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Articles

Enthalpic and Entropic Contributions to the Mutational Changes in the Reduction Potential of Azurin†

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ABSTRACT: The changes in the reduction potential of *Pseudomonas aeruginosa* and *Alcaligenes denitrificans* azurins following point mutations and residue ionizations were factorized into the enthalpic and entropic contributions through variable temperature direct electrochemistry experiments. The effects on the reduction enthalpy due to changes in the first coordination sphere of the copper ion, as in the Met121Gln and Met121His variants of *A. denitrificans* azurin, insertion of a net charge and alteration in the solvation properties and electrostatic potential in proximity of the metal site, as in the Met44Lys and His35Leu variants of *P. aeruginosa* azurin, respectively, and proton uptake/release in wild-type and mutated species could invariably be accounted for on the basis of simple coordination chemistry and/or electrostatic considerations. The concomitant changes in reduction entropy were found in general to contribute to the $E^{\circ\prime}$ variation to a lesser extent as compared to the enthalpy changes. However, their effects were by no means negligible and in some instances were found to heavily contribute to (or even become the main determinant of) the observed change in reduction potential. Several lines of evidence indicate that the entropic effects are notably influenced by reduction-induced solvent reorganization effects. In particular, protein reduction tends to be favored on entropic grounds with increasing exposure of the copper site to the solvent. Moreover, enthalpy−entropy compensation phenomena are invariably observed when residue mutation or pH-induced conformational changes modify the solvent accessibility of the metal site or alter the H-bonding network in the hydration shell of the molecule. Therefore, in these cases, caution must be used in making predictions of $E^{\circ\prime}$ changes simply based on Coulombic or coordination chemistry arguments.

The reduction potential ($E^{\circ\prime}$)[1] of the Cu^{2+}/Cu^{+} couple in blue copper proteins is markedly higher as compared to inorganic Cu complexes in aqueous solution (*1*−*6*). This is one of the peculiar and most thoroughly investigated properties of these species, which serve as biological electron carriers (*1*, *7*−*10*). The degree of hydrophobicity of the protein environment, the nature of axial copper ligation, and

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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; CV, cyclic voltammetry; PGE, pyrolytic graphite edge electrode; SCE, saturated calomel electrode; SHE, standard hydrogen electrode; *P. aer.*, *Pseudomonas aeruginosa*; *A. den.*, *Alcaligenes denitrificans*.

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site exposure to the solvent have been established as the principal effectors of these potentials (*1*−*4*, *11*−*13*).

Further insight into the mechanisms of $E^{\circ\prime}$ regulation can be gained from the factorization of the corresponding enthalpic and entropic components, which can be measured through variable temperature electrochemical measurements (*2*−*4*, *14*, *15*). A comparative analysis of a large number of native blue copper proteins indicated that the main determinant of the high reduction potential of these species is the selective enthalpic stabilization of the reduced over the oxidized state due to ligand binding interactions and electrostatics at the metal site (*2*). In particular, $\Delta H^{\circ\prime}$ turned out to be particularly sensitive to the strength of axial copper ligation. However, also the reduction entropy, which is related to solvation properties of the site and possibly to differences in flexibility of the polypeptide chain in the two redox states, plays a relevant role in the control of $E^{\circ\prime}$.

Here, we have exploited a mutational approach to characterize further the effectors of the reduction thermodynamics in these species. In particular, we measured the enthalpic and entropic contribution to the change in reduction potential due to point mutations and residue ionizations in *Pseudomonas aeruginosa* and *Alcaligenes denitrificans* azurins. Mutations were made at the axial copper ligand (Met121), which was replaced with His and Gln (the latter being the other residue serving as the axial ligand in native blue copper proteins) and at residues in close proximity of the metal site which either belong to the hydrophobic patch involved in association with the ET partner (Met44) or contribute to the electrostatic potential and/or structural features through Coulombic and H-bonding interactions (His35). We found that the enthalpic effects due to the change in the first coordination shell and insertion of net charges in the surroundings of the site can be interpreted by simple arguments based on coordination chemistry and electrostatic considerations, respectively, whereas the reduction entropy is sensitive to changes in the solvation properties of the copper site.

