Proton Linkage of Complex Formation between Cytochrome *c* and Cytochrome b_5: Electrostatic Consequences of Protein–Protein Interactions†

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ABSTRACT: Two potentiometric methods have been used to study the pH-dependent changes in proton binding that accompany complex formation between cytochrome *c* and cytochrome b_5. With one method, the number of protons bound or released upon addition of one cytochrome to the other has been measured as a function of pH. The results from these studies are correlated with the complexation-induced difference titration curve calculated from the titration curves of the preformed complex and of the individual proteins. Both methods demonstrate that complex formation at acid pH is accompanied by proton release, that complex formation at basic pH is accompanied by proton uptake, and that the change in proton binding at neutral pH, where stability of complex formation is maximal, is relatively small. Under all conditions studied, the stoichiometry of cytochrome *c*–cytochrome b_5 complex formation is 1:1 with no evidence of higher order complex formation. Although the dependence of complex formation on pH for interaction between different species of cytochrome *c* and cytochrome b_5 are qualitatively similar, they are quantitatively different. In particular, complex formation between yeast iso-1-cytochrome *c* and lipase-solubilized bovine cytochrome b_5 occurs with a stability constant that is 10-fold greater than observed for the other two pairs of proteins under all conditions studied. Interaction between these two proteins is also significantly less dependent on ionic strength than observed for complexes formed by horse heart cytochrome *c* with either form of cytochrome b_5. This study establishes a role for detailed potentiometric analysis of complex formation between electron transfer proteins that interact in a pH-dependent fashion in providing otherwise inaccessible mechanistic insight concerning proton-linked complex formation.

The electrostatically stabilized complex formed between cytochrome *c* and cytochrome b_5 represents one of the simplest systems for the study of interprotein electron transfer, and as such it has been the subject of considerable experimental (Ng et al., 1977; Stonehuerner et al., 1979; Mauk et al., 1982; 1986; Eley & Moore, 1983; McLendon & Miller, 1984; Holloway & Mantsch, 1988; Kornblatt et al., 1988; Rodgers et al., 1988; Burch et al., 1990; Whitford et al., 1990) and theoretical (Salemme, 1976; Mauk et al., 1986; Wendoloski et al., 1987) investigation. Despite significant advances in our understanding of long-range electron transfer in biological systems (McLendon, 1988; Gray & Malmström, 1989), many factors controlling protein–protein electron transfer remain ill defined. Although recent attention has focused upon the role of the protein–protein interface in the attenuation of electron transfer rates (Peery & Kostić, 1989; Hazzard et al., 1989; Everest et al., 1991; Nocek et al., 1991), no detailed structural information is available concerning the exact interfacial contacts between this or any other pair of electron transfer proteins. The involvement of charged residues in the specificity and stabilization of electrostatic complexes between a variety of electron transfer proteins is well documented (Kostić, 1991), though little is known about the pH dependence of these reactions. This deficiency is surprising because the electrostatic potential surface of a protein is defined by the protonation state of its constitutent titratable groups [for recent studies concerning this relationship, see Wendoloski and Matthew (1989) and Northrup et al. (1990)]. Although no detailed structural characterization of a complex formed by such proteins is available, much effort has gone into evaluating general features of model structures proposed on the basis of crystallographic information and electrostatic calculations concerning the individual components of the complex. The specificity of their binding comes mainly from electrostatic complementarity, while the driving force for binding comes mainly from solvent exclusion at the interface and close packing of side chains within the complex (Mauk et al., 1982; Fisher et al., 1986; Kornblatt et al., 1988; Chothia & Janin, 1990).

For a pH-dependent associative process, there is by definition a pH range over which association is accompanied by the uptake or release of protons that results from the change in the ionization state of one or more ionizable groups upon complexation. This complex-formation-linked change in proton binding arises from perturbation of the electrostatic environment of those ionizable groups. The protons taken up or released upon formation of a complex reflect the difference in net proton charge between the free unbound molecules and the associated bound complex. Therefore, the pH dependence of complex-formation-linked proton binding is equivalent to the difference proton titration curve between bound and unbound states. Embodied in this curve is information concerning the pK_as of the groups that are affected by complex formation as demonstrated for protein–small molecule complexes (D'Albis & Bêchet, 1967; Rupley et al., 1967; Glick, 1968; Valenzula & Bender, 1970; Parsons & Raftery, 1972; Folgel & Biltonin, 1975), protein–metal ion complexes (Coleman & Vallee, 1961), and the oxygenation of hemoglobin (the Bohr effect) (Wyman & Gill, 1990, and references cited therein). In these cases the relatively simple difference curves could be described adequately by a small number (1–3) of binding-linked titratable groups.

Ross and Subramanian (1981) have discussed the effect of proton release on the thermodynamics of protein–protein association, but apart from the trypsin–trypsin inhibitor complex

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(Lebowitz & Laskowski, 1962; Laskowski & Finkenstadt, 1972; Yung & Trowbridge, 1980) little quantitative information is available concerning complex-formation-linked changes in protonation states, especially with regard to the complexes formed between highly charged electron transfer proteins. For example, an electrophoretic technique has been used to detect interaction between cytochrome b_5 and other proteins (Righetti et al., 1978; Livingston, et al., 1985). On the other hand, Guerlesquin et al. (1987) have studied the cytochrome c_3–ferredoxin complex by both potentiometry and calorimetry and suggested that the proton release between pH 6.1 and 7.6 was consistent with a shift in pK_a of a single titratable group upon complexation.

As implied above, proton linkage of protein–protein complex formation can be studied by two potentiometric methods. The method developed by Laskowski can be used to measure proton release or uptake that accompanies protein–protein complex formation upon mixing of the two proteins at a particular pH. Alternatively, continuous proton titrations [e.g., Barker et al. (1991)] of a preformed protein–protein complex and of the corresponding individual proteins can be used to determine the complexation-induced difference proton titration curve over a range of pH. The association between cytochrome *c* and cytochrome b_5 is highly pH dependent (Mauk et al., 1982, 1986). Consequently, formation of the cytochrome *c*–cytochrome b_5 complex can be described by eq 1 in which q can

$$\text{cyt } c + \text{cyt } b_5 \rightleftharpoons \text{cyt } c\text{:cyt } b_5 + q\text{H}^+ \qquad (1)$$

be nonstoichiometric and negative.[1]

We have used both potentiometric methods to study the complexation of horse and yeast mitochondrial cytochromes *c* with trypsin-solubilized (Strittmatter & Ozols, 1967; Reid & Mauk, 1982) and lipase-solubilized bovine hepatic microsomal cytochrome b_5 (Strittmatter, et al., 1967). With the Laskowski technique, interaction between various pairs of these proteins has been analyzed to determine complexation-linked proton binding stoichiometry for the first time and to determine association constants over wide ranges of pH and ionic strength. We have used the alternative method of direct, continuous proton titrations (Barker et al., 1991) to obtain the difference proton titration curves originating from complexation for each of the protein pairs. The results from these two approaches are compared and a combination of the two methods shown to be a powerful tool in the study of protein–protein interaction.

