Control of Metalloprotein Reduction Potential: Compensation Phenomena in the Reduction Thermodynamics of Blue Copper Proteins[†]

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ABSTRACT: The reduction thermodynamics ($\Delta H^{\circ\prime}_{rc}$ and $\Delta S^{\circ\prime}_{rc}$) for native *Paracoccus versutus* amicyanin, for *Alcaligenes faecalis* S-6 pseudoazurin, and for the G45P, M64E, and K27C variants of *Pseudomonas aeruginosa* azurin were measured electrochemically. Comparison with the data available for other native and mutated blue copper proteins indicates that the features of metal coordination and the electrostatic potential due to the protein matrix and the solvent control the reduction enthalpy in a straightforward way. However, the effects on the reduction potential are rather unpredictable owing to the entropic contribution to $E^{\circ\prime}$, which is mainly determined by solvent reorganization effects. Analysis of all the $\Delta H^{\circ\prime}_{rc}$ and $\Delta S^{\circ\prime}_{rc}$ values available for this protein class indicates that enthalpy−entropy compensation occurs in the reduction thermodynamics of wt cupredoxins from different sources, as well as for mutants of the same species. The findings indicate that the reduction enthalpies and entropies for these species are strongly affected by reduction-induced reorganization of solvent molecules within the solvation sphere of the protein. The absence of a perfect enthalpy−entropy compensation is due to the fact that while the differences between reduction entropies are dominated by solvent reorganization effects, those between reduction enthalpies are significantly controlled by intrinsic molecular factors related to the selective stabilization of the reduced form by coordination features of the copper site and electrostatic effects at the interface with the protein matrix.

Blue copper proteins (cupredoxins) are small electron transfer proteins, found in plants and bacteria, whose biological role relies on the presence of a single copper ion in a distorted tetrahedral coordination which switches reversibly between the +1 and +2 redox states (*1*−*5*). The reduction potential ($E^{\circ\prime}$)[1] of the Cu^{2+}/Cu^{+} couple in these species is thus a crucial physicochemical parameter for protein function (*6*−*11*). Extensive experimental and theoretical work has been devoted to the understanding of the molecular factors responsible for its modulation, which include the features of copper ligation, the polarity of the protein environment, and the accessibility of the metal site to solvent (*1*, *6*−*8*, *11*−*15*).

Partitioning the enthalpic ($\Delta H^{\circ\prime}_{rc}$) and entropic contributions ($T\Delta S^{\circ\prime}_{rc}$) to $E^{\circ\prime}$ by means of variable temperature electrochemical measurements proved to be informative on the mechanisms of $E^{\circ\prime}$ regulation in blue copper proteins as well as in other electron transfer metalloproteins (*6*−*8*, *14*−*23*). In particular, the high redox potential of these copper proteins turns out to be the result of the selective enthalpic stabilization of the reduced over the oxidized state ($\Delta H^{\circ\prime}_{rc} < 0$) owing to ligand binding interactions and electrostatics at the metal site. This contribution prevails on the reduction entropy. The latter is related to oxidation state-dependent changes in molecular solvation and polypeptide chain flexibility, which, in general, disfavor reduction ($\Delta S^{\circ\prime}_{rc} < 0$) (*6*, *14*−*18*). In this work, we wanted to address the question of how and to what extent solvation controls the reduction thermodynamics in this protein family, in particular focusing on solvent reorganization effects. The way this problem can be approached is by looking at enthalpy/entropy compensation phenomena. In fact, a number of models for the hydration of nonpolar solutes and biopolymers indicate that the structural reorganization of the hydrogen-bonding network in the hydration sphere of the molecule generally induces largely compensating enthalpy and entropy changes (*23*−*31*). These effects are at the origin of the compensation phenomena observed for the thermodynamics and/or kinetics of series of closely related solution reactions in several chemical contexts (*32*−*36*). They are particularly common

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[1] Abbreviations: $\Delta H^{\circ\prime}_{rc}$, enthalpy change for reduction; $\Delta S^{\circ\prime}_{rc}$, entropy change for reduction; $E^{\circ\prime}$, standard reduction potential; ET, electron transport; CV, cyclic voltammetry; PGE, pyrolytic graphite edge electrode; SCE, saturated calomel electrode; SHE, standard hydrogen electrode; *P. aeruginosa*, *Pseudomonas aeruginosa*; *P. versutus*, *Paracoccus versutus*; *A. faecalis* S-6, *Alcaligenes faecalis* S-6.

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in processes involving biological macromolecules, such as protein unfolding, nucleic acid denaturation, enzyme kinetics and binding (*34*, *35*), and were detected for the reduction thermodynamics of cytochromes *c* in a pioneering work by Bertrand (*23*).

We were prompted to carry out this analysis by the observation that (i) the reduction potentials within the series of native blue copper proteins vary less than the reduction enthalpies and entropies and (ii) point mutations at surface residues in plastocyanin and azurins induce compensating changes in $\Delta H^{\circ\prime}_{rc}$ and $\Delta S^{\circ\prime}_{rc}$ that result in very small alterations of the redox potential (*14*, *15*).

In this work, which follows a preliminary report on azurin variants (*8*), we have looked at the compensation plots derived from all the available reduction thermodynamics measured for native and mutated blue copper proteins. Considered here are not only literature data. We have also measured the $\Delta H^{\circ\prime}_{rc}$ and $\Delta S^{\circ\prime}_{rc}$ values for other species, which are of use for clarifying the influence of first coordination effects, electrostatic interactions between the metal site and the protein matrix, and the solvent as determinants of $E^{\circ\prime}$ in these species. In particular, data have been obtained for native *Paracoccus versutus* amicyanin and *Alcaligenes faecalis* S-6 pseudoazurin, which feature some differences in the metal site geometries. Moreover, we have investigated the changes in reduction thermodynamics due to point mutations in *Pseudomonas aeruginosa* azurin affecting exposition of the metal site to solvent (G45P) (*37*) and involving changes in the electrostatic potential in different regions of the molecule surface. Regarding the latter mutants, Met64 has been replaced with a glutamic acid residue, thereby inserting a negative charge in the hydrophobic patch involved in association with the redox partner (*38*). Moreover, Lys27, which lies on the opposite side of the molecule with respect to the metal center, was replaced with a cysteine. This mutation not only removes a positive charge but can also be used to construct dimers of the protein by the formation of an interprotein disulfide bridge (*39*).

