Chapter 4

Electron transfer and complex formation in a pH sensitive dimer of azurin

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Abstract

A dimer of N42C azurin (P. aeruginosa), formed by intermolecular disulfide bond formation between the introduced surface exposed cysteine residues, was previously shown to exhibit high rates of intermolecular electron self-exchange. The work presented in this chapter aims to render the construct sensitive to pH by introduction of an additional surface mutation (M64E). The effects of ionisable residues in the dimer interface were analysed as a function of pH. A crystal structure of the dimer at pH 3.1 was solved to 2.3 Å resolution. It shows a complex orientation distinct from other known azurin complexes. The dimer exhibits slow intramolecular self-exchange at pH 4.0 ($k_{\text{ese}}^{\text{intra}} \approx 100 \text{ s}^{-1}$), attributed to the large Cu-to-Cu distance of 20.5 Å. Deprotonation of several interfacial residues at high pH results in an approximately 3-fold decrease of $k_{\text{ese}}^{\text{intra}}$. This decrease is tentatively ascribed to an increase of the Cu-to-Cu distance as a result of pH induced conformational changes of the dimer around the site of crosslinking. Intermolecular electron self-exchange was found to be almost absent, irrespective of pH.
**Introduction**

Protein-mediated electron transfer (ET) is a key component of biological processes in which electrons are generated and passed on to acceptor proteins. Often the partners are remote from each other in space and auxiliary electron carrier proteins are required. In order to achieve optimal efficiency, shuttling of electrons should not only proceed with sufficient specificity and affinity but complexes also need to rapidly dissociate after the transfer event. Complex formation is consequently a delicate balance between the various interactions regulating the contact between the proteins involved and many studies of ET complexes are aimed at unravelling these interactions. This is, however, often complicated by the inherently short lifetimes of the complexes.

A common method to render ET complexes more accessible to analysis is covalent crosslinking, for instance by site-directed introduction of cysteine residues on the protein surface. Under oxidizing conditions these cysteines react to form a covalent intermolecular disulfide bond. This technique has, for instance, been applied to achieve dimerization of the small blue copper protein azurin from *P. aeruginosa*. Although the resulting dimer is non-physiological, it serves as a simple and elegant model system for ET complexes. In the case of the N42C azurin dimer it was found that intermolecular disulfide bond formation resulted in a sterically hindered complex incapable of intramolecular electron self-exchange (e.s.e.). However, it was still capable of e.s.e. between separate dimers albeit with significantly reduced efficiency compared to the monomeric WT protein. These e.s.e. properties in solution have been related to the crystal structure of the complex.

Building on these results, we have introduced ionisable groups on the protein surface in the vicinity of the dimer contact region. It has previously been demonstrated that the introduction of a negative charge at position 64, situated in the so-called hydrophobic patch of the protein, results in a marked decrease of \( k_{\text{e.s.e.}} \) at high pH, due to electrostatic repulsion in the contact surface. By simultaneous introduction of ionisable and chemically reactive groups in the protein interface we have attempted to construct an ET complex with pH controlled structural and electronic properties. To this end the double mutant N42C/M64E azurin was constructed. The system’s e.s.e. properties were analysed by monitoring the redox sensitive ¹H NMR methyl resonance of Val31. Since it was found that the Val31 resonance is also sensitive
to pH induced conformational changes in the protein as a result of deprotonation/protonation of His35, an additional H35F mutation was introduced to eliminate any unnecessary variables from the e.s.e. rate analysis. The H35F/N42C/M64E azurin dimer showed low rates of intramolecular e.s.e. that are only mildly pH dependent. A structure of the N42C/M64E dimer at pH 3.1 was solved (2.3 Å) by X-ray diffraction and is discussed in connection to the pH dependent solution behaviour of the H35F/N42C/M64E azurin dimer.

