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The Role of Molecular Crowding in Long-Range Metalloprotein Electron Transfer: Dissection into Site- and Scaffold-Specific Contributions

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Abstract

Here we report the effect of molecular crowding on long-range protein electron transfer (ET) and disentangle the specific responses of the redox site and the protein milieu. To this end, we studied two different one-electron redox proteins that share the cupredoxin fold but differ in the metal centre, T1 mononuclear blue copper and binuclear Cu₂, and generated chimeras with hybrid properties by incorporating different T1 centres within
the Cuₐ scaffold or by swapping loops between orthologous proteins from different
organisms to perturb the Cuₐ site. The heterogeneous ET kinetics of the different proteins
was studied by protein film electrochemistry at variable electronic couplings and in the
presence of two different crowding agents. The results reveal a strong frictional control of
the ET reactions, which for 10 Å tunnelling distances results in a 90% drop of the ET rate
when viscosity is matched to that of the mitochondrial interior (ca. 55 cP) by addition of
either crowding agent. The effect is ascribed to the dynamical coupling of the metal site
and the milieu, which for T1 is found to be twice stronger than for Cuₐ, and the activation
energy of protein-solvent motion that is dictated by the overall scaffold. This work
highlights the need of explicitly considering molecular crowding effects in protein ET.

Keywords
Metalloproteins, Loop Engineering, Electron Transfer, Molecular Crowding, Frictional
Control.

1. Introduction
Most kinetic experimental studies of protein electron transfer (ET) reactions are
performed with close to ideal diluted solutions[1−3] and rationalized within the
framework of Marcus semiclassical equation for long-range (nonadiabatic) ET.[4,5] This
treatment, implies a number of underlying assumptions that are not necessarily fulfilled
for proteins in real biological environments,[6] which are characterized by high
macromolecular crowding[7,8] and strong local electric fields.[9] For example, water-
protein nuclear fluctuations required to equalize donor-acceptor electronic energies before ET, as well as the subsequent electrostatic relaxation, are assumed to be much faster than electron tunnelling. This condition may break down due to either enhanced electron tunnelling probability or to a slowdown (and eventually dynamical arrest) of nuclear modes imposed by the milieu. Indeed, dynamical effects on ET reactions have been theoretically addressed by a number of researchers such as Marcus,[10] Weaver,[11] Zusman,[12] Jortner,[13] Beratan,[14] Waldeck,[15] Matyushov[16] and others, employing different approximations. From the experimental side examples of dynamical effects on protein ET reactions are rather limited and viscosity or crowding studies on protein ET, although very valuable, mainly refer to the effect of crowding agents on the diffusional component of interprotein ET in solution, rather than to the electron tunnelling step.[17,18] Relevant to the present work, different authors studied metalloproteins immobilized on electrodes coated with self-assembled monolayers (SAMs) of functionalized alkanethiols, and found exponential distance-dependencies of the measured ET rate constants ($k_{ET}$) only for thick SAMs, but distance-independent plateaus for thinner spacers.[19–24] For the soluble metalloproteins cytochrome c[25–27] and azurin[28–30] ET rates were found to be sensitive to the medium viscosity. These observations were interpreted as a change of ET regime from nonadiabatic at longer distances to frictional control in terms of Zusman’s equations at thinner films,[25,26,28,29] and to electric field dependent protein-solvent collective motions of large and small amplitude.[31–33] These preceding investigations demonstrate that moderate viscosities may have an impact on long range ET rates. The goal of the present
work is to deepen our understanding of possible kinetic effects of biological molecular
crowding on protein ET and, more specifically, to dissect the contributions to this outcome
of the different structural and dynamical elements of metalloproteins that are relevant to
the ET reaction coordinate. To the best of our knowledge, this crucial issue remains to
date elusive and largely unexplored.

In this context it is pertinent to highlight the structural and dynamical complexity of
proteins, which requires multidimensional and hierarchical free energy landscapes to
describe the conformational substates explored at biologically relevant temperatures.

