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THERMODYNAMIC STUDIES OF THE INSERTION AND FOLDING OF MEMBRANE PROTEINS: YEAST CYTOCHROME C OXIDASE

ERNESTO FREIRE

Department of Biology and Biocalorimetry Center,
The Johns Hopkins University, Baltimore, MD 21218

SUMMARY

Yeast cytochrome c oxidase is a multisubunit protein located in the inner mitochondrial membrane where it serves as the final intermediary in the respiratory chain and as a proton pump. The three largest subunits are synthesized within the mitochondria and the remaining six subunits are encoded by the nuclear genome and then imported to the inner mitochondrial membrane from the cytoplasm. The exact mechanism of assembly and stabilization of this protein complex is only beginning to be elucidated. In order to investigate the stabilizing interactions within the enzyme complex and the mode of association between subunits we have performed structural stability and thermodynamic perturbation experiments using different biophysical techniques including high sensitivity differential scanning calorimetry and differential solubility thermal gel analysis. These studies have suggested the existence of hierarchical layers of interactions between subunits that define three important cooperative structures. The major components of these structures in the yeast enzyme appear to be: a) subunits I and II; b) subunit III; and, c) the imported subunits IV and VI. In order to confirm these assignments and study directly the assembly of the protein complex, subunit dissociation experiments under controlled conditions have been performed.

YEAST CYTOCHROME C OXIDASE

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

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of the enzyme (I, II and III) of 57, 30 and 26 kdaltons respectively are encoded by the mitochondrial genome and probably inserted into the inner mitochondrial membrane during translation by membrane bound ribosomes. The imported subunits, ranging in molecular weight from 17 down to 5 kdaltons, are encoded by the nuclear genome and translated by free ribosomes in the cytoplasm (Sebald et al, 1972; Mason and Schatz, 1973; Power et al, 1984). They are synthesized as precursor proteins carrying amino terminal extensions of 20-40 amino acids called signal or targeting sequences. These signal sequences target the newly synthesized protein to the mitochondria and once in the mitochondria direct the protein to its final destination. Except in few cases (e.g. cytochrome c which carries an internal sequence) the signal sequence is cleaved in the mature protein (for a review see Rapoport, 1986). Figure 1 illustrates the general assembly pathway of cytochrome c oxidase.

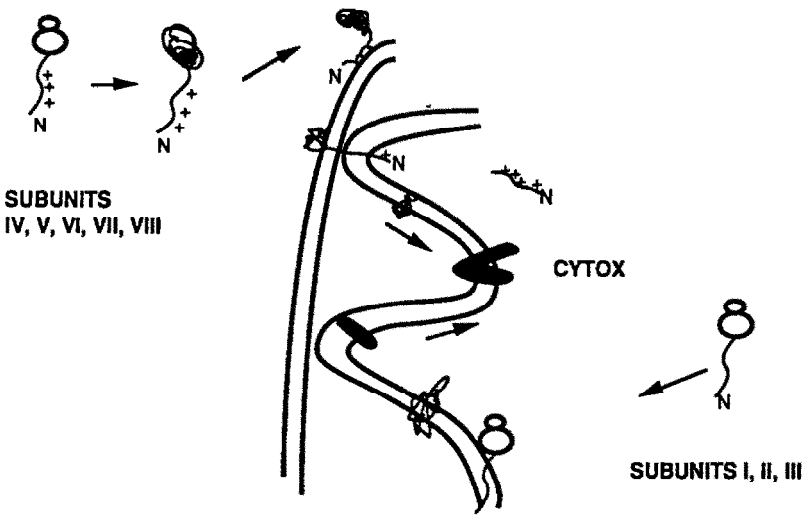

Figure 1: Schematic representation of assembly pathway of cytochrome c oxidase. Subunits I, II and III are of mitochondrial origin. Subunits IV-VIII are imported from the cytoplasm.

The process of assembly of the cytochrome c oxidase complex includes several events: 1) Synthesis of subunits; 2) Targeting, translocation and insertion of subunits into the inner mitochondrial membrane; and, 3) subunit folding, association and assembly. These events do not necessarily occur sequentially, as in the case of cotranslational insertion in which synthesis is coupled to membrane penetration. In this laboratory, we have been primarily concerned with the energetics of protein insertion and the mechanism of subunit folding, association and assembly using thermodynamic perturbation techniques.

