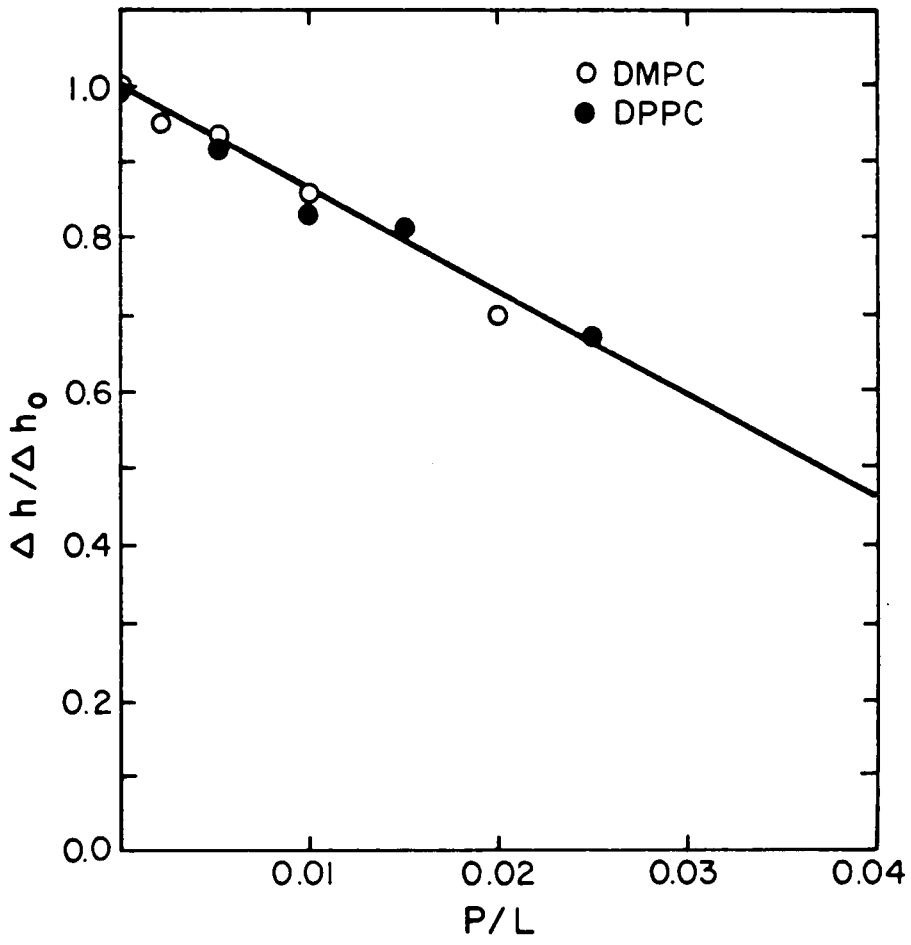


Figure 1-2. Relative decrease in enthalpy change associated with gel-liquid-crystalline transition of DMPC and DPPC as a function of cytochrome b_5/lipid molar ratio.

domain is separated from the hydrophobic domain by a joining segment of approximately nine amino acid residues (Strittmatter & Dailey, 1982). This result is also consistent with the observation that cytochrome b_5 has only a minor effect on the cooperative behavior of the lipid phase transition, as expected from the interaction with the small hydrophobic domain and not from the relatively bulky hydrophilic region.

Excimer formation. The technique of excimer formation using pyrene derivatives embedded within the lipid bilayer matrix is well documented in the literature (see Galla & Hartmann, 1980, for a recent review) and has been used to investigate lipid lateral mobilities as well as phase-separation phenomena. Figure 1-3 shows the temperature dependence of the ratio I'/I of the fluorescence intensities of pyrenedecanoic acid measured at the maxima of the excimer (I') and monomer bands (I) for several cytochrome b_5/DPPC ratios. If the system is cooled from above to below the lipid phase transition temperature, there is an increase in the excimer/monomer ratio at T_m. In the absence of protein this increase is relatively large and is due to the fact that pyrenedecanoic acid is excluded from the gel phase and concentrated in the remaining fluid regions of the bilayer; finally, as the entire bilayer becomes solid, pyrenedecanoic acid aggregates forming static dimers whose properties are not diffusion controlled (Galla & Sackmann, 1974, 1975). As the protein/lipid ratio increases, the amplitude of the change in the excimer/monomer ratio associated with the lipid phase transition gradually

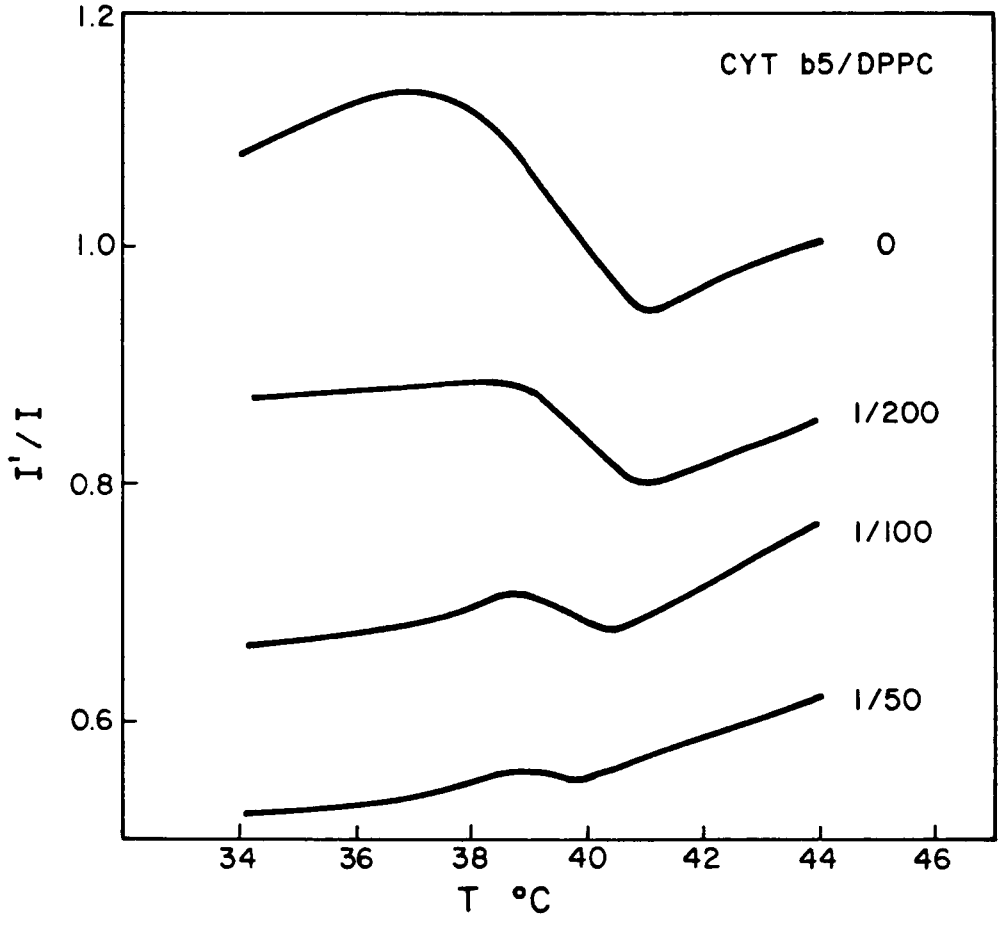


Figure 1-3. Excimer/monomer ratios of pyrenedecanoic acid vs. temperature for DPPC large single lamellar vesicles containing different amounts of cytochrome b_5. In these experiments the samples were excited at 320 nm, and the emissions of monomer and dimer were taken at their maxima at 395 and 480 nm, respectively. The lipid concentration was 50 μM, and the amount of pyrenedecanoic acid was 5 mol %.

decreases. This result is consistent with the calorimetric and fluorescence energy transfer data, which indicate that the presence of protein molecules prevents some of the lipid molecules from participating in the gel-liquid-crystalline transition and that this lipid remains in a disordered configuration at all temperatures. As the concentration of protein increases, more lipid remains in this disordered state below T_m, thus preventing the aggregation of pyrenedecanoic acid. This protein effect is very similar to the one observed after addition of cholesterol into the lipid bilayer (Estep et al., 1978; Galla & Hartmann, 1980).

Above the lipid phase transition the formation of excited dimers is a diffusion-controlled process as demonstrated by Galla & Sackmann (1974). Under these conditions the addition of cytochrome b_5 causes a progressive decrease in the excimer/monomer ratio (see Figure 1-3). Since the ratio (I'/I) is directly proportional to the lateral diffusion coefficient of the lipid probes within the membrane, this result indicates that the presence of cytochrome b_5 slows down the free movement of the probes. Monte Carlo calculations of lipid lateral diffusion (W. Van Osdol & E. Freire, unpublished results) indicate that this effect can be interpreted in terms of the proteins acting as obstacles or barriers to the free diffusion of the lipids without being necessary to assume a major structural perturbation of the lipid bilayer matrix.

Discussion

The calorimetric results presented in this paper indicate that the major effect of cytochrome b_5 on the thermotropic behavior of the lipid bilayer is a progressive reduction in the enthalpy change associated with the gel-liquid-crystalline phase transition. This effect is similar to the one observed with other membrane proteins (Chapman et al., 1979; Curatolo et al., 1977), suggesting the existence of a rather general and nonspecific perturbing effect. The dependence of the enthalpy decrease upon the protein/lipid molar ratio indicates that each cytochrome b_5 molecule prevents 14 ± 1 phospholipid molecules from undergoing the phase transition. This number is equivalent to the number of lipid molecules in the outer monolayer of the vesicle that can be fit in a single layer around a cylinder of 16-Å radius. Vaz et al. (1979) have previously estimated a Stokes radius of ~16 Å for the cytochrome b_5 hydrophobic tail; Visser et al. (1975) calculated a Stokes radius of 17.4 Å from hydrodynamic measurements. Thus, the calorimetric results give support to the idea that the protein perturbation of the bilayer is only local and that it extends to only one layer of lipid around the protein. Furthermore, the calorimetric data suggest that the major perturbation induced by cytochrome b_5 is in the outer layer of the membrane and that the inner layer is affected very little if at all. The existence of two superimposed peaks at high protein/lipid ratios might be the result of such molecular arrangement. If such is the case, the sharp component would correspond to the inner layer of the

bilayer whereas the broad component would correspond to the larger perturbed outer layer. Since cytochrome b_5 is able to rapidly exchange on this time scale between lipid vesicles (Roseman et al., 1977; Leto et al., 1980), it is very unlikely that the two overlapping peaks observed with the calorimeter arise from an uneven distribution of the protein among the lipid vesicles.

The lipid molecules surrounding the protein molecule are unable to undergo the gel-liquid-crystalline transition, remaining in the same physical state below and above the transition temperature of the bulk lipid. The boundary-layer lipid appears to be in a relatively disordered state, judging from the fact that, below T_m, pyrenedecanoic acid is excluded from the bulk lipid and partitions around the protein molecules. This conclusion is consistent with the observations of Kimelman et al. (1979) for the M13 coat protein using trans-parinaric acid as a lipid probe; these authors observed that, below T_m, trans-parinaric acid was excluded from the boundary-layer lipid due to its preferential partitioning in the gel phase. Thus, the protein molecules appear to be surrounded by a relatively disordered layer of lipid whose structural state is somewhat insensitive to the physical state of the rest of the membrane. These conclusions agree with deuterium nuclear magnetic resonance experiments on cytochrome b_5-DMPC bilayers that indicate that, below T_m, the presence of the protein prevents some of the lipid molecules from undergoing crystallization (Oldfield et al., 1978).

Even though the perturbations exerted by individual protein

molecules are only local, the superposition of these local perturbations gives rise to macroscopic effects that affect the properties and behavior of the lipid bilayer as a whole. As discussed above, the incorporation of protein molecules into the lipid bilayer slows down the lateral diffusion of the lipid probes even in the absence of a major structural change in the lipid bilayer. This is due to a reduced collision frequency of the lipid probes induced by the presence of the protein molecules within the bilayer. The presence of protein molecules, acting as obstacles for the free lateral diffusion of the lipid molecules, causes an increase in the mean number of diffusional steps required for the collision of two lipid probes and results in a reduced rate of excimer formation. It must be noted that the incorporation of cytochrome b_5 into the lipid bilayer does not affect the excimer fluorescence lifetime of pyrenedecanoic acid (C. Rigell, S. Georghiou, & E. Freire, unpublished results), in agreement with the interpretation that the reduced rate of excimer formation observed in the presence of the protein is a purely diffusional phenomenon.

The effect of the protein molecules on the collision frequency of other molecules embedded within the bilayer will affect the reaction rates of second-order and higher order reactions within the membrane. Also, since this effect arises from the topological distribution of molecules along the plane of the membrane, changes in the state of aggregation of membrane proteins as well as the formation of phase-separated domains will undoubtedly affect the efficiency and kinetic parameters of membrane-located biochemical reactions.

PART 1

REFERENCES

REFERENCES

Bartlett, G. R. (1959) J. Biol. Chem. 234, 466.

Chapman, D., Gomez-Fernandez, J. C., & Goni, F. M. (1979) FEBS Lett. 98, 211-228.

Correa-Freire, M. C., Freire, E., Barenholz, Y., Biltonen, R., & Thompson, T. E. (1979) Biochemistry 18, 442-445.

Curatolo, W., Sakura, J. D., Small, D. M., & Shipley, G. G. (1977) Biochemistry 16, 2313-2319.

Enoch, H. G., Fleming, P. J., & Strittmatter, P. (1977) J. Biol. Chem. 252, 5656-5660.

Estep, T. N., Mountcastle, D. B., Biltonen, R. L., & Thompson, T. E. (1978) Biochemistry 17, 1984-1989.

Galla, H. J., & Sackmann, E. (1974) Biochim. Biophys. Acta 339, 103-115.

Galla, H. J., & Sackmann, E. (1975) J. Am. Chem. Soc. 97, 4114.

Galla, H. J., & Hartmann, E. (1980) Chem. Phys. Lipids 27, 199-219.

Jarasch, E. D., Jurgen, K., Bruder, G., Fink, A., Morre, D. J., & Franke, W. W. (1979) J. Cell Biol. 80, 37-52.

Kimelmann, D., Tecoma, E. S., Wolser, P. K., Hudson, B. S., Wickner, W. T., & Fimoni, R. D. (1979) Biochemistry 18, 5874-5880.

