Chapter 8

General discussion, conclusions and perspectives
This thesis centres on the study of model systems for biological electron transfer formed by dimerization of the small blue copper protein azurin. Azurin was chosen for this study because of the substantial amount of information that is available on the structure and electron transfer behaviour of the wild type protein and a variety of mutants.

In these studies the so-called electron self-exchange reaction was investigated by which electrons are transferred between a reduced and an oxidized redox centre in a process where the products are identical to the reactants. As the overall equilibrium between the reactants is not affected by this reaction, it is not accessible to many conventional spectroscopic techniques, such as UV-Vis spectroscopy. Instead, electron self-exchange was studied by NMR spectroscopy since this technique is both sensitive to changes in the redox state and to dynamical processes.

Non-adiabatic ET theory emphasizes the relationship between the structure of a protein complex and the rate, $k_{ET}$, by which electrons are transferred as $k_{ET}$ diminishes exponentially with the distance between the redox centres.\[1\] When available, structural information may help to identify distinct ET pathways through the protein. For these reasons we have followed two parallel approaches in the analysis of several azurin based ET constructs: electron self-exchange behaviour of various complexes was monitored in solution by NMR whilst, where possible, their structures were determined by X-ray diffraction.

Direct interpretation of interprotein ET rates can be complicated by the effects of complex formation and dissociation. Co-crystallization of partner proteins, on the other hand, is often hampered by the low affinities and concomitantly short life times inherent to most redox complexes. These problems can be avoided when two proteins are covalently crosslinked. Van Amsterdam et al. have demonstrated that by site-directed introduction of surface-exposed cysteine residues, dimers of azurin can be formed which exhibit high rates of electron self-exchange.\[2,3\]

**Covalent crosslinking: development of tunable redox complexes**

Based on the extensive investigation of the electron self-exchange behaviour of WT and several mutated azurins as well as on that of several crosslinked constructs, one of the main challenges was to use these insights for the development of systems with controlled and tunable ET behaviour. One way in which this may be achieved
is by rendering systems with high electron self-exchange efficiency, such as the crosslinked azurin dimers, sensitive to pH by the introduction of ionisable residues. These novel dimers can serve as useful touchstones to the premises that underlie their construction.

The work described in Chapters 3, 4, and 5 is an extension of the work by van Amsterdam et al. that showed the development of dimers of azurin with high rates of either intermolecular or intramolecular electron self-exchange, dependent on the mode in which the proteins are connected. It was shown by X-ray crystallography that intermolecular disulfide bond formation between N42C azurin molecules results in a dimer in which the Cu-to-Cu distance is too large to allow fast intramolecular electron self-exchange. The intramolecular electron self-exchange, on the other hand, was found to be quite efficient. This is in agreement with the observation that in the crystal structure the hydrophobic patches of the two azurin moieties are largely exposed and available for interdimer contacts. In contrast, the insertion of a spacing molecule (BMME) in between the cysteine residues allowed rearrangement of the N42C-BMME azurin complex and formation of a dimer in which the copper centres are in sufficient proximity to achieve high rates of intramolecular electron self-exchange. In Chapter 3 this electron self-exchange behaviour was further analysed as a function of temperature with the aim of determining the reorganizational energy \( \lambda \). It was found that the line broadening that occurs in the NMR spectra of the complex at lower temperatures only permits determination of a lower limit for \( k \), which similarly decreases with temperature. This did not allow for a determination of the reorganization energy, \( \lambda \).

The Chapters 4 and 5 focus on the further modification of the disulfide and BMME crosslinked dimers of N42C azurin, respectively. The systems were brought under the control of pH by introduction of ionisable glutamate residues (M64E) on the surface of the protein near the presumed site of interaction. It is known that this mutation renders the rate of electron self-exchange, \( k \), strongly pH sensitive and that at high pH the \( k \) for M64E azurin decreases about 100-fold. It was anticipated that a similar pH dependence could be introduced into crosslinked dimers. Although such effects were indeed observed, both the magnitude of the effect and the mechanism of pH dependence were found to differ from our initial expectations (Chapters 4 and 5).
The (H35F/)N42C/M64E disulfide bridged dimer described in Chapter 4 displayed low, only moderately pH dependent, intramolecular e.s.e. rates that are in clear contrast to the fast intermolecular exchange that was observed in the N42C disulfide bridged dimer. This unexpected behaviour may be connected to the conformation that is adopted by the N42C/M64E disulfide bridged dimer at pH 3.1 which is markedly different from that of the N42C disulfide bridged dimer at pH 8.5. As the Cu-to-Cu distance in this complex is much shorter and the interaction sites near the copper centres within the dimer are more shielded with respect to the ‘open’ conformation previously encountered, the (H35F/)N42C/M64E disulfide bridged dimer appears to favour intramolecular exchange instead.

