Temperature Driven Changeover in the Electron Transfer Mechanism of a Thermophilic Plastocyanin

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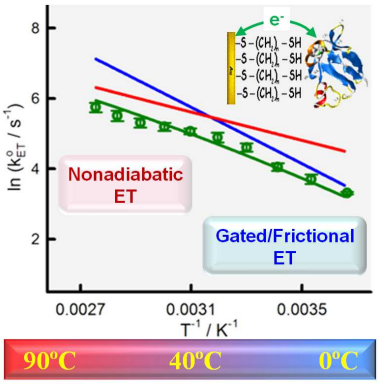
ABSTRACT. Electron transfer kinetics of the thermophilic protein Plastocyanin from *Phormidium laminosum* adsorbed on 1,ω-alkanedithiol self-assembled monolayers (SAMs) deposited on gold have been investigated. The standard electron transfer rate constant has been determined as a function of electrode-protein distance and solution viscosity over a broad temperature range (0 – 90 ºC). For either thin or thick SAMs, the electron transfer regime remains invariant with temperature; whereas for the 1,11-undecanethiol SAM of intermediate chain-length, a kinetic regime changeover from a gated or friction-controlled mechanism at low temperature (0 - 30º C) to a non-adiabatic mechanism above 40 ºC is observed. To the best of our knowledge, this is the first time a thermal-induced transition between these two kinetic regimes is reported for a metalloprotein.

TOC GRAPHICS



KEYWORDS. Thermophilic plastocyanin; protein electron transfer; kinetic regime changeover; 1,ω-alkanedithiol monolayer; temperature; solution viscosity.

Several strategies have been proposed to elucidate the factors that determine the rate of electron exchange between proteins and electrodes. Among them stand out the analysis of the effects of protein-electrode distance, temperature or solution viscosity on the electron transfer (ET) rate constant.[1-5] These studies have revealed two distinct kinetic regimes, in which ET rates are either independent or exponentially dependent on the protein-electrode distance. This last regime is well described by a non-adiabatic electron transfer mechanism, where the ET rate is controlled by the electron tunneling frequency at the top of the activation barrier.[5-15] However, the nature of the rate-limiting event is more ambiguous for the distance-independent kinetic regime, for which two alternatives have been purported in the literature. The first assumes that, as a consequence of the increased electronic coupling at short protein-electrode distances, the ET rate is controlled by a frictional mechanism that involves the protein and its surrounding medium and takes the reactant to the top of the activation barrier.[16-31] The second introduces a preceding barrier-crossing event (gating step), which may involve a conformational fluctuation[32] or a protein reorientation,[33] to optimize the rate of electron transfer.[32-39] Irrespective of the precise nature of the controlling event in the distance-independent kinetic regime, the transition between the two kinetic regimes has been demonstrated by varying systematically the strength of the electronic coupling between electrode and protein by using molecular spacers of variable composition and length. For redox proteins immobilized on thiol monolayers (HS-$(CH_2)_n$-X) the mechanism transition has often been reported to take place for protein-electrode distance corresponding to $n \approx 10$.[12, 15, 24, 25, 31, 33, 39-43] Moreover, each kinetic regime can be further characterized by a distinct dependence of the ET rate constant with temperature and solution viscosity,[25-33, 44, 45] though temperature or viscosity driven mechanism transitions have not yet

been reported for metallo-proteins. To the best of our knowledge, the current work provides the first observation of a temperature-induced mechanism changeover for a redox metallo-protein.