Time scales for such exploration are also hierarchical, ranging from femtoseconds for
bond vibrations, picoseconds to nanoseconds for side-chain rotations and microseconds
to seconds for collective motions of larger protein domains.[34] This flexibility at different
levels proved pivotal for canonical and alternative functions of different proteins and
enzymes.[35–43]

Based on notions adopted from the physical chemistry of glasses, Frauenfelder and
coworkers[44–46] formulated a model that divides protein fluctuations into three types:
\( \alpha \) and \( \beta_h \) and vibrations. The \( \alpha \) motions are associated to changes in the protein shape
and, therefore, are slaved to bulk solvent fluctuations, while \( \beta_h \) fluctuations refer to
internal protein motions and are slaved to the hydration shell. Hence, the surrounding
milieu (solvent) crucially assists protein function through structural and dynamical
modulation.[47,48] One should note, however, that the in vivo milieu seriously departs
from the nearly ideal behaviour of dilute aqueous solutions typically used for in vitro and
in silico studies. Cells present a number of large structures such as membranes and
cytoskeleton, as well as dissolved macromolecules in concentrations that can be as high as
450 g/L and occupy up to 40% of the cytoplasmic volume, in addition to a large variety of
small molecules. In these complex and highly crowded environments fundamental
physicochemical parameters, such as viscosity, diffusion and activity coefficients, may
diverge from ideality by several orders of magnitude. Moreover, crowding is
expected to reshape the free energy landscapes of proteins and, therefore, their
dynamics. This may be particularly true for proteins that partake in respiratory
electron transfer (ET) chains of all living organisms. In eukariotes respiratory complexes
are embedded into the inner mitochondrial membrane (IM), while electron shuttles such
as cytochrome c are present at millimolar levels in the intermembrane space
These species coexist with about 49 other proteins present in the IMS and
481 proteins that are either integral or peripherally bound to the IM. Moreover,
membrane-integral respiratory complexes may form giant supercomplexes (respirosomes)
of variable stoichiometry. Such level of crowding undoubtedly affects the structure
and dynamics of water and, severally, those of the proteins themselves, as documented
for instance for small peptides and large photosynthetic complexes.
The intricate electrostatic description of media such as the IM and the IMS adds
another layer of complexity. The IM is essentially an energy-transduction device that uses
a cascade of downhill ET reactions to drive proton translocation, thus building up a
gradient that energizes ATP synthesis. This proton gradient generates a transmembrane
potential that combined with the membrane surface and dipolar potentials may create
local electric fields of up to 0.1 V Å⁻¹, in addition to local contributions due to protein
surface charges. Experimental and theoretical investigations on model systems show that
electric fields of biologically meaningful magnitude may affect hydration, structure,
dynamics and reactivity of proteins,[61–64] as well as relaxation times, viscosity,
hydrogen bonding and other features of water.[65–72] Conceptually similar
considerations stand for other biological systems based on protein ET, such as in
photosynthesis and bacterial respiration.

As pointed out by Ellis[8] almost two decades ago, the potential of macromolecular
crowding to affect reactivity is obvious but often underappreciated, also for ET reactions.
In the present work, we specifically assess the role of frictional effects in protein ET, i.e.
of the viscosity component of molecular crowding. To this aim, we envisage an approach
that involves the engineering of different metal centers into two protein scaffolds.
Namely, we consider two different types of one-electron copper redox proteins that share
the cupredoxin fold but differ by their redox centers: the type 1 (T1) mononuclear blue
copper site and the purple binuclear CuA center. Protein samples with hybrid properties
were generated by loop engineering to obtain chimeras that incorporate different T1
centers within the CuA scaffold, as well as perturbed CuA sites obtained by loop
replacement without altering the protein scaffold. The heterogeneous ET kinetics of the
different protein variants was studied by protein film electrochemistry at variable
electronic couplings and in the presence of two different crowding agents. The obtained
results reveal metal site- and scaffold-specific frictional control for tunnelling distances
shorter than ca. 24 Å.
2. Experimental

2.1 Protein preparation.

WT and mutant Cu₄-soluble fragments from subunit II of the cytochrome ba₃ from T. thermophilus were produced as described previously [73–76] and stored in 100 mM phosphate buffer (pH 6.0; 100 mM KCl). Azurin from Pseudomonas aeruginosa was purchased from Sigma-Aldrich. Before use, protein samples were buffer exchanged to the desired final condition by thorough filtration with Amicon Ultracel-5K filters employing a refrigerated centrifuge at 4000 rpm and 4 °C (Hermle Z326K).

