Redox Thermodynamics of Blue Copper Proteins

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Abstract: The thermodynamic parameters of protein reduction ($\Delta H^{\circ\prime}_{rc}$ and $\Delta S^{\circ\prime}_{rc}$) were measured for a number of blue copper proteins including spinach plastocyanin, cucumber plastocyanin, *Pseudomonas aeruginosa* azurin, *Rhus vernicifera* stellacyanin, cucumber stellacyanin, and horseradish umecyanin through voltammetric techniques in nonisothermal experiments at neutral pH. Including previous estimates for other members of the same protein family, we discuss here the thermodynamics of the electron-exchange reaction for twelve blue copper proteins from different sources. The enthalpic term ($-\Delta H^{\circ\prime}_{rc}/F$) turns out to be the dominant contribution to the reduction potential in this protein class. However, the entropic term ($T\Delta S^{\circ\prime}_{rc}/F$) heavily affects $E^{\circ\prime}$, especially for the azurins. These data were analyzed in the light of the structural and dynamic information available on protein folding, geometric and electronic features of copper ligation, and solvation properties of the two redox states. It is clearly seen that the reduction enthalpy of the subfamily of the "phytocyanins" is less negative as compared to that of the other cupredoxins, most likely owing to a stronger axial ligation of the copper ion (which results in a nearly tetrahedral coordination geometry) and the greater exposition of the site to the solvent, which are both factors that stabilize the Cu(II) ion. The reduction entropy, which in most cases is negative, is instead apparently related to the solvation properties of the site. In addition, by analogy with class I cytochromes *c*, an increase in protein rigidity could also contribute to the entropy loss on reduction. Finally, it is apparent that the strategy of protein control of the reduction thermodynamics in high-potential electron-transfer metalloproteins (blue copper proteins, class I cytochromes *c*, HiPIPs) is the same: a dominant enthalpic term arising from ligand-binding interactions and electrostatic factors at the metal/protein interface, which strongly stabilizes the reduced state, is most often opposed by a weaker entropic term due to changes in protein dynamics and solvation properties, which disfavors protein reduction.

Introduction

The thermodynamics of the electron-exchange reaction for the members of a given family of redox metalloproteins, analyzed in the light of an extensive information on the individual structural and dynamic properties, may help elucidate how the protein controls the reduction potential of the metal within the series.[1,2] This is of use for the general understanding of the mechanisms which regulate the driving force for the electron flow between metalloredox partners in biological systems. Such an approach can be profitably applied to blue copper proteins which are among the most thoroughly investigated electron-transfer metalloproteins in terms of overall protein structure,[3−5] electronic features of metal ligation,[6−12] and kinetic properties.[5,13−15] Reduction enthalpies and entropies were determined in the past only for a few species by variable temperature spectro-electrochemical measurements and direct electrochemistry at solid electrodes.[2,16−19] The availability of a larger data set should, in principle, allow information to be obtained on how the thermodynamics of the redox reaction are influenced by the electronic and structural properties of metal ligation and the electrostatics at the metal center, including the interaction with the solvent, and by the dynamic and solvation properties of the molecule as a whole. To this end, we have measured the reduction thermodynamics of a number of wild-type blue copper proteins from different sources, most of which are structurally characterized either in solution or in the crystal state. In particular, we have determined the standard enthalpy

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and entropy change for the reduction of the Cu(II) center for six blue copper proteins from bacteria and green plants through variable-temperature direct electrochemistry using nonisothermal cells,[20] in the same conditions (0.1 M phosphate buffer, pH 7). Most of these species, namely spinach plastocyanin, cucumber plastocyanin, cucumber stellacyanin, and horseradish umecyanin were investigated for the first time in this respect. In addition, the reduction thermodynamics were remeasured for *Pseudomonas aeruginosa* azurin and *Rhus vernicifera* stellacyanin.[21] Overall, including the literature values, we have discussed the reduction thermodynamics for twelve blue copper proteins from different sources.