Leto, T. L. (1980) Ph.D. Dissertation, University of Virginia.

Leto, T. L., & Holloway, P. W. (1979) J. Biol. Chem. 254, 5015-5019.

Leto, T. L., Roseman, M. A., & Holloway, P. W. (1980) Biochemistry 19, 1911-1916.

Marsh, D., Watts, A., Pates, R. D., Uhl, R., Knowles, P. F., & Esmann, M. (1982) Biophys. J. 37, 265-271.

Oldfield, E., Gilmore, R., Glaser, M., Gutowsky, H. S., Hshung, J. C., Kang, S. Y., King, T. E., Meadows, M., & Rice, D. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 4657-4660.

Ozols, J. (1974) Biochemistry 13, 426-434.

Roseman, M., Holloway, P. W., Calabro, M. A., & Thompson, T. E. (1977) J. Biol. Chem. 252, 4842-4849.

Roseman, M., Holloway, P. W., & Calabro, M. A. (1978) Biochim. Biophys. Acta 507, 552-556.

Schullery, S., Schmidt, C. F., Felgner, P. L., Tillack, T. W., & Thompson, T. E. (1980) Biochemistry 19, 3919-3923.

Spatz, L., & Strittmatter, P. (1971) Proc. Natl. Acad. Sci. U.S.A. 68, 1042-1046.

Strittmatter, P., & Dailey, H. A. (1982) in Membranes and Transport (Martonosi, A. N., Ed.) pp 71-82, Plenum Press, New York.

Strittmatter, P., Rogers, M. J., & Spatz, L. (1972) J. Biol. Chem. 247, 7188-7194.

Sullivan, M. R., & Holloway, P. W. (1973) Biochem. Biophys. Res. Commun. 54, 808-815.

Vaz, W. L. C., Austin, R. H., & Vogel, H. (1979) Biophys. J. 26, 415-426.

Visser, L., Robinson, N. C., & Tanford, C. (1975) Biochemistry 14, 1194-1199.

Wong, M., Anthony, F. H., Tillack, T. W., & Thompson, T. E. (1982) Biochemistry 21, 4126-4132.

PART 2

ISOLATION OF CYTOCHROME c OXIDASE

Cytochrome c oxidase was isolated and purified from bovine heart. The protocol developed is based on that described by Capaldi and Hayashi (1972). Isolation of cytochrome oxidase includes three major steps: the isolation of beef heart mitochondria, the preparation of the green membrane fraction and the ammonium sulfate precipitation of cytochrome oxidase.

Isolation of Beef Heart Mitochondria

Beef hearts are obtained fresh from the killing floor at the local meat packing plant and immediately packed in ice. Typically, six beef hearts are used for each preparation. All subsequent work is done in a cold room at ~4°C. The beef hearts are trimmed of fat and connective tissue and cut into small pieces (~1 inch cubes).

Approximately 800 gms of cubed beef heart are added to 2400 milliliters of o.25 M sucrose, 10 mM Tris HCl, 0.2 mM EDTA pH 7.8 and 8 milliliters of 4 M NaOH is also added prior to homogenization for 45 seconds at medium speed in a large, four liter capacity, Waring blender. Following homogenization, the slurry is diluted 1:3 with the sucrose buffer in 500 milliliter centrifuge bottles. The homogenate is shaken well and centrifuged 15 minutes at 4,000 rpm in a Beckman J21C centrifuge to remove cellular debris and nuclei. The supernatant is filtered through two layers of cheesecloth to remove floating fat globules and the pellet is discarded. The supernatant is centrifuged for 20 minutes at 13,000 rpm in a GSA

rotor in a Sorvall centrifuge to sediment mitochondria. The resultant brown pellet is collected and washed with four volumes of the sucrose buffer. This pellet is stored overnight at -20°C.

Preparation of the Green Membrane Fraction

The frozen mitochondria pellet is thawed in warm tap water with agitation to ensure that the peripheral mitochondria slurry remains at a temperature less than 4°C. After gentle homogenizaiton of the solution, a representative one milliliter aliquot is removed and the total volume of the slurry is measured. A biuret protein assay (Gornall et al., 1949) is performed on the aliquot and the protein concentration of the entire sample is adjusted to 23 mg/ml with 0.66 M Sucrose, 0.05 M Tris HCl, 0.001 M Histidine HCl pH 8.0.

To this solution deoxycholate is added from a 10% solution w/v pH 10.0 such that there is 0.3 mg of deoxycholate per milligram of protein. (Note--both deoxycholate and cholate (sodium salts) were obtained from Sigma and are recrystallized from ethanol solution at least three times prior to preparation of solutions as described by Wilkstrom, 1979.) After the addition of the deoxycholate solution, crystalline KCl (72 gm/liter of total suspension) is added. Once the KCl is completely dissolved with stirring, the solution is centrifuged at 30,000 rpm for 30 minutes using a Ti60 or 42.1 rotor in a Beckman ultracentrifuge. The pellet obtained is the green membrane pellet described by Fowler et al. (1962).

Ammonium Sulfate Precipitation of Cytochrome Oxidase

After discarding the supernatant, the green membrane pellet is suspended in 0.1 M KPO_4 pH 7.4 using a homogenizer being careful not to add too much buffer so as to keep the protein concentration greater than 19 mg/ml. A small aliquot is removed and the total volume of the solution is measured. Once again the protein concentration is measured using the biuret protein assay and the protein concentration is adjusted to 19 mg/ml with 0.1 M KPO_4 buffer pH 7.4.

To this solution, cholate is added from a 20% cholate w/v pH 7.4 solution to a concentration of 1 mg cholate per mg protein at 0°C (the solution is stirred in an ice bath). A neutralized saturated ammonium sulfate solution is added while stirring to 25% v/v. This solution is stirred for one hour at 0°C, and centrifuged at 37,000 rpm for 15 minutes using a Ti60 or 42.1 rotor. The supernatant, which contains the cytochrome oxidase is removed and the solution is made 0.31 ammonium sulfate using the equation described by Yu et al. (1972).

$$Y = \frac{S_2 - S_1}{1 - S_2}$$

Y is the volume in milliliters of saturated ammonium sulfate solution to be added to a one milliliter solution of initial saturation S_1 to obtain a final saturation of S_2.

After the addition of ammonium sulfate, the solution is stirred for 30 minutes and centrifuged at 37,000 rpm for 15 minutes.

The pellet is saved and the ammonium sulfate concentration of the supernatant is adjusted to 0.37. The supernatant is centrifuged at 37,000 rpm for 15 minutes and another addition is made to obtain 0.41 ammonium sulfate in that supernatant. Each of the pellets is saved separately. If the supernatant is green following the 0.41 ammonium sulfate cut, the solution is made 0.45 ammonium sulfate and centrifuged. The final supernatant should be light yellow and may be discarded. The cytochrome oxidase pellet is an oily dark green precipitate. All pellets are gently homogenized in 0.1 M PO_4 buffer pH 7.4 and dialyzed against the same buffer to remove residual salt and detergent. Both heme a/protein ratios and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) profiles are determined for the pellets to measure the purity of the preparations. SDS-polyacrylamide gel electrophoresis is done using the method of Kadenbach et al. (1983). If either the heme/protein ratio is too low or contaminant bands are present, the ammonium sulfate fractionation is repeated. The protein concentration is readjusted to 19 mg/ml using the biuret procedure and cholate and ammonium sulfate are added as described earlier. The heme a/protein ratios and SDS-PAGE profiles are redetermined to measure the purity of the isolated cytochrome oxidase.

Measurement of Heme a/Protein Ratio

Heme content is measured by dissolving the purified cytochrome oxidase in a 0.1% Tween 80 0.1 M PO_4 pH 7.4 solution, 0.1 mg/ml final

protein concentration and measuring the absorbance at 605 nm for both the oxidased (isolated form) and reduced state. A few grains of sodium hydrosulfite (dithionite) is used to obtain the reduced form of the enzyme. Heme content is calculated using the extinction coefficient of $\varepsilon^{\text{red-ox}}_{605\ \text{nm}} = 13.5\ \text{mM}^{-1}\ \text{cm}^{-1}$ (Yu et al., 1975).

The protein content is measured using the Lowry protein assay. Heme a/protein ratios are expressed as nanomoles heme a per milligram protein and should fall into the range of 9-11 nmoles heme a/mg protein.

Measurement of Cytochrome c Oxidase Activity

To insure that the isolated cytochrome oxidase molecule is competent in the transfer of electrons from cytochrome c to molecular oxygen, the activity of the purified enzyme is measured using the method of Smith (1955). In this procedure, the activity is expressed as the first order velocity constant for the oxidation of reduced cytochrome c.

A cytochrome c solution is prepared from Sigma horse heart cytochrome c type VI. About 2 milliliters of 300 μM cytochrome c in 0.01 m PO_4 buffer pH 7.0 is reduced by adding a small amount of sodium dithionite. The cytochrome c solution will change from dark red to a light orange color.

A rapid gel filtration centrifugation column is prepared as described by Tuszynski et al. (1980). A three milliliter plastic syringe casing is used as the column. A small amount of glass wool

is placed in the bottom of the syringe and lightly tamped into place. Preswollen Sephadex G-25 is added and allowed to settle up to the three milliliter mark. The syringe is placed into a 13 x 100 mm test tube and centrifuged at medium speed (~1400 rpm) in a desk top centrifuge (IEC-HNSII) for 90 seconds. The column is rinsed twice with two milliliters of 0.01 M PO_4 pH 7.0 buffer and the reduced cytochrome c solution is added to the column and centrifuged again for 90 seconds. The resultant cytochrome c solution is adjusted to 90 µM with 0.01 M PO_4 pH 7.0 buffer and stored in a test tube under nitrogen.

Activity is measured spectrophotometrically as a decrease in the absorbance at 550 nm of the cytochrome c solution. The cuvette contains 2.4 milliliters of 0.1 M PO_4 pH 7.0 with 0.6% Tween 80, and 0.5 milliliters reduced cytochrome c solution (90 µM), A 100 µl aliqiot of diluted cytochrome oxidase dissolved in 0.1 M PO_4 pH 7.0 with 0.5% Tween 80 is added such that complete oxidation of cytochrome c occurs between three and ten minutes. Immediately after addition of the oxidase, readings are taken at ten second intervals using a Hewlett Packard 3467A Logging Multimeter interfaced to an LKB Ultrospec 4050 spectrophotometer. A drop of saturated $K_3Fe(CN)_6$ solution is added to determine the absorbance, OD_∞, of the completely oxidized cytochrome c solution. A plot of $\ln(OD-OD_\infty)$ versus time gives the velocity constant which is described in terms of µmoles cytochrome c oxidized per minute. Specific activity is related to the amount of cytochrome oxidase present and

should fall into the range of 8-11 μmoles cytochrome c oxidized min^{-1} (mg of protein)$^{-1}$.

An alternate method of determining cytochrome oxidase activity is described by Yonetani (1966).

PART 2

REFERENCES

REFERENCES

Capaldi, R. A., & Hayashi, H. (1972) FEBS Lett. 26, 261-263.

Fowler, L. R., Richardson, S. H., & Hatefi, Y. (1962) Biochim. Biophys. Acta 64, 170-173.

Gornell, A. G., Bardawill, C. J., & David, M. M. (1949) J. Biol. Chem. 177, 751-756.

Kadenbach, B., Jarausch, J., Hartmann, R., & Merle, P. (1983) Anal. Biochem. 129, 517-521.

Smith, L. (1955) Methods Enzymol. 2, 732-741.

Tuszynski, G. P., Knight, L., Piperno, J. R., & Walsh, P. N. (1980) Anal. Biochem. 106, 118-122.

Wikstrom, M., & Sigel, E. (1979) in Membrane Biochemistry (Carafoli, E., & Semerza, G., Eds.) pp 82-91, Springer-Verlag, New York.

Yonetani, T. (1966) Biochem. Prep. 11, 14-20.

Yu, C. A., Yu, L., & King, T. E. (1972) J. Biol. Chem. 247, 1012-1019.

Yu, C. A., Yu, L., & King, T. E. (1975) J. Biol. Chem. 250, 1383-1392.

PART 3

PROTEIN AND LIPID STRUCTURAL TRANSITIONS IN CYTOCHROME c OXIDASE-DIMYRISTOYLPHOSPHATIDYLCHOLINE RECONSTITUTIONS

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 study, 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 through 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 part address both the effects of cytochrome c oxidase on the thermotropic behavior of the phospholipid molecules and also the conformational stability of the 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 three times in ethanol to remove bile sale contaminants as described by Wilkstrom (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 and 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

$$\varepsilon^{\text{red-ox}}_{605\text{nm}} = 13.5\ \text{mM}^{-1}\ \text{cm}^{-1}.$$

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 (Tuszynski et al., 1980). Typical activities were in agreement with other reports, ranging from 8 to 11 μmol of cytochrome c oxidized min^{-1} $(\text{mg of protein})^{-1}$ (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 at 4°C. 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,t here 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 of 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. For the gel electrophoresis analysis, the same concentration of protein was used for the Tween 80 solubilized samples as for the membrane reconstituted samples.