2.2 Protein film voltammetry.

Cyclic voltammetry (CV) experiments were performed with either a Gamry REF600 or a PAR263A potentiostat using a water-jacketed non-isothermal cell. The cell was placed inside a Faraday cage (Vista Shield) and equipped with a homemade polycrystalline gold bead working electrode, a Pt wire auxiliary electrode and a Ag/AgCl (3 M KCl) reference electrode. All potentials quoted here are referred to NHE. Prior to use Au electrodes were oxidized in 10% HClO₄ applying a 3 V potential for 2 minutes, sonicated in 10% HCl for 15 minutes, rinsed with water and subsequently treated with a 1:3 v/v H₂O₂: H₂SO₄ mixture at 120 °C. The electrodes were then subjected to repetitive voltage cycling between -0.2 and 1.6 V in 10% HClO₄. After thorough rinsing with water and ethanol, Au electrodes were coated with self-assembled monolayers (SAMs) by overnight incubation in ethanolic solutions containing 2mM HS-(CH₂)n-CH₃ and 3mM HS-(CH₂)n-CH₂OH. Before protein adsorption SAM-coated electrodes were routinely cycled at 0.1 V s⁻¹ within the potential...
window required for each protein in 10 mM acetate buffer, pH 4.6, containing 250 mM KNO₃. Electrodes used for subsequent experiments were those that showed a well behaved and stable capacitive response only, with currents lower than 5 nA measured at 0.1 V s⁻¹ for n = 15 and lower than 300 nA measured at 10 V s⁻¹ for n = 5. Effective areas of the working electrodes were obtained by CV before SAM-coating using 20 mM Fe(CN)₆³⁻/⁴⁻ in 0.25 M KNO₃ as redox probe. The obtained values ranged from 2.6 to 7.9 mm², with an average value of 6 mm². The SAM-modified electrodes were finally incubated in 0.1 to 0.5 mM protein solutions during 2 hours for protein adsorption, and then transferred to the electrochemical cell. Measurements were performed in 10 mM acetate buffer, pH 4.6, containing 250 mM KNO₃. The solution viscosity was adjusted by dissolving variable amounts of either sucrose or polyethylene glycol 4000 (PEG4000) in the same buffer/electrolyte mixture, thus maintaining constant ionic strength throughout all the experiments. The temperature of the jacketed cell was varied employing a coupled circulation thermostat (Lauda Alpha RA8) and continuously monitored with a thermocouple (Fluke 51 II). CVs were typically acquired at scan rates between 50 and 500 mV s⁻¹ for the thicker SAMs, and 1 to 60 V s⁻¹ for the thinner films. All the CVs display the shape characteristic of surface-confined redox species and linear variations of the anodic and cathodic currents with the scan rate. Protein films were quite stable for about 100 voltammetry cycles. For longer cycling we observe a small gradual loss of the CV signals without changes in peak positions and FWHM, which suggest slow desorption of the protein film. CV measurements used throughout this work correspond to stable signals. Electrodes were replaced by freshly prepared ones as soon as a small a drop of intensity
was detected. Rate constants were obtained using Laviron’s formalism,[77] and activation
parameters were estimated from Arrhenius plots in a temperature range from ca. 5 °C to
40 °C.

Control kinetic experiments were performed using Creager’s method[78] based on
alternating current voltammetry (ACV). ACVs were acquired in stepped mode every 20 mV
in a potential window of 0.5 V centred on the reduction potential of each sample using an
rms amplitude of 10 mV. The range of frequencies was 1 Hz to 100 kHz for SAMs with n =
3, 5 and 7, 0.3 Hz to 30 kHz for n = 10 and 0.03 Hz to 30 Hz for n = 15.

Uncompensated resistance was routinely determined using the optimized impedance
routine included in the Framework Data Acquisition Software from Gamry (Version 6.33).
For Au electrodes coated with SAMs with n = 6, i.e. the thinnest SAMs that employed for
investigating viscosity effects, Ru values were typically 10 Ω in the absence of thickening
agent and 20 Ω at 5 cP. After protein adsorption Ru slightly increases, reaching values of
up to 40 and 50 Ω for viscosities of 1 and 5 cP, respectively, for the highest protein surface
concentrations employed here of ca. 8 pmol cm⁻², which are attained with azurine. The
highest currents obtained in CV experiments that are used for subsequent quantitative
treatment were achieved for azurine films on SAMs with n = 6 and scan rates of 60 V s⁻¹.
These maximum currents were about 40 µA and independent of the addition of thickening
agents. Based on these numbers, we can establish an upper limit for ohmic losses that is 2
mV for the most demanding conditions employed here, and typically one order of
magnitude lower or less.
CV measurements of bulk protein solutions were performed using a home-made water jacketed non-isothermal three electrode cell that requires ca. 40 μL samples with concentrations around 100 μM (10 mM buffer acetate, pH 4.6, 250 mM KNO₃). Gold working electrodes were coated with HS-(CH₂)₆-OH to prevent protein adsorption.

