Supporting Information

Fluorescence Correlation Spectroscopy of Labeled Azurin Reveals Photoinduced Electron Transfer between Label and Cu Center

**Chemicals and proteins**

Unless stated otherwise, chemicals were purchased from Sigma-Aldrich (Sigma-Aldrich Corp., St. Louis, USA) and used as received. Wild type (wt) Cu-azurin (CuAz) and Zn-azurin (ZnAz) from *Pseudomonas aeruginosa* were expressed and purified as previously described.\(^4\) For the labeling of the azurin (Az) with the dye ATTO655 (ATTO655 NHS ester, ATTO-TEC GmbH, Siegen, Germany) the supplier-provided protocol was used. Labeling resulted in a mixture of products that could be separated by ion exchange chromatography on a MonoQ column.\(^5\) The elution profile is shown in Fig. S1. Peaks 1-5 correspond with unlabeled azurin, azurin labeled at the N-terminus, labeled at Lys122, Lys24 or Lys27 (not further investigated), and a doubly labeled species, respectively.\(^5\) For the experiments described in this study, N-terminally labeled and Lys122 labeled azurin were selected. They represent azurins where the label is either far away from (29.1 Å) or relatively close to (18.5Å) the Cu, respectively.

**UV/Vis absorption and fluorescence spectroscopy**

All optical absorption and fluorescence experiments on bulk solutions were carried out at room temperature in 100 mM HEPES pH 7.0 under stirring. UV/Vis absorption spectra were recorded on a Cary 50 Spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) at a speed of 400 nm/min with a bandwidth of 2 nm and an interval of 1 nm between data points. With the use of a Cary Eclipse Spectrophotometer (Agilent Technologies, Santa Clara, CA, USA), emission spectra of fluorescently labeled azurin samples were recorded in the 600 – 800 nm range by exciting the sample at 590 nm. Excitation and emission slits were set to 5 nm band-pass and suitable optical filters were automatically selected by the instrument to minimize second order diffraction effects of the monochromator.

**Sample Preparation for FCS experiments**

A stock solution of sucrose was prepared by adding 10 ml of a 500 mM HEPES pH 7.0 buffer solution to 37.5 g of high purity (purity >99.5%) D(+) -saccharose powder. The volume was then adjusted to 50 ml by adding ultrapure water. The solution was thoroughly sonicated to achieve complete dissolution of the sucrose and degassed to avoid the presence of air
bubbles. The sucrose concentration of the solution was checked by measuring the refractive index \( n \), at 20ºC, with a thermostated Zeiss Abbe refractometer (Carl Zeiss, Germany). The refractive index amounted to 1.445 (±0.002) corresponding with a content of 61.4% (w/w) sucrose and a viscosity of 75 cP. The sucrose solution was passed through a 0.22 μm Millipore syringe filter (EMD Millipore, USA) to prevent the presence of particulate in the sample.

For each measurement, a fresh sample was prepared by mixing 372 μl of the sucrose stock solution with 4 μl of bovine serum albumin (BSA) stock solution (10 mg/ml), 4 μl of a 100 nM azurin stock solution and between 10 and 20 μl of 2 or 20 mM freshly prepared stock solutions of ascorbate or hexacyanoferrate(II) or (III). The sample volume was adjusted to 400 μl by admixture (≤ 20 μl) of 100 mM HEPES buffer, pH 7.0. The calculated sucrose content of the samples was 58.0% (w/w), corresponding to a viscosity of 42.7 cP at 22ºC. All solutions were passed through a 0.22 μm filter to avoid presence of particulate. The final sample concentration of labeled protein was around 0.4-0.8 nM. Manipulation of the redox potential of the solution was established by employing hexacyanoferrate(III), hexacyanoferrate(II) or ascorbate. In the absence of BSA the amplitude of the ACF curve \((G(0))\) varied strongly depending on the added amount of chemicals like ascorbate or hexacyanoferrate. We suspect that the azurin, adsorbed at the glass surface, (partly) desorbed upon changing the ionic strength or the redox conditions of the solution. This was not further investigated. Admixture of BSA reduced the effect presumably because the BSA covered the glass surface thus preventing adsorption of the azurin; an example of the effect of BSA on the FCS measurements is shown in Fig. S2.

**Calibration of the FCS spectrometer**

Calibration of the confocal volume in water and in sucrose was based on the assumption that the light intensity in the detection volume can be approximated by a 3-dimensional Gaussian profile, in which case the autocorrelation function (ACF) can be calculated analytically:

\[ G(0) = \frac{1}{3} \mu_1^2 \]
\[
G(\tau) = G(0) \cdot \left( 1 + \frac{4D\tau}{w^2} \right)^{-\frac{1}{2}} \left( 1 + \frac{4D\tau}{z_0^2} \right)^{-\frac{1}{2}} = G(0) \cdot \left( 1 + \frac{\tau}{\tau_D} \right)^{-\frac{1}{2}} \left( 1 + \left( \frac{w}{z_0} \right)^2 \frac{\tau}{\tau_D} \right)^{-\frac{1}{2}}
\]

\[
\tau_D = \frac{w^2}{4D}, \quad (S2a)
\]

\[
k = \frac{z_0}{w} \quad (S2b)
\]

where \( G(\tau) \) is the ACF, \( D \) is the diffusion coefficient of the molecule of interest, \( \tau_D \) is the residence time of the molecule in the confocal volume and \( r_0 \) and \( z_0 \) denote the distances from the center of the confocal volume where the intensity has dropped by a factor of \( 1/e^2 \) in the radial and axial direction, respectively.

