Thermodynamics of the Acid Transition in Blue Copper Proteins†

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ABSTRACT: The thermodynamic parameters of the conformational transition occurring at low pH (acid transition, AT) in blue copper proteins, involving protonation and detachment from the Cu(I) ion of one histidine ligand, have been determined electrochemically for spinach and cucumber plastocyanins, *Rhus vernicifera* stellacyanin, cucumber basic protein (CBP), and *Paracoccus versutus* amicyanin. These data were obtained from direct protein electrochemistry experiments carried out at varying pH and temperature. For all species but CBP, the overall conformational change turns out to be exothermic. The entropy change is remarkably species-dependent. In particular, we found that (i) the balance of bond breaking/formation favors the acid transition in plastocyanins, which show remarkably negative $\Delta H^{\circ\prime}_{AT}$ values, and (ii) the transition enthalpy turns out to be much less negative (or even positive) for the two phytocyanins (stellacyanin and CBP): for these species, the transition turns out to be observable thanks to the favorable (positive) entropy change. Thus, it is apparent that the thermodynamic "driving force" for this transition is enthalpic for the plastocyanins and entropic for the phytocyanins. Amicyanin is an intermediate case in which both enthalpic and entropic terms favor the transition. Under the assumption that the transition entropy originates from solvent reorganization effects, which are known to involve compensative enthalpy and entropy changes, the free energy change of the transition would also correspond to the enthalpy change due to bond breaking/formation in the first coordination sphere of the metal and in its immediate environment. Indeed, this term turns out to be very similar for the proteins investigated, in line with the conservation of the Cu(I)−His bond strengths in these species, except for amicyanin, for which the greater exothermicity of the transition can be ascribed to peculiar features of the active site.

Copper and iron proteins serving as biological electron carriers are known to undergo pH-induced conformational transitions involving changes in the first coordination sphere of the metal. In particular, the so-called alkaline transition in oxidized class I cytochromes *c* and the acid transition in reduced blue copper proteins have been thoroughly investigated by spectroscopic and structural means (*1*−*16*). For cytochromes *c*, additional low-spin Fe(III) conformers are known to form when the pH is raised above 8, in which the native axial methionine ligand is replaced by a surface lysine, the ligand exchange being triggered by deprotonation of an as yet unidentified residue (*14*, *15*). In reduced blue coppers at acidic pH values, the solvent-exposed metal-bound histidine undergoes protonation and a series of conformational changes that lead to its dissociation from the cuprous ion and to the establishment of an approximately trigonal planar metal coordination geometry (*3*, *6*, *11*, *17*−*19*). In both cases, ligand replacement/detachment from the metal causes a dramatic change in the reduction potential, responsible for the redox inactivation of the protein, in the sense that it becomes unable to carry out its physiological function. For that reason, hypotheses have been put forward on the possible physiological role of these transitions (which are both reversible) as biological redox switches (*1*, *13*).

The thermodynamics of these processes have been investigated in less detail in the past, although they provide information on the balance of chemical bond formation/disruption and on protein and solvent reorganization effects, which complements the structural and kinetic data and which can be used to more fully characterize these transitions. We recently measured the thermodynamics of the alkaline transition in mitochondrial and bacterial cytochromes *c*, which were found to be remarkably species-dependent, and analyzed their modulation by medium properties (*16*, *20*−*23*). Here, we focus on the thermodynamics of the acid transition in a number of blue copper proteins, which we determined through direct protein electrochemistry experiments carried out at varying pH and temperature. To our knowledge, these data are unprecedented. They help in understanding the molecular factors that control the differences in the apparent pK_a of this transition in different blue copper proteins and that are the subject of some debate. They highlight the role of entropic factors, which are mostly related to solvent reorganization effects.

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EXPERIMENTAL PROCEDURES

Proteins. Native plastocyanins from spinach and cucumber, *Rhus vernicifera* stellacyanin, the copper basic protein from cucumber (CBP),[1] and recombinant *Paracoccus versutus* amicyanin were isolated and purified as described elsewhere (*24*−*28*). All chemicals were reagent-grade and were used without further purification. Nanopure water was used throughout.