Experimental Procedures

Horse heart cytochrome *c* (type VI) was purchased from Sigma Chemical Co. and purified by FPLC cation-exchange chromatography (Pharmacia, Mono-S HR10/10) to remove deamidated and polymeric cytochrome (Mauk & Mauk, 1989). Yeast iso-1-cytochrome *c*, in which the cysteine at amino acid position 102 had been replaced with threonine, was isolated as described previously (Cutler et al., 1987; Rafferty, et al., 1990). Two recombinant forms of bovine hepatic microsomal cytochrome b_5 were expressed in and isolated from *Escherichia coli*. Lipase-solubilized cytochrome b_5 was prepared as described previously (Funk et al., 1990). The gene coding for the tryptic form of the protein had been constructed by deletion of N- and C-terminal sequence from the lipase form (Funk, 1990) and expressed in exactly the same expression system as used for the lipase-solubilized protein (Funk et al., 1990). The trypsin-solubilized form of the recombinant protein has a sequence identical with that of trypsin-solubilized cytochrome b_5 isolated from bovine liver (Reid & Mauk, 1982) and differs from the lipase-solubilized form of the protein by deletion of two residues from the amino terminus and nine from the carboxyl terminus of the protein (Funk et al., 1990). Final purification of these proteins was achieved by FPLC (Pharmacia, Mono-Q HR10/10). Samples of the FPLC-purified proteins were exchanged into glass-distilled, deionized water by centrifugal ultrafiltration in a Centriprep-10 (Amicon). Protein concentrations were determined spectrophotometrically with a Shimadzu 250 or Cary-219 spectrophotometer based on $\epsilon_{409.5} = 106\,100\ M^{-1}\ cm^{-1}$ for ferricytochrome *c* (Margoliash & Frohwirt, 1959) and $\epsilon_{412.5} = 117\,000\ M^{-1}\ cm^{-1}$ for ferricytochrome b_5 (Ozols & Strittmatter, 1964) (0.02 M phosphate, pH 7.2, 25 °C).

Titrations and pH measurements were performed with a Radiometer ABU93 Triburette that was equipped with three 1-mL burettes and a SAM90 sample station housed within an aluminum Faraday cage. A glass electrode (Radiometer G222C) and a double-junction calomel reference electrode (Radiometer K701) were calibrated at 25 °C with standards at pH 4 (Radiometer S1316), pH 7 (Radiometer S1336), and pH 10 (BDH RO1206-74). The lower compartment of the double-junction reference electrode was filled with 0.1 M KCl to avoid an increase in ionic strength during the course of the experiment resulting from leakage of salt from the reference solution. All measurements were made with maximal stirring speed. The autoburette was interfaced to a microcomputer for software control of titrator operation and data acquisition. Stock solutions of base were standardized against potassium acid phthalate (primary standard, Aldrich) and used to standardize stock solutions of acid. Least-squares analyses of data simulations were performed with the programs FITALL (version 4.0, MTR Software, Toronto) and MINSQ (version 3, MicroMath Scientific Software).

Protein–Protein Titrations (Laskowski Method). The four solutions required for each experiment were the titrand protein, the titrant protein, and dilute, standardized acid and base for the titration of protons taken up or released upon addition of the titrant to the titrand. Titrations were performed with either the total amount of ferricytochrome *c* held constant and titrated with ferricytochrome b_5 or vice versa. In either case, approximately 30 mg of the titrant protein was deionized by passage over 1.0 mL of MB-3 mixed bed ion-exchange resin (Sigma A-7518) and collected into a sealed bottle under an argon atmosphere. Approximately 3 mg of the titrand protein was passed over 0.4 mL of the same resin and collected in a similar fashion. When ferricytochrome b_5 is treated in this manner, the protein must immediately be returned to neutral pH from the isoionic pH at which it emerges from the ion-exchange resin to avoid precipitation and denaturation of the protein. Sodium chloride (1 M) was then added to achieve the required ionic strength.

Approximately 6.5 mL (the exact volume for each titration was determined gravimetrically) of the titrant solution (0.3–0.7 mM) was transferred into the sealed, glass titration vessel that was continuously flushed with humidified argon and maintained at 25 ± 0.1 °C by a circulating water bath (RMS-6, Lauda). Traces of CO_2 were removed from the gas stream by passage over KOH pellets and through NaOH solution (4 M) followed by passage through 1 M sodium phosphate and

[1] The rigorous description of eq 1 and derivation of eq 3 refer to a standard state in which all species are fully protonated and require the introduction of Δc as the number of new cationic groups created as a consequence of complex formation (Laskowski & Finkenstadt, 1972). We have chosen to simplify our description of the theory by not referring to this standard state so that Δc is implicit in our description of q.

finally through deionized water that was maintained at 23 ± 2 °C. The pH of the titrant solution was adjusted to approximately the desired value and then allowed to stabilize. Adjustments of pH were made with dilute, CO_2-free HCl and NaOH with NaCl added to maintain the ionic strength of the medium if required. All protein concentrations were corrected for the change in volume resulting from this pH adjustment. Once the pH was stable, this protein was transferred anaerobically to a sealed bottle that was flushed continuously with argon and loaded into one of the burettes. Approximately 2.5 mL of the titrand protein solution (40–80 nmol) was treated in the same manner to adjust its pH to exactly the same value as the titrant protein solution. When the pH of this solution was stable, the titration was started.

Titrations were carried out under computer control in an interactive manner. After each volume of titrant protein was added, the pH of the protein mixture was adjusted to its initial value with either 0.2 mM HCl or NaOH. The pH change that occurred on addition of the titrant protein was generally complete in 2–3 min. A titration curve consisted of 30–50 points that corresponded to the volume of acid or base required to maintain the pH at its starting value versus the volume of the titrant protein added at each point. These data were converted to mole fraction of exchanged protons, H_e^+, versus the ratio of protein concentrations for nonlinear regression analysis fit to eq 2 to obtain the association constant, K_A, and saturating value of H_e^+, q:

$$H_e^+ = \frac{q}{2C}\left[\left(RC + C + \frac{1}{K_A}\right) - \sqrt{\left(RC + C + \frac{1}{K_A}\right)^2 - (4RC)}\right] \quad (2)$$

where C is the concentration of titrant protein at a 1:1 ratio and R is the molar ratio of titrant to titrand. Over certain pH ranges (5.7–6.2 and 7.1–8.5), the saturation binding curves required correction for changes in measured pH upon the addition of the titrant protein to salt solution alone.

Lebowitz and Laskowski (1962) were the first to use potentiometric measurements for quantitative determination of the difference titration curve for a protein–protein complex and to relate it to the pH dependence of the association constant, K_A. It can be shown (Edsall & Wyman, 1958; Laskowski & Finkenstadt, 1972; Wyman & Gill, 1990) that

$$\text{d log } K_A/\text{d pH} = q \quad (3)$$

This equation can be integrated readily to yield

$$(\log K_A)_2 = (\log K_A)_1 + \int_{pH_1}^{pH_2} q \, \text{dpH} \quad (4)$$

which allows K_A to be evaluated at any pH provided that K_A is known at one reference pH and that the difference titration curve is known over the region between pH_1 and pH_2. No specific information concerning the origins of the difference curve is required to make use of this relationship (Lebowitz & Laskowski, 1962; Rupley et al., 1967).

