JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Communication

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Understanding the Mechanism of Short-Range Electron Transfer Using an Immobilized Cupredoxin

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Supporting Information

ABSTRACT: The hydrophobic patch of azurin (AZ) from *Pseudomonas aeruginosa* is an important recognition surface for electron transfer (ET) reactions. The influence of changing the size of this region, by mutating the C-terminal copper-binding loop, on the ET reactivity of AZ adsorbed on gold electrodes modified with alkanethiol self-assembled monolayers (SAMs) has been studied. The distance-dependence of ET kinetics measured by cyclic voltammetry using SAMs of variable chain length, demonstrates that the activation barrier for short-range ET is dominated by the dynamics of molecular rearrangements accompanying ET at the AZ-SAM interface. These include internal electric field-dependent low-amplitude protein motions and the reorganization of interfacial water molecules, but not protein reorientation. Interfacial molecular dynamics also control the kinetics of short-range ET for electrostatically and covalently immobilized cytochrome *c*. This mechanism therefore may be utilized for short-distance ET irrespective of the type of metal center, the surface electrostatic potential, and the nature of the protein−SAM interaction.

Electron transfer (ET) reactions involving metal centers in proteins are of fundamental importance to life.[1−3] Immobilizing an electron shuttling redox metalloprotein such as azurin (AZ), on an electrode in such a way that ensures electrical contact, with the distance tuned by a molecular spacer, reproduces physiological interactions and is therefore an excellent experimental approach for understanding the mechanism of biological ET.[4−12] Azurin has a type 1 copper site with His_2Cys coordination in an approximately trigonal arrangement, plus two weak axial interactions.[13] Three of the ligands at the active site of AZ, including the exposed coordinating His, are situated on the C-terminal loop that makes a major contribution to a hydrophobic patch (Figure 1). Azurin interacts with physiological partners and with alkanethiol SAMs via this hydrophobic region.[6,9,12−20] The ET regime for immobilized AZ has been found to depend on the donor−acceptor distance.[6,18,21,22] Long-range ET follows semiclassical Marcus theory: the rate constant displays an exponential variation with distance, consistent with a non-adiabatic electron tunneling mechanism. However, such distance dependence is lost with short-range ET for which a dynamically controlled mechanism, resulting in an extra activation barrier from molecular reorganization at the SAM−protein interface, becomes rate limiting.[6,7,14,17,23−26] In this work, we identify the cause of this additional, and controversial, energy term.

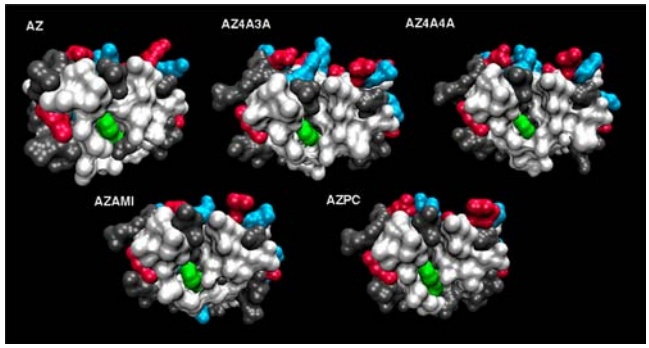

Figure 1. Molecular surface surrounding the solvent exposed copper binding His (green) in wild type and loop mutants of *Pseudomonas aeruginosa* AZ. This portion of the molecular surface, which includes the hydrophobic patch, faces the nonpolar SAM in the electrode-AZ assemblies. Nonpolar residues are colored white, while neutral (hydrophilic), negative, and positive residues (at pH 7) are gray, red, and blue, respectively. The sequences of the C-terminal ligand-containing loops are C^{112}TFPGH^{117}SALM^{121} (AZ), C^{112}AAAAH^{117}AAAM^{121} (AZ4A3A), C^{112}AAAAH^{117}AAAAM^{122} (AZ4A4A), C^{112}TPH^{115}PFM^{118} (AZAMI), and C^{112}SPH^{115}QGAGM^{120} (AZPC). Hydrophobic patch areas of 1603, 1572, 1734, 1424, and 1312 Å^2, respectively, have been calculated from the X-ray structures of AZ,[13] AZ4A3A,[27] AZ4A4A,[27] AZAMI,[28] and AZPC[29] using VMD.[30]

