Long Range Intramolecular Electron Transfer in Azurins

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Abstract: The disulfide bridge linking cysteines-3 and -26 in the blue single copper protein azurin isolated from *Alcaligenes faecalis* (*Alc. faec.*) and *Pseudomonas fluorescens B* (*Ps. fluor.*) reacts with pulse radiolytically produced CO_2^- radical anions to yield the disulfide radical ion, $RSSR^-$. This radical then decays by an intramolecular electron transfer (et) to the Cu(II) center which is at a distance of ~2.6 nm. At 25 °C and pH 7.0, the rate constants of the intramolecular electron transfer reactions are 11 ± 2 and 22 ± 3 s^{-1} in *Alc. faec.* and *Ps. fluor.* azurin, respectively. This intramolecular reaction rate was studied over the 2.0–46.5 °C temperature range. The activation enthalpies derived from the temperature dependence are $\Delta H^* = 54.5 \pm 1.4$ and $\Delta H^* = 36.3 \pm 1.2$ kJ mol^{-1}, and the entropies of activation are $\Delta S^* = -43.9 \pm 9.5$ and $\Delta S^* = -97.7 \pm 5.0$ J K^{-1} mol^{-1} for *Alc. faec.* and *Ps. fluor.* azurin, respectively. Under similar conditions, the specific rate of the intramolecular et between the homologous $RSSR^-$ and the Cu(II) site in *Pseudomonas aeruginosa* (*Ps. aer.*) azurin was found to be 2–4 times higher than in the above two azurins. This difference cannot be rationalized satisfactorily by the differences in either driving force or distance between the electron donor and acceptor in these proteins. Instead the differences in activation parameters and rates may be rationalized in terms of an increased reorganization energy in *Ps. fluor.* and *Alc. faec.* as compared with *Ps. aer.* azurin. The other main pertinent difference among these azurins is that residue Trp 48 present in the *Ps. aer.* protein midway between the electron donor and acceptor is substituted in the *Alc. faec.* and *Ps. fluor.* proteins by a valine and a leucine, respectively. This suggests that the aromatic indole ring system may play a role either in enhancing the mixing of the electron donor and acceptor wave functions or by affecting the reorganization energy of the reaction.

Introduction[1]

Long range electron transfer reactions in proteins provide a major element in biological energy conversion systems.[2] It is by now generally accepted that in addition to the standard free energy difference between the reactants, $\Delta G°$, and the reorganization energy, λ, other factors which determine the rates of these reactions are the distance separating the electron donor–acceptor couple and the nature of the intervening medium.[3] One way of investigating the influence of the microenvironment through which electron migration can take place between the redox sites is by comparing systems where a fixed-distance is maintained between similar donor and acceptor pairs, yet the intervening medium is varied. The blue single copper protein, azurin, is present in various bacteria.[4] Several azurins with well-defined structural and physico-chemical differences are available from these natural sources, hence providing a system for examining the parameters which control the rates of the LRET described below.[4,5]

In all azurins sequenced to date, a disulfide bridge is found at one end of the β-barrel structured protein, at a distance of ~2.6 nm from the copper site.[5] Using sufficiently strong reductants (e.g., CO_2^- radicals produced by pulse radiolysis) this disulfide can be transformed into a radical anion[6] which, in turn, decays by transferring the electron intramolecularly to the copper center.[7] We have earlier observed and studied this intramolecular electron transfer in *Ps. aer.* and *Alcaligenes spp.* (*Alc. spp.*) azurins, both of which have a single tryptophan (residue 48) midway between the electron donor and acceptor. In the former azurin, the specific rate of the intramolecular et at 25 °C was found to be 44 ± 7 s^{-1}, while in *Alc. spp.* azurin it is 8.5 ± 1.5 s^{-1}.[7] This difference in specific rates correlates well with the difference in redox potentials of the copper centers in the two proteins.

We have now shown that similar LRET takes place in azurins isolated from *Alc. faec.* and *Ps. fluor.* where the Trp-48 is substituted by a valine and a leucine residue, respectively.[4] In order to investigate the influence of the reorganization energy and of the microenvironment separating the electron donor and acceptor, we have studied the temperature dependence of the rates of intramolecular electron transfer processes in these proteins.

† Abbreviations: *Alc. faec.*, *Alcaligenes faecalis*; *Ps. aer.*, *Pseudomonas aeruginosa*; *Alc. spp.*, *Alcaligenes spp.*; *Ps. fluor.*, *Pseudomonas fluorescens* biotype B; LRET, long range electron transfer; et, electron transfer; Az[Cu(II)], oxidized azurin.