It is known that the electronic and geometric properties of the metal site in blue copper proteins are imposed by the protein matrix through electrostatic interactions and hydrogen bonding at the metal−protein interface.[3,5,11,12] This way the protein also controls the reduction potential, which previous theoretical work has correlated to the nature and strength of the axial bond to copper.[6,7] We found here that the enthalpic contribution to $E°'$ invariably prevails on the entropic term, in a way which closely parallels that observed previously for class I cytochromes *c*.[1] The first coordination sphere of the metal turns out to control the gross features of the reduction enthalpy in a rather straightforward way. The reduction entropy, which is instead apparently linked to solvation and dynamic properties of the molecule, although contributing to a lesser extent to $E°'$ than the enthalpic term, plays an important role in the control of the reduction potential in these species.

Experimental Procedures

Materials. All chemicals were reagent grade and were used without further purification. Nanopure water was used throughout.

Protein Purification. Plastocyanin from spinach (*Spinacea oleracea),* plastocyanin and stellacyanin from cucumber (*Cucumis sativus*), and *R. vernicifera* stellacyanin were isolated with slight modifications of the literature methods.[22−25] *P. aeruginosa* azurin and umecyanin from horseradish (*Armoracia laphatifolia*) were from Sigma.

Electrochemical Measurements. Cyclic voltammetry experiments (CV) were performed with a potentiostat/galvanostat PAR model 273A. A 1-mm diameter gold disk and a pyrolitic graphite disk (PGE) were used as working electrodes, and a saturated calomel electrode and a 5-mm diameter Pt electrode were used as a reference and counter electrode, respectively. Potentials were calibrated against the MV^{2+}/MV^{+} couple (MV = methyl viologen).[26] All of the redox potentials reported in this paper are referenced 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 using a cell for small volume samples ($V = 0.5$ mL) under thermostatic control. The best electrochemical response for azurin and umecyanin was obtained with a 4-mercaptopyridine surface-modified gold electrode. A pyrolytic graphite electrode was used for the other species. 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 gold electrode was first treated with anhydrous ethanol for 10 min, polished with alumina (BDH, particle size of about 0.015 μm) water slurry on cotton wool for 5 min, and finally treated in an ultrasonic pool for about 10 min. Modification of the electrode surface was performed by dipping the polished electrode into a 1 mM solution of 4-mercaptopyridine for 30 s and then rinsing it with Nanopure water.[27−30] The PGE electrode was subjected to the same cleaning procedure, in which the duration of each step was halved, and used without further treatment. Protein samples were freshly prepared before use, and the concentration of each, in general about 0.1 mM, was checked spectrophotometrically. A single voltammetric wave which was either reversible or quasi-reversible was observed for all species. 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 $E°'$ values. The temperature dependence of the reduction potential was determined with a "nonisothermal" cell,[20,31] in which the reference electrode is kept at constant temperature, while the temperature of the working electrode is varied. For such an experimental setting, the standard entropy change of the reaction center upon reduction ($\Delta S°'_{rc}$) is obtained from the plot of $E°'$ vs temperature. The enthalpy change ($\Delta H°'_{rc}$) is obtained from the Gibbs-Helmholz equation, namely from the slope of $E°'/T$ vs $1/T$ plot. Measurements were invariably performed in 0.1 M phosphate buffer at pH 7. For *P. aeruginosa* azurin, the experiments were run also at pH 5 and 8. For each protein, the experiments were performed at least two times, and the reduction potentials were found to be reproducible within ±2 mV.