### Materials and Methods

#### Mutagenesis
The secondary mutation M64E was introduced into the N42C azurin gene by three step PCR-based site-directed mutagenesis for which two partially complementary oligonucleotides were designed. The forward primer encodes for the replacement of methionine by glutamate: 5'-GGGCGTGCTCACCGACGGCGAGCGGTCTCCG. The reverse primer includes two silent mutations that encode a PstI restriction site for screening purposes: 5'-GTGACCACGCCCTGATGTCTGCAGCGGTGCT. The underlined section shows the regions of complementarity in the two primers, the mutation sites are shown in bold. As a template, the N42C azu containing plasmid pIA02, derived from pUC18, was used. The presence of the N42C/M64E azurin gene in the newly created plasmid pTJ01 was confirmed by sequence analysis and the obtained plasmid was then used as a template for the one-step synthesis of the H35F/N42C/M64E azurin gene in a procedure based on the Stratagene’s ExSite® PCR-Based Site-directed Mutagenesis Kit. Two fully complementary 32 base pair long oligonucleotides encoding the His35Phe mutation were used as primers: (forward primer) 5'-CGGTCAACCTGTCCCTGATGTCTGCAGCGGTGCT. The mutated codon for the H35F substitution is indicated in bold and an additional silent mutation that was introduced for screening by XmaI digestion is underlined and in italics. Sequence analysis confirmed the presence of all desired mutations in the resulting pTJ02 plasmid. The H35F/N42C/M64E azurin gene was subcloned into a pET28a vector for cytoplasmic expression by removal of the signal sequence for localization to the periplasmic space. Gene amplification was performed using primers for N-terminal signal peptide removal (5'-GGTCAGCGCCATGGCAGGTGC TCGGTG) and addition of a C-terminal BamHI/BstYI recognition site (5'-CCAGGATCCGACATCG AGCCCAGGT). Insertion into a pET28a vector was accomplished by digestion of the gene fragment using the restriction enzymes BamYI
and NcoI. The vector was digested by the enzymes BamHI and NcoI, after which the gene and vector fragments were ligated, resulting in the plasmid named pTJ13.

Protein expression and purification
Wild type and H35F azurin were expressed and purified as previously described.\cite{4,5} Non-isotopically labelled N42C/M64E azurin and H35F/N42C/M64E azurin were produced in the periplasmic space of *E. coli* JM109 cells transformed with plasmids pTJ01 or pTJ02 respectively. Cells were cultured on LB medium at 37°C/250 rpm to an OD$_{600}$ of 0.6 before induction by addition of 100 μM IPTG. Cells were harvested by centrifugation typically about 5 hours after induction. Both mutants were isolated and purified from the medium as described for N42C azurin with yields of 10-15 mg/L.\cite{1} Up to 50% of the purified azurin was found to contain zinc in the metal binding site and was thus redox-inactive. Uniformly $^{15}$N labelled H35F/N42C/M64E azurin was expressed in the cytoplasm of BL21 *E. coli* cells transformed with pTJ13 and grown on isotopically labelled OD2-N medium (Silantes GmbH) supplemented with 50 μM kanamycin at 30°C/200 rpm. Once an OD$_{600}$ of 0.5 was obtained, the temperature was lowered to 25°C 30 minutes prior to induction with 1 mM IPTG. Due to lower growth rates, cells grown on OD2-N medium were not harvested until 15 hours after induction. The cytoplasmically expressed protein was released from the cells by resuspension in 20 mM MES, 100 mM NaCl pH 6.5 and disruption of the cells in a French Press cell in the presence of phenylmethylsulfonyl fluoride (PMSF) (1mM) and DNase I (50 mg/mL). After centrifugation, the protein was isolated from the resulting supernatant following a protocol similar to that used for periplasmically expressed azurin. The protein was initially purified in the apo-form and reconstituted with copper before further purification on a DEAE ion exchange column.

Crosslinking
Disulfide linked dimers of N42C/M64E and of H35F/N42C/M64E azurin were spontaneously formed upon addition of a slight molar excess of Cu(NO$_3$)$_2$ and an approximately 5-fold excess of K$_3$Fe(CN)$_6$. Excess Cu(NO$_3$)$_2$ and K$_3$Fe(CN)$_6$ were subsequently removed by repeated rounds of dilution and concentration using a Centricon concentration device (MWCO 10 kDa). Analysis of the protein sample by SDS-PAGE under non-reducing conditions showed nearly complete dimerization.
NMR sample preparation

