Key Role of the Local Hydrophobicity in the East Patch of Plastocyanins on Their Thermal Stability and Redox Properties

José Luis Olloqui-Sariego, Inmaculada Márquez, Estrella Frutos-Beltrán, Irene Díaz-Moreno, Miguel A. De la Rosa, Juan José Calvente, Rafael Andreu, and Antonio Díaz-Quintana

Departamento de Química Física, Universidad de Sevilla, c/ Profesor García González, 1, 41012 Sevilla, Spain
Instituto de Investigaciones Químicas, c/cartuja, Universidad de Sevilla y CSIC, Avd. Américo Vespucio 49, 41092 Sevilla, Spain

ABSTRACT: Understanding the molecular basis of the thermal stability and functionality of redox proteins has important practical applications. Here, we show a distinct thermal dependence of the spectroscopic and electrochemical properties of two plastocyanins from the thermophilic cyanobacterium *Phormidium laminosum* and their mesophilic counterpart from *Synechocystis* sp. *PCC 6803*, despite the similarity of their molecular structures. To explore the origin of these differences, we have mimicked the local hydrophobicity in the east patch of the thermophilic protein by replacing a valine of the mesophilic plastocyanin by isoleucine. Interestingly, the resulting mutant approaches the thermal stability, redox thermodynamics, and dynamic coupling of the flexible site motions of the thermophilic protein, indicating the existence of a close connection between the hydrophobic packing of the east patch region of plastocyanin and the functional control and stability of the oxidized and reduced forms of the protein.

1. INTRODUCTION

Understanding the structural and molecular factors that modulate the thermal stability and activity of redox proteins is of high scientific and technological interest. Plastocyanin (Pc) is a member of the blue copper protein family and acts as an electron carrier in oxygenic photosynthesis. Despite their structural similarities, Pc from the thermophilic cyanobacterium *Phormidium laminosum* (Pho-Pc) displays a higher thermal stability than its counterpart from mesophilic *Synechocystis* sp. *PCC 6803* (Syn-Pc), as well as a different dependence of the melting point on the redox state. Copper ligands are known to play a key role in the thermal stability of blue copper proteins, and they are highly conserved in Pcs. On the other hand, molecular dynamics (MD) simulations suggest that small structural differences in the flexible loop L5 (see Figure 1) can modulate the stability of the active copper site and contribute to the protein’s thermal response. This loop is far from the copper center and takes part in the so-called east patch, also known as site 2 in functional analysis. Further, the triple mutant A44D/D49P/A62L of Syn-Pc, designed by sequence comparison to stabilize the redox behavior of the protein during crystallization trials, displays a conformation of loop L5 similar to that found in the structure in the solution of the wild-type (WT) species (Figure 1).

This indirect control was evident when inducing a thermal destabilization of the thermophilic protein after introducing a single mutation in the east patch. Further X-ray absorption spectroscopy investigations revealed that this mutation induces a subtle change in the local geometry of the Cu-binding site, which then causes the thermal stabilization. Nevertheless, the mechanism that allows this flexible region, located far from the metal active center, to regulate the protein’s thermal stability is not fully understood.

Here, we have investigated the thermostability and redox properties of Pcs by variable-temperature cyclic voltammetry and fluorescence and circular dichroism (CD) spectroscopic measurements. Notably, the thermophilic and mesophilic variants display different thermal dependence of the spectroscopic and electrochemical properties. To dig into the structural factors causing these differences, we have analyzed the effect of replacing the amino acid Val48 of *Syn-Pc* by isoleucine (V48I *Syn-Pc*). Considering that Val48 is located between loops L5 and L7 in the east patch of *Syn-Pc*, this mutation aims at stabilizing the mesophilic variant by introducing an extra methylene group between the two aforementioned loops, thereby trying to mimic the local hydrophobicity of the thermophilic variant (see Figure 1). The loops L1 and L5 are the areas with the highest fluctuations in all Pcs. In the vicinity of the L5 loop of *Syn-Pc*, there is a cavity in which two water molecules are intercalated that interact with the residues of the L5 and L7 loops and form a network of hydrogen bridges. The L5 loop has a three-residue insert that in which two water molecules are intercalated that interact with the residues of the L5 and L7 loops and form a network of hydrogen bridges. The L5 loop has a three-residue insert that allows the formation of a hydrophobic cluster consisting of residues Phe80, Val48, Ile85, Pro81, Pro49, and Tyr85 in *Pho-Pc*. However, such grouping is reduced to Tyr79, Val48, and Pro76 in *Syn-Pc*. The inclusion of an extra methylene group by the substitution of valine 48 in *Syn-Pc* by isoleucine is expected to reduce the gap between the L5 loop and the barrel, thereby increasing the hydrophobic interactions in the cluster.
decreasing its solvation, and reinforcing the van der Waals interactions among the residues in this area. The consequences of this mutation on the flexibility and mobility of the protein structure have been analyzed further by MD.