2.3 Spectroscopic determinations.

UV-vis absorption spectra were acquired at 25 °C with a Thermo Scientific Evolution Array spectrophotometer employing 1 cm or 0.1 cm path length as required, placed into a jacketed cell-holder for temperature control through a circulation thermostat (Fisherbrand FBC620). Raman spectra were acquired in backscattering geometry with 532 nm excitation using a Dilor XY800 Raman microscope equipped with a CCD detector. Prior to measurement ca. 10 μL protein samples were placed and frozen at 77 K in a Linkam THMS 300 thermostat. RR spectra were acquired at 0.5 cm⁻¹ resolution. UV-vis and RR determinations were performed in 10 mM acetate buffer, pH 4.6, containing 250 mM KNO₃, with and without addition of crowding agent.

3. Results and discussion

The present work aims to gain a deeper understanding of molecular crowding effects on long-range protein ET and, specifically, to dissect the responses of the redox site and the protein milieu to viscous media. The proteins selected for this study are: (i) wild type azurin (Azu WT) from P. aeruginosa as a prototypical mononuclear T1 blue copper protein, (ii) the Cu₄-containing soluble domain of the ba₃ O₂-reductase from T. thermophilus (Tt-
Cu₁₉) as a prototypical binuclear purple copper protein and (iii) three chimeric proteins constructed by loop engineering of Tt-Cu₁₉ where the sequences of the three loops that surround the metal site are replaced by those corresponding to other organisms to create novel T1 and Cu₁₉ variants (Figure 1). The structure and spectroscopy of the WT and chimeric proteins has been reported elsewhere. [43,73–76,79,80] Remarkable features relevant to the present work are: (i) Tt-Cu₁₉ and WT Azu share the cupredoxin motif, but with some differences that elicit a higher thermal stability in Tt-Cu₁₉;[80–83] (ii) Azu WT is a canonical T1 blue copper center;[3,84] (iii) Tt-Cu₁₉ is canonical Cu₁₉ centers, while Tt-3L is a slightly distorted Cu₁₉ site that preserves the mixed valence character and the typical purple colour;[43,75] (iv) Ami-Cu₁₉ and Azu-Cu₁₉ are distorted and greenish mononuclear T1 sites.[76]

The heterogeneous ET reactions of the five protein variants were investigated by protein film electrochemistry using Au electrodes coated with self-assembled monolayers (SAMs) of SH-(CH₂)ₙ-CH₃ / SH-(CH₂)ₙ-CH₂OH mixtures in 4/6 proportion and variable length (n = 3, 5, 7, 10 and 15). Proteins were adsorbed on the SAM-coated electrodes and measured at pH 4.6 in 10 mM acetate buffer containing 250 mM KNO₃.

This combination of coating and electrolyte composition was adopted from previous reports, which demonstrate that these conditions optimize adsorption without altering the redox copper centers.[20,73,74,79] Cyclic voltammetry (CV) experiments afford quasi reversible responses in all cases (Figures S1 to S4) with FWHM values close to ideal that, in average, yield charge transfer coefficients between 0.45 and 0.55. Moreover, the reduction potentials are very similar to those obtained in solution under comparable
conditions (Table S1), thus confirming the integrity of the adsorbed proteins. Surface-
enhanced RR spectra of the SAM-coated electrodes recorded before and after protein
adsorption do not exhibit changes in the intensity ratio of the $\Delta \nu_{C-S}$ bands characteristic of
the gauche and trans conformations of the alkanethiols found at 634 and 702 cm$^{-1}$,
respectively (data not shown). [85] The intensity ratio of the $\Delta \nu_{C-S}$ bands is a sensitive
marker of order in SAMs and, therefore, its invariance strongly suggests that the SAM-
protein interactions are relatively weak and non-perturbative, in agreement with the
preservation of the reduction potentials of the adsorbed proteins. From the integration
of the voltammetric peaks we obtain protein coverages that are typically below 4 pmol
cm$^{-2}$ for Azu WT and below 2 pmol cm$^{-2}$ for the other proteins, which represent less than
1/3 and 1/6 of full coverage, respectively, as estimated using a crystallographic diameter
of 40 Å for both scaffolds.