Thermal studies of typical redox proteins are significantly restrained, since proteins become often denatured at moderate temperatures. A general approach to overcome this limitation would involve the use of thermophilic proteins that are likely to keep their structure and functionality intact in a broad temperature range. Within this context, we have previously reported on how Plastocyanin from *Phormidium laminosum* (Pc-PhoWT) adsorbed onto a graphite electrode displays an unusual thermal resistance, retaining its redox activity at temperatures as high as 90ºC.[46] Pc-PhoWT is a blue copper protein with a Type I redox center. The metal atom is buried in a hydrophobic pocket and it is coordinated by two histidines (HisN and HisC), one methionine and one cysteine. The Cu binding site features a distorted trigonal pyramid and HisC is the only solvent-exposed copper-ligand, thus making this group the most probable physiological electron transfer port of the protein (see Supporting Information). Its thermostability has also been probed by spectroscopic techniques in a broad temperature range.[47, 48] Based on these findings, this protein appears to be a good candidate to assess the influence of temperature on its ET kinetics. Herein, we report on the effect of a broad variation of temperature (0 -90 ºC) on the electron transfer kinetics of Pc-PhoWT immobilized on gold electrodes modified with 1,ω-alkanedithiol self-assembled monolayers (SAMs), under variable conditions of protein-electrode distance and solution viscosity. We provide clear experimental evidence of a changeover in the electron transfer regime along a thermal scan when Pc-PhoWT is immobilized on a 1,11-undecanedithiol monolayer. In this case, electron exchange proceeds through the distance-independent kinetic limit at low temperatures, and it switches reversibly into the non-adiabatic regime as the temperature is raised over 40 ºC.

The redox properties of Pc-PhoWT immobilized on distinct 1,ω-alkanedithiol SAMs differing in their hydrocarbon chain length were characterized by recording its voltammetric response as a function of scan rate. Figure S-2 illustrates some typical raw voltammograms. The formal potential (E^0) shifts from 0.421 V to 0.375 V vs. SHE upon increasing the dithiol chain length, remaining somewhat more positive than the reported values for Pc-PhoWT adsorbed onto graphite[46] and in solution.[48] A parallel variation of the reduction entropy (see Supporting Information) towards less negative values suggests that the E^0 shift results from a tighter hydrophobic interaction between protein and monolayer as the alkanedithiol chain-length increases. To determine the standard electron transfer rate constant (k_{ET}^o), the effect of the scan rate on the peak potential separation was assessed at distinct temperatures and electrode-protein distances. Accordingly, upon increasing the scan rate, the two voltammetric peaks depart from each other, producing trumpet plots as those depicted in Figure S-2. Solid lines in these plots are theoretical fits computed in the high reorganization energy limit of the Marcus electron transfer theory (i.e. for $\lambda > F\,|E_p - E^0|$, where λ is the reorganization energy, E_p the peak potential, and F the Faraday's constant).[9] Though significant protein loss (ca. 50 %) was evident after exposing the electrode to the hottest solutions, a full recovery of the initial kinetic and thermodynamic electron transfer parameters (see Supporting Information) was observed after subjecting the immobilized protein to broad changes of temperature and solvent viscosity.

The effect of the electrode-protein distance on k_{ET}^0 at distinct temperatures is illustrated in Figure 1. At a given temperature (Figure 1a), it displays a biphasic behavior: the standard electron transfer rate constant barely depends on the chain length up to ca. 9 methylene units, but decays exponentially for the longer chain lengths ($n \geq 11$). The exponential decay factor is $\beta = 1.14$ per methylene group, consistent with the use of saturated hydrocarbon spacers.[49,50]

Furthermore, Figure 1b shows that the temperature dependence of k_{ET}^{0} is stronger at the plateau than within the exponential decay region.