2. RESULTS AND DISCUSSION

2.1. Thermal Stability of Pcs. The thermal stabilities of oxidized and reduced Pcs are characterized by their melting points ($T_m$). The thermal stabilities of Pcs were investigated by monitoring the change in the intensity of fluorescence of tryptophan 31, a residue located within the hydrophobic pocket occluded by the east patch loop of Pc. The tryptophan fluorescence shift and intensity are, in fact, indicative of the exposure of its indol ring to the external aqueous environment. Complementarily, CD was also used to detect the changes in the secondary structure during thermal unfolding. In particular, we followed the changes in the $\beta$-strand content upon increasing the temperature. Figure 2 illustrates the normalized unfolding curves obtained by the fluorescence and CD measurements of the oxidized and reduced forms of the three Pcs. The normalized thermal unfolding curves were fitted to a simple two-state unfolding model to estimate the melting points ($T_m$) of the Pcs according to the following equation

$$S = \frac{S_f + S_u e^{-\Delta G_f/RT}}{1 + e^{-\Delta G_f/RT}}$$

where $S$ is the protein signal at a given temperature ($T$), $S_u$ and $S_f$ are the signals of protein solutions of equal concentration of either unfolded or folded protein, and $\Delta G_f$ is the free-energy difference of the equilibrium between the unfolded conformation and the native conformation. This free energy is approximated by the integrated Gibbs–Helmholtz equation

$$\Delta G = \Delta H_T \left(1 - \frac{T}{T_m}\right) - \Delta C_p \left(T_m - T + T \ln \left(\frac{T}{T_m}\right)\right)$$

where $T_m$, $\Delta H_T$, and $\Delta C_p$ are the melting temperature, enthalpy change, and heat capacity change of the specific equilibrium, respectively. To fit the thermal unfolding curves of each Pc, we have simplified eq 2 by considering that $\Delta C_p \approx 0$, so that $\Delta G \approx \Delta H_T \left(1 - T/T_m\right)$. Moreover, we have considered that $S_i = S_0 + m_i(T - T_o)$ to account for the linear dependence of the CD signal of the folded protein prior to its thermal unfolding, where $S_0$ is the signal of native molecules at the low temperature $T_o$ and $m_i$ is the slope of the spectroscopic signal showing a linear dependence with temperature.

The fitting parameters used to quantify the thermal unfolding curves of Pcs are listed in Table 1. The estimated $T_m$ values of the oxidized and reduced forms of the protein by the two different spectroscopic techniques are somewhat

![Figure 1](image1.png)

![Figure 2](image2.png)
Table 1. Thermal Unfolding Parameters of Pcs

|          | WT Pho-Pc | V48I Syn-Pc | WT Syn-Pc |
|----------|-----------|-------------|-----------|
| \(T_m\) (°C)/oxidized\(b\) | 81.8 ± 0.4\(c\) | 67.1 ± 0.1 | 59.8 ± 0.1 |
| \(\Delta H_m\) (kJ mol\(^{-1}\))/oxidized\(b\) | 640 ± 50\(e\) | 4500 ± 1000 | 1200 ± 500 |
| \(T_m\) (°C)/reduced\(b\) | 75.7 ± 1.0\(e\) | 68.7 ± 0.3 | 68.5 ± 0.6 |
| \(\Delta H_m\) (kJ mol\(^{-1}\))/reduced\(b\) | 380 ± 10\(e\) | 2000 ± 1000 | 1200 ± 500 |
| \(T_m\) (°C)/oxidized\(b\) | 74.6 ± 0.5\(e\) | 67.0 ± 0.7 | 63.1 ± 0.1 |
| \(\Delta H_m\) (kJ mol\(^{-1}\))/oxidized\(b\) | 380 ± 30\(e\) | 500 ± 50 | 380 ± 30 |
| \(T_m\) (°C)/reduced\(b\) | 73.9 ± 0.5\(e\) | 73.8 ± 0.3 | 72.0 ± 0.3 |
| \(\Delta H_m\) (kJ mol\(^{-1}\))/reduced\(b\) | 400 ± 50\(e\) | 300 ± 30 | 300 ± 30 |
| \(T_m\) (°C)/apo-Pc\(b\) | 49.3 ± 1.3\(e\) | 44.1 ± 0.3 | 43.4 ± 0.7 |
| \(\Delta H_m\) (kJ mol\(^{-1}\))/apo-Pc\(b\) | 200 ± 30 | 200 ± 30 | 200 ± 30 |

\(a\) Determined by fluorescence spectroscopy. \(b\) Determined by CD spectroscopy. \(c\) From refs 6 and 15.