Electrochemical Measurements. Cyclic voltammetry experiments (CV) were performed with a potentiostat/galvanostat PAR model 273A. A 1 mm diameter pyrolitic graphite disk (PGE) was used as working electrode, a saturated calomel electrode as reference electrode, and a 5 mm diameter Pt as a counter electrode. Potentials were calibrated against the MV^{2+}/MV^{+} couple (MV = methyl viologen) (*29*). All the redox potentials reported in this paper are referred to the standard hydrogen electrode. The electric contact between the reference electrode and the working solution is obtained with a Vycor set. All measurements were carried out under argon in a cell for small volume samples ($V = 0.5$ mL) under thermostatic control. Scan rates varied from 0.02 to 0.5 V s^{-1}. The cleaning procedure of the working electrode is crucial to the voltammetric response. The PGE was first treated with anhydrous ethanol for 5 min, then polished with alumina (BDH, particle size about 0.015 μm) water slurry on cotton wool for 7 min; finally the electrode was treated in an ultrasonic pool for about 5 min and used without further treatment. Modification of the electrode surface was performed by dipping the polished electrode into a 1 mM solution of polylysine and morpholine for 30 s and then rinsing it with Nanopure water. Sodium phosphate (10 mM) and 100 mM NaCl were used as base electrolytes. Protein samples were freshly prepared before use and their concentration, in general about 0.1 mM, was checked spectrophotometrically. The pH was changed by adding small amounts of concentrated NaOH or HCl under fast stirring. A single voltammetric wave was observed for all species, which was either reversible or quasi-reversible. Peak separation in CV experiments varied from 60 to 90 mV for scan rates in the range 0.02−0.2 V s^{-1}. Anodic and cathodic peak currents were almost identical and both were proportional to protein concentration and $v^{1/2}$ (v = scan rate), indicating a diffusion-controlled electrochemical process. Given the reversibility or quasi-reversibility of the electrochemical process, the symmetrical shape of the voltammograms, and the almost negligible influence of the scan rate on the half-wave potentials, the $E_{1/2}$ values (taken as the average of the cathodic and anodic peak potentials) can be confidently assumed as the $E^{\circ\prime}$ values. The experiments were performed several times and the reduction potentials were found to be reproducible within ±2 mV. In the pH titrations at different temperatures the reference electrode was kept at constant temperature (21 ± 0.1 °C), while the half-cell containing the working electrode and the Vycor junction to the reference electrode were under thermostatic control, and the temperature was varied from 5 to 35 °C.

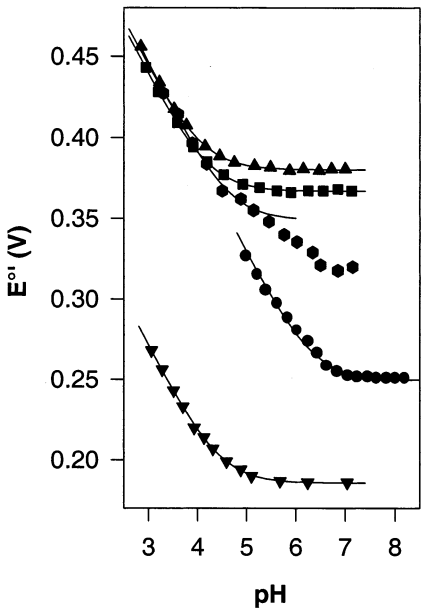

FIGURE 1: pH dependence of the reduction potential of *T. versutus* amicyanin (●), cucumber plastocyanin (■), spinach plastocyanin (▲), *R. vernicifera* stellacyanin (▼), and CBP (⬢). Solid lines are least-squares fits to eq 1. $T = 298$ K.

RESULTS

At pH 7 and 25 °C, cucumber and spinach plastocyanins, CBP, *R. vernicifera* stellacyanin, and *P. versutus* amicyanin yield an electrochemically reversible or quasi-reversible, one-electron and diffusion-controlled voltammetric signal at potentials ($E^{\circ\prime}$) of +370, +382, +320, +187, and +248 mV (vs SHE), respectively, due to the $Cu^{2+/1+}$ equilibrium of the copper center, in agreement with the values obtained elsewhere (*1*, *30*−*32*). As the pH is lowered below 6 (below 7 for amicyanin), the reduction potential invariably increases (Figure 1). In the low-pH region (from below 6 for amicyanin to below 3.8 for CBP), the $E^{\circ\prime}$/pH profile shows a slope of approximately 50−60 mV/pH. This behavior has been observed elsewhere for plastocyanins (*1*, *4*, *10*, *13*), amicyanin (*32*, *33*), and phytocyanins such as CBP, plantacyanin from spinach, and mavicyanin (*31*, *34*, *35*) and indicates that copper(II) reduction is coupled to a protonation process at the active site. Such protonation occurs at the solvent-exposed Cu-binding His residue, which detaches from the metal, as previously shown by crystallographic data for cuprous poplar plastocyanin (*3*). The low-pH region of the curves in Figure 1 was fitted to the following single acid−base equilibrium equation, which applies to the above condition (*1*):

