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Thermodynamic Parameters of Cytochrome c_3–Ferredoxin Complex Formation

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ABSTRACT: The complex formation between cytochrome c_3 and ferredoxin I from *Desulfovibrio desulfuricans* Norway was studied by microcalorimetric and pH-stat titration measurements. The stoichiometry of the complex was found to be one molecule of cytochrome c_3 per monomer of ferredoxin I. The association constant determined at $T = 283$ K in tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer, 10^{-2} M and pH 7.7, was $K_A = 1.3 \times 10^6$ M^{-1}. Though the enthalpy ($\Delta H = 19 \pm 1$ kJ·mol^{-1}) and the entropy ($\Delta S = 183$ J·K^{-1}·mol^{-1}) were positive and consistent with a hydrophobic process involved in the interaction, the analysis of ionic strength dependence exhibited an important electrostatic effect on the association. The use of both Tris-HCl and phosphate buffers during microcalorimetric experiments showed proton release at pH 6.6. The pH-stat study of proton release indicated that one of the charged groups involved in the interacting site underwent a pK shift from 7.35 to 6.05.

Oxidation–reduction reactions between two metalloproteins necessitate the formation of an intermediate complex in which the redox centers of the two proteins are optimally oriented to achieve physiological electron transfer (Poulos & Kraut, 1980). Various protein–protein interaction studies have been recently reported on the basis of crystallographic information, NMR data, chemical modifications, or covalent cross-linking experiments. Cytochrome c is one of the most investigated electron carrier proteins. Complex formation between cytochrome c and both physiological and nonphysiological partners have been studied: cytochrome c–cytochrome c peroxidase (Poulos & Kraut, 1980; Waldmeyer & Bosshard, 1985), cytochrome c–cytochrome b_5 (Salemme, 1976; Eley & Moore, 1983), and cytochrome c–flavodoxin (Simondsen et al., 1982; Hazzard & Tollin, 1985; Dickerson et al., 1985). In these models the prosthetic groups are nearly coplanar, separated by a distance of about 10 Å, and the specificity of the interaction is provided by the ϵ-amino groups on cytochrome c and carboxyl groups on its partner. Moreover, a hydrophobic

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association resulting in the exclusion of water molecules at the interface has been reported (Salemme, 1976).

The complex formation of physiological oxidation reduction proteins is poorly described as structural information is often limited. We undertook the study of the interaction between cytochrome c_3 and ferredoxin I, which are physiological partners in the sulfate-reducing bacterium *Desulfovibrio desulfuricans* Norway. Cytochrome c_3 is a tetraheme cytochrome (M_r 13000) characteristic of all sulfate-reducing bacteria belonging to the *Desulfovibrio* genus. Both primary and tertiary structure determinations of *D. desulfuricans* Norway cytochrome c_3 have been obtained (Bruschi, 1981; Haser et al., 1979). The four hemes exhibit different and low redox potentials (−165, −305, −365, and −400 mV) (Bianco & Haladjian, 1981), localized in nonequivalent protein environments (Bruschi et al., 1984; Gayda et al., 1985; Guerlesquin et al., 1985a) as described by X-ray crystallographic studies (Pierrot et al., 1982).

Ferredoxin I from *D. desulfuricans* Norway has been shown to be a dimeric protein containing a [4Fe-4S] cluster of redox potential of −374 mV per subunit (M_r 6000) (Guerlesquin et al., 1980, 1982). The primary structure of the ferredoxin has been reported (Bruschi et al., 1985), and preliminary crystallographic data have been obtained (Guerlesquin et al., 1983). The elucidation of its three-dimensional structure should provide the structural basis of the architecture of the interacting site as shown by NMR experiments (Guerlesquin et al., 1985b) and should improve understanding of the electron-transfer mechanism between hemes and the iron–sulfur cluster.

In this organism, cytochrome c_3 acts as an obligate intermediate between ferredoxin and hydrogenase. The requirement of cytochrome c_3 for electron transfer between these two proteins instead of direct coupling as occurs in *Clostridia* reveals high specificity between these oxidation–reduction partners. Rapid kinetics studies of electron transfer between cytochrome c_3 and ferredoxin I from *D. desulfuricans* Norway (Capeillere-Blandin et al., 1986) have established a bidirectional electron exchange between the two proteins. NMR experiments have supported a model in which cytochrome c_3 and ferredoxin I combine to form a bimolecular complex and are consistent with one or two hemes being involved in the binding site (Guerlesquin et al., 1985b).