\( G(0) \) is given by

\[
G(0) = \frac{1}{\langle N \rangle} = \frac{1}{c \cdot V_{\text{eff}} \cdot N_A}
\]

where \( \langle N \rangle \) is the average number of particles in the probe volume, \( c \) is the concentration of the sample used for the measurement, \( V_{\text{eff}} \) is the effective probe volume, and \( N_A \) is Avogadro’s constant. \( V_{\text{eff}} \) is larger than the confocal volume, which is calculated on the basis of a Gaussian intensity profile, by a factor of \( 2^{(2/3)} \).9

The autocorrelation curves obtained from a dilution series of ATTO655 in pure buffer (Fig. S3A) were fitted to Eqs. (S1-S2) resulting in values for \( w \) and \( k \) of 385 nm and 7.1, respectively (Fig. S3B). The effective volume was calculated by \( V_{\text{eff}} = \sqrt{\pi^3}kw^3 \) giving \( V_{\text{eff}} = 2.3 \) fL. A similar procedure was used to calibrate \( V_{\text{eff}} \) for the 61.4% (w/w) sucrose stock solution (data not shown) resulting in \( w = 384 \) nm, \( k = 3.5 \), and \( V_{\text{eff}} = 1.1 \) fL, respectively. This result is consistent with the expected reduction of the axial dimension of the confocal volume in solutions with refractive index higher than that of water.10-11

Values for the diffusion time of the dye as obtained from the fits amounted on average to 0.102 ms (water) and 2.9 ms (sucrose stock solution). On the basis of the known diffusion coefficient of ATTO655 in water \( (D = 383 \, \mu m^2 s^{-1}) \) and \( w = 384 \) nm a value of 0.097 ms is calculated for \( \tau_D \) (see Eq. (S2b)), in good agreement with the experimentally determined
value. The measured diffusion correlation time increases by a factor of 29 when 100% water is replaced by 58% (w/w) sucrose solution in water. As the viscosity increases by a factor of 42 and the diffusion time, according to the Stokes-Einstein model, is proportional to the viscosity of the medium, the increase by only a factor of 29 is smaller than expected. This may in part reflect the strong dependence of the viscosity of concentrated sucrose solutions on temperature, the quoted viscosity applying at 20°C while the experiments were performed at a slightly higher temperature. A very rough estimate of the diffusion time of an azurin molecule in the sucrose solution may be obtained from the rule of thumb that $\tau_D$ is proportional to the cube root of the molecular weight. With $M_r = 13998$ for azurin and 887 for ATTO655 the estimated diffusion correlation time for azurin in a 58% (w/w) sucrose solution in water would amount to 7 ms. This is in the preferred time range for the experiments on labeled azurin.

**Autocorrelation function when the azurin in solution is only partly reduced**

When the azurin in a sample is only partly reduced the expression for the ACF has to be adapted from what is described by Eqs. (2) – (4) in the main text. This can be understood by realizing that a partly reduced sample contains reduced as well as oxidized azurin molecules and that these two species exhibit different brightness: in the oxidized form FRET quenches part of the fluorescence, while in the reduced Az the fluorescence of the label is maximal. Consequently, the expression for the ACF has to be adapted.

When denoting the relative brightness of the label in the oxidized (Cu(II)Az) and the reduced (Cu(I)Az) azurin by $\eta_{ox}$ and $\eta_{red}$, respectively, and the fractions of the azurin molecules in the oxidized and reduced form by $\rho_{ox}$ and $\rho_{red}$, respectively, the expression for the ACF can be derived from the more general expression in ref7-8 leading to

$$G(\tau) = \frac{\eta_{ox}^2 \rho_{ox} G_{ox}(\tau) + \eta_{red}^2 \rho_{red} G_{red}(\tau)}{(\eta_{ox} \rho_{ox} + \eta_{red} \rho_{red})^2}$$  \hspace{1cm} (S4)

The ACFs for the oxidized and reduced particles, $G_{ox}(\tau)$ and $G_{red}(\tau)$, respectively, each have the form of Eq. (1) (see main text). They are similar except for the part that corresponds with the intramolecular ET between Cu$^+$ and the label and between the label and Cu$^{2+}$. Here we ignore the latter reaction. It occurs on a much longer time scale than the former reaction and the contribution to the ACF is small unless the degree of reduction is very low which is
not the case. Hence $G_{2,ox} = 1$ whereas

$$G_{2,red}(\tau) = \frac{(1 - F_{2,red} + F_{2,red}e^{-\tau/\tau_2})}{(1 - F_{2,red})}$$

