Electrostatic Effects on the Thermodynamics of Protonation of Reduced Plastocyanin

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The L12E, L12K, Q88E, and Q88K variants of spinach plastocyanin have been electrochemically investigated. The effects of insertion of net charges near the metal site on the thermodynamics of protonation and detachment from the copper(I) ion of the His87 ligand have been evaluated. The mutation-induced changes in transition enthalpy cannot be explained by electrostatic considerations. The existence of enthalpy/entropy (H/S) compensation within the protein series indicates that solvent-reorganization effects control the differences in transition thermodynamics. Once these compensating contributions are factorized out, the resulting modest differences in transition enthalpies turn out to be those that can be expected on purely electrostatic grounds. Therefore, this work shows that the acid transition in cupredoxins involves a reorganization of the H-bonding network within the hydration sphere of the molecule in the proximity of the metal center that dominates the observed transition thermodynamics and masks the differences that are due to protein-based effects.

Introduction

Blue copper proteins (cupredoxins) are electron carriers found in plants and bacteria and contain a four-coordinate copper ion that switches reversibly between the +1 and +2 redox states.[1–4] For several species, the solvent-exposed metal-bound histidine (His87 in spinach plastocyanin) protonates and detaches from the Cu^{I} ion at acidic pH values.[5–17] This triggers conformational changes that lead to the establishment of a favorable trigonal-planar metal coordination geometry.[6, 11, 14, 16] Here, we shall refer to the overall process as "acid transition". The consequent dramatic increase in the reduction potential of the copper center disables the protein functionally.[7, 9, 15] Therefore, the acid transition, which is fully reversible, has been proposed to play a physiological role as a molecular redox switch.[7, 9, 16] The pK_a value of the acid transition in cupredoxins is remarkably species-dependent, and follows the order: amicyanin $\gg$ plastocyanins $\geq$ phytocyanins $\gg$ azurin = rusticyanin.[17] For the last two species, the transition is not observed even at pH values as low as 2–3.[16, 18, 19] There is an open debate on the molecular factors that control the pK_a values, which have been proposed to include sequence features,[5, 9, 20–22] π–π stacking interactions,[7, 9] and solvent accessibility of the metal site.[23]

Gaining insight into the molecular factors that control the thermodynamics of the transition, which include formation/disruption of covalent bonds and weak interactions—the latter also involving solvent reorganization effects—is important for a deep characterization of the process. This information complements structural and kinetic data. We have previously shown that the thermodynamic driving force for the acid transition is enthalpic for the plastocyanins and entropic for the phytocyanins, whereas amicyanin is an intermediate case in which both enthalpic and entropic terms favor the transition.[17]

Here, we report on how the transition thermodynamics are influenced by electrostatic effects. In particular, we have investigated some variants of spinach plastocyanin (pc) in which Leu12 and Gln88 have been replaced with charged residues, Glu and Lys. These amino acids have been selected because of their proximity to the Cu-bound His87, which is involved in the acid transition.[24–26] Gln88 belongs to the acid patch on the eastern side of the protein and flanks His87, whereas Leu12 belongs to the hydrophobic patch on the northern side of the protein and is located exactly above the metal site, facing His87. Most importantly, the present mutations have been shown not to alter appreciably protein folding and the coordination features of the copper site.[26, 27] They are, therefore, appropriate means for probing electrostatic effects.

Results

Recombinant wild-type (wt) spinach plastocyanin and its L12E, L12K, Q88E, and Q88K mutants feature reduction potentials ($E^{\circ\prime}$) of +0.410, +0.393, +0.425, +0.369, and +0.393 V (vs. standard hydrogen electrode), respectively, at pH 7 and 25 °C.[26] For all species, $E^{\circ\prime}$ increases linearly upon lowering of the pH below 6, with a slope of approximately 50–60 mV per unit change in pH (Figure 1). This behavior indicates that Cu^{2+} reduction is coupled to a protonation process at the active site that involves the solvent-exposed Cu-binding His residue, which detaches from the metal.[9, 15, 17, 23, 28] The low-pH region of the $E^{\circ\prime}$/pH profiles was fitted to the following single acid–base equilibrium equation, which applies to the above conditions:[17, 23, 29]