Fully reduced samples were obtained by incubation with 2 mM ascorbate at RT until the sample had turned completely colourless at which point ascorbate was removed from the sample by washing with deaerated 25 mM potassium phosphate (KP), pH 7.0. All NMR samples of unlabelled protein were prepared in 25 mM KP, pH 7.0* (*uncorrected for deuterium isotope effect), 99.9% D$_2$O and concentrated to 1 or 2 mM of protein. For concentration dependent experiments, the sample was diluted several times in 25 mM KP, pH* 7.0. The pH of the samples was adjusted to desired pH by addition of small amounts of NaOD or DCl. Partially oxidized samples were attained by mixing of fully oxidized and fully reduced protein in suitable ratios. The degree of oxidation of the samples was determined by measuring the absorbance at 628 nm relative to the fully oxidized sample of the same concentration. UV-Visible spectra were recorded on a Perkin Elmer lambda 800 spectrophotometer using a special sample holder for NMR tubes equipped with fibre-optic light guides (Hellma).

A sample of fully reduced, monomeric$^{15}$N-H35F/N42C/M64E azurin was prepared in 25 mM KP, pH 5.7, 5 mM dithiotreitol (DTT) supplemented with 6 % D$_2$O and concentrated to 2 mM. The pH of the sample was adjusted by addition of small volumes of NaOD or DCl. After recording of the NMR spectra, DTT was removed by repeated rounds of concentration and dilution with 25 mM KP. Dimerization of the protein was accomplished by addition of a catalytic amount of Cu(NO$_3$)$_2$ and an approximately 10-fold molar excess of K$_3$Fe(CN)$_6$. Once fully dimerized, confirmed by SDS-PAGE analysis, the sample was transferred into 25 mM KP, pH 4.3. Selective reduction to Cu(I) azurin without disruption of the intermolecular disulfide bond was performed by addition of 2 mM ascorbate. Titration to desired pH was achieved by addition of small amounts of either NaOH or HCl.

NMR

All $^1$H NMR experiments were performed at 295 K on a Bruker Avance DMX 600 MHz spectrometer. All $^1$H spectra were calibrated against an internal reference of 200 μM 3-(trimethylsilyl)propionate-d$_4$ (TSP). As the chemical shift of TSP (δ) is sensitive to pH (pK$_a$ = 5.0), its reference position was corrected for pH according to equation 1:

$$\delta = 0.003 - 0.019*(1+10^{5.0-pH})^{-1}$$
In addition to the Val31 $^1$H signal of the oxidized protein, the spectra of the fully oxidized samples indicated the presence of a small amount (~5%) of an additional species for which a low intensity resonance was observed around -0.69 ppm at all pH values. This feature is commonly encountered in samples of azurin and is attributed to the presence of a small amount of redox-inactive azurin. This resonance was subtracted from the experimental spectra shown in Figure 4.2.

Multidimensional NMR experiments were performed at 302K. Data sets were processed in the AZARA program suite (available at http://www.bio.cam.ac.uk) and analyzed with ANSIG for WINDOWS 1.0. Backbone assignments were obtained through standard sequential assignment procedures using the [$^{15}$N, $^1$H] HSQC, [$^{15}$N, $^1$H, $^1$H] NOESY and [$^{15}$N, $^1$H, $^1$H] TOCSY spectra on the basis of assignments available for WT azurin.

*Electron self-exchange rate determination*

E.s.e. rates were determined by simulation of the line shape of the Val31 methyl $^1$H NMR resonance using the MEX/MEXICO program suite developed by Bain and Duns. All possible intermolecular and intramolecular exchange processes were taken into account (see Supplementary Material). The chemical shifts and linewidths of the fully oxidized and fully reduced protein were given as input parameters whilst both the inter- and intramolecular e.s.e. rates were manually iterated until simulations were obtained that most closely resembled the experimental traces as judged by visual comparison.

*Crystallization and structure solution*

The disulfide bridged dimer of N42C/M64E azurin was crystallized by sitting drop vapour diffusion using a reservoir solution containing 30 % (w/v) of polyethylene glycol 4000 and 0.1 M sodium citrate buffer at pH 3.1. The protein was concentrated to 4 mg/ml and mixed in a 1:1 ration with the reservoir solution, and rod-shaped crystals grew within two days. Due to the high content of PEG 4000, the mother liquor turned out to be sufficiently cryoprotective for direct flash-cooling of the crystals in liquid nitrogen without formation of ice. Data were collected on a Rigaku MicroMax 007 rotating anode X-ray generator with a mar345dtb image plate detector. For structure solution, the model of the native azurin from *P. aeruginosa* was used with the program MOLREP. Two monomers were found in the asymmetric
unit, clearly connected through a disulfide bridge between residues 42. The N42C and M64E mutations were modelled with O[11], and refinement was carried out with REFMAC5[12]. Figures 4, 5 and 7 were generated using PyMOL 0.98.[13]

