Spectrophotometric Analysis of the Interaction between Cytochrome b_5 and Cytochrome c†

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ABSTRACT: The interaction between cytochrome c and the tryptic fragment of cytochrome b_5 has been found to produce a difference spectrum in the Soret region with a maximum absorbance at 416 nm. The intensity of this difference has been used to determine the stoichiometry of complex formation and the stability of the complex formed. At pH 7.0 [25 °C (phosphate), μ = 0.01 M], the two proteins were found to form a 1:1 complex with an association constant, K_A, of 8 (3) × 10^4 M^{-1}. The stability of the complex was found to be strongly dependent on ionic strength with K_A increasing to 4 (3) × 10^6 M^{-1} at μ = 0.001 M [25 °C, pH 7.0 (phosphate)]. Analysis of the dependence of K_A on pH from pH 6.5 to 8 demonstrated that this complex is maximally stable between pH 7 and 8 or about midway between the isoelectric points of the two proteins. Analysis of the temperature dependence of K_A revealed that formation of the complex between the two proteins is largely entropic in origin with $\Delta H°$ = 1 ± 3 kcal/mol and $\Delta S°$ = 33 ± 11 eu [pH 7.0 (phosphate), μ = 0.001 M]. This result may be explained either by the model of Clothia and Janin [Clothia, C., & Janin, J. (1975) *Nature (London) 256*, 705] in terms of extensive solvent reorganization upon complexation or by the model of Ross and Subramanian [Ross, P. D., & Subramanian, S. (1981) *Biochemistry 20*, 3096] in which the negative enthalpic and entropic contributions of short-range protein–protein interactions are offset by proton release.

Oxidation–reduction reactions between two metalloproteins are generally thought to involve intermediate formation of a complex of the two proteins in which the redox centers of the proteins are aligned optimally prior to electron transfer (Poulos & Kraut, 1980). Several examples of such protein–protein interaction have been studied: cytochrome c/cytochrome c peroxidase (Leonard & Yonetani, 1974; Erman & Vitello, 1980; Mochan & Nicholls, 1972), cytochrome c/cytochrome c oxidase (Vanderkooi et al., 1977; Staudenmayer et al., 1977; Smith et al., 1977; Ferguson-Miller et al., 1978; Bisson et al., 1978; Glatz et al., 1979), cytochrome c/cytochrome bc_1 (Weiss & Juchs, 1978), cytochrome c/cytochrome c_1 (Bosshard et al., 1979; Chiang et al., 1976; Konig et al., 1980), and cytochrome c/cytochrome b_2 (Baudras et al., 1971). In most of these cases, studies between proteins that are physiological oxidation–reduction partners have been limited by the unavailability of detailed structural information for at least one of the two proteins.

For this reason, the interaction between cytochrome b_5 and cytochrome c has been of particular interest. Cytochrome b_5 reduces cytochrome c rapidly in vitro (Strittmatter, 1964), and recent evidence indicates that these proteins function as physiological redox partners in vivo (Bernardi & Azzone, 1981). The three-dimensional structures of both proteins are well characterized (Mathews et al., 1979; Takano & Dickerson, 1980). On the basis of crystallographic information, Salemme (1976) has proposed a detailed model for the complex formed between cytochrome b_5 and cytochrome c. In this model, the two heme groups are nearly coplanar and are separated by a distance of approximately 8.4 Å (heme edge to heme edge). The specificity of the interaction depicted in this model is provided by interaction of ϵ-amino groups on cytochrome c with carboxyl groups on cytochrome b_5. As this model has served as a prototype for studies on other heme-protein pairs, we have undertaken a study designed to quantify the interaction between these two proteins directly to learn more about the factors stabilizing such interaction and to identify conditions that favor complex formation.

Experimental Procedures

Glass-distilled water passed through a Barnstead NANOpure water purification system (resistivity 17–18 MΩ·cm) was used throughout. All chemicals were reagent grade. Phosphate buffers were prepared at known ionic strengths based on the pK_a values of Bates & Acree (1943). Measurements of pH were made with a Radiometer Model PHM 84 pH meter and a Model GK2321C combination electrode.

The tryptic fragment of cytochrome b_5 was purified from fresh beef liver to an $A_{412.5}/A_{280}$ ratio of 5.7–6.0 as previously described (Reid & Mauk, 1982). Horse heart cytochrome c (type VI) was obtained from Sigma and was used without further purification. Both proteins were dialyzed into the desired buffer with Spectrapor dialysis tubing (6000–8000 MW cutoff) to minimize loss of protein on the dialysis membrane at low ionic strengths. The concentration of cytochrome c was determined on the basis of ϵ_{410} = 106 100 M^{-1} cm^{-1} (Margoliash & Frohwirt, 1959), and the concentration of cytochrome b_5 was determined at 25 °C on the basis of $\epsilon_{412.5}$ = 117 000 M^{-1} cm^{-1} (Ozols & Strittmatter, 1964).