TARGETING SEQUENCES

The targeting sequences of imported subunits usually contain a high preponderance of basic amino acids interspersed with neutral or hydrophobic residues. The basic amino acids are at positions such that, when the peptide is in an alpha helix conformation, they are located on the same side of the helix forming an amphiphilic helical structure (von Heijne, 1986). The aminoacid sequence of the targeting peptide of yeast cytochrome c oxidase subunit IV (cytox IV), the largest of the imported subunits is:

$$\overset{+}{\text{Met}}\text{LeuSerLeu}\overset{+}{\text{Arg}}\text{GlnSerIle}\overset{+}{\text{Arg}}\text{PhePheLys}\overset{+}{\text{Pro}}\text{AlaThr}\overset{+}{\text{Arg}}\text{ThrLeuCysSer....}$$

This peptide, as well as other mitochondrial targeting peptides, interact very strongly with lipid bilayer membranes containing negatively charged phopspholipids, e.g. inner mitochondrial membrane (Myers et al, 1987). Twenty percent of the total lipid of the inner mitochondrial membrane is the di-anionic lipid cardiolipin (diphosphatidyl glycerol) and confers a relatively high negative charge density to its surface. Calorimetric studies in this laboratory (Kodama and Freire, 1989) indicate that the cytox IV peptide binds to negatively charged membranes with an association constant on the order of $10^{6}\ M^{-1}$ at 25 °C and a binding enthalpy of -90 kcal/mole. The association is about five to ten times stronger to the gel than to the fluid phase. In fact, binding of the signal peptide to the membrane shifts the lipid transition to higher temperatures and induces a local phospholipid rigidification as indicated by differential scanning calorimetry and fluorescence anisotropy measurements. The preference of the signal sequence for the gel phase is most likely due to the better structural matching existing between the helix dimensions and the phospholipid headgroup separation in the gel phase. Since the

natural state of the membrane is the fluid state, the ability of the signal sequence to induce localized gel-phase membrane microdomains provides a potential driving force for insertion. For example, the cross sectional area occupied per phospholipid molecule in the fluid phase is on the order of 60 A^2 and decreases to about 50 A^2 in the gel phase (Janiak et al, 1979; MacDonald and Simon, 1987). A local change affecting ~25 lipid molecules (as deduced from structural and binding studies) will create a region of high lateral compressibility with the potential to generate an insertion area of up to 250 A^2. These structural defects can be utilized by the newly synthesized proteins to penetrate the lipid bilayer or to undergo conformational transitions under conditions of enhanced membrane compressibility. In this respect, it has been previously observed that the rate of insertion of proteins into membranes is maximal at the phase transition temperature of the lipid bilayer (Pownall et al, 1979, Pownall and Massey, 1982).

ENZYME STABILITY AND SUBUNIT INTERACTIONS

The final assembly of the cytochrome c oxidase complex involves subunit folding and subunit association. At this time the exact linkage between these two events is still not completely understood. Our general strategy to address this issue has been: 1) to study the structural stability of the intact enzyme complex under different conditions in order to identify the degree of energetic coupling existing between the various subunits; and, 2) to selectively dissociate specific subunits and study their structural stability in isolation and their reassociation.

The structural stability of the yeast enzyme has been examined by high sensitivity differential scanning calorimetry, differential detergent solubility thermal gel analysis and enzyme activity measurements (Morin et al ,1989). These studies have shown that the phospholipid moiety affects the stability of the enzyme as judged by the dependence of the denaturation temperature on the lipid composition of the bilayer. The enzyme is more stable when reconstituted with the 18-carbon, unsaturated phospholipid dielaidoyl phosphatidylcholine (DEPC) than with the 14-carbon saturated phospholipid dimyristoyl phosphatidylcholine (DMPC). In calorimetric scans performed at a scanning rate of 45

°C/hour the protein transition is centered at 60.5 °C in DEPC and 54.0 °C in DMPC. In addition, the shapes of the calorimetric transition profiles are different in the two lipid systems indicating that not all of the protein subunits are affected equally by the lipid moiety. The overall enthalpy change for the enzyme denaturation is essentially the same for the two lipid reconstitutions (405 kcal/mole of protein for the DMPC and 425 kcal/mole for the DEPC reconstituted enzyme). Analysis of the data indicate that the unfolding of the enzyme cannot be represented as a two state process and that it involves multiple transitions. Molecular assignment of the calorimetric peaks has been done by differential detergent solubility thermal gel experiments. These experiments indicate that the major contributors to the main transition peak observed calorimetrically are subunits I and II and that the transition temperature of subunit III is the most affected by the phospholipid moiety.