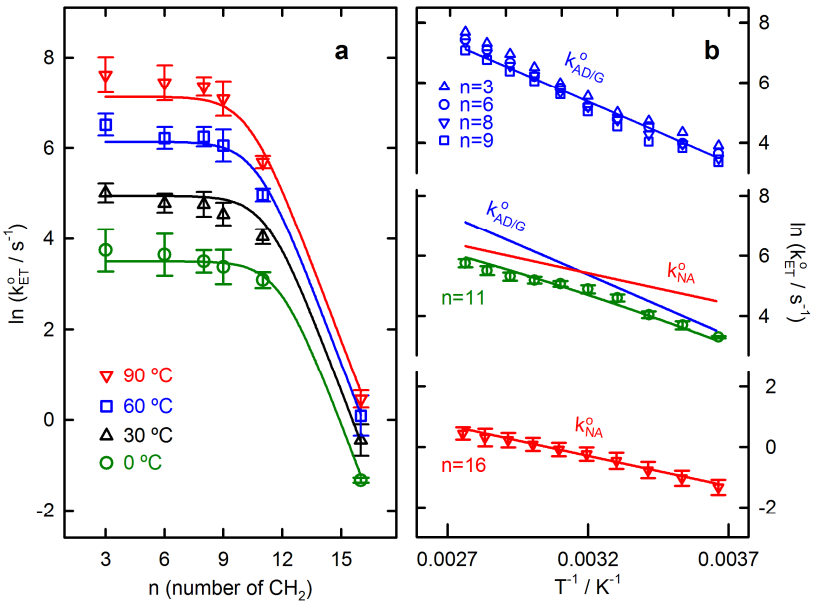


Figure 1. (a) Dependence of the logarithm of the standard electron transfer rate constant on the number of methylene groups per thiol molecule for Pc-PhoWT immobilized on 1,ω-alkanedithiol SAMs, obtained in a 0.1 M sodium phosphate buffer of pH 7 at the indicated temperatures. Solid lines have been computed from eqs 1-4 in the text. (b) Arrhenius plots in the 0 - 90 ºC temperature range. The green line in the n = 11 plot was computed from the corresponding red and blue lines according to eq 4, as indicated in the text. Symbols are experimental values, and error bars are standard deviations for at least three replicated measurements for each data point.

In the non-adiabatic limit (distance-dependent regime), the standard rate constant k_{ET}^{0} is limited by the electron tunneling probability and can be expressed as:

$$k_{NA}^{0} = \frac{|H_{AB}^{o}|^{2}\rho_{m}}{\hbar}\left(\frac{\pi^{3}R}{\lambda}\right)^{1/2}\sqrt{T}\,exp(-\beta n)\,exp\left(-\frac{\lambda/4}{RT}\right) \quad (1)$$

where H_{AB}^{o} is the electronic coupling energy for n = 0 and ρ_m is the density of electronic states of the metal. Fit of eq 1 to experimental $\ln k_{ET}^{o}$ vs. T^{-1} data for n = 16 with $\beta = 1.14$ and $\rho_m = 0.28$ eV^{-1}, [25] gives rise to $\lambda = 61.7$ kJ mol^{-1} (or 0.64 eV) and $H_{AB}^{o} = 7\text{x}10^{-3}$ eV. The obtained value for λ falls within the range of accepted values for blue copper proteins.[46, 51-54]

On the other hand, the standard rate constant under the distance-independent regime can be expressed as:

$$k_{AD}^{0} = \frac{1}{\tau_{eff}^{o}}\left(\frac{\lambda}{\pi^{3}R}\right)^{1/2}\frac{1}{\sqrt{T}}exp\left(-\frac{(\lambda/4)-|H_{AB}^{o}|e^{-\beta n}+\Delta H_{\eta}^{\#}}{RT}\right) \quad (2)$$

when the electron transfer kinetics are controlled by a frictional mechanism, or simply as:

$$k_{G}^{0} = A_{G}\,exp\left(-\frac{\Delta H_{G}^{\#}}{RT}\right) \quad (3)$$

when they are controlled by a gating step. τ_{eff}^{o} is the high-temperature limit of the characteristic polarization relaxation time of the solvent/protein/SAM environment, $\Delta H_{\eta}^{\#}$ is the activation enthalpy associated with solvent/protein/SAM friction, and A_G, $\Delta H_{G}^{\#}$ are the Arrhenius preexponential factor and height of the activation barrier of the gating step, respectively.