Solvent accessibility and interface areas
The solvent-accessible surface area (ASA) was calculated using the program NACCESS 2.1.1 with a probe radius of 1.4 Å. The interface area was defined as the sum of the ASAs of the individual proteins minus the ASA of the complex. The interface in disulfide bridged dimers of N42C azurin was defined as the average of the two dimers in the asymmetric unit of the structure deposited in the protein data bank under access code 1JVO.[3]

Results

pH dependence of the Val31 methyl 1H resonance
In the NMR spectra of WT and N42C azurin, the proton resonances of the methyl groups of Val31 are known to be sensitive to the redox state of the protein despite the fact that the residue is not situated in the immediate coordination sphere of the redox centre. This has enabled the determination of intermolecular and intramolecular e.s.e. rates in dimers of azurin by simulation of the lineshape as a function of exchange rates at different degrees of oxidation. Unambiguous determination of e.s.e. rates however requires the number of input parameters to be restricted. The pH dependent splitting of the Val31 proton resonance introduces several additional parameters into the model, so that the straightforward analysis of the e.s.e. properties of the N42C/M64E azurin dimer is severely complicated.

This pH dependence is evident for WT azurin as a splitting of the signal into a ‘high pH’ and a ‘low pH’ signal [Figure 4.1A]. The equilibrium between the two signal’s succession is characterized by a pK$_{a,\text{ox}}$ of 6.3 ± 0.1 and a pK$_{a,\text{red}}$ of 7.0 ± 0.1 [Figure 4.1B] and reflects the protonation/deprotonation equilibrium of the non-coordinating His35 residue.[14] Deprotonation of H35 is known to induce a peptide-bond flip between residues 36 and 37.[15] Mutation of H35 to a non-protonatable Phe residue was expected to eliminate this pH dependence of the Val31 proton signal.[5] Analysis of the Val31 proton resonance of the H35F mutant at varying pH shows that indeed the pH dependence has been removed. The splitting of the signal which
is observed for reduced H35F azurin at pH < 5 presumably is the result of another pH induced, unidentified conformational change. Although H35 was once thought to be involved in the e.s.e. reaction of azurin, it was later shown that mutagenesis of H35 to residues such as Phe, Gln and Leu did not significantly affect the protein’s ability to carry out e.s.e.[5,16] Also, these substitutions showed only marginal effects on the global protein-fold and more specifically on the copper-binding site itself. It is therefore assumed that the H35F mutation does not significantly influence the properties of the construct, whilst greatly facilitating determination of e.s.e. rates as a function of pH. All NMR experiments were therefore performed on dimers of H35F/N42C/M64E azurin.

**Figure 4.1:** A) Upfield regions of the $^1$H NMR spectra of WT and H35F azurin at increasing pH showing the Val31 $^1$H$^2$ resonance. B) Normalized intensity of the high pH resonance of Cu(II) ($\bullet$) and Cu(I) ($\circ$) WT azurin vs. pH. Solid lines represent fits to the Henderson-Hasselbalch equation from which $pK_{a,ox} = 6.3$ and $pK_{a,red} = 7.0$ were determined. At pH<5 Cu(I)-H35F azurin shows signs of a conformational change affecting Val31 that is not connected to the P36-G37 bond flip.
Electron self-exchange

The Val31 resonances of the fully oxidized and fully reduced forms of the H35F/N42C/M64E azurin dimer are independent of pH, in agreement with predictions based on H35F azurin [Figure 4.2A,B]. The spectra of partially oxidized protein show line broadening, but not coalescence, of the Val31 resonance of the oxidized and reduced signals indicating that any exchange processes are in the slow exchange regime [Figure 4.2C]. In the slow exchange regime, i.e.:

\[ k_{\text{ese}} \ll \frac{\pi^* \Delta v}{\sqrt{2}} \]