$$E^{\circ\prime} = E^{\circ\prime}_{\mathrm{lim}} + 2.3\frac{RT}{F}\log\left(1 + [\mathrm{H}^{+}]/K_{\mathrm{a}}\right) \qquad (1)$$

where $E^{\circ\prime}_{\mathrm{lim}}$ is the limit $E^{\circ\prime}$ value at high pH and K_a is the apparent His proton dissociation constant for the reduced protein. For all species the voltammetric response deteriorates at low pH (measurements could be extended down to pH 3 for the two plastocyanins, CBP and Sc, whereas no reliable data could be collected below pH 5 for amicyanin). Therefore, especially for those species containing a more acidic

[1] Abbreviations: $\Delta H^{\circ\prime}_{\mathrm{AT}}$, overall enthalpy change for His protonation and associated conformational change; $\Delta S^{\circ\prime}_{\mathrm{AT}}$, overall entropy change for His protonation and associated conformational change; $\Delta H^{\circ\prime}_{\mathrm{rc}}$, enthalpy change for reduction; $\Delta S^{\circ\prime}_{\mathrm{rc}}$, entropy change for reduction; $E^{\circ\prime}$, standard reduction potential; CV, cyclic voltammetry; PGE, pyrolytic graphite edge electrode; SCE, saturated calomel electrode; SHE, standard hydrogen electrode; pc, plastocyanin; CBP, copper basic protein from cucumber, Sc, *Rhus vernicifera* stellacyanin.

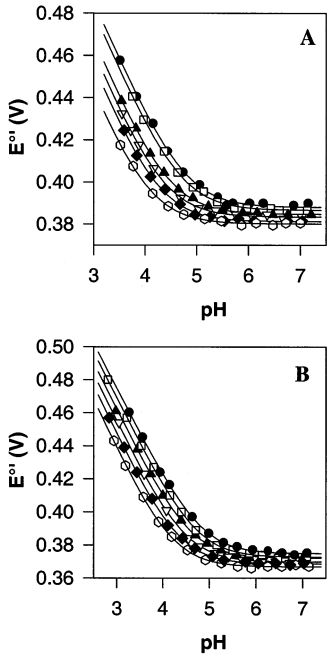

FIGURE 2: pH dependence of the reduction potential for plastocyanins from spinach (A) and cucumber (B) at different temperatures in 10 mM phosphate and 100 mM NaCl. T = 5 (●), 10 (□), 15 (▲), 20 (▽), 25 (◆), and 30 (⬠) °C. The pH values were corrected for the temperature (*56*). Solid lines are least-squares fits to eq 1.

binding His, the apparent pK_a values determined here must be affected by a rather conspicuous error, which we estimate to be ±0.2 for CBP and the two plastocyanins [which is much higher than that (±0.1 pH units) determined from the standard deviation of the data fitting] and ±0.1 for amicyanin and *R. vernicifera* stellacyanin.

An analogous increase in E°′ at low pH has been observed for *Alcaligenes faecalis* S-6 pseudoazurin. The data (not shown) are consistent with those reported previously by Dennison et al. for the species from *Achromobacter cycloclastes* (*10*, *36*). An equilibrium with pK_a^{red} and pK_a^{ox} values of 6.9 ± 0.1 and 6.5 ± 0.1, respectively (at 298 K), has been observed, which is likely due to the conserved uncoordinated surface His6 residue (*36*). The presence of this equilibrium, which apparently also affects the axial Cu−Met bond (*36*), and the deterioration of the voltammetric signal below pH 4.5 prevented a safe determination of the pK_a value of the acid transition for this species, occurring at lower pH, at least at the level of confidence needed for the variable temperature study.