Continuous Proton Titrations. These experiments were carried out as previously described (Barker et al., 1991) with the following modifications. The ionic strength of the medium was adjusted to 0.01 M with NaCl, and the titrants used were CO_2-free 0.01 M HCl or NaOH. The reference electrode was the double-junction electrode described above. The individual proteins were exchanged into 0.01 M NaCl (pH 6.5) by ultrafiltration (Centriprep-10, Amicon).

For each pair of proteins, cytochrome solutions of known concentration were mixed to give a solution containing a defined ratio of the proteins. The final concentration of ferricytochrome b_5 was between 75 and 100 μM while the total ferricytochrome c concentration used was 200–450 μM depending on the particular pair of proteins studied. Approximately 2.5 mL (the exact volume for each titration was determined gravimetrically) of this solution was transferred to the titration vessel. After the pH of this solution had stabilized, it was titrated to pH 5.7 followed by titration to pH 8.5 and then back to pH 5.7. Each protein was also titrated alone in exactly the same manner. The association constants determined from the protein–protein titrations established that a 1:1 mixture of the two proteins would not ensure quantitative cytochrome c–cytochrome b_5 complex formation over the range of pH studied. Therefore, we performed the continuous proton titrations on mixtures of each protein pair with one of the proteins in significant excess in an attempt to assure quantitative complexation of the other protein over the entire titration range. Consequently, the raw data are the sum of the titration of the complex plus the titration of the excess component. For each protein pair, cytochrome c was added in excess because it has fewer titratable groups in the pH range 5.7–8.5 than does cytochrome b_5, which leads to less dilution of the proteins during the experiment.

Titrations were performed under computer control as described previously (Barker et al., 1991), and the raw data were corrected for the titration of an identical volume of 0.01 M NaCl before conversion to proton equivalents vs pH. For the proteins titrated individually, these data were further converted to net proton charge ($\bar{Z}$) vs pH. The following relationship can then be used to obtain the net proton charge ($\bar{Z}_{complex}$) for the 1:1 complex.

$$\bar{Z}_{complex} = \left[\frac{H_{mix} - (\bar{Z}_{cytc} \times M_{cytc})}{M_{complex}}\right] \quad (5)$$

where H_{mix} is the total number of proton equivalents titrated in the mixture of proteins, $\bar{Z}_{cytc}$ is the net proton charge of unbound cytochrome c, M_{cytc} is the moles of *excess* cytochrome c present in the mixture, and $M_{complex}$ is the moles of complex present in the mixture (equal to the moles of cytochrome b_5). The difference titration curve ($\Delta\bar{Z}$ vs pH) between bound and unbound cytochromes is simply described by

$$\Delta\bar{Z} = \bar{Z}_{complex} - \bar{Z}_{cytc} - \bar{Z}_{cyt b_5} = -q \quad (6)$$

where $\bar{Z}_{cyt b_5}$ is the net proton charge of unbound ferricytochrome b_5.

Results

Protein–Protein Titrations. For the interaction between lipase-solubilized ferricytochrome b_5 and horse heart ferricytochrome c, the effect of pH on the exchange of protons (H_e^+) between protein and solution is depicted in Figure 1. Data obtained from the other pairs of proteins are qualitatively similar. Below pH 7.3, the reaction is characterized by a release of protons to the medium, while above pH 7.7 complexation results in the uptake of protons from the medium. The range of pH available for study is limited by the stability of cytochrome b_5 below pH 5.7 and cytochrome c above pH 9. These titration data can all be analyzed satisfactorily by nonlinear least-squares fitting to eq 2, which assumes a 1:1 binding stoichiometry. This analysis determines the best value for both the association constant, K_A, and for the saturating value of H_e^+, q, the stoichiometric number of protons ex-

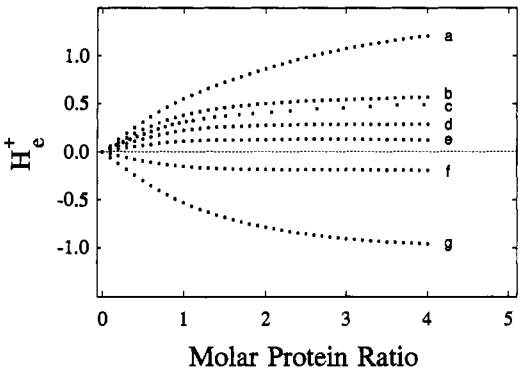

FIGURE 1: Effect of pH upon proton release during the titration of horse heart ferricytochrome *c* with lipase ferricytochrome b_5 (curves a, c, d, e, and g) or vice versa (curves b and f) at an ionic strength of 0.01 M (KCl). The pH of each experiment was (a) 5.77, (b) 6.35, (c) 6.60, (d) 7.10, (e) 7.27, (f) 8.04, and (g) 8.47. H_e^+ is the number of protons released to the medium per mole of complex formed. A negative value of H_e^+ means protons were taken up from the medium. The molar protein ratio is the ratio of the titrant to titrand concentrations (i.e., either [cytochrome b_5]/[cytochrome *c*] or [cytochrome *c*]/[cytochrome b_5]).

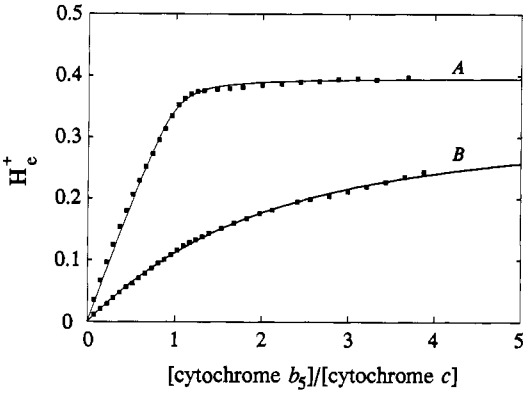

FIGURE 2: Titration of yeast iso-1-ferricytochrome *c* with tryptic ferricytochrome b_5 at pH 6.82. (A) μ = 0.01 M, $[\text{cytochrome } c]_{\text{initial}}$ = 21.12 μM; (B) μ = 0.05 M, $[\text{cytochrome } c]_{\text{initial}}$ = 20.89 μM. The lines are unweighted nonlinear fits to eq 2.

changed per mole of complex formed. Figure 2 shows the best fits to data collected at pH 6.8 and ionic strengths of 0.01 and 0.05 M for the binding of yeast iso-1-cytochrome *c* to tryptic cytochrome b_5. For each pair of proteins, the results were independent of the protein chosen as the titrant. In some experiments, the ratio of [titrant protein] to [titrand protein] was taken as high as 10:1 (data not shown). In no case did the titration data suggest the occurrence of higher order protein–protein complex formation.

The pH dependencies of both *q* and K_A were obtained from data collected at an ionic strength of 0.01 M for each pair of proteins studied here as shown in Figure 3. For all pairs of proteins, little or no proton release or uptake was observed between pH 7.3 and 8 (i.e., *q* was close to zero). In this region, *q* could be determined to ±0.05 (equivalent to about 2.5 nmol of protons at the protein concentrations used), but this small response leads to very large errors in the determination of K_A by curve fitting and precludes the use of these association constants in the data analysis. In addition, for the complexes formed between yeast iso-1-cytochrome *c* and either tryptic or lipase cytochrome b_5 at 0.01 M ionic strength and at the protein concentrations used in these experiments, the stability of complex formation is sufficiently great that all log K_A values greater than ~6.4 give equivalent fits with the same *q* values.