EXPERIMENTAL PROCEDURES

Proteins. Wild-type *Ps. aeruginosa* and *A. denitrificans* azurins and the variants H35L, M44K (*P. aer.*), and M121H and M121Q (*A. den.*) were isolated according to the literature (*16*−*19*). 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 pyrolitic graphite disk (PGE) was used as the 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) (*20*). All 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. 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 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. 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 assumed as the $E^{\circ\prime}$ values. The temperature dependence of the reduction potential was determined with a "nonisothermal" cell (*21*, *22*) 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 (*21*−*23*)

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

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 (*21*−*24*). The pH of the samples was changed by adding small amounts of concentrated NaOH or HCl under fast stirring. 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 thermodynamics of Cu^{2+} reduction for wild-type and mutated *P. aeruginosa* and *A. denitrificans* azurins were determined from the temperature dependence of $E^{\circ\prime}$ in nonisothermal experiments (Figures 1 and 2). The $\Delta H^{\circ\prime}_{rc}$ and $\Delta S^{\circ\prime}_{rc}$ values are listed in Table 1 along with the enthalpic and entropic contributions to $E^{\circ\prime}$ ($-\Delta H^{\circ\prime}_{rc}/F$ and $T\Delta S^{\circ\prime}_{rc}/F$, respectively).

Substitution of the Axial Methionine Ligand. The copper site of *A. denitrificans* azurin possesses 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 as weak axial ligands (*25*). In the M121H and M121Q variants, substitution of the axial

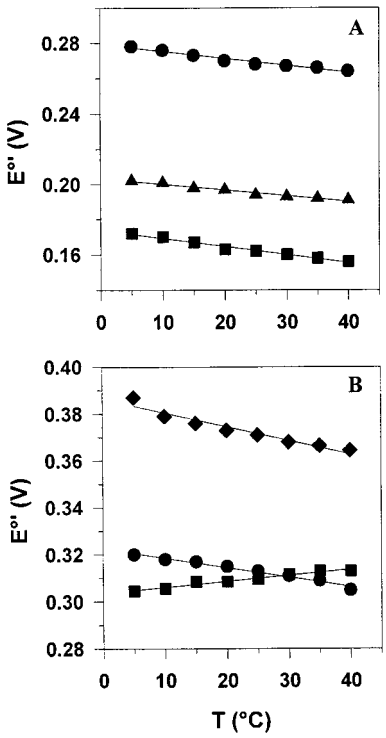

FIGURE 1: $E^{\circ\prime}$ vs T plots for wild-type and mutated azurins at pH 7. The slope of the plots yields the $\Delta S^{\circ\prime}_{rc}$ values. (A) *A. denitrificans* azurin: wild type (●), M121Q (■), and M121H (▲). (B) *P. aeruginosa* azurin: wild type (●), H35L (■), and M44K (◆). Conditions: protein concentration, 0.1 mM; base electrolyte, 0.1 M phosphate, pH 7. Solid lines are least-squares fits to the data points. Error bars have the same dimensions of the symbols.

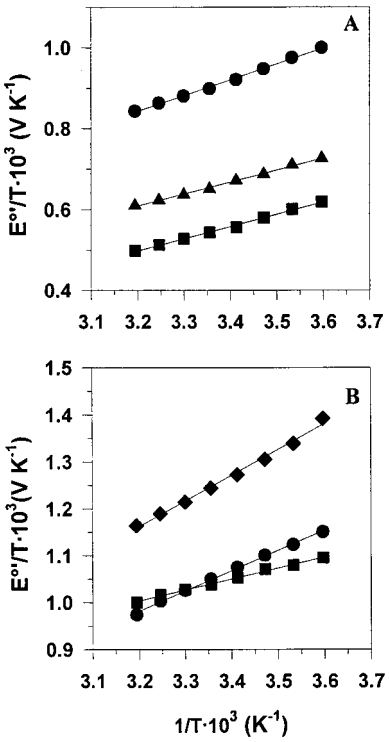

FIGURE 2: $E^{\circ\prime}/T$ vs $1/T$ plots for wild-type and mutated azurins at pH 7. The slope of the plots yields the $-\Delta H^{\circ\prime}_{rc}$ values. (A) *A. denitrificans* azurin: wild type (●), M121Q (■), and M121H (▲). (B) *P. aeruginosa* azurin: wild type (●), H35L (■), and M44K (◆). Conditions: protein concentration, 0.1 mM; base electrolyte, 0.1 M phosphate, pH 7. Solid lines are least-squares fits to the data points. Error bars have the same dimensions of the symbols.