in which \( \Delta v \) represents the chemical shift difference between the positions of the analysed resonances in the fully oxidized and fully reduced protein, inter- and intramolecular e.s.e. exert very similar effects on the linewidths of the spectra, rendering unambiguous discrimination between the two processes solely based on the spectra obtained at a fixed protein concentration rather difficult. The experimental spectra were most closely reproduced by simulations that assumed the simultaneous occurrence of both processes. The different effects of inter- and intramolecular exchange on the Val31 proton resonance of azurin are demonstrated in Figure II of the Supplementary Material. From these simulations \( k_{\text{ese} \text{ intra}} \) is estimated as \( \approx 100 \, \text{s}^{-1} \). It is only slightly dependent on pH with a less than 2.4-fold decrease in exchange rate towards high pH [Table 4.1]. The intermolecular rate \( k_{\text{ese} \text{ inter}} \) on the other hand is found to be largely independent of pH with \( k_{\text{ese} \text{ inter}} \leq 3 \times 10^4 \, \text{M}^{-1} \text{s}^{-1} \).

**Table 4.1**: \( k_{\text{ese} \text{ intra}} \) and \( k_{\text{ese} \text{ inter}} \) corresponding to the simulations of the Val31 
H resonance shown in Figure 4.2. The intermolecular rate \( k_{\text{ese} \text{ inter}} \) is given by the relationship \( k_{\text{ese} \text{ inter}} = k_{\text{ese} \text{ inter}} \ast 0.5 \ast [\text{dimers}] \) (see Supplementary Material).

<table>
<thead>
<tr>
<th>pH</th>
<th>( k_{\text{ese} \text{ intra}} \left(10^4 , \text{M}^{-1} , \text{s}^{-1}\right))</th>
<th>( k_{\text{ese} \text{ inter}} \left(\text{s}^{-1}\right))</th>
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<tr>
<td>4.0</td>
<td>2.8 ± 0.4</td>
<td>90 ± 10</td>
</tr>
<tr>
<td>4.7</td>
<td>2.0 ± 0.4</td>
<td>60 ± 5</td>
</tr>
<tr>
<td>6.5</td>
<td>2.8 ± 0.4</td>
<td>30 ± 5</td>
</tr>
<tr>
<td>7.4</td>
<td>3.0 ± 0.6</td>
<td>40 ± 5</td>
</tr>
<tr>
<td>8.2</td>
<td>2.6 ± 0.4</td>
<td>40 ± 5</td>
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A more detailed analysis makes use of the concentration dependence of the e.s.e. rates at pH 4.5. Starting at an initial protein concentration of 2 mM, the sample was stepwise diluted until a dilution factor of 10 was reached. Simulation of the spectra shows that the exchange contributions on the line broadening consist of a weak, concentration dependent, intermolecular exchange contribution \( k_{\text{ese \ inter}} = (1.8 \pm 0.1) \times 10^4 \text{M}^{-1}\text{s}^{-1} \) and a more dominant, concentration independent, intramolecular contribution [Figure 4.3]. The thus observed \( k_{\text{ese \ intra}} \) is about 3-fold lower than that determined in the pH dependency experiment at pH 4.7 and is in fact much closer to the rates determined at pH > 7. This discrepancy could be connected to small differences in line width of the Val31 resonances of the fully oxidized and reduced protein (\( \Delta w = 2.5 \text{ Hz for reduced and } \Delta w = 0.6 \text{ Hz for oxidized samples} \) due to field inhomogeneities. The conclusion that can be drawn from these experiments is that, regardless of the exact exchange rates, both the inter- and intramolecular electron self-exchange are in the slow-exchange regime.

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**Figure 4.2:** Val31 methyl resonance in the 'H NMR spectra of H35F/N42C/M64E di-sulfide azurin at (A) 100%, (B) 0% and (C) 50-60% oxidation (1mM protein in 25 mM KP, T=295K). The upper traces represent the experimental data whereas the simulated spectra generated using the MEXICO software program are shown in the lower traces. The experimental spectra have been corrected for the presence of a small (~5%) amount of redox-inactive azurin with a resonance around -0.69 ppm.
Crystal structure of disulfide bridged N42C/M64E azurin

