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Effects of pH on Protein Association: Modification of the Proton-Linkage Model and Experimental Verification of the Modified Model in the Case of Cytochrome *c* and Plastocyanin

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Abstract: Effects of pH on protein association are not well understood. To understand them better, we combine kinetic experiments, calculations of electrostatic properties, and a new theoretical treatment of pH effects. The familiar proton-linkage model, when used to analyze the dependence of the association constant K on pH, reveals little about the individual proteins. We modified this model to allow determination not only of the numbers of the H^+ ions involved in the association but also of the pK_a values, in both the separate and the associated proteins, of the side chains that are responsible for the dependence of K on pH. Some of these side chains have very similar pK_a values, and we treat them as a group having a composite pK_a value. Use of these composite pK_a values greatly reduces the number of parameters and allows meaningful interpretation of the experimental results. We experimentally determined the variation of K in the interval $5.4 \leq \text{pH} \leq 9.0$ for four diprotein complexes, those that the wild-type cytochrome *c* forms with the wild-type plastocyanin and its mutants Asp42Asn, Glu59Gln, and Glu60Gln. The excellent fittings of the experimental results to the modified model verified this model and revealed some unexpected and important properties of these prototypical redox metalloproteins. Protein association causes a decrease in the pK_a values of the acidic side chains and an increase in the pK_a values of the basic side chains. Upon association, three carboxylic side chains in wild-type plastocyanin each release a H^+ ion. These side chains in free plastocyanin have an anomalously high composite pK_a value, ~6.3. Upon association, five or six side chains in cytochrome *c*, likely those of lysine, each take up a H^+ ion. Some of these side chains have anomalously low pK_a values, less than 7.0. The unusual pK_a values of the residues in the recognition patches of plastocyanin and cytochrome *c* may be significant for the biological functions of these proteins. Although each mutation in plastocyanin markedly, and differently, changed the dependence of K on pH, the model consistently gave excellent fittings. They showed decreased numbers of H^+ ions released or taken up upon protein association and altered composite pK_a values of the relevant side chains. Comparisons of the fitted composite pK_a values with the theoretically calculated pK_a values for plastocyanin indicated that Glu59 and Asp61 in the wild-type plastocyanin each release a H^+ ion upon association with cytochrome *c*. Information of this kind cannot readily be obtained by spectroscopic methods. Our modification of the proton-linkage model is a general one, applicable also to ligands other than H^+ ion and to processes other than association.

Introduction

Association of proteins with other biomolecules and with each other is an important step in various biological processes. Specific recognition between hormones and receptors, enzymes and inhibitors, antibodies and antigens, and electron carriers and redox enzymes depends on their selective association. The charge, and therefore the protonation state, of titratable groups plays a decisive role in biomolecular interaction.[1] When their

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association involves release or uptake of H^+ ions, the association constant will depend on pH.[2,3]

Although this dependence is crucial for many biological functions, studies of the effects of pH on protein−protein association are scarce. Among the few systems examined to date are cytochrome *c* and cytochrome b_5,[4,5] proteinases and their inhibitors,[6−8] and cytochrome *c* and cytochrome *c* peroxidase.[9] Dependence of association constant on pH has been studied sporadically. A theoretical method needed for a systematic study is not yet available.

To develop a general method for this study, we chose a pair of well-characterized prototypical electron carriers: negatively charged blue copper protein plastocyanin (pc) and positively charged heme protein cytochrome *c* (cyt). Each of them contains many charged amino acid residues suited for electrostatic interactions. The negative charge in high-plant plastocyanins is localized mostly in the so-called acidic patch, which consists of the lower cluster, residues Asp42, Glu43, and Glu44, and the upper cluster, residues Glu59, Glu60, and Asp61 (numbered as in the protein from spinach). The positive charge in mitochondrial cytochromes *c* is concentrated in the basic patch surrounding the heme edge exposed at the protein surface. These patches in the two proteins have been implicated in their reactions with charged metal complexes,[10−14] physiological partners,[15−18] other proteins,[19−23] and each other.[24−27] One goal of our study of the system in eq 1 and Figure 1 is to find out more about these prototypical heme and blue copper proteins, which are indispensable in the study of electron-transfer reactions and mechanisms. The other goal is to develop a general method for studying noncovalent protein association and test this method on this interesting pair of proteins.

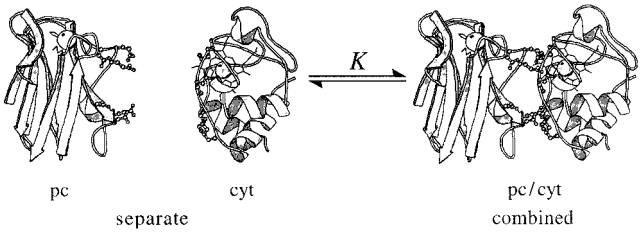

Figure 1. Reversible association between the blue copper protein plastocyanin (pc) and the heme protein cytochrome *c* (cyt). The copper and iron atoms are shown as spheres, while the four ligands to the copper atom and the porphyrin ring are shown as wire-frame models. The acidic residues Asp42, Glu43, Asp44, Glu59, Glu60, and Asp61 in plastocyanin, and the basic residues Lys13, Lys25, Lys27, and Lys86 in cytochrome *c* are highlighted as ball-and-stick models. The protein structures were drawn with the program MolScript v2.1.[77]

$$\text{cyt} + \text{pc} \overset{K}{\rightleftharpoons} \text{cyt/pc} \qquad (1)$$

To determine the association constant, *K*, we take advantage of the redox activity of the two proteins. The bimolecular rate constant k_{obs} for their electron-transfer reaction depends on the association constant; by determining k_{obs} at different pH values, we can precisely obtain *K* at these pH values. These kinetic studies with native proteins would require use of external redox agents, which would interact with the proteins and perturb their association. We avoid these complications by studying the photoinduced reaction in eq 2, for which external agents are not needed. Replacement of iron(II) by zinc(II) in the heme does not markedly affect the structure of cytochrome *c* and its association with other proteins.[28−30] The slant in the formulas in eq 2 represents this association. The oxidation of the triplet (excited) state of the zinc derivative, ^{3}Zncyt, by copper(II) plastocyanin, pc(II), has been extensively studied in our laboratory.[25,27,31−35] Kinetics is a convenient method for determining the dependence of *K* on pH.

$$^3\text{Zncyt} + \text{pc(II)} \overset{K}{\rightleftharpoons} {}^3\text{Zncyt/pc(II)} \rightarrow \text{Zncyt}^+\text{/pc(I)} \qquad (2)$$

The binding of H^+ ions is an example of ligand binding to the macromolecule. The so-called linkage model relates the free energy of a reaction (such as association) that the macromolecule undergoes and the chemical potential of the external ligand.[36]

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When the ligand is the H^+ ion, we have the proton-linkage model. The number (q) of H^+ ions released or taken up during a reaction is related to the pH dependence of the free energy of this reaction. The system to which the H^+ ion (or some other ligand) binds need not be a single macromolecule; it can be a pair of them, and the reaction of interest may be association of this pair. If association constant K at one pH value and the number q are known, then K at another pH value can be calculated with eq 3. (This known equation is derived in the Supporting Information, on pp S11−S13.)

$$\log K(\mathrm{pH}_2) = \log K(\mathrm{pH}_1) + \int_{\mathrm{pH}_1}^{\mathrm{pH}_2} q \, \mathrm{dpH} \qquad (3)$$

The standard proton-linkage model has been applied to protein−protein association,[4,9,37] protein unfolding,[38] and redox reactions.[39,40] The main shortcoming of the standard model is that it treats the reacting system as a featureless donor or acceptor of H^+ ions. Although correct and useful, the standard model does not give information about the individual macromolecules and the titratable groups affecting their reaction.