For the case of horse heart cytochrome *c* binding with lipase cytochrome b_5 (Figure 3A), the pH region between 7.3 and 7.9, where there is minimal net change of protonation upon binding, encompasses the region where maximum complex stability is observed as predicted by eq 3. The shapes of the pH dependence of *q* for all three pairs of proteins are qualitatively similar (Figure 3) in that they describe the first derivative with respect to pH of a parabolic curve. The solid lines in the lower panels of Figure 3 represent simulations of proton exchange produced by shifts in the pK_a of titratable groups in either protein and are described by eq 7.[1]

$$q = \sum_{i=1}^{i} [f(pK_a^b)_i - f(pK_a^f)_i] \tag{7a}$$

where

$$f(pK_a) = \frac{10^{(pH-pK_a)}}{10^{(pH-pK_a)} + 1} \tag{7b}$$

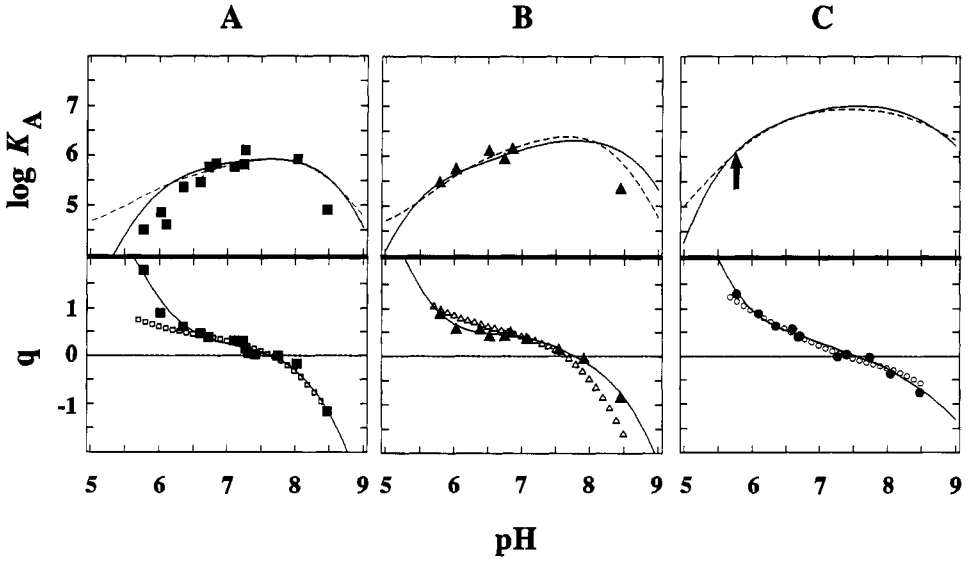

FIGURE 3: pH dependence of *q* and log K_A at an ionic strength of 0.01 M for the association of (A) horse heart ferricytochrome *c* with lipase ferricytochrome b_5; (B) horse heart ferricytochrome *c* with tryptic ferricytochrome b_5; (C) yeast iso-1-ferricytochrome *c* with lipase ferricytochrome b_5. The solid symbols represent data obtained by the protein–protein method, while the data described by the open symbols were obtained by the continuous proton titration of the mixed proteins. The origin of the lines is described in the text. The moles of each protein in the continuous titration experiments is the same as indicated in the legend for Figure 5.

Table I: Parameters Used To Simulate the pH Dependence of Change in Proton Binding That Accompanies Interaction between Cytochrome c and Cytochrome b_5 (Figure 3)[a]

	horse heart cytochrome c + lipase cytochrome b_5			horse heart cytochrome c + tryptic cytochrome b_5			yeast iso-1-cytochrome c + lipase cytochrome b_5		
i	pK_a^f	pK_a^b	no.[b]	pK_a^f	pK_a^b	no.	pK_a^f	pK_a^b	no.
				Protein–Protein Titration Data[c]					
1	5.4	4.7	8	5.0	3.5	6	5.2	3.5	6
2	9.4	11.6	8	9.4	11.5	6	10.0	11.5	6
3	7.6	7.3	1	7.2	6.6	1	6.9	6.4	1
4	8.8	11.3	1	9.0	11.0	1	8.4	11.0	1
				Continuous Proton Titrations[d]					
1	5.9	5.2	2	5.9	5.3	3	5.9	1.0	1
2	8.5	14.0	2	8.5	14.0	3	5.8	4.2	1
3	7.5	7.0	1	7.7	7.0	1	6.6	6.3	1
4	9.1	13.0	1	9.1	13.0	1	8.4	13.0	1

[a] pK_a^f refers to the pK_a of the group(s) in the free protein(s), and pK_a^b refers to the pK_a of the group(s) in the bound protein(s). [b] Values in these columns represent the number of groups given identical parameters. [c] Solid curves in Figure 3. [d] Curves, not shown, through open symbols in Figure 3.

and $(pK_a^f)_i$ and $(pK_a^b)_i$ are the pK_as of titratable group i in free and bound states, respectively. The lines through the log K_A data represent the integrated form of eq 7 and can be described by

$$\log K_A = X + \sum_{i=1}^{i} [f(pK_a^b)_i - f(pK_a^f)_i] \quad (8a)$$

where

$$f(pK_a) = \log [1 + 10^{(pH-pK_a)}] \quad (8b)$$

Equation 8 involves the same parameters as eq 7 with the addition of the integration constant X that is obtained by evaluating at least one point on the log K_A curve experimentally. In Figure 3A, the solid line simulating the pH dependence of log K_A for the horse heart cytochrome c–lipase cytochrome b_5 binding was generated from the same parameters as the solid curve, which provides a reasonable simulation of the pH dependence of q for that pair. The maximum log K_A is calculated to be 5.9 at pH 7.65 (μ = 0.01 M). The association constant log K_A for horse heart cytochrome c binding to tryptic cytochrome b_5 reaches a maximal value of 6.2 at pH 7.8 (μ = 0.01 M). The maximum stability constant for the complex formed by yeast iso-1-cytochrome c and lipase cytochrome b_5 is approximately an order of magnitude greater than that for the complex formed with horse heart cytochrome c but exhibits a similar broad pH maximum centered around pH 7.4. For this pair of proteins, only one measured value for log K_A fell below our cutoff of 6.4, and that was a value of 6.1 obtained at pH 5.77. This value (indicated by the arrow) was used to establish the position of the log K_A curve in Figure 3C, which predicts a maximum value of 7.0 for log K_A (pH 7.4). This maximum is approximately an order of magnitude greater than that for the complex formed by lipase cytochrome b_5 with horse heart cytochrome c. All pairs of proteins exhibit similar broad pH maxima. The values of the parameters used with eqs 7 and 8 to generate the curves in Figure 3 are given in Table I. Both the association constant and q for lipase cytochrome b_5 association with horse or yeast cytochrome c are dependent on ionic strength, as shown in Figure 4. The curves shown in Figure 4 represent unweighted fits of the experimental data to the van Leeuwen (1983) relationship. The protein radii and dipole moments for each protein used in this analysis were as described by Eltis et al. (1991). However, unrealistic values for the charge product for each protein pairing were required to the fit the experimental data to the van Leeuwen equation.[2]