Spectrophotometric measurements were made with a Cary 219 spectrophotometer equipped with a thermal isolation accessory and thermostated sample and reference cell holders. The temperature was controlled with a Laude Model RC3 refrigerated, circulating water bath. Thermal equilibration was monitored to ±0.2 °C with a Fluke Model 2175A digital thermometer equipped with a subminiature copper–constantan thermocouple.

Difference spectra, Job plots, and spectrophotometric titrations were obtained by the method of Erman & Vitello (1980) using matched tandem cuvettes (Hellma). Titration data were fitted to the function derived by these authors (eq 1) with the nonlinear regression program of Duggleby (1981).

Results

The difference spectrum that results from the interaction of cytochrome b_5 with cytochrome c is shown in Figure 1. The absorbance maximum of the spectrum was found to occur at

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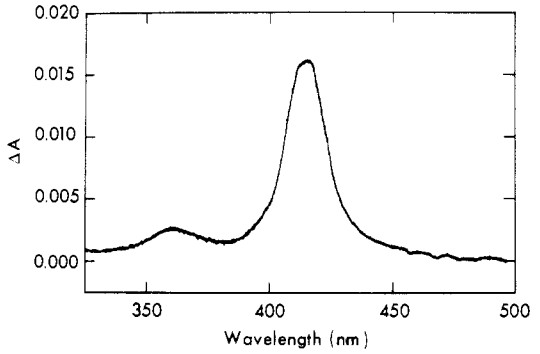

FIGURE 1: Difference spectrum due to complex formation in a solution containing 4.51 μM cytochrome c and 3.62 μM cytochrome b_5 [4 °C, pH 7.0 (phosphate), μ = 1 mM]. This spectrum has been corrected for the instrument base line; the path length was 0.874 cm.

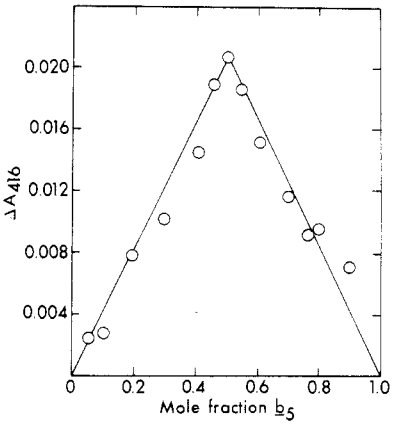

FIGURE 2: Plot of the change in absorbance at 416 nm upon complex formation as a function of the mole fraction of cytochrome b_5 and cytochrome c. The total concentration of the two proteins was held constant at 10.54 μM [25 °C, pH 7.0 (phosphate), μ = 1 mM]; the path length was 0.879 cm.

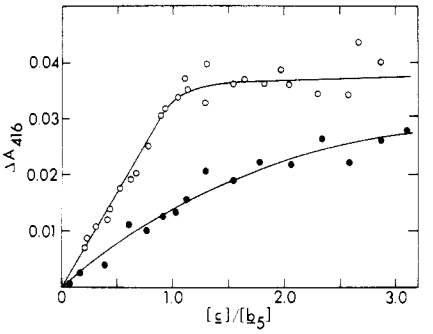

FIGURE 3: Spectrophotometric titration of cytochrome b_5 with increasing concentrations of cytochrome c and cytochrome b_5 concentration of 9.91 μM [25 °C, pH 7.0 (phosphate)]: (open circles) μ = 1 mM; (closed circles) μ = 10 mM. The path length was 0.879 cm.

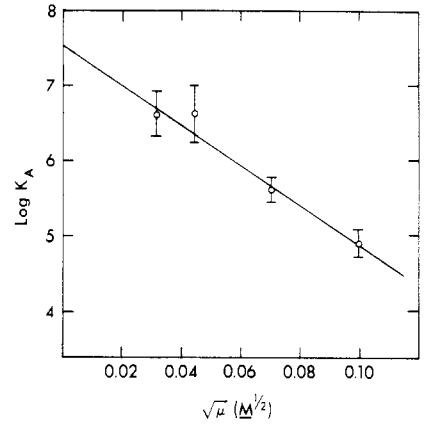

FIGURE 4: Ionic strength dependence of the association constant for the formation of the complex between cytochrome b_5 and cytochrome c [25 °C, pH 7.0 (phosphate)]. The solid line is a weighted linear least-squares fit to the data.