methionine by histidine and glutamine, respectively, induces a remarkable strengthening of the Cu−ligand bond (which shortens from 3.11 Å to 2.24 and 2.26 Å), the displacement of the copper ion from the trigonal N_2S plane toward the strong axial ligand, and the establishment of a distorted tetrahedral coordination geometry (*18*, *19*, *26*). The metal site of the M121Q mutant closely resembles that of native cucumber stellacyanin (*27*), which shares the same ligand set.

With respect to the wild type, both mutants show a less negative reduction enthalpy but only modest differences in reduction entropy (note that the error affecting $\Delta S^{\circ\prime}_{rc}$ is ±6 J mol^{-1} K^{-1}) (Table 1). The enthalpic effect, which therefore turns out to be mainly responsible for the mutation-induced decrease in $E^{\circ\prime}$, can be ascribed to the selective stabilization of the oxidized over the reduced state induced by the stronger electron donor properties of the substituting ligand. It is noteworthy that the $\Delta H^{\circ\prime}_{rc}$ value of M121Q (−29 kJ mol^{-1}) is very similar to that of the stellacyanins from *Rhus vernicifera* (−25 kJ mol^{-1}), cucumber (−32 kJ mol^{-1}), and horseradish umecyanin (−33 kJ mol^{-1}) (*2*), which all involve axial glutamine ligation.

The absence of significant changes in the reduction entropy for these mutants can be justified with the rigidity of type 1 copper sites, which in the wild-type species are known to undergo only small changes in the coordination shell on passing from the cupric to the cuprous state. This rigidity is conferred by a tight network of hydrogen bonds and van der Waals interactions (*25*, *28*−*33*), which therefore appear to be largely unaffected by the replacement of the axial ligand. The results indicate that, in general, first coordination sphere effects on the redox potential mainly derive from $\Delta H^{\circ\prime}_{rc}$.

Table 1: Thermodynamic Parameters for Cu^{2+} Reduction in Wild-Type and Mutated 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)
P. aer.					
wt	−42	−39	+315	+435	−120
H35L	−22	+25	+309	+228	+77
M44K	−51	−50	+371	+528	−154
A. den.					
wt	−38	−39	+268	+393	−120
M121Q	−29	−45	+162[c]	+300	−139
M121H	−28	−32	+194	+290	−99

[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] This value is in good agreement with that obtained recently for the same species with CV experiments using a gold electrode surface modified with 4,4′-dipyridyl disulfide (+147 ± 5 mV) (*52*); the modest difference might be related to the different buffer systems used.

Changes in the Accessibility of the Copper Site to the Solvent. The changes in reduction enthalpy and entropy for *P. aeruginosa* H35L azurin with respect to the wt species are almost exactly compensatory. Interestingly, it is known that substitution of this residue induces the opening of a cavity that becomes filled with water molecules in the vicinity of the metal site; hence this mutation should enhance the accessibility of the metal site to the solvent (*32*). The sizable mutation-induced increase in ΔH°_{rc} (Table 1) is

consistent with the enhanced interaction of the cupric ion with the water dipoles in its environment. An additional contribution arises from the fact that His35 is partly protonated at pH 7 ($pK_a^{ox} = 6.2$, $pK_a^{red} = 7.2$): its substitution with a neutral Leu residue implies the deletion of a partial positive charge, hence a stabilization of the oxidized state (*34*). The concomitant increase in reduction entropy can be interpreted in a similar vein: the increased electrostatic interaction of the metal ion in its oxidized form with the water molecules in the cavity around Leu35 leads to increased ordering that is reflected in a decrease in oxidation entropy or, equivalently, an increase in reduction entropy. This nicely confirms the observation that emerged previously from the analysis of the redox thermodynamics of a number of native species, namely, that a greater exposure to the solvent of the metal site results in an increase of the reduction entropy (*2*). In the present case, this even leads to a positive $\Delta S^{\circ\prime}_{rc}$ (Table 1). Other contributions to the reduction entropy, namely, oxidation state-dependent changes in the flexibility of the polypeptide chain, including creation and/or suppression of vibrational and torsional degrees of freedom (which are largely uncharacterized for this protein class), and the differential binding of ions to surface sites of the oxidized and reduced protein (*14*), could be operative, but their role cannot be established safely at present. However, the presence of enthalpy−entropy compensation effects strongly suggests that the effect of mutation-induced alterations of the redox potential is dampened by the solvent. In fact, 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 in general largely compensating enthalpy and entropy changes (*35*−*42*).