In this study, the reactants are two prototypical proteins, cytochrome *c* and plastocyanin, much studied in electron-transfer reactions. The reaction is their association. We modify the familiar proton-linkage model so that it can give information about individual proteins and even about amino acid residues involved in the protein association. Our procedure consists of four parts. First, we determine (by kinetic experiments) dependence of the association constant on pH. Second, we extract from these experimental results the composite pK_a values, both in separate and in combined proteins, of groups of amino acid residues that change their protonation state upon protein association. Third, we theoretically calculate individual pK_a values of all titratable amino acid residues in proteins. Fourth, we study the effects of several plastocyanin mutations on the pH dependence of its association with cytochrome *c* and explain these effects in terms of the modified proton-linkage model. We compare the calculated effects of the mutations on individual pK_a values and the observed effects of these mutations on protein association. We then identify some of the amino acid residues in plastocyanin whose deprotonation is triggered by association with cytochrome *c*. We discovered anomalous pK_a values of certain amino acid residues in both cytochrome *c* and plastocyanin, which may be significant for their biological functions. Moreover, we verified our modified proton-linkage model, which is generally applicable to pH-dependent interactions.

Materials and Methods

Chemicals. Distilled water was demineralized to resistivity greater than 16 MΩ cm. Chromatography gels were purchased from Sigma Chemical Company. Triethanolammonium chloride was purchased from Aldrich Chemical Co. Hydrogen fluoride, nitrogen, and ultrapure argon were purchased from Air Products Co. All other chemicals were purchased from Fisher Chemical Company.

Buffers. The buffers kept the ionic strength constant at 2.50 ± 0.10 mM over the entire pH range, from 5.4 to 9.0.[41] This full range was covered with a buffer made by dissolving 2.50 mmol (0.4641 g) of triethanolammonium chloride and 2.50 mmol (142.8 μL) of glacial acetic acid in 1.000 L of water. The pH interval from 5.4 to 7.2 was covered also with a 2.50 mM solution of sodium cacodylate, and the pH interval from 6.6 to 9.0 was covered also with a 2.50 mM solution of triethanolammonium chloride. The basic component of the first two buffers and the acidic component of the third were created in situ by adding 0.100 M solutions of NaOH or HCl, respectively. These solutions of base and acid were used also to adjust the pH, which was measured with a Fisher Accumet 805 MP pH meter equipped with an Aldrich combination microelectrode. The values of the association constant (K) in the overlap region, from pH 6.6 to 7.2, were the same regardless of the buffer chosen.

Proteins. Horse-heart cytochrome *c* was purchased from Sigma Chemical Co. The iron-free (so-called free base) form was made, purified, and reconstituted with zinc(II) by a modification[30] of the original procedure.[42,43] The product, zinc cytochrome *c*, was handled at 4 °C, in the dark. The criteria of purity were the absorbance ratios $A_{423}/A_{549} > 15.4$ and $A_{549}/A_{585} < 2.0$. The absorptivity at 423 nm is 2.43×10^5 $M^{-1}cm^{-1}$.[42] Spinach plastocyanin and its three single mutants were prepared by overexpression in *E. coli*[44] with the vector pUG223t_r[19] and purified first with a DE32 column and then with a 26/10 Q Sepharose high-performance FPLC (fast protein liquid chromatography) column from Pharmacia. The blue fraction was concentrated by dialysis against dry poly(ethylene glycol) (PEG 20 000) and passed through a gel-filtration column, Sephacryl S-100 HR. The amount of copper(II) plastocyanin was determined spectrophotometrically in the presence of $K_3[Fe(CN)_6]$, on the basis of the absorptivity at 597 nm, which is 4700 M^{-1} cm^{-1}.[44] The UV−vis spectra were recorded with a Perkin-Elmer Lambda 18 spectrophotometer.

Flash Kinetic Spectrophotometry. The so-called laser flash photolysis on a microsecond time scale was done with a standard apparatus.[45] Argon for deaeration was first passed through water and then gently through the buffered solution of the proteins for 30 min. The sample cuvette was kept at 293.0(2) K with the 30-L circulating bath Forma 2067. Concentration of Zncyt was kept at 10.0 μM, while concentration of pc(II) was varied from 2.0 to 30.0 μM. Initial volume of the protein solution was 1.000 mL. Upon each addition of a small portion (1.00−4.00 μL) of a 0.100 M solution of NaOH or HCl, the pH was measured with the freshly calibrated pH meter, and the solution was deaerated for 10 more minutes. Calibration of the pH meter was checked after the last adjustment of pH. Formation and decay of the triplet sate, ^{3}Zncyt, was monitored by the change in the absorbance at 460 nm, ΔA_{460}. For each concentration of pc(II) at a given pH value, four or more laser pulses were delivered, and kinetic traces were recorded.

Regression Analysis of the Rate Constant k_{obs} and Association Constant K. The kinetic traces were fitted to eq 4 by a least-squares nonlinear regression method, with the program SigmaPlot v4.01, purchased from SPSS Inc.

$$\Delta A_{460} = a_1 \exp(-k_F t) + a_2 \exp(-k_{obs} t) \qquad (4)$$

We confirmed[25,26,31,32,46] that the first-order rate constant k_F (whose amplitude is a_1) does not depend on the concentration of pc(II) and corresponds to unimolecular quenching of ^{3}Zncyt by pc(II), while the pseudo-first-order rate constant k_{obs} (whose amplitude is a_2) depends on the concentration of pc(II) and corresponds to bimolecular quenching of ^{3}Zncyt by pc(II). Fitting of k_{obs} to eqs 5 and 6 gave the rate constants for association, k_{on}, and dissociation, k_{off}, of the Zncyt/pc(II) complex, as in our previous studies.[25,33,47] A typical fit is shown in Figure S1 in the Supporting Information. In this study, for the first time, we analyze the pH dependence of the association constant K, calculated from eq

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7. The error margins for all reported values correspond to two standard deviations and the confidence limit greater than 95%.

$$k_{obs} = \frac{k_{on}k_F[pc(II)]}{k_{off} + k_F + k_{on}[pc(II)]} \quad (5)$$

$$[pc(II)] = [pc(II)]_0 - \frac{1}{2}\left\{[Zncyt]_0 + [pc(II)]_0 + \frac{k_{off}}{k_{on}} - \sqrt{\left([Zncyt]_0 + [pc(II)]_0 + \frac{k_{off}}{k_{on}}\right)^2 - 4[Zncyt]_0[pc(II)]_0}\right\} \quad (6)$$

$$K = \frac{k_{on}}{k_{off}} \quad (7)$$

Calculations of pK_a Values of Individual Residues. The crystal structures of horse-heart cytochrome *c* (designated 1HRC)[48] and spinach plastocyanin (designated 1AG6)[49] were taken from the Protein Data Bank. Because the latter structure is known only for the Gly8Asp mutant, we created the wild-type form by changing Asp8 back to Gly with the program CHARMM.[50] We then created the mutants Asp42Asn, Glu59Gln, and Glu60Gln by the same method. In these slight mutations of the carboxylic acid to the corresponding amide, the coordinates of the non-hydrogen atoms in the side chains were kept fixed. The hydrogen atoms were added by the HBUILD method,[51] and their energy was minimized in 3000 steps of steepest descent in the CHARMM program.