To further understand the nature and function of this complex, we must be able to estimate the energetics involved. We report here the microcalorimetric study of the factors stabilizing the cytochrome c_3–ferredoxin I complex, as this method seems particularly accurate for obtaining thermodynamics data of interacting systems and protein recognition.

Experimental Procedures

Cytochrome c_3 and ferredoxin I were purified as previously reported by Bruschi et al. (1977) from *Desulfovibrio desulfuricans* Norway strain (NCIB 8310). All the experiments were performed at 283 K, as ferredoxin I is a thermolabile protein. The ferredoxin concentrations were calculated on the basis of a subunit molecular weight (6000), and all the protein concentrations were initial values. In order to obtain ferredoxin I and cytochrome c_3 under identical buffering conditions, the protein solutions were extensively dialyzed together in the same vessel.

Microcalorimetric Measurements. Calorimetric experiments were carried out at 283 K in an LKB Bioactivity Monitor flow apparatus, at several pH and ionic strength values, with tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) or phosphate buffers. The apparatus was calibrated both electrically and chemically. The chemical calibration was accomplished by the neutralization of Tris with HCl. The sensitivity used was 10 μW full scale, and the background noise was less than ±0.05 μW. The appropriate buffer and cytochrome c_3 at varying concentrations (in the range 10–100 μM) in the same buffer were introduced at equal flow rates (20×10^{-3} L·h^{-1}) into the mixing chamber. Consequently, the final protein concentrations were equal to half of the initial concentrations. The base line recorded corresponds to the heat of dilution of cytochrome c_3; 2 mL was necessary to reach steady state. For each cytochrome c_3 concentration, an aliquot, equal to 2 mL, of ferredoxin I at constant concentration (in the range 10–20 μM) was inserted into the continuous buffer flow. The reaction heat was proportional to the recorded steady-state value (maximum deviation) or the area of the peak. When necessary, the heat effect was corrected by the dilution heat of the same aliquot of ferredoxin in the buffer alone (less than 10% of the binding heat at the lower cytochrome c_3 concentration).

In the case of binding of two ligands with only one class of binding site, an enthalpic titration method previously described (Caossolo et al., 1980) using several heat measurements enabled stoichiometry, association constant (K_A), and enthalpy change (ΔH) of the complex formation to be determined. However, K_A values lower than 10^3 M^{-1} or higher than 10^7 M^{-1} cannot be reliably obtained from enthalpic titration data in the range of ferredoxin concentrations used. Nevertheless, microcalorimetry is always an accurate method to determine stoichiometry and ΔH values for high-affinity complex formation (Sari & Belaich, 1973).

When the complex formation reaction is associated with one or more pK shifts of ionizable groups that interfere in the binding process, protons are exchanged with the buffered medium. So the apparent ΔH value (ΔH_{app}) must be corrected to obtain the true ΔH value. In our case, if we call R_{sat} the ratio of the number of moles of released H^+ at ferredoxin saturation per mole of ferredoxin and ΔH_n the heat of neutralization of 1 mol of H^+ by the buffer, we can write

$$\Delta H = \Delta H_{app} - R_{sat}\Delta H_n \quad (1)$$

R_{sat} can be calculated from two ΔH_{app} values determined in two buffers having different ΔH_n values:

$$R_{sat} = \frac{\Delta H^1_{app} - \Delta H^2_{app}}{\Delta H^1_n - \Delta H^2_n} \quad (2)$$

pH-Stat Measurements. A Radiometer RTS 822 apparatus was used for pK shift measurements and binding studies. This apparatus consisted of a PHM 84 Research pH meter, equipped with a G222C glass electrode and a K 4112 calomel electrode, a TTT 80 titrator with a TTA assembly, an ABU 80 autoburet (the accuracy of the delivered value was better than 0.1 μL), and a REA 260 recorder with a REA 270 pH-stat unit. The pH-stat titration method is based on the fact that when one or several pK shifts occur during a complex formation reaction, the number of moles of released H^+ (or OH^-) is proportional to the concentration of complex formed (Sari et al., 1973). The released protons were neutralized at constant pH, with a dilute sodium hydroxide solution (in the range 10^{-4} to 5×10^{-4} N). The volume of the liquid phase used for all experiments was about 1 mL. Volume variations due to the reactant adducts were taken into account for all calculations. All the reactants were adjusted to the experimental pH value with a precision of 10^{-3} pH unit. Ionic strength was adjusted with Na_2SO_4. The reaction vessel was thermostated at 283 K. A stream of N_2 was used to avoid