Since Chidsey’s seminal work,[86] SAM-coated electrodes are broadly used for kinetic
studies because they allow the systematic variation of protein-electrode electronic
coupling through the chain length of the thiols. Hence, we measured the ET rate
constant, $k_{ET}$, for the different protein variants as a function of the SAM thickness
employing two different and independent experimental approaches: Laviron’s
formalism[77] based on the peak separation of CVs obtained at variable scan rates and
Creager’s method[78] based on variable frequency ac voltammetry. Figures S5 and S6
show typical Laviron’s working curves and trumpet plots, respectively, while a
representative Creager’s plot is presented in Figure S7. The two methods yield almost
identical $k_{ET}$ values with a nearly 1:1 correspondence (Figures S8 and S9).
The results are summarized in Figures 2 and S10, and are characterized by an exponential distance dependence of $k_{ET}$ at thicker SAMs ($n \geq 10$) and a softer dependency at thinner films that tends to a plateau. Qualitatively similar results have been reported by other authors for Azu WT and Tt-CuA, although with slightly lower rate constants ascribable to the different experimental conditions.[20,28,30,87–89] The long distance exponential decay, with tunnelling decay factor $\beta = 1$ per methylene group, is the fingerprint of a nonadiabatic ET mechanism.[90] The softer variation at shorter distances, on the other hand, suggests a change of regime associated to the stronger electronic coupling.

Waldeck et. al. observed a similar distance dependence for cytochrome $c$ coordinatively bound to pyridinyl-terminated alkanethiols and for Azu WT adsorbed on hydrophobic SAMs, and ascribed it to a friction-controlled ET mechanism at shorter distances.[25,26,91] Under the working hypothesis that Waldeck’s proposal is correct, we reason out that the construction of chimeras as those summarized in Figure 1 offer a unique opportunity to advance for the first time in assessing and dissecting biologically relevant crowding effects on the ET reaction coordinate at different levels of the hierarchical protein architecture and dynamics. To this end, we first investigated the impact on the ET kinetics of adding variable amounts of sucrose and PEG4000 (Figures 3, S11 and S12). These two chemicals were selected because are commonly used as crowding agents in biological studies due to their ability to emulate in-cell high viscosities while minimizing specific interactions.[8] The concentrations of the crowding agents were adjusted to produce viscosities up to 4 cP, i.e. a typical cytosolic value, and control experiments were performed up to 60 cP, which
correspond to the typical intramitochondrial viscosity.[90–92]

In all these experiments electrolyte and buffer composition were kept constant, thus fixing the ionic strength at \( I = 250 \text{ mM} \) that, as shown in Figure S13, is sufficiently high to warrant \( I \)-independent \( k_{ET} \) values, even at high viscosities.

For all the protein variants we observe a drop of \( k_{ET} \) upon addition of either crowding agent. When plotted against the solution viscosity \( \eta \), we obtain a power law dependence of the form \( k_{ET} \propto \eta^{-\gamma} \) (Figure 3) which, for a given protein at a given SAM thickness, is nearly identical for the two crowding agents, thus pointing out that the kinetic effect most likely arises from the increase of the bulk viscosity rather than due to specific interactions of sucrose and PEG4000 with the different components of the SAM/protein systems.

Similar results are obtained in control experiments with extended viscosity range of up to ca. 60 cP (Figure S14).

It is important to point out that additions of large amounts of sucrose or PEG4000 do not affect the active sites, as resonance Raman and electronic absorption spectra remain unaffected for all the protein variants studied here (Figures S15 and S16). In agreement with these observations, CV responses are not affected by the crowding agents aside from the increased peak separations (Figures S11 and S12). Indeed, reduction potentials remain largely constant over a broad concentration range of crowding agents, with only some minor variations for some of the protein variants and no changes for the others (Figure S17), which may reflect slightly different sensitivity to changes in the dielectric constant.

Note that the addition of both crowding agents not only rises the viscosity but also decreases the static dielectric constant \( (\varepsilon_s) \) and increases the optical dielectric constant.
(ε_{op}) of the solution, which results in a reduction of the Pekar factor $\varepsilon_{op}^{-1} - \varepsilon_{s}^{-1}$ (Figure S18). In terms of classical Marcus theory this would imply lower outer sphere reorganization energies and, therefore, slightly faster ET. The results shown in Figure 3 suggest that the accelerating effect of a lower Pekar factor is overcompensated by the slowing down effect of the higher viscosity, thus pointing out to a friction-controlled ET reaction.