Results and Discussion

The positive reduction potentials of blue copper proteins are, in general, accounted for by the selective stabilization of the Cu(I) ion by ligand-binding interactions and the rather hydrophobic environment and the small solvent accessibility of the metal site.[4,5,9] Analogous arguments apply to the heme iron of class I cytochromes *c*.[1] Analysis of the enthalpic and entropic components of the free energy change of Cu(II) reduction may contribute to the understanding of the origin of the relative stabilization of the two oxidation states. The thermodynamics of Cu(II) reduction of a large number of blue copper proteins from different sources, determined from the temperature dependence of $E°'$ in nonisothermal experiments, are listed in Table 1, along with those obtained in the past by us and by others.[2,16−19,21] The present data for *P. aeruginosa* azurin and *R. vernicifera* stellacyanin are in excellent agreement with those reported elsewhere from spectro-electrochemical experiments.[18,21] Reduction enthalpies, as well as most of the entropies, are negative as shown in Figures 1 and 2, respectively. The enthalpic and entropic contributions to $E°'$ ($-\Delta H°'_{rc}/F$ and $T\Delta S°'_{rc}/F$, respectively) are also reported. The favorable enthalpy change for Cu(II) reduction appears to be the determinant of the high $E°'$ values of blue copper centers. On the contrary, Cu(II) reduction turns out to be almost invariably disfavored on entropic grounds, with the only exceptions being those of the cucumber basic protein (CBP), spinach basic protein (SBP), and fungal laccase. The entropic contribution to $E°'$, although

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Table 1. Thermodynamic Parameters for the Reaction Center in Blue Copper Proteins[a]

protein	$\Delta H^{\circ\prime}_{rc}$ (kJ mol^{-1})	$\Delta S^{\circ\prime}_{rc}$ (J mol^{-1} K^{-1})	$E^{\circ\prime b}$ (mV)	$-\Delta H^{\circ\prime}_{rc}/F$ (mV)	$T\Delta S^{\circ\prime}_{rc}/F^{b}$ (mV)	ref
plastocyanin (*Spinacea oleracea*)	−46	−36	366	477	−111	this work
plastocyanin (*Phaseolus vulgaris*)	−38	−10	360	391	−31	18
plastocyanin (*Cucumis sativus*)	−45	−30	374	466	−92	this work
azurin (*Pseudomonas aeruginosa*)	−49	−65	307	508	−201	this work
azurin (*Pseudomonas aeruginosa*)	−50	−68	307	516	−208	18
azurin (*Alcaligenes denitrificans*)	−36	−32	276	374	−98	19
azurin (*Alcaligenes faecalis*)	−43	−58	266	447	−181	19
stellacyanin (*Rhus vernicifera*)	−25	−22	187	259	−68	this work
stellacyanin (*Rhus vernicifera*)	−23.7	−17.6	191	246	−54	18
stellacyanin (*Cucumis sativus*)	−32	−21	265	330	−65	this work
CBP (*Cucumis sativus*)	−20	+31	306	207	96	2
SBP (*Spinacea oleracea*)	−31	+7	345	321	22	16
umecyanin (*Armoracia laphatifolia*)	−33	−17	290	342	−52	this work
fungal laccase (*Polyporus versicolor*)	−73.1	+7.1	780	757	15	17

[a] All values obtained in 0.1 M phosphate buffer at pH 7, except those for fungal laccase which refer to pH 5.4, 0.2 M phosphate. The literature values are from spectro-electrochemical data obtained from nonisothermal OTTLE experiments. Average errors on $\Delta H^{\circ\prime}_{rc}$ and $\Delta S^{\circ\prime}_{rc}$ values, are ±2 (kJ mol^{-1}) and ±6 (J mol^{-1} K^{-1}), respectively. [b] At 25 °C.