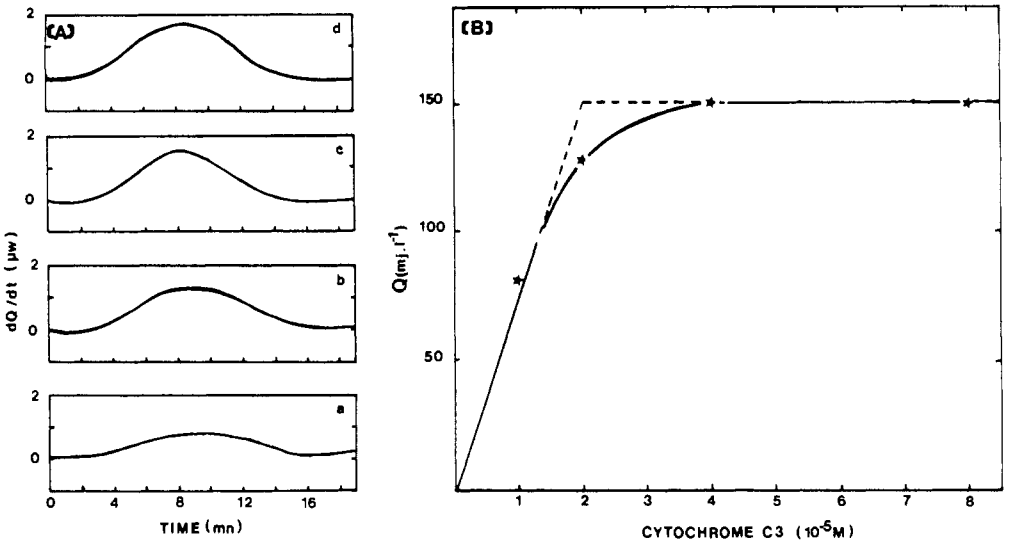

FIGURE 1: (A) Microcalorimetric diagrams of the cytochrome c_3–ferredoxin I complex formation titration. The experiment was performed at 283 K in 10^{-2} M Tris-HCl buffer, pH 7.7. Ferredoxin concentration was 2×10^{-5} M (monomer), and cytochrome c_3 was at increasing concentrations: (a) 10^{-5} M (Q = 323.8 μJ); (b) 2×10^{-5} M (Q = 515.6 μJ); (c) 4×10^{-5} M (Q = 605.0 μJ); (d) 8×10^{-5} M (Q = 612.2 μJ). (B) Titration curve obtained from the experiment of (A). The broken line shows the stoichiometry of the reaction (one molecule of cytochrome c_3 per ferredoxin I subunit).

carbonation of the medium. In the pH range of 6–8, where the best accuracy of the method is reached, 5×10^{-11} mol of H^+ could be titrated.

R, the ratio of the number of moles of released H^+ per mole of ferredoxin, was determined at constant ferredoxin concentration (about 10 μM) while the cytochrome c_3 concentration was increased (until 100 μM) by the addition of 2-μL aliquots. From the titration curve, $R = f$(cytochrome c_3 concentration), it was possible to determine both the stoichiometry and the K_A value of the complex. The value R_{sat} corresponded to the ferredoxin saturation. Moreover, if only one pK was affected, the curve $R_{sat} = f$(pH) was symmetrical, and the maximum R_{sat} value was a function of the pK shift. The maximum R_{sat} value was centered for a pH value between the two pK values before and after complexation. Thus, knowledge of the pK shift and the maximum R_{sat} value allowed us to estimate charged-group pK values before and after complex formation (see Appendix).