The pK_a values of amino acid residues were calculated by a published method,[3,52] starting from the pK_a values of the model compounds, *N*-formyl methylamide derivatives of amino acids.[53] The partial charges were taken from AMBER94[54] force field for atoms in side chains and from a quantum-mechanical calculation[55] for the blue copper site. If all the residues in the protein were electroneutral, a titratable side chain would have the so-called intrinsic pK_a value. The intrinsic pK_a values and the interaction energies between titratable groups were obtained by the MEAD program,[56] solving the linearized Poisson−Boltzmann equation on a grid in two focusing steps.[57,58] The dielectric constants of the protein interior and of water were set to 4.0 and 80.0, respectively.[59] The ionic strength was set to 2.50 mM, as in the experiments. The solvent-accessible and the ion-exclusion layers were probed with spheres having radii of 1.4 and 2.0 Å, respectively. The grid was centered on the geometric center of the model compound or the protein in the first step and on the titratable group in the second. The first grid had a spacing of 1.0 Å; the second, of 0.25 Å. The two grids had the same length in all three dimensions: 61 Å for the calculations of the model compounds and 81 Å for the calculations of the proteins. Calculated $\Delta G°$ values for transferring the titratable group from aqueous solution to the protein in which all other residues are electroneutral were added to the pK_a value of the appropriate model compound. We wrote a program that uses the output of the program MEAD and determines the protonation probabilities of all titratable residues in a protein on the basis of free energies of protonation calculated by a hybrid of a statistical-mechanical method and the Tanford−Roxby method.[60] The uncertainty in the pK_a values is ∼1 pH unit.

The pK_a value of an isolated titratable side chain is the pH value at which the protonation probability, $\langle x \rangle$, is 0.50. Because an isolated side chain obeys the Henderson−Hasselbalch equation, pK_a = p$K_{1/2}$. Because, however, a protein exists in multiple protonation states, the p$K_{1/2}$ value of a side chain no longer reflects the probability of its protonation.[3] Consequently, the pK_a value depends on pH, as Figure S2 in the Supporting Information shows. Fortunately, this dependence is very small over the pH range in our experiments.[61] For the sake of accuracy, $\langle x \rangle$ was calculated over the entire range 5.4 ≤ pH ≤ 9.0, in steps of 0.05 pH units. At each step, the pK_a result was obtained with eq 8. An average of all the pK_a results corresponding to $\langle x \rangle > 0.0010$ for a given titratable side chain is the pK_a value of this side chain.

$$pK_a = pH + \log\frac{\langle x \rangle}{1 - \langle x \rangle} \quad (8)$$

Modified Proton-Linkage Model. The original linkage model[36] is exact. We modify it to reduce the number of adjustable parameters in a way that reveals more about the system and its pH-dependent reaction. For a detailed derivation, see the Supporting Information, pp S11−S16. When m_k amino acid residues each lose a H^+ ion in the same pH interval, we designate them *isoacidic residues* and treat them as a group. With the modified model, we extract not only the number of H^+ ions released or taken up upon association, but also the composite pK_a values for each group of isoacidic residues involved in (and therefore affected by) association. The width of the pH interval and other criteria for residues to be considered isoacidic will be analyzed in the Results section. The system (protein pair) has *M* groups of isoacidic residues, which are indexed with *k*. Residues in each group act collectively, and each group has one composite pK_a value in the separate (p$K^s_{a_k}$) and another composite pK_a value in the combined (p$K^c_{a_k}$) proteins; note the superscripts s and c, for separate and combined.

Our experiments detect only those residues, and only those groups of isoacidic residues, that are affected by the protein association in the pH range covered. Each group of isoacidic residues can lose m_k hydrogen ions. When a total of $\sum_{k=1}^{M} m_k$ of H^+ ions are bound, all residues in all isoacidic groups are protonated, and only one protonation state is possible. We take it as the reference protonation state of the protein pair, for which the association constant is K_0. The other protonation states are obtained by deprotonation of this one.

When *i* of the *M* groups of isoacidic residues are deprotonated, the number of possible protonation states is p_i, given by eq 9. The protonation states of the system (protein pair) with the same number of deprotonated groups of isoacidic residues are indexed with *j*, which runs from 1 to p_i. The two indexes, *i* and *j*, designate a protonation state of the protein pair. Going from the reference (fully protonated) state to the *i*,*j* protonation state, the protein pair loses a_i^j H^+ ions, the number given by eq 10.

$$p_i = \binom{M}{M-i} = \frac{M!}{(M-i)!i!} \quad (9)$$

$$a_i^j = \sum_{k=1}^{M} x_{ik}^j m_k \quad (10)$$

The component x_{ik}^j of a protonation-state vector shows whether the *k*-th group of *M* isoacidic residues is protonated (0) or not (1). The constant L_i^j, defined for separate (superscript s) proteins in eq 11 and for combined (superscript c) proteins in eq 12, is the equilibrium constant between the reference protonation state and the *i*,*j* protonation state. (For a detailed explanation, see Supporting Information.) These

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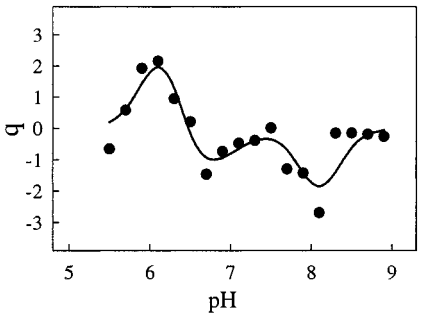

Figure 2. Dependence on pH of the number of H^+ ions (q) released to the buffer by the protein pair shown in Figure 1 upon protein association at ionic strength of 2.50 mM and 293 K. The line is calculated with eq 14 on the basis of the fitted line from Figure 3a.

equilibrium constants can be related to the $pK^s_{a_k}$ and $pK^c_{a_k}$ values, as the right-hand sides of eqs 11 and 12 show.

$$L_i^{js} = \prod_{k=1}^{M} x_{ik}^{j} \,(K^s_{a_k})^{m_k} = 10^{-\sum_{k=1}^{M} x_{ik}^{j} m_k \, pK^s_{ak}} \tag{11}$$

$$L_i^{jc} = \prod_{k=1}^{M} x_{ik}^{j} (K^c_{a_k})^{m_k} = 10^{-\sum_{k=1}^{M} x_{ik}^{j} m_k \, pK^c_{a_k}} \tag{12}$$

Let the system (a protein pair) contain n titratable residues. If these residues are treated individually, as in the standard proton-linkage model, there are 2^n values of L_i^{js} and 2^n values of L_i^{jc}. In a realistic case of, say, n=8, there are a total of $2 \times 2^8 = 512$ equilibrium constants; any fitting with them would be futile. We avoid this proliferation of adjustable parameters by recognizing a group of titratable residues that are deprotonated more or less in concert, over a relatively narrow pH range. We call them *isoacidic residues* and recognize multiple groups of these residues. In our modified proton-linkage model (eq 13), there are only M values of m_k, M values of pK^s_a, and M values of pK^c_a. With this relatively small number of parameters, a fitting of the pH dependence of the association constant becomes possible. This fitting is not merely a numerical procedure; its results are very informative.

