Chapter 7: The role of copper ligands on electron transfer in azurins

Abstract
The long-range intramolecular electron transfer (ET) rates between the disulfide radical anion and the copper site in azurin from *Alcaligenes denitrificans* and three mutants of azurin from *A. denitrificans* and *Pseudomonas aeruginosa* have been measured. In the three mutants a copper ligand of azurin was substituted by a glycine (His46Gly and His117Gly azurin) or a histidine (Met121His azurin).

Met121His azurin exhibits an ET rate at 298 K which is half that of wt azurin from *A. denitrificans* (21±4 vs. 42±4 s⁻¹). His46Gly and His117Gly azurin both exhibit slower ET rates (15±2 and 7±3 s⁻¹, resp.) compared to wt azurin from *P. aeruginosa* (44±7 s⁻¹). When the oxidised copper site of His117Gly azurin is reconstituted with imidazole the ET rate increases to 149±17 s⁻¹. From the temperature dependence the activation enthalpy and entropy have been determined.

The ET rates are discussed in terms of the Marcus equation. The similar ET rates of His46Gly and His117Gly suggests that ET does not proceed via a pathway that includes the imidazole ring of His46. The importance of a pathway via the internally positioned tryptophan is discussed taking into account the observed enthalpy-entropy compensation for a range of azurins (mutants).
Introduction
Electron transfer (ET) plays an important role in many biological systems and a central question is whether specific structural features of proteins promote ET [Beratan et al., 1992; Broo et al., 1992; Farid et al., 1993; Gray et al., 1996; Moser et al., 1992; Siddarth et al., 1993]. The blue copper protein azurin has become an effective model for examination of intramolecular long range ET (LRET) in proteins [Farver et al., 1989; Farver et al., 1992a; Farver et al., 1992b; Farver et al., 1993; Farver et al., 1996a; Farver et al., 1996b; Farver et al., 1997b]. It consists of a rigid β-sheeted polypeptide, and three-dimensional structures have been determined for a large number of wild type (WT) and single site mutated azurins [Baker, 1988; Hammann et al., 1996; Nar et al., 1991b; Nar et al., 1991a; Romero et al., 1993]. Furthermore, in order to study internal ET, no modification by an external redox group is needed, since azurin contains two potential redox centres, (1) the copper ion coordinated directly to amino acid residues and (2) a disulfide bridge (RSSR) at the opposite end of the molecule. It has been previously demonstrated that LRET between these two centres can be induced by pulse radiolytic reduction of RSSR [Farver et al., 1989; Farver et al., 1992a; Farver et al., 1992b; Farver et al., 1993; Farver et al., 1996a; Farver et al., 1996b; Farver et al., 1997b].

Using both wild type and single-site mutated azurins, the effect of specific structural changes on the rate of intramolecular ET has been studied. Blue copper proteins like azurin serve as electron mediators with their mono-nuclear copper site, the so-called type-1 copper site, as the active site. In type-1 copper sites the copper ion is strongly coordinated by three ligands, the Sγ of a cysteine and the Nδ's of two histidines. Additionally, one or, in the case of azurin, possibly two, weaker axial ligands are present. The resulting trigonal pyramidal or distorted tetrahedral geometry is thought be intermediate between that preferred by Cu(I) and Cu(II) [Malmström, 1994; Williams, 1995]. This has been thought to make the type-1 copper sites ideally suited for electron transfer. In the used mutants specific features of the type-1 copper site are disrupted and it can be studied how the ET properties are affected.

By applying modifications directly in the copper coordination sphere, another issue could be addressed. In order to understand better the role of the polypeptide matrix separating D and A, relevant ET routes have been identified using the structure-dependent pathway model developed by Beratan and Onuchic [Regan et al., 1993; Skourtis et al., 1994]. Two pathways have been recognised in the past which are about equally effective. In the present
work I have used azurin mutants in order to establish the efficiencies of the two previously determined pathways going to the copper(II) centre via different ligands.