The subscripts $ox$ and $red$ refer to the oxidized and reduced azurin molecules and the subscript 2 denotes the parts in the ACF that correspond with the ET reaction between label and Cu. After some rearrangement Eq. (S4) becomes

$$G(\tau) = \frac{1}{\langle N \rangle} \alpha G_{\text{diff}}(\tau)G_1(\tau)(1 + \beta Ke^{-\tau/\tau_2})$$

(S5)

With

$$K = \frac{F_{2,red}}{1 - F_{2,red}}$$

$$\alpha = \frac{\eta_{ox}^2\rho_{ox} + \eta_{red}^2\rho_{red}}{(\eta_{ox}\rho_{ox} + \eta_{red}\rho_{red})^2}$$

$$\beta^{-1} = 1 + \left(\frac{\eta_{ox}}{\eta_{red}}\right)^2 \frac{\rho_{ox}}{\rho_{red}}$$

Eq. (S5) is similar to the Eqs. (1)-(4) in the main text, that were used to fit the data of K122-labeled CuAz except for the factors $\alpha$ and $\beta$. It is clear now why $F_2$ did not come out as a constant from this fit since the fit equation did not contain the factor $\beta$. Also $G(0)$ will vary with the degree of reduction when Eqs (1)-(4) are used because of the factor $\alpha$. When the reduction proceeds, $\rho_{ox}/\rho_{red}$ diminishes and $\alpha$ and $\beta$ will approach $1/\rho_{red}$ and 1, respectively, and become constant. Both $\alpha$ and $\beta$ depend on the ratio between oxidized and reduced azurin, i.e., on the redox potential of the solution.
Calculation of the work term in the Rehm-Weller equation

The electrostatic work term (Eq. (7)) contributes an amount of +144 meV (Scheme 1) or -288 meV (Scheme 2) to the driving force and eventually contributes an amount of 60 meV (Scheme 1) or -210 meV (Scheme 2) to \( \lambda \). In both cases, this gave a slightly better agreement with the expected value for \( \lambda \) (950 meV) than when the electrostatic term would have been neglected. The work term was evaluated by setting the distance between donor and acceptor at 10Å, which is the distance between Cu and the aromatic part of the label when the ATTO655 moiety is modelled as being in van-der-Waals contact with the protein surface in the K122 labeled Azu variant. In this configuration, the effective \( \varepsilon \) will be intermediate between that of the pure protein (\( \varepsilon = 4 \)) and that of water (\( \varepsilon = 86 \))\(^{13-14} \) and for calculation purposes it was set at 10.

Fluorescence anisotropy and possible association of the dye with the protein surface

One way to establish the rotational correlation time of a fluorophore in solution is by measuring its fluorescence anisotropy as a function of the viscosity.\(^{15} \) In the present study anisotropy was measured by inserting a polarizer and an analyzer in the excitation and emission paths of the fluorophore, respectively, and measuring the intensity of the components of the emitted light polarized parallel (\( I_\parallel \)) and perpendicular (\( I_\perp \)) to the polarization of the incoming beam. The fluorescence anisotropy, \( r \), is defined as \( r = (I_\parallel - I_\perp) / (I_\parallel + 2 I_\perp) \). According to Perrin the relation between \( r \) and the solution viscosity \( \eta \) is given by

\[
\frac{1}{r} = \frac{1}{r_0} + \frac{1}{r_0} \frac{\tau}{\eta V}
\]

\((S7)\)

with \( r_0 \) the fundamental anisotropy of the chromophore, \( V \) its hydrodynamic volume, \( \tau \) the fluorescence life time of the chromophore and \( \eta \) the viscosity of the solution. Denoting the rotational correlation time of the chromophore in pure water and the viscosity of pure water by \( \theta \) and \( \eta_0 \), respectively, one obtains \( \theta^{-1} = \frac{RT}{\eta_0 V} \) and
When plotting $1/r$ against $1/\eta$, with $\eta$ in cP, the intercept divided by the slope provides a value for $\theta/\tau$ (notice that $\eta_0 = 1$ cP). For our experiments the viscosity was varied by changing the sucrose content of the solution. The precise composition of the solution was determined by measuring the index of refraction and reading the sucrose content from the tables in ref 6.

The data for Nt-ZnAz and K122-ZnAz are presented in Fig. S15, panels A and B, respectively. Intercepts and slopes amount to (uncertainties in parentheses): 4.73 (.22) and 5.08 (.39) cP for K122 labeled ZnAz, respectively, and 4.71 (.27) and 5.40 (.50) cP for Nt labeled ZnAz. The fluorescence life time of ATTO655 attached to ZnAz was determined in a separate experiment as $\tau = 2.7$ ns. The averaged rotational correlation time of the labeled ZnAz in water, as determined from the regression lines in Figs. S15A and B, is $(2.4 \pm 0.3)$ ns, which should be compared with the rotational correlation time of the protein of 4.75 ns. Extrapolating the regression lines in Figs. S15A and S15B to $\eta^{-1} = 1$ one finds for the anisotropy of the labeled azurin in water a value of 0.101. Were the fluorophore completely immobilized in the protein one would calculate from Eq. (S8) with $r_0 = 0.227$, $\tau = 2.7$ ns and $\theta = 4.75$ ns a value of 0.145. Both the decrease in the rotational correlation time (from 4.75 to 2.4 ns) as well as the decrease in anisotropy (from 0.145 to 0.101) show that the dye is not tightly associated with the protein by complex formation, but retains some mobility.