Addition or Suppression of Net Protein Charges. Met44 is a conserved residue belonging to the hydrophobic surface patch that surrounds the Cu ligand His117. This patch is thought to be important for making contact with redox partners prior to electron transfer (*17*, *43*−*46*). At pH <8 the reduction potential of the M44K mutant of *P. aeruginosa* azurin is about 60 mV higher than that of the wild-type species (Table 1) (*17*). EPR, NMR, and electronic spectra show that M44K mutation does not affect appreciably the protein structure, including the features of the metal site, although at pH >8 when deprotonation of Lys44 sets in, the optical and EPR features of the Cu site are diagnostic of small structural changes (*17*). The enthalpic effect of this residue substitution is that expected for the introduction of one positive charge near the metal center, namely, a more negative $\Delta H^{\circ\prime}_{rc}$ as a result of the preferential stabilization of the cuprous state. This effect is opposite to that observed for the H35L mutant in which we have a deletion of one (partial) positive charge, indeed. Analogous results have been obtained on a number of plastocyanin mutants subject to charge alterations on residues in the vicinity of the copper center (*47*). Hence, the enthalpic effects of these mutations can be straightforwardly interpreted on simple electrostatic grounds. The much greater $|\Delta\Delta H^{\circ\prime}_{rc}|$ value for H35L (20 kJ mol^{-1}) as compared to M44K (9 kJ mol^{-1}) is thought to be due to the solvation effects discussed above for the former species and to the fact that the residue in position 44 is located on the protein surface and the electrostatic effect is partly suppressed by the high dielectric constant of water

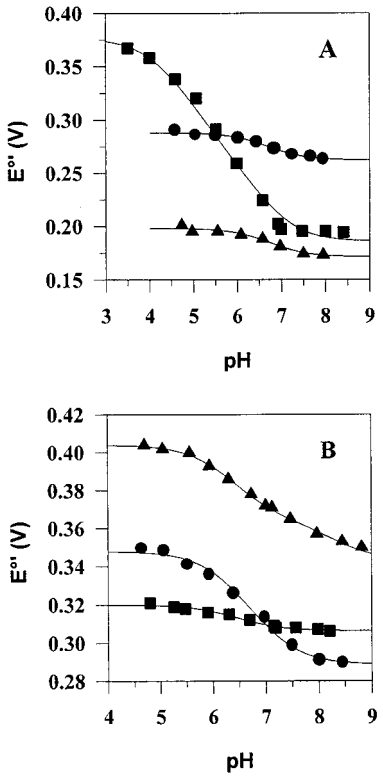

FIGURE 3: pH dependence of the reduction potential of (A) *A. denitrificans* azurin, wild type (●), M121H (■), and M121Q (▲), and (B) *P. aeruginosa* azurin, wild type (●), H35L (■), and M44K (▲). Condition: protein concentration, 0.1 mM. Solid lines are least-squares fits to the equation given in footnote *a* of Table 2.

(*17*, *34*). As noted above for H35L, also for the M44K variant the $\Delta\Delta S^{\circ\prime}_{rc}$ term partly compensates the $\Delta\Delta H^{\circ\prime}_{rc}$ term. Since the entropic effect is likely to be related to solvation effects, it is apparent that the solvent exerts a dual role in quenching the electrostatic effects. First, the high dielectric constant of the solvent lowers the Coulombic interaction, and, second, the entropic effects of the reorganization of the solvent H-bonding network tends to compensate the enthalpic change due to addition/deletion of charge.