We have found that compensation effects occur in the reduction thermodynamics of native (or recombinant wild-type) cupredoxins from different sources, as well as for mutants of the same species. Therefore, reduction-induced reorganization of solvent molecules within the solvation sphere of the proteins appears to strongly affect the changes in enthalpies and entropies among these species but not the variations in $E^{\circ\prime}$ (*23*).

EXPERIMENTAL PROCEDURES

Proteins. Wild-type *P. versutus* amicyanin, *A. faecalis* S-6 pseudoazurin, and the variants M64E, G45P, and K27C of *P. aeruginosa* azurin were isolated as described elsewhere (*37*, *38*, *40*−*42*). All chemicals were of 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 pyrolytic graphite disk (PGE) was used as a working electrode, and a saturated calomel electrode and a 5 mm diameter Pt were used as a reference and counter electrode, respectively. Potentials were calibrated against the MV^{2+}/MV^{+} couple (MV = methyl viologen) (*43*). All of 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 using a cell for small volume samples ($V = 0.5$ mL) under thermostatic control. The cleaning procedure of the working electrode is crucial to the voltammetric response. The PGE was first treated with anhydrous ethanol for 5 min and then polished with alumina (BDH, particle size of 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. Phosphate buffer (100 mM) was used as the base electrolyte. Protein samples were freshly prepared before use, and their concentration, in general about 0.1 mM, was checked spectrophotometrically. 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 $\upsilon^{1/2}$ (υ = 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 taken as the $E^{\circ\prime}$ values. The temperature dependence of the reduction potential was determined with a "nonisothermal" cell (*44*, *45*) in which the reference electrode is kept at constant temperature, while the half-cell containing the working electrode and the Vycor junction to the reference electrode is under thermostatic control with a water bath. The temperature was varied from 5 to 40 °C. With this experimental configuration, the reaction entropy for reduction of the oxidized protein ($\Delta S^{\circ\prime}_{rc}$) is given by (*7*, *44*, *45*)

$$\Delta S^{\circ\prime}_{rc} = S^{\circ\prime}_{red} - S^{\circ\prime}_{ox} = nF(dE^{\circ\prime}/dT) \qquad (1)$$

Thus, $\Delta S^{\circ\prime}_{rc}$ was determined from the slope of the plot of $E^{\circ\prime}$ versus temperature, which turns out to be linear under the assumption that $\Delta S^{\circ\prime}_{rc}$ is constant over the limited temperature range investigated. With the same assumption, the enthalpy change ($\Delta H^{\circ\prime}_{rc}$) was obtained from the Gibbs−Helmholtz equation, namely, as the negative slope of the $E^{\circ\prime}/T$ versus $1/T$ plot. The nonisothermal behavior of the cell was carefully checked by determining the $\Delta H^{\circ\prime}_{rc}$ and $\Delta S^{\circ\prime}_{rc}$ values of the ferricyanide/ferrocyanide couple (*7*, *44*−*46*). For each protein, the experiments were performed at least two times, and the reduction potentials were found to be reproducible within ±2 mV.

Protein Modeling. The crystallographic structure of *P. aeruginosa* azurin (*47*) was obtained from the Brookhaven Protein Data Bank (PDB entry: 4AZU). Model building for the G45P mutant was obtained by SWISS-MODEL, a fully automated protein structure homology−modeling server, accessible via the ExPASy web server.

RESULTS AND DISCUSSION

The thermodynamics of Cu^{2+} reduction for wild-type *P. versutus* amicyanin, *A. faecalis* S-6 pseudoazurin, and mutated *P. aeruginosa* azurins were determined from the

Table 1: Thermodynamic Parameters of Cu^{2+} Reduction for Native and Recombinant Wild-Type Blue Copper Proteins[a]

protein	$\Delta H^{\circ\prime}_{rc}$ (kJ mol^{-1})	$\Delta S^{\circ\prime}_{rc}$ (J K^{-1} mol^{-1})	$E^{\circ\prime}$ [b] (mV)	$-\Delta H^{\circ\prime}_{rc}/F$ (mV)	$T\Delta S^{\circ\prime}_{rc}/F$ [b] (mV)
plastocyanin (*Spinacea oleracea*)[c]	−46	−36	+366	+477	−111
plastocyanin (*Cucumis sativus*)[c]	−45	−30	+374	+466	−92
azurin (*P. aeruginosa*)[c]	−49	−65	+307	+508	−201
azurin (*A. denitrificans*)[d]	−36	−32	+276	+374	−98
amicyanin (*P. versutus*)[e]	−29	−12	+255	+297	−37
pseudoazurin (*A. faecalis* S-6)[e]	−16	+37	+275	+161	+114
CBP (*Cucumis sativus*)[f]	−20	+31	+306	+207	+96
SBP (*Spinacea oleracea*)[g]	−31	+7	+345	+321	+22
stellacyanin (*Rhus vernicifera*)[c]	−25	−22	+187	+259	−68
stellacyanin (*Cucumis sativus*)[c]	−32	−21	+265	+330	−65
umecyanin (horseradish)[c]	−33	−17	+290	+342	−52
mavicyanin (zucchini)[h]	−18	+9	+215	+186	+28

[a] All values obtained in 0.1 M phosphate buffer, pH 7. Average standard errors on $\Delta H^{\circ\prime}_{rc}$ and $\Delta S^{\circ\prime}_{rc}$ are ±2 kJ mol^{-1} and ±6 J mol^{-1} K^{-1}, respectively. The sum $-\Delta H^{\circ\prime}_{rc}/F + T\Delta S^{\circ\prime}_{rc}/F$ often does not close exactly to $E^{\circ\prime}$ since, because of the experimental error, the $\Delta H^{\circ\prime}_{rc}$ and $\Delta S^{\circ\prime}_{rc}$ values are rounded to the closest integer. [b] At 25 °C. [c] From ref *6*. [d] From ref *15*. [e] This work. [f] From ref *16*. [g] From ref *17*. [h] From ref *18*.