Results

Microcalorimetric Titration of Cytochrome c_3–Ferredoxin I Complex Formation. The heat of binding of ferredoxin I to cytochrome c_3 was measured as a function of cytochrome c_3 concentration (Figure 1). From the titration curve the stoichiometry of the complex formation was calculated to be one molecule of cytochrome c_3 per monomer of ferredoxin I (four hemes per [Fe-S] cluster). The association constant, K_A, determined from this plot was 1.2×10^6 M^{-1} at 283 K, 10^{-2} M Tris-HCl, and pH 7.7. The heat of binding is endothermic, the apparent enthalpy change value is $\Delta H_{app} = 13.9 \pm 1.0$ kJ·mol^{-1}, and the apparent entropy change value is $\Delta S_{app} = 165$ J·K^{-1}·mol^{-1}. The ΔH_{app} and ΔS_{app} values are consistent with a hydrophobic interaction contributing to the complex formation.

The dependence of the association constant, K_A, as a function of ionic strength was studied with a series of titration curves (Figure 2) and was determined by altering the ionic strength of the buffer while keeping the pH constant. The association constant decreased from $K_A = 1.2 \times 10^6$ M^{-1} at 10^{-2} M Tris-HCl, to $K_A = 0.86 \times 10^6$ M^{-1} at 2×10^{-2} M Tris-HCl, and to $K_A = 0.20 \times 10^6$ M^{-1} at 10^{-1} M Tris-HCl. The enthalpy changes measured at these ionic strengths were

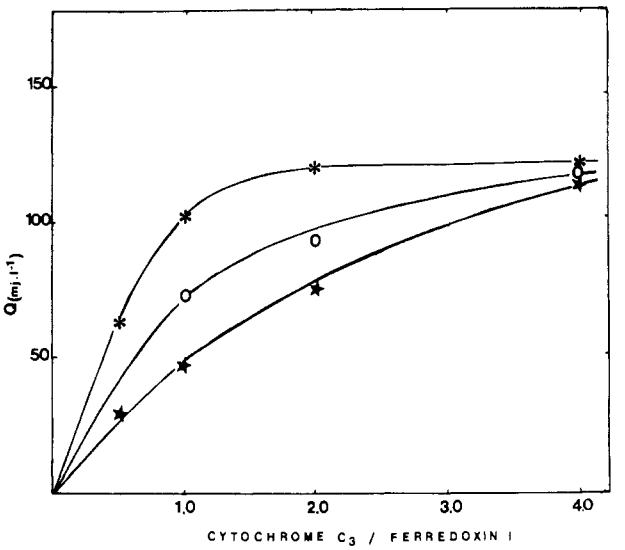

FIGURE 2: Ionic strength dependence of the microcalorimetric titration curves of the complex formation. These experiments were performed at 283 K and in Tris-HCl buffer, pH 7.7. The ionic strengths were respectively (*) 10^{-2}, (O) 2×10^{-2}, and (★) 10^{-1} M. Ferredoxin concentration was 1.6×10^{-5} M (monomer).

respectively $\Delta H_{app} = 13.9 \pm 1.0$ kJ·mol^{-1}, $\Delta H_{app} = 15.3 \pm 1.0$ kJ·mol^{-1}, and $\Delta H_{app} = 16.5 \pm 1.0$ kJ·mol^{-1}. At 0.5 M Tris-HCl and 1.6×10^{-5} M ferredoxin concentration, the K_A value was too weak to be determined by the enthalpic titration method. As the association constant was strongly inhibited by increasing ionic strength, we could conclude that electrostatic interactions played an important role in the binding site of the two proteins.

The pH dependence of the cytochrome c_3–ferredoxin association is illustrated in Figure 3. These results indicate that the complex was more stable at pH 6.6 ($K_A = 2 \times 10^7$ M^{-1}) than at pH 7.7 ($K_A = 1.2 \times 10^6$ M^{-1}) and pH 8.45 ($K_A = 3 \times 10^5$ M^{-1}). This effect would be amplified if the variation of ionic strength of 10^{-2} M Tris-HCl buffer at these different pH were considered. Moreover, during these experiments a reverse heat of binding was observed at pH 6.6 in Tris-HCl buffer ($\Delta H_{app} = -8 \pm 1$ kJ·mol^{-1}). As the heat of binding in PO_4 buffer, at pH 6.6, was always endothermic ($\Delta H_{app} = 16.5$