Fit of eq 2 or 3 to the experimental $\ln k_{ET}^{o}$ vs. T^{-1} data for $n \leq 9$ (Figure 1b) gives rise to $\tau_{eff}^{o} = 7\text{x}10^{-9}$ s and $\Delta H_{\eta}^{\#} = 19.1$ kJ mol^{-1} or $A_G = 1.2\text{x}10^{8}$ s^{-1} and $\Delta H_{G}^{\#} = 34.5$ kJ mol^{-1}, respectively. Bearing in mind that $\tau_{eff} = \tau_{eff}^{o}\exp(\Delta H_{\eta}^{\#}/RT)$, τ_{eff} shows a tenfold decrease from ~ 32 μs at 0 ºC to ~ 3.9 μs at 90 ºC. The magnitude of these relaxation times is much larger than those of typical liquids,[55] and c.a. tenfold the characteristic relaxation time reported by

Khoshtariya et al.[25] for the adiabatic electron transfer of cytochrome *c* adsorbed on thiol monolayers. On the other hand, this ~ 10 μs time scale lies well within the 1 - 10^3 μs range that has been determined[56] for the mobility of the β_7 - β_8 loop of the Plastocyanin from *Anabaena variabilis*. It should be noted that this loop contains three of the four copper ligands and, therefore, seems to be relevant for the electron transfer process.

When the protein is adsorbed on a SAM with an intermediate alkyl chain length (n = 11), the $\ln k_{ET}^{o}$ vs. T^{-1} slope changes from that characteristic of the distance-independent regime, at low temperatures, to that of the non-adiabatic regime at high temperatures. This transition can be accounted for with the following series combination of k_{NA}^{o} and $k_{AD/G}^{o}$ (green solid line in Figure 1b):

$$\frac{1}{k_{ET}^{0}} = \frac{1}{k_{NA}^{0}} + \frac{1}{k_{AD/G}^{0}} \qquad (4)$$

which is known to describe the transition from the non-adiabatic to the adiabatic kinetic regimes,[25] but it can also account for the presence of a gating step[32] (see Supporting Information). As the crossing point of the two individual k_{NA}^{o} and $k_{AD/G}^{o}$ contributions to k_{ET}^{o} shows (Figure 1b), the transition between the two limiting kinetic regimes takes place at ca. 40 ºC for the n = 11 monolayer. It should be noted that this transition is predicted to be outside the available temperature range for n < 11 or n > 11 (Figure S-7 in Supporting Information).

As a test of consistency, eq 4 was able to reproduce the experimental $\ln k_{ET}^{o}$ vs. *n* dependence in Figure 1a with the same parameter values that were determined from the analysis of Figure 1b.

In order to corroborate the temperature-induced mechanistic changeover found for Pc-PhoWT adsorbed on the n = 11 SAM, use was made of the distinct sensitivity of the non-adiabatic and distance-independent kinetic regimes to changes in the solution viscosity η. Previous studies

have shown that k_{NA}^{o} is independent of η, while $k_{AD/G}^{o}$ is proportional to $\eta^{-\gamma}$ where $\gamma > 0$.[25, 33, 57] Thus, according to eq 4, k_{ET}^{o} is expected to be proportional to $\eta^{-\gamma_{ap}}$, where the value of the γ_{ap} exponent ($0 < \gamma_{ap} \leq \gamma$) depends on the relative contributions of k_{NA}^{o} and $k_{AD/G}^{o}$ to k_{ET}^{o}.