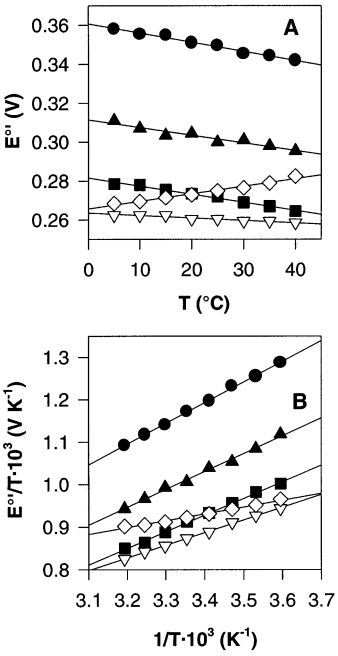

FIGURE 1: $E^{\circ\prime}$ vs T (A) and $E^{\circ\prime}/T$ vs $1/T$ (B) plots at pH 7 for wild-type *P. versutus* amicyanin (▽) and *A. faecalis* S-6 pseudoazurin (◇) and for the *P. aeruginosa* azurin variants G45P (●), M64E (■), K27C (▲). The slope of the plots in panels A and B yields the $\Delta S^{\circ\prime}_{rc}$ and $-\Delta H^{\circ\prime}_{rc}$ values, respectively. Conditions: protein concentration, 0.1 mM; base electrolyte, 0.1 M phosphate, pH 7. Solid lines are least-squares fits to the data points. The size of the symbols indicates the experimental uncertainty of the data points.

temperature dependence of $E^{\circ\prime}$ in nonisothermal experiments (Figure 1). The $\Delta H^{\circ\prime}_{rc}$ and $\Delta S^{\circ\prime}_{rc}$ values, along with the enthalpic and entropic contributions to $E^{\circ\prime}$ ($-\Delta H^{\circ\prime}_{rc}/F$, $T\Delta S^{\circ\prime}_{rc}/F$), are listed in Tables 1 and 2 for wild-type and mutated proteins, respectively. Tables 1 and 2 also list data from work reported previously to facilitate discussion. It is worthy of note that active site protonation coupled to protein reduction in amicyanin could affect (increase) the $E^{\circ\prime}$ values at low temperature, since the pK_a for this equilibrium in amicyanin increases from 6.2 at 35 °C to 6.5 at 5 °C (*48*). However, this equilibrium appears to exert a minor, if any, influence on the measure of the reduction thermodynamics for this species, since no deviations from linearity of the $E^{\circ\prime}/T$ profile at low temperature are observed (Figure 1).

Table 2: Thermodynamic Parameters of Cu^{2+} Reduction for Wild-Type and Mutated *P. aeruginosa* Azurins[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)
wt[c]	−42	−39	+315	+435	−120
H35L[c]	−22	+25	+309	+228	+77
M44K[c]	−51	−50	+371	+528	−154
M64E[d]	−38	−40	+271	+394	−123
G45P[d]	−47	−45	+349	+487	−139
K27C (dimer)[d]	−41	−38	+303	+420	−117

[a] All values obtained in 0.1 M phosphate buffer, pH 7. Average standard errors on $\Delta H^{\circ\prime}_{rc}$ and $\Delta S^{\circ\prime}_{rc}$ are ±2 kJ mol^{-1} and ±6 J mol^{-1} K^{-1}, respectively. The sum $-\Delta H^{\circ\prime}_{rc}/F + T\Delta S^{\circ\prime}_{rc}/F$ often does not close exactly to $E^{\circ\prime}$ since, because of the experimental error, the $\Delta H^{\circ\prime}_{rc}$ and $\Delta S^{\circ\prime}_{rc}$ values are rounded to the closest integer. [b] At 25 °C. [c] From ref *15*. [d] This work.

Redox Thermodynamics for Wild-Type Amicyanin and Pseudoazurin. Amicyanin and pseudoazurin show comparable $E^{\circ\prime}$ values, resulting from remarkably different enthalpic and entropic contributions. In particular, amicyanin is characterized by negative $\Delta H^{\circ\prime}_{rc}$ and $\Delta S^{\circ\prime}_{rc}$ values (−29 kJ mol^{-1} and −12 J mol^{-1} K^{-1}, respectively), as most blue copper proteins (Table 1) (*6*, *16*−*18*), whereas pseudoazurin displays the least negative reduction enthalpy and the most positive reduction entropy ($\Delta H^{\circ\prime}_{rc}$ = −16 kJ mol^{-1}; $\Delta S^{\circ\prime}_{rc}$ = +37 J mol^{-1} K^{-1}) so far determined for this protein class. The differences in reduction enthalpy are noticeable since the two proteins possess a high degree of structural homology and the same set of copper ligands (two histidines, a cysteine, and a methionine) (*40*, *49*−*54*). However, the differences in thermodynamic values can be accounted for on the basis of the differences in copper site geometry between these two proteins, according to a model proposed by us previously (*6*, *15*, *18*). In fact, it has been observed that rhombic sites (with tetragonal or tetrahedral distortions), such as that of pseudoazurin (*47*, *55*−*58*), enthalpically stabilize the Cu(II) ion more efficiently than the axial ones, like that of amicyanin (*40*, *53*, *58*), most likely by virtue of a stronger axial ligation (*6*, *15*, *18*). Hence, the present cases of amicyanin and pseudoazurin (*49*, *54*) can be framed within this model and provide further support to it. However, the differences in reduction enthalpy among mutants of the same protein or different proteins containing structurally similar redox centers (*14*−*16*, *18*) cannot be accounted for only by