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 x 18 x 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 nomenclature 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 15000g 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 3-1 shows the excess heat capacity function associated with the main transition of dimyristoylphosphatidylcholine 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

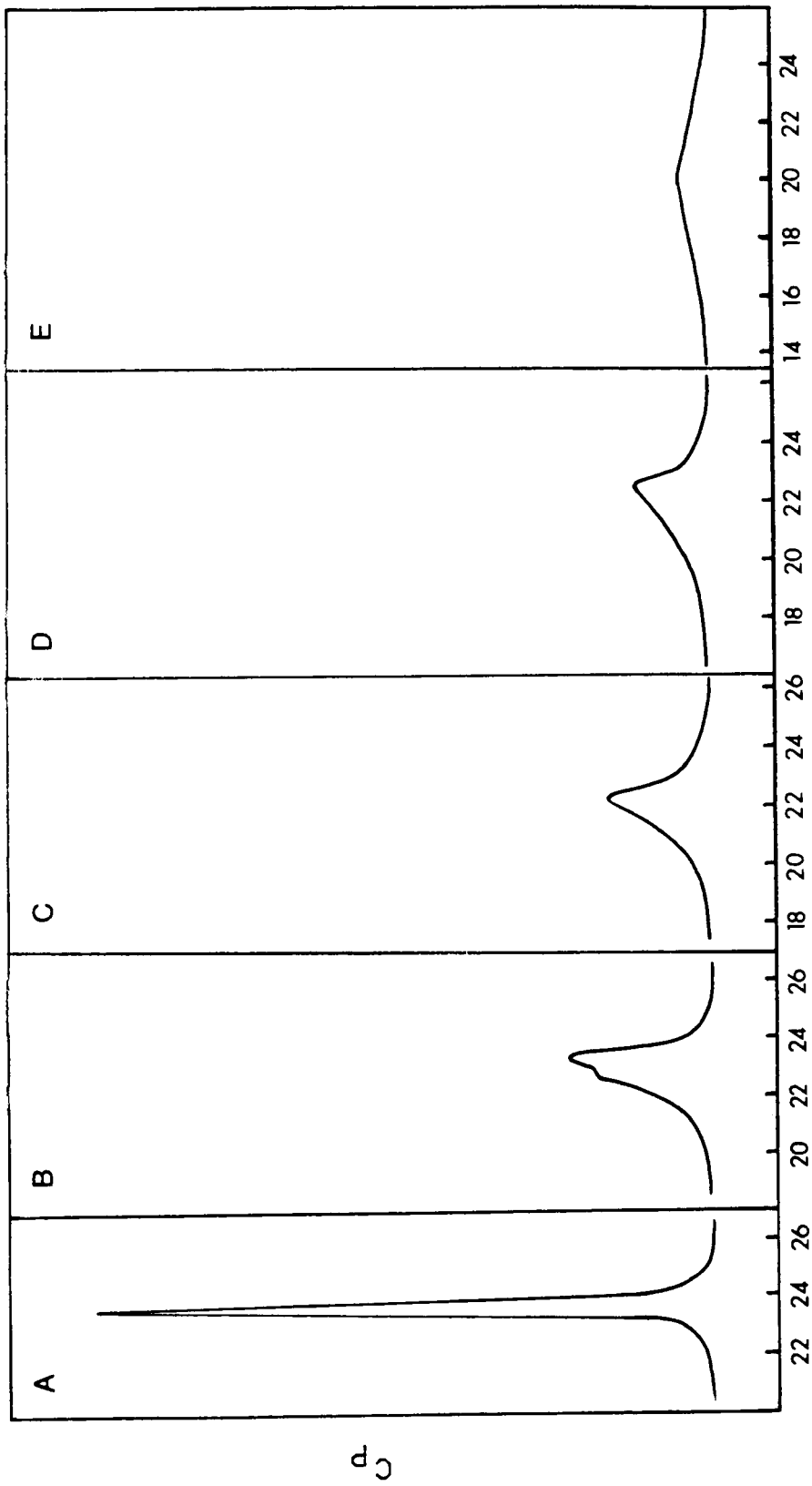


Figure 3-1. Excess heat capacity function of cytochrome c oxidase--DMPC reconstitutions at different protein:lipid molar ratios: (A) P:L = 0; (B) P:L = 1:1200; (C) P:L = 1:800; (D) P:L = 1:400; (E) P:L = 1:200. All the scans were run at a scanning rate of 15°C/h in 10 mM Tricine, pH 7.5. Sample concentrations in the calorimeter cell were 5 mg of DMPC/mL.

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 3-2. The decrease in ΔH upon increasing the protein:lipid ratio indicates that the protein molecules are preventing some of

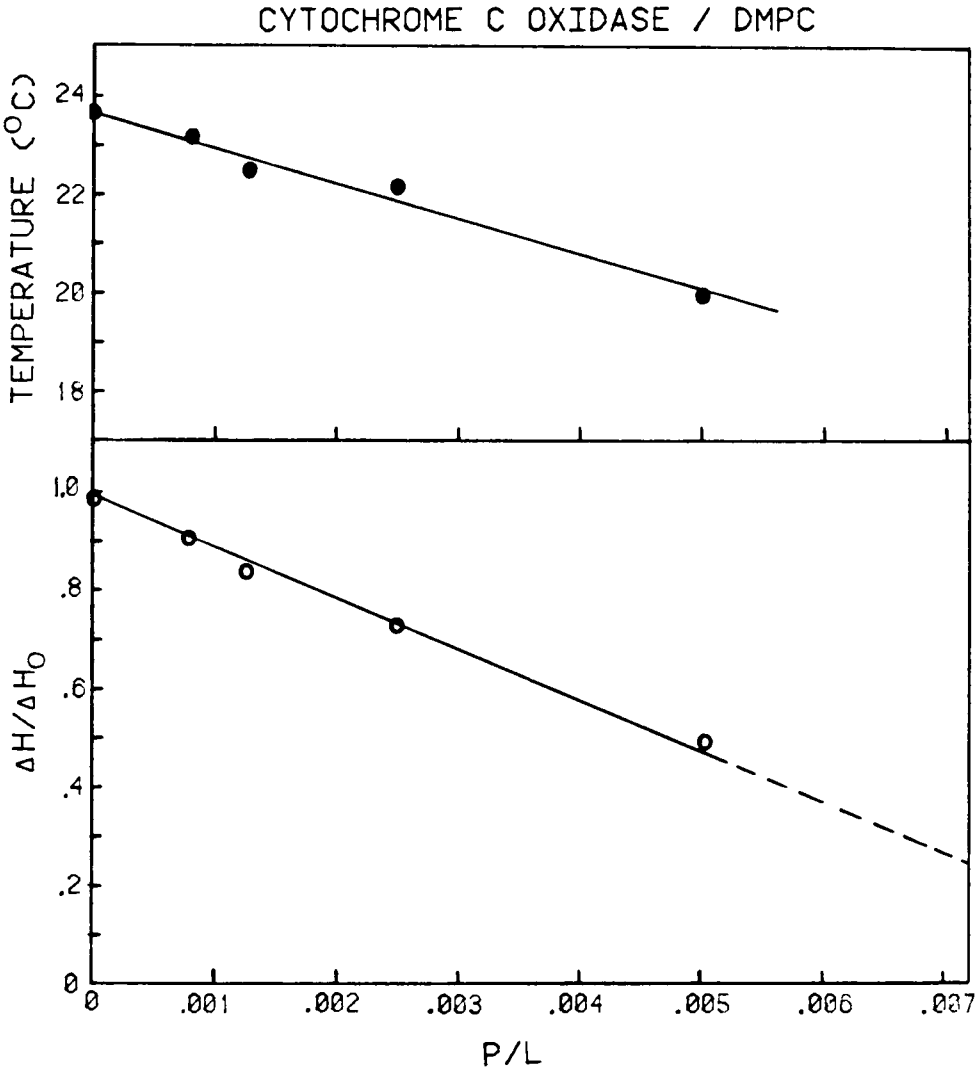


Figure 3-2. Dependence of thermodynamic parameters for the DMPC main gel-liquid-crystalline transition on the protein: lipid molar ratio.

the phospholipid molecules 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-cyrstalline transition per protein molecule. As shown in Figure 3-2, 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 3-3. In agreement with the calorimetric results, the fluorescense 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

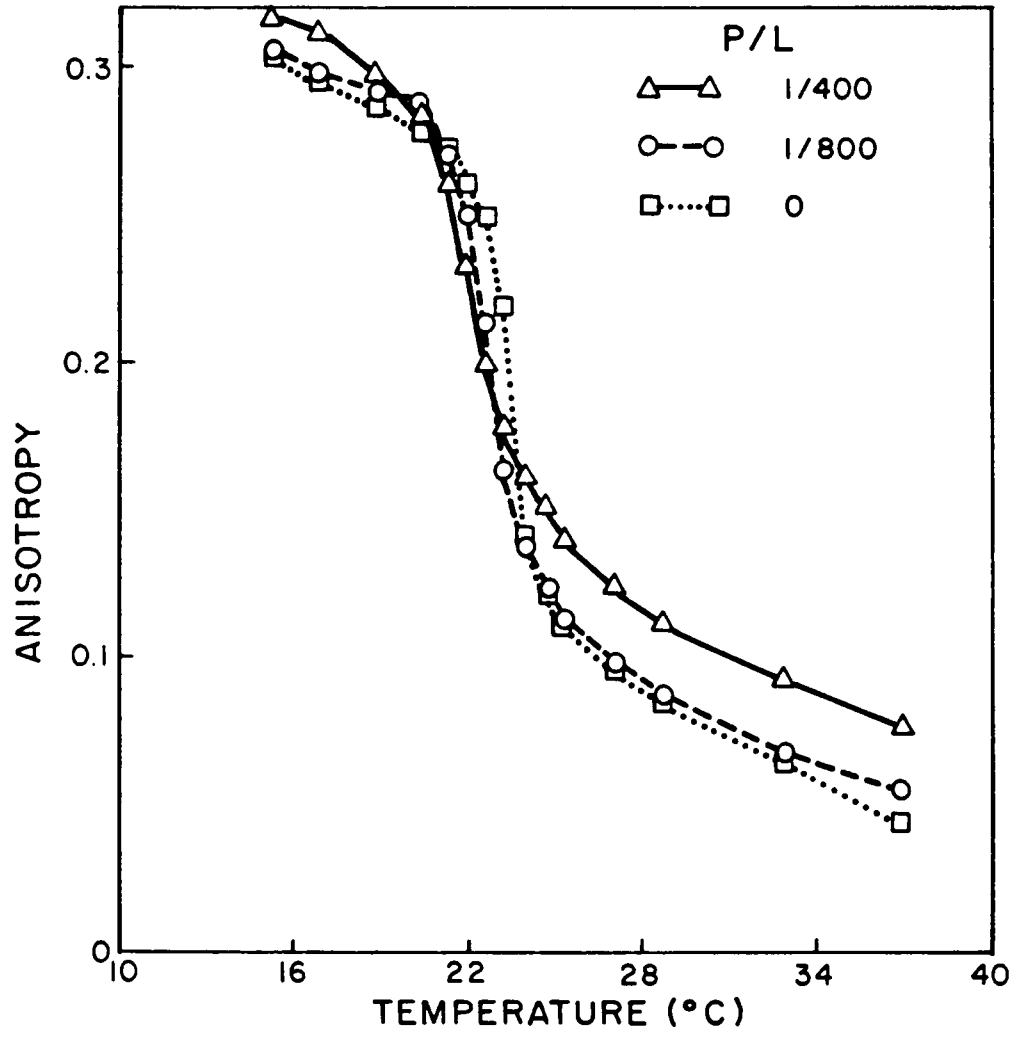


Figure 3-3. Steady-state fluorescence anisotropy of DPH as a function of temperature for pure DMPC vesicles and for DMPC vesicles containing cytochrome c oxidase at molar ratios of 1:800 and 1:400.

ordered or less fluid 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-sensitivity differential scanning calorimetry in both detergent-solubilized and membrane-reconstituted samples. As shown in Figure 3-4, 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 ($\Delta H:\Delta H_{VH}$) 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 $\Delta H:\Delta H_{VH}$ 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

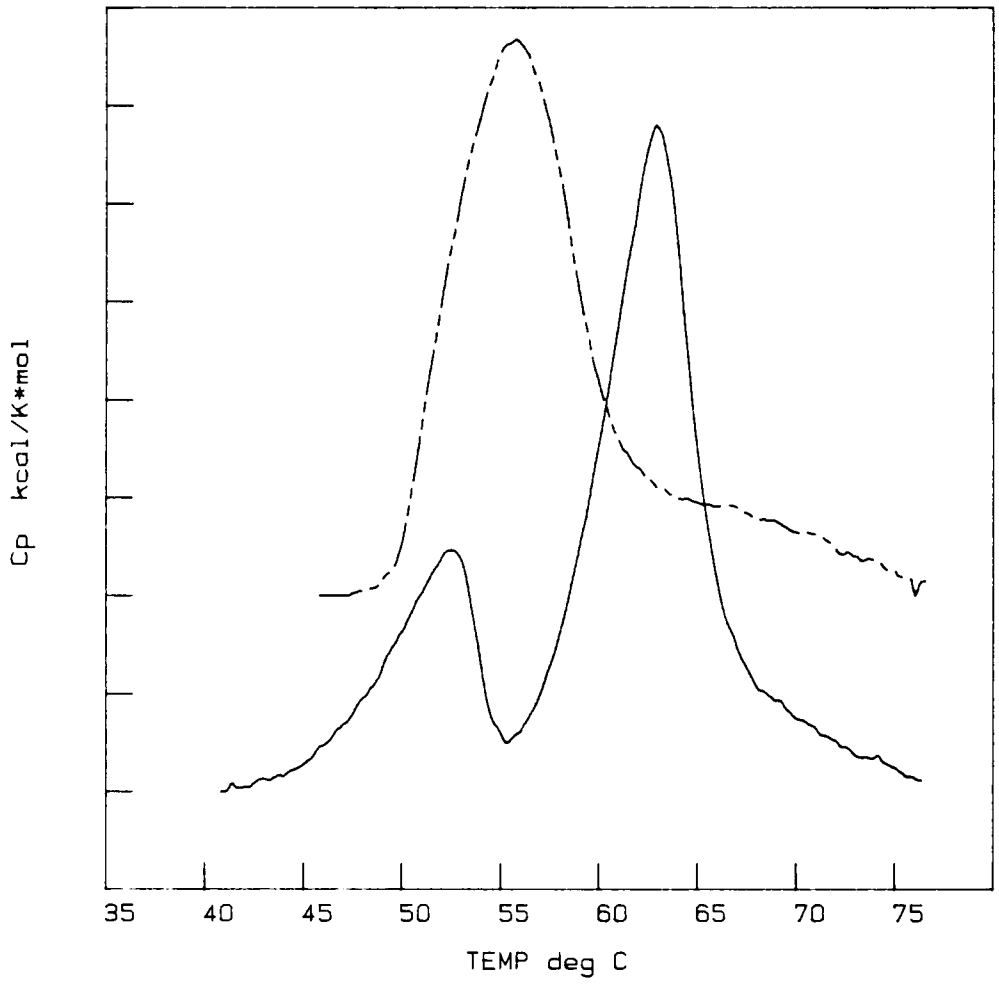


Figure 3-4. Excess heat capacity function for cytochrome oxidase solubilized in 0.1% Tween 80 and 0.1 M PO_4, pH 7.4 (dashed line), and reconstituted in DMPC vesicles at a protein:lipid ratio of 1:130 (solid line). The scanning rate for this experiment was 41.9°C/h.

