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ORIGINAL PAPER

Thermodynamics of the alkaline transition in phytocyanins

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Abstract The thermodynamics of the alkaline transition which influences the spectral and redox properties of the type 1 copper center in phytocyanins has been determined spectroscopically. The proteins investigated include *Rhus vernicifera* stellacyanin, cucumber basic protein and its Met89Gln variant, and umecyanin, the stellacyanin from horseradish roots, along with its Gln95Met variant. The changes in reaction enthalpy and entropy within the protein series show partial compensatory behavior. Thus, the reaction free energy change (hence the pK_a value) is rather variable. This indicates that species-dependent differences in reaction thermodynamics, although containing an important contribution from changes in the hydrogen-bonding network of water molecules in the hydration sphere of the protein (which feature enthalpy–entropy compensation), are to a large extent protein-based. The data for axial ligand variants are consistent with the hypothesis of a copper-binding His as the deprotonating residue responsible for this transition.

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Introduction

A number of pH-dependent active-site conformational changes have been identified in electron transfer (ET) metalloproteins. Such transitions have been extensively studied for *c*-type cytochromes and blue copper proteins, which possess easy-to-follow physicochemical properties [spectral features, reduction potential ($E°$), kinetics, etc.] [1–33]. These pH-induced alterations are of great importance since they may act as molecular switches for ET reactivity in physiological processes [3, 5, 11, 17, 20, 28, 34–36] and could possibly induce protein changes similar to those occurring during folding or aggregation [14, 22, 28] and also upon interaction with natural redox partners [12, 33, 37–39].

The phytocyanins form a subclass of the single-domain blue (type 1) copper proteins, the so-called cupredoxins [40, 41], and have been divided into the stellacyanins, plantacyanins and uclacyanins [41]. For the present study we focus on stellacyanins and plantacyanins, with the important difference between these being the presence of an axial Gln ligand [42–44] in the former in place of Met in plantacyanins [45, 46] and almost all other cupredoxins (Fig. 1) [40]. All stellacyanins and plantacyanins that have been studied to date have been found to undergo an alkaline transition which influences the absorption [1, 4, 10, 16, 23, 24, 28, 29], electron paramagnetic resonance and electron–nuclear double resonance [1, 6, 28], NMR [10, 16, 26, 28] and resonance Raman [29] spectra, and thus must alter the active-site structure. In all cases this is a completely reversible transition and is a different process from

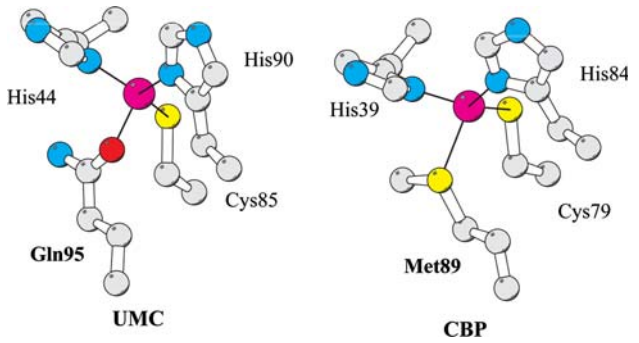

Fig. 1 Copper site structures of umecyanin (*UMC*; the stellacyanin from horseradish roots) [43] and cucumber basic protein (*CBP*; a plantacyanin) [45] highlighting the differences in axial ligation in these two phytocyanins. The structure of the stellacyanin from *Rhus vernicifera* (RST) has not been determined but the stellacyanin from cucumber [42] has an active-site geometry almost identical to that of UMC. In the phytocyanins both of the His ligands are solvent-exposed but His90 in UMC and His84 in CBP are more accessible and are therefore the most likely deprotonating groups responsible for the alkaline transition

