EPR and NMR spectroscopy of spin-labeled proteins

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Chapter I

Introduction

This thesis explores novel routes of structure determination for dynamic and flexible protein systems, such as transient protein-protein complexes. To do so, a magnetic resonance approach is chosen in which specifically introduced spin probes play the main role. Such spin probes make the approach general, as systems devoid of natural paramagnetic centres can be investigated as well. Electron paramagnetic resonance (EPR) and nuclear magnetic resonance (NMR) are employed. For the EPR part of the investigation, biologically relevant models have been made and new high-field EPR methods were applied. In the NMR part, transient protein complexes have been studied with paramagnetic NMR. In the following, the background of both approaches is explained and an overview of the contents of this thesis is provided.

Proteins and protein interactions

Proteins are an important class of biological macromolecules present in all forms of life. These large and complex molecules show excellent functional flexibility, allowing them to play key roles in a great number of activities essential for the living world. No other type of biological macromolecule could perform all of the functions that proteins have gathered over billions of years. The characteristics of proteins permit to arrange their spatial structure so that specific chemical groups may be placed in definite positions. This mechanism allows them to act as catalysts in a number of reactions, and to carry out important structural, transport, and regulatory functions. Proteins normally perform these functions together with other biomolecules, rather than in isolation; indeed, a change in their quaternary state is often coupled with some particular function, or activity. Proteins bind frequently other proteins, as well as copies of the same protein, with which they form dimers or higher-order oligomers. The interaction with the biomolecular partner may occur either in relative isolation or within protein interaction networks and chains[1,2]. Therefore, it can be claimed that the study of proteins and in particular of how they interact is essential to understand countless biological processes.

Lifetime and strength of the protein complex are tightly coupled to the function performed by the complex. The affinity between the proteins that constitute the complex is a thermodynamic property expressed by the dissociation constant K_d, equal to the ratio between the dissociation rate constant k_{off} and the association rate constant k_{on}; it is therefore linked to the lifetime of the complex. The values for K_d may vary between 10^{-2} and 10^{-16} M and with it the nature of the protein complexes also gradually varies between the two extremes, of static complexes on the one hand, and of transient complexes on the other[3]. Static complexes are those where proteins are bound tightly to each other in a single, well-defined orientation. The value for K_d in this type of

complexes[3] is in the order of 10^{-15} – 10^{-16} M; examples are complexes formed between antigens and antibodies or between enzymes and inhibitors (e.g. the barnase-barstar complex[4]).

Opposite characteristics are found for transient complexes; these are typical of processes where a rapid rate of reaction is requested, which permits chained reactions to happen in an efficient way. Binding specificity, i.e. binding in a well-defined orientation, is needed; for instance, in the case of electron transfer processes, in which a minimal distance between the redox centres is necessary[5,6], since the reaction efficiency decreases exponentially with this distance. At the same time, affinity must be low so that, once the reaction has happened, the proteins can rapidly dissociate and a new partner can be found. For this to happen, the binding surfaces must have characteristics such that an efficient reaction is possible, without them being perfectly complementary as is observed for the interaction surfaces of static complexes.

A compromise between good specificity and low affinity is therefore necessary in transient complexes. A high dissociation rate constant is usually combined with a high association rate constant resulting in K_d values[7] in the order of 10^{-3} – 10^{-6} M. Studies on electron transfer systems provided evidence for the existence of the encounter complex. This is the initial complex formed between proteins (or between proteins and other macromolecules like DNA), which precedes the formation of the specific complex. In the encounter complex, the partners sample each others surface through a series of micro-movements, until the more stable active complex is formed. The current work provides a contribution to the investigation of the nature and characteristics of encounter complexes. The structure and dynamics of protein complexes are explored using proteins to which spin labels have been attached; these complexes are analyzed using both EPR and NMR techniques.

Spin labels for structural and dynamic protein-protein studies

A spin label is a stable radical, in which the unpaired electron is shared almost equally between the nitrogen and the oxygen atoms. Such a spin label can be attached covalently and specifically to a native or engineered cysteine in a protein. Introducing a spin label as a probe permits to explore structural and dynamic aspects of the protein by measuring the EPR observables of the spin label.

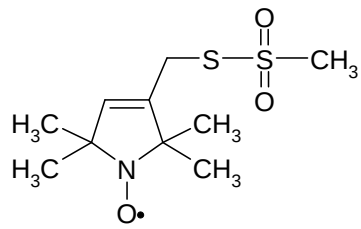

Figure 1.1 MTSL: the paramagnetic label used in this study.