arguments of coordination chemistry (*6*, *14*−*18*). A significant role is also played by electrostatic interactions between the charge of the metal center and permanent and induced protein and solvent dipoles, and nearby net protein charges (*6*, *14*−*18*), as shown by quantitative theoretical estimates of the electrostatic contributions to the reduction enthalpy for a number of electron transfer metalloproteins, based on the PDLD (protein dipoles Langevin dipoles) approach (*59*−*62*). The only calculation available for cupredoxins regards *A. faecalis* S-6 pseudoazurin (*49*) and suggests that an important contribution to the moderately negative reduction enthalpy for this protein comes from solvation effects. In this vein, it is likely that the less negative $\Delta H^{\circ\prime}_{rc}$ value of amicyanin compared to plastocyanin, despite an almost identical axial copper site, is due to the greater solvent exposition of the metal center in the former protein (*40*, *53*, *63*), which should enthalpically favor the oxidized state.

The reduction entropy is controlled mainly by oxidation state-dependent changes in the solvation properties and the flexibility of the polypeptide chain (*6*, *14*−*18*). In particular, it has been observed that, for ET proteins with positively charged metal centers (such as cupredoxins and cytochromes *c*), the reduction entropy invariably increases (i.e., becomes less negative or more positive) with increasing solvent accessibility of the metal center (*6*, *14*−*18*, *20*−*23*). This general qualitative rule can be easily justified with electrostatic arguments: the greater the exposition of the site to solvent, the more effective the mechanism that leads to a decreased ordering of the water molecules in the surroundings of the site following the one-unit drop of the positive charge on reduction. For cupredoxins these ordering phenomena are likely to be influenced by the hydrophobicity of the environment surrounding the imidazole ring of the solvent-exposed histidine(s). It is apparent that the entropy values for amicyanin and pseudoazurin follow this qualitative rule. In fact, the less negative $\Delta S^{\circ\prime}_{rc}$ value for amicyanin as compared to that of the structurally related spinach plastocyanin (−12 vs −36 J mol^{-1} K^{-1}) is in agreement with the greater solvent exposition of the metal site for the former species (*63*). For *A. faecalis* S-6 pseudoazurin, the same solvation effects proposed to be responsible for the enthalpic effects (see above) may help to explain the remarkable entropy increase on reduction.

Redox Thermodynamics of P. aeruginosa Azurin Mutants. The copper site of azurin displays a distorted trigonal-bipyramidal coordination formed by three strong equatorial ligands, one Cys and two His, plus one Met and the oxygen of the peptide group of a Gly acting as weak axial ligands (*23*, *64*−*67*). The presence of the weakly coordinating axial carbonyl group and the ensuing pentacoordination are peculiar to azurins (*1*, *2*, *5*). Replacement of the axial methionine by histidine and glutamine in *Alcaligenes denitrificans* azurin substantially modifies the structural and electronic properties of the copper site (*68*−*71*), inducing a remarkable decrease in the redox potential for both mutants, which is totally enthalpic in origin (*15*). Substitution of Gly45 with proline produces no or only minor alterations in the properties of copper coordination (*37*). The $E^{\circ\prime}$ of the G45P mutant is 34 mV more positive than that of the wild-type protein. While the mutation lowers both reduction enthalpy and entropy (Table 2), the change in $E^{\circ\prime}$ is dominated by the change in enthalpy. Analysis of a computer-generated three-dimensional model of the mutant indicates that replacement of the native glycine with proline does not alter the bond between copper and the peptide oxygen. Hence, the selective enthalpic stabilization of the reduced over the oxidized state originates from outside the first coordination sphere of the metal. Visual inspection of computer-generated structures shows that insertion of the bulky proline side chain (whose γ and β carbons are partially exposed to the solvent) further diminishes exposition of the metal site to the solvent, which is consistent with the decrease in $\Delta H^{\circ\prime}_{rc}$ (*6*, *15*). This effect is expected also to affect the reduction entropy because of partial suppression of the electrostatic mechanism of oxidation state-dependent ordering of water molecules in proximity of the metal site discussed above, leading to a more negative $\Delta S^{\circ\prime}_{rc}$ as compared to the wild-type protein. The hypothesis that solvation effects play a major role in determining the redox thermodynamics of this mutant is in agreement with the existence of enthalpy−entropy compensation effects (see discussion below). Furthermore, it is possible that the bulkiness of the proline side chain and the mutation-induced deletion of the strong H-bond (2.7 Å) between the peptide nitrogen of residue 45 and the peptide oxygen of Lys41 could affect the oxidation state-dependent changes in the flexibility of the polypeptide chain, influencing the reduction entropy in a way that cannot be established safely at present.

Met64 belongs to the hydrophobic surface patch surrounding the solvent-exposed His117, which is thought to play an important role in azurin ET complex formation (*40*, *72*). At pH 7, substitution of Met64 with a glutamic acid, which is known not to affect appreciably either the protein structure or the properties of the metal site (*37*, *72*, *73*), causes a 44 mV decrease in the reduction potential (Table 2). The observed increase in reduction enthalpy is what one expects for the introduction of one negative charge in proximity of the metal center, owing to the preferential stabilization of the cupric state. This effect parallels that observed previously for the H35L variant, in which deletion of a partial positive charge does occur, and is opposite to that determined for the M44K variant, which instead involves addition of a positive charge (*15*). Moreover, the smaller $|\Delta\Delta H^{\circ\prime}_{rc}|$ value determined for M64E (4 kJ mol^{-1}) as compared to M44K (9 kJ mol^{-1}) is consistent with the greater distance between the net charge and the metal center in the former case (11.4 vs 7.2 Å) (*38*). Analogous results have been obtained for a number of plastocyanin mutants subjected to charge alterations on residues in the vicinity of the copper center (*14*). Therefore, the effects of charge changes *on the enthalpic portion* of the reduction potential in these species can be justified on simple electrostatic grounds.