The structure of the N42C/M64E azurin dimer at pH 3.1 was solved to 2.3 Å resolution [Table 4.2]. Comparison of each of the subunits with WT azurin (PDB access code 1E5Y) shows that they are largely unaffected by the introduction of the mutations with an overall backbone atom RMSD of 0.6 Å, excluding the N- and C-terminal residues. The regions directly around the sites of mutation are relatively unperturbed. The two subunits share a contact surface, with the contacts between the two surfaces consisting mainly of weak hydrophobic interactions, in a manner that is clearly distinct from the classical hydrophobic packing in crystals of WT azurin [Figure 4.4]. In the dimer interface two water molecules are found that form several hydrogen bonds with the protein. A hydrogen bonding network across the interface is formed which stabilises the conformation of the dimer by forming hydrogen bonds between each of the waters to Glu64 O\(^-\) or the backbone oxygen atoms of Cys42 on both protein chains [Figure 4.5]. The shortest Cu-to-Cu distance observed is the intramolecular distance of 20.5 Å. All Cu-to-Cu distances between dimers in adjacent asymmetric units within the crystal exceed 25 Å. These
intermolecular interactions can therefore be considered irrelevant for the electron self-exchange analysis.

**Figure 4.4:** Stereo tube representation of the structure of the disulfide linked dimer of N42C/M64E azurin at pH 3.1 (dark) (2.3 Å) superimposed on the crystal packing orientation of wild type azurin at pH 5.5 (light) (1E5Y, 1.98 Å). Copper atoms are depicted as spheres, the 42C-42C disulfide bond is shown in stick rendering. [Full colour image shown on page 182].

**Table 4.2: Data collection and refinement statistics**

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<th>Value</th>
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<tr>
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</tr>
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<td>r.m.s.d. angles</td>
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</tr>
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Chapter 4

Chemical shift perturbation analysis of the dimer interface

The $^1$H, $^{15}$N amide assignments for monomeric Cu(I)-H35F/N42C/M64E azurin at pH 5.7 were obtained by comparison to assignments for WT azurin. Additional information was obtained by the use of sequential NOEs in the $^{15}$N-$^1$H HSQC NOESY spectra and sidechain spin systems obtained from the $^{15}$N-$^1$H HSQC TOCSY spectrum were used to confirm assignments. Residues G37, L39, G45, I87, G90 and K122 could not be assigned. With the exception of K122, all of these are situated on flexible loops in the vicinity of the crosslinking site. Assignments of monomeric protein at pH 4.4, 5.9 and 8.0 and of the dimeric complex at pH 4.3, 5.7, 7.0 and 8.4 were derived by comparison with the assigned spectrum of monomeric H35F/N42C/M64E azurin at pH 5.7.

**Figure 4.5:** Crystal structure of the N42C/M64E azurin disulfide-bridged dimer at pH 3.1. The copper centres are depicted as large spheres (black). Close-up: Interface of the dimer showing the ordered water molecules (small grey spheres). All residues involved in hydrogen bonding with these water molecules are shown (sticks) and labelled. The hydrogen bonds are shown as dashed lines. The letters between brackets indicate the individual protein chains. Also shown are the Cu ligands H117 (sticks). [Full colour image shown on page 182].
Figure 4.6A,B: $^{15}$N,$^1$H HSQC spectra of H35F/N42C/M64E azurin. Black contours represent monomeric azurin, grey contours the corresponding disulfide bridged dimers. A) monomer at pH 4.4, dimer at pH 4.3, B) monomer at pH 5.7, dimer at pH 5.6. Close-ups show the regions around resonances 42, 43 and 44. Residues that are shifted in the dimer are indicated with arrows. [Full colour image shown on page 183].
**Figure 4.6C,D:** $^{15}$N,$^1$H HSQC spectra of H35F/N42C/M64E azurin. Black contours represent monomeric azurin, grey contours the corresponding disulphide bridged dimers. C) monomer at pH 5.9, dimer at pH 7.0, D) monomer at pH 8.0, dimer at pH 8.4. Close-ups show the regions around resonances 42, 43 and 44. Residues that are shifted in the dimer are indicated with arrows. [Full colour image shown on page 184].
Superposition of the spectra of the dimer at pH 4.3 and that of the monomer at pH 4.4 shows few significant changes. Residues C42, V43 and M44 have disappeared completely from the spectrum upon dimerization, as have two additional unidentified resonances, suggesting that in the dimer these residues are involved in an exchange process that broadens their signals beyond detection [Figure 4.6A]. At pH 5.7 and above, these resonances have reappeared at slightly shifted positions [Figure 4.6B-D]. Above pH 6, low intensity resonances at these same positions start to appear for V43 and M44 in the spectra of monomeric H35F/N42C/M64E azurin. It seems that the shifts occur as a result of an unidentified protonation equilibrium that in the dimerized form of the protein has been shifted towards lower pH. It can not be distinguished whether the exchange broadening at pH ≤ 4.4 is due to conformational exchange or a protonation equilibrium. It is, however, evident that these effects do not affect a large part of the protein surface and that the H35F/N42C/M64E dimer does not form a very tight interface at any pH.
Discussion & Conclusion