that recently described for the cupredoxins azurin and plastocyanin [47]. The alkaline transition also influences $E°$ and the observed pK_a value is species-dependent and ranges from 9 to 10 [15, 16, 23, 28]. Identification of the deprotonating group responsible for this effect has proved to be less than trivial. Among the ligands, the axial Gln found in the stellacyanins could switch from $O^{\varepsilon 1}$ to $N^{\varepsilon 2}H^-$ coordination at high pH [6, 48]. However, this possibility has been excluded by NMR studies of Ni(II) umecyanin (UMC; the stellacyanin from horseradish roots) [26], the investigation of site-directed mutants [28], and also the observation of this effect in the plantacyanins [28]. A conserved Lys residue adjacent to the axial ligand was also considered as a likely candidate for triggering the alkaline transition [16, 49–51], but again mutagenesis studies have disproved this idea [28]. The $N^{\varepsilon 2}H$ moiety of one of the two solvent-exposed His ligands was suggested as the most likely deprotonating group [28], and resonance Raman experiments carried out on UMC highlighted a shortening of a Cu(II)–N(His) bond at alkaline pH [29]. This quite small effect coincides with a weakening of the M(II)–S(Cys) bond and also the axial interaction. However, the alkaline transition does not have a dramatic influence on the isotropic shifts of His ligand resonances in the NMR spectra of Cu(II), Co(II) and Ni(II) stellacyanins [10, 16, 23, 24, 26, 28].

Measuring the thermodynamics of pH-induced conformational transitions in proteins provides an insight into the balance of the bond breaking/formation processes affecting the enthalpy change. Furthermore, transition-induced changes in protein conformational degrees of freedom and solvent reorganization effects which determine the reaction entropy [13, 18, 19, 30, 33, 52] are highlighted. These data complement spectroscopic and structural information, contributing to a deeper characterization of a particular chemical event. Herein, we report measurements of the thermodynamics of the alkaline transition for native stellacyanin from *Rhus vernicifera* (RST), wild-type (WT) cucumber basic protein (CBP; a plantacyanin) and the Met89Gln variant and also UMC and its Gln95Met variant in order to obtain a deeper understanding of this effect. These WT proteins were chosen because they exhibit different active-site structures (the copper sites of RST and UMC are probably alike, but the former was included as it is the most-studied stellacyanin; Fig. 1), while the mutants allow the possibility to assess the axial ligand differences in detail.

Materials and methods

Protein isolation and purification

RST was isolated from *Rhus vernicifera* acetone powder (Saito, Osaka, Japan) according to a literature method [1]. WT and Gln95Met UMC and WT CBP and the Met89Gln variant were isolated and purified as described previously [24, 28].

Protein samples for UV–vis studies

Proteins were fully oxidized using a solution of potassium ferricyanide. Excess oxidant was removed by gel filtration on a Sephadex G15 column (GE Healthcare) or using ultrafiltration, and the proteins were usually exchanged into 10 mM potassium phosphate buffer. Protein concentrations, determined using published extinction coefficients [24, 28, 53], were in the range from 10 to 40 μM. The pH of the samples was changed by adding small amounts of 1 M NaOH or HCl while rapidly stirring the solution.

UV–vis spectrophotometry

Electronic spectra were recorded using either Varian Cary C50 or PerkinElmer λ35 spectrophotometers. The cell (1-cm path length) was under thermostatic control with the temperature within the cell measured with a Cu–costan microthermocouple.

Results

The alkaline transition in oxidized phytocyanins is known to result in a blueshift of the main S(Cys) → Cu(II) ligand to metal charge transfer (LMCT) band at approximately

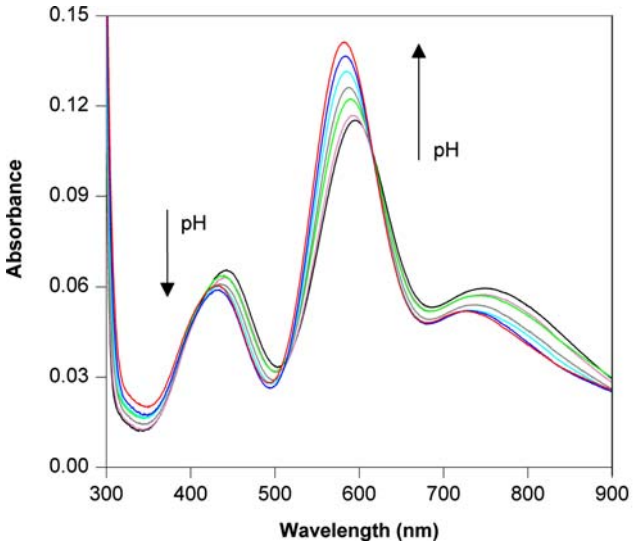

Fig. 2 UV–vis spectra (25 °C) of CBP (36 μM) in 10 mM phosphate buffer at various pH values. Spectra are shown in the pH range from 7.3 (*black line*) to 10.9 (*red line*) and as the pH is raised the main S(Cys) → Cu(II) LMCT band at approximately 600 nm increases in intensity and shifts to higher energy (the less intense band at approximately 450 nm shifts in a similar manner but decreases in intensity)