2.2. Redox Thermodynamics of Immobilized Pc. On the basis of the above findings, we have investigated the influence of the remote east patch region on the redox function of Pcs. In particular, we have assessed the temperature dependence of the interfacial electron transfer of immobilized Pcs on a cysteamine-modified gold electrode. To this end, the protein-modified electrode was subjected to a thermal cycle that lasted ~6 h, where the temperature was first increased from 0 to 90 °C and then lowered back to 0 °C, while recording the cyclic voltammograms every 10 °C. Figure 3a illustrates some typical cyclic voltammograms recorded at 0 and 80 °C for the three Pcs. The thermodynamics of Pc redox conversion was characterized by its formal potential, which is determined as the midpoint potential (\(E_{1/2}\)) of the anodic and cathodic voltammetric waves at low scan rates. Figure 3b shows the temperature dependence of the midpoint potentials for the three Pcs. The temperature dependence of the \(E_{1/2}\) values was found to be different for Pho-Pc and Syn-Pc. At low...
temperatures, the $E_{1/2}$ values for Pho-Pc are more positive than for Syn-Pc, indicating a higher stabilization of the reduced Pho-Pc. However, as the temperature raises over 30 °C, the $E_{1/2}$ values of Pho-Pc become more negative than those of Syn-Pc, suggesting a further stabilization of the oxidized form of Pho-Pc as the temperature is increased.

The values of the redox thermodynamic parameters $\Delta S_{\text{rc}}$ and $\Delta H_{\text{rc}}$ associated with the electron exchange between the protein and the electrode, were estimated from the slopes of the $E_{1/2}$ versus $T$ (Figure 3b) and the $E_{1/2}/T$ versus $1/T$ (Figure 3c) plots, respectively, and are listed in Table 2. The

|                  | WT Pho-Pc | V48I Syn-Pc | WT Syn-Pc |
|------------------|-----------|-------------|-----------|
| $E_{1/2,298K}$ (mV) vs NHE | 369 ± 5  | 369 ± 5  | 365 ± 5  |
| $\Delta S_{\text{rc}}$ (J mol$^{-1}$ K$^{-1}$) | −65 ± 4  | −67 ± 9  | −41 ± 7  |
| $\Delta H_{\text{rc}}$ (kJ mol$^{-1}$) | −54 ± 3  | −54 ± 4  | −47 ± 3  |

$E_{1/2}$ values for these proteins are found to be modulated by both enthalpic and entropic contributions. In particular, the redox thermodynamics of Pho-Pc and Syn-Pc is characterized by the highly negative $\Delta S_{\text{rc}}$ and $\Delta H_{\text{rc}}$ values, in agreement with previous results reported for the other proteins of the same family, revealing significant differences in the protein solvation and/or structure upon changing its oxidation state. Thus, the larger absolute $\Delta S_{\text{rc}}$ and $\Delta H_{\text{rc}}$ values of Pho-Pc, as compared to those of Syn-Pc, would point to a higher flexibility of the protein matrix of Pho-Pc. Notably, the $E_{1/2}$ values of the V48I mutant are similar to those of the thermophilic protein in the whole temperature range we have explored (Figure 3b). The thermodynamic analysis of the $E_{1/2}$ values shows that the $E_{1/2}$ differences between the Syn-Pc mutant and its WT counterpart come from a competitive balance between the enthalpic and entropic contributions (see Table 2). These results reveal that the hydrophobic packing in the east patch region of Pc is crucial to control the relative stability and function of the oxidized and reduced forms of the protein.

Besides, the fact that the oxidized form of the V48I Syn-Pc mutant is more thermostable than the oxidized form of Syn-Pc, combined with the fact that the $E_{1/2}$ values of the V48I Syn-Pc mutant are negatively shifted, up to 20 mV at the highest temperature, suggests the existence of a relationship between the redox function and protein thermal stability.

2.3. MD of Pc. Bearing in mind that the thermostability and functionality of redox proteins are related to their structural flexibility, we have analyzed the influence of the local hydrophobicity of the east patch of these Pcs on their mobility and flexibility by MD. We have performed a series of MD simulations at 298 K (see Figure 4).