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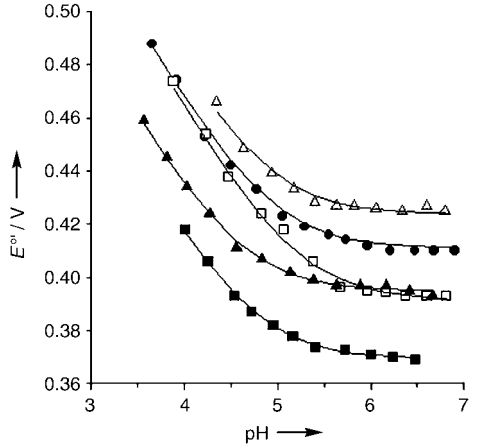

Figure 1. *pH dependence of the reduction potential for recombinant spinach plastocyanins: wt (●); L12E (□); L12K (△); Q88E (■); Q88K (▲). Solid lines are least-squares fits to Equation (1).* T = *298 K.*

$$E^{o\prime} = E^{o\prime}_{lim} + 2.3\,\frac{RT}{F}\log\left(1 + \frac{[H^+]}{K_a}\right) \quad (1)$$

where $E^{\circ\prime}_{lim}$ is the $E^{\circ\prime}$ limit value at high pH, and K_a (apparent, being measured at finite ionic strength) is the His proton dissociation constant for the reduced protein. Because of the deterioration of the voltammetric signals at low pH, measurements could not be performed below pH 3.5–4.

For all species, the pH dependence of $E^{\circ\prime}$ was measured at different temperatures in the range 5–35 °C. As an example, the families of $E^{\circ\prime}$ versus pH curves for wt plastocyanin and its Q88E variant are shown in Figure 2. Similar behaviors have been observed for all the other mutants. The corresponding van't Hoff plots are invariably linear and feature slopes that are in some cases remarkably different (Figure 3). The transition thermodynamics have been evaluated by using the integrated van't Hoff equation:

$$pK_a = \frac{\Delta H^{o\prime}_{AT}}{2.3\,R}\,\frac{1}{T} - \frac{\Delta S^{o\prime}_{AT}}{2.3\,R} \quad (2)$$

To facilitate discussion, it is convenient to refer to the *protonation* reaction. Therefore, the $\Delta H^{\circ\prime}_{AT}$ and $\Delta S^{\circ\prime}_{AT}$ values (where AT stands for acid transition) obtained from the least-squares fits of the pK_a versus $1/T$ plots to Equation (2) are listed in Table 1 with the *sign changed*.

The $\Delta S^{\circ\prime}_{AT}$ values for the recombinant species are always positive, whereas the sign of $\Delta H^{\circ\prime}_{AT}$ varies. Mutations at both positions invariably result in a remarkable increase in $\Delta H^{\circ\prime}_{AT}$ and $\Delta S^{\circ\prime}_{AT}$ as compared to the wild-type protein. However, the entropic contributions to the free-energy change of the acid transition at 298 K ($-T_{298}\Delta S^{\circ\prime}_{AT}$) are linearly correlated to the enthalpic terms ($-\Delta H^{\circ\prime}_{AT}$; Figure 4), with a slope of −1.04 (r = 0.998). This indicates that these changes almost perfectly compensate, thus resulting in only modest changes in the free energy of the transition. Since the intervals of variation of the entropy and enthalpy values are much greater than the experimental errors, this compensation effect is real and has no stat-