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 becomes 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 $\Delta H:\Delta H_{VH}$ 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 a 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 3-5. 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

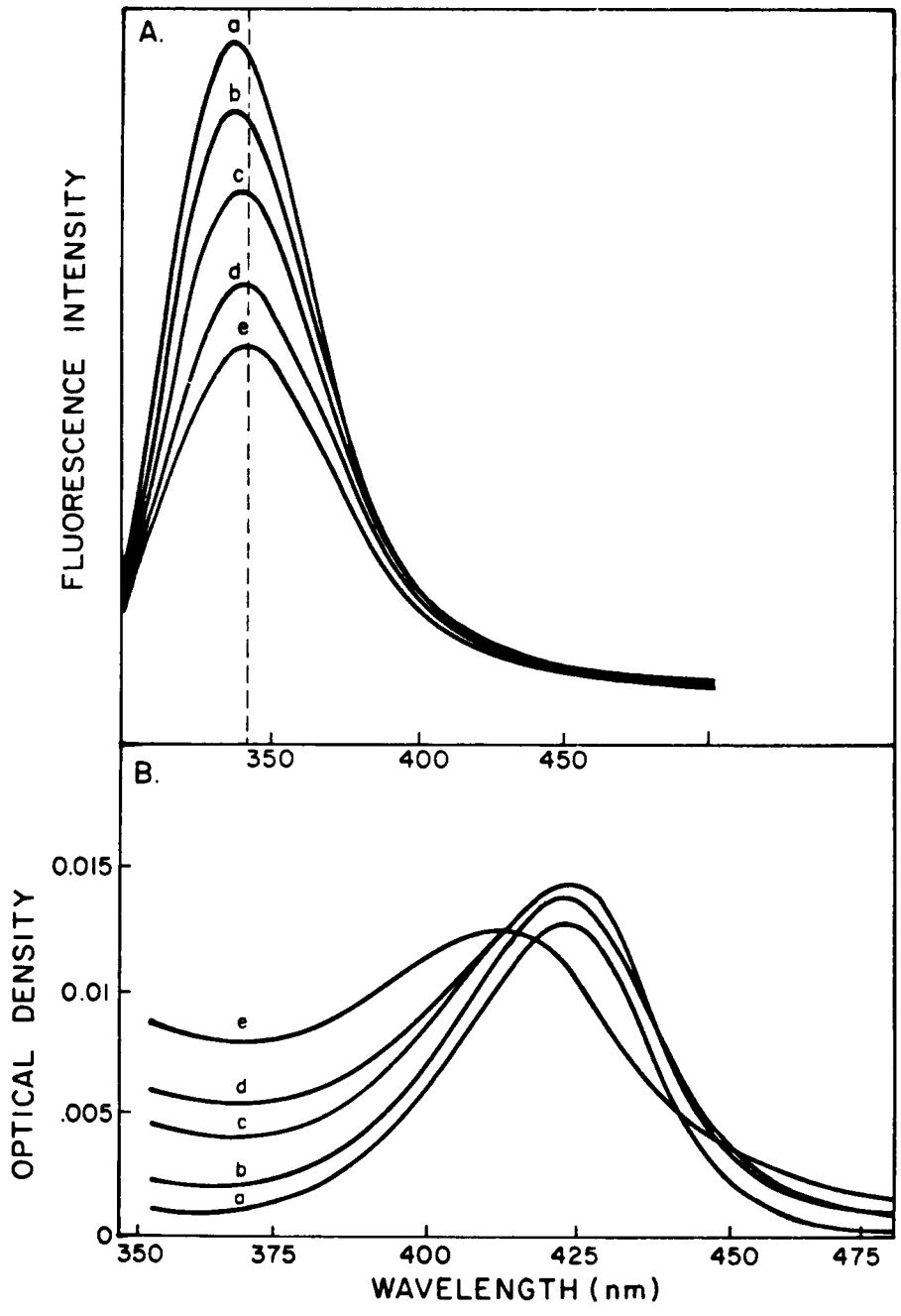


Figure 3-5. Steady-state tryptophan fluorescence spectra (A) and heme absorption spectra (B) vs. temperature for cytochrome c oxidase reconstituted into DMPC vesicles. The fluorescence spectra were measured at the following temperatures: (a) 36, (b) 43, (c) 51, (d) 61, and (e) 66°C. The heme absorption spectra were measured at (a) 17, (b) 21, (c) 37.5, (d) 55.5, and (e) 72.2°C.

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 analyses are shown in Table 3-1. Figures 3-6 and 3-7 show the deconvoluted curves as well as the theoretical curves obtained with the thermodynamic parameters in Table 3-1. For the detergent-solubilized enzyme, the standard deviation between the theoretical and experimental curves is 1.0 kcal K^{-1} and for the membrane-reconstituted samples, 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

Table 3-1. Deconvolution of the Heat Capacity Function of Cytochrome c Oxidase.*

	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

*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.

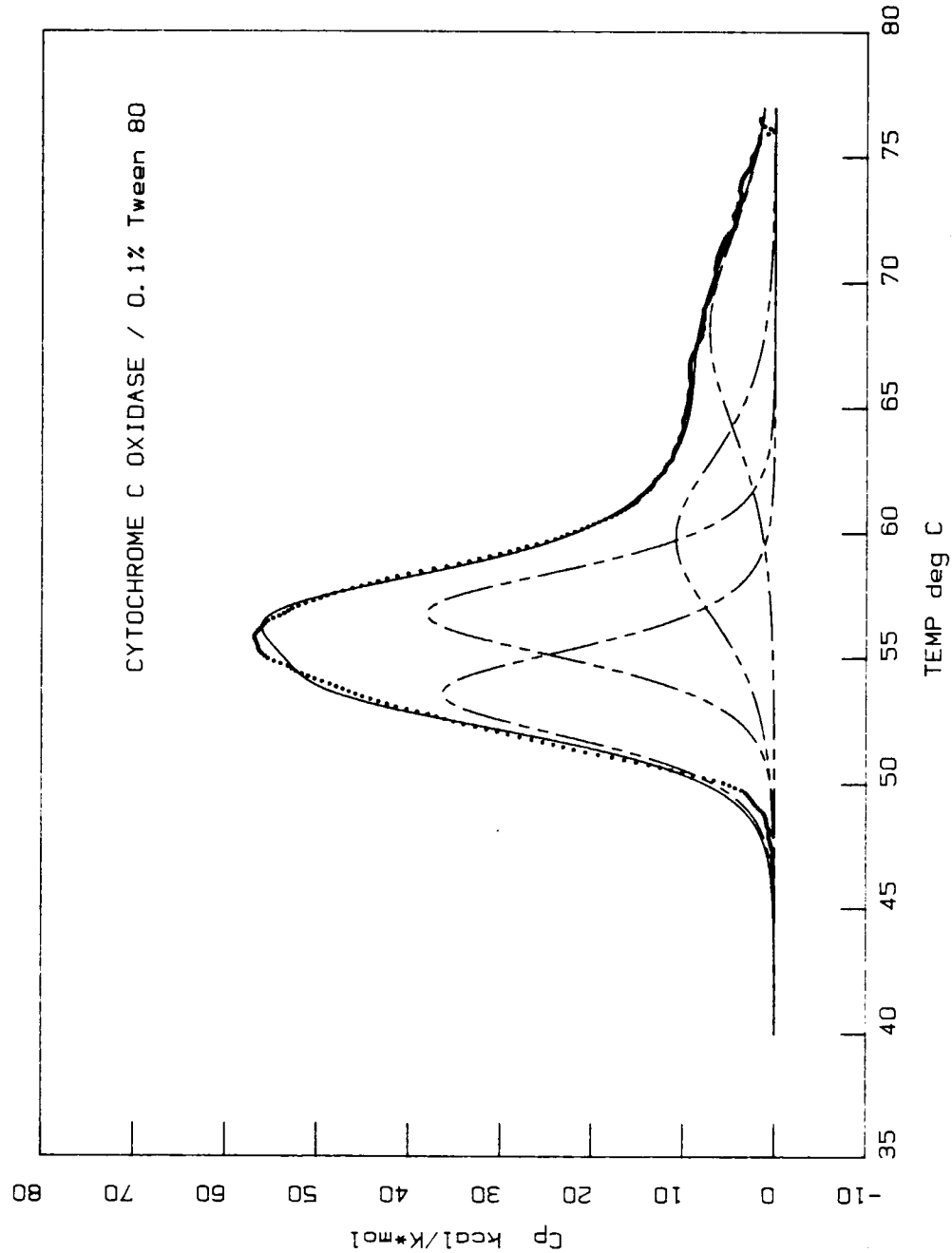


Figure 3-6. Deconvolution of the heat capacity function of detergent-solubilized cytochrome oxidase.

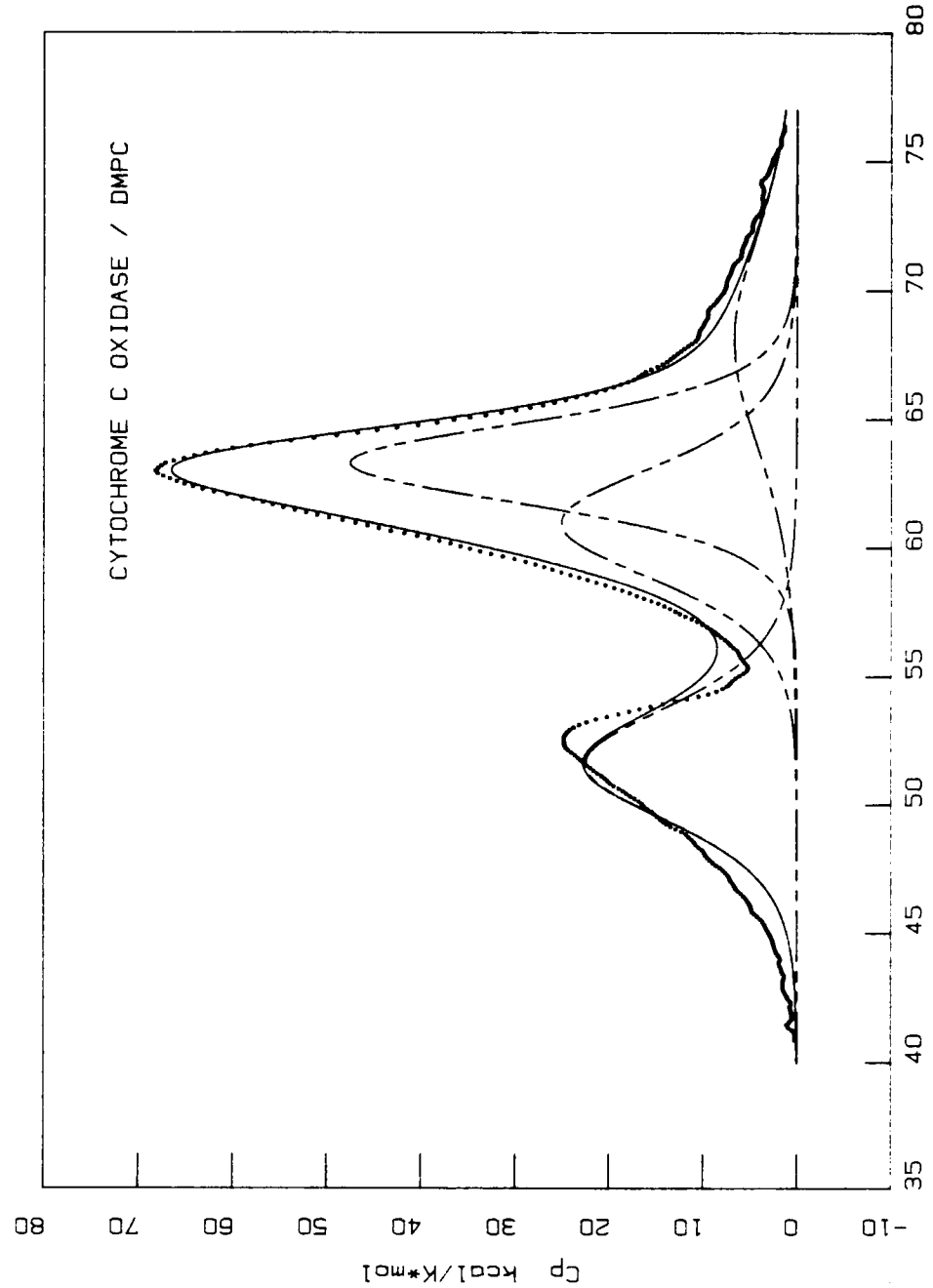


Figure 3-7. Deconvolution of the heat capacity function for membrane-reconstituted (DMPC) cytochrome c oxidase.