416 nm at all values of pH and temperature examined. At pH 7.0 (μ = 0.001 M and $[c]/[b_5]$ = 1), the change in absorbance produced by the interaction between these proteins represents an increase of approximately 2.0% over the total absorbance before mixing. At higher temperatures and at pH greater than 7, the difference spectrum exhibits a broad trough at 395–400 nm. At higher concentrations (each protein approximately 50 μM), the difference spectrum was found to exhibit additional maxima in the visible region at approximately 562 and 526 nm (pH 7.0, 25 °C, μ = 5 mM).

Using the intensity of this difference as a measure of complex formation, it was possible to determine the stoichiometry of the complex and its stability. The Job plot shown in Figure 2 indicates that the observed difference spectrum arises from the formation of a 1:1 complex between the two proteins. This finding is confirmed by titration curves as shown in Figure 3. Titration data obtained under the conditions used for recording the spectrum shown in Figure 1 indicate that approximately 83% of the cytochrome b_5 is complexed with cytochrome c under these circumstances.

The dependence of K_A on ionic strength as determined from a series of titration curves is shown in Figure 4. From this plot we estimate an association constant of 3 (2) × 10^7 M^{-1} at zero ionic strength. Consistent with earlier kinetic studies (Ng et al., 1977; Stonehuerner et al., 1979), the interaction between these proteins is strongly inhibited by increasing ionic strength. The range of ionic strength accessible to this study was limited in two ways. Use of lower ionic strength was prevented by the limited buffer capacity of phosphate at lower concentrations while higher ionic strengths were inaccessible because the stability of the complex was sufficiently decreased under these conditions that the scatter in the titration data prevented reliable calculations.

Analysis of the temperature dependence of complexation between the two proteins was complicated by a variation in the electronic absorption spectrum of cytochrome b_5 between 4 and 32 °C. Although the spectrum of cytochrome b_5 has been shown to change at elevated temperatures (e.g., Sugiyama et al., 1980), an effect at more moderate temperatures does not appear to have been reported previously. To compensate for this change, we used the following molar absorptivities (M^{-1} cm^{-1}) to determine cytochrome b_5 concentration in these measurements: 115 700 at 32 °C, 118 100 at 18 °C, 119 200 at 10 °C, and 120 300 at 4 °C.

The temperature dependence of K_A for formation of the cytochrome b_5–cytochrome c complex is shown as a van't Hoff plot in Figure 5. From this plot, $\Delta H°$ has been estimated to be 1 ± 3 kcal/mol and $\Delta S°$ to be 33 ± 11 eu. Although these values are approximate owing to the relatively large error that is inherent in these measurements, it does appear that the interaction between cytochrome b_5 and cytochrome c is largely entropic in nature. The possible meanings of this observation are discussed further below.

The effect of pH on K_A has been studied over a limited range of pH as shown in Figure 6. The occurrence of a pH optimum in complex formation approximately midway between the isoelectric points of the two proteins is clearly consistent with the expected behavior. In principle, it would be possible to analyze these data as a sum of two titration curves, assuming one ionizable group for each protein. As this simple model seems unlikely to be a reasonable description of

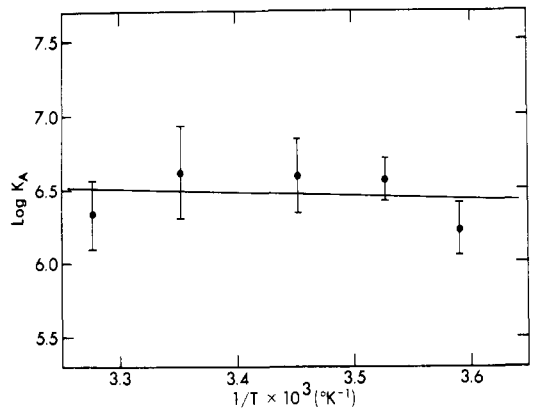

FIGURE 5: Temperature dependence of K_A for formation of the cytochrome b_5–cytochrome c complex [pH 7.0 (phosphate), μ = 1 mM]. The solid line is a weighted linear least-squares fit to the van't Hoff equation.

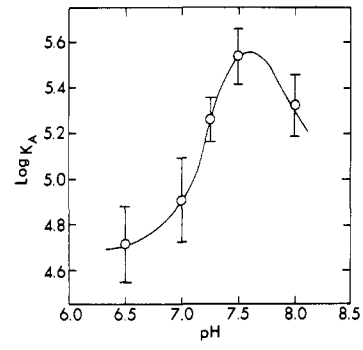

FIGURE 6: pH dependence of K_A for formation of the cytochrome b_5–cytochrome c complex [25 °C (phosphate), μ = 10 mM].

the pH dependence and as insufficient information is available on which to base a more complex analysis, we have not attempted to fit these data to any model at present.