A spin label is commonly attached to a protein through the site-directed spin labelling technique. In site-directed spin labelling, a nitroxide side chain is introduced *via* cysteine substitution mutagenesis, followed by

modification of the unique sulfhydryl group with a specific nitroxide reagent[8]. Measurements of the spectral properties of the paramagnetic nitroxide probe with EPR spectroscopy provide a wealth of information on the environment of the spin label in the protein.

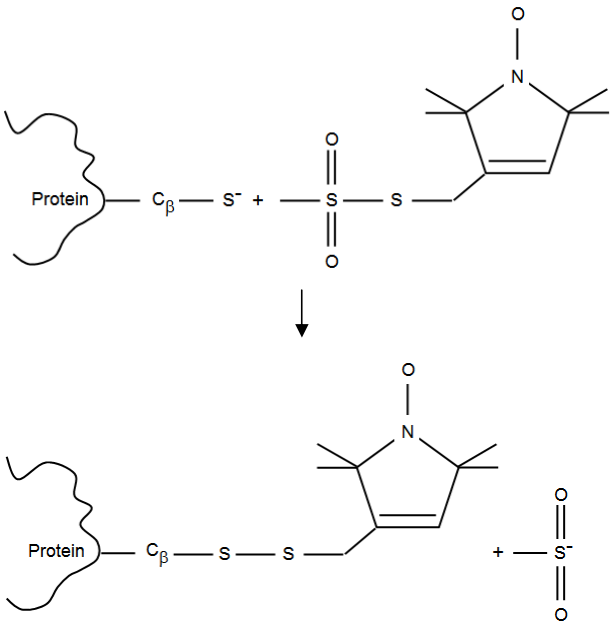

Figure 1.2 Reaction scheme of MTSL with a thiolate group of a cysteine residue of a protein.

Figure 1.1 shows the (1-oxyl-2,2,5,5-tetramethyl-3-pyrroline-3-methyl)-methanethiosulfonate spin label (MTSL), which is the nitroxide spin label used in the present work.

Figure 1.2 shows the reaction scheme of MTSL with a thiolate group of a protein.

The site-directed spin labelling technique has been successfully employed for the characterisation of protein structure[9-11] and was shown to work even for membrane proteins[10,12]. For surface-exposed spin labels, perturbation of the protein structure should be minimal, giving reliable information on the structure and dynamics at the site of the spin label [13-15]. Such information can be relevant for the study of protein-protein interactions, because these are determined by the surface properties of the interacting surfaces. Two EPR observables of the spin label reflect the polarity and proticity of the environment of the spin label, where proticity refers to the propensity of the protein environment to donate hydrogen bonds. The influence of solvent polarity and hydrogen bonding on the EPR parameters of a nitroxide spin label can therefore be used to extract information on the microenvironment[16].

The EPR techniques are also helpful for determination of the distance between two spin labels attached to the protein, permitting to solve structural problems that are not easily accessible by standard structural techniques. Usually, two spin labels are introduced so that their distance reflects the structural property of interest. The distance distributions that are obtained contain information about the structure of the molecule and the flexibility of the spin label linker. These parameters can help to understand dynamics of the macromolecules, which is particularly important in a biological context.

Basic aspects of the EPR technique

Important parameters obtained in EPR experiments are the *g*-tensor (**G**) and the hyperfine coupling tensor (**A**). To explain how they can be read from an EPR spectrum a brief introduction is given. In an EPR experiment, the sample, a spin labeled-protein in the present work, is placed in a strong magnetic field and exposed to electromagnetic radiation in the microwave range. The EPR permits to measure the energy separation between the spin states of an unpaired electron in the environment of other magnetic species which perturb the external magnetic field, B.

Resonance condition: g- tensor and electron-nuclear hyperfine interaction

In the simple case of a free electron, the spin, and the magnetic moment associated with it, is quantized to be parallel or antiparallel to the external field. The energy separation ΔE between the two states is:

$$\Delta E = g_e \mu_B B = h\nu \qquad \text{(Eq. 1.1)}$$

where g_e is the free electron *g*-factor (≈ 2.0023) and ß is the Bohr magneton (≈ $9.3*10^{-27}$ J/mT). The populations of the energy levels are determined by Boltzmann statistics. Irradiation with electromagnetic radiation of frequency ν, satisfying the resonance condition, can induce transitions between the two levels (Figure 1.3). The population difference caused by the energy separation can then be detected as absorption[17].