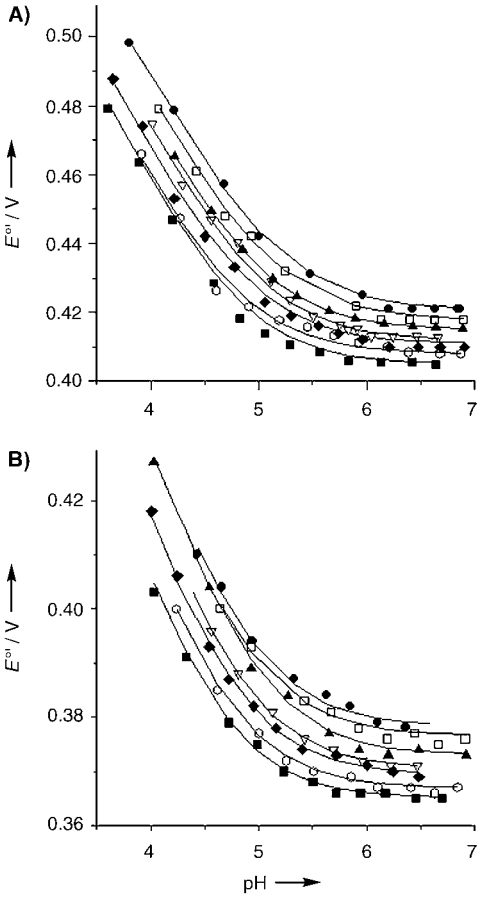

Figure 2. *E°′ versus pH for A) wt spinach plastocyanin and B) its Q88E mutant at different temperatures in 10 mM phosphate and 100 mM NaCl.* T = *5 (●), 10 (□), 15 (▲), 20 (▽), 25 (◆), 30 (open hexagon), and 35 °C (■). The pH values were corrected for the temperature effects.[41] Solid lines are least-squares fits to Equation (1).*

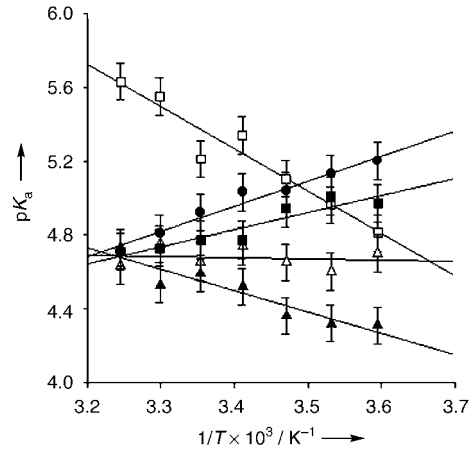

Figure 3. *Apparent pK_a values for the acid transition of spinach pc mutants as a function of 1/T (van't Hoff plot): wt (●); L12E (□); L12K (△); Q88E (■); Q88K (▲). Solid lines are least-squares fits to the data points. Please note that the transition enthalpies obtained from the slope of these plots refer to the deprotonation reaction. Those reported in Table 1 instead refer to the protonation reaction, hence the signs are reversed.*

Table 1. *Thermodynamic parameters for the acid transition (AT), which involves protonation at the metal site, for mutants of spinach platocyanin (pc).*[a]

	$\Delta H^{\circ\prime}_{AT}$[b] [kJ mol^{-1}]	$\Delta S^{\circ\prime}_{AT}$[b] [J K^{-1} mol^{-1}]	$-T\Delta S^{\circ\prime}_{AT}$[c] [kJ mol^{-1}]	$\Delta G^{\circ\prime}_{AT}$ [kJ mol^{-1}]	$\Delta\Delta G^{\circ\prime}_{AT}$[c,d] [kJ mol^{-1}] (mutant−wt)	pK_a[e]
Native pc[f]	−47	−77	+23	−24		4.2
Wild type pc	−26	+6	−2	−28	−4[g]	4.9
L12E	+44	+250	−75	−31	−3	5.2
L12K	+1	+93	−28	−27	+1	4.7
Q88E	−18	+32	−10	−28	0	4.8
Q88K	+22	+162	−48	−26	+2	4.6

[a] Values were obtained in 10 mM phosphate buffer, 100 mM sodium chloride. [b] Average error for $\Delta H^{\circ\prime}_{AT}$ and $-T\Delta S^{\circ\prime}_{AT}$ values is ±2 kJ mol^{-1}. [c] At 298 K. [d] These values would correspond to $\Delta\Delta H^{\circ\prime}_{conf}$ (see text). [e] The error affecting the pK_a values is ±0.1 pH units (determined from the standard deviation of the data fitting). [f] From ref. [17]. [g] This value refers to (wt−native).