Interestingly, ET rates are sensitive to the solution viscosity not only in the plateau region of the $k_{ET}$ vs distance curves but, essentially for all chain lengths with $n < 15$, which approximately corresponds to tunnelling distances $< 23$ Å.[95] As shown in Figure 4 for two representative examples, the $\gamma$ factors exhibit a sigmoidal distance dependence that denotes the interplay between through-SAM electron tunnelling times and friction-controlled protein dynamics.

Note that at the sub-monolayer coverages employed here, $k_{ET}$ and activation free energy values are insensitive to protein surface concentration (Figure S19), thereby confirming that the observed kinetic effects are ascribable to the external crowding agent. For SAMs with $n = 5$, which roughly corresponds to tunnelling distances of ca 10 Å, we observe a 30-45 % drop of $k_{ET}$ at $\eta = 4$ cP, and more than 90% drop at 55 cP. Interestingly, in-cell local microviscosities have been reported to vary between 1 to 400 cP,[92] with values of around 35-63 cP in healthy mitochondria and one order of magnitude higher values under apoptotic conditions.[93,94]

The ET rate is given by the product of two terms: a Franck–Condon factor, which accounts for the probability of achieving donor-acceptor energetic degeneracy through
thermal fluctuations, and the electronic coupling, which decays exponentially with distance and represents the probability of electron tunnelling between degenerate states. Both terms may be affected by molecular crowding through the increased viscosity. As discussed in detail by Matyushov and coworkers,[96,97] the first term is sensitive to the ratio between the reactant-product relaxation time ($\tau_{\text{relax}}$) and the reaction time ($\tau_r$). Marcus theory works under the assumption that $\tau_{\text{relax}} \ll \tau_r$, which may not be valid in slowly relaxing media, thus leading to a reduced phase sub-space accessible to thermal exploration within the reaction time scale. This may result in a decrease of the apparent Gibbs energy barrier and reorganization energy with respect to the Marcus equilibrium values. Moreover, Matyushov et. al.[98] concluded that for the electrochemical ET of cytochrome c the pre-exponential term of the ET rate constant is not affected by solvent dynamics. In this scenario, one should expect an increase of $k_{\text{ET}}$ upon raising the solvent viscosity, as a result of lowering the activation barrier. The experimental results obtained for the copper proteins studied here show the opposite trend, which strongly suggests that the system does not reach the limiting case of $\tau_{\text{relax}} \ll \tau_r$ but, instead, the reaction proceeds in an intermediate regime where the viscosity effect is dominated by the pre-exponential factor. These experimental conditions are better described by Zusman equation for electrochemical reduction at zero overpotential of adsorbed species:[91,99]

$$k_{\text{ET}} = \left(\frac{1}{\pi^2 \tau_s^2 k_B T}\right)^{1/2} \left(\frac{4 \hbar^2}{\pi^2 \hbar^2 k_B T}\right) \ln \left(\frac{\rho |H_{DA}|^2 \pi^2 k_B T}{\lambda \hbar}\right)$$  

(1)

where $\lambda$ is the classical reorganization energy, $\tau_s$ is the solvent relaxation time, $k_B$ is Boltzmann constant, $H_{DA}$ is the electronic coupling matrix element, $\rho$ is the density of
states at the electrode surface and \( h \) is the reduced Planck constant. In a usual first order approximation, \( \tau_s \) can be expressed in terms of the longitudinal relaxation time \( \tau_L \) and, thus, as a function of the solvent viscosity \( \eta \) and molar volume \( V_m \):

\[
\tau_s = \frac{\epsilon_s}{\epsilon_{op}} \tau_L = \frac{\epsilon_s}{\epsilon_{op}} \frac{3\eta V_m}{RT} \quad (2)
\]

Assuming a simple Debye solvent model, the viscosity can be described in terms of Andrade’s empirical equation:

\[
\eta = A \exp \left( \frac{\Delta G_s^\#}{RT} \right) \quad (3)
\]

where \( A \) is an empirical pre-exponential parameter and \( \Delta G_s^\# \) is the activation free energy for the solvent viscous flow.