**Materials & Methods**

*Proteins*

WT azurins of *Pseudomonas aeruginosa* [van de Kamp et al., 1990b] and *Alcaligenes denitrificans* [Kroes et al., 1996b], *P.a.* His117Gly azurin [den Blaauwen et al., 1991], *P.a.* His46Gly azurin [van Poudereyn et al., 1996a] and *A.d.* Met121His azurin [Kroes et al., 1996b] were isolated as described before. His117Gly and His46Gly azurin were isolated as apo-forms and reconstituted shortly before the experiments by adding 1 equivalent of Cu(NO$_3$)$_2$. The absorptions at 280 nm were used to determine the amount of the apo-form of His46Gly and His117Gly azurin ($\varepsilon_{280} = 9.1$ mM$^{-1}$cm$^{-1}$ (see chapter 4)). After adding copper, His46Gly azurin was used immediately while His117Gly azurin was incubated for 20 – 30 min. at room temperature. When imidazole was used, it was added to His117Gly azurin to an end-concentration of 0.5 mM after the incubation with Cu(NO$_3$)$_2$ and left to equilibrate for at least another 10 minutes.

Protein (5 or 10 µM) solutions, in 0.1 M sodium formate and 10 mM phosphate (pH 4.0), were deaerated and saturated with N$_2$O in glass syringes. For the His46Gly azurin the formate concentration was decreased to 10 mM (in stead of 100 mM) to minimize ligation of formate to the copper ion. For His46Gly azurin a dissociation constant of 0.25 M was measured for the formate complex [van Poudereyn et al., 1996a]. Thus, under the conditions used for the experiments, the formation of the formate complex can be neglected.

After the pH was adjusted to pH 7.0 by titration with 0.5 or 0.05 M NaOH, the concentrated protein stock solution was added. N$_2$O bubbling was continued for another 5 min and the solution was then transferred into the pulse radiolysis cell under anaerobic conditions.

*Kinetic measurements*

Pulse radiolysis experiments were carried out using the Varian V-7715 linear accelerator of the Hebrew University in Jerusalem. Electrons accelerated to 5 MeV were employed, using pulse lengths in the range from 0.1 to 1.5 µs, equivalent to 0.6-10 µM of CO$_2^-$ radical ions. All optical measurements were carried out anaerobically, under purified argon at a pressure slightly in excess of 1 atm in a 4x2x1 cm Spectrosil cuvette. Three light
passes were applied, which resulted in an overall optical path-length of 12.3 cm. A 150 W xenon lamp produced the analysing light beam, and appropriate optical filters with cut-off at 385 nm were used to avoid photochemistry and light scattering. The data acquisition system consisted of a Tektronix 390 A/D transient recorder and a PC. The temperature of the sample solutions was controlled by a thermostating system, and continuously monitored by a thermocouple attached to the cuvette. Practically all 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.

Results

WT azurin from *Alcaligenes denitrificans* and the Met121His azurin mutant

Pulse radiolytically produced CO$_2^-$ radicals rapidly reduce the two redox-active sites of the azurins: the copper(II) ion and the disulfide bridge (fast phase) [Farver et al., 1989]. In WT azurins the Cu(II) → Cu(I) reduction results in an absorption decrease around 630 nm while the formed RSSR$^-$ radicals absorb at 410 nm. The two reactions, \( (i) \) and \( (ii) \), are depicted below.

\[
\begin{align*}
\text{RSSR-Az[Cu(II)]} + \text{CO}_2^- & \rightarrow \text{RSSR-Az[Cu(I)]} + \text{CO}_2 \\
\text{RSSR-Az[Cu(II)]} + \text{CO}_2^- & \rightarrow \text{RSSR}^-\text{-Az[Cu(II)]} + \text{CO}_2 \\
\text{RSSR}^-\text{-Az[Cu(II)]} & \rightarrow \text{RSSR-Az[Cu(I)]}
\end{align*}
\]

