Long-Range Electron Transfer in Engineered Azurins Exhibits Marcus Inverted Region Behavior

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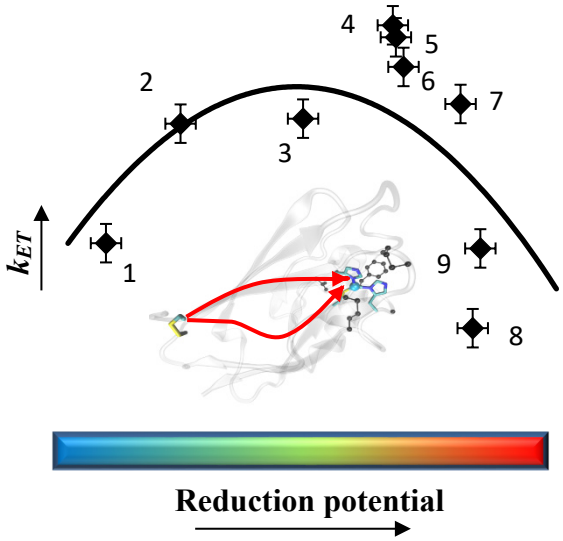
ABSTRACT: The Marcus theory of electron transfer (ET) predicts that, while the ET rate constants increase with rising driving force until it equals a reaction's reorganization energy, at higher driving force the ET rate decreases, having reached the Marcus inverted region. While experimental evidence for the inverted region has been reported for organic and inorganic ET reactions, as well as for proteins conjugated with ancillary redox moieties, evidence for the inverted region in a "protein-only" system has remained elusive. We herein provide such evidence in a series of non-derivatized proteins. These results may facilitate design of ET centers for future applications such as advanced energy conversions.

TOC GRAPHICS



KEYWORDS: Blue copper, electron transfer, Marcus theory, reorganization energy, protein engineering

Understanding the parameters that control electron transfer (ET) rates in biomolecules is of fundamental importance in chemistry and biology.[1-9] Nature has optimized the structures, separation distances and driving forces of donors and acceptors to ensure facile and efficient ET in biological processes[10-12] and a large number of studies have focused on understanding the control of these rates.[13-19] The semi-classical Marcus theory states that three parameters determine the rate of ET between a donor and an acceptor held at a fixed distance and orientation; the electronic coupling between the reactants, their reorganization energy and the driving force of the reaction.[20,21] A counter-intuitive result of the theory is its prediction of a Gaussian dependence of the ET rates on driving force; namely that while the ET rate constants increase with rising driving force until it equals the reorganization energy, at higher driving force the rate constants decrease, reaching the "Marcus inverted region". Three decades elapsed until the inverted region was experimentally observed by Miller, Calcaterra, and Closs in 1984[22] using a series of organic donor-acceptor molecules. This was further established in an inorganic model system of an iridium(I) dimer by Gray *et al*.[23] The search for an inverted region within proteins was pioneered by Durham, Millett and coworkers[24] using ruthenium-labelled cytochrome b_5, and by Gray and coworkers employing singlet and triplet excited states of a zinc-substituted cytochrome *c*.[25]

Since these reports were published, experimental evidence for the "inverted region" behavior in a non-derivatized protein has remained elusive. Such experimental evidence is of considerable interest because the inverted region has been proposed to be responsible for a number of crucial biological ET processes in proteins, such as the charge separation in photosynthetic reaction

centers, and was experimentally observed through replacement of the native quinones at the QA site of the reaction center protein from *Rhodobacter spheroides*.[26,27]

An optimal system for pursuing such a challenge is the bacterial ET protein azurin (Az) that contains a blue type 1 copper center coordinated by three strong ligands, His46, Cys112 and His117 in a trigonal planar geometry around the Cu center. A large number of biochemical and biophysical studies, including spectroscopic, X-ray crystallographic and computational studies of both wild type (WT) Az and its mutants have resulted in thorough understanding of three dimensional and electronic structures responsible for its ET function.[28-35] Of particular interest are those Az mutants whose reduction potentials have been tuned to allow a wide range of driving forces for ET reactions.[36-41] Recently we produced Az mutants where this primary coordination sphere is kept intact while the weaker axial ligand, Met121, was replaced by Gln or Leu in order to tune the hydrophobicity around the copper.[42] In addition, Asn47 was replaced by Ser and Phe114 by Asn or Pro to modify the hydrogen bonding networks around the Cys112 and His117 copper ligands, respectively.[42] These changes modulated the reduction potential of the Cu site over a wide range (> 500 mV) without significantly disrupting the coordination site. In order to investigate how the changes in the Cu(II) site's potential influence its reorganization energy, we have previously measured the intramolecular ET rates in Az from a disulfide radical-anion, produced by pulse radiolysis, to the Cu(II) center in several of these mutants.[43] Indeed we found that the ET proceeds with lower reorganization energy than in WT Az.

