Aromatic Residues May Enhance Intramolecular Electron Transfer in Azurin

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Electron transfer (ET) plays an important role in many biological systems, and a central question is whether specific amino acid residues may promote ET.[1] The semiclassical Marcus theory for nonadiabatic processes predicts that intramolecular ET in proteins is governed by the standard free energy of reaction ($\Delta G°$), the nuclear reorganization energy (λ), and the electronic coupling (H_{DA}) between electron donor (D) and acceptor (A) at the transition state:[2]

$$k = \frac{2\pi}{\hbar}\frac{H_{DA}^2}{(4\pi\lambda RT)^{1/2}}e^{-(\Delta G°+\lambda)^2/4\lambda RT} \quad (1)$$

The electronic coupling energy, H_{DA}, is expected to decay exponentially with the distance between D and A as

$$H_{DA} = H_{DA}^{\circ}\, e^{-\beta/2(r - r_0)} \quad (2)$$

For protein ET the distance between D and A may be considerable ($\geq$1.0 nm), leading to a very small electronic coupling. Still, intramolecular ET over distances of 2.0 nm or more has been observed.[3]

The blue single-copper protein azurin is engaged in biological ET and serves as an ideal system for examination of intramolecular long range ET (LRET) in proteins.[4] It consists almost exclusively of a rigid β-sheet polypeptide, and three-dimensional structures have been determined for a large number of wild-type (WT) and single site mutated azurins.[5] Furthermore, no attachment of external redox group is needed, since it contains two potential redox centers, the copper ion coordinated directly to amino acid residues and a disulfide bridge (RSSR) in the opposite end of the molecule. We have previously demonstrated that intramolecular LRET between the two centers can be induced by pulse radiolysis.[4]

Using both WT and single site mutated azurins, we have studied the effect of specific amino acid substitutions on the rate of intramolecular ET. In order to understand better the effect of the polypeptide matrix between D and A, we have used the structure-dependent pathway model developed by Beratan and Onuchic for identifying the relevant ET routes.[6] In this model, the total coupling of a pathway is given as a repeated product of the couplings of the individual links. The optimum pathway between the two redox sites, $\Pi\epsilon$, is then identified.

Pathway calculations for the above intramolecular ET were performed using the high-resolution three-dimensional structures of *Pseudomonas aeruginosa* azurin and of its mutants, when available.[5] For other mutants, structures based on 2D NMR studies and energy minimization calculations were employed. The calculations predict two major electron transfer routes in all of the azurins[4] listed in Table 1: one longer path through the peptide chain to the copper-ligating imidazole of His46 and one shorter path through the buried residue 48 (usually a tryptophan), necessitating a through-space jump from Val31 to this side chain, and further to the copper ligand, Cys112. The electronic coupling factors were found to be $\Pi\epsilon = 2.5 \times 10^{-7}$ and 3.0×10^{-8}, respectively.[4] However, in this analysis the electronic interaction between the Cu(II) ion and its ligands was not included. It has been demonstrated that the high degree of anisotropic covalency in the blue single-copper protein, plastocyanin, would enhance ET through the Cys ligand.[7] By similar arguments, from the ligand coefficients of Ψ_{HOMO} in azurin obtained by Larsson *et al.*,[8] it can be estimated that ET through Cys would be enhanced by a factor of ~150 over ET via one of the His ligands. This means that the two pathways would be about equally important.

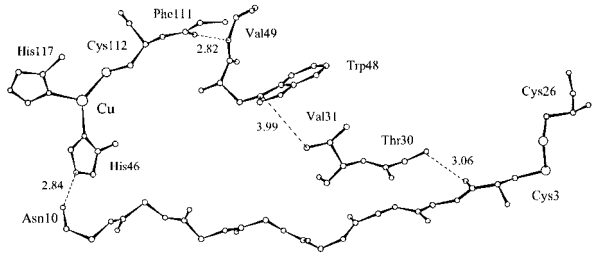

Figure 1. Calculated pathways for ET from the sulfur of Cys3 to the copper center in WT *P. aeruginosa* azurin. Some interconnecting distances (three H-bonds and one van der Waals contact) are given (Å). In the V31W mutant, the closest distance between the two tryptophans (3.5 Å) occurs between W48 $C^{\zeta 3}$ and W31 $C^{\epsilon 3}$. The coordinates were obtained from ref 5b.

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Table 1. Kinetic and Thermodynamic Data for Intramolecular Reduction of Cu(II) by $RSSR^-$ at 298 K and pH 7.0

azurin	k_{298} (s^{-1})	$E^{o\prime}$ (mV)	$-\Delta G^{\circ}$ [a] (kJ mol^{-1})	$\Delta H^{\ddagger}$ (kJ mol^{-1})	$\Delta S^{\ddagger}$ (J K^{-1} mol^{-1})	H_{DA} [b] (10^7eV)
P. aeruginosa[c]	44(7)	304	68.9	47.5(22)	−56.5(35)	0.8
W48A	35(4)	301	68.6	46.3(59)	−58.3(60)	0.7
W48F	80(5)	304	68.9	43.7(67)	−61.9(97)	1.0
W48L[d]	40(4)	323	70.7	48.3(19)	−51.5(57)	0.7
W48M[d]	33(5)	312	69.7	48.4(13)	−50.9(74)	0.7
W48S	50(5)	314	69.9	49.8(49)	−44.0(35)	0.8
W48Y	85(5)	323	70.7	52.6(69)	−30.2(36)	1.0
V31W	285(18)	301	68.6	47.2(24)	−39.7(25)	2.1

[a] Calculated from the measured Cu(II)/Cu(I) electrode potentials and assuming $E^{o\prime} = -410$ mV for the RSSR/$RSSR^-$ couple in all of the azurins studied here. [b] 1 eV = 96.49 kJ mol^{-1}. [c] Reference 4a. [d] Reference 4d.