Unlike other blue copper mutants studied previously (*14*, *15*), the $\Delta S^{\circ\prime}_{rc}$ of the M64E variant is almost identical to that of the wild-type protein. Since the entropic effect is likely to be related to solvation effects, which for mutations inducing charge changes has been found to be such as to at least partially compensate the enthalpy-based change in $E^{\circ\prime}$ (*14*, *15*), it appears that for this mutation such solvent reorganization effects do not affect appreciably $\Delta S^{\circ\prime}_{rc}$, possibly because of their localization at a remarkable distance from the redox center (*38*).

Substitution of Lys27 with a cysteine involves removal of a positive charge and protein dimerization due to the

formation of an intermolecular disulfide bridge (*39*). It is apparent that the reduction thermodynamics of the resulting dimer are identical to wild-type azurin. The invariance of reduction enthalpy is consistent with the absence of mutation-induced alterations in copper coordination (*39*) and with the location of Lys27 at a great distance from the metal center, on the opposite side of the protein (*14*, *15*, *39*). The constancy of the $\Delta S^{\circ\prime}_{rc}$ value can be justified by the type of protein dimerization which, considering the metal site as the "head" of the molecule, can be defined as "tail to tail". In fact, such protein association hardly affects the reduction-induced solvent reorganization effects in proximity of the metal site, which are the main effectors of the reduction entropy, and the possible oxidation state-dependent changes in protein flexibility as well. These results are consistent with the significant changes in redox thermodynamics observed for blue copper proteins and cytochromes upon formation of ET protein complexes (*74*, *75*), in which the interacting molecules are oriented so as to minimize the distance between the redox centers. Such interactions are indeed likely to affect the organization of water molecules in the hydration sphere in proximity of the metal sites.

Enthalpy−Entropy Compensation Phenomena. Compensation phenomena in the kinetics and/or thermodynamics of series of homologous reactions are known to occur in several chemical contexts (*24*−*36*). Compensation in the reaction thermodynamics implies the existence of a linear relationship between the enthalpy and entropy changes within the series, according to the equation:

$$\Delta H_i = a + b(T_i \Delta S_i)$$

where *a* and *b* are constants, the latter being a measure of the extent of enthalpy−entropy compensation (*26*, *32*−*35*). An exact compensation would imply a straight line of unit slope at fixed temperature.

The discovery and importance of these so-called "extra-thermodynamic" relationships (for example, in host−guest and biological recognition) has attracted the interest of many researchers toward the comprehension of their physicochemical origin and functional implications (if any). Although a unifying view has not been reached, it appears that enthalpy−entropy compensations are related to reaction-induced creation or alteration of inter- or intramolecular interactions, whose strength is much smaller that that of a typical covalent bond (*31*−*33*). This can be rather intuitively justified by considering that in structurally adjustable systems, such as proteins and nucleic acids, the greater the strengthening of inter- and intramolecular interactions, reflecting a decrease in the reaction enthalpy, the more pronounced the reduction of the thermally accessible states of the system, which therefore results in an entropy decrease (*31*−*33*, *35*, *36*). Most importantly, for solution reactions it is now generally accepted that the main processes responsible for enthalpy−entropy compensation phenomena are solvent reorganization effects (*25*, *26*, *28*−*32*, *34*−*36*). This hypothesis is based on a thermodynamic model which divides any solution reaction in a nominal process, i.e., the pure chemical event, and in an environmental process describing the associated reorganization of solvent molecules (*26*, *32*, *35*). It can be demonstrated thermodynamically that the latter term does not contribute to the free energy of the overall process, although the corresponding entropy and enthalpy changes are nonzero (*26*, *32*, *35*). Hence, they must offset each other exactly. If these contributions are comparable to (or greater than) those accompanying the nominal process, an approximate enthalpy−entropy compensation is observed (*26*, *32*, *35*). These conclusions have a general validity, which is independent of the solution properties. However, in hydrogen-bonding solvents, such as water, the effects of solvent reorganization are more pronounced (*26*, *32*), thus the compensation patterns can be observed more clearly. This model has been applied successfully to the analysis of the enthalpy−entropy compensation patterns for several reactions involving biological macromolecules in solution, such as protein and nucleic acid folding, drug binding to receptor sites in membrane proteins, and enzyme catalysis (*26*, *33*, *35*, *36*).

Although redox reactions play a key role in biology, only limited information is available to date on whether enthalpy−entropy compensation phenomena occur in the thermodynamics of electron transfer processes involving biological macromolecules (*8*, *23*). The large amount of data obtained in these last years on the reduction thermodynamics of native and mutated blue copper proteins (*6*, *14*−*18*) allows the existence of such compensation to be probed on a statistically meaningful data set. We were prompted to carry out this analysis by the observation that in general the $E^{\circ\prime}$ values of these species are composed of opposite enthalpic and entropic contributions (*6*, *14*−*18*).

The redox potentials and the entropic contributions to $E^{\circ\prime}$ at 298 K ($T\Delta S^{\circ\prime}_{rc}/F$) plotted against the enthalpic terms ($-\Delta H^{\circ\prime}_{rc}/F$) for native blue copper proteins and plastocyanin and azurin mutants are shown in panels A, B, and C of Figure 2, respectively. Invariably, the data points exhibit reasonably good linear behavior. Since it has been shown that a compensation pattern is real provided the confidence region for each data point (indicated by the error bars) is much smaller than the overall range covered by the data (*26*, *32*, *76*), the knowledge of the experimental uncertainties is essential. Hence, to prevent any possible false compensation pattern of statistical origin, we will consider only the data obtained by us through variable temperature electrochemical measurements in the same experimental conditions (*6*, *14*−*18*) (however, we note that the data obtained by others nicely fall within the correlation, as shown in Figure 2A). It is apparent that the data of Figure 2 cover ranges which are much greater than the experimental uncertainties, indicating that the resulting compensation patterns are significant (*26*, *32*, *76*). Moreover, enthalpy−entropy compensation is also immediately apparent from the observation that the range of variation of the redox potential in each series turns out to be invariably much smaller than those of both the enthalpic and entropic terms (Figure 2).