The ET reactions of immobilized AZ loop variants whose hydrophobic patch area is systematically altered (Figure 1)[27−29,31−34] have been studied. The distance-dependence of the kinetics and activation parameters have been obtained from cyclic voltammetry of the proteins adsorbed on gold electrodes functionalized with different alkanethiolates. We find that the rate of short-range ET is controlled by the dynamics of

Received: April 10, 2012
Published: July 12, 2012

dx.doi.org/10.1021/ja303425b | *J. Am. Chem. Soc.* 2012, 134, 11848−11851

molecular rearrangements accompanying ET at the recognition interface. These include internal electric field-dependent low-amplitude protein motions and the reorganization of interfacial water molecules. The ET event is not gated by protein reorientation on the surface.

Cyclic voltammograms of AZ and its loop variants adsorbed on alkanethiolates $[-S(CH_2)_nCH_3]$ with different chain lengths (1-pentanethiol, PT, n = 4; 1-heptanethiol, HPT, n = 6; 1-decanethiol, DT, n = 9; 1-tetradecanethiol, TDT, n = 13; and 1-hexadecanethiol, HDT, n = 15) consist of a single well-defined quasi-reversible signal due to the one-electron reduction/oxidation process of the type 1 copper center (Figure 2). The

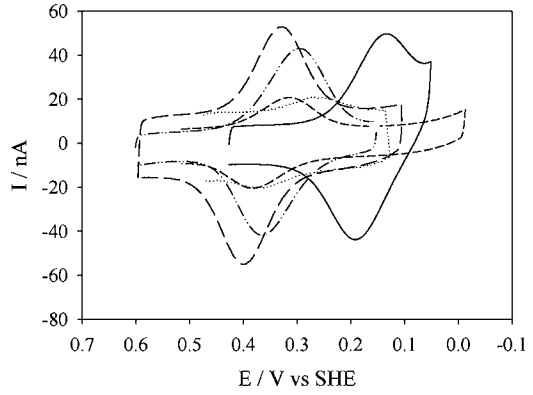

Figure 2. Cyclic voltammograms (293 K) of AZ (— ·· —), AZAMI (—), AZPC (- - - -), AZ4A3A (·····), and AZ4A4A (— — —), adsorbed on a gold electrode coated with a 1-hexadecanethiol SAM dipped in a working solution made up in 5 mM Tris buffer plus 10 mM sodium perchlorate at pH 7.5 (5 mM acetate buffer at pH 4.6, for AZ) at a scan rate of 0.05 V s^{-1}. The different current intensities for the various immobilized species are due to the different surface areas of the gold electrodes.

peak-to-peak separation varies over the scan rates investigated (at 20 °C) and is also influenced by temperature. Peak currents increase linearly with increasing scan rate, as expected for an adsorbed species, and are almost constant from 5 to 30 °C. Under these conditions, the responses are reproducible for all species and persist for several cycles. Current intensities decrease at higher temperatures, most likely due to protein desorption. The formal reduction potentials ($E^{\circ\prime}$ values) are independent of scan rate in the range studied and are hardly affected by the nature of the SAM (Table S1). The ET rate constants at zero driving force, k_s, were determined according to Laviron (Table S2).[35−38]

The influence of temperature on k_s was measured, and activation enthalpies ($\Delta H^{\#}$) were determined using the Arrhenius equation:

$$\mathrm{d}\ln k_s/\mathrm{d}(1/T) = -\Delta H^{\#}/R \quad (1)$$

namely from the slope of the plot of ln k_s versus $1/T$ (Table 1, Figure S1). The plots of ln k_s versus n (Figures 3 and S3) are biphasic for all loop variants, as observed previously for AZ.[6] For n > 9, k_s decreases exponentially with n, consistent with long-range ET for immobilized metalloproteins occurring via a nonadiabatic (tunneling) mechanism.[15,18,19,39] For n < 9, k_s is scarcely affected by n.[6,7,14,17,23−26] For such short-range ET, the kinetics of the process is strongly affected by the electric field of the charged electrode surface and a gating dynamic mechanism involving reorientation of the protein on the SAM surface prior