This conclusion is reinforced by ongoing research on AzATTO655 constructs in which the label is at positions 27, 41, 42, or 92 in the amino acid chain. Together with the labeling positions (122 and Nt) used in the present two constructs (see above) these places cover a large part of the protein surface. Preliminary data show that within experimental uncertainty the rotational correlation times and anisotropies in water of these six Az-ATTO constructs are similar and amount to $(2.4 \pm 0.2)$ ns and $0.106 \pm 0.06$, respectively. Thus, there appears to be no particular area on the protein surface with which the label preferentially associates to form a stable complex.

The question remains about the nature of the resting motion of the label in the Az-ATTO655 constructs. Lakowicz has provided examples where a relatively modest reduction in
anisotropy is still compatible with fast motion of the label provided the accessible volume for the label is limited.\textsuperscript{15} The size of this volume may depend critically on the length and nature of the linker.\textsuperscript{17} A linker containing a maleimide moiety, for instance, may severely limit mobility as compared to a simple $C_\alpha$ linker.\textsuperscript{17} Stacking between label and the end-base of an oligonucleotide may enhance stable complex formation.\textsuperscript{18} Or, when used to label a short peptide the label may stack with a tryptophan in the amino acid sequence of the label.\textsuperscript{19} In labeled proteins hydrophobic interactions may promote complex formation between label and protein surface.\textsuperscript{20} In the present case information on this subject must await further experimental (NMR, ESR and DEER spectroscopy, time domain fluorescence anisotropy) and theoretical (MD) efforts. What can be remarked at this stage is that in the measured ACFs of the Az-ATTO655 constructs only a single decay can be distinguished that can be ascribed to intramolecular ET. If equilibrium would exist between transient complexes (including the dissociated form), the equilibrium kinetics would have to be fast on the time scale of the ET reaction, otherwise additional decays or a stretched exponential would appear in the ACF.

**Cyclic Voltammetry**

NH$_2$- and COOH-forms of the ATTO655 dye were obtained directly from ATTO-TEC GmbH (57076 Siegen, Germany), K122 NHS-ATTO655-labeled ZnAz was obtained as described for the Cu containing construct\textsuperscript{5}. Cyclic voltammetry experiments were performed at ambient temperature in a Hagen cell\textsuperscript{21} equipped with a carbon glass working electrode, a Pt counter electrode and an Ag/AgCl/3M KCl reference electrode. The glassy carbon electrode was polished with 6 $\mu$m diamond slurry on a polishing cloth, cleaned with water, incubated for 5 mins in 65% nitric acid and washed with 1M aqueous phosphate buffer pH 7, subsequently rinsed with water and then dried with tissue paper. Sample volumes were between 20-50 $\mu$L and protein and dye concentrations amounted to 20-40 $\mu$M. The cell was gently sparged with argon during measurements. Solutions were buffered with 20 mM Hepes pH 7. Voltammograms were recorded with the help of a $\mu$Autolabel potentiostat (Metrohm, Autolab, Utrecht, The Netherlands) at 10mV/sec scan speed (1 or 100 mV/sec in special cases). The potential window (vs. Ag/AgCl) varied from -1.1/-0.05 V (dye-COOH) to -0.65/0.0 V (dye-NH2) and -0.6/+0.3 V (dye-azurin). Representative examples of measured cyclic
voltammograms are shown below.

Cyclic voltammograms of 50–100 μM solutions of ATTO655 constructs in 10 mM HEPES buffer pH7 at room temperature. Scan rate 10 mV/sec. Top left: dye bound by maleimide chemistry to S122C azurin at position 122. Top right: free NH2-form of ATTO655; bottom left: free COOH form of ATTO655. Potential scale on x-axis is with respect to the Ag/AgCl (sat KCl) electrode (+202.5 mV wrt NHE electrode).

Peak positions were determined with NOVA 2.0 software (Metrohm Autolab). The results of the analysis are presented below:

<table>
<thead>
<tr>
<th></th>
<th>ATTO655-NH₂</th>
<th>ATTO655-COOH</th>
<th>ATTO655-Azurin c)</th>
<th>Azurin d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_m$, mV a)</td>
<td>-129</td>
<td>-142</td>
<td>-183</td>
<td>291</td>
</tr>
<tr>
<td>$\Delta E$, mV b)</td>
<td>59</td>
<td>47</td>
<td>54</td>
<td>95</td>
</tr>
</tbody>
</table>

a) Midpoint potential in mV vs SHE  
b) Distance in mV between anodic and cathodic peak  
c) Label attached at position 122 in the amino acid chain  
d) Cu-containing wild type azurin

The midpoint potential of the carboxy-form of the dye is slightly more negative (by 13 mV) than that of the amine-form which means that replacing the carboxy-group by the amnio-group slightly stabilizes the reduced form of the dye, in agreement with simple charge
considerations. The midpoint potential of ATTO655 in water is appreciably lower than that reported for MR121 (-0.42V vs SCE) in acetonitrile (AN)\(^{22-23}\). Remarkably the difference almost disappears when the dye is attached to azurin: ATTO655 attached to Az has the same reduction potential in water (-0.183V vs SHE) as MR121 in AN (-0.18V vs SHE). We have assumed, therefore, that the value of the oxidation potential of ATTO655 in water (which is needed for the Rehm-Weller equation (see main text)) likewise will be similar to that of MR121 in AN (1.55V vs SHE\(^{23}\)). The anodic/cathodic peak-peak difference is slightly less than the theoretical value of 59 mV which may point to a possible two electron reaction. This was not further investigated.