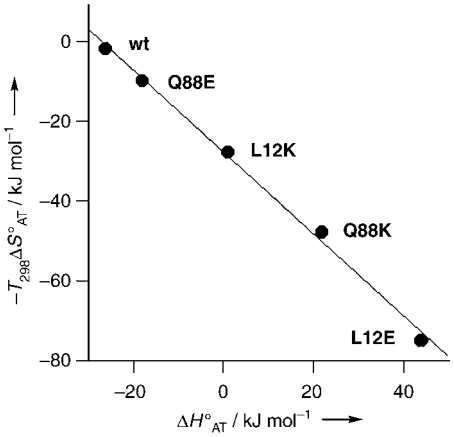

Figure 4. *Enthalpy versus entropy compensation plot at 298 K for the acid transition in spinach pc mutants. Error bars have the same dimension of the symbols. Solid lines are least-squares fits to the data points.*

istical origin.[30] A similar compensation behavior has been found previously for a number of native cupredoxins.[17]

Discussion

The acid transition in cupredoxins might or might not occur at physiologically meaningful pH values depending on kinetic and thermodynamic factors. The former originate from rotational barriers in the isomerization process, which mainly involve the imidazole ring of the histidine detached from the Cu^{I} ion.[31] The latter include the enthalpic and entropic changes that are due to covalent-bond breaking/formation, and changes in weak interactions that accompany the conformational changes of the polypeptide chain.[32] Other important contributions come from processes of solvent reorganization that involve changes in the hydrogen-bonding network within the hydration sphere of the molecule,[17] which are mainly localized in the hydrophobic patch that surrounds the solvent-exposed metal-binding histidine(s), as recently indicated by molecular-dynamics and -mechanics calculations.[26] The transition thermodynamics can be split into two terms that separate the protein-based contributions ($\Delta X^{\circ\prime}_{conf}$, where $X = G, H, S$) from solvent-reorganization effects ($\Delta X^{\circ\prime}_{sol}$):[19]

$$\Delta X^{o}\prime_{AT} = \Delta X^{o}\prime_{conf} + \Delta X^{o}\prime_{sol}$$

This approach is the key to interpreting the charge effects on the transition thermodynamics detected here, which—as rough data—are difficult to justify. In fact, several of the changes in $\Delta H^{\circ\prime}_{AT}$ are counterintuitive if treated with simple electrostatic considerations. These, in fact, would predict that a positive charge disfavors the protonation reaction, thereby inducing an increase in $\Delta H^{\circ\prime}_{AT}$, whereas a negative charge should induce the opposite effect. However, while the increase in $\Delta H^{\circ\prime}_{AT}$ observed for all mutants, as compared to the wt protein, is in agreement with the electrostatic expectation when lysines, which bear a positive charge, are introduced in position 12 and 88, this is not so for glutamic-acid insertion, the carboxylic side chains of which are characterized by at least a partial negative charge in the pH range studied. Regarding the mutation-induced changes in transition entropy, these differ remarkably and cannot be in any way approached in terms of protein-based effects.

These difficulties can be handled by exploiting an interpretative route that starts from the compensation pattern shown in Figure 4.[17] It has been demonstrated that for solution reactions, especially for those that occur in hydrogen-bonding solvents, solvent-reorganization effects, which are sensitive to the surface features of the molecule, do not contribute to the free energy of the overall process because the corresponding nonzero entropy and enthalpy changes *offset each other exactly, at any temperature* ($\Delta H^{\circ\prime}_{sol} = T\Delta S^{\circ\prime}_{sol}$; $\Delta G^{\circ\prime}_{sol} = 0$).[30,33–36] It follows that the observation of a compensation pattern for the *measured* $\Delta H^{\circ\prime}_{AT}$ and $\Delta S^{\circ\prime}_{AT}$ values for our protein series indicates that either both terms are dominated by solvent-reorganization effects (namely, $\Delta H^{\circ\prime}_{sol} > \Delta H^{\circ\prime}_{conf}$ and $\Delta S^{\circ\prime}_{sol} > \Delta S^{\circ\prime}_{conf}$) or that the solvent-based terms are comparable or even smaller than the protein-based terms, but the latter are highly conserved within the series. In both cases, however, solvent-reorganization effects dominate the *changes* in transition enthalpy and entropy along the series, but, because of compensation, they do not contribute to $\Delta\Delta G^{\circ\prime}_{AT}$. Therefore:

$$\Delta\Delta G^{o}\prime_{AT} = \Delta\Delta H^{o}\prime_{AT} - T\Delta\Delta S^{o}\prime_{AT} = \Delta\Delta H^{o}\prime_{conf} - T\Delta\Delta S^{o}\prime_{conf}$$