$$K = K_0 \frac{\sum_{i=0}^{M}\sum_{j=1}^{p_i} [H^+]^{-a^j} L_i^{jc}}{\sum_{i=0}^{M}\sum_{j=1}^{p_i} [H^+]^{-a^j} L_i^{js}} \tag{13}$$

Protein association (within the system) can trigger release and uptake of H^+ ions (by the system). Therefore, association constant K depends on the concentration of H^+ ions. This dependence can be fitted to eqs 9−13 with a reasonable approximation that the individual titratable residues can be collected into groups of isoacidic residues, each isoacidic group corresponding to one m_k value. The initial values of these fitting parameters m_k in eqs 11 and 12 can be estimated from the experimentally determined dependence of K on pH. According to eq 3, the slope of the plot of log K versus pH is q, defined in eq 14. This plot is shown in Figure 2.

$$q = \frac{d \log K}{d \,\mathrm{pH}} \tag{14}$$

Positive and negative values of q correspond to release and uptake, respectively, of H^+ ions upon protein association. We round the maxima and minima in Figure 2 (because m_k must be an integer) and use their absolute values. In this way, we satisfy the convention that the reference protonation state has a maximum number of H^+ ions bound and all other protonation states are formed by loss of H^+ ions by the protein pair (to the buffer). In the program SigmaPlot, m_k values had to be

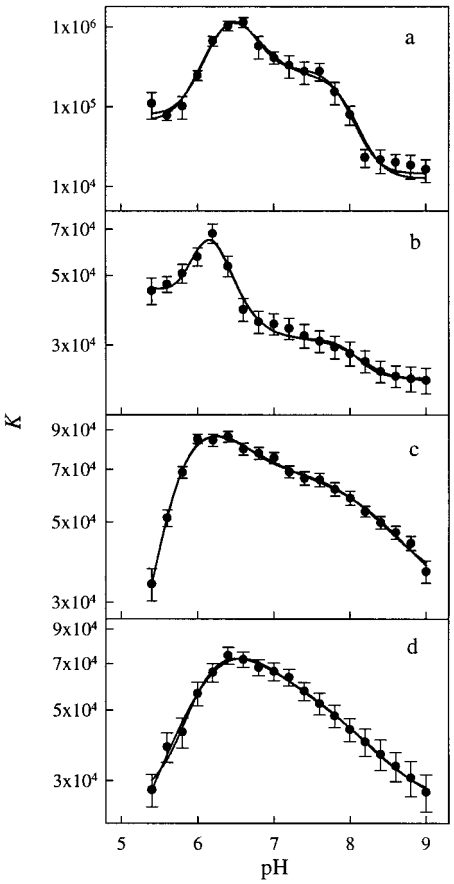

Figure 3. Dependence on pH of association constant K, for association of cytochrome c with the wild-type plastocyanin (a) and the following three mutants: Asp42Asn (b), Glu59Gln(c), and Glu60Gln(d) at ionic strength of 2.50 mM and 293 K. Error bars for the experimental results include two standard deviations. The two nearly overlapping lines in each case are fittings to eqs 9−13. The two sets of fitted parameters are given in paired columns in Table 1.

treated as constants and changed manually, one at the time. We changed them in unit steps and allowed all the pK_a values and K_0 to float.

Because the experimental plot in Figure 3a shows three inflection points, we recognize three groups of isoacidic residues, corresponding to m_1, m_2, and m_3. Fittings of the experimental pH-dependent association constants to eqs 9−13 yielded the following results: K_0; three $pK^s_{a_k}$ values, for three groups of isoacidic residues in separate (free) proteins; and three $pK^c_{a_k}$ values, for these groups in combined (associated) proteins. We considered the fitting successful if the fitted line passed through all 19 experimentally determined points (within the error bars); all such fittings gave the correlation coefficient $R^2 \geq 0.990$. The fits were easily "spoiled" by small changes in the parameters. This is further evidence that the fits are meaningful. The fitted values of m_k, $pK^s_{a_k}$, and $pK^c_{a_k}$ are reasonable and clearly related to the known properties of the proteins under study, as will be discussed below. That reasonableness, and not merely the high statistic quality of the fits, reassured us about the soundness of these results.

A detailed derivation of the modified proton-linkage model and an example of its application to our experimental results are given in the Supporting Information.

Results

Oxidative Quenching of ^{3}Zncyt by pc(II). In the absence of copper(II) plastocyanin, the excited state ^{3}Zncyt decays with the rate constant of 80 ± 10 s^{-1}. In the presence of copper(II) plastocyanin, this excited state is oxidatively quenched. As the concentration of copper(II) plastocyanin increases, the rate constant increases as well, as shown in Figure S1 in the

Table 1. Parameters with Which the pH Dependence of Association Constants K in Figure 3a−d Were Fitted to Eqs 9−13[a]

	pc form							
parameter[b]	wild-type[c]		Asp42Asn[d]		Glu59Gln[e]		Glu60Gln[f]	
m_1	3	3	3	3	2	2	1	2
m_2	2	3	2	2	1	2	1	1
m_3	3	3	2	3	1	1	1	1
$pK^s_{a_1}$	6.4	6.3	6.1	6.1	5.7	5.6	6.3	6.0
$pK^s_{a_2}$	6.3	6.6	6.0	6.0	6.4	6.6	6.3	6.8
$pK^s_{a_3}$	7.9	7.8	8.1	8.1	8.6	8.3	8.2	8.1
$pK^c_{a_1}$	5.8	5.9	5.9	5.9	5.2	5.1	5.0	5.7
$pK^c_{a_2}$	7.0	6.9	6.4	6.4	6.6	6.7	7.0	7.0
$pK^c_{a_3}$	8.3	8.3	8.2	8.1	8.9	8.7	8.6	8.4
$10^{-4}\,K_0$	8(1)	7(1)	4.7(6)	4.7(6)	1.3(6)	0.9(4)	0.9(3)	2.6(3)

[a] Association of cytochrome *c* with each of the four forms of plastocyanin was successfully fitted with two sets of parameters: m_k is the number of isoacidic residues in a group; pK^s_a and pK^c_a are composite pK_a values for isoacidic residues in, respectively, separate (free) and combined (associated) proteins; and K_0 is the association constant for fully-protonated proteins. Subscript 1 designates plastocyanin; subscripts 2 and 3, cytochrome *c*. [b] Margin of error for the pK_a values is ±0.10. [c] Fittings shown in Figure 3a. [d] Fittings shown in Figure 3b. [e] Fittings shown in Figure 3c. [f] Fittings shown in Figure 3d.

Supporting Information. These findings confirm the previous detailed studies in this laboratory and were analyzed as before.[26,31,46] In this study, we are interested in the bimolecular reaction between the two proteins (eq 2), whose rate constant is k_{obs} and the amplitude of whose kinetic trace is a_2.