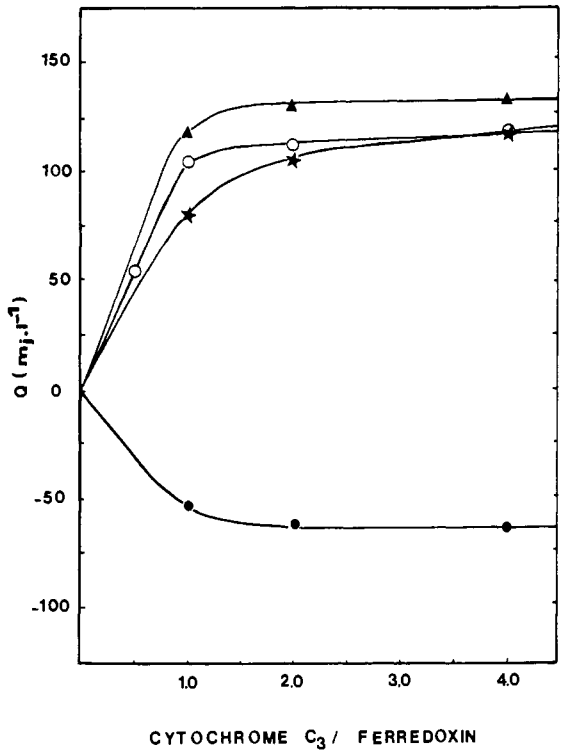

FIGURE 3: pH dependence of the microcalorimetric titration of the cytochrome c_3–ferredoxin I complex. These experiments were performed at 283 K, and ferredoxin I concentration was 1.6×10^{-5} M. Tris-HCl buffer (10^{-2} M) was used at (●) pH 6.6 ($\mu = 10^{-2}$), (○) pH 7.7 ($\mu = 0.5 \times 10^{-2}$), and (★) pH 8.45 ($\mu = 10^{-3}$). The association constants were respectively found to be $K_A = 2 \times 10^7$ M^{-1} (pH 6.6), $K_A = 1.4 \times 10^6$ M^{-1} (pH 7.7), and $K_A = 3 \times 10^5$ M^{-1} (pH 8.45). Phosphate buffer (10^{-2} M) was used at pH 6.6 ($\mu = 2 \times 10^{-2}$) (▲), $K_A = 2.1 \times 10^7$ M^{-1}.

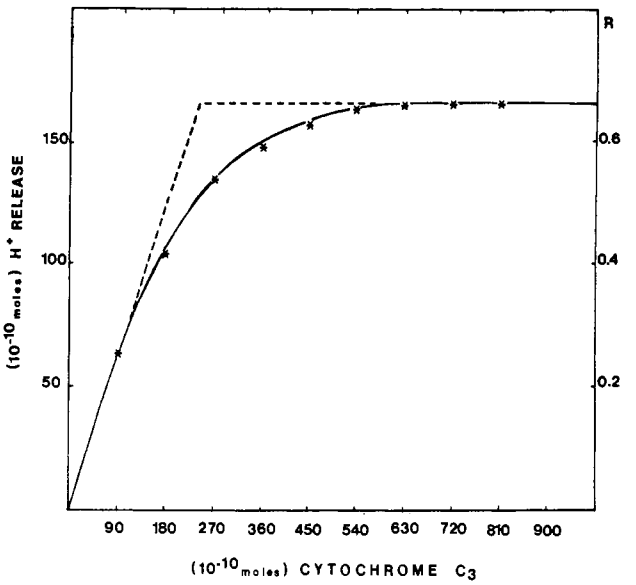

FIGURE 4: pH-stat titration of ferredoxin I–cytochrome c_3 complex formation. The experiment was performed in a volume of 1 mL, pH 6.6, at 283 K and in Na_2SO_4 (3×10^{-3} M). Ferredoxin I concentration was 2.4×10^{-5} M (monomer). The broken line shows the stoichiometry: one molecule of cytochrome c_3 per ferredoxin I subunit.