The least-squares fitting of the $T\Delta S^{\circ\prime}_{rc}/F$ vs $-\Delta H^{\circ\prime}_{rc}/F$ plot for native blue copper proteins yields a slope (b) of -0.7 ± 0.1 and a regression coefficient (r) of 0.89 (Figure 2A). Similar parameters are obtained for the corresponding plots for the variants of spinach plastocyanin ($b = -0.7 \pm 0.1$, $r = 0.89$) (Figure 2B) and *P. aeruginosa* azurin ($b = -0.8 \pm 0.1$, $r = 0.94$) (Figure 2C). Observation of slopes close to 1 indicate that the contributions to the reaction enthalpy and entropy within the series arising from solvent reorganization effects (which are fully compensative at any temperature) (*26*, *32*, *35*) are much greater than the non-

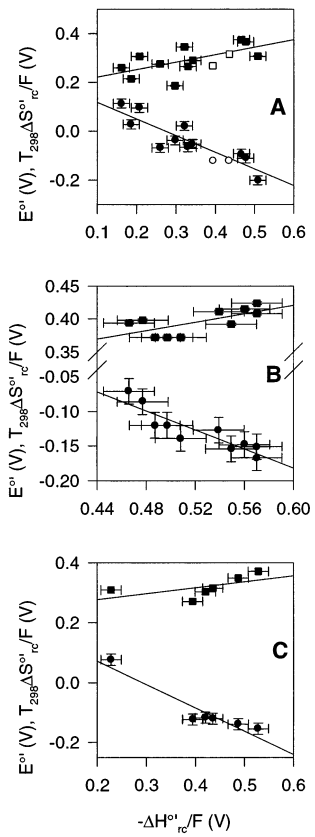

FIGURE 2: Compensation plots for the reduction thermodynamics determined for native and recombinant wild-type blue copper proteins (A) and for the series of spinach plastocyanin (B) and *P. aeruginosa* azurin (C) mutants (*14*, *15*; this work). Plotted are the $(T\Delta S^{\circ\prime}_{rc})/F$ (circles) and the $E^{\circ\prime}$ values (squares) as a function of $-\Delta H^{\circ\prime}_{rc}/F$. $T = 298$ K. Filled symbols correspond to data obtained from the authors in this and previous work (*6*, *15*−*18*). Open symbols indicate literature data (*7*, *80*). Solid lines are least-squares fits to the data points.

compensating terms associated with the intrinsic chemical process, as reported, for example, for protein and nucleic acid unfolding (*26*, *32*, *35*, *36*). When the enthalpic and entropic contributions arising from intrinsic and solvent reorganization processes become more similar, deviations of the slope from unity and larger scattering of the data points are expected (*26*). The slopes of approximately 0.7 observed for the native and mutated blue copper proteins considered here indicate that entropic and enthalpic contributions to $E^{\circ\prime}$ at 298 K compensate, but not perfectly. In fact, the entropic term varies within the series to a lesser extent than the enthalpic term. However, this behavior demonstrates the great importance of the reduction-induced changes in H-bonding within the solvation sphere of the molecule in controlling the redox properties of blue copper proteins. This nicely confirms the validity of previous observations, based on smaller data sets, which indicated solvation as the main process responsible for the opposing enthalpy and entropy changes induced by point mutations on the protein surface (*8*, *14*, *15*). An analogous compensation behavior is found for native proteins (Figure 2A).

The absence of perfect compensation is most likely related to the fact that changes in reduction entropy are most likely dominated by solvent reorganization phenomena (consistent with the similar reduction-induced conformational changes shown by recent NMR data for different blue copper proteins (*77*−*79*), whereas the variations in reduction enthalpy remarkably depend also on the contribution from the nominal part of the reaction (*34*), namely, electron uptake by the solvated oxidized protein. In fact, it has been shown that the changes in $\Delta H^{\circ\prime}_{rc}$ between different proteins are significantly linked to intrinsic molecular factors, such as ligand binding interactions and electrostatics at the metal site (*6*, *14*−*18*), with the solvent playing a more limited role.