The aim of the work presented here was to construct an artificial protein complex with pH dependent structural and ET properties. To this end, a covalently crosslinked homodimer of the blue copper protein azurin was created by intermolecular disulfide bond formation between engineered surface exposed cysteines. The pH dependence was introduced by substitution of the interfacial M64 by an ionisable Glu residue. Analysis of the system’s electron transfer rate as a function of pH required additional replacement of the protonatable H35 residue by Phe. It was shown that over the entire pH range studied, the complex exhibited slow electron self-exchange. Although no unambiguous distinction could be made between the inter- and intramolecular contributions to the e.s.e. process, the limited concentration dependence of the observed e.s.e. rate identifies intramolecular exchange as the more dominant process \( k_{\text{ese}}^{\text{intra}} \leq 100 \text{ s}^{-1} \).

Despite the presence of several ionisable groups in the interface, the electron self-exchange behaviour of the complex shows only very moderate pH dependence. The slow rates of exchange observed for the H35F/N42C/M64E dimer are in clear contrast to that of the N42C azurin dimer for which a much higher \( k_{\text{ese}}^{\text{inter}} \) was determined \( (2.8 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}) \). It is tempting to relate this difference to the clear difference between the crystallographic structures of the two complexes. Whereas the N42C dimer had adopted a rather ‘open’ dimer conformation \( (1.8 \times 10^2 \text{ Å}^2) \) with an intramolecular Cu-to-Cu distance >25 Å, the N42C/M64E dimer at pH 3.1 forms a much more closed conformation \( (7.0 \times 10^2 \text{ Å}^2) \) in which the intramolecular Cu-to-Cu distance is only 20.5 Å. Although this distance is still rather large, it is now within the limits that will allow for intramolecular e.s.e., albeit with very low rates.

A framework in which biological ET rates are often discussed is the model described by Dutton et al. that assumes an exponential decay of \( k_{\text{ET}} \) with the distance separating the redox centres through a homogeneously packed medium following the empirically derived equation:\(^{[17,18]}\)

\[
\log k_{\text{ET}} = 13.0 - (1.2 - 0.8\rho)(d-3.6) - (\Delta G^0 + \lambda)/\lambda
\]

Eq. 2
in which $\rho$ denotes the packing density, $d$ the distance separating the donor and acceptor sites, $\lambda$ is the reorganization energy and $\Delta G^\circ$ is the standard free energy of the reaction (energies are expressed in eV). For the N42C azurin dimer, these parameters are $\lambda = 0.7$ eV, $\rho = 0.75$ and $\Delta G^\circ = 0$ (e.s.e.). Based on these parameters and a Cu-to-Cu distance of 20.5 Å, a value of $k_{\text{ intra}} \approx 1.4 \times 10^7$ s$^{-1}$ is predicted, in reasonable agreement with the experimentally determined rates.

Alternatively, ET can be viewed as proceeding along defined tunnelling pathways (TP). Analysis of such pathways in the crystal structure of the N42C/M64E dimer identifies a path that involves residues H117, G116 and P115 on both chains and a large through-space jump of 4.0 Å between the C$\beta$ and C$\gamma$ atoms of Pro115 on either chain respectively [Figure 4.7]. The coupling efficiency of this pathway is extremely low ($\Pi < 10^4$) and, in fact, is considered too low for ET to occur at a discernible rate. The pathway algorithm, however, calculates couplings in static structures with fixed distances between atoms. In reality, protein motions may temporarily reduce bond lengths and through-space distances, increasing the coupling strength. Also, although in the crystal structure no ordered water molecules were observed within hydrogen bonding distance of the P115 residues, in solution such solvent interactions could help to promote the electronic coupling and mediate the electron self-exchange.