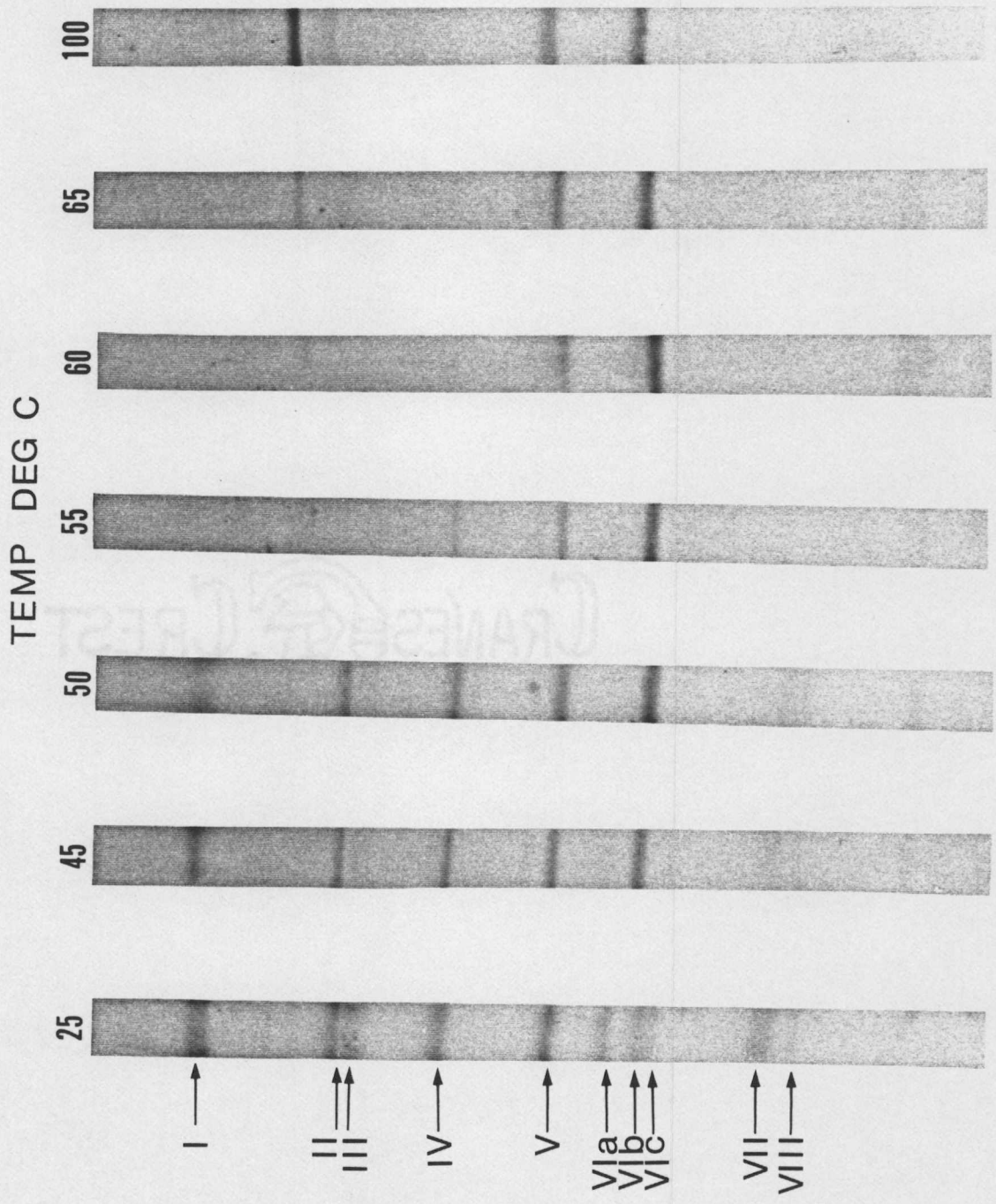
more than one subunit. As shown in Table 3-1, the enthalpies of two of the melting steps (steps 2 and 4) appear to be unchanged in both the detergent-solubilized and membrane-reconstituted enzymes, suggesting that they probably involve the same subunits in both samples. This is not the case for the first 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 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 definition 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 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 3-8 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,

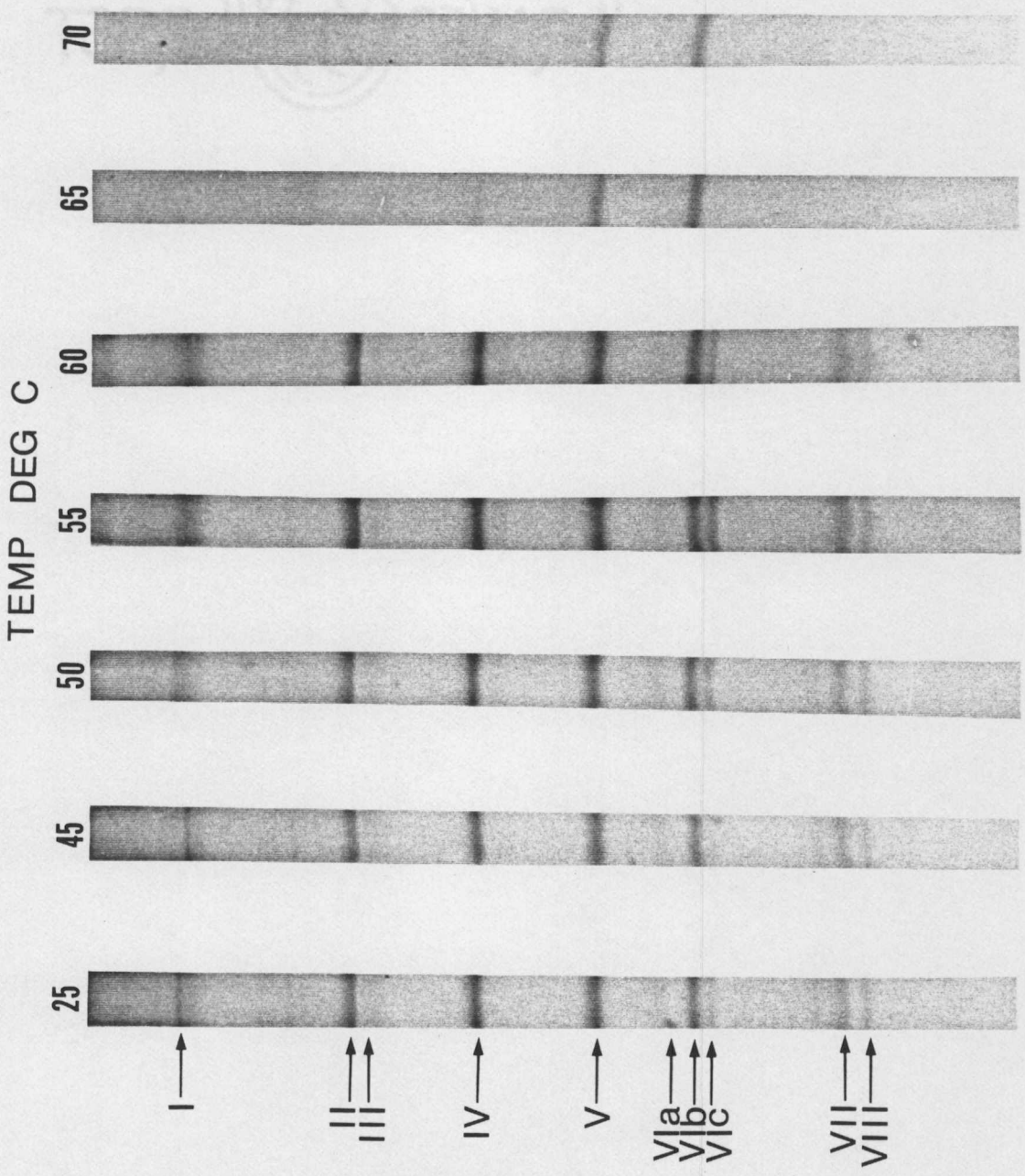
Figure 3-8. SDS-polyacrylamide gel of the detergent-soluble fraction following thermal denaturation of detergent-solubilized cytochrome c oxidase. Samples were heated to the indicated temperatures, and the postcentrifugation supernatant was run on an 18.75% polyacrylamide gel as described under Experimental Procedures.


TEMP DEG C
25
45
50
55
60
65
100
I
II
III
IV
V
VIa
VIb
VIc
VII
VIII

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 temperature.

A second series of samples containing cytochrome oxidase reconstituted with DMPC was subjected to the same procedure. Figure 3-9 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.

Figure 3-9. SDS-polyacrylamide gel of detergent-soluble subunits following thermal denaturation of cytochrome c oxidase reconstituted with DMPC.


TEMP DEG C
25
45
50
55
60
65
70
I
II
III
IV
V
VIa
VIb
VIc
VII
VIII

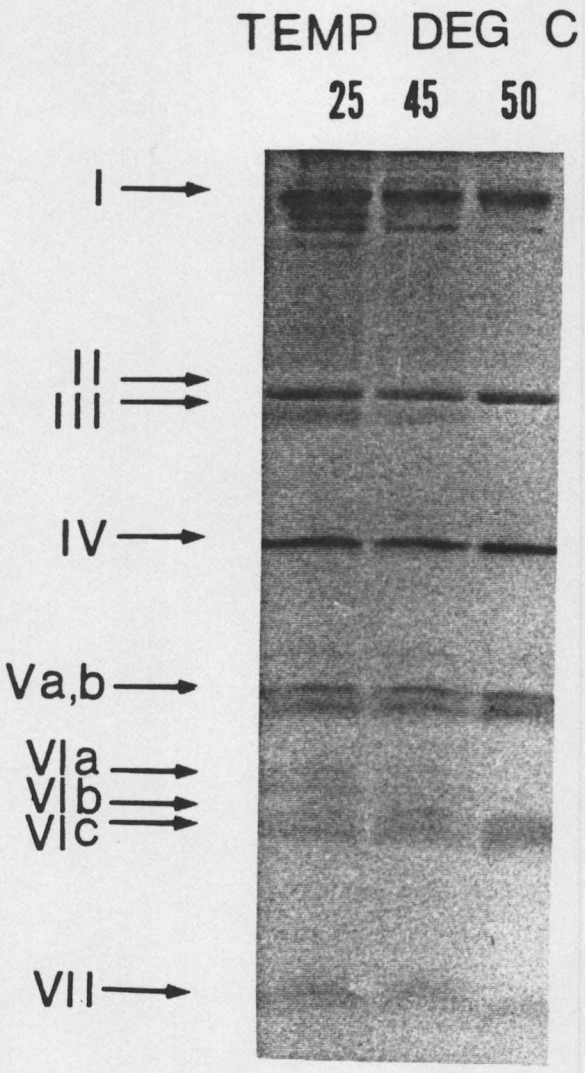
As shown in Figure 3-10, for a gel run in the presence of 1% v/v β-mercaptoethanol to improve subunit resolution (Kadenbach et al., 1983), subunit III is clearly present in the 25°C temperature sample, becomes fainter at 45°C, and is no longer discernible in the gel at 50°C. This melting sequence for subunit III is in good agreement with the location of the first deconvoluted peak in the calorimetric results (see Figure 3-7) 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

Figure 3-10. 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.


TEMP DEG C
25 45 50
I
II
III
IV
Va,b
VIa
VIb
VIc
VII

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 embedded portion of the protein. Knowles et al. (1979) and Jost et al. (1973), using phospholipid spin-label probes, have estimated a boundary layer of lipid of ~50 lipid molecules which are restricted in regard to order and mobility. 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 characteristics 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]

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[1]Subunit VII according to the nomenclature of Downer et al.

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 sub-mitochrondrial 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 thermo-dynamic behavior of cytochrome oxidase is dependent on the nature

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(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.

of the hydrophobic environment and appears to be the same in membrane-reconstituted samples as in submitochondrial particles.

PART 3

REFERENCES

REFERENCES

Alonso, A., Restall, C. J., Turner, M., Gomez-Fernandez, J. C., Goni, F. M., & Chapman, D. (1982) Biochim. Biophys. Acta 689, 283-289.

Biltonen, R. L., & Freire, E. (1978) CRC Crit. Rev. Biochem. 5, 85-124.

Capaldi, R. A., & Hayashi, H. (1972) FEBS Lett. 26, 261-263.

Chapman, D., Gomez-Fernandez, J. C., & Goni, F. M. (1979) FEBS Lett. 98, 211-228.

Corbley, M. J., & Azzi, A. (1984) Eur. J. Biochem. 139, 535-540.

Correa-Freire, M. C., Freire, E., Barenholz, Y., Biltonen, R. L., & Thompson, T. E. (1979) Biochemistry 18, 442-445.

Curatolo, W., Sakura, J. D., Small, D. M., & Shipley, G. G. (1977) Biochemistry 16, 2313-2319.

Deatherage, J. F., Henderson, R., & Capaldi, R. A. (1982a) J. Mol. Biol. 158, 487-499.

Deatherage, J. F., Henderson, R., & Capaldi, R. A. (1982b) J. Mol. Biol. 158, 501-514.

Downer, N. W., Robinson, N. C., & Capaldi, R. A. (1976) Biochemistry 15, 2930-2936.

Freire, E., & Biltonen, R. L. (1978) Biopolymers 17, 463-479.

Freire, E., Markello, T., Rigell, C., & Holloway, P. W. (1983) Biochemistry 22, 1675-1680.

Fry, M., & Green, D. E. (1980) Biochem. Biophys. Res. Commun. 93, 1238-1246.

Fry, M., Vande Zande, H., & Green, D. E. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 5908-5911.

Hesketh, T. R., Smith, G. A., Houslay, M. D., McGill, K. A., Birdsall, N. J. M., Metcalfe, J. C., & Warren, G. B. (1976) Biochemistry 15, 4145-4151.

Jost, P. C., Griffith, O. H., Capaldi, R. A., & Vanderkooi, G. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 480-484.

Kadenbach, B., Jarausch, J., Hartmann, R., & Merle, P. (1983) Anal. Biochem. 129, 517-521.

Kang, S. Y., Gutowsky, H. S., Hsung, J. C., Jacobs, R., King, T. E., Rice, D., & Oldfield, E. (1979) Biochemistry 18, 3257-3267.

Kimelman, D., Tecona, E. S., Wolser, P. K., Hudson, B. S., Wickner, W. T., & Somoni, R. D. (1979) Biochemistry 18, 5874-5880.

Kinosita, K., Kawato, S., Ikegami, A., Yoshida, S., & Orii, Y. (1981) Biochim. Biophys. Acta 647, 7-17.

Knowles, P. F., Watts, A., & Marsh, D. (1979) Biochemistry 18, 4480-4487.

Knox, B. E., & Tsong, T. Y. (1984) Biophys. J. 45, 296a.

Kornblatt, J. A., & Hui Bon Hoa, G. (1982) Biochemistry 21, 5439-5444.

Krebs, J. J. R., Hauser, H., & Carafoli, E. (1979) J. Biol. Chem. 254, 5308-5316.

Lentz, B. R., Clubb, K. W., Alford, D. E., Hoechli, M., & Meissner, G. (1985) Biochemistry 24, 433-442.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.

Lysko, K. A., Carlson, R., Taverna, R., Snow, J., & Brandts, J. F. (1981) Biochemistry 20, 5570-5576.

Marinetti, G. V. (1962) J. Lipid Res. 3, 1-20.

Marsh, D., Watts, A., Pates, R. D., Uhl, R., Knowles, P. F., & Esmann, M. (1982) Biophys. J. 37, 265-271.

Penttila, T. (1983) Eur. J. Biochem. 133, 355-361.

Privalov, P. L. (1982) Adv. Protein Chem. 35, 1-104.

Privalov, P. L., & Khechinashvili, N. N. (1974) J. Mol. Biol. 86, 665-684.

Robinson, N. C., & Capaldi, R. A. (1977) Biochemistry 16, 375-381.

Saraste, M., Penttila, T., & Wikstrom, M. (1981) Eur. J. Biochem. 115, 261-268.

Smith, L. (1955) Methods Enzymol. 2, 732-741.

Tuszynski, G. P., Knight, L., Piperno, J. R., & Walsh, P. N. (1980) Anal. Biochem. 106, 118-122.