When the concentration of copper(II) plastocyanin is kept constant and the pH is raised from 5.4 to 9.0, the rate constant k_{obs} increases, reaches a maximum, and then decreases, as seen in Figure S3 in the Supporting Information. Simultaneously, the amplitude a_2 decreases, reaches a minimum, and increases, as seen in Figure S4 in the Supporting Information.

Dependence of the Association Constant *K* on pH. We experimentally determined K at 19 pH values in the interval from 5.4 to 9.0. We did so for four protein pairs, those that cytochrome *c* forms with the wild-type plastocyanin and its three mutants; see Figure 3a−d. As the pH is raised (from 5.4 to 9.0), association constant K increases, reaches a maximum, and then decreases. The wild-type plastocyanin and all three mutants show the same qualitative trend, but quantitative differences among them are important. Both the similarities and dissimilarities among the four plastocyanin variants are revealing and will be discussed in detail below.

Fitted m_k Values. All four plots in Figure 3 were successfully fitted in the same way. Reasonable initial values of m_k (absolute values of the q extrema in Figure 2) were available from experimental data, as explained above. For example, the initial m_k values for fitting of results in Figure 3a were $m_1 = 2$, $m_2 = 1$, and $m_3 = 3$. We changed them, as outlined in the preceding section, and an excellent fit was quickly achieved. That further changes of the m_k values spoil the fit proves that the prior success was not accidental. Some of these controls are shown in Figure S5 in the Supporting Information. If a set of m_k values was unsatisfactory, adjustment of the six pK_a values could not produce a good fit. Of the approximately 300 attempted fittings, only eight were satisfactory. These eight sets of fitted parameters are the eight columns in Table 1, two columns each for wild-type plastocyanin and its three mutants.

Fitted Composite pK_a Values. Criterion for Isoacidic Residues. The estimated error in the pK_a values is ±0.10 pH units. Changes of this magnitude in the pK_a values were more than sufficient to divert the fitted line from the error bars of some of the experimentally determined points, as seen in Figures S6−S8 in the Supporting Information.

Table 2. Calculated pK_a Values of Selected Amino Acid Residues in the Acidic Patch of Plastocyanin, at Ionic Strength of 2.50 mM and 293 K

	pc form			
residue[a]	wild-type	Asp42Asn	Glu59Gln	Glu60Gln
Asp42	4.4	*b*	4.3	4.3
Glu43	6.2	5.8	6.0	6.1
Asp44	6.2	5.4	6.1	6.1
Glu59	5.5	5.4	*b*	4.9
Glu60	4.3	4.3	3.7	*b*
Asp61	6.3	6.3	6.0	5.2

[a] Numbering for the protein from spinach. [b] Residue absent in the mutant.

We define *isoacidic residues* as those that lose their H^+ ions in the same pH interval. Upon protein association, isoacidic residues act in concert. Treating them as a group is our main modification of the proton-linkage model. The width of the pH interval (i.e., the criterion for residues to be considered as isoacidic) depends on the number, precision, and "density" of the experimental data points on the pH scale. In this study, the individual pK_a values of the isoacidic residues belonging to the same group differ by ±0.30 pH units or less. We determined this interval by imposing spreads of various magnitude on these individual pK_a values. When the spread was within ±0.30 pH units, the fitted lines passed through the error bars of all experimentally determined points for each of the four forms of plastocyanin.

Because association of cyt with all four forms of pc (wild-type and three mutants) obeys the same equations, because the fits were excellent, and because the fitted parameters were quite reasonable, we accepted the success in fitting as verification of our modified proton-linkage model.

Calculated Individual pK_a values. We used a continuum electrostatic model to calculate the pK_a values of all titratable residues in all four forms of plastocyanin and also in wild-type cytochrome *c*; see Tables S1 and S2 in the Supporting Information. The pK_a values for the residues in the acidic patch of plastocyanin, which will be discussed in detail, are given in Table 2. The pK_a values for the propionic acid substituents in the free heme molecule fall in the interval 4.4−4.8. These pK_a values likely decrease when the heme is incorporated into cytochrome *c*, a positively charged protein. Because our experiments are done in the interval $5.4 \leq pH \leq 9.0$, the propionic acid substituents need not be considered in the interpretation of these experiments.

Discussion

Protein Association. Protein association involves shape complementarity and hydrophobic interactions, which do not depend on pH, and electrostatic (ionic and polar) interactions, including hydrogen bonding, which do.[62] By changing the pH value, one can adjust the protonation state of titratable side chains and study the dependence of the association constant, K, on this state.

Association constants can be determined by NMR spectroscopy or calorimetry, but there are many pitfalls in these experiments. Exchange of H^+ ions, nonspecific perturbation of NMR resonances, interactions of the proteins with the buffer as well as with each other, and deviations from ideal behavior when the protein concentrations are relatively high are all factors

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that interfere with the experiments.[62] To complicate matters further, these interactions and deviations may also depend on pH.

Even if the technical problems in the experimental approach are solved, association constants *K* may be difficult to interpret. If protein association is a step in a chemical reaction, and especially if multiple protein complexes are possible, it may not be clear whether the resulting *K* corresponds to the reactive complex. For all of these reasons, we determined *K* by a kinetic method, which requires relatively low concentrations of proteins and can give precise results.

Protein Pair. To study in detail the effect of pH on association between proteins, we chose a pair of stable and well-characterized metalloproteins having complementary electrostatic properties.[12,15,63−67] Chemical modification of cytochrome *c*,[68] saturation kinetics in the electron-transfer reaction,[25,31,46] NMR spectroscopy,[69] site-directed mutations in plastocyanin,[33,35] and computational simulation of docking[70,71] all agree that the acidic patch in plastocyanin abuts the basic patch of cytochrome *c* upon association. It is not a purpose of our study to verify this established fact. We are interested in the dependence of association constant on pH, both in its own right and for the new information that the pH dependence provides about these important proteins.

Dependence of Association Constant, *K*, on pH. We covered the widest possible pH interval, from 5.4 through 9.0. Further extension of it would have compromised the stability of plastocyanin (at the low end) and cytochrome *c* (at the high end). Fortunately, the association constants varied greatly in this pH interval, and these variations provided stringent tests of our modified proton-linkage model. The same pH interval was covered in a recent, but very different, study of pH effects on a pair of redox proteins.[73] As Figures 3a−d show, affinity of cytochrome *c* for each of the four forms of plastocyanin follows the same general trend: increase from 5.4 to ∼6.5 and then decrease to 9.0. We will first explain this general increase and decrease in *K* and will later analyze the inflections in the plots.

The initial increase in *K* cannot be due to weakening repulsion between the two proteins, because the acidic patch in plastocyanin lacks cationic side chains. The increase is due to deprotonation of carboxylic groups in the acidic patch in plastocyanin, a process that favors salt bridges with the basic patch in cytochrome *c*. The decrease in *K* as the pH is raised further cannot be due to strengthening repulsion, because the basic patch in cytochrome *c* lacks acidic side chains. The decrease is due to deprotonation of cationic residues in this basic patch, a process that disfavors salt bridges with the acidic patch in plastocyanin. Formation of a salt bridge is thermodynamically favored over burying one or two charged residues in the protein interface, especially so if one or both participating side chains are already in the ionic form. Salt bridges may also contribute to the specificity of interactions.[72]

To explain quantitatively the dependence of *K* on pH, we needed a suitable theoretical model for this dependence. Only one such model seems to be commonly used in chemistry and biochemistry.

