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THERMODYNAMIC CHARACTERIZATION OF THE INTERACTION BETWEEN CYTOCHROME b_5 AND CYTOCHROME c

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In recent years many studies have been directed toward the elucidation of the interaction mechanisms within protein-protein complexes. One of the best studied protein - protein complexes has been the cytochrome b5 and cytochrome c electron transfer pair. Thermodynamic information about the association process has been obtained through methods which indirectly measure the binding between the proteins. We report here the use of Isothermal Titration Calorimetry to characterize the association of Rat cytochrome b5 and Horse cytochrome c. The association is accompanied by an unfavorable enthalpy change (+1.0 +/- 0.1 Kcal/mole) and a large stabilizing change in entropy (33.9 +/- 0.6 eu).

A model for the interaction between cytochrome b5 and cytochrome c has been proposed by Salemme and coworkers (1). This "docked complex" involves the formation of four inter-protein salt bridges between the interacting surfaces of the proteins. The association of these proteins has been studied experimentally by difference spectroscopy, NMR, and chemical crosslinking with all results suggesting that salt bridges are important in complex formation (2-4). The role of salt linkages has also been probed using site directed mutagenesis and high pressure techniques which assay the change in overall volume of dissociation upon removal of charged residues at the surface of cytochrome b_5 (5). The removal of groups implicated in the formation of salt bridges was found to decrease the overall negative volume change upon dissociation, suggesting an

Abbreviations: cyt c, cytochrome c; cyt b_5, cytochrome b_5; ITC, Isothermal Titration Calorimetry.

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involvement of charge-charge interactions. Mutagenesis also demonstrated that even with all the charged residues proposed to form salt bridges on cyt b5 altered, there still exists a large negative free energy change, implying that the complex still formed in the absence of the proposed salt linkages. It has been shown that the main contribution to the free energy difference in many electron transfer systems results from an increase in entropy (2, 5). It is currently believed that the large entropy change originates from the exclusion of water from the protein - protein interface. Calculations of the surface area sequestered at the interface would give rise to the favorable free energy changes seen in the b5 mutants (5). Other modeling exercises have been carried out and suggest that the role of electrostatics in protein-protein complex formation is to "steer" the proteins into the proper orientation from long distances such that when collision occurs they are in a conformation that is favorable for electron transfer to occur (6).

The effect of ionic interactions on the electron transfer kinetics has also been studied by several groups (2, 6, 7). It has long been noted that the reaction between cytochrome b5 and cytochrome c is strongly ionic strength dependent (2). Very recently however, it has been found that a simple exponential kinetic model cannot adequately describe the cyt b5 -cyt c complex (7). The concept of conformational gating in protein-protein complexes has been demonstrated in many systems where at low ionic strength, the electron transfer partners are tightly bound and exhibit biphasic kinetics (7-9). As the ionic strength increases, the complex becomes "loose" and the fast phase decreases while the slow phase increases. This has been explained by the existence of a fast reacting conformer and a slow reacting conformer which interconvert slowly at low ionic strength. Increasing the ionic strength allows for rapid equilibration between the two states resulting in monophasic kinetics at higher ionic strengths. At even still higher ionic strength the reactions become second order. We have conducted isothermal titration calorimetry experiments on cytochromes c and b_5 in an attempt to shed light on the binding of these two proteins at low ionic strength.

Methods

Protein Preparation

Horse heart cytochrome c was purchased from Sigma and used without further purification. Soluble rat liver cytochrome b_5 was overexpressed and purified from *Escherichia coli* as reported by von Bodman et al (10). The pure proteins were dialyzed into 2 mM Tris pH 7.4 at 25 C with at least three buffer changes. Cytochrome c was then concentrated to > 1 mM using Centricon-3 from Amicon. The final concentration was determined by the absorbance 410 nm (ε = 106.1 $mM^{-1}cm^{-1}$) (11). Cytochrome b5 was diluted with a portion of the final dialysis buffer to obtain a concentration between 20 and 50 μM. The final concentration was determined by the absorbance at 412 nm (ε = 130 $mM^{-1}cm^{-1}$) (10).

Isothermal Titration Calorimetry

The Microcal ITC cell was employed for all titrations (12). Cytochrome b5 at a concentration of 20 to 50 μM was degassed under vacuum 5 min. and loaded into the ITC cell. Cytochrome c was also degassed and loaded into an ITC syringe at a concentration of >1 mM. The injection volume was determined by calculating the volume of cytochrome c needed to titrate the cyt b5 to a two fold molar excess. Experiments were conducted at 25 C in 2 mM Tris-HCl pH 7.4. Titrations were carried far past the inflection point in order for subtraction of the heat due to mixing cytochrome c into a protein solution. The heat of dilution of cyt c was assumed to be linear and represented by the last three or four injections of the titrant. Enthalpy changes, binding constants, and binding stoichiometry were determined by the curve fitting program Origin from Microcal inc.