In order to probe the possible influence of aromatic residues on ET, we have now produced single site mutated azurins in which Trp48 has been substituted by other amino acids, both aromatic and nonaromatic residues, and determined the rate constants for intramolecular ET as a function of temperature. The results are shown in Table 1 together with the standard free energies of reaction (ΔG°), the activation enthalpy ($\Delta H^{\ddagger}$), and activation entropy ($\Delta S^{\ddagger}$). Also shown is the electronic coupling energy, $H_{DA,}$ calculated on the basis of the Marcus equation (eq 1) assuming that the reorganization energy for the mutant azurins has the same value as earlier determined for the wild-type protein (99 kJ mol^{-1}).[4e]

Table 1 demonstrates that substitution of Trp48 with other amino acids only has a small effect on the kinetic parameters after correcting for changes in driving force. However, another mutant was constructed in which Val31 was substituted with Trp, thus producing a "double-Trp" mutant (V31W azurin) in which the two indole rings are placed in neighboring positions (cf., Figure 1).

To study the spatial relationship between the two indole rings, 2D NOESY and TOCSY experiments were carried out on V31W. Two spin systems consisting of four peaks (tryptophans) could immediately be identified from the TOCSY spectra which were assigned to residues 31 and 48. A large number of residues exhibited chemical shift values identical to those of the corresponding residue in the WT protein.[9] The chemical shifts of the four protons of the Trp48 side chain are within 0.1 ppm the same as in the WT protein indicating similar orientation.

Both tryptophans have NOEs between their side chains and methyl groups of an isoleucine and a valine, probably Ile7 and Val95. These NOEs put further constraints on the orientation of the Trp31 side chain. The two ring systems are not stacked in a parallel fashion, but the indole rings of the two tryptophans form an oblique angle relative to each other.

Thus, on the basis of the NMR data, we conclude that the regions in the mutant protein located behind Trp 48 (relative to Trp 31) have the same structure as the equivalent regions in the wild-type. Energy minimization calculations on this mutant have also been performed and show a close (van der Waals contact) of the two indole rings consistent with the observation of NOEs between the ring protons.

LRET in the V31W azurin mutant takes place with a rate constant of 285 s^{-1} at 298 K and pH 7.0 which is considerably faster than for any other azurin studied so far.[4] This strongly suggests, that the main ET route is the "Trp48" pathway, since the other pathway through His46 would not be affected by this mutation. In order to understand better the parameters that control the rate of ET, the factors which determine the activation enthalpy and entropy have been examined. The activation enthalpy is given by the following relation:[2]

$$\Delta H^{\ddagger} = \frac{\lambda}{4} + \frac{\Delta H^{\circ}}{2}\left(1 + \frac{\Delta G^{\circ}}{\lambda}\right) - \frac{(\Delta G^{\circ})^2}{4\lambda} \qquad (3)$$

In this study of azurins, $\Delta H^{\ddagger}$ is constant within experimental error, which supports our previous assumption that the reorganization energies do not change significantly in this series.

The entropy of activation includes a contribution from the distance dependence of the electronic coupling[2] (cf. eq 2)

$$\Delta S^{\ddagger} = \Delta S^{*} - R\beta(r - r_0) \qquad (4)$$

and ΔS^{*} is related to the standard entropy of reaction, ΔS°:

$$\Delta S^{*} = \frac{\Delta S^{\circ}}{2}\left(1 + \frac{\Delta G^{\circ}}{\lambda}\right) \qquad (5)$$

The increase in rate in V31W azurin originates in a more advantageous entropy of activation (Table 1) which is larger by 16.8 J K^{-1} mol^{-1} compared with WT azurin. Since ΔS° can safely be assumed to be the same for intramolecular ET in WT and V31W azurin, the increase in entropy would, according to eq 4, correspond to a *decrease* in $\beta(r - r_0)$ from the previously determined value of 24.6 in WT to 22.6 in V31W azurin. A smaller electronic decay factor, β, in the mutant is also reflected in the electronic coupling energy, H_{DA}, between electron donor and acceptor which was found to be 2.1×10^{-7} eV. This is an improvement of a factor 2.6 relative to WT azurin ($H_{DA} = 0.8 \times 10^{-7}$ eV). In contrast, a calculation of the electronic coupling factor which treats all covalent bonds equally gave $\Pi\epsilon = 0.9 \times 10^{-8}$ for V31W azurin compared with 3.0×10^{-8} for WT azurin (*vide supra*).

We suggest that the relative positions of Trp31 and Trp48 may enhance the interaction between D and A since the ring systems are in van der Waals contact which will provide a large electronic overlap and give rise to a resonance-type tunneling through the indole rings. Aromatic residues placed in appropriate positions may enhance ET through proteins by a more effective coupling through their extended π^*-orbitals since the energy gap between that of the tunneling electron and the aromatic π-system is significantly smaller than between the electron tunneling energy and σ-orbitals. A single aromatic residue placed midway between D and A in a predominantly σ-ET pathway is not advantageous by itself however, since $\sigma \rightarrow \pi \rightarrow \sigma$ ET will be energetically unfavorable. However, several aromatic residues placed in successive positions or aromatic molecules in direct contact with either D or A would act as an extended relay which could enhance the electronic coupling.

In systems which have been selected by evolution for efficient electron transfer, aromatic residues have been found in positions that probably enhance the electronic coupling. Examples are the tryptophan-mediated reduction of quinone in the photosynthetic reaction center,[10] in the MADH/amicyanin system where a Trp residue is placed at the interface between the two proteins,[11] and in the [cytochrome *c* peroxidase/cytochrome *c*] complex where a Trp seems to have a similar function.[12]

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