For all species, the pH dependence of $E^{\circ\prime}$ was measured at varying temperatures in the range 5−35 °C at constant ionic strength (10 mM phosphate + 0.1 M NaCl). The families of $E^{\circ\prime}$/pH curves determined at different temperatures for some of the species under investigation are shown in Figure 2. The van't Hoff plots are illustrated in Figure 3. The pK_{app} values increase linearly with increasing temperature for all species but CBP. The transition thermodynamics can then be evaluated from the integrated van't Hoff equation:

$$pK_{app} = \left(\frac{\Delta H^{\circ\prime}_{AT}}{2.3R}\right)\left(\frac{1}{T}\right) - \frac{\Delta S^{\circ\prime}_{AT}}{2.3R} \qquad (2)$$

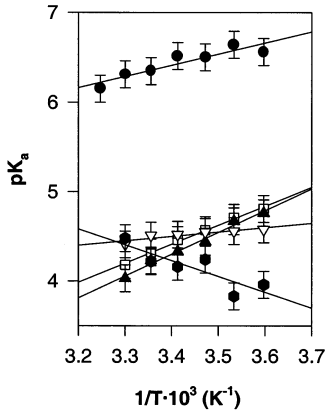

FIGURE 3: Apparent pK values for the acid transition of various blue copper proteins as a function of 1/T (van't Hoff plot): *P. versutus* amicyanin (●); cucumber plastocyanin (□); spinach plastocyanin (▲); *R. vernicifera* stellacyanin (▽); CBP (⬢). Solid lines are least-squares fits to the data points. Please note that the transition enthalpies obtained from the slope of these plots refer to the deprotonation reaction. Those reported in the text instead refer to the protonation reaction, hence the signs are reversed.

Since we find it more convenient to refer to the *protonation* reaction, we here report the transition enthalpies and entropies obtained from the least-squares fits of the pK_a vs 1/T plots to eq 2 with the *sign changed*. Thus, the $\Delta H^{\circ\prime}_{AT}$ and $\Delta S^{\circ\prime}_{AT}$, where AT stands for acid transition, refer to the overall conformational change occurring upon lowering the pH. The $\Delta H^{\circ\prime}_{AT}$ and $\Delta S^{\circ\prime}_{AT}$ values for the various species are listed in Table 1. The acid transition turns out to be exothermic for all species but CBP, whereas the sign of the entropy change is more variable.

DISCUSSION

The various cupredoxins show different susceptibilities to low-pH redox inactivation due to the coupling of electron and proton uptake at the copper site. Such a propensity is directly related to the apparent pK_a value of the solvent-exposed C-terminal Cu-binding histidine, which follows the order amicyanin ≫ plastocyanins ≥ phytocyanins ≫ azurin = rusticyanin. In particular, the latter two species appear not to undergo pH-induced changes in copper(I) coordination at pH values as low as 2−3 (*1*, *17*, *37*, *38*). Whether the acid transition takes place or not (at pH values that allow preservation of a reasonable degree of protein integrity) depends on thermodynamic and kinetic factors. The latter, which have been recently proposed to play a dominant role, originates from rotational barriers in the isomerization process (*39*). Transition thermodynamics are related to the enthalpic and entropic balance of bond breaking/formation and protein and solvent reorganization processes. For those species in which the transition occurs at meaningful pH values (in the sense stated above), comparison of transition enthalpies and entropies helps gaining insight into the molecular factors that underlie the variability in the apparent pK_a values. This issue has been extensively debated since the seminal contribution by Freeman and co-workers (*3*), which showed that reduced poplar plastocyanin at pH 3.8 exhibits at the nearly trigonal planar copper site formed by the His37, Cys84 and Met92 ligands, as a result of protonation, detachment from the metal and rotation of the imidazole ring of His87. Attempts have been made to correlate the apparent pK_a values to sequence features

Table 1: Thermodynamic Parameters for the Acid Transition (AT) (Involving *Protonation* at the Metal Site) of Blue Copper Proteins from Different Sources[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})	$\Delta G^{\circ\prime}_{AT}$[c,d] (kJ mol^{-1})	pK_a, calc[c]	pK_a, exp[c]
P. versutus amicyanin	−24	+42	−12	−36	6.4	6.4
cucumber plastocyanin	−41	−54	+16	−25	4.4	4.4
spinach plastocyanin	−47	−77	+23	−24	4.2	4.2
R. vernicifera stellacyanin	−11	+49	−15	−27	4.5	4.5
CBP	+34	+197	−59	−25	4.3	4.2