REFERENCES

1. Sykes, A. G. (1991) *Adv. Inorg. Chem. 36*, 377−408.
2. Baker, E. N. (1994) in *Encyclopedia of Inorganic Chemistry* (King, R., Ed.) pp 883−905, Wiley, Chichester.
3. Farver, O., and Pecht, I. (1994) in *Copper Proteins and Copper Enzymes* (Lontie, R., Ed.) Vol. 1, pp 183−214, CRC Press, Boca Raton, FL.
4. Farver, O. (1996) in *Protein Electron Transfer* (Bendall, D. S., Ed.) pp 161−188, Bios, Oxford, U.K.
5. Messerschmidt, A. (1998) *Struct. Bonding 90*, 37−68.
6. Battistuzzi, G., Borsari, M., Loschi, L., Righi, F., and Sola, M. (1999) *J. Am. Chem. Soc. 121*, 501−506.
7. Taniguchi, V. T., Sailasuta-Scott, N., Anson, F. C., and Gray, H. B. (1980) *Pure Appl. Chem. 52*, 2275−2281.
8. Canters, G. W., Kolczak, U., Armstrong, F. A., Jeuken, L. J. C., Camba, R., and Sola, M. (2000) *Faraday Discuss. 116*, 205−220.
9. Malmström, B. G. (1994) *Eur. J. Biochem. 223*, 711−718.
10. Williams, R. J. P. (1995) *Eur. J. Biochem. 234*, 363−381.
11. Gray, H. B., Malmström, B. G., and Williams, R. J. P. (2000) *J. Biol. Inorg. Chem. 5*, 551−559.
12. Solomon, E. I., Penfield, K. W., Gewirth, A. A., Lowery, M. D., Shadle, S. E., Guckert, J. A., and LaCroix, L. B. (1996) *Inorg. Chim. Acta 243*, 67−78.
13. Randall, D. W., Gamelin, D. R., La Croix, L. B., and Solomon, E. I. (2000) *J. Biol. Inorg. Chem. 5*, 16−19.
14. Battistuzzi, G., Borsari, M., Loschi, L., Menziani, M. C., De Rienzo, F., and Sola, M. (2001) *Biochemistry 40*, 6422−6430.
15. Battistuzzi, G., Borsari, M., Canters, G. W., de Waal, E., Loschi, L., Warmerdam, G., and Sola, M. (2001) *Biochemistry 40*, 6707−6712.
16. Battistuzzi, G., Borsari, M., Loschi, L., and Sola, M. (1997) *J. Biol. Inorg. Chem. 2*, 350−359.
17. Battistuzzi, G., Borsari, M., Loschi, L., and Sola, M. (1998) *J. Inorg. Biochem. 69*, 97−100.
18. Battistuzzi, G., Borsari, M., Loschi, L., Ranieri, A., Sola, M., Mondovì, B., and Marchesini, A. (2001) *J. Inorg. Biochem. 83*, 223−227.
19. Battistuzzi, G., D'Onofrio, M., Borsari, M., Sola, M., Macedo, A. L., Moura, J. J. G., and Rodrigues, P. (2000) *J. Biol. Inorg. Chem. 5*, 748−760.
20. Battistuzzi, G., Borsari, M., Sola, M., and Francia, F. (1997) *Biochemistry 36*, 16247−16258.
21. Battistuzzi, G., Borsari, M., Cowan, J. A., Loschi, L., and Sola, M. (1999) *Biochemistry 38*, 5553−5562.
22. Battistuzzi, G., Borsari, M., Cowan, J. A., Ranieri, A., and Sola, M. (2002) *J. Am. Chem. Soc. 124*, 5315−5324.
23. Bertrand, P., Mbarki, O., Asso, M., Blanchard, L., Guerlesquin, F., and Tegoni, M. (1995) *Biochemistry 34*, 11071−11079.
24. Blokzijl, W., and Engberts, J. B. N. F. (1993) *Angew. Chem., Int. Ed. Engl. 32*, 1545−1579.
25. Lumry, R., and Rajender, S. (1970) *Biopolymers 9*, 1125−1227.
26. Grunwald, E., and Steel, C. (1995) *J. Am. Chem. Soc. 117*, 5687−5692.
27. Krug, R. R., Hunter, W. G., and Grieger, R. A. (1976) *J. Phys. Chem. 80*, 2335−2351.
28. Lumry, R. (1971) *Electron and Coupled Energy Transfer in Biological Systems*, Marcel Dekker, New York.
29. Ben-Naim, A. (1975) *Biopolymers 14*, 1337−1355.
30. Lee, B., and Graziano, G. (1996) *J. Am. Chem. Soc. 118*, 5163−5168.
31. Grunwald, E. (1986) *J. Am. Chem. Soc. 108*, 5726−5731.
32. Liu, L., and Guo, Q.-X. (2001) *Chem. Rev. 101*, 673−695.
33. Searle, M. S., Westwell, M. S., and Williams, D. H. (1995) *J. Chem. Soc., Perkin Trans. 2*, 141−151.