The structure of the low pH form of the dimer is rather unexpected as it was originally believed that the Cys42-Cys42 bond was too rigid to allow the dimer halves to approach each other sufficiently to bring the redox centres within ET distance. The present structure indicates that this bond is more flexible than anticipated, although the resulting complex still does not display high rates of exchange. The small but nonetheless significant pH dependency of $k_{\text{ intra}}$ suggests an electrostatically

![Figure 4.7: Calculated tunnelling pathway between the copper centres of the N42C/ M64E disulfide bridged dimer of azurin (HARLEM). Those atoms on residues 115-117 on both protein chains which are part of the TP are shown as sticks. The through-space jump between Pro115(A)-C$\beta$ and Pro115(B)-C$\gamma$ is indicated by a dashed line.](image)
induced increase of the Cu-to-Cu distance as a result of a small change in the geometry of the dimer. Chemical shift perturbation analysis of the dimer at different pH values indicates that the dimer interface is not strongly interacting throughout the observed pH range. At lower pH some localised exchange effects are present which are abolished at higher pH. Although it is tempting to attribute these pH dependent exchange effects to conformational switching, the current data are insufficient to sustain such a claim. Resolution of a crystal structure at higher pH could help to elucidate the mechanism of pH dependence.
Supplementary material

A model for determination of electron self-exchange rates in protein dimers

For the determination of electron self-exchange rates in dimers of azurin using simulation of the Val31 $^1$H$^2$ resonance in the MEX software, a model was developed that defines the various interaction sites and the potential processes between them.\[10\]

In partially oxidized samples 4 distinct types of redox centres can be identified: 0) a Cu(II) site within a fully oxidized dimer, 1) a Cu(I) site within a semi-oxidized dimer, 2) a Cu(II) site within a semi-oxidized dimer and 3) a Cu(I) site within a fully reduced dimer. The population of each of these sites is determined by the level of oxidation of the sample $x$ and the total concentration of dimers. A model is constructed that takes into account all possible exchange processes between them, both intermolecular and intramolecular. The bimolecular rates for $k_{\text{inter}}$ are defined by the chance of a reduced site (sites 1 and 3) to become oxidized through intermolecular hole transfer from an oxidized site (sites 0 and 2) [Figure I]:

\[ k_{\text{obs,inter}} = k_{\text{ese,inter}} \cdot 0.5 \cdot [\text{dimer}] \]

At $x = 0.5$, such as is approximately the case in the experiments described in Figures 4.2 and 4.3, all sites are equally populated, assuming that the redox potentials of the two half-reactions for reduction of the dimer are equal, and the different values for $k_{\text{inter}}$ can be reduced to a single $k_{\text{ese,inter}}$ so that $k_{\text{obs,inter}} = k_{\text{ese,inter}} \cdot 0.5 \cdot [\text{dimer}]$.\[ Figure I: Schematic representation of the possible intermolecular and intramolecular hole transfer routes (indicated with arrows) that comprise the model used for MEX based simulation of the Val31 $^1$H$^2$ resonance in disulfide-linked dimers of H35F/N42C/M64E azurin. The different sites are labelled 0, 1, 2 and 3. The labels $k_{\text{inter}}$ and $k_{\text{ese,intra}}$ represent the first order rates that are manually iterated to produce a simulation of the experimental spectra. $k_{\text{ese,inter}}$ is given by the dependency of $k_{\text{ese,inter}}$ on the concentration of the oxidized sites involved ([0] or [2]).\]
The different effects of intermolecular and intramolecular electron self-exchange on the lineshape of the Val31 $^1$H resonance in partially oxidized samples of azurin can easily be visualised [Figure II]:

**Figure II**: Simulations of the Val31 $^1$H resonance of H35F/N42C/M64E disulfide linked azurin at 57% oxidation, pH 4.7 as a function of exchange rate when A) only intermolecular exchange occurs or B) only intramolecular exchange occurs. The numbers shown represent $k_{\text{obs,inter}}$ ($s^{-1}$) and $k_{\text{obs,intra}}$ ($s^{-1}$) respectively. The combined effects of both modes of action are shown in Figure II-C: spectra obtained at different combinations of intermolecular and intramolecular electron self-exchange rates are superimposed on the experimental spectrum (black solid line).
References


Electron transfer and complex formation in a pH sensitive dimer of azurin