**Power dependence of f(I)**

The dependence of \(f(I)\) on the excitation light intensity \(I\) was checked by measuring \(k_2f\) as a function of \(I\) for K122-CuAz under reducing conditions (see main text). According to Eq. (7)

\[
\frac{F_2}{\tau_2} = f(I) \frac{k_1^I}{[k_{01}/(k_{01} + k_{10})]} = \frac{k_2f}{[\sigma I/(\sigma I + k_{10})]} = \frac{\sigma I}{[\sigma I + k_{10}]} \cdot k_2f
\]

or

\[
\tau_2/F_2 = (1/k_2f) [1 + k_{10}/\sigma I] = (1/k_2f) [1 + k_{10}/\gamma I_p].
\]

Here, \(I\) is the power in photons/cm\(^2\),msec whereas \(I_p\) is the total power in \(\mu W\) measured directly after the objective and \(\gamma\) is a conversion factor, which amounts to \(\gamma = 9.0 \times 10^1 \, \mu W^{-1} m sec^{-1}\) for the present experimental set-up. When plotting \(\tau_2/F_2\) vs \(1/I_p\) the data points should lie on a straight line from the slope of which a value of \(k_2f\) can be extracted. The ACFs were measured for a series of \(I_p\) values between 1 and 80 \(\mu W\) (see Fig. S10A). They were fit as described in the main text and from the fits values for \(F_2, \tau_1, F_2\) and \(\tau_2\) were obtained (Fig. S10B). Subsequently \(\tau_2/F_2\) was plotted as a function of \(1/I_p\) (Fig. S10C). The points lie on a straight line, indeed, the slope of which provides \(k_2f = 3.7 \times 10^6 \, s^{-1}\), in agreement with the value quoted in the main text (calculated for \(I_p = 20 \, \mu W\)).

**TD-DFT calculations**

TD-DFT calculations were performed on the ground and electronically excited states of oxazine-1 and of the dye ATTO655 (see below for structures and atom numbering). The Gaussian09 rev. E01 program suite was used together with the B3LYP functional and the 6-
31G* basis set. The calculations were performed for the gas phase; to account for the effects of the solvent (i.e., water) the calculations were repeated with the Polarizable Continuum Model.

**Oxazine-1.** Geometry optimization of the molecule in the electronic ground state leads to a geometry with $C_{2v}$ symmetry. Calculation of the harmonic force field shows that this is indeed a stable geometry, giving rise to a Mulliken charge distribution as shown in Table S1. Calculation of the vertical excitation energies shows that the lower-lying excited singlet states are found at (shown in parentheses are the values obtained in water)

<table>
<thead>
<tr>
<th>State</th>
<th>Excitation energy (nm)</th>
<th>Oscillator strength</th>
</tr>
</thead>
<tbody>
<tr>
<td>$S_1$</td>
<td>479 (570)</td>
<td>0.88 (1.30)</td>
</tr>
<tr>
<td>$S_2$</td>
<td>430 (443)</td>
<td>0.00 (0.00)</td>
</tr>
<tr>
<td>$S_3$</td>
<td>368 (357)</td>
<td>0.00 (0.00)</td>
</tr>
<tr>
<td>$S_4$</td>
<td>330 (337)</td>
<td>0.03 (0.08)</td>
</tr>
<tr>
<td>$S_5$</td>
<td>322 (323)</td>
<td>0.01 (0.02)</td>
</tr>
</tbody>
</table>

which agrees within the computational accuracy of excited-state energies of this type of calculation with the $S_1$ gas-phase results of Fleming et al. (2.59 eV here, 2.52 eV previously) and the solvent-phase results (2.18 eV here, 2.12 eV previously)\(^24\). Optimization of the geometry of the molecule in $S_1$ with $C_{2v}$ symmetry restrictions leads to a Mulliken charge distribution that for all practical purposes remains the same.