Materials and Methods

Purification of Azurins. The *Ps. fluor.* type B was a kind gift of Dr. R. P. Ambler, while the *Ps. aer.* and *Alc. faec.* were obtained from the American Type Culture Collection, Bethesda, MD, U.S.A. Azurin was isolated from *Alc. faec.* using the following modified procedure of Ambler and Wynn for *Ps. aer.* azurin.[8] Bacteria (1.5 kg) were blended with 4 L of cold potassium phosphate buffer (0.02 M, pH 6.5). The suspension was then sonicated for 5 min in 300-mL portions. After addition of 25 mg of DNAse, the lysate was centrifuged for 1 h at 50000 g, and the supernatant was dialyzed overnight against cold 0.05 M CH_3COONH_4, pH 4.0. After centrifugation at 20000 g for 30 min, the clear supernatant was loaded on a Whatman CM-52 column equilibrated with the same buffer. From this point onwards the Ambler–Wynn procedure was followed, with the exception that the *Alc. faec.* azurin was eluted at a higher pH (4.9) than the *Ps.* protein. At the latter step, the protein was applied on a Whatman DEAE-52 column and was eluted with 0.075 M Tris buffer, pH 8.7. The isolated azurin had at this stage an absorption ratio (A_{625}/A_{280}) of 0.50, and no further purification was deemed necessary. The molar extinction coefficient of the oxidized *Alc. faec.* protein, $\epsilon_{625} = 4000$ M^{-1} cm^{-1}[9] and the midpoint

(1) Abbreviations, see p 1.

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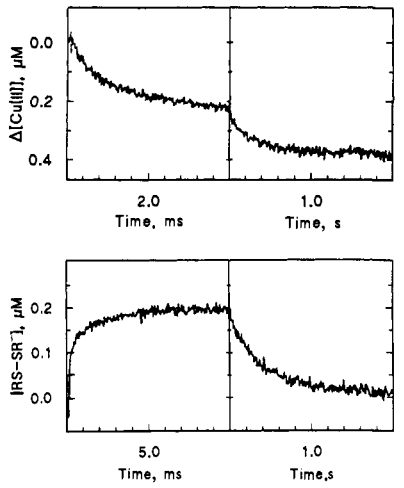

Figure 1. Time resolved absorption changes of 9.5 μM *Alc. faecalis* azurin following pulse radiolysis in N_2O saturated 0.1 *M* sodium formate solution, pH 7.0, pulse width, 0.3 μs, T = 11.8 °C: a. monitored at 625 nm and b at 410 nm.

potential at pH 7.0 is 266 mV.[10]

Ps. fluor. B azurin was isolated following the procedure of Ambler–Wynn for *Ps. aer.* azurin.[8] The $A_{625/280}$ ratio of the final product was 0.27 and ϵ_{625} = 5700 M^{-1} cm^{-1}.[11] The midpoint potential at 25 °C, pH 7.0, 0.1 M formate, 0.01 M phosphate was determined by titrating Az[Cu(II)] (0.06 mM) with ascorbate in the presence of $Fe(CN)_6^{3-/4-}$ at two different concentrations (0.5 and 1.0 mM). Extrapolating to zero mediator concentration yields E' = 347 mV.

Kinetic Measurements. Pulse radiolysis experiments were carried out using a Varian V-7715 linear accelerator of the Hebrew University in Jerusalem. Five megaelectronvolts accelerated electrons were employed using pulse lengths in the range from 0.1 to 1.5 μs equivalent to 0.6–10.0 μM of CO_2^- radical ions. All measurements were carried out anaerobically, under purified argon at a pressure slightly in excess of 1 atm in a 4 × 2 × 1 cm spectrosil cuvette, using three light passes which result in an overall optical pathlength of 12.3 cm. A 150-W xenon lamp produced the analyzing light beam, and appropriate optical filters with cut-off at 385 nm or 590 nm were used to avoid photochemistry and light scattering. The data acquisition system consisted of a Tektronix 390 A/D transient recorder and a Mini PDP1123 computer. The temperature of the reaction solutions was controlled by a thermostating system and was continuously monitored by a thermocouple attached to the cuvette. Most reactions were performed under pseudo-first-order conditions, with typically a 10-fold excess of oxidized protein over reductant. Each kinetic run was repeated at least three times.