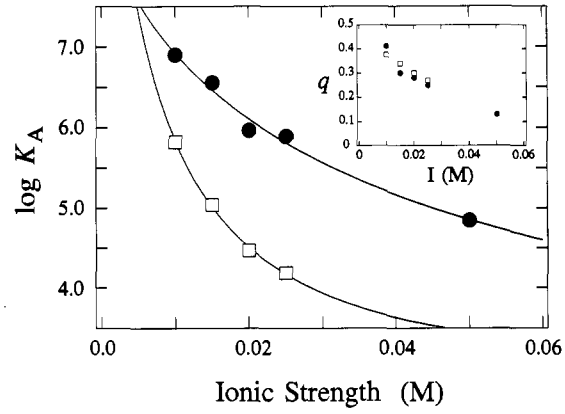

FIGURE 4: Ionic strength dependence of log K_A at pH 6.70 for the association of lipase ferricytochrome b_5 with horse heart ferricytochrome c (□) and yeast iso-1-ferricytochrome c (●) [the stippled circle was calculated from the integral shown in Figure 3C]. The lines are unweighted nonlinear fits to the van Leeuwen equation (1983). (Inset) The ionic strength dependence of q for the same protein pairs. The symbols are the same as above.

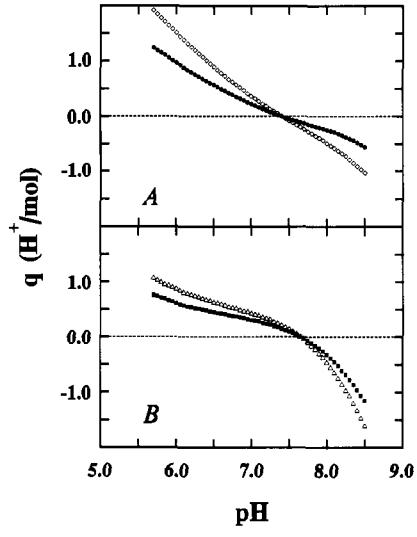

FIGURE 5: Comparison of the difference proton titration curves (q vs pH) between free and complexed proteins, obtained by the continuous proton titration method, for the following protein pairs: (A) yeast iso-1-ferricytochrome c (0.528 μmol) with lipase ferricytochrome b_5 (0.263 μmol) in 2.5225 mL (●) and yeast iso-1-ferricytochrome c (0.466 μmol) with tryptic ferricytochrome b_5 (0.259 μmol) in 2.5114 mL (◇); (B) horse heart ferricytochrome c (1.65 μmol) with lipase ferricytochrome b_5 (0.186 μmol) in 2.5078 mL (■) and horse heart ferricytochrome c (1.08 μmol) with tryptic ferricytochrome b_5 (0.141 μmol) in 2.5053 mL (△).

Continuous Proton Titrations. Titrations of the mixtures of ferricytochrome *c* and ferricytochrome b_5 were reversible within experimental error over the pH range 5.7–8.5. For each protein pair, *q* vs pH was obtained from the relationship given in eq 6. As the point of zero net charge for both proteins and the complexes could not be obtained from these experiments, the absolute position of the *q* vs pH curve with respect to the *q*-axis was determined by optimization of overlap with results generated from the protein–protein titration curves (Figure 3, solid symbols). The continuous curves are described by the open symbols in Figure 3 and are compared in Figure 5. As for the protein–protein titrations, we have used eq 7 to generate curves (not shown) that simulate the *q* vs pH data obtained by this technique. The values of the parameters used for this purpose are also given in Table I. These same values were used in eq 8 to generate the log K_A vs pH curves described by the dashed lines in the top panels of Figure 3. The integration constants were evaluated from the log K_A values obtained from the protein–protein titrations. Only in the case of tryptic cytochrome b_5 binding to horse heart cytochrome *c* was the predicted maximum value for log K_A significantly different when compared to that predicted from the protein–protein titrations. By the continuous method, we calculate that log K_A reaches 6.5 at pH 7.65, compared to 6.2 at pH 7.8 from protein–protein titrations. For the pairing of tryptic cytochrome b_5 and yeast iso-1-cytochrome *c*, a complete *q* vs pH curve has not been measured by the protein–protein method. However, on the basis of a value for log K_A of 6.4 at pH 6.8 (Figure 2, μ = 0.01 M) and the *q* vs pH curve from the continuous titration (Figure 5), log K_A for this complex is predicted to reach a maximal value of 6.9 at pH 7.4.

The values of the parameters used in eq 7 to simulate the *q* vs pH curves obtained by either method do not represent unique solutions to the equation in each case. To the extent possible, we have tried to limit the values used for $i = 3$ and $i = 4$ to describe known properties of some titratable groups on either protein (vide infra). For other *i* values, parameters were varied systematically until reasonable qualitative agreement between experimental and simulated data was obtained. Comparison of the two sets of parameters can therefore only be qualitative and is a first step toward understanding the origins of such curves. This in no way compromises the validity of the integral (log K_A) curves, which are independent of the parameters used to simulate the *q* curves.

Discussion

Proton involvement in protein–protein reactions has been studied for very few pairs of proteins but may serve to have two significant effects upon the interaction of small oppositvely charged electron transfer proteins. The protonation states of the titratable groups contribute to the electrostatic potential surface of each protein and thereby make a major contribution to the exact arrangement and orientation of the protein–protein complex. Additionally, proton exchange with the medium resulting from changes in pK_a upon association contribute unfavorably to the overall thermodynamics (Yung & Trowbridge, 1980; Ross & Subramanian, 1981). Therefore, the pH dependence of these binding reactions provides important mechanistic information.

We have studied the proton linkage of 1:1 cytochrome *c*–cytochrome b_5 complex formation through use of two complementary potentiometric methods that allow us to define the complex-formation-linked proton difference titration curve and thereby deduce the dependence on pH of K_A and proton binding stoichiometry (*q*) for this interaction. We find no evidence of higher order complex formation as reported previously (Miura et al., 1980; Whitford et al., 1991) on the basis of NMR studies employing much higher protein concentrations. One property of the system under study in this report is that at maximum stability of the complex the net change in proton binding that accompanies complex formation is minimal. However, complex formation over this pH range can be evaluated if it is considered as part of a broader collection of data spanning a wide pH range. With these data, the association constant at maximum stability can be calculated as demonstrated by our analysis of the results shown in Figure 3. Qualitatively, the shape of the pH dependence of log K_A determined experimentally is similar to that predicted from the pH dependence of *q*. The relative maximal stabilities of complexes formed between the pairs of proteins studied here follows the following order: yeast iso-1-cytochrome *c*/lipase cytochrome $b_5 \simeq$ yeast iso-1-cytochrome *c*/tryptic cytochrome $b_5 >$ horse heart cytochrome *c*/tryptic cytochrome $b_5 >$ horse heart cytochrome *c*/lipase cytochrome b_5. The greater stability of complexes formed by yeast cytochrome *c* relative to those formed by the horse heart protein is difficult to rationalize solely on the basis of electrostatic considerations insofar as the distribution of surface charges on the yeast protein are similar to those of the horse heart cytochrome. Consequently, it seems likely that stabilizing hydrophobic interactions may be of greater importance in complexes formed by yeast cytochrome *c*. One likely reason for the difference between the binding behavior of the cytochrome b_5 proteins is the C-terminal extension on the lipase form that interacts with the acidic electrostatic binding surface of the protein (Funk et al., 1990).