Proton-Linkage Model. In this study, the system is a pair of proteins, cytochrome *c* and plastocyanin considered together. As Figure 1 shows, the system can exist in two association states: the proteins are either separate or combined. When the system changes its association state, the system may take up or release H^+ ions to the buffered solution. If a titratable side chain is affected by the change of the association state of the system, this side chain will have different individual pK_a values in the separate and combined states. Consequently, the change of the association state may cause (de)protonation of this side chain. A swap of H^+ ions within the system (i.e., between the two proteins) cannot be detected. We are interested in those cases in which protein association triggers uptake or release of H^+ ions by the protein pair (viewed as a whole). If this uptake (from the buffer) or release (to the buffer) occurs in the experimental pH range, it can be detected in the plot of *K* versus pH.

The proton-linkage model is correct, but it treats the system simply as a donor or acceptor of H^+ ions and yields only the total number of these ions involved in a pH-dependent process. Because the model does not recognize the specific amino acid residues that release or take up the H^+ ions, it cannot give detailed information about the triggering process. If the pK_a values of the responsible side chains could be determined, the model would become a useful tool for the study of chemical and biochemical reaction mechanisms. We modified the standard model and achieved this goal: we obtained composite pK_a values of groups of amino acid residues that are involved in the protein association.

Modified Proton-Linkage Model. In principle, our analysis holds equally for increasing or decreasing pH, association or dissociation of the protein pair, and uptake or release of H^+ ions by the protein pair. For the sake of consistency and clarity, we consider the changes in the direction of increasing pH, where protein association may trigger release or uptake of H^+ ions.

We introduce the concept of *isoacidic residues*, those that lose a H^+ ion each in the same pH interval and therefore can be treated together, as a group. This concept and definition apply equally to residues such as glutamic acid and to residues such as protonated lysine, which is an acid but is conventionally classified as a basic residue.

In this study, we distinguish several types of pK_a values. The *calculated* ones are the result of theoretical calculations. The *individual* values for particular residues cannot be deduced form the experiments. A group of isoacidic residues is characterized by its *composite* pK_a value, which is read from the corresponding inflection point in Figure 3. The composite value is made up of individual values, whose contributions to the composite value remain unknown. The unknown individual pK_a values of isoacidic residues in this study are clustered within ±0.30 pH units, as explained above. The width of this interval depends on the specific system under study and precision of the experimental measurements and results. A combination of these individual values is a composite pK_a value for the group of isoacidic residues.

Scheme 1 shows the effects of protein association and of pH change on a carboxylic side chain in plastocyanin; this side chain may belong to a group of isoacidic aspartic acid or glutamic acid residues. This conceptual scheme applies regardless of the

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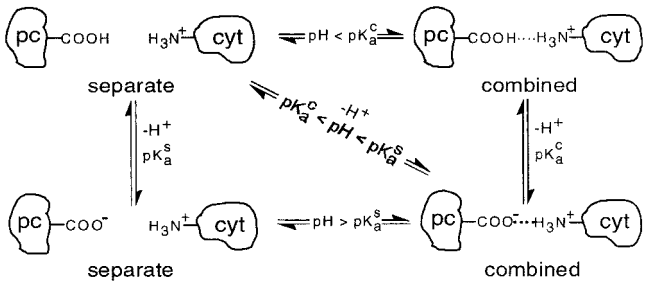
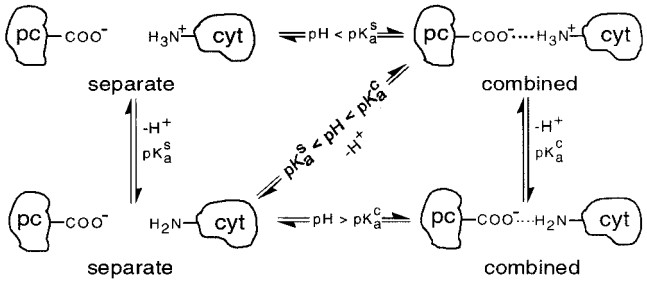
Scheme 1. Effect of pH (Vertical Arrows) and of Association (Horizontal Arrows) on Acidic Residues in Plastocyanin that Is Separate from (Superscript s) and Combined with (Superscript c) Cytochrome *c*[a]



[a] One carboxylic side chain represents a group of several such isoacidic residues. Protein association in a certain pH interval triggers the release of H^+ ions to the buffer. Boldface dots represent a salt bridge; lightface dots, a hydrogen bond.

Scheme 2. Effect of pH (Vertical Arrows) and of Association (Horizontal Arrows) on Basic Residues in Cytochrome *c* that Is Separate (Superscript s) from and Combined (Superscript c) with Plastocyanin[a]



[a] One ammonium side chain represents a group of several isoacidic residues. Protein association in a certain pH interval triggers uptake of H^+ ions to the buffer. Boldface dots represent a salt bridge; lightface dots, a hydrogen bond.

protonation state of the basic residues in cytochrome *c*; in this case, they happen to be protonated, as cations. Association and dissociation of the two proteins are represented by horizontal arrows. Loss of H^+ ions to the buffer is represented by vertical arrows; the release of H^+ ions triggered by association, by the downward diagonal arrow. When pH < pK_a^c, the neutral carboxylic group predominates in both separate and combined proteins, so that upon association (the upper horizontal) the system does not release a H^+ ion (to the buffer). A salt bridge cannot form, but a hydrogen bond may form. At pK_a^c < pH < pK_a^s, the side chain is predominantly neutral when the proteins are separate but predominantly charged when they are combined. Association (the diagonal) triggers the release of a H^+ ion, which can be detected. A salt bridge can form. At pH > pK_a^s, the carboxylate anion predominates in both separate and combined proteins, so that upon association (the lower horizontal) the system does not release a H^+ ion, and a salt bridge can form.

Scheme 2 shows the effects of protein association and of pH change on an ammonium side chain in cytochrome *c*, a member of a group of isoacidic lysine residues. The acidic residues in plastocyanin happen to be deprotonated. As before, association and loss of H^+ ions are represented by rightward and downward arrows, respectively, whereas uptake of H^+ ions triggered by association is represented by the upward diagonal arrow. As before, the protonation state, and therefore the charge, of the group of isoacidic residues depends on the relation among the pH, pK_a^s, and pK_a^c values. The main consequence of these relations is the possibility for forming salt bridges or mere hydrogen bonds.

When the oppositely charged interaction surfaces in separate proteins are hydrophilic and titratable residues are present, as in this diprotein system, acidic residues tend to release H^+ ions upon association, while basic residues tend to take up H^+ ions. For this reason, the diagonal arrows in Schemes 1 and 2 are placed along one diagonal only; the arrows along the other diagonal are omitted for the sake of clarity. The modified proton-linkage model, however, is equally applicable to the processes shown in Schemes 1 and 2 and those not shown there.

Application of the Modified Proton-Linkage Model to Wild-Type Plastocyanin and Cytochrome *c*. Number of Isoacidic Residues. The results are shown in Figure 3a; namely, 19 points covering the pH interval from 5.4 to 9.0. The general trend was explained above: As pH is raised, *K* increases owing to the acidic residues in plastocyanin and then decreases owing to the so-called basic (actually, acidic when protonated) residues in cytochrome *c*. Now we will analyze subtler, but more revealing, features of the curve: the inflection points. There are three of them, corresponding to three groups of isoacidic residues. Each group contains m_k residues and has a pK_a^s value in the separate proteins and a pK_a^c value in the combined proteins. As explained in the Materials and Methods section, the fitting yields 10 parameters: one K_0 value and triplets of m_k, pK_a^s, and pK_a^c values.