The total simulated time was 50 ns in each run. This assures a sufficient conformation sampling, as we showed for Pho-Pc in the previous 10 ns computations. The structure of the three proteins hardly changed along the computations, according to the behavior of the radius of gyration ($R_g$) and the RMS deviations (RMSD) of the backbone atom coordinates along the trajectories (Figure 4A). In fact, the RMSD between the average structure of the trajectories and the coordinates in the PDB (Protein Data Bank) were 1.04 Å for Pho-Pc (pdb code: 1baw) and 1.2 Å for Syn-Pc (pdb code: 1pcs), and the largest differences were located at the mobile loops L1 and L5. The analysis of the per-residue fluctuations carried out to test the flexibility of the different parts of the protein showed similar patterns for the three Pcs (Figure 4B). Nevertheless, Syn-Pc showed slightly higher values than the two other proteins for the three sequence stretches corresponding to the loops L1, L5, and the C-terminal loop (CTL), which provides three of the ligands to the metal. To test whether such minimal changes would have a functional meaning, the covariance matrix of atomic coordinates for Syn-Pc and the V48I mutant was obtained (Figure 4C). Notably there are substantial differences between the inter-residual covariances, suggesting a change in the coupling of motions of flexible regions within the proteins.

![Figure 4](image-url)
Then, the quasi-harmonic analysis of the mass-weighted covariance of main chain atoms allowed us to determine the concerted motions within the three proteins. Figure 5 displays the RMS fluctuation ellipsoids for the first nontrivial modes.

![Figure 5](image)

**Figure 5.** Thermal ellipsoids for the first internal motion modes of the main chain atoms. The ellipsoid amplitudes are proportional to the atomic fluctuations along the trajectory for each mode. The ellipsoid colors follow the CPK scheme of the corresponding atoms. The arrows highlight the differences in the coupling of the flexible site motions.

For Syn-Pc, this “essential dynamics” analysis shows a strong correlation of motions of the N-terminus with loops 1 and C (black arrows in Figure 5). This coupling of motions involving the CTL is much weaker for Pho-Pc and V48I Syn-Pc. This is also consistent with our previous analysis by X-ray absorption near-edge spectroscopy, showing a 0.15 Å increase in the Cu–SγCys bond length in the reduced Syn-Pc with respect to that in Pho-Pc.16 Instead, both Pho-Pc and V48I Syn-Pc show an increased coupling between the motions of loop 1 and the other regions of the protein far from the copper site, including loop 5 in the east patch (green and orange arrows in Figure 5). These results strongly suggest a relationship between the coupling of the internal motions of the CTLs containing the copper ligands and the packing of the hydrophobic residues located in the east patch of Pc.

Figure 6B illustrates how the V48I mutation decreases the interplay among K59, E84, and R87, and, as a result, the fluctuations in the side chain of R87 decrease. In Pho-Pc, R93 (R87 in Syn proteins) is restrained through its interaction with D44 (the average distance between the polar heads is ca. 6 Å). Moreover, Figure 6 also shows an overall change in the behavior of the rest of the charged residues, in particular those at the L5 loop, which may also influence the behavior of R87.

![Figure 6](image)

**Figure 6.** Side-chain effects of the V48I mutation. (A) Electrostatic potentials at the surface of oxidized WT Syn-Pc, V48I Syn-Pc, and Pho-Pc. (B) Thermal ellipsoids for the first internal motion mode of side chains of ionizable residues and amino acids near the copper site. Ellipsoid amplitudes are the atomic fluctuations along the trajectory for this mode. All atoms are colored according to the CPK profile. (C) Box diagrams corresponding to the MD-derived statistics of the geometrical parameters associated to the copper site properties at 25 °C, as described in the main text.
In agreement, the mutations in this region decrease the redox potential measured in solution, depending on their impact on the electrostatic potential.\textsuperscript{31} The observed changes affect the geometry of the first metal coordination sphere and its environment. Figure 6C displays the average of three parameters related to the stability and other properties of the oxidized copper site.\textsuperscript{21,32} Cu(I) tends to form a trigonal planar geometry, whereas Cu(II) tends to form tetrahedral coordinates.\textsuperscript{25} Thus, the height of Cu above the equatorial plane relates to the stability of the oxidized species. The other two parameters, the angle $\Phi$ between the $\text{N} \cdots \text{Cu} \cdots \text{N}$ and $\text{S} \cdots \text{Cu} \cdots \text{S}$ planes and the angle $\alpha$ between the $\text{N} \cdots \text{Cu} \cdots \text{N}$ plane and the $\text{Cu} \cdots \text{S}_{\text{Cys}}$ bond, relate to the overlap between the metal and ligand orbitals, again affecting the stability of the active site. Notably, we found small but significant differences (all $p$ values in the Student t test below 10$^{-19}$) among the three proteins in these geometric parameters, V48I Syn-Pc displaying a value between those of WT Syn-Pc and Pho-Pc. Notably, according to these data, the oxidized form at 25 °C is less stable in the last one, in agreement with the slightly higher redox potential observed experimentally.