± 1.0 kJ·mol^{-1}), while the K_A value ($K_A = 2.1 \times 10^7$ M^{-1}) is analogous, we estimated that the exothermicity, at pH 6.6 in Tris-HCl buffer, was due to a buffering proton neutralization effect. With $\Delta H_n^{Tris} = -46.8$ kJ·mol^{-1} and $\Delta H_n^{PO_4} = -8.2$ kJ·mol^{-1} (personal calorimetric determination values at 283 K), at ferredoxin saturation, R_{sat} was calculated as equal to 0.63 mol of released proton per mole of complexed ferredoxin at pH 6.6. So the enthalpy changes obtained in figures 1 and 2 are apparent values, without R_{sat} factor correction. A further attempt was thus made to characterize proton release with a pH-stat titration study of the complex formation.

pH-Stat Titration of Cytochrome c_3–Ferredoxin I Complex Formation. The proton release that occurs during the cytochrome c_3–ferredoxin I complex formation was observed as a function of cytochrome c_3 concentration. From the titration curve (Figure 4), the stoichiometry was found to be one molecule of cytochrome c_3 per monomer of ferredoxin I, with an association constant $K_A = 2 \times 10^7$ M^{-1} at pH 6.6 and 3×10^{-3} M Na_2SO_4 ($\mu = 0.9 \times 10^{-2}$). These K_A values are in excellent agreement with those determined by the microcalorimetric method. The R_{sat} value, $R_{sat} = 0.68$ mol of released proton per mole of complex formed at saturation, was in good agreement with the R_{sat} value calculated by microcalorimetric experiments using relationship 2. R_{sat} values were determined at various pH with constant ionic strength. The R_{sat} values measured were $R_{sat} = 0.54$ at pH 6.1, $R_{sat} = 0.68$ at pH 6.6, $R_{sat} = 0.45$ at pH 7.3, and $R_{sat} = 0.1$ at pH 7.6. From the proton-release pH dependence we could conclude that proton release might be associated with a pK shift of one or several charged groups at the complex interface. However, the fact that the curve obtained from these values was symmetrical implied that only one group was involved in the proton release. The maximum R_{sat} value equal to 0.68 is compatible with a pK shift of 1.3 pH units. Moreover, the maximum R_{sat} value was around pH 6.7; thus, we could estimate an approximate value for the pK shift from $pK_{free} = 7.35$ to $pK_{bound} = 6.05$ (see Appendix).

We could also observe that the R_{sat} value decreased significantly with ionic strength; for example, $R_{sat} = 0.15$ at $\mu = 0.9 \times 10^{-1}$ and pH 6.6. The R_{sat} decrease with ionic strength could be correlated to a reduction of the pK shift between the respective pK values of the free and the bound charged group.

Thermodynamic Parameters of Cytochrome c_3–Ferredoxin I Complex. From the pH-stat measurements we concluded that proton release has to be taken in account when correcting the thermodynamic parameters obtained from microcalorimetric measurements, using relationship 1. Thus at 10^{-2} M Tris-HCl, pH 6.6, the enthalpy change was largely affected by proton release ($R_{sat} = 0.68$): $\Delta H_{app} = -8 \pm 1$ kJ·mol^{-1} instead of $\Delta H = 24 \pm 1$ kJ·mol^{-1}, whereas in phosphate buffer the $\Delta H_{app} = 16.5 \pm 1.0$ kJ·mol^{-1} instead of $\Delta H = 22 \pm 1$ kJ·mol^{-1} is less modified by the proton release. At pH 7.7 the proton release effect was not so great ($R_{sat} = 0.1$): $\Delta H_{app} = 13.6 \pm 1.0$ kJ·mol^{-1} instead of $\Delta H = 18.3 \pm 1.0$ kJ·mol^{-1}. At pH 7.7 for high ionic strength and at pH 8.45, the R_{sat} factor was so weak that corrections were neglected. Table I summarizes the effect of the pH on the thermodynamic parameters of the reaction.

Discussion

Despite knowledge of structural data concerning *Desulfovibrio desulfuricans* Norway cytochrome c_3 and ferredoxin I, the role of the four hemes in cytochrome c_3 and the physiological purpose of the ferredoxin dimerization are not yet understood. The two metabolic pathways (reduction of sulfate and oxidation of pyruvate) are coupled at the cytochrome c_3–ferredoxin complex level. A bidirectional electron exchange has been described in this complex, and it is essential to determine the number of interacting sites participating in these reactions. The present results argue unambiguously for a stoichiometry of 1/1 (one molecule of cytochrome c_3 per monomer of ferredoxin I). The establishment of this stoichiometry reveals the reactivity of the ferredoxin monomer