In order to enable a quantitative examination of the relationship between the ET driving force and the rate constant in a protein-only system without any ancillary foreign donor or acceptor, we have now expanded this series and prepared several other mutants (F114N,

N47S/F114S/M121L, and M44F/N47S/F114N/G116F) with higher potentials than that of WT Az (Figure 1 and Table 1).

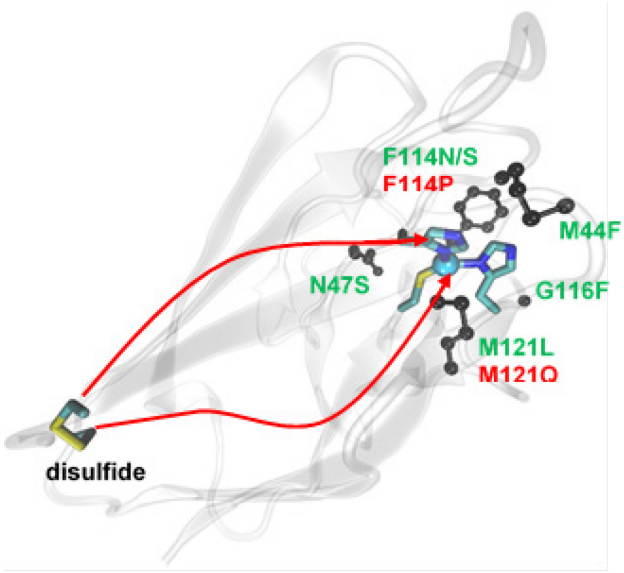


Figure 1. Schematic presentation of Az and the designed mutants. Calculated paths (see below) of electron transfer from the disulfide to Cu are illustrated by the red arrows. The mutated residues are shown in black stick and ball. The green mutations cause increase in reduction potential while the red ones decrease it.

Kinetic measurements of the intramolecular ET in these nine different Az mutants, including three previously unreported ones with reduction potentials from 0.39 to 0.61 V vs. NHE have been investigated. Pulse radiolytically produced CO_2^- radicals reduce both the disulfide bridge (Cys3-Cys26) and the Cu(II) site in Az with similar, essentially diffusion controlled rate constants ($k_1 \approx 10^9$ $M^{-1}s^{-1}$), (Figure 2). Since an excess of protein is employed over the reducing CO_2^- radicals, only a fraction of either disulfide or Cu(II) is reduced, allowing for the disulfide radical produced ($RSS^{\bullet}R^-$) to transfer an electron to the Cu(II) center in a second, slower and concentration independent, intramolecular ET process (Figure 2):

$$\text{Az[Cu(II)RSS}^{\bullet}\text{R}^{-}] \xrightarrow{k_{ET}} \text{Az[Cu(I)RSSR]}$$

The rate constants, k_{ET} at 25ºC, were determined by monitoring both the oxidation of the radical (410 nm, Figure 2A) and reduction of Cu(II) (600-635 nm, depending on the mutant's absorption maximum, Figure 2B). The k_{ET} values and calculated activation parameters are presented in Table 1 together with earlier results.