The term $\Delta\Delta S^{\circ\prime}_{conf}$ includes contributions that arise from equilibria among different rotamers of the protonated His side chain,[12] the increased mobility of chain segments in proximity to the metal site,[16] and formation of an additional H-bond of the protonated His in reduced plastocyanin.[6] In the present case, it is reasonable to assume that such structural alterations

and/or changes in the conformational degrees of freedom of the molecule hardly contribute to the differences in transition entropy between the mutants and the native protein. In fact, the mutated residues, Leu12 and Gln88, are located on the protein surface, with their side chains protruding toward the solvent,[24,26,27] and their replacement does not significantly alter the structural properties of the protein environment surrounding the metal.[26,27] Hence, to a first approximation, the term $\Delta\Delta S^{\circ\prime}_{conf}$ can be neglected. It follows that:

$$\Delta\Delta G^{o\prime}_{AT} = \Delta\Delta H^{o\prime}_{conf}$$

Therefore, the difference in transition free energy would coincide with the difference in transition enthalpy that is due to bond-formation/breaking processes and changes in the electrostatics at the metal site.

Several considerations apply here. The $\Delta\Delta G^{\circ\prime}_{AT}$ ($=\Delta\Delta H^{\circ\prime}_{conf}$) values (Table 1) are in agreement with simple electrostatic considerations. In fact, the introduction of a negative charge enthalpically favors the protonation reaction. This induces negative $\Delta\Delta G^{\circ\prime}_{AT}$ ($=\Delta\Delta H^{\circ\prime}_{conf}$) values, as is the case for the L12E mutant compared to wt pc and of wt pc compared to native pc (recombinant wt pc indeed contains an Asp residue in position 8 in place of a glycine in the native protein[37]). The opposite effect is observed upon insertion of a positive charge (see L12K and Q88K). We note that although the individual $\Delta\Delta G^{\circ\prime}_{AT}$ values are comparable to the uncertainty that affects the free-energy change (Table 1), the above analysis holds for all the data, with no exceptions. This means that we are observing significant effects. Analogous results have been obtained from the analysis of the effects of these mutations on the thermodynamics of the reduction reaction.[26] Moreover, these findings tell us that the enthalpic balance of the other processes of bond breaking/formation in the transition is very similar in the mutated and wt species. Finally, we note that the $\Delta\Delta G^{\circ\prime}_{AT}$ ($=\Delta\Delta H^{\circ\prime}_{conf}$) values are small (or null, as for Q88E). This is rather surprising if we consider the proximity of the mutated residues to the metal center, but it is certainly the result of the suppression of the electrostatic interaction between the solvent-exposed charge and the metal due to the high dielectric constant of water. The present observations are in agreement with previous experimental data and theoretical calculations on the changes in pK_a of blue copper proteins due to electrostatics effects.[38,39]

In conclusion, this work shows that the acid transition in cupredoxins involves a reorganization of the H-bonding network within the hydration sphere of the molecule in the proximity of the metal center that dominates the observed transition thermodynamics but does not contribute to the resulting free-energy change due to exact *H*/*S* compensation. These contributions mask the differences in transition thermodynamics that are due to protein-based effects, which can be recognized only after the solvent-based terms are factorized out. The pK_a value of this transition in cupredoxins thus appears to be ultimately controlled only by the features of the coordination sphere of the copper ion and its immediate environment, which determine the protein-based enthalpy change. The solvent accessibility of the metal site might play a role as one of the factors that influence the relative affinity of the imidazole nitrogen of the C-terminal histidine ligand toward the proton and the Cu^{I} ion. Transition-induced changes in conformational degrees of freedom of the peptide spacers among the copper ligands are likely to dominate the modest protein-based entropy change.

Experimental Section

Recombinant wt spinach pc and the L12E, L12K, Q88E, and Q88K mutants were isolated and purified as described elsewhere.[26,40] Cyclic voltametry experiments were performed at varying pH and temperature as described in ref. [17].

Abbreviations

$E^{\circ\prime}$: standard reduction potential; $\Delta H^{\circ\prime}_{AT}$, $\Delta S^{\circ\prime}_{AT}$: overall enthalpy and entropy changes, respectively, for His protonation and associated conformational change.

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