Sodium formate (0.1 M) aqueous solutions (pH 4.0) were deaerated and saturated with N_2O by extensive bubbling with the gas for ~30 min in glass syringes. The protein was then added, and the pH was adjusted to the required value by titration with 0.5 or 0.05 M NaOH. N_2O bubbling was continued for 5 min further, and the solution was then transferred into the pulse radiolysis cell under anaerobic conditions.

Results

In a solution of 0.1 M sodium formate saturated with N_2O (0.02 M), the radiation pulse leads to the production of CO_2^- radicals.[12] In the *Alc. faec.* Az[Cu(II)] solutions, two fast processes were observed at 625 (Figure 1a) and at 410 nm, respectively (Figure 1b). The Cu(II) site has its absorption maximum at 625 nm, (ϵ_{625} = 4000 M^{-1} cm^{-1}[9]), while it has practically no absorption at 410 nm, where the disulfide radical anion has an intense band (ϵ_{410} ~ 10000 M^{-1} cm^{-1}[6]). The fast reduction steps were found to be first order in both protein and CO_2^- radical concentrations. Therefore, these processes are most probably direct bimolecular reductions of the two different redox-active sites in azurin by the CO_2^- radicals: (1) the Cu(II) center and (2) the single S–S bond connecting Cys-3 with Cys-26. The rate constant for the fast direct reduction of Cu(II) (cf. Figure 1a) at pH 7.0 and 298 K is (1.5 ± 0.2) × 10^8 M^{-1} s^{-1}. The specific rate of the RS–SR^- radical formation is (1.8 ± 0.3) × 10^8 M^{-1} s^{-1}. The combined yield of reduced protein is rather low, probably due to the competing dimerization of CO_2^- radicals to produce oxalate (e.g., a 0.4 μs pulse producing 2.6 μM CO_2^- only led to a total of 0.4 μM reduced protein (15% yield), as monitored by the final reduction yield of Cu(II)).

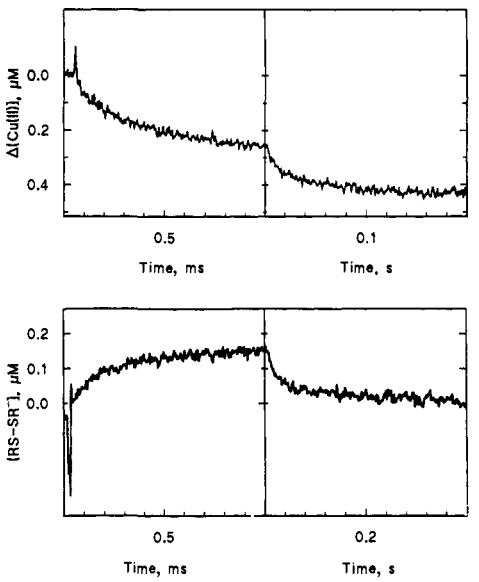

Figure 2. Time resolved absorption changes of 10.0 μM *Ps. fluorescens* azurin following pulse radiolysis in N_2O saturated 0.1 M sodium formate solution, pH 7.0, pulse width, 0.3 μs, T = 12.5 °C: a. monitored at 625 nm and b. monitored at 410 nm.

In solutions of *Ps. fluor.* azurin, the same reaction pattern was observed, only the specific rates differed (cf. Figure 2). Thus, under the same experimental conditions as above, the rate constant of the direct reduction of Cu(II) is (2.1 ± 0.3) × 10^8 M^{-1} s^{-1} and that of the disulfide radical formation is (3.5 ± 0.4) × 10^8 M^{-1} s^{-1}.

Following these two fast bimolecular processes in the *Alc. faec.* protein, a slower further reduction of Cu(II) to Cu(I) was observed at 625 nm (Figure 1a). This occurred concomitantly with the decay of the RS–SR^- radical as monitored at 410 nm (Figure 1b). The rates of both these processes were found to be identical: At 298 K and pH 7.0 the specific rate is 11 ± 2 s^{-1}, independent of protein and disulfide radical-ion concentrations (Figure 3). Furthermore, the reduction yield of the blue copper site in this slow phase was, within experimental error, the same as that of the RS–SR^- produced in the fast step. Taken together, all these observations clearly suggest that the slow step is an intramolecular electron transfer from RS–SR^- to Cu(II). A similar slow intramolecular electron transfer from the disulfide radical to the Cu(II) center was also observed in the *Ps. fluor.* protein (cf. Figure 2). The specific rate of this process was independent of the protein and disulfide radical concentrations (Figure 3) throughout the whole temperature range examined. At 298 K, pH 7.0 the rate constant is 22 ± 3 s^{-1}.