Modulation of the Reduction Thermodynamics by Acid−*Base Equilibria.* The pH dependence of $E^{\circ\prime}$ for recombinant wild-type *P. aeruginosa* azurin and its M44K and H35L mutants and for recombinant wild-type *A. denitrificans* azurin and its M121H mutant is shown in Figure 3. The data for the former group of proteins are in agreement with those obtained previously from potentiometric titrations with the ferro/ferricyanide couple (*17*, *48*) and from spectroelectrochemical measurements (*49*). The $E^{\circ\prime}$ for wt *P. aeruginosa* azurin titrates with apparent pK_a values of $pK_a^{ox} = 6.2$ and $pK_a^{red} = 7.2$ (Table 2). The remarkable suppression of the pH dependence of $E^{\circ\prime}$ for H35L confirms that His35 is mainly responsible for the modulation of $E^{\circ\prime}$ of the wt species in the pH range investigated, as proposed elsewhere (*17*, *48*). The residual pH dependence ($pK_a^{ox} = 6.3$, $pK_a^{red} = 6.6$) can be attributed to His83, which is located farther from the Cu (*17*, *34*, *48*, *50*). A closely similar $E^{\circ\prime}$/pH profile has been obtained for M44K ($pK_a^{ox} = 6.1$, $pK_a^{red} = 6.7$). Introduction of the additional positive charge of K44 lowers slightly the apparent pK_a for His35 deprotonation, as noted elsewhere (*17*, *48*). The $E^{\circ\prime}$ measurements for this mutant could be performed only up to pH 9, due to the worsening of the

Table 2: Apparent pK_a Values for Wild-Type and Mutated Azurins[a]

protein	pK_{ox}	pK_{red}	protein	pK_{ox}	pK_{red}
P. aer. wt	6.2	7.2	*A. den.* wt	6.5	6.9
P. aer. H35L	6.3	6.6	*A. den.* M121Q	6.5	7.0
P. aer. M44K	6.1	6.7	*A. den.* M121H	4.0	7.2

[a] The pK_{app} values were obtained from a fit of the experimental data to the conventional one-proton equilibrium equation $E^{\circ\prime} = E_a^{\circ} - 2.303(RT/nF)\log[([H^+] + K_{ox})/([H^+] + K_{red})]$ in which $E_a^{\circ\prime}$ is the limit $E^{\circ\prime}$ value for the protein containing the ionizing residue in the fully protonated form and K_{ox} and K_{red} are the proton dissociation constants for the oxidized and reduced proteins, respectively (*55*). The pK_a values are affected by an uncertainty of ±0.1 unit.

voltammetric response at high pH; hence the additional titration step due to the deprotonation of Lys44 could not be fully detected, although its onset is visible at the high pH end of the titration curve in Figure 3A (*17*, *48*).

Table 3 lists the $\Delta H^{\circ\prime}_{rc}$ and $\Delta S^{\circ\prime}_{rc}$ values determined at the two extremes of pH for the various species. It turns out that for wt *P. aeruginosa* azurin the enthalpic effect of His35 deprotonation [$\Delta\Delta H^{\circ\prime}_{rc} = (\Delta H^{\circ\prime}_{rc})_{pH=8} - (\Delta H^{\circ\prime}_{rc})_{pH=5} = +7$ kJ mol^{-1}] is that of stabilizing the oxidized state, as expected on simple electrostatic grounds. If we consider the $\Delta\Delta H^{\circ\prime}_{rc}$ values of +20 and −9 kJ mol^{-1} determined by the partial deletion of one positive charge in H35L (which also includes the effect of the solvent dipoles, as noted above) and the addition of one positive charge in M44K, respectively, the electrostatic effects of charge variation in proximity of the metal center turn out to affect the enthalpic term in a qualitatively straightforward way. The $\Delta\Delta S^{\circ\prime}_{rc}$ for His35 deprotonation is almost negligible. This is consistent with the crystallographic data obtained for this species at pH 5.5 and 9, which show that the acid−base equilibrium of His35 induces a conformational reorganization and a H-bonding change at low pH which are localized at residues 36−37, whereas the coordination geometry and the rest of the protein structure are largely unaffected (*28*). The data for H35L indicate that the acid−base equilibrium of His83 exerts a very small influence in both reduction enthalpy and entropy of the protein.