Table I: pH Dependence of the Thermodynamic Parameters of Cytochrome c_3–Ferredoxin I Complex[a]

buffer	pH	K_A (M^{-1})	ΔH_{app} ($kJ \cdot mol^{-1}$)	ΔH ($kJ \cdot mol^{-1}$)	ΔG ($kJ \cdot mol^{-1}$)	ΔS ($J \cdot K^{-1} \cdot mol^{-1}$)
phosphate	6.60	2.1×10^7	16.5 ± 1.0	22 ± 1	−39.6	217
Tris-HCl	6.60	2.0×10^7	−8.0 ± 1.0	24 ± 1	−39.5	224
	7.70	1.3×10^6	14.2 ± 1.0	19 ± 1	−33.0	183
	8.45	3.0×10^5	16.0 ± 1.0	16 ± 1	−29.6	161

[a] Each value is the mean of two different calorimetric experiments performed in phosphate or Tris-HCl buffer (10^{-2} M), at 283 K. The ΔH corrections have been done with the R_{sat} value obtained from pH-stat measurements ($R_{sat} = 0.68$). The confidence intervals for ΔH_{app} values were calculated from the variances of the linear regression coefficients (Coassolo et al., 1980). We have given the same confidence intervals for ΔH_{app} and ΔH because R_{sat} value confidence intervals cannot be significantly established.

and the presence of a high-affinity site on the cytochrome c_3. The three other hemes would be involved in the interaction with other partners of the electron-transfer chain (hydrogenase or flavodoxin), assuming an intramolecular electron exchange in the molecule. The enthalpic and potentiometric titration measurements are in good agreement with preliminary ^1H NMR data of the complex (Guerlesquin et al., 1985b). From NMR experiments the two highest potential hemes (−165 and −305 mV) seem to be more affected by the complex formation. However, the association constant value determined by NMR titration ($K_A = 10^4\ M^{-1}$) was smaller than the value we have obtained in the present work. The protein concentration used for NMR experiments (10^{-3} M) in the case of ferredoxin I (pI = 3.9) provides a high ionic strength, decreasing the complex affinity.

The low positive enthalpy change value, and the fact that the complex is entropy driven, could suggest a possible hydrophobic contribution. However, the large effect of the ionic strength on the protein association unambiguously shows the electrostatic interaction to be involved in the protein recognition.

Our thermodynamic results are comparable to those reported by Mauk et al. (1982) on cytochrome c–cytochrome b_5 complex. The weak enthalpy change, ΔH, and the large positive value of ΔS indicate an entropy-driven effect, which would be compatible with the Chothia and Janin (1975) model. However, the marked ionic strength effect demonstrates that Coulombic forces make the determining contribution to the interaction. It is possible that the large positive entropy changes arise from exclusion of water molecules at the interface, accompanying the Coulombic interactions. The large positive ΔS arising from the loss of water molecules can more than compensate for the negative ΔS due to ionic interactions and to possible van der Waals contacts or hydrogen-bond formation as discussed by Ross and Subramanian (1981).

The fact that the K_A value is significantly increased by proton release implies that the phenomenon is an additional driving force in the interaction. Proton release combined with water molecule exclusion dominates to produce the net increase in the enthalpy and entropy. The identity of the protein and the nature of the proton-dissociating group cannot be determined from our results.

Protein association processes are sometimes accompanied by proton release (Ross & Subramanian, 1981), but the identity of the proton-dissociating groups is poorly described. Perutz et al. (1969) reported an alkaline Bohr effect in hemoglobin, ascribed to a decrease in pK of conjugate bases from 7.7 in deoxyhemoglobin to 6.2 in oxyhemoglobin. By analogy in the cytochrome c_3–ferredoxin I complex, the pK shift from 7.35 to 6.05 associated with the proton release could possibly be assigned to either histidine-9 in ferredoxin I or one of the α-NH_2 groups of cytochrome c_3 (alanine-1) or ferredoxin I (threonine-1). On the other hand, as described by Mathews (1985), heme propionate groups of *Pseudomonas aeruginosa* cytochrome c-551 present a pK shift (6.2 to 7.3) between the oxidized and reduced forms. A possible role of heme propionates in the binding site must also be considered when describing this proton release.