3. CONCLUSIONS

The comparison of the thermal and functional behaviors of a thermophilic Pc from \textit{Phormidium laminosum} with that of its mesophilic counterpart from \textit{Synechocystis} sp. PCC 6803, in a wide temperature range (0–90 °C), reveals a marked difference in their redox properties and thermal stability. A single point mutation, trying to replicate the local hydrophobicity of the thermophilic variant in the east patch, single point mutation, trying to replicate the local hydrophobicity of the thermophilic variant in the east patch, with an acid residue, will increase the thermal stability of the oxidized copper site.\textsuperscript{31} The observed changes a reference in their redox properties and thermal stability. A single point mutation, trying to replicate the local hydrophobicity of the thermophilic variant in the east patch, with an acid residue, will increase the thermal stability of the oxidized copper site.\textsuperscript{31}

4. EXPERIMENTAL SECTION

4.1. Protein Production. The expression of the petE genes from \textit{Synechocystis} sp. and \textit{P. laminosum}—using pBlue-script II SK+ constructs already published—followed the procedures previously described.\textsuperscript{5,6} Site-directed mutagenesis was performed with the “quick change” method (Stratagen Inc., La Jolla, CA, USA). The WT and V48I Syn-Pc proteins were produced in \textit{Escherichia coli} DH5$\alpha$ strains, whereas the expression of the Pho-Pc coding gene took place in the electroverted K12 cells. As described previously, the proteins were purified by ion-exchange chromatography across a diethylaminoethy cellulose column, using a 5 mM tris-(hydroxymethyl)aminomethane (Tris)/HCl buffer and a 10–180 mM NaCl concentration gradient. The WT and V48I Syn-Pc species were further purified by chromatofocusing in a Polybuffer Exchanger 94 (Sigma-Aldrich) column equilibrated in 5 mM Tris/acetate, pH 7.0, and a subsequent size exclusion step in Sephadex G50. In its turn, Pho-Pc purification required an additional size exclusion chromatography step in a HiLoad 16/60 Sephadex 75 (Pharmacia) column equilibrated in 100 mM NaCl and 10 mM sodium phosphate buffer, pH 7.0. The protein concentrations and purity were determined by measuring the light absorption at 280 nm (protein) and 597 nm (oxidized blue copper site), using a 4500 M$^{-2}$ cm$^{-1}$ extinction coefficient for the copper site at 597 nm.

4.2. Variable-Temperature Cyclic Voltammetry. Polycrystalline gold disk electrodes had a geometric area of 0.0314 cm$^2$. Prior to measurements, the gold surface was cleaned by successively polishing with 0.3 and 0.05 μm alumina and rinsed with Millipore water, and then it was sonicated in absolute ethanol to remove the residual alumina. The surface was then dried with argon and chemically cleaned using a “piranha” solution (7:3 mixture of concentrated H$_2$SO$_4$ and 30% H$_2$O$_2$). The cysteamine self-assembled monolayers were prepared by immersing the gold electrode in a 1 mM cysteamine solution in ethanol for 2 h at 4 °C. Protein immobilization was carried out by depositing onto the thiol-modified electrode surface of a 10 μL drop 100 μM protein Pc, 10 mM sodium phosphate buffer solution of pH 7 for 16 h at room temperature. Then, the electrodes were thoroughly rinsed with water and washed with the working buffer solution.

Linear scan voltammetric measurements were performed with an Autolab PGSTAT 30 from Eco Chemie B.V., in a three-electrode undivided glass cell, equipped with a gas inlet and thermostated with a water jacket. The counter and reference electrodes were a Pt bar and an Ag/AgCl/NaCl saturated electrode, respectively. The reference electrode was connected to the cell solution via a salt bridge and kept at room temperature (23 ± 2 °C) in a nonisothermal configuration. The reported potential values have been corrected to the normal hydrogen electrode (NHE) potential scale by adding +192 mV to the experimental potential values. All measurements were carried out under argon atmosphere. The working solutions contained 0.1 M sodium phosphate buffer at pH 7.0. To assess the influence of temperature on the midpoint potential, the electrode with the adsorbed protein was kept inside the electrochemical cell for ~6 h, while the temperature was first increased from 0 to 90 °C, and then lowered back to 0 °C, with stops to record the voltammograms at different potential scan rates (from 0.01 to 0.1 V s$^{-1}$), every 10 °C.

4.3. Fluorescence Spectroscopy. The thermal titration curves derived from the fluorescence measurements were obtained according to the protocol described previously.\textsuperscript{6} The measurements were performed with a Cary (Varian) fluorimeter coupled to a Polystat cc2 (Huber) bath for temperature control. The temperature of the sample was recorded using a Digitron 2008 system equipped with a flexible thermocouple that was inserted inside the cuvette and in contact with the sample. The protein concentration was 20 μM in a 10 mM pH 7 phosphate buffer solution. To maintain the protein in a single redox state throughout the experiment, equimolar concentrations of sodium ascorbate or potassium ferricyanide were added. The temperature in the cuvette was programmed to increase at a rate of 1 °C min$^{-1}$, from 20 to 95 °C. The sample was subjected to continuous stirring throughout the process to achieve temperature homogenization. The samples were excited with 275 nm light, and the fluorescence emission was monitored at 350 nm. As a control before and after each temperature ramp, an emission spectrum
from 300 to 600 nm was recorded, whereas the sample was being excited with a 275 nm ultraviolet light. The fluorescence and temperature data were recorded every 30 s and exported to the Origin Version 8.0 program for analysis.