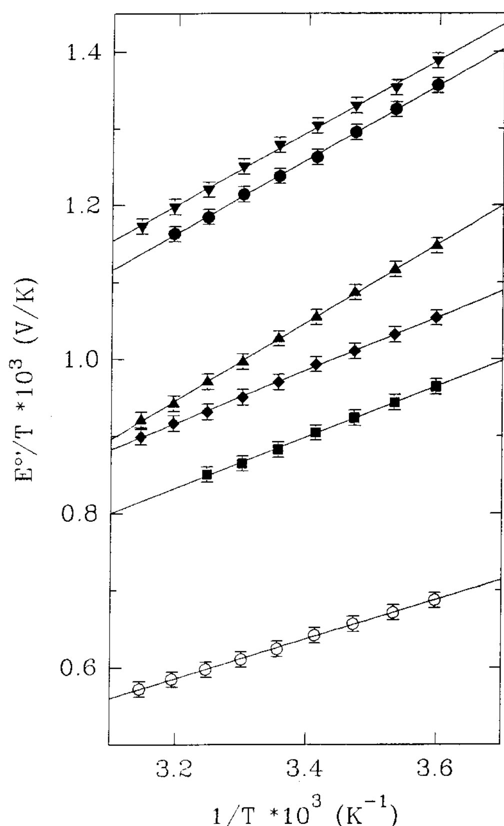

Figure 1. $E^{\circ\prime}/T$ vs $1/T$ plots for blue copper proteins from various sources. The slope of the plots yields the $-\Delta H^{\circ\prime}_{rc}$ values. (▼) cucumber (*Cucumis sativus*) plastocyanin; (●) spinach (*Spinacea oleracea*) plastocyanin; (▲) *Pseudomonas aeruginosa* azurin; (◆) horseradish (*Armoracia laphatifolia*) umecyanin; (■) cucumber (*Cucumis sativus*) stellacyanin; (○) *Rhus vernicifera* stellacyanin. Protein concentration, 0.1 mM; base electrolyte, 0.1 M phosphate, pH 7. Solid lines are least-squares fits to the data points.

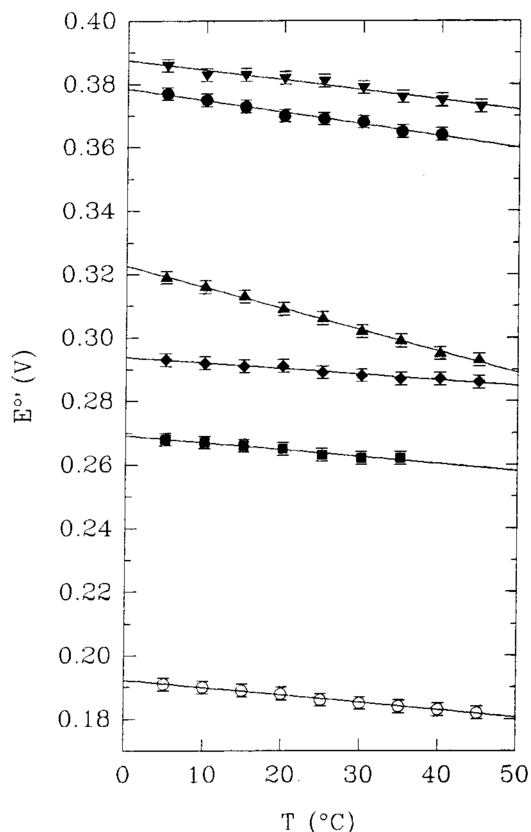

Figure 2. Temperature dependence of the reduction potential of blue copper proteins from various sources. The slope of the plots yields the $\Delta S^{\circ\prime}_{rc}$ values. (▼) cucumber (*Cucumis sativus*) plastocyanin; (●) spinach (*Spinacea oleracea*) plastocyanin; (▲) *Pseudomonas aeruginosa* azurin; (◆) horseradish (*Armoracia laphatifolia*) umecyanin; (■) cucumber (*Cucumis sativus*) stellacyanin; (○) *Rhus vernicifera* stellacyanin. Protein concentration, 0.1 mM; base electrolyte, 0.1 M phosphate, pH 7. Solid lines are least-squares fits to the data points.

invariably smaller than the enthalpic term, heavily affects the $E^{\circ\prime}$ of most species, in particular that of the azurin family. It is noteworthy that this behavior closely parallels that of class I cytochromes *c*.[1]

The Enthalpic Term. The most notable correlation between the reduction enthalpy ($\Delta H^{\circ\prime}_{rc}$) and the coordination features of the copper center involves the strength of axial ligation. In particular, it turns out that the species with an axial copper site, such as azurins and plastocyanins, in which the metal resides in, or in close proximity to, the NNS plane formed by the His_2-Cys ligands, and is weakly coordinated by the axial ligand(s),[3] show more negative reduction enthalpies than those species, such as cucumber stellacyanin and CBP, which possess a more tetrahedral site as a result of a stronger axial ligation.[32,33] Clearly, in the former sites the weak donor interaction of the axial ligand favors the reduced over the oxidized site,[6,7] despite the prefer-