There are important similarities in the behavior of the yeast enzyme and the beef heart enzyme (Rigell et al, 1985; Rigell and Freire, 1987). In both cases the thermal denaturation of the enzyme is characterized by three important features: 1) The bulk of the enzyme (subunits I and II) melt together and comprise the principal contributors to the main peak observed by differential scanning calorimetry; 2) Subunit III behaves somewhat independently and gives rise to a separate low temperature peak in the beef heart enzyme and a shoulder in the low temperature side of the yeast enzyme denaturation profile in the DMPC reconstituted enzyme; additionally, experiments using two different phospholipid moieties suggest that the stability of subunit III can be affected differently than subunits I and II; 3) The cytoplasmically imported subunits IV and VI in the yeast enzyme and the homologous subunits Va and Vb in the beef heart enzyme (using the nomenclature of Kadenbach et al,1983) dissociate from the enzyme complex at the denaturation temperature and stay in aqueous solution after denaturation of the bulk of the enzyme complex.

The thermal stability experiments, in both the beef heart and yeast enzyme, are consistent with the existence of hierarchical intersubunit interactions that define three primary behavioral structures. Apparently, subunits I and II, subunit III, and the imported subunits IV and VI in yeast or Va and Vb in beef heart define the main constituents of these structures. These findings are consistent with other observations in the literature. For example, subunit III in the beef heart enzyme can be selectively removed by either detergent solubilization at high pH

(Saraste et al, 1981; Pentilla, 1983) or chymotryptic digestion (Capaldi et al , 1983) resulting in an enzyme that retains electron transfer activity. Subunits IV and VI in the yeast enzyme can be dissociated from the rest of the enzyme by GuHCl at concentrations below those required for denaturation of the bulk of the enzyme (Montgomery, D. unpublished results). After removal of the GuHCl by dialysis, these subunits show secondary structure and undergo a reversible thermally induced unfolding transition as demonstrated by CD and DSC experiments. The calorimetric unfolding profile at neutral pH is characterized by two peaks centered at 33 and 45^{o} C respectively. These studies demonstrate that these imported subunits are only marginally stable in solution and that they are stabilized by their association to the membrane bound enzyme subunits. Concomitantly, the subunit IV, V, VI-depleted enzyme denatures approximately ten degrees below the intact enzyme (as determined by temperature inactivation experiments) indicating that the minor subunits also contribute to the stability of the bulk of the enzyme (Montgomery, D. unpublished results). Subunit Va in the beef enzyme has been shown to be largely exposed to the aqueous phase by its tryptic removal from the reconstituted enzyme complex (Zhang et al, 1984). Also, examination of the amino acid sequences of subunits Va and Vb in the beef heart enzyme indicate that they are largely water soluble subunits (Tanaka et al, 1979) as is the case for subunits IV and VI in the yeast enzyme (Gregor and Tsugita, 1982; Maarse et al, 1984). The similarities found between the yeast and beef heart enzyme complexes suggest the conservation of fundamental behavioral patterns through evolution despite variations in subunit composition between the two systems.