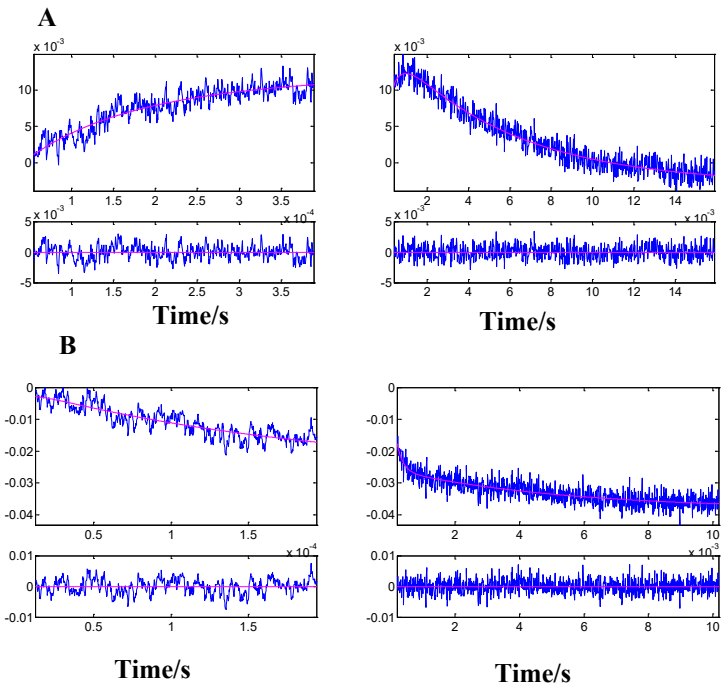


Figure 2. (A). Time resolved absorption changes at 410 nm of the F114N azurin mutant, monitoring formation and decay of the $RSS^{\bullet}R^{-}$ radical ions. (B) Time resolved absorption changes (625 nm) of the F114N Az mutant monitoring reduction of Cu(II). The protein concentration was 93 μM in an N_2O saturated solution; temperature 25 ºC. All other technical details are described in the Experimental methods section.

Calculations of ET pathways between the RSS$^{\bullet}$R^{-} radical ion and Cu(II) in these mutants showed that the same two pathways are operating in them and in the WT. Neither pathway involves the mutated residues.

The semi-classical Marcus theory for ET reactions between spatially fixed and oriented donors and acceptors provides a framework for analysis of rate constants in the non-adiabatic regime, Eq. 1:[20]

$$k_{ET} = \kappa(r)\,\nu\,\exp(-\Delta G^{*} / RT) \qquad (1)$$

where

$$\Delta G^{*} = \frac{\lambda_{TOT}}{4}\left[1 + \frac{\Delta G^{0}}{\lambda_{TOT}}\right]^{2} \qquad (2)$$

In Eq. 1, κ(r) is the transmission coefficient at a separation distance, r, while ν is the frequency of nuclear motions. (In the non-adiabatic regime, κ(r)ν is independent of this frequency). R and T are the gas constant and temperature (in K) respectively. ΔG^{*} and ΔG^{0} (cf. Eq. 2) are the activation free energy and standard free energy of reaction, respectively, and λ_{TOT} is the total reorganization free energies of both the ET donor and acceptor. When the driving force of the reaction equals the total reorganization energy, the rate constant reaches a maximum value, k_{MAX}. Since κ(r)ν decays exponentially with the separation distance, we can calculate k_{MAX} by Eq. 3 below:

$$k_{MAX} = \frac{k_B T}{h}\exp\left[-\beta(r - r_0)\right] \qquad (3)$$

where k_B and h, respectively are Boltzmann's and Planck's constants, r is the donor-acceptor

separation distance and r_0 is the value of r for direct (van der Waals) contact; the generally accepted value for r_0 is 0.3 nm. The timetable for activationless electron tunneling in β-sheet proteins of Gray *et al*. provides a decay constant of $\beta = 10\ nm^{-1}$.[1]

Attempts to experimentally determine the reduction potential for the formation of the $RSS^{\bullet}R^{-}$ radical in azurin have failed so far. Since the cystine is partly solvent exposed, we have been using a value of -0.41 V vs. NHE, determined from hybrid disulfide between a nitroaromatic and a protein cysteine thiol.[44] It is noteworthy that any change in the value for the reduction potential of the disulfide primarily adds or subtracts a constant from the abscissa values of the plot in Figure 2, and thus the reorganization energy by the same value.

The activation free energies, ΔG^{*} of the ET reactions in each mutant can be calculated from the activation parameters presented in Table 1. However, the experimentally determined activation entropy , $\Delta S^{\ddagger}$, includes a contribution from the electronic coupling:[20]

$$\Delta S^{\ddagger} = \Delta S^{*} + R\ln(\kappa\nu / 10^{13}) = \Delta S^{*} - R\beta(r - r_0) \qquad (4)$$

where the symbols have already been defined above.

Table 1. Rate constants, activation parameters of the intramolecular ET reactions, and the potentials of the copper site.