Van Zoelen, E. J. J.; Van Dijck, P. W. M., De Kruijff, B., Verkleij, A. J., & Van Deenen, L. L. M. (1978) Biochim. Biophys. Acta 514, 9-24.

Vik, S. B., & Capaldi, R. A. (1977) Biochemistry 16, 5755-5759.

Wikstrom, M., & Sigel, E. (1979) in Membrane Biochemistry (Carafoli, E., & Semenza, G., Eds.) pp 82-91, Springer-Verlag, New York.

Wikstrom, M., Krab, K., Saraste, M. (1981) Annu. Rev. Biochem. 50, 623-655.

PART 3

APPENDIX

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

PART 4

THERMAL GEL ANALYSIS CHARACTERIZATION OF CONFORMATIONAL TRANSITIONS OF CYTOCHROME c OXIDASE

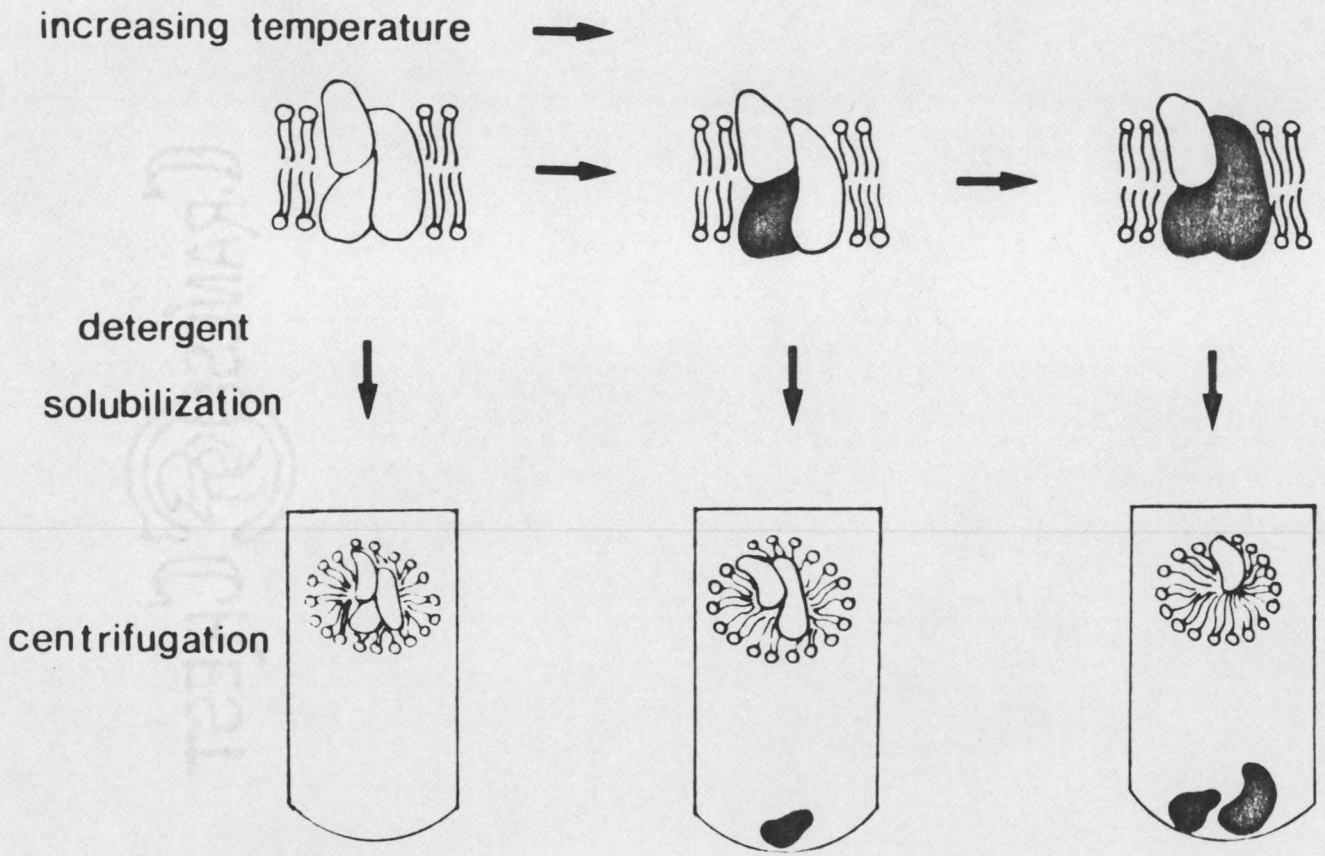
Introduction

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 today, however, very little information is available on the thermal denaturation of heterologous multisubunit proteins or integral membrane proteins. In a previous communication we have studied by 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. Figure 4-1 represents the method of differential solubility thermal gel analysis for studying the denaturation of a complex membrane protein reconstituted into a lipid bilayer. For the enzyme in the native state, all of the subunits are soluble in an appropriate detergent concentration. Thus, following detergent addition, sonication and centrifugation, all of the subunits of the

Figure 4-1. Schematic diagram of differential solubility thermal gel analysis. White globules represent the native protein subunits and blackened globules represent the denatured subunits.

THERMAL GEL ANALYSIS

SAMPLE PREPARATION



enzyme complex remain soluble in the supernatant. If the reconstituted enzyme is heated to a temperature at which one or more subunits are denatured and consequently lose solubility in detergent solution then they are removed from solution by centrifugation. A further increase in temperature, resulting in the denaturation and loss of detergent solubility of other subunits of the enzyme complex, permits the removal of these denatured subunits by centrifugation. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the supernatants of a series of samples, each representing a different temperature point, yields a denaturation profile of the enzyme complex for the individual subunits.

In Part 3 the technique of differential solubility thermal gel analysis was introduced and in this part we describe a further development in the application of differential solubility thermal gel analysis to the study of protein melting in membranes. Here the technique is standardized in a rigorous manner with regard to protein concentration, detergent concentration, internal standard addition, sonication time and centrifugation (see Experimental Procedures for precise conditions).

In this part, 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 single subunit melting profiles can be used to calculate apparent equilibrium constants, as well as apparent

enthalpy and entropy changes 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 thirteen subunits (Kadenbach et al., 1983), the three 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 the thermodynamic parameters for the thermal unfolding of cytochrome c oxidase. Calorimetry of the enzyme was 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 subunits of the enzyme complex in a detergent containing medium (Rigell et al., 1985). In this part 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 oxidase. This technique when applied to the membrane-reconstituted

enzyme allows a quantitative determination of transition temperatures, apparent equilibrium constants and Van't Hoff enthalpies of denaturation for individual subunits of the enzyme.

Experimental Procedures

Materials

Dimyristoyl phosphatidylcholine (DMPC) was obtained from Avanti Biochemicals (Birmingham, AL) and used without further purification. Tween 80 was purchased from Fisher Scientific (Fair Lawn, NJ). Acrylamide, N. N' methylene bis acrylamide and sodium cholate were obtained from Sigma (St. Louis). Sodium cholate was recrystallized three times in ethanol to remove bile salt contaminants as described by Wilkstrom (1979).

Cytochrome c oxidase was isolated from bovine heart as described before (Rigell et al., 1985). Briefly, this method followed the procedure described by Capaldi and Hayashi (1972). The cytochrome oxidase pellet was repurified by dialyzing away ammonium sulfate and cholate against 0.1M 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 oxidase: 9-11 nmoles/mg protein for heme a/protein ratio and 8-11 μmoles of cytochrome c oxidized min^{-1} (mg of protein)$^{-1}$ for activity measurements.

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

SDS Polyacrylamide Gel Electrophoresis

SDS Polyacrylamide gel electrophoresis was performed using the method of Kadenbach et al. (1983). A Bio-Rad Protein vertical slab gel apparatus, (16 x 18 x 0.15 cm) was used to run the gels. An 18.75% acrylamide separation gel (32:1 acrylamide: n,n'-methylene bis acrylamide) with an 8.3% acrylamide stacking gel (same ratio) was used with the buffers described in the protocol. Electrophoresis was run for two hours at 80V (constant) and then for ten hours at 200V (constant). Gels were prefixed in a 50% methanol 10% acetic acid solution for three hours at ambient temperature and stained in a 0.2% Cooumassie blue/5:4:1 methanol: water: acetic acid solution at 45°C with shaking for one hour. Destaining was accomplished by incubation overnight at room temperature in an 8:1:1 water: methanol: acetic and acid solution.

Cytochrome Oxidase Reconstitution

Cytochrome 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 dessicated overnight to remove residual solvent. The dried lipid was suspended in 10mM 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 seconds in a bath sonicator (Laboratory Supplies, Hicksville, NY). Protein was then added to obtain a protein lipid ratio of 1/400 assuming a molecular weight of 165,000 for cytochrome oxidase (Deatherage, 1982). This solution was gently bath sonicated for ten seconds at 4°C before dialysis against four liters of 10mM Tricine pH 7.5 for 24 hours at 4°C.

Thermal Denaturation of Cytochrome Oxidase

A separate aliquot of reconstituted cytochrome oxidase was prepared for each temperature point of the thermal denaturation. Each aliquot contined 150 µl of the cytochrome oxidase-DMPC reconstitution (containing 5 mg/ml DMPC, P/L = 1/400). The samples were placed in a RTE-8 Neslab Refrigerated Circulating water bath equipped with the Neslab Temperature Programmer ETP-3. The temperature of the samples was monitored using a thermistor connected to a Hewlett Packard 3467A Logging Multimeter. The thermistor was placed adjacent to the sample test tubes in a test tube contianing 150 µl of 10mM Tricine pH 7.5. Thermal denaturation was performed by scanning the temperature at a 20°C/hr heating rate. At the desired temperature the samples were immediately placed on ice. Ten minutes later, 100 µl of 1.25% Tween 80 in 10mM Tricine pH 7.5 was added to make the resultant solution 0.5% Tween 80. Also 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 seconds at 1400 rpm to remove condensate from the walls of the test tubes. Each sample was sonicated for 30

seconds in a bath type sonicator, recentrifuged at 1400 rpm and transferred to airfuge tubes. Samples were centrifuged in a Beckman airfuge at ~178,000xg for 10 minutes to pellet insoluble protein. Following this last centrifugation, 150 µl of each supernatant was diluted to 300 µl with electrophoresis sample buffer (62.5 mM Tris base, 20% glycerol, 4% Sodium Dodecyl Sulfate pH 6.8). Samples were then made 1% B-mercaptoethanol to improve resolution of the subunits.

Laser Densitometry and Data Acquisition

Densitometry of the destained gels was performed using an LKB 2202 Ultroscan Laser Densitometer interfaced to an IBM PC using 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. Subsequent data manipulation to obtain normalized peak intensities and areas was performed using software developed in this laboratory.

Results

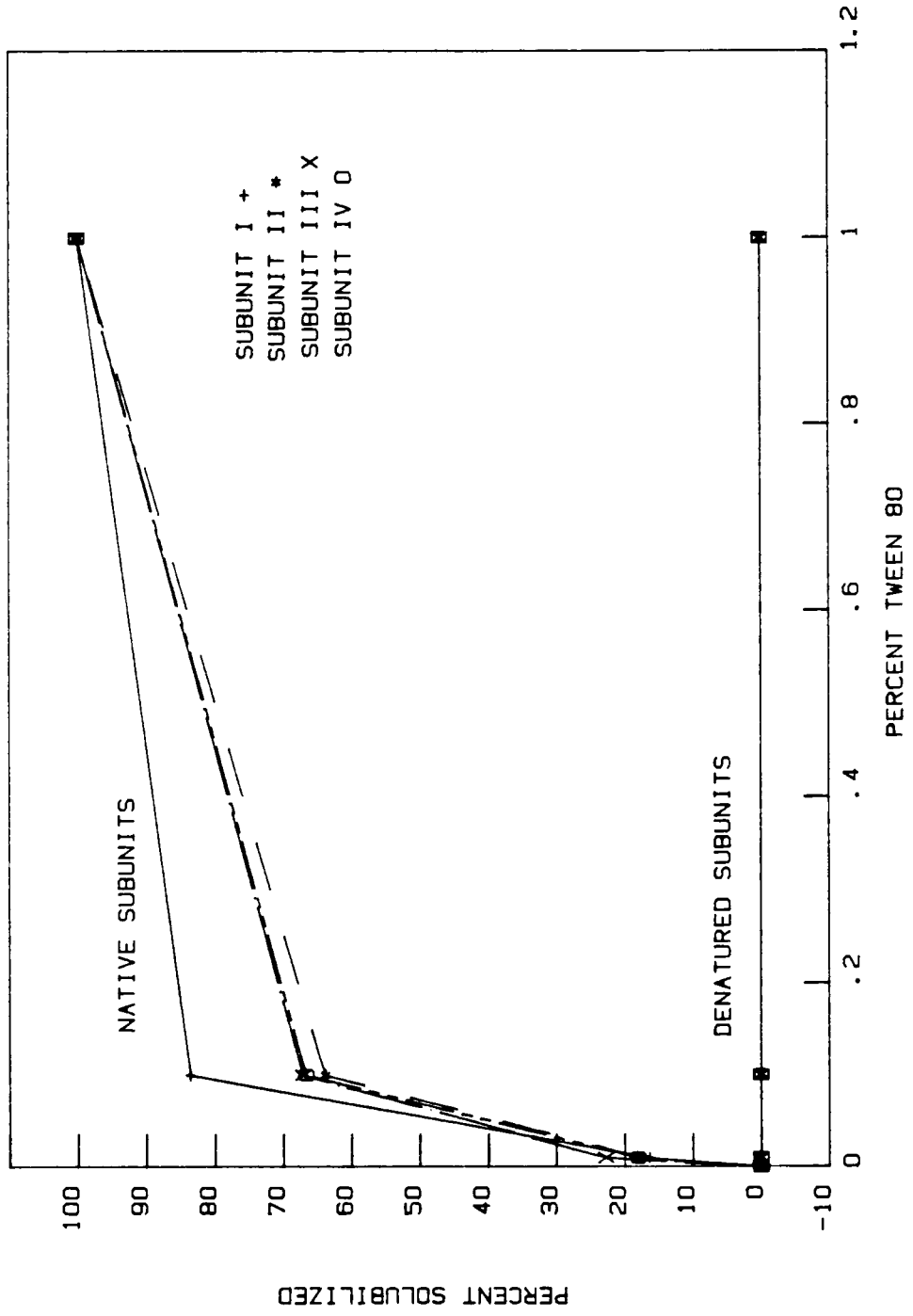
Temperature Induced Change in Cytochrome Oxidase Subunit Solubility

Cytochrome oxidase is an integral membrane protein and as such, solubilization of the protein complex can only be achieved by the addition of detergent. The thermal denaturation of cytochrome 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, non-denatured, form results in the solubilization of all of the subunits. However, when detergent is added to the reconstituted enzyme following 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 this change in subunit solubility as a function of temperature (Rigell et al., 1985).