As proposed by Waldeck and co-workers, the empirical power law \( k_{ET} \propto \eta^{-\gamma} \), together with equations 1-3 leads to the following approximated expression for the electrochemical ET rate constant at zero driving force:

\[
k_{ET} = \frac{\epsilon_{op}}{3A\gamma V_m \epsilon_s} \sqrt{\frac{RT}{4\pi}} e^{-\frac{\Delta G_{ET}^\#}{k_BT}} \exp \left( -\frac{\Delta G_{ET}^\# + \gamma \Delta G_s^\#}{k_BT} \right) \quad (4)
\]

where \( \Delta G_{ET}^\# = \lambda/4 \) is the classical Marcus activation free energy for ET. This equation predicts that for a friction-controlled ET reaction the overall apparent activation free energy, \( \Delta G_{app}^\# \), contains a first term that represents the intrinsic ET reorganization energy of the system and a second term, \( \gamma \Delta G_s^\# \), which accounts for the temperature dependence of the medium relaxation dynamics. \( \Delta G_{app}^\# \) can be estimated from the temperature dependence of \( k_{ET} \). Arrhenius and Eyring treatments of these data yield essentially identical results, in agreement with previous observations for similar copper proteins[20,74,87] that the entropic contribution is negligibly small and, therefore,
\[ \Delta G^\#_{\text{app}} \approx \Delta H^\#_{\text{app}}. \]  
Arrhenius plots obtained in the absence of crowding agents for the five protein variants adsorbed on SAMs with \( n = 5 \) and \( n = 15 \) are shown in Figure S20, and the results are summarized in Table S1. Note that for all the protein variants \( k_{ET} \) is independent of solvent viscosity when measured using the thickest SAM, i.e. \( \gamma = 0 \) for \( n = 15 \), hence we obtain \( \Delta H^\#_{\text{app}} \approx \Delta G^\#_{ET} = \lambda/4 \) in these cases. \( \Delta G^\#_s \) values can then be obtained by subtracting \( \Delta G^\#_{ET} \) measured at the thickest SAM from the \( \Delta G^\#_{app} \) values measured with thin films in the absence of crowding agents, and dividing by the corresponding \( \gamma \) factors obtained from viscosity experiments with the thin SAMs. The two relevant frictional parameters \( \gamma \) and \( \Delta G^\#_s \) obtained for SAMs with \( n = 5 \) are plotted in Figure 5. Interestingly, the value of \( \gamma \) seems to be a property of the metal site, with an average value of 0.54 for the different native and non-native T1 centers, and an average value of 0.29 for Tt-Cu\(_A\) and Tt-3L. \( \Delta G^\#_s \), in contrast, seems to be a specific property of the protein scaffold, with an average value of 0.037 eV for the Tt-Cu\(_A\) fold independently of the metal center incorporated, and a value of 0.120 eV for the native azurin fold.

The parameter \( \gamma \) is a measure of the degree of frictional control, i.e. of the influence of solvent/protein relaxation on the ET rate.[25,26] It is also an empirical correction that accounts for the distribution of relaxation times not contemplated in the Debye solvent model.[13] Limiting values of 0 and 1 correspond to the fully nonadiabatic and adiabatic regimes, respectively, while values in between denote an intermediate regime with overdamped solvent/protein dynamics, which is consistent with the observed distance dependence of \( \gamma \) (Figure 4). The site specificity of \( \gamma \) revealed in the present work suggests a more distinct interpretation for this parameter as a measure of the dynamic coupling.
between the redox metal site and the protein/solvent milieu. This observation is consistent with the structural rigidity of the Cu$_2$S$_2$ diamond core in Cu$_A$ sites, which includes a Cu-Cu covalent bond, compared to the more easily distorted T1 sites.[84] On the other hand, $\Delta G^\#_S$ is similar, within experimental error, for all the protein variants that share the Tt-Cu$_A$ fold, with an average value of 0.037 eV. This value rises to 0.120 eV for Azu WT. While both types of proteins are characterized by a high rigidity that is regarded crucial to ensure efficient ET,[80,81] the lower melting temperatures of T1 proteins and backbone mobility studies by NMR reveal lower rigidity compared to Cu$_A$. [80,82,83] At first sight this suggests the counterintuitive idea that the activation parameter $\Delta G^\#_S$ is higher for more flexible proteins, which is reinforced by the even larger value reported in literature for the highly flexible cytochrome c (Figure 5).[26] Our proposal is that $\Delta G^\#_S$ is not reporting on the overall protein flexibility. Instead, this scaffold-specific parameter is a measure of the temperature-dependence of protein-solvent motions that are relevant to the ET reaction coordinate.