4.4. CD Spectroscopy. To monitor the changes in the secondary structure during unfolding, the CD spectra were recorded in the far ultraviolet region (190–240 nm) with a Jasco J-815 spectropolarimeter, while increasing the temperature from 25 to 95 °C at a rate of 1 °C min⁻¹ with a Peltier unit. Three spectra were recorded every 5 °C with a scanning speed of 200 nm min⁻¹. The samples contained 15 μM protein in a 10 mM pH 7 phosphate buffer solution. The unfolding curves were obtained from the CD values recorded at 219 nm.

4.5. Computational Analysis. The MD trajectories were computed with the Amber 16 package, using the 14SB force field, except for the copper site for which the parameters set by Comba and Remenyi and the restrained electrostatic potential charges computed by Muñoz-López were applied. The simulations were run under periodic boundary conditions in orthorhombic boxes, wherein the initial minimum distance from the protein to cell faces was 10 Å. The particle mesh Ewald electrostatics was set with the Ewald summation cutoff at 9 Å. The counterions neutralized the charges of the system. The structures were solvated with SPC water molecules. The protein side chains were energy-minimized (100 steepest descent and 1400 conjugate gradient steps) down to an RMS energy gradient of 0.01 kJ mol⁻¹ Å⁻¹. Afterward, the solvent was subjected to 1000 steps of steepest descent minimization, followed by 500 ps NPT-MD computations using isotropic molecule position scaling and a pressure relaxation time of 2 ps at 298 K. The temperature was regulated with Berendsen’s heat bath algorithm, with a coupling time constant equal to 0.5 ps. The density of the system reached a plateau after ca. 114 ksteps.

The SHAKE algorithm was used to constrain the bonds involving hydrogen atoms. The coordinate files were processed using CPPTRAJ. Further processing was made in Origin (Originlab), and the graphic displays were built in UCSF Chimera. The electrostatic potentials were computed with the APBS software.

**AUTHOR INFORMATION**

**Corresponding Authors**

*E-mail: jolloqui@us.es (J.L.O.-S.).
*E-mail: qzaida@us.es (A.D.-Q.).

**ORCID**

José Luis Olloqui-Sariego: 0000-0002-3737-9814

**Present Address**

‡Sygnis Pharma AG; Parque Científico de Madrid, c/ Faraday, 7, 28049 Madrid, Spain.

**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

J.L.O.-S., I.M., J.J.C., and R.A. acknowledge the financial support from the Spanish Ministry of Economy and Competitiveness and the European Union FEDER (grants CTQ2014-52641-P and CTQ2015-71955-REDT (ELECTRIONET)), I.D.-M., M.A.D.I.R., and A.D.-Q. thank funds from the Spanish Ministry of Economy, Industry and Competitiveness (BFU2015-71017/BMC), Ramon Areces Foundation (2015–2017), and the Andalusian Government (BIO-198). E.F.-B. was hired with funds from the Andalusian Government to A.D.-Q. (P06-CVI-01713).

**REFERENCES**

1. Szlágyi, A.; Závodszky, P. Structural differences between mesophilic, moderately thermophilic and extremely thermophilic protein subunits: results of a comprehensive survey. *Structure 2000*, 8, 493–504.

2. Chan, C.-H.; Yu, T.-H.; Wong, K.-B. Stabilizing salt-bridge enhances protein thermostability by reducing the heat capacity change of unfolding. *PLoS One* 2011, 6, No. e21624.

3. Sen, S.; Nilsson, L. *Thermotolerant Proteins: Structural Stability and Design*; CRC Press, Taylor & Francis Group, LLC, 2012.

4. Gromiha, M. M.; Pathak, M. C.; Saraboji, K.; Ortlund, E. A.; Gaucher, E. A. Hydrophobic environment is a key factor for the stability of thermophilic proteins. *Proteins: Struct, Funct., Bioinf.* 2013, 181, 715–721.

5. Redinbo, M. R.; Yeates, T. O.; Merchant, S. Plastocyanin: structural and functional analysis. *J. Bioenerg. Biomembr.* 1994, 29, 46–66.