Fitting of Figure 3a to eqs 9−13 is very sensitive to the choice of m_k values and sensitive to the choice of the other parameters. We often tested fits by deliberately spoiling them, changing m_1, m_2, or m_3 by one unit, to see if the fit will markedly worsen. A few of these tests are shown in Figure S5 in the Supporting Information. In the end, out of nearly 100 attempts at fitting with different m_k triplets, only two met all the criteria. They are given in the first two columns in Table 1.

The m_k values show that association involves a group of three isoacidic residues in wild-type plastocyanin and also one group of two or three isoacidic residues and another group of three isoacidic residues in cytochrome *c*. The apparent mismatch between the salt bridge partners, three in plastocyanin versus five or six in cytochrome *c*, is only apparent. The other residues in plastocyanin that form salt bridges have typical, relatively low, pK_a values and therefore are deprotonated already at pH < 5.4. They are undetectable in the pH range covered in our experiments.

A direct titration of cytochrome *c* showed that this protein loses ∼5 H^+ ions over this pH range.[74] Indeed, our fitting gave the sum $m_2 + m_3$ of 5 or 6. Evidently, the modified proton-linkage model gives realistic numbers of isoacidic residues.

Application of the Modified Proton-Linkage Model to Wild-Type Plastocyanin and Cytochrome *c*. pK_a Values. Our fittings gave $pK_{a_1}^s$ of 6.4 and 6.3 for the first group of isoacidic residues in separated proteins. To test the sensitivity of the fits to composite pK_a values, we systematically changed these values, one at the time, in the first two columns in Table 1. Some of these attempts are shown in Figures S6−S8 in the Supporting Information. Deviations from the best values by as little as ±0.10 clearly spoil the fits. Evidently, the error margin for the composite pK_a values in this study is approximately ±0.10 pH units. In judging success of the fitting, one must consider not only the fidelity of the line to the data points but also the number and precision of these experimental results.

The two successful fittings in Table 1 are quite consistent; only the $pK_{a_2}^s$ values in the two columns differ by more than 0.10 pH units. Because the difference is small, the following

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interpretation is consistent with both columns in Table 1. That $pK^{s}_{a_1} > pK^{c}_{a_1}$, whereas $pK^{s}_{a_2} < pK^{c}_{a_2}$ and $pK^{s}_{a_3} < pK^{c}_{a_3}$, means that association favors carboxylate anions in plastocyanin and ammonium (lysine) cations in cytochrome *c*. This finding is symptomatic of salt bridges formed in a polar environment.[2]

The possibility of extracting composite pK_a values of groups of residues and analyzing their shifts upon protein association is the chief benefit of our modification of the standard proton-linkage model. In the reminder of this study, we examine the first inflection point in Figure 2a, the one due to plastocyanin residues. This analysis will demonstrate the new possibilities opened by the modified method for the study of biomolecular interactions.

Application of Modified Proton-Linkage Model to Plastocyanin Mutants and Cytochrome *c*. In each of the three mutants, one carboxylic group in the acidic patch is converted to the corresponding carboxamide, Asp to Asn or Glu to Gln. This noninvasive[19,23] change alters electrostatic but not steric properties of the side chain.[34] We want to know how association with cytochrome *c* will change when a plastocyanin residue that can form a salt bridge is noninvasively replaced by another that cannot. We will observe the changes in m_1, $pK^{s}_{a_1}$, and $pK^{c}_{a_1}$ parameters upon each mutation in plastocyanin.

Out of some 200 attempts at fitting, each of the plots in Figure 3b−d was accurately reproduced (correlation coefficients $R^2 \geq 0.990$) with only two sets of parameters. The consistency of the two fits for each plot can be assessed by comparing parameters in each pair of columns in Table 1. The quantitative results shown there can be summarized as follows. For Asp42Asn, the m_3 values differ by 1, and all the pK_a values agree. For Glu59Gln, the m_2 values differ by 1, and only the $pK^{s}_{a_3}$ values differ slightly. For Glu60Gln, the m_3 values differ by 1, and two of the pK_a values differ by more than 0.3 pH units. Although these differences are relatively small, we interpret the parameters cautiously.

Table 1 shows that mutations in plastocyanin affect not only the parameters for the combined proteins but also the parameters for cytochrome *c*, namely m_2, m_3, $pK^{s}_{a_2}$, and $pK^{s}_{a_3}$; the last two parameters describe cytochrome *c* when this protein is separate from (not associated with) plastocyanin. For example, the m_2 value changes from 3 for wild-type plastocyanin to 1 for the Glu60Gln mutant, and the $pK^{s}_{a_2}$ value changes from 6.3−6.6 for the wild-type plastocyanin to 6.0 (in both fittings) for the Asp42Asn mutant. Although these changes may at first appear artifactual, they are in fact legitimate and expected.

The modified proton-linkage model (just like the standard model) recognizes only those amino acid residues in separate proteins that will became perturbed when the proteins combine (i.e., associate with each other). Amidation (by mutation) of an acidic residue in plastocyanin prevents it from forming a salt bridge with cytochrome *c*. A smaller number of residues in cytochrome *c* become perturbed upon association, and consequently, the m_2 and m_3 values decrease. As the number of residues in a group of isoacidic residues in cytochrome *c* varies, so does their composite pK_a value. For example, the aforementioned second group of isoacidic resides in cytochrome *c* seems to be made up of fewer residues when cytochrome *c* interacts with Asp42Asn mutant than when it interacts with the wild-type plastocyanin. In the former case, the composite of the individual pK_a values in free cytochrome *c* is 6.0; in the latter case, this composite is 6.3 or 6.6.

These changes in the $pK^{s}_{a_2}$ and $pK^{s}_{a_3}$ values upon plastocyanin mutation are relatively small, 0.1−0.6 pH units. That fitting to the modified proton-linkage model reveals these expected perturbations is further evidence for the soundness of the modified model.

Plastocyanin Residues Involved in Association with Cytochrome *c*. In these experiments we could not go below pH 5.4 to observe the pK_a values of ordinary acidic residues, because these conditions would have damaged plastocyanin.[75] The isoacidic residues belonging to the group m_1 have unusually high pK_a values, a symptom of their mutual interactions within plastocyanin. Deprotonation of one carboxylic group makes the deprotonation of another more difficult, and their pK_a values increase.