Given that the long-range ET rates for the systems studied here are within a range of ca. 1 to 8000 Hz (Figures 2 and S10), low frequency protein-solvent motions may be critical for the ET reaction. Moreover, protein-solvent dynamics may be affected by local electric fields at the SAM/protein interface,[61,100] which are similar in magnitude to those estimated for biological membranes,[101,102] thus possibly increasing $\Delta G^\#_S$ under in vivo conditions with respect to diluted protein solution.

4. Conclusions
The present results highlight the need of explicitly considering molecular crowding effects in protein ET reactions. Highly crowded environments, such as the mitochondrial intermembrane space, are characterized by microviscosities that are one to two orders of magnitude higher than for diluted aqueous solutions, and by the presence of high local electric fields imposed by the membrane potentials that may affect protein-solvent dynamics. Under these conditions nuclear motions relevant to the ET reaction coordinate are overdamped, thus breaking down some of the underlying assumptions of Marcus theory, and leading to a friction-controlled mechanism for electron tunnelling distances shorter than ca. 24 Å. The degree of frictional control is determined by two parameters: (i) the frictional activation barrier, which we show to be specific for the protein scaffold, and (ii) the dynamical coupling between the redox site and the surrounding protein-solvent milieu, which reports on the electronic structure and the functional features of the electron transfer site. The first and second parameters resemble Frauenfelder’s $\alpha$ and $\beta_h$ fluctuations, respectively, whose characteristic time scales are modulated by the local electric fields and by the high viscosity prevailing in biological electron-proton energy transduction. Overall, our strategy allows a dissection of the effects that impact on ET under crowding conditions, as well as it opens new possibilities of studying in detail the impact of these empirical parameters in a central biological phenomenon under physiological conditions.

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Appendix A. Supplementary data

Supplementary data related to this article can be found athttps://doi.org/xxxxx

References


Figure 1.

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<tr>
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Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 1. Top: Crystal structure of the Cu₄-containing soluble domain of the ba₃ O₂-reductase from Thermus thermophilus (Tt-Cu₄; pdb 2CUA) and of the metal sites of the Tt-Cu₄ and Ami-Cu₄ variants (pdb 2CUA and 5U7N, respectively). Bottom: sequences of the three engineered loops. Letters in light blue denote first sphere ligands of the copper ions. The last column indicates the organism from which the loop sequence was adopted for the chimeras: Thermus thermophilus (Tt), Homo sapiens (Hs), Paracoccus denitrificans (Pc) and Pseudomonas aeruginosa (Pa).

Figure 2. Normalized ET rate constants as a function of the SAM thickness. Each data point is the average of at least three independent experiments performed at 25°C in 10 mM acetate buffer, pH 4.6, containing 0.25 M KNO₃. Absolute $k_{ET}$ values, as obtained by Laviron’s method, are displayed in Figure S10.

Figure 3. Normalized ET rate constant as a function of the relative viscosity for proteins adsorbed on SAMs with $n = 5$ (25°C; pH 4.6, 0.25 M KNO₃). (A) Monocuclear T1 centres. (B) Binuclear Cu₄ sites. (C) Comparison of Azu WT and Tt-Cu₄ for experiments performed with sucrose (empty symbols) or PEG4000 (filled symbols). The lines are fittings to $k_{ET}(\eta) = k_{ET}(\eta_0)\eta^{-\gamma}$.

Figure 4. Frictional parameter $\gamma$ as a function of SAM thickness for Azu WT and Tt-Cu₄ using sucrose as crowding agent. All measurements were performed at 25°C in 10 mM acetate buffer (pH 4.6, 0.25 M KNO₃).
Figure 5. Empirical frictional parameter ($\gamma$) and activation free energy for the milieul frictional motion ($\Delta G^\#_f$) for the copper proteins and wired Cyt c. Blue, green, red and orange bars are data obtained in this work at 25°C in 10 mM acetate buffer (pH 4.6, 0.25 M KNO$_3$) and using HS-(CH$_2$)$_5$-CH$_3$/HS-(CH$_2$)$_5$-CH$_2$OH SAMs. Gray bars are data taken from literature for WT azurin on HS-(CH$_2$)$_5$-CH$_3$ SAMs (Azu WT (CH$_3$)) and cytochrome c coordinated to a pyridinyl-terminated SAM (Cyt c (Py-SAM)).