![Structure and atom numbering of oxazine-1.](image-url)
It can thus be concluded that there are no major changes in the Mulliken charge distribution upon excitation from $S_0$ to $S_1$. The same holds for the off-diagonal Mulliken populations involving N18 and C20 (and equivalently N15-C17), which is where the dye connects with the aliphatic linker:

<table>
<thead>
<tr>
<th></th>
<th>$S_0$ C$_{2v}$ gas</th>
<th>$S_0$ C$_{2v}$ water</th>
<th>$S_1$ C$_{2v}$ gas</th>
<th>$S_1$ C$_{2v}$ water</th>
</tr>
</thead>
<tbody>
<tr>
<td>gas</td>
<td>0.294</td>
<td></td>
<td>0.298</td>
<td>0.296</td>
</tr>
<tr>
<td>water</td>
<td>0.294</td>
<td></td>
<td>0.298</td>
<td>0.296</td>
</tr>
</tbody>
</table>

*Off-diagonal Mulliken populations involving N18 and C20.*

**ATTO655.** The calculations were repeated for ATTO655. To minimize the effect of spurious charge transfer states the B3LYP functional was replaced by the CAM-B3LYP functional. The same conclusion as for oxazine-1 was found to apply to the Mulliken charge distributions and the N15-C17 off-diagonal elements: little change occurs upon excitation from the ground state to the lowest excited singlet state. Examples of calculated charge distributions are presented below for oxazine-1 and ATTO655.
**Intramolecular reorganization energies**

The inner shell contribution to the reorganization energy of the dye has been assessed by TD-DFT calculations on oxazine-1 (indicated as L), which is the relevant chromophore of ATTO-655. These contributions have been calculated as the difference between the vertical and adiabatic energy differences of the two states of interest. For example, the reorganization energy of the L$^+$ $\rightarrow$ L transition is calculated as $L^+(L) - L^+(L^+)$ with $L^+(L)$ the energy of the ground state of $L^+$ at the equilibrium geometry of L and $L^+(L^+)$ the energy of the ground state of $L^+$ at the equilibrium geometry of $L^+$. The calculated intramolecular reorganization energies are reported below. It can be concluded that the differences between the intramolecular contributions to the reorganization for the forward and backward ET reactions (see Schemes 1 and 2) are on the order of 100 meV or less.

<table>
<thead>
<tr>
<th>Transition</th>
<th>Reorganization energy (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$L^* \rightarrow L^-$</td>
<td>0.048</td>
</tr>
<tr>
<td>$L^- \rightarrow L$</td>
<td>0.155</td>
</tr>
<tr>
<td>$L^* \rightarrow L^+$</td>
<td>0.118</td>
</tr>
<tr>
<td>$L^+ \rightarrow L$</td>
<td>0.035</td>
</tr>
</tbody>
</table>
Table S1. Mulliken charge distributions for Oxazine-1 and ATTO655. For atom numbering see above.
**Pathway calculations**

For the pathway calculations the following form of the Marcus equation is often employed:\(^{2,3,25}\)

\[
k_{et} = A \ T_{DA}^2 \exp \left(-[(\Delta G + \lambda)^2]/(4\lambda kT)\right)
\]

in which the distance dependence of the matrix element of the electronic coupling between donor and acceptor is incorporated in \(T_{DA}\). The coupling is calculated by considering a chain of atoms (pathway) that runs from to donor to acceptor. For each step in the chain the coupling is attenuated by a particular factor. The total attenuation is the product of the individual attenuation factors. Three types of atom to atom steps are considered: steps between atoms that are bound covalently, between atoms that are linked by a H-bond and between atoms that have no bond. For each type of step a semi-empirical value for the attenuation factor is used. Details can be found for instance in \(^3\).

As the pathway forms a bridge between donor and acceptor, an important consideration relates to the coupling between bridge and donor/acceptor. This coupling depends on the molecular details of bridge and donor/acceptor and its magnitude is not easily cast in a generalized form. The ensuing uncertainty is absorbed in the pre-factor \(A\). The values for \(A\) circulating in the literature mostly lie between \(10^{13}\) and \(10^{14}\). A value of \(3 \times 10^{13}\) is often found to give acceptable results. This is the value chosen in the present work.

A second point of interest is that more than one path may be envisaged. The overall probability of a transition of an electron from donor to acceptor should then be summed over the probabilities corresponding with the individual paths. However, the paths may interfere mutually and calculating the details of the phases along each path is a challenge that falls outside the scope of the present analysis. In general, it is not known how fast the sum of the transition probabilities converges. Often, only the pathway with the largest coupling is taken into consideration. Predictions of \(k_{et}\) on the basis of the pathway model therefore carry an intrinsic margin of uncertainty. Mutual comparison of ET rates, on the other hand, can be made with more confidence since the influence of the pre-factor \(A\) cancels out then.

As an example, the calculation of \(T_{DA}\) is illustrated by Table S2 for the case where the label is attached to the NZ atom of Lys122 in azurin. The ‘Pathway’ program \(^{26}\) finds the pathway...
with the largest coupling between NZ and Cu and calculates the magnitude of $T_{DA}$ by multiplication of the attenuation factors corresponding with the individual steps (see Table S2 and Fig. S16). The overall attenuation amounts to $T_{DA} = 2.176 \times 10^{-3}$; the linker adds an extra five covalent bonds, which means an extra attenuation of $(0.6)^5$ and a final value of $T_{DA}$ of $1.692 \times 10^{-4}$. In a similar way $T_{DA}$ was calculated for Nt-Az (see main text). For the calculations the protein structure as documented in PDB file 1AZU was used.