Further structural studies by construction of models with computer-aided graphics or covalent cross-linking of cytochrome c_3-ferredoxin I complex would enable a better interpretation of the thermodynamic parameters of the complex.

ACKNOWLEDGMENTS

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APPENDIX

pH-Stat Titration Method to Determine Affinity Constant for Biomolecular Complex (AB) Associated to a pK Shift. Let $[A^-]$ and [AH] be the concentrations of nonprotonated and protonated species that are going to bind to the B species and $[BA^-]$ and [BAH] be the concentration of the corresponding complexed forms; x is the total complex concentration expressed by $x = [A^-] + [AH]$ or $x = [BA^-] + [BAH]$. $[A_t]$ and $[B_t]$ are respectively the total concentrations of the A and B species. If the acidic dissociation constant $K_1 = [A^-][H^+]/[BAH]$ is different from $K_2 = [BA^-][H^+]/[BAH]$, the complex formation reaction would be associated with the proton exchange in the reaction medium.

The absolute value of exchanged proton concentration (Φ) could be written as

$$\Phi = [A^-] - [BA^-] = [AH] - [BAH] = \left(\frac{1}{1 + 10^{pK_1 - pH}} - \frac{1}{1 + 10^{pK_2 - pH}}\right)x$$

or

$$\Phi = R_{sat}x \tag{3}$$

with

$$R_{sat} = \frac{1}{1 + 10^{pK_1 - pH}} - \frac{1}{1 + 10^{pK_2 - pH}} \tag{4}$$

R_{sat} is the quantity of exchanged protons per mole of complex formed. The apparent association constant of the complex AB can be written as

$$K_A = \frac{x}{([A_t] - x)([B_t] - x)} \tag{5}$$

From the relationships 3 and 5 we can calculate

$$\Phi = R_{sat} \times \frac{\frac{1}{K} + [B_t] + [A_t] - \sqrt{(1/K + [B_t] + [A_t])^2 - 4[A_t][B_t]}}{2}$$

and from the titration curves $\Phi = f([A_t])$ or $R = \Phi/[B_t] = f([A_t]/[B_t])$. With $[B_t]$ constant, it is then possible to estimate K_A and R_{sat} values (Caossolo et al., 1980), like for calorimetric measurements, and to check the complex stoichiometry.

For each pH value, the corresponding R_{sat} value enables K_2 to be calculated if K_1 is known. Moreover, if K_1 and K_2 are

unknown, it is still possible to calculate these values from the curve $R_{sat} = f(pH)$ (relationship 4), as the curve is symmetrical with a maximum value at pH = $(pK_1 + pK_2)/2$. From this pH value and the corresponding R_{sat} value, pK_1 and pK_2 can be established.

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Purification and Characterization of a Tissue Plasminogen Activator–Inhibitor Complex from Human Umbilical Vein Endothelial Cell Conditioned Medium

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ABSTRACT: Tissue plasminogen activator–inhibitor complexes were purified from the conditioned medium of human umbilical vein endothelial cells by affinity chromatography followed by gel filtration. It was found that a single complex was isolated which can exist in two distinct interconvertible conformations. These may be separated by electrophoresis into a form with a 105 000 apparent molecular weight and a form with an 88 000 apparent molecular weight. The particular conformation which predominates may be altered by changing the pH at which preparations are incubated or by including dithiothreitol in incubation buffers. Plasminogen activator enzymatic activity may be partially recovered from purified complexes by incubation in the presence of fibrin. Incubation in 1.5 M NH_4OH results in the dissociation of the complex into two major polypeptides of 67 and 40 kilodaltons (kDa). The 40-kDa protein was isolated by gel filtration high-pressure liquid chromatography. N-Terminal amino acid analysis of this protein revealed three distinct sequences. Two of these were nearly identical and matched the N-terminal sequence recently reported for the native plasminogen activator inhibitor from endothelial cells. The third sequence exactly matched an internal portion of the same protein. The results suggest that the internal sequence is located at the site where the inhibitor is cleaved by tissue plasminogen activator.

During the last few years, evidence has accumulated which indicates that there are specific inhibitors which modulate the activity of the plasminogen activator serine proteases in vivo (Ogston & Walker, 1980; Korninger et al., 1985; Wiman et al., 1984a; Kruithof et al., 1984; Verheijen et al., 1984). Deviations from normal plasma levels of plasminogen activator

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