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ence of the Cu(I) ion for a tetrahedral coordination. These thermodynamic data are in line with the role proposed by Solomon et al. for the weak Cu–L(Met or Gln) axial bond imposed by the protein matrix as the key electronic contribution to the high reduction potential in blue copper proteins.[6,7] Such a control is clearly exerted on the enthalpic contribution to $E°'$. Indeed, *P. aeruginosa* azurin and CBP show almost the same $E°'$ despite very different strengths of axial ligation [Cu–S(Met) = 3.12 and 2.61 Å, respectively], owing to the compensating effects of the reduction entropies (Table 1). However, it must be pointed out that since the interaction of the metal site with solvent dipoles also influences $\Delta H°'_{rc}$,[2] the less negative $\Delta H°'_{rc}$ of cucumber stellacyanin and CBP as compared to that of the other species could also involve a contribution of the greater stabilization of the oxidized site by the solvent, since it is known that these species possess the most solvent-accessible metal sites in cupredoxins.[32,33]

It is worth noting that the above two protein groups identified on enthalpic grounds coincide with those recognized on the basis of the *overall* electronic properties of metal ligation in blue copper sites. Indeed, from geometry and energy calculations on model complexes, blue copper sites were grouped into (*a*) axial sites characterized by a trigonal geometry with a π Cu–(S)Cys bond, and (*b*) rhombic sites with a tetragonal geometry containing all σ bonds to the strong His and Cys ligands.[12] These two geometries turn out to be very similar in energy; thus, it was proposed that the choice of either structure is made by the protein through the interactions of the metal coordination set with the immediate environment.[12] The results of the two sets of electronic structure calculations[6,7,11,12] can be reconciled by considering the strength of axial ligation as the most effective device for the modulation of the electronic and geometric structure of the site by which the protein builds the most convenient metal geometry in the context of the electrostatics, hydrogen bonding, and hydrophobicity of the environment. Within this framework, we believe that singling out whichever coordination feature as the ultimate determinant of the reduction enthalpy would be rather academic. The $\Delta H°'_{rc}$ values are linked to the properties of the metal coordination set as a whole, not of an individual bond. Indeed, the strength of axial ligation is known to be linked to the Cu–S(Cys) bond strength,[6,7,12] as indicated by the correlation found between the decrease in the stretching frequency of the Cu–S(Cys) bond in resonance Raman spectra and the increase in the tetrahedral character of the site, as a result of a stronger interaction of the axial ligand.[34] Thus, the previous model proposed on the basis of a much smaller data set which attributed the control of the reduction enthalpy of this protein class to the electronic properties of the trigonal $N(His)_2S(Cys)$ binding set, and particularly of the Cu–S(Cys) bond,[2,35] appears to provide only a partial picture of the role of metal coordination in this respect.

Differences in $\Delta H°'_{rc}$ among proteins with the same site geometry (either axial or rhombic), cannot be rationalized easily by simple arguments of coordination chemistry. Thus, while the longer Cu–S(Cys) bond in oxidized *P. aeruginosa* azurin as compared to that in *Alcaligenes denitrificans* azurin (2.25 vs 2.14 Å),[36,37] is in line with the more negative $\Delta H°'_{rc}$ value for the former species (Table 1) [at long bond distances, the shorter Cu–O(Gly) bond for the former species (2.95 vs 3.13 Å), which should stabilize the oxidized state, would appear to play a minor role], the more negative reduction enthalpies for stellacyanins as compared to CBP (Table 1) are opposite to what one could expect from the greater stabilization of the Cu(II) ion by the binding of the amide oxygen of Gln in stellacyanin at a shorter distance (2.21 Å)[33] as compared to the methionine sulfur in CBP (2.61 Å)[32] (as noted elsewhere,[2] the lower $E°'$ of stellacyanin as compared to CBP is entirely entropic in origin). Thus, additional electrostatic factors from outside the immediate environment of the metal clearly affect $\Delta H°'_{rc}$, although they could not be identified from a simple inspection of the X-ray structures.[32,33] The changes in $E°'$ due to point mutations involving residues of the second coordination sphere in azurins support this view,[38–40] although in some instances entropic contributions to the change in potential cannot be excluded.[39] On the other hand, we note that the reduction thermodynamics for cucumber and *R. vernicifera* stellacyanins are very similar despite the relatively low sequence identity (34%).[33] The extremely negative $\Delta H°'_{rc}$ value for fungal laccase cannot be discussed on safe bases owing to the absence of structural information, although the arrangement of the four copper ions is believed to be closely similar to that of ascorbate oxidase,[41] in which a plastocyanin-like type 1 center lies in proximity of a type 2 and type 3 centers clustered near each other. The matter is further complicated by the fact that intramolecular electron transfer occurs between the type 1 and the type 2 or type 3 coppers,[42] and that these redox centers are likely to influence each other. Kinetic data suggest that the blue copper center is exposed to the solvent.[43]