Discussion

This study provides a quantitative characterization of the interaction between cytochrome b_5 and cytochrome c and an initial characterization of its dependence on ionic strength, temperature, and pH. Our results are fully consistent with the model for the complex proposed by Salemme (1976).

Previous reports have also dealt with analysis of the cytochrome b_5–cytochrome c complex. Millett and co-workers (Ng et al., 1977; Stonehuerner et al., 1979; Smith et al., 1980) have studied the kinetics of native and specifically trifluoroacetylated derivatives of cytochrome c reduction by microsomal preparations and have obtained results that are largely consistent with the model proposed by Salemme (1976). These same workers (Stonehuerner et al., 1979) have also demonstrated the existence of a complex, using gel permeation [K_D = 20 μM, pH 7.5 (10 mM Tris-HCl)[1]] and sedimentation velocity experiments, and have shown that steady-state reduction of cytochrome c by cytochrome b_5 is highly dependent on ionic strength. Our findings are clearly in accord with this report.

Miura et al. (1980) have studied the stoichiometry of interaction between these two proteins using ^{1}H NMR spectroscopy. Under conditions similar to ours, they observed a b_5:c ratio of 1:2, which they attributed to formation of a b_5–c complex similar to that proposed by Salemme but modified by binding of a second cytochrome c to the back of the first. As we see no evidence for any stoichiometry other than 1:1, we are unable to rationalize their findings with ours except to note that their experiments are performed at much higher protein concentrations. The possibility of higher order protein aggregation under these conditions cannot be ruled out. The suggestion that cytochrome c binds to cytochrome c in a b_5–c–c trimer, however, seems unlikely to us as cytochrome c is not known to aggregate in solution as this model suggests it should.

The thermodynamic parameters for formation of the b_5–c complex are best considered in terms of recently developed models for protein–protein interaction. The model of Clothia and Janin ascribes the stability of protein association to an extensive change in solvent structure accompanying the reduction of protein surface area exposed to solvent that occurs on interaction between proteins. According to this model, specificity of protein interaction (i.e., which proteins may interact with each other) is determined by complementarity of protein structures that permits close packing. The forces involved in this recognition (e.g., hydrogen bonds, van der Waals contacts) are not thought to contribute significantly to the stability of the complex. For the b_5–c complex, recognition would be provided by the electrostatic charge complementarity of the type described by Salemme's model while changes in solvation would provide its stability. The effect of this complexation on solvation has already been pointed out by Salemme (1976).

More recently, however, Ross & Subramanian (1981) have noted that most protein–protein association reactions are, in fact, characterized by negative enthalpies and entropies. To account for this observation, they presented an augmented version of the Clothia–Janin model that consists of two steps. The first step involves solvent reorganization and formation of a "hydrophobically associated complex" of the type described earlier (Clothia & Janin, 1975). The second step involves an internal rearrangement of the complex with formation of short-range or van der Waals interactions to produce an "interacting complex". This latter step does not involve solvent reorganization to a significant extent and is argued to make negative contributions to $\Delta H°$ and $\Delta S°$ that can offset the positive contributions from the first step. Ross and Subramanian further argue that those few protein–protein association reactions that are characterized by positive entropies and enthalpies probably involve concomitant proton release. It is this proton release in combination with the solvent reorganization occurring in the first step that dominates to produce a net positive enthalpy and entropy.

In terms of these models, two possibilities exist for explaining the thermodynamic parameters reported here for formation of the b_5–c complex. The first and simplest is that the Clothia–Janin model is sufficient in this case or, in other words, that the second step of the Ross–Subramanian model does not (always) occur. The second possibility is that interaction of cytochrome b_5 with cytochrome c is best described by the Ross–Subramanian model accompanied by release of a proton or protons that has not yet been reported. Given the nature of the proposed sites of contact between the two proteins, proton release may indeed be a feature of this interaction. Although the present studies do not address this issue, the conditions identified here for optimal complex formation should facilitate future experiments designed to evaluate this possibility.

It is assumed in this and previous studies that the interaction between ferricytochrome b_5 and ferricytochrome c is similar to that between ferrocytochrome b_5 and ferricytochrome c. There is no means of studying the complex formed between the latter forms of the proteins at equilibrium. As crystallo-

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; ^{1}H NMR, proton nuclear magnetic resonance.

graphic studies indicate minimal conformational differences between the reduced and oxidized forms of these proteins, this assumption seems reasonable. From consideration of the structure of ferrocytochrome b_5 it would seem that if this form of the protein does interact differently with cytochrome *c* it does so with a larger K_A than the oxidized protein since the principal difference would be Coulombic. In this case, then, the present results define the lower limits for stability of the ferrocytochrome b_5–ferricytochrome *c* complex. It seems most likely, however, that the ionic strength, pH, and temperature dependences of this complex are at least qualitatively similar to those presented here.

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