Since Met121His azurin contains two strong Cu(II) absorption bands (at 439 and 593 nm), rather than one as in WT azurin (at ~630 nm), the response after pulse radiolysis is slightly different from the WT case. At 593 nm a decrease in absorption is still observed in the fast phase, due to direct copper reduction by CO$_2^-$ \( (i) \). At 410 nm, however, a decrease or an increase in absorbance was observed, depending on the starting conditions before the pulse. This is due to overlap of the Cu(II) and RSSR$^-$ absorption bands and, thus, the simultaneous detection of reactions \( (i) \) and \( (ii) \) at 410 nm. In a fully oxidised protein sample the absorbance at 410 nm decreases, while if the copper ions are only partially oxidised the absorbance may increase in time. Since the reduction by CO$_2^-$ is a bimolecular process, its rate will be dependent on the concentration of the disulfide bridges and the oxidised copper sites. The ratio of RSSR to Cu(II) will determine the rate of reactions \( (i) \) and \( (ii) \), and therefore whether the absorption at 410 nm will increase or decrease in the fast phase.
In protein molecules where copper is reduced, obviously no subsequent electron transfer (ET) will be observed. However, where the RSSR$^-$ anion has been produced in Az[Cu(II)], the electron can be transferred to the copper ion in a slower process (iii above). This reaction manifested itself both in WT and in Met121His azurin as a concomitant absorption decrease at 410 and around 600 nm. In WT azurin, the absorption at 410 nm eventually returns to its starting value. Since oxidised Met121His azurin also absorbs at 410 nm, the absorption at this wavelength ends below the baseline (Figure 1).

Electron transport (ET) may occur either within the same protein (intramolecular ET, (iii) in the above reaction scheme) or between two proteins (intermolecular ET, not shown in the scheme). In order to distinguish between these two possibilities the rates of RSSR$^- \rightarrow$ Cu(II) ET were determined as a function of protein concentration, typically between 1 and 10 µM oxidised azurin. The ET rate constants for WT azurin of A. denitrificans showed a slight concentration dependence at protein concentrations above ~ 5 µM, indicating a small

**Figure 1:** Time course of the observed absorbance changes and their exponential decay fittings of the mutant Met121His following a pulse of accelerated electrons. Left. Changes at 410 nm at which wavelength the Cu(II) and RSSR$^-$ both absorb (see text). Right: Changes in Cu(II) concentration monitored at 596 nm. Bottom: Difference plots between the fitting - using two exponentials - and the observed data. Conditions were: 30°C pH 7.0, pulsewidth 0.5, Oxidised Met121His azurin concentration: ~7 µM (Left) and ~ 4 µM (Right) of in total 10 µM protein.
contribution from intermolecular ET. Intermolecular ET has also been observed with some other azurins [Farver et al., 1996a]. By analysing the dependence of the rate constant on concentration the true intramolecular rate constant can easily be extracted, however. At pH 7.0 and 298 K the rate constant for the intramolecular ET constant of A.d. WT azurin is 42 ± 4 s\(^{-1}\) (see Table 1). Interestingly, the ET rate constant for Met121His azurin was independent of protein concentration between 1 and 10 µM and is half that in WT azurin, i.e. 21 ± 4 s\(^{-1}\). For both proteins the activation parameters have been determined by measuring the ET rate as a function of temperature (see Figure 2, Table 1).

**Table 1.** Kinetic and thermodynamic data for intramolecular reduction of Cu(II) by RSSR\(^-\) at 298 K and pH 7.0

<table>
<thead>
<tr>
<th>Azurin</th>
<th>(k_{298}) s(^{-1})</th>
<th>(E^0) mV vs SHE</th>
<th>(-\Delta G^0)(^a) kJ mol(^{-1})</th>
<th>(\Delta H^#) kJ mol(^{-1})</th>
<th>(\Delta S^#) J K(^{-1})mol(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ps. aer.</em>(^b)</td>
<td>44±7</td>
<td>304</td>
<td>68.9</td>
<td>47.5±2.2</td>
<td>-56.5±3.5</td>
</tr>
<tr>
<td><em>Alc. den.</em></td>
<td>42±4</td>
<td>305</td>
<td>69</td>
<td>43.5±2.5</td>
<td>-67±9</td>
</tr>
<tr>
<td>H46G</td>
<td>15±2</td>
<td>&lt;300</td>
<td>&lt;68.5</td>
<td>42.1±3.5</td>
<td>-81±5</td>
</tr>
<tr>
<td>H117G,(aq)</td>
<td>7±3</td>
<td>&lt;300</td>
<td>&lt;68.5</td>
<td>22.0±3.2</td>
<td>-155±11</td>
</tr>
<tr>
<td>H117G,(im)</td>
<td>149±17</td>
<td>~240(^c)</td>
<td>62.7</td>
<td>54.5±3.9</td>
<td>-22±1</td>
</tr>
<tr>
<td>M121H</td>
<td>21±4</td>
<td>194(^d)</td>
<td>58.3</td>
<td>28.0±2.1</td>
<td>-127±8</td>
</tr>
</tbody>
</table>