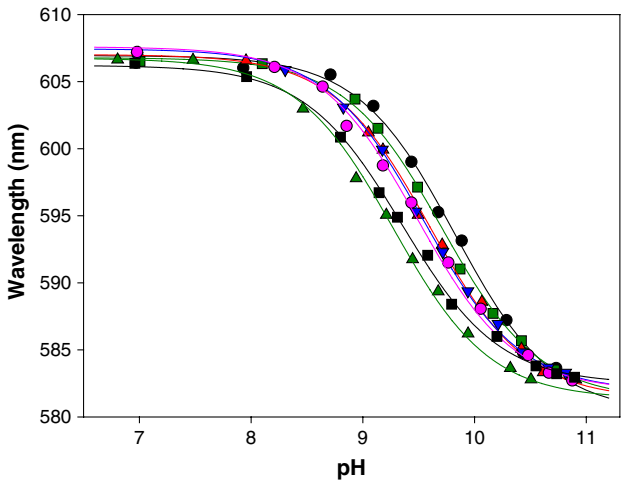

Fig. 3 pH dependence of the main S(Cys) → Cu(II) LMCT band at approximately 600 nm of UMC (23 μM) in 10 mM phosphate buffer at 10 °C (*black circles*), 15 °C (*green squares*), 20 °C (*red triangles*), 25 °C (*inverted blue triangles*), 30 °C (*pink circles*), 35 °C (*black squares*) and 40 °C (*green triangles*). *Solid lines* are least squares fits of the data to Eq. 1

600 nm in the UV–vis spectrum by about 20 nm (Fig. 2) [1, 4, 10, 16, 23, 24, 28, 29]. A second, less intense, band at about 450 nm shifts in a similar pH-dependent manner (Fig. 2). For all of the proteins investigated, the apparent equilibrium constant for the transition, K_{app}, can be determined by fitting the sigmoidal pH dependence between 7 and 11 of the main LMCT band to a the conventional one-proton equilibrium equation (Eq. 1):

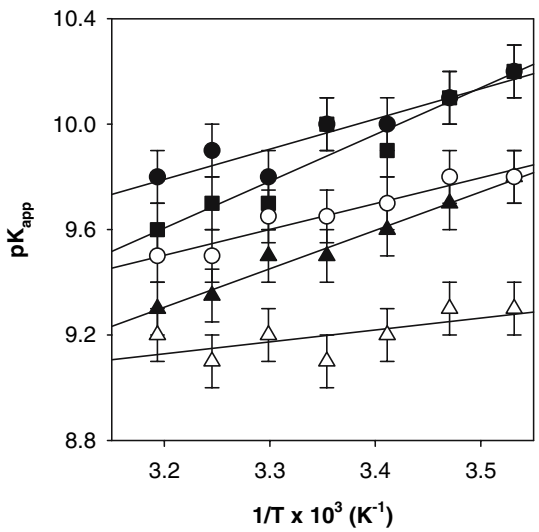

Fig. 4 pK_{app} values for the alkaline transition as a function of $1/T$ in 10 mM phosphate buffer for RST (*squares*), CBP (*filled circles*), UMC (*filled triangles*), Met89Gln CBP (*open circles*) and Gln95Met UMC (*open triangles*). *Solid lines* are least-squares fits of the data to Eq. 2

$$\lambda = \frac{\left(K_{app}\lambda_H + [H^+]\lambda_L\right)}{\left(K_{app} + [H^+]\right)}, \quad (1)$$

where λ_H and λ_L are the wavelengths of the band at high and low pH, respectively. The transition thermodynamics parameters ($\Delta H^{\circ\prime}_{AT}$ and $\Delta S^{\circ\prime}_{AT}$, where AT stands for alkaline transition) were determined by measuring the apparent equilibrium constant at varying temperature (from 10 to 40 °C; Fig. 3). These data were analyzed with the integrated van't Hoff equation (under the hypothesis that $\Delta H^{\circ\prime}_{AT}$ is constant over the limited temperature range investigated), utilizing a plot of pK_{app} versus $1/T$ (Eq. 2):

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

The van't Hoff plots for all the proteins investigated are shown in Fig. 4 and the $\Delta S^{\circ\prime}_{AT}$ and $\Delta H^{\circ\prime}_{AT}$ values are listed in Table 1. The measured pK_{app} values at 25 °C, listed in Table 1, are in good agreement with those reported previously [16, 23, 28].