Table 1. Activation Enthalpy $\Delta H^{\#}$ (kJ mol^{-1}) for Heterogeneous Protein-Electrode ET of AZ Loop Mutants Immobilized on a Gold Electrode Coated with Alkane-1-thiolate SAMs $[-S(CH_2)_nCH_3]$ of Different Lengths[a]

protein	n = 4 (PT)	n = 6 (HPT)	n = 9 (DT)	n = 13 (TDT)	n = 15 (HDT)
AZAMI (pH 5.7)	17.6	15.8	9.1	8.1	8.1
AZAMI (pH 7.5)	17.4	15.3	9.0	7.8	7.7
AZPC (pH 5.1)	19.5	16.2	11.3	8.7	8.7
AZPC (pH 7.4)	19.4	16.1	11.0	8.3	8.3
AZ4A3A (pH 4.9)	15.4	13.2	8.0	7.7	7.7
AZ4A3A (pH 7.6)	15.7	13.3	8.3	8.1	8.0
AZ4A4A (pH 4.9)	13.9	11.6	7.9	7.5	7.5
AZ4A4A (pH 7.6)	13.9	11.5	7.9	7.4	7.4
AZ (pH 4.6)	14.4	13.1	9.0	7.6	7.6
AZ (pH 4.6)[b]	15.4	n.d.	n.d.	n.d.	7.6

[a]Experiments performed in 5 mM buffer plus 10 mM sodium perchlorate at 293 K. The error on $\Delta H^{\#}$ is ±0.2 kJ mol^{-1}. [b]At 298 K, from ref 6.

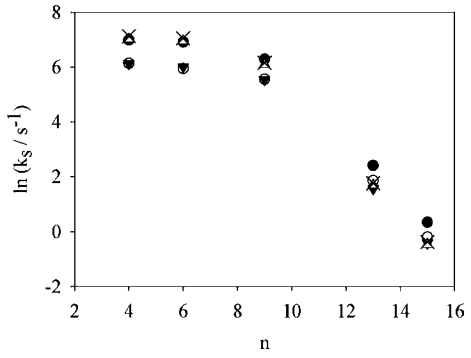

Figure 3. Plots of ln k_s against the number of methylene units in the SAM alkanethiolate chain (n) of electrode-immobilized AZ variants (293 K) at pH values at which the side chains of both His35 and His83 are in the imidazolium form (see Supporting Information). Data are shown for AZAMI at pH 5.7 (○), AZPC at pH 5.1 (▼), AZ4A3A at pH 4.9 (Δ), AZ4A4A at pH 4.9 (×), and AZ at pH 4.6 (●) in 5 mM acetate plus 10 mM sodium perchlorate. Error bars are smaller than the symbols. A very similar plot has been obtained from data at pH values at which the side chains of both His35 and His83 are in the imidazole form (Figure S3).

to ET has been suggested for both AZ and cytochrome c.[4,7,17,18] Alternatively, a 'frictional' mechanism, in which the relaxation component is thermally activated, has also been proposed.[6,22]

Altering the hydrophobicity of the surface of AZ that interacts with the SAM (Figure 1) will influence the strength of AZ−SAM binding (greater hydrophobicity enhancing the binding and rigidity of the protein adsorbed layer).[40] For *short-range* ET (n < 9; PT and HPT SAMs), the plot of $\Delta H^{\#}$ versus the area of the hydrophobic patch (A_{hp}) of AZ (Figure 4) *demonstrates that a weakening of this hydrophobic interaction results in an increase in* $\Delta H^{\#}$. Therefore, protein reorientation on the surface and high-amplitude protein motions to yield a configuration competent for efficient ET (gating) *cannot* be responsible for the activation barrier affecting short-range ET, as if this were the case a weaker AZ−SAM interaction would give a decrease in $\Delta H^{\#}$. A weaker hydrophobic interaction, enhancing the flexibility of the AZ−SAM construct, results in an increase in the local internal dynamic fluctuations of protein and water molecules, affected by the electric field at the AZ−SAM interface, which must be the main determinant of the