	Azurin Mutant	k_{ET} (s^{-1}) at 298 K	E° (mV)	ΔH‡ (kJ/mol)	ΔS‡ (J/K·mol)
1.	**F114P/M121Q***	81 ± 11	122 ± 6	36.6 ± 7.5	-86 ± 14
2.	**F114P***	191 ± 26	220 ± 18	~29	-106
3.	**F114N****	198 ± 14	381 ± 8	29.9 ± 0.2	-101 ± 1
4.	**N47S/F114N***	387 ± 59	499 ± 3	33.7 ± 2.5	-82 ± 4
5.	**N47S/M121L***	355 ± 51	503 ± 5	44.0 ± 2.1	-48 ± 1
6.	**F114N/M121L***	287 ± 34	513 ± 4	~ 39	~ -66
7.	**M44F/N47S/ F114N/G116F****	220 ± 13	588 ± 20	26.8 ± 2.3	-110 ± 7
8.	**N47S/F114S/ M121L****	44 ± 5	604 ± 14	30.4 ± 4.3	-110 ± 12
9.	**N47S/F114N/ M121L***	78 ± 12	614 ± 11	41.7 ± 5.9	-71 ± 8

*Data from Ref.[43]. **This study.

Figure 3 shows a plot of the ET rate constants, k_{ET}, vs. driving force, for the nine Az mutants fitted to the theoretical curve calculated using Eqs. (1) and (2) above. A non-linear least squares analysis of the data yields a value of k_{MAX} = 249(+56/-44) s^{-1} and a reorganization free energy of λ_{TOT} = 0.78(+0.04/-0.04) eV. The broken lines result from using the method of support planes,[45] the limit of each parameter that produces a 10% change in chi squared while the other parameter is allowed to optimize. The two broken lines represent the limits of k_{MAX}. In this analysis, k_{MAX} is the pre-exponential term in Eq. 1, treated as a constant, and is consistent with a k_{MAX} = 286 s^{-1} calculated directly from Eq. 3. Also, the reorganization free energies of the individual mutants were calculated independently using the experimental activation parameters, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$. Using $\beta(r\text{-}r_0)$ = 23.8 (*cf.* Eq. 3) we obtain $R\beta$ $(r\text{-}r_0)$ = 198 $JK^{-1}mol^{-1}$ (or 2.05 meV K^{-1}). We then calculate ΔG^{*} using the activation enthalpy and the corrected activation entropy values. Finally,

the reorganization free energy, λ_{calc} is calculated from Eq. 2. The results are presented in Table 2.

Table 2. Reorganization free energies, $\lambda_{calc.}$ for the Az mutants.

Azurin Mutant	$-\Delta G^0$ (eV)	ΔG^* (eV)	$\lambda_{calc.}$ (eV)
1. F114P/M121Q*	0.532 ± 0.006	0.033 ± 0.007	0.87
2. F114P*	0.630 ± 0.023	~0.016	0.87
3. F114N**	0.791 ± 0.008	0.009 ± 0.001	0.98
4. N47S/F114N*	0.909 ± 0.003	-0.009 ± 0.001	0.91
5. N47S/M121L*	0.913 ± 0.005	-0.007 ± 0.006	0.91
6. 114N/M121L*	0.923 ± 0.004	~ -0.003	0.92
7. M44F/N47S/F114S/G116F**	0.998 ± 0.020	0.006 ± 0.001	0.85
8. N47S/F114S/M121L**	1.014 ± 0.014	0.043 ± 0.005	0.67
9. N47S/F114N/M121L*	1.024 ± 0.011	0.040 ± 0.006	0.69

*Data from (Ref.[43]). **This study.

As illustrated in Figure 3, the experimentally determined rate constant values of the intramolecular ET in the Az mutants fit the theoretical parabola calculated using Marcus theory in the above plot of the ET rate constants vs. their driving force reasonably well, considering the redox potential range of mutants studied and the requirement of k_{MAX} and λ_{TOT} being constant for the entire set of examined mutants. The reorganization energy includes contributions from both the T1 Cu site and the disulfide-radical ion. From previous pulse radiolysis studies, we have calculated a λ_{SS} = 1.2 eV[46,47] which yields a λ_{Cu} = 0.4 eV, a significantly lower value than that (λ_{Cu} = 0.82 eV) previously determined for WT *Pseudomonas aeruginosa* Az.[37] In a previous

study, we have attributed such lowering of the reorganization energy to increased flexibility of the T1 copper center caused by changes in non-covalent interactions such as hydrogen bonding and hydrophobicity in the secondary coordination sphere of the T1 copper site.[43] It has been shown before that changing hydrogen bonds and collective perturbation of the protein dynamics can affect the λ values.[48] The deviations from the fit line at the highest driving force would, if attributed only to variation in the reorganization energy, correspond to a variation of up to 0.25 eV from the average or fit value.