**Table S2:** Output of the Pathways algorithm$^{26}$. Reproduced here is the pathway that connects the Cu atom and the NZ of Lys122 and that provides the largest coupling. The columns on either side of the arrows show, respectively: atom number, chain, residue name, residue number and atom name for the initial (left of the arrow) and the final (right of the arrow) atom in each step along the pathway. The last three columns show the step type (CB, covalent bond; HB, hydrogen bond; TS, through-space jump), step distance and the running distance along the pathway at the current step.$^{27}$

PATH 1: net decay 2.17678e-03

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Figures

Figure S1. Chromatogram showing the separation of dye-labeled azurin species. The peaks are labeled from 1 through 5 and correspond with unlabeled azurin(1), N-terminally labeled azurin(2), Lys122 labeled azurin(3), a mixture of differently labeled species(4), and a doubly labeled species (5), respectively.
**Figure S2.** Illustration of the effect of addition of BSA on the ACF of a 10 nM solution of Cu-azurin labeled at Lys122 with ATTO655. The upper panels show the count rate as a function of time in the absence (left) and in the presence (right) of 10 mg/ml BSA. The lower panel shows the effect on the autocorrelation function: in the absence of BSA the value of $G(0)$, which is inversely proportional to the concentration of the labeled protein in solution, is greater than in the presence of BSA.
Figure S3. Calibration of the confocal volume. A) ACF data collected for solutions containing different concentrations of ATTO655 in water as indicated in the figure. The data points are the autocorrelation values at each lag time. The error bars represent the standard deviation of each point. The dashed lines are fits to Eq. S2. B) Structural parameters of the confocal volume as obtained by fitting the ACF data of free ATTO655 in water (see Eq. S2). The parameter $k$ (left vertical axis) is the shape factor of the detection volume. The parameter $w$ (right vertical axis) is the axial radius of the confocal volume.
Figure S4. Depiction of azurin (gray) modified with ATTO655 (red) on the two positions studied in the present work. The Cu ion and the 5 residues coordinating it are depicted, respectively, as an orange sphere and green sticks. A) Label linked to the N-terminal residue Ala1 (blue). B) Label attached to the side chain of Lys122 (blue). The figures were prepared in silico by using the available crystal structure of azurin²⁸-³⁰ and the molecular structure of the label ATTO655. The molecule in panel B has been rotated counterclockwise over 90° with respect to panel A around a vertical axis in the plane of the page.
Figure S5. Experimentally observed ACFs of Zn azurin labeled at the N-terminus with ATTO655. Datasets were obtained on samples containing 10 (A) and 500 µM (B) potassium hexacyanoferrate(III). The red lines are fits according to Eq. (2) with $G(\tau) = G(0) G_{\text{off}}(\tau)$. The insets show the residuals of the fits. (C) Diffusion times observed at various concentrations of hexacyanoferrate(III). Vertical bars here (panel C) and elsewhere denote 95% confidence intervals.
Figure S6. Experimentally observed ACFs of Zn azurin labeled at the N-terminus with ATTO655 for samples containing 25 (A), 100 (B) and 500 µM (C) potassium hexacyanoferrate(II) (top to bottom). The red lines are fits according to Eq. (2) with \( G(\tau) = G(0) G_{\text{diff}}(\tau) \). The insets show the residuals of the fits. (D) Diffusion times derived from the fits for various concentrations of hexacyanoferrate(II).
Figure S7. Experimentally observed ACFs of Zn azurin labeled at the N-terminus with ATTO655 for samples containing 25 (A), 70 (B) and 500 (C) µM ascorbate. The red lines are fits according to Eq. (2) with $G(t) = G(0) \cdot G_{\text{diff}}(t) \cdot G_1(t)$ and $\tau_0 = 12$ ms. The insets show the residuals of the fits. (D) Parameters obtained from the fits. The green squares and red dots show the values for $\tau_1$ and $F_1$, and correspond with the left and right y-axis, respectively. Vertical bars denote confidence intervals.
Figure S8. Parameters obtained by fitting the ACFs of Zn azurin labeled at K122 at various concentrations of ascorbate. The equation used for the fits was $G(\tau) = G(0) \cdot G_{\text{diff}}(\tau) \cdot G_1(\tau)$ with $\tau_d = 12$ ms. The green squares are the data for $\tau_1$, and correspond with the vertical axis at the left. The red circles are the data for $F_1$, and correspond with the vertical axis at the right.
**Figure S9.** Parameters obtained by fitting the ACFs of Cu azurin labeled at the N-terminus at various concentrations of hexacyanoferrate(II) (panel A) and ascorbate (panel B). The equation used to fit the ACF’s was $G(t) = G(0) G_{diff}(t) G_1(t)$ with $\tau_0 = 12$ ms. The green squares are the data for $\tau_1$ (left vertical axis). The red circles are the data for $F_1$ (right vertical axis).
Figure S10. The dependence of the ACF on the excitation light intensity. A. ACF curves were measured for solutions of K122-CuAz (\sim 1 nM) under reducing conditions at total excitation light intensities between 1 and 80 μW as measured directly behind the objective. Intensities are indicated in the figure. Conditions: 58% (w/w) sucrose in 20 mM HEPES, pH 7.0 (for further details: see main text). B. Autocorrelation parameters $F_1$, $\tau_1$, $F_2$ and $\tau_2$ as obtained from fits of the ACFs (see main text for further details). C. $\tau_2/F_2$ as a function of the inverse incident light power. The straight line is a least squares fit to the equation $\tau_2/F_2 = (1/k_f^f) [1 + k_{10}/\gamma I_p]$. 