dx.doi.org/10.1021/ja303425b | J. Am. Chem. Soc. 2012, 134, 11848−11851

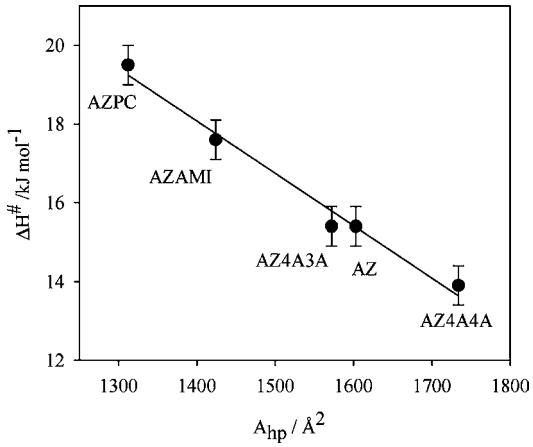

Figure 4. Activation enthalpy ($\Delta H^{\#}$) for short-range ET of AZ loop mutants immobilized on a gold electrode coated with 1-pentanethiol (n = 4) at pH values at which the side chains of both His35 and His83 are in the imidazolium form plotted against the area of the hydrophobic patch (A_{hp}) involved in the interaction with the SAM.

dominant dynamic contribution to $\Delta H^{\#}$. A decreased hydrophobic interaction will allow more water molecules to access the protein−SAM interface, which may also contribute to the increase in $\Delta H^{\#}$.

The $\Delta H^{\#}$ values for all the proteins studied here decrease with increasing ET distance up to n = 9 (Table 1), as expected from the decreasing influence of the electric field at the AZ−SAM interface on protein dynamics. The $\Delta H^{\#}$ values level off at larger n and are the same (for each n) at low and high pH (Table 1); i.e., they are independent of the protonation state of surface residues such as His35 and His83 (see Supporting Information). This confirms that $\Delta H^{\#}$ depends only on the protein−SAM interaction occurring at the hydrophobic patch.

Under the tunneling regime (long-range ET, n > 9) the reorganization energy (λ) and the tunneling factor (TF) can be determined (see Supporting Information) and are in good agreement with previously measured values.[1−3,6,9] The $\Delta H^{\#}$, λ, and TF values are almost unaffected by the area of the hydrophobic patch (Table S3), indicating that the processes occurring at the AZ−SAM interface do not appreciably influence the kinetics of long-range ET.

The $E^{\circ\prime}$ values and the changes in $E^{\circ\prime}$ upon immobilization (Table S1) are not dependent on A_{hp} for both short- and long-range ET. However, for short-range ET, the reduction entropy ($\Delta S^{\circ\prime}_{rc}$) increases linearly with increasing A_{hp} (see Supporting Information and Figure S4). $\Delta S^{\circ\prime}_{rc}$ is known to be primarily influenced by the reduction-induced reorganization of water molecules within the hydration sphere of the protein.[41−43] For the adsorbed AZ mutants under investigation, a key role is played by those molecules close to the hydrophobic patch, whose number and organization (clustering) are related to A_{hp}. These interfacial water molecules thus affect both the kinetics of ET and the thermodynamics of reduction.

In conclusion, we show that internal electric-field-dependent low-amplitude protein dynamics and the reorganization of interfacial water molecules control the rate of short-range ET for immobilized AZ. Such interfacial molecular dynamics most likely also constitute the rate-limiting step for short-range ET in other redox metalloproteins under a variety of conditions, irrespective of the type of metal center, the surface electrostatic potential, and the nature of the protein−SAM interaction.[22,23,44] This mechanism has physiological importance, particularly when a protein is associated with a charged membrane where it experiences high electrostatic forces.

■ ASSOCIATED CONTENT

Supporting Information

Protein purification, electrochemical measurements, the pH dependence of $E^{\circ\prime}$, and the determination of the kinetic parameters of ET. 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 interest.

■ ACKNOWLEDGMENTS

This work has been supported by the Fondazione Cassa di Risparmio di Modena (Prot. N. 1297.08.8C) [M.S.], the Commission of the European Communities (Contract No. MRTN-CT-2006-035649) [M.S. and C.D.], the MIUR (PRIN 2009 prot. N. 20098Z4M5E_002) [M.B.], and the Biotechnology and Biological Sciences Research Council Grant BB/C504519/1 (C.D.). The University of Modena and Reggio Emilia is acknowledged for a fellowship to S.M.

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