The strong ionic strength dependence of K_A (Figure 4) is consistent with electrostatic stabilization of complex formation between oppositely charged reactants. As indicated previously, the difference in behavior observed for the yeast and horse heart cytochromes is not readily explained by differences in the charged surface residues of the two proteins and probably involves a greater contribution of hydrophobic interactions to the stabilization of complexes formed by the yeast cytochrome. The inability of the van Leeuwen analysis to fit the data to

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[2] The van Leeuwen relationship is defined by

$$\ln k = \ln k_\infty - [Z_1Z_2 + ZP(1 + \kappa R) + PP(1 + \kappa R)^2][q^2/4\pi\epsilon_0\epsilon k_B TR]f(\kappa)$$

where

$$ZP = \frac{Z_1P_2 + Z_2P_1}{qR}$$

$$f(\kappa) = \frac{1 - e^{-2\kappa R_2}}{2\kappa R_2(1 + \kappa R_1)}$$

and where $R = R_1 + R_2$, $PP = P_1P_2/(qR)^2$, $\kappa = 0.329(I)^{1/2}$, Z_1 and Z_2 are the monopole charges on ferricytochrome *c* and ferrocytochrome b_5, R_1 and R_2 are the radii of the two proteins, *q* is the elementary charge, ϵ_0 is the dielectric permittivity, ϵ is the dielectric constant of water, k_B is Boltzmann's constant, and P_1 and P_2 are the components of the dipoles of the proteins through their respective exposed heme edges. For cytochrome b_5, the heme edge was taken to be the γ methine carbon, and in cytochrome *c* the heme edge was taken to be the methyl carbon of the heme pyrrole most exposed to solvent. To generate the curves shown in Figure 4, the net protein electrostatic (monopolar) charges required for each protein were −1.5 and 130 for lipase cytochrome b_5 and horse heart cytochrome *c*, respectively, and −71 and 0.2 for lipase cytochrome b_5 and yeast iso-1-cytochrome *c*, respectively. The parameters used for cytochrome b_5 and horse heart cytochrome *c* were described previously (Eltis et al., 1991). For yeast iso-1-ferricytochrome *c*, P_1 = 455 (S. H. Northrup, personal communication).

known parameters[2] may reflect the narrow ionic strength range of these experiments. Alternatively, this relatively simple relationship, in which the Debye–Hückel theory is modified by both monopolar and dipolar interactions, is not applicable to this system. This finding is consistent with our previous observations concerning the kinetics of electron transfer between these two proteins (Eltis et al., 1991). We note that van Leeuwen (1983) obtained reasonable parameters from analysis of our previous binding data (Mauk et al., 1982). However, these previous data are not quantitatively reproduced by our current measurements (vide infra), and the sequence of cytochrome b_5 on which his electrostatics calculations were based is now known to have been incorrect (Cristiano & Steggles, 1989).

Comparison of the q vs pH curves obtained by each technique (Figure 3) shows that over most of the range of pH studied here there is good agreement between continuous and discontinuous measurements of q. However, *absolute* evaluation of this curve by the continuous method requires at least one, and preferably more, values of q obtained from the protein–protein titrations. The largest discrepancy between the two methods is observed below pH 6.2 for the complexation between horse heart cytochrome c and lipase cytochrome b_5 (Figure 3A, lower panel). Under these conditions, these two proteins associate with the lowest affinity observed (Figure 3A, upper panel) in this study, and we therefore suggest that the cytochrome b_5 was not fully associated with cytochrome c in this region of the continuous titration experiment. The concentration of cytochrome c and/or the total protein concentration required to achieve quantitative complex formation over the entire pH range for these cases is greater than can be accommodated by this experimental technique. We conclude, therefore, that these pairs of proteins define the lower limit of complex stability for which the continuous titration method can be reasonably useful.

Although the dependence of K_A on pH is qualitatively similar for each pair of proteins studied here, more quantitative comparison reveals subtle differences between them (Figure 5). For example, the complexation-induced difference proton titration curves shown in Figure 5B for complexes formed by horse heart cytochrome c are relatively independent of the identity of the cytochrome b_5 with which it interacts compared to the corresponding titration curves determined for yeast iso-1-cytochrome c. As noted above, the difference observed between the two titration curves at acid pH in Figure 5B is even smaller if the results from protein–protein titrations are considered (Figure 3A,B). In addition, complexes formed by horse heart cytochrome c exhibit greater proton release upon binding either cytochrome b_5 between pH 6.8 and 7.6 (vide infra).

The changes in pK_a values responsible for the results shown in Figure 3 result from changes in the dielectric of titratable groups on the interacting proteins. Three mechanisms by which these dielectric perturbations may arise can be envisioned. Clearly, those residues directly involved in salt-bridge formation undergo significant changes in their dielectric environment. In addition, residues adjacent to the protein–protein interface but not directly involved in salt-bridge formation or more distant residues (Russell & Fersht, 1987) can undergo binding-linked pK_a shifts through perturbation of the dielectric constant of their environment. Finally, residues remote from the protein–protein interface could undergo pK_a shifts as the result of binding-induced conformational changes. The amino acid residues responsible for the pH dependence of q, K_A, and the differences in binding behavior exhibited by different species or derivatives of the cytochromes studied here cannot be described definitively without additional information.

To explain the origin of the proton release and uptake, we refer to information currently available concerning the individual proteins and the complexes that they form with each other, and we suggest that all three mechanisms described above can be observed in this system. There are a total of five surface histidines in horse heart cytochrome c and bovine cytochrome b_5. Yeast iso-1-cytochrome c possesses one additional histidyl residue. In an attempt to assess the effect of electrostatics in the cytochrome c–cytochrome b_5 system, electrostatic calculations were performed (Mauk et al., 1986) with model complex structures (Salemme, 1976) to establish the effect of complexation on the titratable groups. This analysis predicted that the pK_a of His-26 on cytochrome b_5 is decreased by complexation with cytochrome c, that the protonation state of His-26 influences the exact structure of the complex, and that more than one cytochrome c–cytochrome b_5 complex occurs in solution, the distribution between which is a function of solution conditions (viz., pH, ionic strength, and temperature). The change in the protonation state of His-26 of cytochrome b_5 is assigned to the shifts in pK_a around neutrality (Table I, $i = 3$) that are required to simulate the positive values of q between pH 6.5 and 7.5 for cytochrome b_5 binding to horse heart cytochrome c. It is also possible that the proton release in the region of pH 6–6.5 is due to a shift in the pK_a of one (or more) of the histidines on cytochrome c, although this has not been explicitly included in the modeling. Notably, His-33 has a pK_a of 6.6 in unbound horse heart cytochrome c (Cohen & Hayes, 1974). This residue has a similar value in the yeast protein as does His-39 (Cutler et al., 1989). The other two histidines on cytochrome b_5 are not predicted to be affected by the electrostatic field generated by cytochrome c (Mauk et al., 1986). Systematic assignment of the histidyl residues responsible for these effects will be undertaken through characterization of appropriate variant cytochromes constructed by site-directed mutagenesis.