\(^a\) Calculated from the measured Cu(II)/Cu(I) electrode potentials and assuming \(E^0 = -410\) mV vs SHE for the RSSR/RSSR\(^-\) couple in all the azurins studied here. \(^b\) Taken from [Farver et al., 1989] \(^c\) Estimated value at pH 7.0 on basis of 270 mV at pH 6.0, See chapter 4 \(^d\) Taken from [Kroes et al., 1996b]
His46Gly and His117Gly azurin mutants from Pseudomonas aeruginosa

Pulse radiolytic reactions were performed on His117Gly azurin in the presence and absence of imidazole and on His46Gly azurin. Due to a high dissociation constant of the His46Gly azurin-imidazole complex (2.7 mM [van Pouderoyen et al., 1996a] compared to 24 µM for His117Gly azurin, see chapter 4) pulse radiolytic experiments on His46Gly azurin copper site reconstituted with imidazole could not be performed.

The reaction pattern for His117Gly azurin with imidazole is quite similar to P.a. WT azurin (vide supra). No intermolecular ET was observed, and a rate constant for intramolecular ET from RSSR\(^-\) to Cu(II) of 149 ± 17 s\(^{-1}\) was determined at 25 °C, which is higher than for WT azurin (Table 1). The activation parameters have also been determined (see Figure 2, Table 1). Data from P.a. WT azurin [Farver et al., 1989] have been included in Table 1.

The reaction pattern for His117Gly azurin in the absence of imidazole shows similarities to Met121His azurin, in the sense that both mutants exhibit considerable absorption at 410 nm. In contrast to Met121His azurin, however, a discrepancy was found in His117Gly azurin between the ET rate constants determined at 410 nm and at 628 nm, the first wavelength giving higher values at temperatures above 15 °C. Since the rate constants have decreased six-fold compared to WT azurin, this is most likely due to a competing RSSR\(^-\) dismutation reaction increasing the observed ET rate at 410 nm. In this reaction two RSSR\(^-\) anions react to produce a reduced disulfide (2 RSH) and RSSR. Therefore, the parameters presented in Table 1 are based on 628 nm
data, only. His46Gly azurin also exhibits slow intramolecular ET rates. Since His46Gly azurin only has very weak absorption at 630 nm, the Cu(II) reduction could not be monitored independently from the RSSR\(^-\) anion oxidation and/or dismutation. However, at temperatures above 15 °C two separate exponential decays could be distinguished for the absorption traces at 410 nm (see Figure 3). The slower exponential decay (at ~ 2 s\(^{-1}\) at 30 °C) was still observed in samples being fully reduced, ruling out the possibility that this signal is due to ET from RSSR\(^-\) to Cu(II). Using the faster exponential decay (for 20 °C and higher) the parameters were obtained that are presented in Table 1. Like for His117Gly azurin the ET rate is lower than in WT azurin.

**Discussion**

The semiclassical Marcus theory for non-adiabatic processes predicts that intramolecular ET in proteins is governed by the driving force of the reaction (\(\Delta G^0\)), the nuclear reorganization energy (\(\lambda\)) and the electronic coupling (\(H_{DA}\)) between electron donor (D) and acceptor (A) at the transition state [Marcus et al., 1985]:

\[
k_{ET} = \frac{4\pi^3}{h^2 \lambda RT} H_{DA}^2 \exp \left( -\frac{(\Delta G^0 + \lambda)^2}{4\lambda RT} \right)
\]

(1)

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

\[
H_{DA} = H_{DA}^0 \exp \left[ -\frac{\beta}{2} (r_{DA} - r_{\text{van der Waals}}) \right]
\]

(2)

Using the experimentally determined rate constants and activation parameters of 23 different WT and mutated azurins (including the values reported here), while assuming that the reorganisation energy among these azurins is similar, values for \(\lambda\) (119±29 kJ/mol) and \(\beta(r-r_0)\) (24.0±1.9) are calculated (see Figure 4). [Farver et al., 1993; Farver et al., 1996a]