The temperature dependence of the intramolecular electron transfer rate constants in both azurins was measured independently at both 625 nm (Cu(II) reduction) and at 410 nm (RS–SR^- decay) over a temperature range from 2.0 to 46.5 °C. The Eyring plots derived from these experimental data are shown in Figure 4. Enthalpies of activation calculated from the slopes were as follows: ΔH^* = 54.5 ± 1.4 (*Alc. faec.*) and ΔH^* = 36.3 ± 1.2 kJ mol^{-1} (*Ps. fluor.*). The entropies of activation (which include the en-

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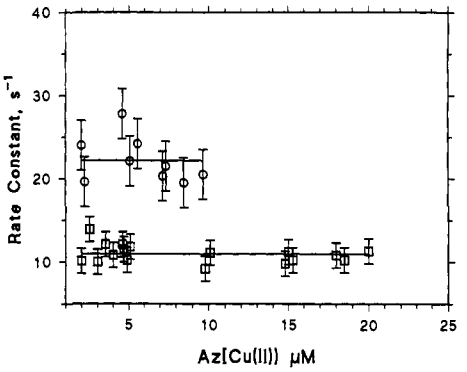

Figure 3. Concentration dependence of the first order specific rate determined for the slow reduction step of Cu(II) and the slow oxidation step of $RSSR^-$ in *Alc. faec.* azurin (□) (T = 25.0 °C) and *Ps. fluor.* azurin (○) (T = 24.2 °C).

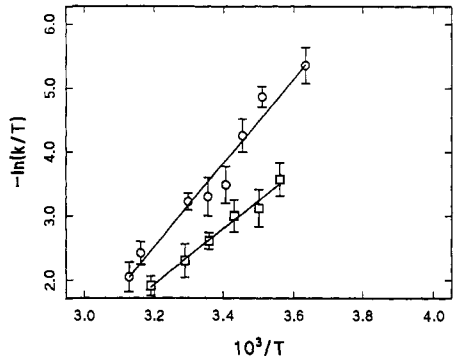

Figure 4. Eyring plots of the slow intramolecular electron transfer from $RSSR^-$ to Cu(II) in *Alc. faec.* (○) and *Ps. fluor.* (□) azurin.

Table I. Kinetic[a] and Thermodynamic Parameters for the Intramolecular Reduction of Cu(II) by $RSSR^-$ in Azurins

azurin	k, s^{-1}	ΔH^*, kJ mol^{-1}	ΔS^*, J K^{-1} mol^{-1}	$\Delta G°$, kJ mol^{-1} [a]
Ps. aer.	44 (7)	47.5 (40)	−56.5 (70)	−68.9
Ps. fluor.	22 (3)	36.3 (12)	−97.7 (50)	−73.0
Alc. faec.	11 (2)	54.5 (14)	−43.9 (95)	−65.2

[a] T = 298 K, pH = 7.0.

tropic contribution from the transmission coefficient) were determined from the intercepts: ΔS^* = −43.9 ± 9.5 (*Alc. faec.*) and −97.7 ± 5.0 J K^{-1} mol^{-1} (*Ps. fluor.*). These experimental results are summarized in Table I.

Discussion

At pH 7.0 and 298 K the specific rate of the intramolecular electron transfer from the disulfide radical anion to the blue Cu(II) center in *Alc. faec.* azurin is 11 ± 2 s^{-1}, while for *Ps. fluor.* azurin it is 22 ± 3 s^{-1}. For *Ps. aer.* azurin, under the same experimental conditions the corresponding intramolecular rate constant was found to be 44 ± 7 s^{-1}.[7] The homology in amino acid sequence among the three copper proteins is high (~70%) with mostly conservative substitutions.[4] In particular conserved are residues constituting or surrounding the copper redox center. The conspicuous difference between the *Ps. aer.* azurin, on the one hand, and those of *Ps. fluor.* and *Alc. faec.* azurins, on the other hand, is that the latter two proteins do not contain a tryptophan at position 48.[4]