Discussion

The alkaline transition in all of the phytocyanins studied is endothermic and occurs with an entropy loss and, thus, the free-energy change is remarkably positive (Table 1). In this protein series the reaction shows a certain degree of

Table 1 Thermodynamic parameters for the alkaline transition (*AT*) of phytocyanins

Protein	$\Delta H^{\circ\prime}_{AT}$ (kJ mol^{-1})	$\Delta S^{\circ\prime}_{AT}$ (J mol^{-1} K^{-1})	$T\Delta S^{\circ\prime}_{AT}$ (kJ mol^{-1})	$\Delta G^{\circ\prime}_{AT}$ (kJ mol^{-1})	pK_{app}[a]
RST	34	–75	–22	56.9	10.0
CBP	22	–117	–35	56.9	10.0
Met89Gln CBP	19	–122	–36	55.0	9.7
Met89Val CBP	ND	ND	ND	50.7	8.9[b]
UMC	28	–89	–27	54.1	9.5
Gln95Met UMC	9	–147	–44	51.8	9.1

Values were obtained in 10 mM phosphate buffer. Average errors on $\Delta H^{\circ\prime}_{AT}$, $\Delta S^{\circ\prime}_{AT}$ and $\Delta G^{\circ\prime}_{AT}$ values are ±2 kJ mol^{-1}, ±6 J mol^{-1} K^{-1} and ±0.2 kJ mol^{-1}, respectively

RST the stellacyanin from *Rhus vernicifera*, *CBP* cucumber basic protein, *UMC* umecyanin, the stellacyanin from horseradish roots, *ND* not determined

[a] At 25 °C

[b] Data from [28]

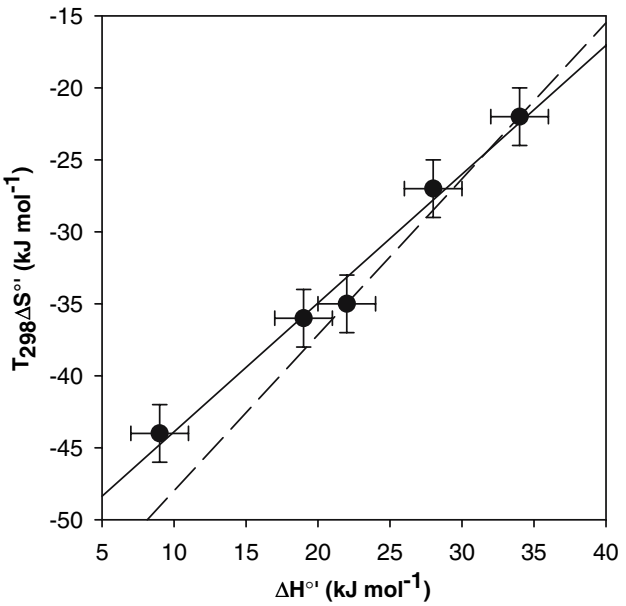

Fig. 5 Enthalpy–entropy compensation plot at 25 °C for the thermodynamics of the alkaline transition of phytocyanins. The *solid line* is the least-squares fit of the data (slope 0.895 and $R^2 = 0.983$). The *dotted line* shows the perfect compensation plot (slope 1) for a constant $\Delta G^{\circ\prime}_{AT} = 56.9\,\text{kJ mol}^{-1}$ (corresponding to a pK_{app} of 10) throughout the series