[a] Values were obtained in 10 mM phosphate buffer with 100 mM sodium chloride. [b] Average errors on $\Delta H^{\circ\prime}_{AT}$ and $\Delta S^{\circ\prime}_{AT}$ values are ±2 kJ mol^{-1} and ±6 J mol^{-1} K^{-1}, respectively. [c] At 298 K. [d] These values would also correspond to the enthalpic effect of transition-induced changes in protein structure, under the assumption that solvent reorganization effects yield perfectly compensating enthalpy and entropy changes (see text).

involving the copper ligands, bonding properties of the first coordination sphere of the metal, and solvent accessibility of the copper site. In particular, the presence of only two residues between the cysteine and the C-terminal histidine has been proposed to be a requisite for the acid transition to occur (*2*, *10*), although recent loop-directed mutagenesis studies on amicyanin have shown that this might represent an oversimplification of the real effectors *(40)*. Moreover, following the results obtained for phytocyanins, the importance of solvent accessibility of the binding histidine(s) has been pointed out (*31*, *35*). It has also been suggested that the number of N−H···S(Cys) H-bonds formed by the Cu-binding cysteine affect the acid transition. In particular, the presence of two such bonds would stabilize the high-pH four-coordinate Cu(I) site, disfavoring the low-pH His dissociation (*41*). However, additional factors related to peculiar structural properties of the species may affect this transition, such as the stabilizing $\pi-\pi$ stacking interaction involving the C-terminal His and an adjacent Phe residue, which has been suggested to be responsible for the absence of detectable low-pH change in Cu(I) coordination (at pH 4) in a plastocyanin from the fern *Dryopteris crassirhizoma* (*4*).

According to the structural data obtained for poplar plastocyanin (*3*), the processes of covalent bond breaking/formation in the immediate copper(I) environment involved in the acid transition include dissociation of the solvent-exposed histidine ligand from the Cu(I) ion, its protonation, and the strengthening of the Cu(I)−S(Met) bond. Regarding noncovalent interactions, rotation of the protonated imidazole ring of the above His residue induces the formation of an additional H-bond. Changes in weak interactions involving internal residues elsewhere in the protein are very small or negligible (*3*). Most importantly, it turns out that most of the residues showing (limited) pH-induced changes are solvent-accessible. Thus, a reorganization of the H-bonding network in the hydration sphere of the molecule must occur. Therefore, the transition thermodynamics can be partitioned into two main terms arising from changes in protein conformation, highly localized at the metal center ($\Delta G^{\circ\prime}_{conf}$), and solvent reorganization effects ($\Delta G^{\circ\prime}_{solv}$).

Transition Enthalpy. For all species but CBP, the overall process results to be exothermic. No data are available at present that would allow partition of the overall transition enthalpy ($\Delta H^{\circ\prime}_{AT}$) into the individual contributions ($\Delta H^{\circ\prime}_{solv}$ and $\Delta H^{\circ\prime}_{solv}$). It is a fact that the $\Delta H^{\circ\prime}_{AT}$ values of Table 1 are difficult to rationalize with arguments of coordination chemistry. For example, the two phytocyanins CBP and Sc turn out to be the species for which the acid transition is more enthalpically disfavored. This result cannot be accounted for clearly in terms of metal coordination features. In fact, the stronger axial Cu bond formed by the methionine sulfur and the glutamine oxygen in CBP (2.6 Å) (*42*) and Sc (2.2 Å) (*43*) as compared to that in amicyanin (2.8 Å) (*44*) and plastocyanin (2.9 Å) (*3*) should weaken the Cu−His bond. Moreover, solvent exposition of both metal-binding histidines in the phytocyanins would be expected to facilitate protonation. Therefore, this would suggest that the determinants of the differences in transition enthalpy among the cupredoxins do not involve the first coordination sphere of the copper ion. Net protein charge is expected to play a role. Indeed, the highly basic nature of the two phytocyanins is consistent with the greater transition enthalpy with respect to the other cupredoxins. However, we believe that solvent reorganization effects are center stage here. The discussion below will strengthen this view. It is also worthy of note that the transition enthalpy determined for these plastocyanins is very similar to the activation enthalpy measured for oxidation of the acid form of reduced ruthenium-modified green algae *Scenedesmus obliquus* plastocyanin ($\Delta H^{\#} = +43$ kJ mol^{-1}) due to intramolecular electron transfer from Cu^+ to the Ru^{3+} bound to a surface histidine (*45*). This activation barrier is due to the transition of the Cu^+ structure from trigonal to distorted tetrahedral (upon proton loss), which likely precedes fast $Cu^+ \rightarrow Ru^{3+}$ electron transfer and hence oxidation to the Cu^{2+} blue state (*45*). The close similarity of these data indicates that the activation barrier for the backward process (reduction plus protonation), which we are considering here, must be small for plastocyanins. This support the hypothesis that observation of the acidic transition in cupredoxins is controlled by kinetics (*39*).