![Figure S10.A](image-url)
Figure S10.B

Figure S10.C
Figure S11. Examples of experimentally observed ACFs of Cu azurin labeled at K122 with ATTO655. Datasets were obtained on samples containing 40, 200 and 500 µM hexacyanoferrate(III) (top to bottom). The red lines are fits according to Eq. (2) with $G(\tau) = G(0) G_{\text{diff}}(\tau) G_{1}(\tau)$ and $\tau_D = 12$ ms. The insets show the residuals of the fits.
Figure S12. FCS experiments on Cu azurin labeled at K122 with ATTO655. A) ACF curves measured in the presence of 5 (black), 55 (green) and 500 (blue) μM of hexacyanoferrate(II). The 55 and 500 μM traces have been normalized so that their G(0)-values coincide with the 5 μM trace. The red lines are fits according to Eq. (2) with $G(t)/G(0) = G_{\text{diff}}(t) G_1(t) G_2(t)$ with $\tau_D = 12$ ms. The inset at the top represents the residuals corresponding to the black colored ACF. The residuals of the other two curves were similar. The inset at the right is a cartoon of K122-labeled azurin. The ATTO655 label is depicted in red, Cu in orange and the Cu ligands in green. Panels A-D: parameters obtained from the fits of the ACFs. The equation used for the fits was $G(t) = G(0) G_{\text{diff}}(t) G_1(t) G_2(t)$ with $\tau_D = 12$ ms. Shown are, as a function of the hexacyanoferrate(II) concentration: $F_1$ (B), $\tau_1$ (C), $F_2$ (D), and $\tau_2$ (E).
Figure S13. Analysis of the ACFs of K122-labeled Cu azurin. ACFs were fitted with the equation $G(\tau) = G(0) G_{off}(\tau) G_1(\tau) G_2(\tau)$ with $\tau_0 = 12$ ms. A. Parameters $\tau_1$ and $F_1$, as obtained from the fits, correspond with $G_1(\tau)$. A and C: $(F_1/\tau_1)/(1 - F_2) (= k_f)$ as a function of the concentration of ascorbate (A) or hexacyanoferrate(II) (C); B and D: $(1 - F_1)/\tau_1 (= k_b)$ as a function of the concentration of ascorbate (B) or hexacyanoferrate(II) (D). The straight lines are least squares fits to the data points.
FRET and PET rates and quenching efficiencies for labeled azurin as a function of length parameters. 

**A.** FRET (black) and PET quenching rates as a function of the distance $R$ between Cu center and chromophore. The FRET rates were calculated with Förster’s formula with a Förster radius $R_0 = 37$ Å and life time of the dye in the absence of quenching of $\tau = 2.7$ nsec. PET rates were calculated from $10 \log k_{PET} = 13 - 0.6(R-3.6) - (\Delta G+\lambda)^2/4\lambda kT$ with $\lambda= 0.76$ eV, and $\Delta G = -0.476$ (red solid line); $\lambda = 1.16$ eV, and $\Delta G = -1.67$ (red dashed line); $\lambda = 0.76$ eV, and $\Delta G = -1.384$ (blue solid line); $\lambda = 1.16$ eV, and $\Delta G = -0.19$ (blue dashed line); see main text for choice of parameters. 

**B.** PET quenching rates as a function of the number of covalent bonds $n$ between chromophore and Cu site. Rates were calculated from $k_{PET} = 3 \times 10^{13} e^{-\beta_n n} e^{-(\Delta G+\lambda)^2/4\lambda kT}$ with $\beta_n = 1$ per bond, $n$ the number of covalent bonds. Different lines refer to similar cases as in panel A. 

**C.** Quenching efficiency $QE \equiv (F_0 - F)/F_0 = k/(k_0 + k)$ calculated as a function of distance between chromophore and Cu center. Here $k_0 = 1/\tau$ and $k$ is either the PET or the FRET rate. FRET rates were calculated as in panel A. PET rates were calculated as in panel A with $\lambda = 0.90$ eV and $\Delta G = -0.9$, $-0.6$ and $-0.3$ eV (green, blue and red lines, respectively).

The data in panel C clearly show that quenching by PET is short ranged while FRET quenching extends over a much longer range.

**Figure S14.**
Figure S15: Room temperature fluorescence anisotropy measurements on ZnAz labeled with ATTO655 at the N-terminus (A) and at Lys122 (B) in buffered sucrose solutions. Measurements were performed at $\lambda = 635$ nm on the FCS spectrometer described in Materials and Methods with a polarizer and an analyzer inserted in the excitation and emission paths, respectively.
Figure S16: Calculated electronic coupling “pathway” connecting the Cu (brown) in azurin with the NZ atom of Lys122 (blue). The pathway providing the strongest coupling is indicated in red. Two other pathways with weaker coupling, coinciding in part with the first pathway, are colored orange and yellow.
Reference List