We ascribe the large values of q observed and predicted at the extremes of pH in these experiments to deprotonation and protonation of the carboxylates and lysines, respectively, that are believed to participate in salt-bridge formation during association. Although the pK_as of these groups are well outside of the range of pH described here, calculations suggest that when several (4–6) of each type of titratable group are involved in the electrostatic interaction, their combined pK_a shifts affect each end of the region of pH range studied. However, some of the pK_as and numbers of groups required (Table I, $i = 1$ and 2) to simulate the significant proton exchange observed at the high and low pH are outside the bounds of currently accepted values for the pK_as of these titratable groups (Edsall & Wyman, 1958; Tanford, 1964) and for the numbers of groups proposed to be involved in salt-bridge formation (Salemme, 1976; Mauk et al., 1986; Hollaway & Mantsch, 1988; Kornblatt et al., 1988; Rodgers et al., 1988; Burch et al., 1990).

An effect of ionic strength on q for the binding of lipase-solubilized cytochrome b_5 to both horse and yeast cytochrome c is expected as many of the pK_as of groups involved are dependent on ionic strength. In particular, we note that the pK_a of cytochrome b_5 residue His-26 that we have implicated above as undergoing a significant binding-induced pK_a shift is also predicted to be acutely sensitive to ionic strength (Mauk et al., 1986; Altman et al., 1989).

The two mitochondrial cytochromes c studied here undergo a conformational change that is linked to the deprotonation of a group at pH 11 (Davis et al., 1975; Pearce et al., 1989).

For the yeast cytochrome, this pH-linked conformational change occurs with an apparent pK_a of 8.5. Therefore, above pH 8.0, a significant fraction of this protein exists in the alkaline conformational state. However, the apparent pK_a for the corresponding conformational transition of the horse heart protein is 9.1, so the influence of the alkaline conformational change is expected to be mostly outside of the range of this study. If cytochrome b_5 binds differentially to the native and alkaline forms, the resulting shift in conformational equilibrium will result in proton uptake or release. We observe that the formation of complexes involving either horse heart or yeast iso-1-cytochrome *c* and cytochrome b_5 above pH 8 results in proton uptake. Although the proton uptake occurring for complexes involving yeast cytochrome *c* is consistent with preferential binding of cytochrome b_5 to the native conformation of cytochrome *c*, the proton uptake is greater for the complexes involving the horse heart protein. This appaernt contradiction could be resolved in two ways. One possibility is that the native conformation of yeast cytochrome *c* binds preferentially to cytochrome b_5 and that some other mechanism is responsible for the proton release observed for binding of cytochrome b_5 to horse heart cytochrome *c*. Alternatively, an additional proton-uptake process, possibly involving lysyl residues (vide supra), occurs in the binding of both species of cytochrome *c* to cytochrome b_5, and this is attenuated in the case of yeast cytochrome *c* by the preferential binding of the alkaline conformation to cytochrome b_5, which would be a proton-releasing phenomenon. The relative simplicity of the second mechanism leads us to prefer it in the absence of additional information. The availability of cytochrome *c* variants with significantly altered alkaline pK_a values (Pearce et al., 1989) provides an avenue for future clarification of this issue.

The current results are similar to but not quantitatively identical with our previous results in which cytochrome *c*–cytochrome b_5 complex formation was monitored by electronic difference spectroscopy. The advantages provided by the potentiometric method include greatly improved precision and sensitivity. As a result, the range of pH and ionic strength over which binding can be measured is much greater with the potentiometric method. The potentiometric results concerning complex formation between horse heart cytochrome *c* and trypsin-solubilized cytochrome b_5 differ from results based on electronic difference spectroscopy in that the maximum log K_A observed is 0.5 (protein–protein titration) to 0.8 (continuous titration) greater than that obtained optically, and the optimal pH for complex formation is found to be 0.3 (continuous titration) to 0.5 (protein–protein titration) pH units greater than obtained optically. These differences may result from the relatively great uncertainty inherent in the spectroscopic measurements. We note that these two experimental techniques may differ in their ability to detect various putative forms of the cytochrome *c*–cytochrome b_5 complex. It seems reasonable to expect, for example, that the electronic difference spectrum produced by complex formation between these two proteins may not be equivalent in line shape or intensity for all forms of the complex that occur in solution as a function of pH. Nevertheless, as the putative orientational variants of 1:1 complex that may be present in solution are presumably in equilibrium with each other, this differential sensitivity of the difference spectrum should not in itself compromise the validity of stability constants determined by this spectroscopic method. Finally, aside from the unique mechanistic information the potentiometric technique provides concerning complexation-linked proton binding, this method also permits assessment of protein–protein association at much higher protein/protein ratios than can be studied effectively by any spectroscopic technique.

Conclusions

Formation of 1:1 complexes between cytochrome *c* and cytochrome b_5 is linked to proton binding to one or both of the interacting proteins. The complex formed by the two proteins exhibits maximum stability at a pH where there is minimal net change in protonation of the proteins. While unequivocal assignment of the amino acid residues responsible for this behavior may be theoretically possible through this type of analysis for the wild-type proteins, the availability of single-site mutants of both cytochrome *c* (Mauk, 1991) and cytochrome b_5 (von Bodman et al., 1987; Funk et al., 1990) will allow a more systematic analysis of the residues at the interface between these proteins. Studies of this type are currently in progress.

Potentiometric measurement of protons exchanged upon complex formation between cytochrome *c* and cytochrome b_5 is a sensitive, precise, and noninvasive method of obtaining the association constant that does not require the modification of either protein. This characteristic is essential if any meaningful analysis of the role of electrostatics using site-specific mutants is to be achieved. *The use of mutant proteins in this manner must also involve the measurement of binding over a wide pH range; mutations that alter the electrostatic potential surface of either protein will affect the pH of maximum stability, the maximum stability constant, and the distribution between alternative docking geometries.*

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References

Altman, J., Lipka, J. J., Kuntz, I., & Waskell, L. (1989) *Biochemistry 28*, 7516–7523.

Barker, P. D., Mauk, M. R., & Mauk, A. G. (1991) *Biochemistry 30*, 2377–2283.

Burch, A. M., Rigby, S. E. J., Funk, W. D., MacGillivray, R. T. A., Mauk, M. R., Mauk, A. G., & Moore, G. R. (1990) *Science 247*, 831–833.

Chothia, C., & Janin, J. (1990) *J. Biol. Chem. 265*, 16027–16030.

Cohen, J. A., & Hayes, M. B. (1974) *J. Biol. Chem. 249*, 5472–5477.

Coleman, J. E., & Vallee, B. L. (1962) *J. Biol. Chem. 236*, 2244–2249.

Concar, D. W., Whitford, D., Pielak, G. J., & Williams, R. J. P. (1991) *J. Am. Chem. Soc. 113*, 2401–2406.

Cristiano, R. J., & Steggles, A. W. (1989) *Nucleic Acids Res. 17*, 799.