Introduction of negatively charged residues in the dimer interface of a BMME crosslinked dimer of azurin resulted in a, slightly pH dependent, depopulation of the dimer species that is involved in fast intramolecular exchange (Chapter 5). Comparison of the crystal structures of the N42C-BMME and M64E/N42C-BMME azurin dimers at pH 8.5 - 9 indicates that the main difference between the two constructs at high pH resides in the apparent absence of a water-based hydrogen bond network in the interface of the latter. As this network is believed to mediate the electronic coupling between the redox centres of azurin through hydrogen bonding with the copper ligands His117, its breakdown offers a plausible explanation for the loss of redox activity. These results offer support for the notion that ordered water molecules which are hydrogen bonded to His117 form an integral part of the electron tunnelling pathway for the electron self-exchange in azurin.

The effect of negatively charged residues in the complex interface
For M64E azurin, the observed pH dependent reduction of $k_{\text{exe}}$ at high pH has been attributed to electrostatic repulsion between the partners. However, in the crystal packing of wild type azurin the M64 groups on interacting monomers are found positioned approximately 20 Å apart. As this orientation is generally equated with that of the electron self-exchange complex in solution, it is unlikely that direct electrostatic repulsion between the deprotonated E64 residues is sufficient to account for the observed effect. Further inspection of the interface of this association complex learns that closer by, on the opposing monomer, residue D11 is situated. The $pK_a$ of aspartate and glutamate residues free in solution are comparable. Simultaneous deprotonation of both residues produces a total of four negative charges in the interface, arranged in two pairs, which provides a far more credible explanation
for the encountered pH dependence. The crystal structure of N42C/M64E-BMME azurin reveals that in this crosslinked complex the electrostatic repulsion between the D11-E64 pairs is reduced, not by rotation around the site of crosslinking but by a positioning of the E64 sidechain such that the D11-E64 distance is maximized. Likewise, in the disulfide bridged dimer of N42C/M64E azurin, E64 is found positioned nearly flat on the protein surface instead of pointing into the interface. At pH 3.1, where the crystals of N42C/M64E disulfide bridged azurin were grown, most of the E64 residues can be assumed to be protonated and thus uncharged. The position of the E64 side chain in these crystals is therefore probably not so much due to electrostatic repulsion as it is favoured by hydrogen bonding to neighbouring residues.

Ligand reconstitution as a means of hotwiring redox centres
Redox proteins are prime candidates for applications in biotechnology owing to their ability to selectively and efficiently convert a chemical signal into an electrical response which can then be transduced to an electrode. However, as the protein matrix in which redox centres are encapsulated is a naturally insulating medium, the electrical contacting of proteins and electrodes poses a significant obstacle to the construction of devices with high efficiency. First generation biosensors to this end employed relay stations such as ferrocenes which were anchored to the protein. An elegant alternative is offered by the malleability of many redox proteins that allows native cofactors and ligands to be substituted for functionalized derivatives thereby enabling direct ‘hotwiring’ of the redox centres. Ideally, the redox centre is directly contacted by insertion of highly conductive molecular wires. The use of molecular wires has attracted much attention in more conventional nanoscale electronics but is still largely unexplored in bionanotechnology.

The H117G mutant of azurin is a clear example of a protein that allows hotwiring. In this mutant, one of the copper ligands is substituted for a non-coordinating glycine residue by site-directed mutagenesis, leaving behind a solvent-exposed aperture in the copper coordination sphere that is accessible to a variety of exogenous ligands. In this way the spectroscopic and electronic properties of the protein can be modified.