Table 2. Thermodynamics of Protein Reduction for *Pseudomonas aeruginosa* Azurin at Different pH Values

pH	$E°'$ (mV)	$\Delta H°'_{rc}$ (kJ mol^{-1})	$\Delta S°'_{rc}$ (J mol^{-1} K^{-1})	ref
5	338	−52	−65	this work
5	348	−48	−47	21
7	307	−49	−65	this work
7	307	−50	−68	18
8	286	−47	−65	this work
8	292	−45	−56	21

The reduction potential of *P. aeruginosa* azurin is known to be modulated by an acid–base equilibrium with a $pK_{a,red} = 7.31$ and $pK_{a,ox} = 6.26$.[21] The X-ray structures of this species at the acidic and alkaline pH limits unequivocally identifies His35, which is located in the vicinity of the metal site, as the residue responsible of this equilibrium.[36] We have remeasured the thermodynamics of protein reduction at pH 5 and 8 (Table 2). The $\Delta H°'_{rc}$ values are in good agreement with previous estimates from spectro-electrochemical measurements,[18,21] but the entropy values differ to some extent. The present data indicate that the decrease in $E°'$ with increasing pH for this species is an entirely enthalpic effect. This observation, in principle, fits with the near

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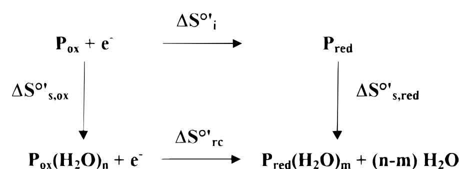
absence of pH-induced changes in the overall protein structure, the only significant change being a highly localized conformational rearrangement involving residues 35−37.[36] Since the metal site also turns out to be unaffected by the pH change, the relative stabilization of the oxidized over the reduced state with increasing pH would appear to be simply the result of a general electrostatic effect related to the increase in the negative charge of the protein due to His35 deprotonation.

The Entropic Term. Table 1 unequivocally shows that the reduction entropy plays an important role in the control of $E°'$ in most blue copper proteins. Information on the molecular bases of the entropy change can, in principle, be obtained from the comparison of the three-dimensional structures of the two redox states for the various species. Of the proteins considered in this work, the azurins from *A. denitrificans*[37,44,45] and *P. aeruginosa*[36,46,47] are the only ones for which both structures are available. We also considered the structures of poplar plastocyanin,[48] which is closely related to French bean and spinach plastocyanins in terms of sequence (about 80% identity) and overall structure.[49,50] The determinants of the reduction entropy clearly do not reside within the first coordination sphere of the metal. In fact, the crystal data show that the metal site is rather rigid, as a consequence of a network of hydrogen bonds and van der Waals interactions. The only change of the Cu(II) site on reduction is a slight increase in metal−ligand distances (from 0.05 to 0.1 Å).[36,37,44−48] This conserved copper site geometry is in line with the requisite of minimizing the reorganization energy for fast electron transfer.[51] Likewise, for all species the secondary structure turns out to be closely similar in the two redox states (with only a few significant changes far from the metal center) as well as the atomic temperature factors and the distribution of the ordered water molecules on the protein surface.[36,37,44,46,48] We noted elsewhere that the greater exposition to the solvent of the metal site of CBP, stellacyanin, and fungal laccase as compared to plastocyanins and azurins is associated with remarkably higher reduction entropies (which become positive for CBP, SBP, and laccase).[2] The present data strongly suggest that these two features are correlated. In fact, relatively high $\Delta S°'_{rc}$ values are obtained also for umecyanin and SBP which belong, along with stellacyanin and CBP, to the cupredoxin subfamiliy of the phytocyanins.[16,32,33] This group of proteins shows common sequence and structural features, including the solvent accessibility of both Cu-binding histidines,[32,33] instead of only one for azurins and plastocyanins. This effect cannot be interpreted unambiguously, although it is likely to be related to a reduction-induced reorganization of water molecules onto the protein surface near the copper site influenced by the hydrophobicity of the environment (see below). That the solvent effects are important in modulating the $E°'$ values of blue copper proteins was demonstrated recently