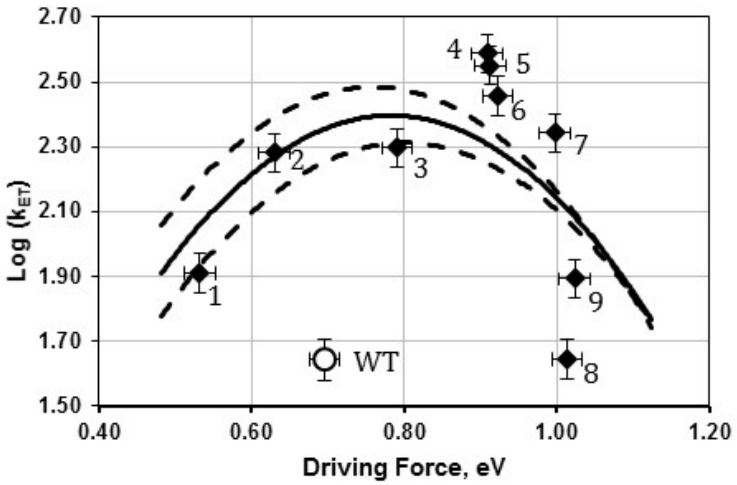


Figure 3. The Marcus plot of log(k_{ET}) of intramolecular ET in the Az mutants as function of the driving force. The fitted line is calculated using k_{MAX} = 249 (+56/-44) s^{-1} and λ_{TOT} = 0.78 (+0.04/-0.04 eV. The points are labelled 1 – 9, as in Tables 1 & 2. WT Az is the open circle symbol.

The exponential decay constant, β (*cf.* Eqs. 3 and 4) is another important parameter determining the ET rates. Differences in β would affect both the ET rates and activation entropies even when a common ET pathway is operating. These differences may be due to subtle changes in the electronic coupling between the copper ion and its ligands, particularly upon slight differences in the covalency of the Cu^{2+}–S(Cys) bond. Calculations of the Fermi contact

term presented in Table S1 show only small differences in the anisotropic covalency, suggesting that the mutations have only a minor effect on the total electronic coupling along the ET pathway. Furthermore, the electronic coupling between electron donor and acceptor does not change significantly as a result of the mutations (an average β = 10.0 +/- 0.2 nm^{-1}) and is unlikely to cause the observed steep decrease in rate constants in mutants with the highest driving force illustrated in Figure 3. Comparing mutants 7 and 8, the five-fold decrease in rate constant would require an unlikely change in the decay constant, β, from 10.0 to 10.7. However, minor changes in the electronic coupling could be responsible for the scatter of the points in Figure 3 (beside experimental errors). Examination of the distances in the ET pathways of the different mutants using their crystal structure or molecular dynamics (MD) simulations showed minor differences that do not correlate with the observed rate constants (Table S2).

Taken together the present results provide rare[49] and compelling evidence that, in this set of Az mutants, the Marcus inverted region has been reached at driving forces greater than ~0.8 eV (Figure 3). However, the ET rates observed for mutants with the highest driving force lie considerably below the calculated curve. Marcus theory in its semi-classical form attributes the inverted region to an increasing activation energy in the exponential term of the rate equation, but nuclear tunneling may cause a decrease in the pre-exponential factor leading to the considerably lower rates observed.[50]

These mutants reach the "inverted region" in the internal ET process, since they possess a lower reorganization energy than most other Az mutants. When the reorganization energy is higher, as in the case of WT Az (cf. Figure 3), even greater driving force would be required to reach the "inverted region", a rather difficult task to fulfill with modifications of the copper environment. Moreover, the inverted region could be achieved in this system because both ET

products, Cu(I) and disulfide, have closed shells and their electronic excited states are too high to be populated after the long range ET. The inverted region in a bimolecular ET reaction with closed shell products has indeed been demonstrated before.[51] The present results provide the first demonstration of the Marcus inverted region in a non-derivatized protein-only system. It has been hypothesized that the dramatically different ET rates in the photosynthetic reaction center are due to the lower reorganization free energy and large activation barrier for the reverse processes that result from the inverted region.[27] The inverted region, therefore, allows for the charge separation required for unidirectional ET through that system and is critical for efficient energy conversion processes.

ASSOCIATED CONTENT

Supporting Information. Details of experimental procedures, including protein expression and purification, kinetic measurements, cyclic voltammetry analyses, and spectroscopic studies are provided in the Supplemental Information. 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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