enthalpy–entropy compensation, i.e., the $\Delta H^{\circ\prime}_{AT}$ and $T\Delta S^{\circ\prime}_{AT}$ values are linearly correlated with a slope of approximately 0.9 (Fig. 5). This has important implications for the interpretation of these data. In fact, it is known that changes in the hydrogen-bonding network between water molecules in the hydration sphere induced by a particular reaction in a protein result in perfect compensatory enthalpy and entropy changes; thus, they strongly affect the individual $\Delta H^{\circ\prime}$ and $\Delta S^{\circ\prime}$ values, but do not influence the free energy of the overall process [19, 30, 52, 54–57]. The observation of perfect enthalpy–entropy compensation (indicated by a plot with a unit slope, and an invariance of $\Delta G^{\circ\prime}$) signifies that the protein-based free-energy change *is the same* in all reactions and that the differences in the individual enthalpy and entropy values are *entirely* due to solvent reorganization effects. However, if differences in $\Delta H^{\circ\prime}$ and $\Delta S^{\circ\prime}$ among the series due to protein-based effects (which are not compensatory) are increasingly important, compensation is not perfect or may not be observed, and increasing differences in $\Delta G^{\circ\prime}$ are found, as is the case for the thermodynamic data reported in this study. The deviation from enthalpy–entropy compensation observed for the alkaline transition data for phytocyanins is greater than that exhibited by the alkaline transition in *c*-type cytochromes [18, 33] and the acid transition in cupredoxins [19, 30, 52], which indeed feature a much lower variability of the reaction free energy within a series of proteins. Therefore, protein-based effects, namely, transition-induced changes in bond strengths and conformational degrees of freedom of the polypeptide chain, strongly contribute to the variability of the thermodynamics of the alkaline transition in the phytocyanins.

The significant transition-induced increase in protein volume of UMC at alkaline pH (the diameter increases by approximately 50% [29]) is consistent with the importance of protein-based effects. In fact, differences within the protein series are conceivable in terms of reaction-induced changes in conformational (vibrational and/or torsional) degrees of freedom of the polypeptide chain, and in the strength of intramolecular interactions, thus resulting in protein-based entropic and enthalpic factors which might contribute to the variability of the reaction free energy. The extent of these differences cannot be quantified at present, but is likely to be much larger than for the alkaline

transition in *c*-type cytochromes, which gives rise to highly localized conformational changes at the protein surface in the proximity of the heme [18, 21, 33].

The imidazole to imidazolate ionization of one of the two copper binding His residues has previously been proposed to be responsible for the alkaline transition in phytocyanins [28, 29]. The deprotonation of a His ligand should strengthen its coordination with the copper atom, and result in a weakening of the Cu–S(Cys) bond and also the axial interaction [28, 29]. If this is the case, the present data indicate that the enthalpic terms associated with the deprotonation reaction, and the weakening of some of the coordination bonds at the copper, overcome that due to the increased strength of the Cu–N(imidazolate) bond, thus determining the endothermicity of the process. Other contributions could arise from transition-induced changes in the hydrogen-bonding network and electrostatic interactions between the copper site and the protein matrix. Similar behavior has been observed for the alkaline transition in *c*-type cytochromes, in which the greater affinity of a Lys amino nitrogen for the ferric ion as compared to the thioether sulfur of Met, indicates that ligand substitution is not responsible for the positive enthalpy change, which instead must be determined by the deprotonation process [13, 18, 33].

It is noteworthy that CBP and the Gln95Met UMC variant, which both possess an axial Met ligand, feature a transition enthalpy which is less positive than that for UMC and RST, in which the copper is axially bound by the $O^{\varepsilon 1}$ of a Gln residue. This is consistent with one of the two Cu(II) binding His ligands being the group responsible for the transition, since the presence of the weaker electron donating Met as the axial ligand would be expected to favor His deprotonation (resulting in a less positive transition enthalpy). The fact that the lowest pK_{app} is exhibited by the Met89Val variant of CBP, which lacks an axial ligand, is consistent with this hypothesis. However, replacement of the axial Met ligand with a Gln in the Met89Gln variant of CBP results in little change in the thermodynamics of the alkaline transition. An enthalpy-based pK_{app} decrease of 0.3 units is observed and the resulting Cu–O(Gln) bond in CBP may actually be weaker than the native Cu–S(Met) interaction. This small influence on the thermodynamics of the alkaline transition is consistent with the Met89Gln mutation having a more limited effect on the spectroscopic properties, and presumably active-site structure, of CBP than the Gln95Met mutation in UMC [28, 58, 59].

Conclusions

Active-site features, including the nature of the axial copper ligand, metal–ligand bond strengths and transition-induced changes in conformational degrees of freedom of the polypeptide are important determinants of the enthalpy, entropy and free-energy changes for the alkaline transition in phytocyanins. Deprotonation of a His ligand would appear to be an active-site event consistent with the observed mutation-induced and species-dependent differences in transition thermodynamics.

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