The feasibility of direct hotwiring of H117G azurin was explored in Chapters 6 and 7. As was clearly demonstrated by the results presented in Chapters 4 and...
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5, an accurate description of the structure of redox complexes is essential for the understanding of their ET behaviour. Determination of a crystal structure of ligand-coordinated H117G azurin has, however, thus far been impaired by the oxidative damage sustained by the protein during crystallization. Although H117G azurin is well characterized and information on the copper coordination sphere and the protein dynamics are available from EXAFS and NMR studies, at the time of these studies, no structures were available of intact holo-H117G azurin. In order to circumvent oxidation of the metal-coordinating residues, a ligand-coordinated complex of H117G azurin was prepared in which the copper centre was substituted for a redox-inactive zinc ion. Chapter 6 describes the structure determination of a non-covalent dimer of Zn-H117G azurin, formed by coordination of a bifunctional linker with zinc-coordinating imidazole moieties at both ends of a flexible alkane chain (1,6-dih). Several interesting features with regards to protein structure and flexibility of H117G azurin were observed.

The structure of the non-covalent \((\text{Zn-H117G})_2-1,6\text{-dih}\) azurin complex indicates that the H117G substitution causes a pronounced increase of flexibility in the loop region between residues 116 and 120 resulting in its displacement from the protein interior (Chapter 6). The loop displacement grants easy access to the metal centre to ligands such as (derivatized) imidazoles or pyridines. The structure of the dimer formed by linker-coordination to Zn-H117G is distinct from that of other dimers of azurin, including those described in Chapter 4 and 5. Although the dimer interface involves interaction between the hydrophobic patches of the individual subunits, the relative orientation of the subunits deviates from the crystal packing found for wild type azurin in that one of the subunits is rotated approximately 180 degrees around its longitudinal axis.

By analogy to 1,6-dih, a series of ligand wires was synthesized consisting of two pyridine rings connected by a bridge of varying electronic and chemical composition (Chapter 7). Like imidazole, a homologue of the native histidine ligand, pyridine is capable of coordination to the copper site of H117G azurin with restoration of its Type-1 characteristics. The bifunctional linkers allow formation of dimers in which the electronic coupling between the copper centres can be assessed by EPR spectroscopy. The EPR spectra obtained for several of these dimers showed clear effects of spin-exchange, consistent with an increased electronic coupling.
Important tools in the investigation of H117G azurin are the spectroscopic changes (UV-Vis, EPR) associated with ligand binding which provide useful indicators of the electronic structure of the metal and its ligands. When sufficient overlap exists between the metal and linker orbitals, the system can approach ‘ohmic’ contact allowing electrons to flow easily to and from the copper site at extremely high rates. Although dimerization of H117G azurin by non-covalent linking allows us to study the systems in solution, the main applications of hotwired redox proteins should be sought in the contacting between proteins and electrodes. A logical extension of the work described in this thesis therefore concerns the immobilization of the protein-wire complexes on electrode surfaces. One particularly interesting application could be the construction of a biosensor for nitric oxide (NO), an important biological signalling molecule, based on copper containing nitrite reductase (NiR). Wijma et al. have demonstrated that under certain conditions this enzyme can be made to operate in the reverse direction, converting NO into nitrite, and that a H145A mutant of NiR, analogous to the H117G mutation in azurin, is accessible to exogenous imidazole based ligands.\[14,15\]

Determinants of conformation in crosslinked complexes
The structures of the dimers examined in Chapters 4, 5 and 6 differ from each other in mode of crosslinking and/or amino acid composition and display clearly different complex conformations. It had already been noted that the inclusion of spacer molecules such as BMME can have major consequences for the relative orientation of the linked proteins. Chapter 4 shows that although the short 42C-42C disulfide bond impedes formation of ET complexes of high efficiency, complexes linked in this way are still capable of adopting conformations with shorter Cu-to-Cu distances than expected based on the results reported for N42C azurin. The complexes described in Chapters 5 and 6, on the other hand, have sufficient motional freedom to adopt conformations suitable for fast intramolecular ET. Given the conservation of the ‘wild type’ conformation in the BMME crosslinked dimer, it was somewhat surprising to find that the H117G azurin dimer had arranged itself in a different fashion. The strong hydrophobic interaction that drives the formation of the wild type orientation may have been attenuated by the described loop displacement. It is also possible that the length of the 1,6-dih linker itself prevents the proteins from approaching each other sufficiently to achieve shielding of the hydrophobic contact regions. Attempts were made to crystallize the protein dimers discussed in Chapter 7 but these proved unsuccessful. It is clear from all the dimers investigated in this
thesis that, even when much is known about the free proteins, the behaviour of their crosslinked complexes can not always be envisaged beforehand and such systems still require detailed analysis.

References


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