6. Feio, M. J.; Navarro, J. A.; Teixeira, M. S.; Harrison, D.; Karlsson, B. G.; De la Rosa, M. A. A Thermal Unfolding Study of Plastocyanin from the Thermophilic Cyanobacterium *Phormidium laminosum*. *Biochemistry 2004*, 43, 14784–14791.

7. Feio, M. J.; Díaz-Quintana, A.; Navarro, J. A.; De la Rosa, M. A. Thermal Unfolding of Plastocyanin from the Mesophilic Cyanobacterium *Synechocystis*. PCC 6803 and Comparison with Its Thermophilic Counterpart from *Phormidium laminosum*. *Biochemistry 2006*, 45, 4900–4906.

8. Poznyakova, I.; Wittung-Stafshede, P. Biological relevance of metal binding before protein folding. *J. Am. Chem. Soc.* 2001, 123, 10135–10136.

9. Poznyakova, I.; Guidry, J.; Wittung-Stafshede, P. Probing copper ligands in denatured Pseudomonas aeruginosa azurin: Unfolding His117Gly and His46Gly mutants. *J. Biol. Inorg. Chem.* 2001, 6, 182–188.

10. Alcaraz, L. A.; Donaire, A. Rapid binding of copper(I) to folded apourostacin. *FEBS Lett.* 2005, 579, 5223–5226.

11. Muñoz-López, F. J.; Raugei, S.; De la Rosa, M. A.; Díaz-Quintana, A. J.; Carloni, P. Changes in non-core regions stabilise plastocyanin from the thermophilic cyanobacterium *Phormidium laminosum*. *J. Biol. Inorg. Chem.* 2010, 15, 329–338.

12. Bond, C. S.; Belland, D. F.; Freeman, H. C.; Guss, J. M.; Howe, C. J.; Wagner, M. J.; Wisce, M. C. J. The structure of plastocyanin from the cyanobacterium *Phormidium laminosum*. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* 1999, 55, 414–421.

13. Romero, A.; De la Cerda, B.; Varela, P. F.; Navarro, J. A.; Hervías, M.; De la Rosa, M. A. The 2.15 Å crystal structure of a triple mutant plastocyanin from the cyanobacterium *Synechocystis* sp. PCC 6803 I Edited by R. Huber. *J. Mol. Biol.* 1998, 275, 327–336.

14. Bertini, I.; Bryant, D. A.; Ciurli, S.; Dikiy, A.; Fernández, C. O.; Luchinat, C.; Safarov, N.; Vila, A. J.; Zhao, J. Backbone Dynamics of Plastocyanin in Both Oxidation States. *J. Biol. Chem.* 2001, 276, 47217–47226.

15. Muñoz-López, F. J.; Beltrán, E. F.; Díaz-Moreno, S.; Díaz-Moreno, I.; Subías, G.; De la Rosa, M. A.; Díaz-Quintana, A. Modulation of copper site properties by remote residues determines the stability of plastocyanins. *FEBS Lett.* 2010, 584, 2346–2350.

16. Chaboy, J.; Díaz-Moreno, S.; Díaz-Moreno, I.; De la Rosa, M. A.; Díaz-Quintana, A. How the Local Geometry of the Cu-Binding Site Determines the Thermal Stability of Blue Copper Proteins. *Chem. Biol.* 2011, 18, 25–31.

17. Royer, C. A. Probing protein folding and conformational transitions with fluorescence. *Chem. Rev.* 2006, 106, 1769–1784.
(18) Greenfield, N. J. Using circular dichroism collected as a function of temperature to determine the thermodynamics of protein unfolding and binding interactions. *Nat. Protoc. 2007*, 2, 2527–2535.

(19) Sancho, J. The stability of 2-state, 3-state and more-state proteins from simple spectroscopic techniques... plus the structure of the equilibrium intermediates at the same time. *Arch. Biochem. Biophys.* 2013, 531, 4–3.

(20) Wittung-Stafshede, P. Role of cofactors in folding of the blue-copper protein azurin. *Inorg. Chem. 2004*, 43, 7926–7933.

(21) Pavella, M.; Burda, J. V. Computational study of redox active centres of blue copper proteins: a computational DFT study. *Mol. Phys. 2008*, 106, 2733–2748.

(22) Battistuzzi, G.; Borsari, M.; Loschi, L.; Righi, F.; Sola, M. Redox Thermodynamics of Blue Copper Proteins. *J. Am. Chem. Soc. 1999*, 121, 501–506.

(23) Battistuzzi, G.; Bellei, M.; Borsari, M.; Canters, G. W.; de Waal, E.; Jeuken, L. J. C.; Ranieri, A.; Sola, M. Control of Metalloprotein Reduction Potential: Compensation Phenomena in the Reduction Thermodynamics of Blue Copper Proteins*. *Biochemistry 2003*, 42, 9214–9220.