We do not use the theoretically calculated pK_a values of individual residues in Table 2 to interpret the inflection points in Figures 3a−d, because individual residues are subsumed in the group of isoacidic residues. Each group, with its composite pK_a value, corresponds to an inflection point in Figure 3. The calculated individual values in Table 2 are, however, necessary for detection and qualitative assessment of the effects of mutations in plastocyanin. Because the theoretical method allowed for interactions among the residues, the calculated individual pK_a values can show indirect effects that the mutation of a given residue has on other residues in plastocyanin. In the following discussions we use results from both Tables 1 and 2.

a. Effects of the Asp42Asn Mutation. Residue 42 belongs to the lower cluster in the acidic patch. Because its individual pK_a in all the plastocyanin forms is 4.3 or 4.4, well outside the pH range covered, this residue is unlikely to contribute to the group of isoacidic residues having composite pK^{s}_{a} of 6.3−6.4 in wild-type plastocyanin. We conclude that Asp42 cannot directly contribute to the dependence in Figure 3a. Indeed, when ionization of residue number 42 is prevented (by mutation to Asn), the m_1 value remains unchanged. This is evidence that this wild-type residue is not deprotonated upon association with cytochrome *c* (in the suitable pH interval, according to Scheme 1). But this residue can contribute to the pH dependence of *K* indirectly, through its interactions with other residues. As Table 2 shows, upon mutation Asp42Asn, individual pK_a values of the neighboring residues Glu43 and Asp44 are lowered by 0.4 and 0.8 units, respectively. This change probably contributes to the slight lowering, by 0.2 or 0.3 units (see Table 1), of the composite $pK^{s}_{a_1}$ value upon mutation.

b. Effects of the Glu59Gln Mutation. Residue Glu59 belongs to the upper acidic cluster. Considering the uncertainty of the calculated individual pK_a value of 5.5, Glu59 probably contributes to the isoacidic group having composite $pK^{s}_{a_1}$ of 6.3−6.4 in wild-type plastocyanin. The mutation Glu59Gln appreciably changes the $pK^{s}_{a_1}$ and $pK^{c}_{a_1}$ values. As Table 1 shows, m_1 is 2 for this mutant, as opposed to 3 for the wild-type plastocyanin. Evidently, when ionization of Glu59 is prevented (by mutation), the number m_1 of ionizable residues in plastocyanin decreases by 1, and the collective behavior of the first group of isoacidic residues is altered. It is tempting to conclude that Glu59 is one of the three isoacidic residues in wild-type plastocyanin. But mutation of Glu59 could perhaps act indirectly, by lowering the pK_a value of some proximate residue, so that this other residue stays deprotonated over the entire pH range and can no longer be detected in Figure 3c. Because mutation Glu59Gln lowers the individual pK_a values of Glu60 and Asp61 relatively little, by 0.6 and 0.3 units, respectively (see Table 2), a large indirect effect is unlikely. We return to the straightforward conclusion that Glu59 itself is a member of the group of isoacidic residues in plastocyanin.

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To test this conclusion, we mutated the adjacent residue, Glu60, in an attempt to affect Glu59 indirectly. If our reasoning is correct, ionization of Glu59 in plastocyanin upon association with cytochrome *c* could be suppressed by lowering the individual pK_a value of Glu59.

c. Effects of the Glu60Gln Mutation. As Table 1 shows, this mutant contains fewer isoacidic residues (m_1) than the wild-type plastocyanin does. A simple explanation would be that Glu60 in the wild-type protein is one of the residues whose ionization is triggered by association with cytochrome *c*. The $pK^s_{a_1}$ value, however, is essentially the same as in the wild-type plastocyanin, and $pK^c_{a_1}$ is little perturbed. These last results argue against ionization of Glu60 itself upon association.

Calculated pK_a of Glu60 is 4.3, probably too low to make this residue a member of the first group of isoacidic residues. Although our experiments alone cannot detect ionization of Glu60, if it occurs upon association, our experiments in combination with calculated individual pK_a values can reveal effects of Glu60 on other acidic residues in plastocyanin. As Table 2 shows, mutation of Glu60 to Gln lowers the individual pK_a of Glu59 by 0.6 and of Asp61 by 1.2 units. The values of 4.9 and 5.2 are probably low enough to preclude detectable ionization of Glu59 and Asp61 (as members of the group of isoacidic residues) in the experimental pH range, which begins at pH 5.4.

The m_1 value of 1 or 2 (in the last two columns in Table 1) is smaller than the m_1 value of 3 (in the first two columns). Upon the mutation Glu60Gln, one or two acidic residues in plastocyanin, most probably residues other than Glu60, cease contributing to the release of H^+ ions upon association with cytochrome *c* in the pH range covered. According to our evidence, one of these residues likely is Asp61; the other, if there are two of them, probably is Glu59, as explained in the preceding paragraphs.

Implications for the Function of Plastocyanin and Cytochrome *c*. Plastocyanin carries electrons from cytochrome *f* to photosystem I in the thylakoid of the chloroplasts, which has pH $\approx$ 5.0.[76] Our experimental results (Table 1) and theoretical calculations (Table 2) consistently show that three of the acidic side chains in plastocyanin retain the H^+ ions, and remain electroneutral, even at pH $\approx$ 5.0. If this is correct, these side chains surely remain electroneutral under physiological conditions. When considering electrostatic properties of proteins, acidic side chains are routinely assumed to be deprotonated and therefore anionic under biological conditions. We just showed that this assumption would cause mistakes in the study of interaction of plastocyanin with its physiological partners.

Results in Tables 1 and 2 also suggest that certain basic residues in cytochrome *c* have anomalously low pK_a values. This finding may be important, because various cytochromes *c* interact with various physiological partners via the basic patch.

Conclusions

We modified the familiar proton-linkage model so that it can reveal important molecular details about pH-dependent phenomena in chemical and biochemical systems. The modified model, in combination with theoretical calculations of pK_a values of individual titratable side chains, can be used to determine composite pK_a values of those side chains that act concertedly or approximately so in releasing or taking up H^+ ions upon some change of the system. This information cannot be obtained by NMR and IR spectroscopic methods. The familiar spectroscopic titrations yield only the protonation probabilities of individual residues, the number $\langle x \rangle$ in eq 8. Our fittings to the modified model yield pK_a values, which are free-energy quantities. To identify isoacidic residues in a protein by spectroscopic pH titration, one would have to perform a great many experiments. Our determination of pH dependence of association constant is simpler and faster.

This study, the first application of the modified proton-linkage model, concerns the dependence on pH of the association between two proteins. The association constant intricately depends on pH, and these dependencies are different in the cases of wild-type and mutant forms of one of the proteins. All these dependences and their differences were consistently explained and easily fitted with only one or two sets of reasonable parameters in each case. Interpretation of these parameters gave much information about the protein−protein interaction. In particular, side chains with anomalous pK_a values were found in both proteins. Even though cytochrome *c* and plastocyanin are not physiological partners, study of their association revealed these properties that are relevant to their biological function.

The modified proton-linkage model is general. Besides protein association, it is applicable to various pH-dependent interactions, such as binding of ligands to receptors: substrates and cofactors to enzymes, proteins to nucleic acids and membranes, antigens to antibodies, metal ions and small molecules (such as dioxygen and nitric oxide) to proteins for transport and storage, and others. Encouraged by the outcome of this study, we will explore some of these new applications of the modified proton-linkage model.

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Supporting Information Available: Two tables showing the pK_a values of titratable residues in copper(II) plastocyanin and iron(II) cytochrome *c*. Plot showing the dependence of the rate constant k_{obs} on the Glu59Gln concentration, plot showing the dependence on pH of the calculated pK_a value, two plots showing the dependence on pH of the rate constant k_{obs} and relative amplitude of the bimolecular reaction, and several plots showing how variation in any of the fitted parameters dramatically spoils the fitting. This material is available free of charge via the Internet at http://pubs.acs.org.

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