The redox potential of the copper center in *Ps. aer.* azurin is 304 mV.[11] For *Ps. fluor.* we have determined the midpoint potential to be 347 mV (this work), while that of *Alc. faec.* azurin is 266 mV,[10] all under the above similar experimental conditions. For the disulfide group we use a single electron reduction potential of −410 mV.[6] For spatially fixed and oriented reactants, the Marcus theory[3] provides the following relationship among the free energy of the reaction, $\Delta G°$, the free energy of activation, ΔG^*, and the reorganization energy, λ:

$$\Delta G^* = \lambda/4[1 + \Delta G°/\lambda]^2$$

Earlier, we estimated the reorganization energy accompanying the intramolecular electron transfer from $RS\text{–}SR^-$ to Cu(II) in *Ps. aer.* azurin to be λ = 117 kJ mol^{-1}.[7] The copper ligands are the same in all three azurins. Also, most of the amino acid residues surrounding the disulfide center in *Ps. aer.* azurin (Asp23, Lys24, Lys27, Asp98, Val99) are conserved in all azurins sequenced so far.[4] The only difference in amino acid sequence around the disulfide center is the substitution of Ser25 in *Ps. aer.* azurin by Ala25 in *Ps. fluor.* and Thr25 in *Alc. faec.*, respectively. If we now assume that the reorganization energies of the solvent molecules and of the surrounding spheres of both the electron donor and acceptor are the same in the different azurins and that no significant structural changes occur because of the sequence differences, we can calculate, using the above expression, the expected differences in activation free energy due to the different driving forces ($-\Delta G°$) alone. These calculations yield an expected ~30% decrease in the reaction rate constant for *Alc. faec.* azurin compared with that of *Ps. aer.* azurin. However, the experimentally observed rate constant is 4-fold lower. The reaction rate constant of the *Ps. fluor.* protein is found to be 2-fold lower than that of *Ps. aer.* azurin, while calculations based on the difference in driving force predict that the rate constant should *increase* by 60%. Thus, clearly the differences in driving force cannot account for the marked differences between the calculated and the observed specific rates. The decrease in reaction rate constants can be explained in terms of different reorganization energies among the azurins if these energies would be larger (132 kJ mol^{-1}) for *Alc. faec.* and *Ps. fluor.* azurins as compared with λ = 117 kJ mol^{-1} for the *Ps. aer.* protein.[7] However, changes in reorganization energies alone are not sufficient for explaining the decrease in specific rates, since the experimentally determined activation enthalpies of the two azurins studied here differ by 18 kJ mol^{-1}, and ΔH^* for *Ps. fluor.* azurin is *smaller* than for *Ps. aer.* azurin. From the approximation[3a]

$$\Delta H^* \sim \frac{\lambda}{4} + \frac{\Delta H°}{2}$$

where λ is the overall reorganization energy and $\Delta H°$ is the standard enthalpy change of the reaction, we would expect a *smaller* reorganization energy for *Ps. fluor.* rather than a larger one. The observed difference in activation enthalpies between *Ps. fluor.* and *Alc. faec.* azurins is also noteworthy. It could be a reflection of the different et pathways since the reorganization energy, λ, contains contributions not only from structural changes in the redox centers but also in the microenvironment separating electron donor and acceptor. Significant variation in the intramolecular et pathways among the three examined azurins are also implied by the observed differences in activation entropies, since the latter also depends on the structure of the relevant pathway.[3a]

Two of the four main parameters which determine the rate of the intramolecular electron transfer (driving force; reorganization energy) have now been considered. The third parameter, the distance between the two redox centers cannot differ significantly in these rather homologous azurins. (In *Ps. aer.* azurin the through-space distance from S_γ to the copper center is 2.6 nm.[5]) The last parameter to be considered is the nature of the microenvironment separating the electron donor and acceptor. The two latter parameters obviously depend on the employed pathway. We have used the method of Beratan and Onuchic[13] to calculate the electronic coupling decay and identify the amino acids that may provide the pathway with the strongest coupling. The calculations were performed on *Ps. aer.* azurin for which the three-dimensional structure has been determined.[5] The predicted pathway emerging from these calculations is shown in Figure 5. It consists of 23 covalent bonds, 2 hydrogen bonds, and 1 through-space (van der Waals contact) jump (0.35 nm) and the overall distance becomes 3.8 nm. Comparison of the amino acid sequences of *Ps. fluor.*