**Met121His azurin**

Spectroscopic and structural studies have revealed the simultaneous occurrence of multiple conformations of Met121His azurin [Kroes et al., 1996b; Messerschmidt et al., 1998; Salgado et al., 1998]. However, at neutral pH a species with a tetrahedral copper site is
dominant. In this tetrahedral copper site His121 coordinates directly to the copper ion with a Cu-N$^{δ1}$ bond distance of 2.22 Å. This is in contrast to the Cu-S$^{δ}$ (Met121) distance of 3.11 Å in WT azurin [Messerschmidt et al., 1998].

Spectroscopic studies show that the novel structure in Met121His azurin perturbs the electronic character of the copper ion, creating a so-called type 1.5 site at neutral pH [Kroes et al., 1996b]. Instead of the normal single intense absorption band around 600 nm observed for blue copper proteins, this mutant exhibits two strong absorption bands at 439 and 593 nm, which results in the green colour of the protein. Like the 593 nm transition, the band at 439 nm results from a ligand to metal charge transfer transition [LaCroix et al., 1996; Pierloot et al., 1998]. Usually the intensity of this band is low in native blue copper proteins, but in the Met121His variant the two bands both have appreciable intensity. The EPR spectra show an increase in hyperfine splitting ($A_d$) from 62 cm$^{-1}$ in WT azurin [Groeneveld et al., 1987] to 102 cm$^{-1}$ in Met121His azurin [Kroes et al., 1996b].

The *A. d.* Met121His azurin kinetics should be compared with those of *A. d.* WT azurin. In Met121His the rate is about half that of WT azurin. According to eq. 1, the smaller rate constant can be accounted for by the lower driving force of Met121His azurin (~58.3 kJ/mol as opposed to ~69.0 kJ/mol for *A. d.* WT azurin, see Table 1). This would mean that the mutation does not alter $λ$ or $H_{DA}$ to a large extent. This is visualised by Figure 4, in which the rates are plotted against the driving force of a large number of (mutant) azurins: His121Met azurin falls within the spread of the experimental points. Apparently, going from an axial type-1 to a rhombic type-1 or type-1.5 copper site is of little influence to the reorganisation energy of the copper site.

**Figure 4:** The ET rate constants at 298 K ($k_{298K}$) plotted against the driving force ($-ΔG^0$) at 298 K for several azurins and mutant azurins. Data from table 1 and from Ref. [Farver et al., 1997b] and references therein. The numbered data points are from: 1. Val31Trp azurin; 2. His117Gly$_{im}$. The line represents the best fit to the equation:

$$k_{298K} = 10^{13} e^{-\beta(r\cdot τ_0)} e^{-\frac{(ΔG^0 + λ)}{4kRT}}$$

with $λ = 119$ and $β(r\cdot τ_0) = 24.0$. The numbered data points (1 and 2) were not used to fit the data.
Electron transfer in azurin mutants

**His46Gly and His117Gly azurin**

Pathway calculations for the above intramolecular ET were performed according to the structure-dependent pathway model developed by Beratan and Onuchic [Regan et al., 1993; Skourtis et al., 1994] using the high-resolution three-dimensional structures of *Pseudomonas aeruginosa* and *Alcaligenes denitrificans* azurins [Baker, 1988; Nar et al., 1991a]. In this model, the overall electronic coupling of a “pathway” is given as a repeated product of the couplings of the individual links ($\varepsilon_i$). The optimum electronic coupling between the two redox sites is then identified as the “pathway” with the largest coupling ($\Pi\varepsilon_i$). Previous calculations have predicted similar electron transfer routes in both azurins [Farver et al., 1992b; Farver et al., 1993]: One longer path proceeds through the peptide chain from Cys3 to the copper-ligating imidazole of His46 (the “His46” pathway) and a second, shorter, path starts from Cys3 and goes through the buried indole ring of Trp48 to the copper ligating thiolate of Cys112 (the “Trp48” pathway). The electronic coupling decay factors were found to be $\Pi\varepsilon_i = 2.5\times10^{-7}$ and $3.0\times10^{-8}$, respectively [Farver et al., 1993]. However, in this analysis the electronic interactions between the Cu(II) ion and its ligands were not included. Thus, a high degree of anisotropic covalency has been shown to exist in the blue single-copper protein, plastocyanin, that would enhance ET through the Cys ligand [Christensen et al., 1990; Lowery et al., 1993]. By similar arguments, from the ligand coefficients of $\psi_{\text{HOMO}}$ in azurin calculated by Larsson et al. [Larsson et al., 1995] it can be estimated that ET through the Cys112 ligand would be enhanced by a factor of ~150 over ET via one of the His ligands. This would suggest that the “Trp48” is more effective than the “His 46” pathway. I have therefore studied the influence of substituting His46 with a glycine on the LRET reactivity since this would effectively block one of the pathways where the connections to the copper ion occur via a hydrogen bond from Asn10 to His46 in the WT protein. At the same time, however, this mutation does also modify considerably the coordination sphere of the copper ion and its properties, and therefore, probably also the rate of ET to the copper site. To further investigate the influence of the change in the electronic properties, the kinetics of ET in a similar mutant, His117Gly azurin, were studied.