Transition Entropy. From the data of Table 1, it is clearly apparent that the entropic contribution to the free energy change of the acid transition is very important. The magnitude of the entropic term compared to the enthalpic contribution ranges from approximately 10% for spinach pc to 50% for cucumber pc and amicyanin, up to approximately 200% for CBP. The sign of $\Delta S^{\circ\prime}_{AT}$ is variable among the various species. The entropic term disfavors the transition in plastocyanins but favors it in amicyanin and, mostly, in phytocyanins. In particular, it turns out that the thermodynamic driving force for this transition is enthalpic for the plastocyanins and entropic for the phytocyanins. Amicyanin is an intermediate case in which both enthalpic and entropic terms favor the transition. The determinants of the reaction entropy cannot be determined safely at present. Contributions arising from events occurring at the site, such as the equilibrium between different rotamers of the protonated His side chain found for amicyanin (*7*), the increased mobility of chain

segments in proximity of the site, as observed in the reduced H117G azurin mutant (*17*), or the formation of an additional H-bond of the protonated His in reduced plastocyanin (*3*), may reasonably play a role. By analogy with the structural data obtained for reduced plastocyanin (*3*), pH-induced structural changes occurring elsewhere in the molecule are likely to be small, and hence they hardly contribute per se to the reaction entropy. The large variability of the transition entropies along the series of proteins considered here is difficult to reconcile with the rather conserved gross features of the site and its immediate environment. The fact that the pH-induced changes involve residues which are, or become, accessible to the solvent suggests that the main determinants of the reaction entropy are likely to be changes in the H-bonding within the hydration sphere of the molecule, probably localized for the most part on the hydrophobic patch surrounding the solvent-exposed metal-binding histidine(s). Thus, on qualitative grounds, we may tentatively relate the positive transition entropy for the phytocyanins to the greater exposition of the copper site to the solvent for these species, in which both metal-binding histidines are accessible to the solvent.

Interpretation of the Thermodynamic Data. Following the considerations offered above, we believe that it is not unreasonable to consider, to a first approximation, the measured entropy changes as contributed entirely by solvent reorganization effects occurring at the protein surface. It has been shown that changes in the H-bonding network within the hydration sphere of the molecule associated with chemical events occurring in biomolecules in general involve compensative enthalpic and entropic changes at any temperature (*46*−*53*). Therefore, if we assume perfect compensating behavior for the portion of transition enthalpy and entropy due to solvation effects, the $\Delta G^{\circ\prime}_{AT}$ values would correspond to the contribution of first coordination sphere effects to the overall transition enthalpy ($\Delta H^{\circ\prime}_{conf}$). These positions can be summarized as follows:

$$\Delta H^{\circ\prime}_{AT} = \Delta H^{\circ\prime}_{conf} + \Delta H^{\circ\prime}_{sol}$$

$$\Delta S^{\circ\prime}_{AT} = \Delta S^{\circ\prime}_{conf} + \Delta S^{\circ\prime}_{sol} \approx \Delta S^{\circ\prime}_{sol}$$

$$\Delta G^{\circ\prime}_{AT} = (\Delta H^{\circ\prime}_{conf} + \Delta H^{\circ\prime}_{sol}) - T\Delta S^{\circ\prime}_{sol}$$