The decrease in $E^{\circ\prime}$ upon raising the pH from 5 to 8 for wt *A. denitrificans* azurin (p$K_a^{ox} = 6.5$, p$K_a^{red} = 6.9$) is much smaller that that for wt *P. aeruginosa* azurin (28 vs 65 mV), in line with that found previously from spectroelectrochemical measurements (*49*). This effect is most probably due to the fact that His83 and His32, which are likely responsible of the pH dependence of $E^{\circ\prime}$ in the former species, are far away from the metal center (*49*). It is interesting to note that in this case the effect of residue deprotonation is to enthalpically stabilize the reduced state (Table 3), which is opposite to the expected electrostatic effect, and to also induce a remarkable change in reduction entropy. This behavior cannot be interpreted safely with the data at hand. It has been argued that the hydrogen-bonding network in the surface patch around His35 in *P. aeruginosa* azurin is distinctly different than in the *A. denitrificans* azurin and that this difference is responsible for the difference in pH titration behavior of His35 in the two azurins (*51*). It is conceivable that a change in redox state of the copper affects the structural organization of this patch differently in the two proteins. The pH dependence of the redox potential of the M121Q azurin variant parallels that of the wt protein (see Figure 3) and can be ascribed to the same causes. The pH dependence has been observed before (*52*) and was thought to be linear with pH. The present more accurate data allow for the sigmoidal character of the variation in redox potential to be recognized.

Table 3: $\Delta S^{\circ\prime}_{rc}$ and $\Delta H^{\circ\prime}_{rc}$ Values at Extremes of pH for Wild-Type and Mutated Azurins[a]

	pH	$\Delta H^{\circ\prime}_{rc}$ (kJ mol^{-1})	$\Delta S^{\circ\prime}_{rc}$ (J K^{-1} mol^{-1})
P. aer. wt	5.0	−43	−33
	8.0	−36	−28
P. aer. H35L	5.0	−23	+27
	8.0	−23	+23
A. den. wt	5.0	−29	−6
	7.5	−37	−39
A. den. M121H	3.5	−20	+51
	7.0	−29	−32

[a] All measurements were done in 0.1 M phosphate buffer. 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 $E^{\circ\prime}$ of the M121H mutant of *A. denitrificans* azurin titrates with a pK_a^{ox} value of 4.0 and a pK_a^{red} value of 7.0. The X-ray data of this mutant at pH 3.5 and 6.5 (*19*) clearly show that the residue responsible of this remarkable $E^{\circ\prime}$/pH profile is the Cu ligand His121, which at low pH protonates and detaches from the metal. The pK_a^{ox} value is very close to that determined from electronic and NMR spectra (*26*, *53*, *54*). The thermodynamics measured at the two extremes of pH (Table 3) can be nicely explained on the basis of the crystallographic data. In particular, the detachment of His121 at low pH would be expected to induce a large stabilization of the cuprous state (hence a more negative reduction enthalpy) which, however, is contrary to the experimental result. In fact, the $\Delta H^{\circ\prime}_{rc}$ value at pH 3.5 is slightly less negative than that at pH 7 (Table 3). Yet, this observation can be explained on the basis of X-ray data that shows that at low pH the empty coordination site is occupied by a buffer ion or water molecule (*19*). This effect, coupled with the enhanced solvent accessibility of the metal site at low pH, would account for the stabilization of the oxidized state. Thus, the large increase in $E^{\circ\prime}$ with decreasing pH turns out to be totally an entropic effect, most probably due in part to the increase in solvent accessibility of the metal site (which we have invariably found to be associated with an entropy increase, as noted above). In addition, the Cys112−Met121 loop acquires additional mobility as a result of the mutation (*19*), which may be more enhanced in the reduced than in the oxidized protein.

CONCLUSIONS

Mutations which affect the coordination sphere of the copper ion or the solvation properties and the electrostatic potential in proximity of the metal site alter the enthalpy of reduction in a way which is easily accounted for on the basis of simple coordination chemistry and/or electrostatic considerations. However, the effects on the free energy, i.e., *on the reduction potential*, are rather unpredictable owing to the entropic contribution to $E^{\circ\prime}$, which is mainly determined by reduction-induced solvent reorganization, although it is apparent that the entropy changes tend to compensate the enthalpic effects. Thus the solvent may quench the effects

of a change in the net surface charge on the reduction potential of redox metalloproteins. Further, changes in the state of protonation of a ligand may heavily influence the entropy of reduction.

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