Results and Discussion

Titration calorimetry allows for the direct determination of the enthalpy of complex formation between two proteins. A typical titration observed is shown in Fig 1. Cytochrome b5 and cytochrome c exhibit an unfavorable positive enthalpy change suggesting that the increase in entropy is the major contribution to the overall free energy change. We find ΔG = 9.1 +/- 0.2 Kcal/mole, ΔH = 1.0 +/- 0.1 Kcal/mole and ΔS = 33.9 +/- 0.6 eu. It has been speculated that the large increase in entropy results from the exclusion of water from the protein - protein interface. There are four negatively charged residues that are involved in

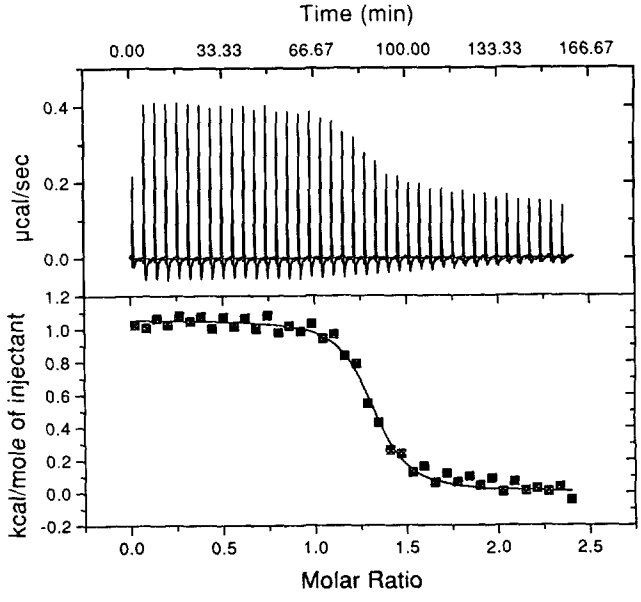

Fig.1. A representative titration experiment. The top graph shows raw data in μcal/sec and the bottom shows the corrected integrated area of each peak as a function of molar ratio.

Table 1

	ΔH Kcal/mole	ΔS eu
Calorimetric	1.0 +/- 0.1	33.9 +/- 0.6
Spectrophotmetric[a]	1 +/- 3	33 +/- 11

a) Mauk et al 1982 Biochemistry, 21, 1843-1846.

salt links in the cyt c-cyt b5 complex (5). These residues are thought to define the region of surface area that is buried in the protein-protein interface. It is also postulated that the ionic interactions serve as a steering mechanism for complex formation (6). We find good agreement in overall free energy changes with that previously determined by indirect optical titrations. (Table 1) (2). The advantage of calorimetry is that the enthalpy of all processes upon binding are measured as opposed to spectroscopic determinations which may be sampling only part of the association.

Another method that has recently been employed to study the role of electrostatics in protein-protein titrations is a potentiometric technique which measures uptake or release of protons upon complex formation (13, 14). Results for the cyt b5 - cyt c interaction using this technique suggest that at pH 7.4, which is near the point of highest affinity, almost no protons are released or taken up during complex formation. This is an important finding for our study since correction for the heat of protonation of the buffer is not required for our conditions. This also points to a limitation of the potentiometric technique for this system. Information on binding constant cannot be obtained at this pH where binding is tightest.

Curve fitting of our data suggests there is only one binding site with a single binding constant. We see no indication that any higher order complexes form. Also we note that we do not see multiple conformers. This does not mean they do not exist, only that calorimetrically they are identical and cannot be distinguished. Kinetic data has shown the existence of these multiple conformers at low ionic strength(7, 15). We conclude from our findings that, if present at low ionic strength, both conformers must have the same thermodynamic characteristics. This suggests that the conformers represent only very subtle changes in the protein-protein geometry.

Conclusion

We have demonstrated here the utility of titration calorimetry to study the interaction of cytochrome b5 and cytochrome c in analogy with similar experiments on other systems (16, 17). This technique does not rely on indirect methods to fully determine all of the thermodynamic parameters in a single experiment. We suggest that ITC is more reliable and more versatile than other

methods which have heretofore been utilized. We have shown that the association of cytochrome b5 and cytochrome c is driven largely by entropic factors, which supports the theory that exclusion of water at the binding interface is a major source of the total binding free energy. We see no evidence of two types of binding sites in our data, suggesting that the multiple conformers seen in the kinetic data are thermodynamically indistinguishable.

Acknowledgments

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