since

$$\Delta H^{\circ\prime}_{sol} = T\Delta S^{\circ\prime}_{sol}$$

then

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

In other words, this approach corresponds to considering the influence of solvation changes on the overall free energy change of the transition to be negligible, because of compensative enthalpy−entropy changes. We realize that this may represent an oversimplification. However, we note that the resulting $\Delta H^{\circ\prime}_{conf}$ ($=\Delta G^{\circ\prime}_{AT}$) values are similar for plastocyanins and phytocyanins, in keeping with the gross similarity of copper−His ligation in these species and the environment of the metal site. It is apparent that dissociation of the Cu(I)−His bond turns out to be somewhat more favored in stellacyanin, consistent with the weakening effect of Gln on the C-terminal His−Cu(I) bond noted elsewhere (*54*). Such an effect due to the strong axial ligation is not apparent for CBP, but this might be due to the roughness of the above approximation. *Therefore, solvent reorganization effects influence the individual values of* $\Delta H^{\circ\prime}_{AT}$ *and* $\Delta S^{\circ\prime}_{AT}$ *but have no net effect on the free energy of the transition, which is mainly linked to the strength of the Cu*−*His bond to be broken and to its immediate environment.* This is clearly supported by the H/S compensation plot shown in Figure 4, which demonstrates the overriding influence of solvent reorganization effects on *the variations* in transition thermodynamics among the different blue coppers. In fact, the almost perfect enthalpy−entropy correlation (indicated by the slope close to unity) demonstrates that the *changes* in transition entropy and enthalpy along the series are dominated by solvent reorganization effects at $T = 298$ K, namely

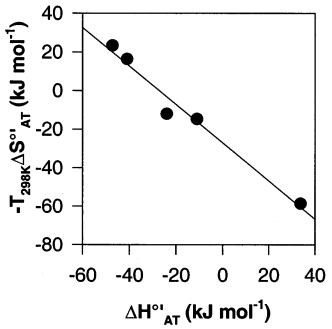

FIGURE 4: Enthalpy/entropy compensation plot at 298 K for the acid transition in blue copper proteins. Error bars have the same dimension of the symbols. Solid lines are least-squares fits to the data points.

$$\Delta\Delta H^{\circ\prime}_{AT} = \Delta\Delta H^{\circ\prime}_{sol} \; (\gg \Delta\Delta H^{\circ\prime}_{conf})$$

$$\Delta\Delta S^{\circ\prime}_{AT} = \Delta\Delta S^{\circ\prime}_{sol} \; (\gg \Delta\Delta S^{\circ\prime}_{conf})$$

This compensation effect is real and has no statistical origin, since the range of variation of the entropy and enthalpy values is much greater than the experimental errors associated with their determination (*51*, *52*).

Amicyanin falls somewhat off the plot. Thus, the overall process involving bond breaking/formation localized at the copper(I) site turns out to be more exothermic and responsible for the higher apparent pK_a. The determinant of this effect cannot be clearly defined at present, and further investigations are needed. The composition of the medium may play a role, through specific protein−ion interactions; in fact, it has been observed that protonation of His96 of amicyanin is influenced by phosphate (*55*).

CONCLUSIONS

The free energy change of the acid transition for plastocyanins and phytocyanins, and hence their apparent pK_a values, are very similar. However, transition enthalpies and entropies differ sensibly. Due to the lack of structural data in solution and in the crystal state for both high- and low-pH forms for most species, the molecular determinants of the $\Delta H^{\circ\prime}_{AT}$ and $\Delta S^{\circ\prime}_{AT}$ values are at present intractable. However, the most striking correlation between the transition thermodynamics and structural and sequence features of cupredoxins is that the subclass of phytocyanins undergo the acid transition at pH values comparable to those of plasto-

cyanins, despite the unfavorable enthalpy change, thanks to a remarkably favorable (positive) entropy change, possibly related to the greater solvent accessibility of the metal site. The observation that $\Delta H^{\circ\prime}_{AT}$ can be hardly explained in terms of properties of the first coordination sphere of the metal, in which bond breaking and formation are localized, suggest that solvent-based enthalpy/entropy compensation effects are operative. Therefore, one possible interpretation of the data is that transition-induced solvent reorganization effects are different in the different species: they induce different values of $\Delta H^{\circ\prime}_{AT}$ and $\Delta S^{\circ\prime}_{AT}$, but they are likely to be compensative. Therefore, the ultimate determinants of the free energy change reside mostly in the immediate metal environment. In fact, once the solvent-based enthalpy changes are factored out, the $\Delta H^{\circ\prime}_{conf}$ values turn out to be comparable for the various species. This is why there are small differences in the apparent pK_a values for the various species, in which the copper−histidine bond strengths are rather conserved, as well as the immediate protein environment. An analogous behavior has been observed for oxidized class I cytochromes *c*, for which the variability of the ΔG° of the alkaline transition among species from different sources is much smaller than that of the enthalpic and entropic terms (*22*). Thus, also in this case solvent reorganization effects dominate the changes in transition thermodynamics within a homologous protein series.

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