(24) Radestock, S.; Gohlke, H. Exploiting the Link between Protein Rigidity and Thermostatibility for Data-Driven Protein Engineering. *Eng. Life Sci. 2008*, 8, 507–522.

(25) Teilum, K.; Olsen, J. G.; Kragelund, B. B. Functional aspects of protein flexibility. *Cell. Mol. Life Sci. 2009*, 66, 2231–2247.

(26) Radestock, S.; Gohlke, H. Protein rigidity and thermostable adaptation. *Proteins: Struct., Funct., Bioinf. 2011*, 79, 1089–1108.

(27) Teilum, K.; Olsen, J. G.; Kragelund, B. B. Protein stability, flexibility and function. *Biochim. Biophys. Acta, Proteins Proteomics 2011*, 1814, 969–976.

(28) Karshikoff, A.; Nilsson, L.; Ladenstein, R. Rigidity versus flexibility: the dilemma of understanding protein thermal stability. *FEBS J. 2015*, 282, 3899–3917.

(29) Díaz-Moreno, I.; Muñoz-López, F. J.; Frutos-Beltrán, E.; De la Rosa, M. A.; Díaz-Quintana, A. Electrostatic strain and concerted motions in the transient complex between plastocyanin and cytochrome f from the cyanobacterium *Phormidium laminosum*. *Bioelectrochemistry 2009*, 77, 43–52.

(30) Molina-Heredia, F. P.; Hervás, M.; Navarro, J. A.; de la Rosa, M. A. A Single Arginyl Residue in Plastocyanin and in Cytochrome c551 of the Cyanobacterium *Anabaena PCC 7119* Is Required for Efficient Reduction of Photosystem I. *J. Biol. Chem. 2001*, 276, 601–605.

(31) De la Cerda, B.; Navarro, J. A.; Hervás, M.; De la Rosa, M. A. Changes in the Reaction Mechanism of Electron Transfer from Plastocyanin to Photosystem I in the Cyanobacterium *Synechocystis PCC 6803* As Induced by Site-Directed Mutagenesis of the Copper Protein†. *Biochemistry 1997*, 36, 10125–10130.

(32) Donaire, A.; Jiménez, B.; Fernández, C. O.; Pierattelli, R.; Nizeki, T.; Moratal, J.-M.; Hall, J. F.; Kohzuma, T.; Hasnain, S. S.; Vila, A. J. Metal–Ligand Interplay in Blue Copper Proteins Studied by 1H NMR Spectroscopy: Cu(II)–Pseudoazurin and Cu(II)–Rustycyanin. *J. Am. Chem. Soc. 2002*, 124, 13698–13708.

(33) Case, D. A.; Cheatham, T. E., III; Darden, T.; Gohlke, H.; Luo, R.; Merz, K. M., Jr.; Onufriev, A.; Simmerling, C.; Wang, B.; Woods, R. J. The Amber Biomolecular Simulation Programs. *J. Comput. Chem. 2005*, 26, 1668–1688.

(34) Maier, J. A.; Martinez, C.; Kasavajhala, K.; Wickstrom, L.; Hauser, K. E.; Simmerling, C. ff14SB: Improving the accuracy of protein side chain and backbone parameters from ff99SB. *J. Chem. Theory Comput. 2015*, 11, 3696–3713.

(35) Comba, P.; Remenyi, R. A new molecular mechanics force field for the oxidized form of blue copper proteins. *J. Comput. Chem. 2002*, 23, 697–705.

(36) Berendsen, H. J. C.; Postma, J. P. M.; van Gunsteren, W. F.; DiNola, A.; Haak, J. R. Molecular dynamics with coupling to an external bath. *J. Chem. Phys. 1984*, 81, 3684–3690.

(37) Ryckaert, J.-P.; Ciccotti, G.; Berendsen, H. J. C. Numerical integration of the cartesian equations of motion of a system with constraints: molecular dynamics of n-alkanes. *J. Comput. Phys. 1977*, 23, 327–341.

(38) Roe, D. R.; Cheatham, T. E., 3rd PTRAJ and CPPTRAJ: Software for Processing and Analysis of Molecular Dynamics Trajectory Data. *J. Chem. Theory Comput. 2013*, 9, 3084–3095.

(39) Pettersen, E. F.; Goddard, T. D.; Huang, C. C.; Couch, G. S.; Greenblatt, D. M.; Meng, E. C.; Ferrin, T. E. UCSF Chimera–A visualization system for exploratory research and analysis. *J. Comput. Chem. 2004*, 25, 1605–1612.

(40) Baker, N. A.; Sept, D.; Joseph, S.; Holst, M. J.; McCammon, J. A. Electrostatics of nanosystems: application to microtubules and the ribosome. *Proc. Natl. Acad. Sci. U.S.A. 2001*, 98, 10037–10041.