Scheme 1. Separation of the Measured Entropy ($\Delta S°'_{rc}$) for Protein (P) Reduction into an Intrinsic Term ($\Delta S°'_{i}$) and Solvation Terms ($\Delta S°'_{s,ox}$, $\Delta S°'_{s,red}$)[a]



[a] Clearly, this also applies on $\Delta H°'_{rc}$.

by NMR experiments and studies on mutated proteins.[39,52] The X-ray data for all blue copper proteins show that there are no internal water molecules but that several are bound to surface pockets with relatively low thermal parameters and also on the hydrophobic patch that surrounds the solvent-exposed imidazole ring of the Cu-binding histidine(s).[32,33,37,47,48]

The binding of an electron to an oxidized protein can be decomposed, like any other binding event in solution, into solute- and solvent-associated processes (Scheme 1).[53] Solvent release or binding by the solute can promote substantial entropy and enthalpy changes.[53−55] Thus, the measured reduction enthalpy and entropy for the present proteins, $\Delta H°'_{rc}$ and $\Delta S°'_{rc}$, can be represented as

$$\Delta H°'_{rc} = \Delta H°'_{i} + \Delta H°'_{s} \quad (1)$$

$$\Delta S°'_{rc} = \Delta S°'_{i} + \Delta S°'_{s} \quad (2)$$

where subscripts i and s denote the intrinsic thermodynamic parameters of the electron binding to the protein and those arising from solvent reorganization effects, respectively. In terms of Scheme 1, $\Delta S°'_{s} = \Delta S°'_{s,red} - \Delta S°'_{s,ox}$ and $\Delta H°'_{s} = \Delta H°'_{s,red} - \Delta H°'_{s,ox}$. A number of models for the hydration of nonpolar solutes and biopolymers recognize that the structural reorganization of the hydrogen-bonding network in the hydration sphere of the molecule induces largely compensating enthalpy and entropy changes[56−65] (although such a compensation turns out not to be a strict requisite of the thermodynamics of solvation or ligand-binding processes).[66] The entropy of transfer of a water molecule from the bulk solution to the hydration sphere of a protein ($\Delta S°'_{s,red}$ and $\Delta S°'_{s,ox}$ in Scheme 1) is positive and increases with increasing the binding strength, up to a limit of about +29 J mol^{-1} K^{-1} for water binding to an high-affinity site such as a metal center or polar groups.[67] Thus, the negative