34. Rekharsky, M., and Inoue, Y. (2000) *J. Am. Chem. Soc. 122*, 4418−4435.
35. Liu, L., Yang, C., and Guo, Q.-X. (2000) *Biophys. Chem. 84*, 239−251.
36. Strazewski, P. (2002) *J. Am. Chem. Soc. 124*, 3546−3554.
37. Jeuken, L. J. C. (2001) Ph.D. Thesis, Chapter 9, University of Leiden, The Netherlands.
38. van Pouderoyen, G., Mazumdar, S., Hunt, N. I., Hill, H. A. O., and Canters, G. W. (1994) *Eur. J. Biochem. 222*, 583−588.
39. van Amsterdam, I. M. C., Ubbink, M., and Canters, G. W. (2002) *Inorg. Chim. Acta 331*, 296−302.
40. Kalverda, A. P., Wymenga, S. S., Lommen, A., van der Ven, F. J., Hilbers, C W., and Canters, G. W. (1994) *J. Mol. Biol. 240*, 358−371.
41. Kukimoto, M., Nishiyama, M., Ohnuki, T., Turley, S., Adman, E. T., Horinouchi, S., and Beppu, T. (1995) *Protein Eng. 8*, 153−158.
42. van de Kamp, M., Hali, F. C., Rosato, N., Finazzi-Agrò, A., and Canters, G. W. (1990) *Biochim. Biophys. Acta 1019*, 283−292.
43. Kuwana, T. (1977) in *Electrochemical studies of biological systems* (Sawyer, D. T., Ed.) ACS Symposium Series, No. 38, American Chemical Society, Washington, DC.
44. Yee, E. L., Cave, R. J., Guyer, K. L., Tyma, P. D., and Weaver, M. J. (1979) *J. Am. Chem. Soc. 101*, 1131−1137.
45. Yee, E. L., and Weaver, M. J. (1980) *Inorg. Chem. 19*, 1077−1079.
46. Koller, K. B., and Hawkridge, F. M. (1985) *J. Am. Chem. Soc. 107*, 7412−7417.
47. Nar, H., Messerschmidt, A., Huber, R., van de Kamp, M., and Canters, G. W. (1991) *J. Mol. Biol. 221*, 765−772.
48. Battistuzzi, G., Borsari, M., Canters, G. W., de Waal, E., Leonardi, A., Ranieri, A., and Sola, M. (2002) *Biochemistry 41*, 14293−14298.
49. Libeu, C. A. P., Kukimoto, M., Nishiyama, M., Horinouchi, S., and Adman, E. T. (1997) *Biochemistry 36*, 13160−13179.
50. Adman, E. T., Turley, S., Bramson, R., Petratos, K., Banner, D., Tsernoglou, D., Beppu, T., and Watanabe, H. (1989) *J. Biol. Chem. 264*, 87−99.
51. Petratos, K., Banner, D. W., Beppu, T., Wilson, K. S., and Tsernoglou, D. (1987) *FEBS Lett. 218*, 209−214.
52. Vakoufari, E., Wilson, K. S., and Petratos, K. (1994) *FEBS Lett. 347*, 203−206.
53. Romero, A., Nar, H., Huber, R., Messerschimdt, A., Kalverda, A., Canters, G. W., Durley, R., and Methews, F. S. (1994) *J. Mol. Biol. 236*, 1196−1211.
54. Guss, J. M., Harrowell, P. R., Murata, M., Norris, V. A., and Freeman, H. C. (1986) *J. Mol. Biol. 192*, 361−387.
55. Dennison, C., and Kohzuma, T. (1999) *Inorg. Chem. 38*, 1491−1497.
56. Kakutami, T., Watanabe, H., Arima, K., and Beppu, T. (1981) *J. Biochem. 89*, 463−472.
57. Susuki, S., Sakurai, T., Shidara, S., and Iwasaki, H. (1989) *Inorg. Chem. 28*, 802−804.
58. Durley, R., Chen, L., Louis, L. W., Mathews, F. S., and Davidson, V. I. (1993) *Protein Sci. 2*, 739−752.
59. Warshel, A., Papazyan, A., and Muegge, I. (1997) *J. Biol. Inorg. Chem. 2*, 143−152.
60. Jensen, G. M., Warshel, A., and Stephens, P. J. (1994) *Biochemistry 33*, 10911−10924.
61. Stephens, P. J., Jollie, D. R., and Warshel, A. (1996) *Chem. Rev. 96*, 2421−2513.
62. Muegge, I., Qi, P. X., Wand, A. J., Chu, Z. T., and Warshel, A. (1997) *J. Phys. Chem. B 101*, 825−836.
63. Diederix, R. E. M., Canters, G. W., and Dennison, C. (2000) *Biochemistry 39*, 9551−9560.
64. Baker, E. N. (1988) *J. Mol. Biol. 203*, 1071−1095.
65. Nar, H., Messerschmidt, A., Huber, R., van de Kamp, M., and Canters, G. W. (1991) *J. Mol. Biol. 218*, 427−447.
66. van de Kamp, M., Canters, G. W., Wijmenga, S. S., Lommen, A., Hilbers, C. W., Nar, H., Messerschmidt, A., and Huber, R. (1992) *Biochemistry 31*, 10194−10207.
67. Jeuken, L. J. C., Ubbink, M., Bitter, J. H., van Vliet, P., Meyer-Klauke, W., and Canters, G. W. (2000) *J. Mol. Biol. 299*, 737−755.
68. Romero, A., Hoitink, C. W. G., Nar, H., Huber, R., Messerschmidt, A., and Canters, G. W. (1993) *J. Mol. Biol. 229*, 1007−1021.
69. Messerschmidt, A., Prade, L., Kroes, S. J., Sanders-Loher, J., Huber, R., and Canters, G. W. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 3443−3448.
70. Kroes, S. J., Hoitink, C. W. G., Andrew, C. R., Al, J., Sanders-Loher, J., Messerschmidt, A., Hagen, W. R., and Canters, G. W. (1996) *Eur. J. Biochem. 240*, 342−351.
71. Salgado, J., Kroes, S. J., Berg, A., Moratal, J. M., and Canters, G. W. (1998) *J. Biol. Chem. 273*, 177−185.
72. Sokerina, E. V., Ullmann, G. M., van Puoderoyen, G., Canters, G. W., and Kostič, N. M. (1999) *J. Biol. Inorg. Chem. 4*, 111−121.
73. van Pouderoyen, G., Cigna, G., Rolli, G., Cutruzzolà, F., Malatesta, F., Silvestrini, M. C., Brunori, M., and Canters, G. W. (1997) *Eur. J. Biochem. 247*, 322−331.
74. Unpublished results from our laboratory.
75. Qian, W., Sun, Y.-L., Wang, Y.-H., Zhuang, J.-H., Xie, Y., and Huang, Z.-X. (1998) *Biochemistry 37*, 14137−14150.
76. Kita, F., Adam, W., Jordan, P., Nau, W. N., and Wirz, J. (1999) *J. Am. Chem. Soc. 121*, 9265−9275.
77. Bertini, I., Bryant, D. A., Ciurli, S., Dikiy, A., Fernandez, C. O., Luchinat, C., Safarov, N., Vila, A. J., and Zhao, J. (2001) *J. Biol. Chem. 276*, 47217−47226.
78. Kalverda, A., Ubbink, M., Gilardi, G., Wymenga, S. S., Crowford, A., Jeuken, L. J. C., and Canters, G. W. (1999) *Biochemistry 38*, 12690−12697.
79. Thompson, G. S., Leung, Y.-C., Ferguson, S. J., Radford, S. E., and Redfield, C. (2000) *Protein Sci. 9*, 846−858.
80. Ainscough, E. W., Bingham, A. G., Brodie, A. M., Ellis, W. R., Gray, H. B., Loehr, T. M., Plowman, J. E., Norris, G. E., and Baker, E. N. (1987) *Biochemistry 26*, 71−82.

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