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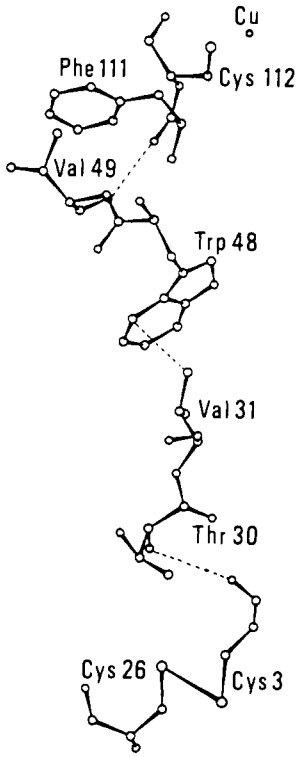

Figure 5. Calculated electron transfer pathway from $RSSR^-$ to Cu(II) in *Ps. aeruginosa* azurin. This tentative model consists of 23 covalent bonds, 2 hydrogen bonds (Cys3-Thr30, Val49-Phe111), and 1 through-space (van der Waals contact) jump (Val31-Trp48). The calculation was based on Beratan and Onuchic's procedure.[12]

and *Alc. faec.* azurins with that of *Ps. aer.* shows that except for one conservative substitution (of Val 31 by an Ile in *Alc. faec.* azurin) there is only one important difference in the calculated pathway: Trp48 of *Ps. aer.* azurin is substituted by a Val and Leu in *Alc. faec.* and *Ps. fluor.* azurins, respectively. Neither the copper ligands nor the residues around the disulfide center differ significantly among the three azurins.[4]

The indole ring of Trp-48 in *Ps. aer.* azurin is part of the calculated LRET pathway leading from Cys-3/26 to the blue copper center. In the *Alc. faec.* and *Ps. fluor.* azurins this tryptophan residue is substituted by the above aliphatic residues. Broo and Larsson[14] have recently performed quantum mechanical calculations of the electronic coupling between the two redox centers in *Ps. aer.* azurin for two potential pathways. In one model they have used an "aromatic pathway" including Trp48, while in the other calculation, a pathway along a peptide backbone only, without side chains, was examined. The calculations yielded the largest electronic coupling factor for the "aromatic pathway", and using the experimentally determined activation parameters[7] they obtained a rate constant of 16 s^{-1} in reasonable agreement with our experimental k = 44 s^{-1}.[7] Removing Trp48 caused the electronic coupling to decrease only by a factor of 2.5. This is also in agreement with our experimental observation since the intramolecular rate constants of the two azurins which lack Trp48 (*Ps. fluor.* and *Alc. faec.*) are 2–4 times slower than for *Ps. aer.* azurin. On the basis of their calculations, Broo and Larsson suggest that the indole side chain of Trp48 has only a bridging role between separate parts of the polypeptide chain without involvement of its particular electronic structure.[14]

Thus, the observed increase in rate suggests that if an aromatic group participates in an electron transfer pathway[15] it would lead to a relatively small effect. Still, it has been suggested[16] that the intracomplex electron transfer rate from cytochrome *c* to zinc cytochrome *c* peroxidase is enhanced by aromatic residues at position 82, while its substitution by aliphatic residues decreases the rate constant by a factor of 10^4. This was proposed to be a result of an aromatic superexchange pathway. Recent studies, however, have shown that superexchange through position 82 does not significantly enhance the reaction rate.[17] Instead, the difference in rates is now proposed to reflect conformational changes accompanying electron transfer. It should be noted, however, that the fastest intracomplex electron transfer is still observed with aromatic residues in position 82.[17] The increase in rate constant is 2–3-fold, i.e., similar to our present observations. Certainly, more experimental work is needed before the role of aromatic residues in electron transfer is settled.

It is also noteworthy that in *Ps. aer.* azurin, Trp48 is midway between electron donor and acceptor. Since a wave function decreases with the distance, r, as $\exp[-\beta r]$,[3] the electronic coupling is very sensitive to the increasing distance between separate amino acid side chains. Thus, if an aromatic residue is not directly bonded to the electron donor or acceptor, it has to be in close proximity to other components or bridging parts of the peptide chain in the same way as for example hydrogen bonds do. This could be the case in systems that were evolutionarily optimized for efficient et, where aromatic groups may be well positioned so as to considerably increase the electronic coupling.

In order to further refine the present structure–reactivity analysis, we are currently studying electron transfer in single-site mutated *P. aer.* azurins (e.g., where Trp48 has been substituted with nonaromatic residues). In these proteins the structural differences are much more restricted, and hence a more concrete comparison among the LRET rates would become possible.

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Registry No. Trp, 73-22-3.

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