It has been shown that the oxidized copper ion in both mutants is accessible to external ligands, which, upon coordination to Cu(II), change the spectroscopic features of the mutants [den Blaauwen et al., 1993b; den Blaauwen et al., 1993a; van Poudroyen et al., 1996a]. For example, without external ligands, His117Gly azurin exhibits pronounced optical absorption-bands at 420 nm and 628 nm, which results in a green colour. His46Gly
Azurin also exhibits bands at 400 and 630 nm, although the absorption at 630 is very weak and cannot be used for monitoring the copper oxidation state in the pulse radiolytic experiments. If imidazole is added, the mutants turn blue with main absorption bands at 628 (His117Gly) and 621 nm (His46Gly), which is almost identical to WT-azurin (626 nm) [den Blaauwen et al., 1993b; den Blaauwen et al., 1993a; van Pouderoyen et al., 1996a]. Also other spectroscopic features are restored, like EPR features and Resonance Raman spectra [Coremans et al., 1995; den Blaauwen et al., 1993a; den Blaauwen et al., 1993b; van Gastel et al., 1998; van Pouderoyen et al., 1996a]. This implies that the structures of the mutants and the geometry of their metal site are maintained despite the replacement of the histidine. If no external ligands are present, water molecules are coordinated to the copper ions [Kroes et al., 1996a].

From Table 1 and Figure 4 it is quite obvious that the rate of LRET is not seriously perturbed by the absence of His46. The comparable rate constants in the two mutants where a His ligand has been substituted by a non-ligating Gly (15 s\(^{-1}\) for His46Gly and 7 s\(^{-1}\) for His117Gly,\(\text{aq}\)) suggest that the dominating pathway for LRET does not proceed via the imidazole ring of His46. This supports the previous hypothesis that in azurin the “Trp48” route from Cys3 to the copper ligand, Cys112, is dominant [Farver et al., 1996a]. These results illustrate the importance of the anisotropic covalency at the blue copper sites of electron transfer: although the electronic coupling decay factor is calculated to be ~10 times lower for the “Trp48” route, electron transport is still preferred via the Cys112.

The lower rate constants compared to WT azurin could be caused by a decreased driving force or an increased reorganisation energy. Even the electronic coupling constant may be important since spectroscopy of His46Gly and His117Gly,\(\text{aq}\) suggests that the electronic structure of the (Cys)\(^2\)-Cu(II) bond is altered (vide supra). However, since the driving force of His117Gly,\(\text{aq}\) and His46Gly is not known nothing can be concluded as yet. From Figure 4 it can be seen that if the absolute value of the driving force (\(\Delta G^0\)) would be less than ~65 kJ/mol, the rate is still with the spread observed of previously determined rates of (mutant) azurins.