ASSEMBLY MECHANISM

All the experiments presented in this paper for the yeast enzyme as well as those published previously for the beef heart enzyme indicate that the thermotropic behavior of subunits I and II cannot be uncoupled from each other under the experimental conditions studied, suggesting that the interactions between these two subunits are stronger than those existing with or between other subunits. A plausible, albeit speculative, assembly mechanism consistent with the above observations is shown in Figure 2. According to this mechanism, the core of the enzyme is first formed by the association of subunits I and II. Probably, folding and subunit association are strongly coupled for these two subunits judging from the apparent inability of

these subunits to dissociate in native form. Once the core is formed, subunit III and the imported subunits IV and VI in yeast (Va and Vb in beef heart) are able to associate to the enzyme. Additional experiments in this laboratory are also consistent with this mechanism and indicate that removal of subunit III does not significantly affect the thermotropic behavior of the beef heart cytochrome c oxidase (Rigell C., unpublished results) and that the dissociated subunits IV and VI from the yeast enzyme possess a tertiary structure and are able to undergo a folding/unfolding transition in the absence of the remaining subunits (Montgomery, D. and Semo, N., unpublished results). The minor imported subunits have not been assigned yet and their role in the overall structural stability of the enzyme is still unknown.

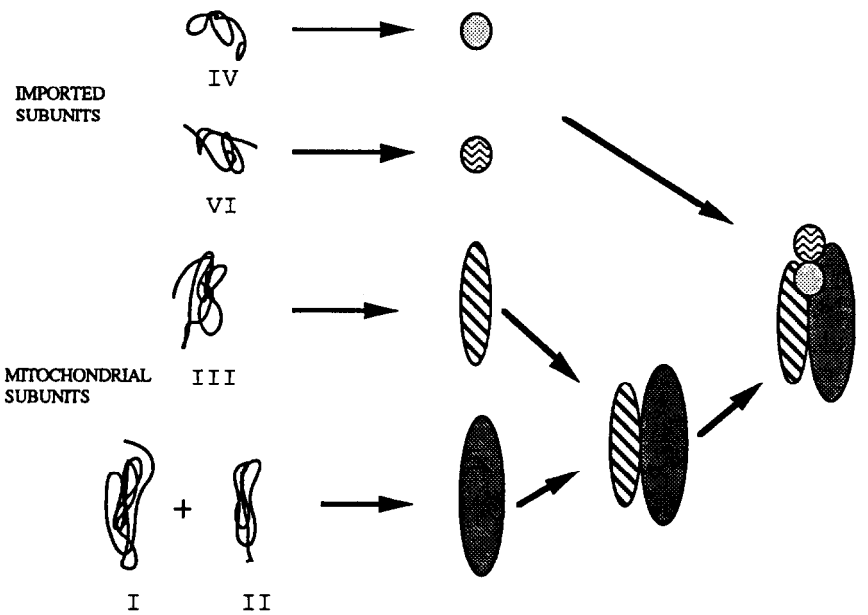

Figure 2 : Tentative assembly mechanism of major subunits of yeast cytochrome c oxidase derived from thermodynamic perturbation experiments. Subunits V, VII and VIII have not been determined at the present time.

KINETICS OF THERMAL UNFOLDING

The thermal denaturation of yeast cytochrome c oxidase is a kinetically restricted process as demonstrated by the dependence of the unfolding profiles on the scanning rate. Recently, a rigorous formalism to extract kinetic information from calorimetric scans performed at different scanning rates has been developed (Sanchez-Ruiz et al, 1988; Freire et al, 1989) and applied to the thermal unfolding of yeast cytochrome c oxidase (Morin et al, 1989). At low temperatures, the rate constants are very small and as such the enzyme is stable in the native conformation for long periods of time. The rate constants are temperature dependent and characterized by activation energies on the order of 37 kcal/mole for the DMPC reconstituted enzyme and 42 kcal/mole for the DEPC reconstituted enzyme. For example, at 25°C the rate constant is 6.5×10^{-5} sec^{-1} for the DMPC reconstituted enzyme and 9.2×10^{-6} sec^{-1} for the DEPC reconstituted enzyme. These rate constants are consistent with relaxation times of 4.5 hours and 30 hours respectively. As the temperature increases the rate constants increase in magnitude as dictated by the positive activation energies. So, for example, at 60°C the rate constants are 0.04 sec^{-1} for the DMPC and 0.013 sec^{-1} for the DEPC reconstituted enzyme, respectively. Under the conditions studied, the rate of irreversible thermal denaturation is faster for the DMPC than for the DEPC reconstituted enzyme, resulting in a reduced enzyme stability in the former lipid. These observations suggest the intriguing possibility that the stabilization of certain membrane proteins might not be entirely a thermodynamic phenomenon but a process modulated by kinetic constraints.

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