Figure 4-2 shows the detergent (Tween 80) concentration dependence of the degree of solubilization of the four largest subunits of cytochrome oxidase in both the native and denatured state. In the absence of detergent the reconstituted enzyme, in the native state (25°C) can be removed entirely from solution by centrifugation at 178,000xg for 10 minutes. SDS-polyacrylamide gel electrophoresis of the resulting supernatant exhibited no subunit bands after staining with coummasie 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 this experiment, (DMPC = 5 mg/ml, cytochrome oxidase = 3 mg/ml) addition of between 0.1% and 1.0% Tween 80 resulted in maximal solubilization of the enzyme complex. However, if the reconstituted enzyme was first denatured by incubation at 90°C for fifteen minutes, none of these subunits were solubilized at any of the concentrations of Tween 80 tested. Under

Figure 4-2. 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).


SUBUNIT I +
SUBUNIT II *
SUBUNIT III X
SUBUNIT IV O
NATIVE SUBUNITS
DENATURED SUBUNITS
PERCENT SOLUBILIZED
PERCENT TWEEN 80
100
90
80
70
60
50
40
30
20
10
0
-10
0
.2
.4
.6
.8
1
1.2

the conditions of these experiments, detergent concentrations ranging between 0.1 - 1% selectively solubilize only the native components of the enzyme. This constitutes the basis of the technique of differential solubility thermal gel analysis, that is, the use of appropriate detergent concentrations to separate native subunits from the denatured subunits of the enzyme complex.

Determination of Thermodynamic Parameters for individual subunits of Cytochrome c Oxidase

Figure 4-3 shows a family of laser densitometer profiles of 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 up to the indicated temperature. The additional band labelled CA., 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 following thermal denaturation. 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 thermodynamic parameters:

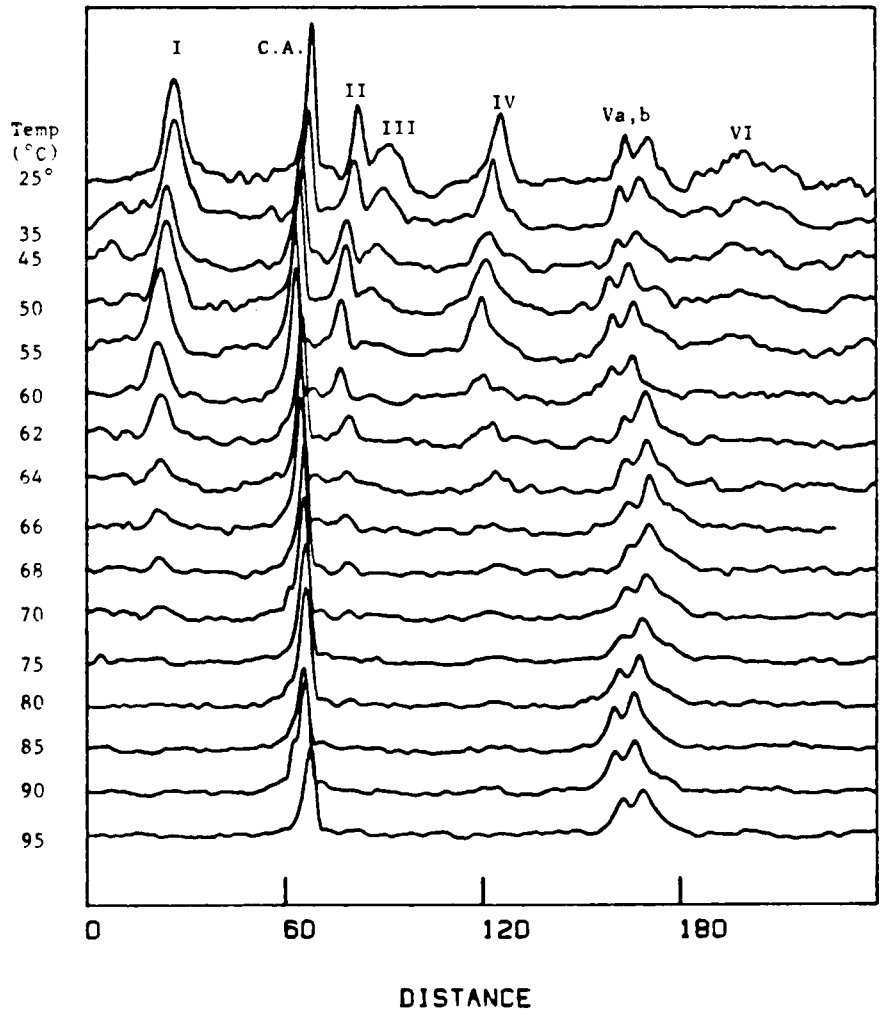


Figure 4-3. Laser densitometer tracing of the differential solubility thermal gel analysis of cytochrome c oxidase reconstituted into DMPC vesicles. Carbonic anhydrase (C.A.) was added as an internal concentration standard.

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

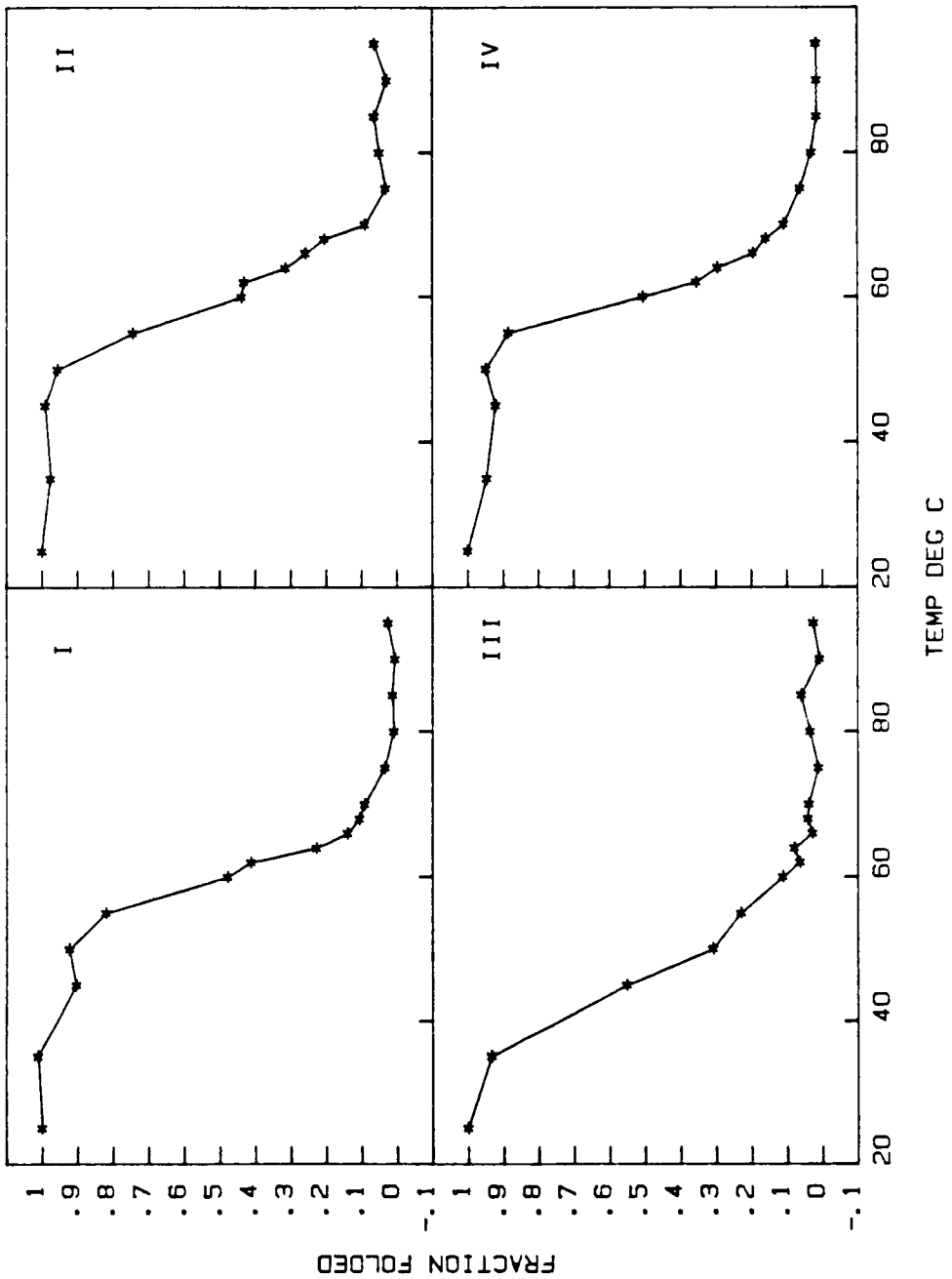
The densitometer tracing in Figure 4-3 represent the unnormalized data for the denaturation of cytochrome oxidase. Over the temperature range examined, it is evident that there is a decrease in all the cytochrome oxidase subunit peak areas with the exception of subunits Va and Vb which remain detergent soluble even after heating the enzyme complex at 95°C.

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

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

where A(T) and (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 4-4 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. For each subunit a melting temperature, Tm, 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 60-63°C, however,

Figure 4-4. The fraction of folded subunit as a function of temperature (°C) for each of the four largest subunits of cytochrome oxidase as determined from the peak areas in Figure 4-3 normalized to the area of the carbonic anhydrase peak area.


I
II
III
IV
FRACTION FOLDED
TEMP DEG C
1
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
-.1
20
40
60
80

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 this technique truly distinguishes the native state from the denatured state. Using the data shown in Figure 4-4, apparent equilibrium constants, Kapp, for the thermal denaturation of each subunit may be obtained as a function of temperature using the equation

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

The Van't Hoff plot of the calculated apparent equilibrium constant for each subunit in Figure 4-4 is presented in Figure 4-5. Data points for the Van't Hoff analysis were obtained from the fitted unfolding curve for each subunit at one degree intervals around the center of the transition. 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 suggests that the unfolding of these subunits may be interdependent. In fact, the melting of these subunits corresponds to the main calorimetric peak (see Figure 4-6) and constitutes the melting of the bulk of the enzyme complex. Subunit III, on the other hand, melts at a lower

Figure 4-5. Van't Hoff plots (lnK vs 1/T) for the thermal denaturation of the four largest subunits of cytochrome oxidase.