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$\Delta S^{\circ\prime}_{rc}$ values for most blue copper proteins (and for all cytochromes *c* as well)[1] could be due, at least in part, to a negative $\Delta S^{\circ\prime}_{s}$ term arising from a reduction-induced release of water molecules from the hydration sphere of the protein, as noted elsewhere.[68,69] This would be accompanied by a compensating (negative) $\Delta H^{\circ\prime}_{s}$ value in eq 1). The correlation of $\Delta S^{\circ\prime}_{rc}$ with the degree of solvent exposition of the site mentioned above would suggest that hydration of the protein surface surrounding the exposed imidazole ring of the Cu-binding histidine(s) is a major determinant of $\Delta S^{\circ\prime}_{s}$. A simplistic interpretation of the less negative reduction entropy of phytocyanins as compared to azurins and plastocyanins would imply that fewer water molecules are released from the hydration sphere of the former protein family upon reduction, which could in some way be consistent with the strong hydrogen bonds formed by water molecules with the distal imidazole nitrogens of both the copper-binding histidines which protrude from the protein surface.[32,33] Moreover, differences in the extension and amino acid composition of the hydrophobic patch around the exposed histidine(s) could also be responsible for a different reduction-induced change in the degrees of freedom of the water structure in its proximity. However, we believe also that oxidation state-dependent changes in the flexibility of the polypeptide chain, including creation and suppression of vibrational and torsional degrees of freedom, which contribute to $\Delta S^{\circ\prime}_{i}$ and are still largely unknown for this protein class, likely play a significant role in the reduction entropy, as recently proposed for cytochromes *c*.[1,70,71] Moreover, for the positively charged phytocyanins, such as CBP, SBP, and stellacyanin, a positive contribution to $\Delta S^{\circ\prime}_{rc}$ could arise from a differential binding of anions to surface sites of the oxidized and reduced protein. Indeed, oxidized CBP was shown to specifically bind different anions with greater stoichiometries as compared to the reduced form.[2] Thus, protein reduction would induce a release of anions and, hence, an increase in entropy. The compensating enthalpy change likely associated with the above changes in the hydrogen-bonding arrangement of water molecules upon protein reduction would constitute only a minor fraction, though significant, of the $\Delta H^{\circ\prime}_{rc}$ value for these species (and for cytochromes *c* as well),[1] which instead turns out to be mainly related to binding interactions at the metal center and to electrostatic effects at the metal−protein interface (see above).

The present data would also indicate on thermodynamic grounds that phytocyanins are a distinct cupredoxin subclass. Their $E^{\circ\prime}$ values span a range that is comparable with that of plastocyanins and azurins ($E^{\circ\prime}$ = 187−345 mV and 266−360 mV, respectively) as a result of the compensating effects of less negative reduction enthalpies and entropies.

Unifying Characters with Other High-Potential ET Proteins. The relative contributions of the enthalpic and entropic terms to $E^{\circ\prime}$ in blue copper proteins and class I cytochromes *c*[1] turn out to be similar. In both families the $E^{\circ\prime}$ would be remarkably positive (as high as +500 mV) if it were only for the enthalpic contribution arising from ligand-binding interactions and electrostatic factors at the metal−protein interface. Such a high value is invariably decreased (with the exception of a few blue copper species) by an entropic term which disfavors protein reduction. It is noteworthy that this thermodynamic picture also applies to high-potential iron−sulfur proteins (HiPIP).[72,73] This is unlikely to be fortuitous and could possibly indicate that the polypeptide matrix adopts the same strategy for the control of the reduction potential in these electron-transport metalloproteins which is likely to be facilitated by the presence of two opposing contributions.

We may note that in blue copper proteins and cytochromes *c* the equatorial metal-binding set is conserved (a parallellism can be set between the four pyrrole nitrogens of the porphyrin ring in cytochromes *c* and the N_2(His)S(Cys) set in blue copper species), at variance with the axial bond to the metal. The strength of axial ligation and the nature of the axial donor atom(s), which are imposed by the protein and may vary in different species for blue copper proteins or change as a function of pH in cytochromes *c*, are important for the tuning of the structural and electronic features of the metal site and, hence, of the enthalpic contribution to $E^{\circ\prime}$. Additional electrostatic factors due to the protein environment and the solvent (net charges, atom polarity, and polarizability) contribute to this term in a fashion that it is still not understood in detail on quantitative bases. Thermodynamic studies of mutated proteins would be illuminating in this respect. The entropy change for protein reduction has a strong effect on $E^{\circ\prime}$ for all of these electron-transport metalloproteins. The conserved structure of the metal site in the two redox states indicates that changes in the dynamic and solvation properties of the protein are the likely determinants of $\Delta S^{\circ\prime}_{rc}$. Hence, this term would appear to be related to properties of the metalloprotein as a whole, which can thus be modulated by the properties of the medium such as ionic strength,[2,30,74,75] and dielectric constant.[76] The latter factor may play a role since most of these species exert their role in the proximity of the cell membrane.

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