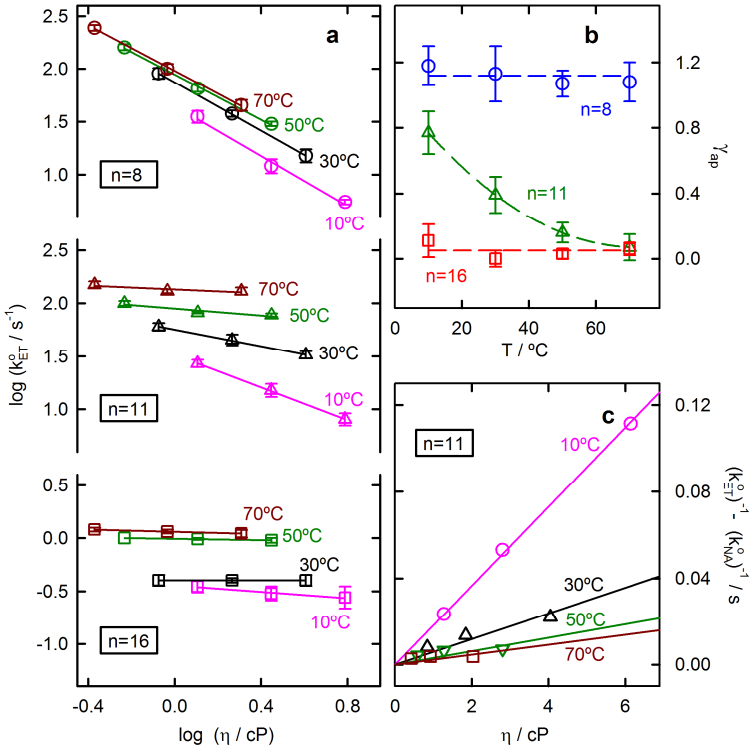


Figure 2. (a) Double logarithmic plot of the overall standard electron transfer rate constant for Pc-PhoWT immobilized on 1,ω-alkanedithiol SAMs, obtained in a 0.1 M sodium phosphate buffer of pH 7, against solution viscosity for the indicated temperature and spacer's number of methylene groups. (b) Slopes of the $\log k_{ET}^{o}$ vs. $\log \eta$ plots in Figure 2a as a function of temperature. (c) Difference between the reciprocals of the observed and calculated non-adiabatic standard electron transfer rate constants as a function of solution viscosity for n = 11 and the indicated temperatures. Solid lines are least squares linear fits passing through the origin.

Figure 2a illustrates the observed dependence of k_{ET}^{0} on the viscosity of aqueous glucose solutions, under variable conditions of electrode-protein distance and temperature. It can be observed that the slope of the logarithmic plot (i.e. γ_{ap}) decreases upon increasing the SAM thickness, from $\gamma_{ap} \approx 1.1$ for n = 8 to $\gamma_{ap} \approx 0.0$ for n = 16, in agreement with the change of kinetic regime discussed previously. More importantly, the slopes corresponding to the intermediate n = 11 SAM display a continuous variation with temperature from $\gamma_{ap} = 0.77$ at 10 ºC to $\gamma_{ap} = 0.09$ at 70 ºC (Figure 2b), as expected for a changeover of the electron transfer mechanism from the frictional/gating regime to the non-adiabatic one. In order to quantify the γ value, corresponding to the $k_{AD/G}^{0}$ term, we have plotted in Figure 2c the difference between observed $1/k_{ET}^{0}$ and calculated $1/k_{NA}^{0}$, as a function of solution viscosity, where the k_{NA}^{0} values have been computed from eq 1 with the parameter values determined previously. According to eq 4, the ordinate values in these plots correspond to those of $1/k_{AD/G}^{0}$. The linearity of these plots strongly suggests that the frictional coupling to the medium is very strong, with a real γ value close to 1.

Previous reports describing the transition between frictional/gating and non-adiabatic electron transfer mechanisms for immobilized cytochrome c[25] and azurin[31] took advantage of the high sensitivity of the non-adiabatic electron transfer rate to the electrode-protein distance. Herein, we have shown that the same transition can be achieved by exploiting the difference in the activation enthalpies of these two mechanisms. Once an adequate tunneling distance is selected, so that the values of the frictional/gating and non-adiabatic rate constants become similar at intermediate temperatures, the kinetic control can then be switched reversibly between the frictional/gating and non-adiabatic limits by just varying the temperature. In the case of the thermophilic

plastocyanin from *Phormidium laminosum* adsorbed on a 1,11-undecanedithiol SAM, the mechanistic changeover takes place at 40 ºC. This transition results from the interplay between different contributions to the observed ET rate, and our previous analysis helps to identify some physical prerequisites, such as the necessary difference in activation enthalpy between the two kinetic regimes or the importance of an adequate choice of the tunneling distance, to observe similar transitions for other redox proteins.

ASSOCIATED CONTENT

Supporting information

Experimental Method, Materials and Methods. Structure of Plastocyanin from *Phormidium laminosum*. Typical Cyclic Voltammograms and Trumpet Plots. Thermal Stability of the SAM and the Immobilized Pc-PhoWT. Expressions for the Rate Constants. Individual Contributions to the Electron Transfer Rate Constant. Thermodynamics of the Immobilized Pc-PhoWT. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interests.

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