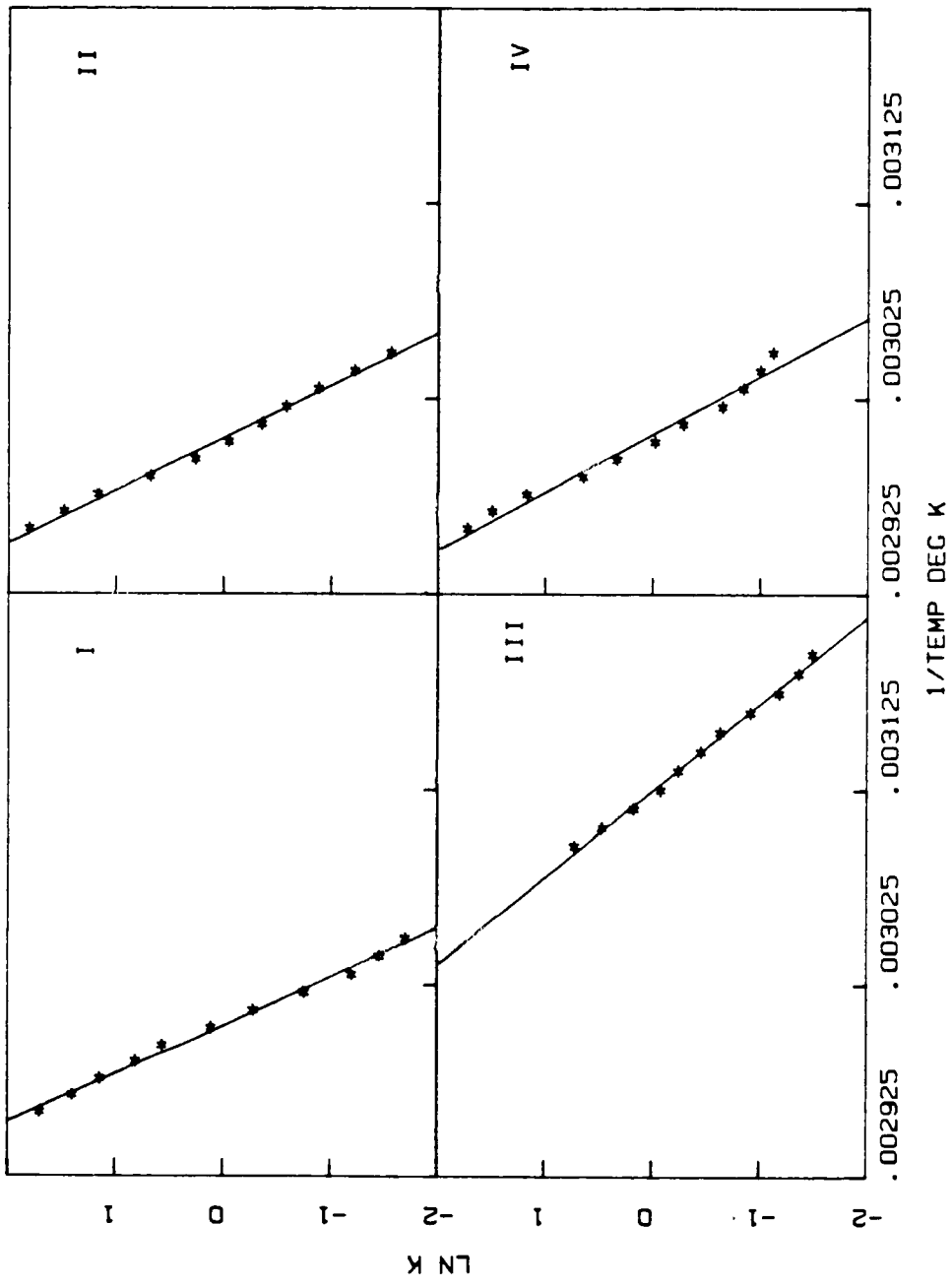
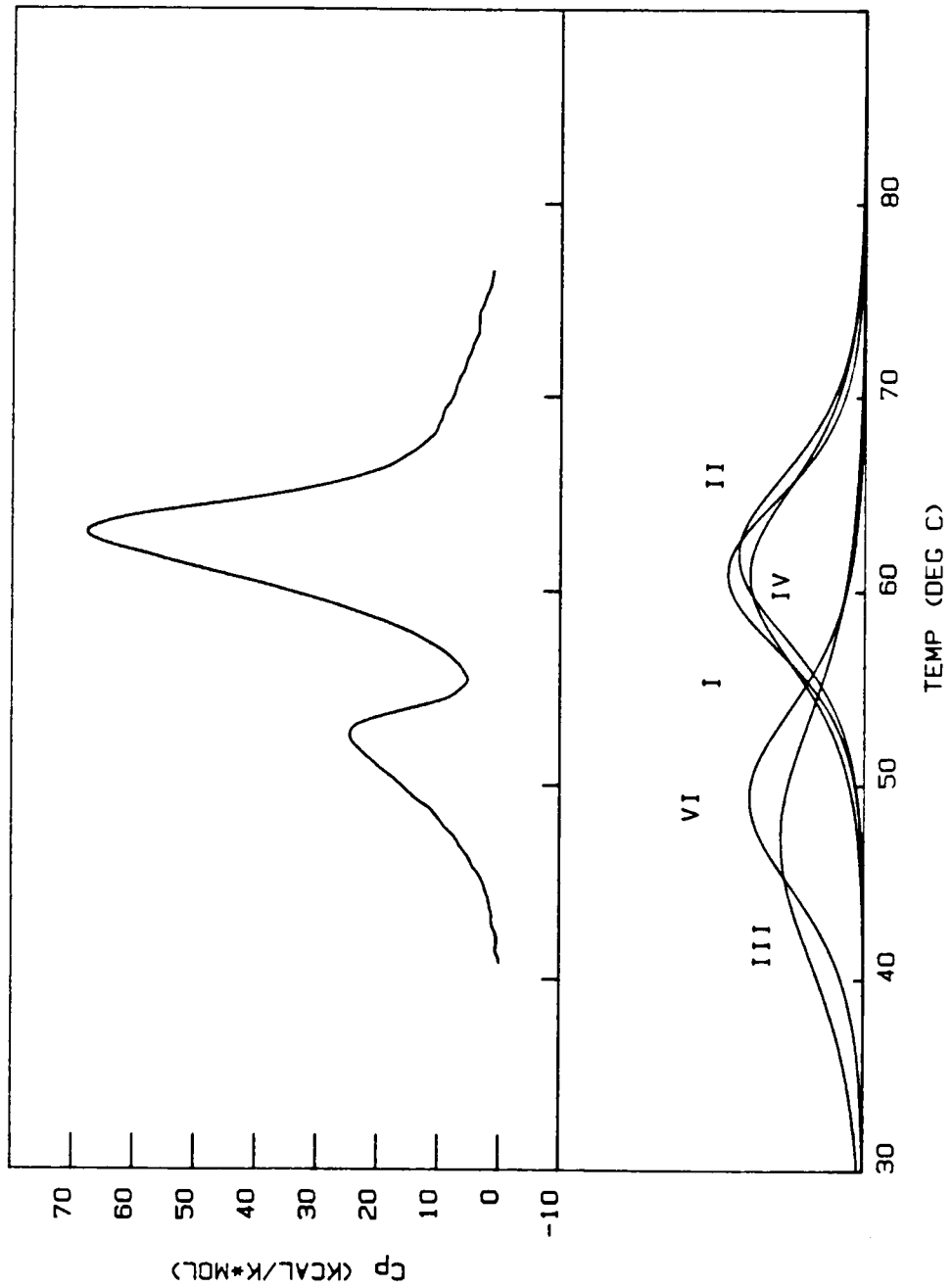

II
IV
I
III
LN K
1
0
-1
-2
.002925
.003025
.003125
1/TEMP DEG K

Figure 4-6. Heat capacity profile (top) and individual subunit differential melting profiles calculated from thermal gel analysis (bottom) for the denaturation of reconstituted cytochrome oxidase.


70
60
50
40
30
20
10
0
-10
Cp (KCAL/K*MOL)
30
40
50
60
70
80
TEMP (DEG C)
I
II
III
IV
VI

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 temperture peak in the calorimetric profile.

Individual Subunit Melting Profiles for Cytochrome Oxidase

In a previous communication we demonstrated that the unfolding 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 ($\Delta H/\Delta H_{V.H.}$) of 1.17 and that the high temperature peak is characterized by a $\Delta H/\Delta H_{V.H.}$ ratio of 2.5 indicating that neither of these peaks represent 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 4-1 presents a comparison of the thermodynamic parameters obtained from the deconvolution of the calorimetric data to the parameters obtained from the differential solubility thermal gel analysis. Although the fractional melting profile data is not shown, subunit VIa (see Figure 4-3, page 99) melts at a temperature similar to that observed

Table 4-1. Thermodynamic Parameters for Thermal Unfolding of Cytochrome c Oxidase Reconstituted into DMPC vesicles.

Transition	Calorimetry		Thermal Gel Analysis		
	Tm(°C)	ΔH(kcal/mol)	Tm(°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	ND	ND

for subunit III, with a Van't Hoff enthalpy of 70 kcal/mole. The melting of these two subunits contribute 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 thermal gel analysis corresponds with the third melting step. The other low molecular weight subunits which were not well resolved in the SDS-polyacrylamide gels, but were observed to denature with the bulk of the enzyme around 62°C, 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 solubility (Va and Vb) may contribute to this melting step.

A comparison of the melting steps obtained in Part 3 from the deconvolution of the excess heat capacity function associated with the detergent-solubilized enzyme denaturation to those of the membrane-reconstituted enzyme indicates that the same subunits contribute to each melting step in each case with the exception of some of the low molecular weight subunits. In Part 3, subunits VII and VIII, which were not well resolved in this part, appear to denature with subunit III and VIa in the Tween 80 solubilized enzyme, whereas in the membrane reconstituted sample these subunits melt with the bulk of the enzyme. This corresponds with the shift in enthalpy from the first melting step to the third melting step in the detergent-

solubilized and membrane reconstituted samples, respectively (see Table 3-1, page 59).

Figure 4-6 shows a comparison of the observed heat capacity profile for the membrane reconstituted enzyme to the individual subunit differential melting profiles generated from the thermodynamic parameters obtained from the thermal gel analysis. These curves were calculated using the following representation of the unfolding process for each subunit,

$$\frac{-\partial Fi}{\partial T} = \frac{K_i}{(1+K_i)^2} \quad \frac{\Delta H_{V.H,i}}{RT^2} = F_i(1-F_i) \quad \frac{\Delta H_{V.H,i}}{RT^2} \qquad (4)$$

where F_i is the fraction of subunit remaining in the native state at temperature T, and K_i is the equilibrium constant evaluated at temperature T. The individual subunit differential melting profiles obtained by thermal gel analysis provide an independent way of identifying and locating the contributions of each individual subunit of the enzyme complex to the heat capacity curve. It must be noted, however, that thermal gel analysis, like any other non-calorimetric 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 unless the transition is of the two-state type.

Discussion

Differential 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 or protein subunits undergoing thermal denaturation. In addition, it permits the calculation of the apparent thermodynamic parameters associated with the thermal denaturation of individual subunits of a multisubunit membrane protein like cytochrome oxidase. Additional studies in this laboratory using cholera toxin (B. Goins, to be published) and erythrocyte membranes (C. Wortman, to be published) 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 determined by thermal 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 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 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 oxidase indicates that subunits I and IV melt within two degrees of subunit II and that all three subunits exhibit approximately the same Van't Hoff enthalpy of denaturation. Subunits Va and Vb remain soluble in detergent solution even after heating the enzyme complex to 95°C. Since these subunits show no temperature dependent change in solubility, no thermodynamic 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 animo 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 method of 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.

PART 4

REFERENCES

REFERENCES

Capaldi, R. A., & Hayashi, H. (1972) FEBS Lett. 26, 261-263.

Capaldi, R. A., Malatesta, F., & Darley-Usmar, V. M. (1983) Biochim. Biophys. Acta 726, 135-148.

Casey, R. P., Thelen, M., & Azzi, A. (1980) J. Biol. Chem. 255, 3994-4000.

Deatherage, J. F., Henderson, R., & Capaldi, R. A. (1982) J. Mol. Biol. 158, 487-499.

Kadenbach, B., Jarausch, J., Hartmann, R., & Merle, P. (1983) Anal. Bioch. 129, 517-521.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.

Penttila, T. (1983) Eur. J. Biochem. 133, 355-361.

Privalov, P. L. (1979) Adv. Prot. Chem. 33, 167-241.

Privalov, P. L. (1982) Adv. Prot. Chem. 35, 1-104.

Prochaska, L. J., Steffens, G. C. M., Buse, G. M., Bisson, R., & Capaldi, R. A. (1981) Biocim. Biophys. Acta 637, 360-373.

Rigell, C. W., de Saussure, C., & Freire, E. (1985) Biochemistry 24, 5638-5646.

Saraste, M., Penttila, T., & Wikstrom, M. (1981) Eur. J. Biochem. 115, 261-268.

Schwal, A. J., Sebald, W., & Weiss, H. (1972) Eur. J. Biochem. 30, 511-516.

Sebald, W., Weiss, H., & Jackl, G. (1972) Eur. J. Biochem. 30, 413-417.

Sone, N., & Nicholls, P. (1984) Biochemistry 23, 6550-6554.

Tanaka, M., Haniu, M., Yasunobu, K. T., Yu, C., Yu, C., Yu, L., Wei, Y., & King, T. E. (1979) J. Biol. Chem. 254, 3879-3885.

Zhang, Y., Georgevich, G., & Capaldi, R. A. (1984) Biochemistry 23, 5616-5621.

CONCLUSIONS

The protein-lipid interactions of two integral membrane proteins, cytochrome b_5 and cytochrome c oxidase, reconstituted into phosphatidylcholine lipid vesicles was investigated to determine the effect of integral membrane protein on the physical properties of the phospholipid. The thermal denaturation of cytochrome c oxidase was examined using high sensitivity differential scanning calorimetry and the effect of the hydrophobic environment on the thermal stability of the enzyme complex was studied. Also, a new technique, differential solubility thermal gel analysis, was developed to identify the subunits of the multisubunit enzyme complex which contribute to the melting steps of the complex denaturation transition.

High sensitivity differential scanning calorimetry and steady state fluorescence measurements were used to examine the effect of the reconstituted integral membrane proteins on the phospholipid environment. Calorimetric results indicated that the effect of both cytochrome b_5 and cytochrome c oxidase on phosphatidylcholine lipid phase transitions were: a reduction in the associated enthalpy change, a lowering of the melting temperature and a decrease in the cooperativity of melting of the phospholipids. From the observed dependence of the enthalpy change associated with the phospholipid phase transition on the protein to lipid ratio, the number of lipids removed from the phase transition per integral membrane protein was calculated. Cytochrome b_5 perturbs 14 ± 1 phospholipid molecules

which indicates that the hydrophobic portion of this enzyme interacts with only one leaflet of the lipid bilayer. Cytochrome oxidase perturbs 99 ± 5 phospholipid molecules per protein corresponding to a very local perturbation of the lipid environment by this transmembrane enzyme complex.

Steady-state fluorescence measurements of the excimer forming probe, pyrene decanoic acid, indicated that integral membrane protein acts as a barrier to the lateral mobility within the bilayer. The temperature dependence of diphenylhexatriene fluorescence anisotropy supported the protein dependent decrease in the phospholipid melting temperature as observed with the high sensitivity differential scanning calorimeter. The anisotropy measurements also indicated that the protein increases the order of the phospholipid above the phase transition temperature. Thus, the effect of integral membrane protein on phosphatidylcholine lipid bilayers is a local phenomenon, maintaining the perturbed lipid in a conformation intermediate between the gel and lipid crystalline state.

The thermal denaturation of cytochrome oxidase was studied with high sensitivity differential scanning calorimetry and is characterized by an enthalpy change of 550 ± 50 kcal/mole protein. This denaturation was centered at 56°C for the detergent solubilized enzyme but was shifted to 63°C when the enzyme was reconstituted into a phosphatidylcholine bilayer indicating the phospholipid bilayer stabilized the enzyme complex. Deconvolution of the excess heat capacity function of the membrane reconstituted enzyme indicated

four melting steps contribute to this complex melting process. A new technique called differential solubility thermal gel analysis was developed based on the observed temperature dependent change in detergent solubility of the individual subunits of the enzyme complex. Using this technique, it was also demonstrated that a stabilization of the enzyme complex occurs when the enzyme is reconstituted into a lipid bilayer as compared to the detergent solubilized enzyme. Differential solubility thermal gel analysis was further developed to give a quantitative description of the denaturation of the enzyme complex, providing apparent enthalpy changes and melting temperatures for individual subunits of the enzyme complex thus permitting the identification of the subunits which contribute to each melting step involved in the thermal denaturation of the enzyme complex. This technique is currently being applied in conjunction with high sensitivity differential scanning calorimetry to investigate the thermal stability of band III of the erythrocyte membrane (Wortman and Freire, to be published) and may be applied to other biological membranes to determine the contribution of individual protein components of the membrane to observed complex melting profiles.

Suggested further experiments with this system include an examination of the role of membrane phospholipid chain length on the stability of cytochrome oxidase and a study of the role of cytochrome c binding to the enzyme stability. Also, those subunits which retain detergent solubility at high temperature (95°C) could be examined for thermal denaturation using high sensitivity differential scanning calorimetry following their isolation from the enzyme complex.

VITA

Christopher White Rigell was born in Baltimore, Maryland, on October 4, 1957. He graduated from Baltimore Polytechnic Institute in June 1975. In June 1979, he received a Bachelor of Arts in Chemistry and Mathematics from Maryville College in Maryville, Tennessee. From July 1979 to June 1981, he worked as an analytical chemist for Kenwill, Inc., in Maryville, Tennessee.

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