Interestingly, the rate of His117Gly azurin, in which the copper site has been restored by the external ligand imidazole (His117Gly,\(\text{im}\)), is about three fold higher than wt azurin (149 vs 44 s\(^{-1}\)). For His117Gly,\(\text{im}\) a reduction potential of ~240 mV at pH 7.0 is estimated on basis of the rapid cyclic voltammetry experiments reported in chapter 4. This
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corresponds to a decrease in the absolute value of the driving force ($|\Delta G^\circ|$) from 68.9 to ~63 kJ/mol, which according to Eq(1) results in a slower rate constant when $\lambda$ and $H_{DA}$ would remain constant. This is opposite of what is observed for His117Gly,im, thus, the rate of His117Gly,im clearly deviates from other observed rates (see Figure 4). This would suggest that in His117Gly,im a change in the electronic coupling is responsible for the observed rate constant. This is supported by the observed change in the entropy of activation (see Table 1), which term is dependent on the electronic coupling. The increase from 44 to 149 s$^{-1}$ in the ET rate can be accounted for by reducing the exponential term in Eq.2, $\beta (r - r_0)$, from 24.0 for wt azurin [Farver et al., 1992b] to 22.3 for His117Gly azurin.

The spectroscopic properties as well as the (Cys)$S^\gamma$-Cu(II) distance (see chapter 5) of His117Gly,im are comparable to wt azurin. This indicates that the electronic character of the (Cys)$S^\gamma$-Cu(II) bond of both proteins is the same, ruling out that this bond is responsible for a change in the electronic coupling. Therefore, a structural change of or close to the electron pathway seems responsible for the increase in the rate constant. A similar conclusion has been made for the observed rate constants of wt azurin at pH 4 [Farver et al., 1996b] and Val31Trp azurin [Farver et al., 1997b]. However, the crystal structures that are available for wt azurin at low pH do not show any conformational change in the calculated electron pathway region compared to structures at neutral or high pH [Nar et al., 1991a].

Entropy-enthalpy compensation

In Figure 5A the activation enthalpy is plotted against entropy for a range of azurins and azurin mutants. As is clearly visible, the entropy and enthalpy show a linear relationship. This enthalpy-entropy compensation is commonly found for ‘ranges of similar reactions’, while its slope is observed to be between 250-315 K when the reactions are performed in water [Leffler, 1955; Lumry et al., 1970]. Hypotheses explaining entropy-entropy compensation include (a) shifts in concentrations of two phenomenologically significant species of water and (b) solvent (=water) reorganization [Grunwald et al., 1995; Lumry et al., 1970]. While the linear behaviour is thought to be due to a property of water, deviation of the linear relationship of a certain data-point is explained by a diverging property of that typical reaction.
Care must be taken with detecting enthalpy-entropy compensation, since during the determination of enthalpy and entropy via the Eyring-plot, the errors in both parameters are connected. To circumvent this problem one can plot the independent parameters: activation energy and activation enthalpy ($\Delta G^\#$ and $\Delta H^\#$) which is shown in Figure 5B. By implementing the enthalpy-entropy compensation condition ‘$\Delta H^\# = \alpha + \beta \Delta S^\#$’ into $\Delta G^\#_T = \Delta H^\# - T \Delta S^\#$, the following equation can be derived:

$$\Delta G^\#_T = \alpha(T/\beta) + \Delta H^\#(1-T/\beta)$$ (3)

With this equation the linear enthalpy-entropy compensation with a slope of 252 ± 5 K is confirmed. However, from this plot also a few data-points are recognised which do not correspond to the relationship. The activation parameters that most clearly deviate are A. faecalis wt azurin and from Val31Trp azurin. Val31 is part of the “Trp48” electron tunnelling pathway from disufhde bridge to the copper site (vide infra). Interestingly, wt azurin from A. faecalis is special among azurins in that it has an Ile at position 31 instead of a Val. It also does not contain a Trp at position 48, but a Val. This supports the
hypothesis that ET proceeds via the “Trp48” pathway. Besides these two data-points also Asp23Ala, Ile7Ser and Phe110Ser azurin do not comply to the linear relationship, although their deviation is less extreme. Ile7 and Phe110 are two residues which are in close contact to the Trp48, while Asp23 is in close contact with the disulfide bridge.

**Conclusion**

Evidently, single site mutations at the metal coordination produce multiple changes in the ET reactivity and will affect more than one of the four significant parameters that govern LRET: Driving force, reorganisation energy, distance and nature of the protein matrix. Still, taken together the above results indicate that the Trp48 ET pathway seem to be dominating relative to the His46 pathway.