Cutler, R. L., Davies, A. M., Creighton, S., Warshel, A., Moore, G. R., Smith, M., & Mauk, A. G. (1989) *Biochemistry 28*, 3188–3197.

D'Albis, A., & Bêchet, J.-J. (1967) *Biochim. Biophys. Acta. 140*, 435–458.

Davis, L. A., Schejter, A., & Hess, G. P. (1974) *J. Biol. Chem. 249*, 2624–2632.

Edsall, J. T., & Wyman, J. (1958) *Biophysical Chemistry*, Chapters 9 and 11, Academic Press, New York.

Eley, G. C. S., & Moore, G. R. (1983) *Biochem. J. 215*, 11–21.

Eltis, L. D., Herbert, R. G., Barker, P. D., Mauk, A. G., & Northrup, S. H. (1991) *Biochemistry 30*, 3663–3674.
Everest, A. M., Wallin, S. A., Stemp, E. D. A., Nocek, J. M., Mauk, A. G., & Hoffman, B. M. (1991) *J. Am. Chem. Soc. 113*, 4337–4338.
Fisher, M. T., White, R. E., & Sligar, S. G. (1986) *J. Am. Chem. Soc. 108*, 6835–6837.
Flogel, M., & Biltonen, R. L. (1975) *Biochemistry 14*, 2603–2609.
Funk, W. D. (1990) Ph.D. Dissertation, University of British Columbia, Vancouver, BC.
Funk, W. D., Lo, T. P., Mauk, M. R., Brayer, G. D., MacGillivray, R. T. A., & Mauk, A. G. (1990) *Biochemistry 29*, 5500–5508.
Glick, D. M. (1968) *Biochemistry 7*, 3391–3396.
Gray, H. B., & Malmström, B. G. (1990) *Biochemistry 28*, 7499–7505.
Guerlerquin, F., Sari, J.-C., & Bruschi, M. (1987) *Biochemistry 26*, 7438–7443.
Hazzard, J. T., McLendon, G., Cusanovich, M. A., & Tollin, G. (1988) *Biochem. Biophys. Res. Commun. 151*, 429–434.
Holloway, P. W., & Mantsch, H. H. (1988) *Biochemistry 27*, 7991–7993.
Kornblatt, J. A., Hui Bon Hoa, G., Eltis, L. E., & Mauk, A. G. (1988) *J. Am. Chem. Soc. 110*, 5909–5911.
Kostić, N. (1991) *Metal Ions Biol. Syst. 27*, 129–182.
Laskowski, M., & Finkenstadt, W. R. (1972) *Methods Enzymol. 26*, 193–227.
Lebowitz, J., & Laskowski, M. (1962) *Biochemistry 1*, 1044–1055.
Livingston, D. J., McLachlan, S. J., La Mar, G. N., & Brown, W. D. (1985) *J. Biol. Chem. 260*, 15699–15707.
Margoliash, E., & Frohwirt, N. (1959) *Biochem. J. 71*, 570–572.
Mauk, A. G. (1991) *Struct. Bonding (Berlin) 75*, 132–157.
Mauk, M. R., & Mauk, A. G. (1989) *Eur. J. Biochem. 186*, 473–486.
Mauk, M. R., Reid, L. S., & Mauk, A. G. (1982) *Biochemistry 21*, 1843–1846.
Mauk, M. R., Mauk, A. G., Weber, P. C., & Matthew, J. B. (1986) *Biochemistry 25*, 7085–7091.
McLendon, G. L. (1988) *Acc. Chem. Res. 21*, 160–167.
McLendon, G. L., & Miller, J. R. (1985) *J. Am. Chem. Soc. 107*, 7811–7823.
Miura, R., Sugiyama, T., Akasaka, K., & Yamano, T. (1980) *Biochem. Int. 1*, 532–538.
Ng, S., Smith, M. B., Smith, H. T., & Millett, F. (1977) *Biochemistry 16*, 4975–4978.
Nocek, J. M., Stremp, E. D. A., Finnegan, M. G., Koshy, T. I., Johnson, M. K., Margoliash, E., Mauk, A. G., Smith, M., & Hoffman, B. M. (1991) *J. Am. Chem. Soc. 113*, 6822–6831.
Northrup, S. H., Wensel, T. G., Meares, C. F., Wendoloski, J. J., & Matthew, J. B. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 9503–9507.
Ozols, J., & Strittmatter, P. (1964) *J. Biol. Chem. 239*, 1018–1023.
Parsons, S. M., & Raftery, M. A. (1975) *Biochemistry 11*, 1633–1638.
Pearce, L. P., Gärtner, A. L., Smith, M., & Mauk, A. G. (1989) *Biochemistry 28*, 3152–3156.
Peery, L. M., & Kostić, N. (1989) *Biochemistry 28*, 1861–1874.
Rafferty, S. P., Pearce, L. L., Barker, P. D., Guillemette, J. G., Kay, C. M., Smith, M., & Mauk, A. G. (1990) *Biochemistry 29*, 9365–9369.
Reid, L. S., & Mauk, A. G. (1982) *J. Am. Chem. Soc. 104*, 841–845.
Righetti, P. G., Gacon, G., Gianazza, E., Lostanlen, D., & Kaplan, J.-C. (1978) *Biochem. Biophys. Res. Commun. 85*, 1575–1581.
Rodgers, K. K., Pochapsky, T. C., & Sligar, S. G. (1988) *Science 240*, 1657–1659.
Ross, P. D., & Subramanian, S. (1981) *Biochemistry 20*, 3096–3102.
Rupley, J. A., Butler, I., Gerring, M., Hartdegen, F. J., & Pecoraro, R. (1967) *Proc. Natl. Acad. Sci. U.S.A. 57*, 1088–1095.
Russell, A. J., & Fersht, A. R. (1987) *Nature 328*, 496–500.
Salemme, F. R. (1976) *J. Mol. Biol. 102*, 563–568.
Stonehuerner, J., Williams, J. B., & Millett, F. (1979) *Biochemistry 24*, 5422–5427.
Strittmatter, P. (1967) *Methods Enzymol. 10*, 553–556.
Strittmatter, P., & Ozols, J. (1966) *J. Biol. Chem. 241*, 4787–4792.
Tanford, C. (1964) *Physical Chemistry of Macromolecules*, Wiley & Sons, New York.
Valenzuela, P., & Bender, M. L. (1970) *Biochemistry 9*, 2440–2446.
van Leeuwen, J. W. (1983) *Biochim. Biophys. Acta 743*, 408–421.
von Bodman, S. B., Schuler, M. A., Jollie, D. R., & Sligar, S. G. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 9443–9447.
Wendoloski, J. J., & Matthew, J. B. (1989) *Proteins: Struct., Funct., Genet. 5*, 313–321.
Wendoloski, J. J., Matthew, J. B., Weber, P. C., & Salemme, F. R. (1987) *Science 238*, 794–797.
Whitford, D., Concar, D. W., Veitch, N. C., & Williams, R. J. P. (1990) *Eur. J. Biochem. 192*, 715–721.
Wyman, J., & Gill, S. J. (1990) *Binding and Linkage. Functional Chemistry of Macromolecules*, University Science Books, Mill Valley, California.
Yung, B. Y. K., & Trowbridge, C. G. (1980) *J. Biol. Chem. 255*, 9274–9730.