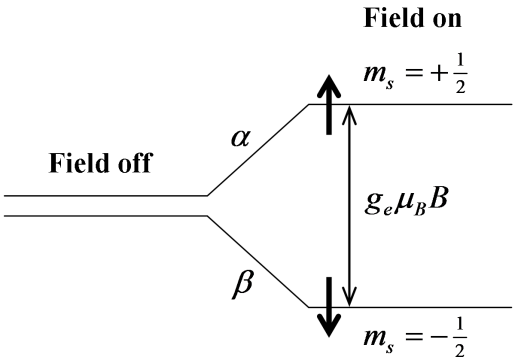

Figure 1.3 Free electron energy levels separation and transitions in presence of a magnetic field.

The interaction of the electronic spin S with an external magnetic field B (or **B**) (Zeeman term) and a magnetic nucleus having nuclear spin *I* can be described by the spin Hamiltonian H_s:

$$H_s = \mu_B S \cdot \mathbf{g} \cdot \mathbf{B} + S \cdot \mathbf{A} \cdot I \qquad \text{(Eq. 1.2)}$$

The orbital angular momentum of the electron in a molecule gives a contribution to the total magnetic moment, which produces a shift in the g-factor from the free electron value and can also be the cause for g to become anisotropic. The resonance is then described by the g-tensor (**G**), with the principal components g_{xx}, g_{yy} and g_{zz}. The isotropic g-value is defined as $g_{iso}=(g_{xx}+g_{yy}+g_{zz})/3$.

The principal directions of the g tensor of a nitroxide are shown in Figure 1.4. In nitroxides the magnitude of g_{xx} is particularly sensitive to hydrogen bonding to the oxygen atom.

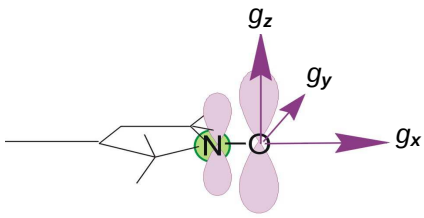

Figure 1.4 The principal directions of the g tensor of a nitroxide.

The second term in Eq. 1.2 describes the hyperfine interaction between the electronic spin *S* and the nuclear spin *I* through the hyperfine coupling tensor **A**, with the principal components A_{xx}, A_{yy} and A_{zz}. The isotropic hyperfine coupling constant is defined as: $A_{iso}=(A_{xx}+A_{yy}+A_{zz})/3$.

The magnitude of A_{zz} gives an indication about the polarity of the environment[16]. The isotropic hyperfine coupling is due to the Fermi contact term. It is caused by the spin density in the s-orbitals of the atom and reflects the distribution of the unpaired electron spin over the molecule. The anisotropy of A derives from the classical dipolar interaction between nuclear and electronic magnetic spin moments[17]. It is a measure for the distance between the electron spin and the nuclei[17] (dipolar interaction).

The internal magnetic fields derived from the nuclei can shift and/or split the basic resonance line into several components. The specific number, separation and relative intensities of these lines give information on the number of magnetic nuclei, their spin and the strength of the hyperfine interactions in the radical.

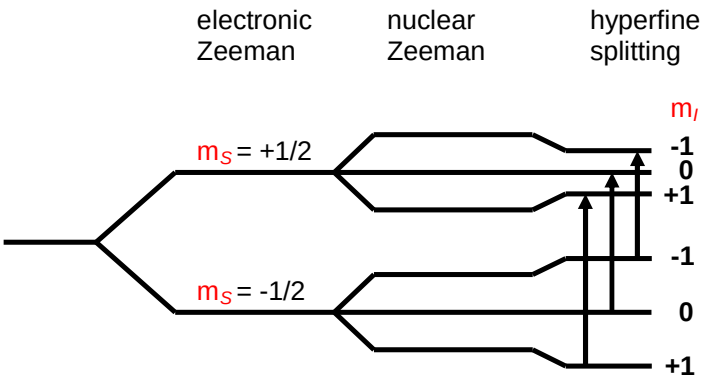

Figure 1.5 Energy level scheme and allowed transitions for S=1/2 and *I*=1.

In the MTSL molecule, the interaction between the nitrogen nucleus (^{14}N (*I*=1) in MTSL) and the electronic spin (S=1/2) results in the energy level scheme shown in Figure 1.5. The resonance is split into three lines.

High Field EPR

To improve the spectral resolution, EPR spectroscopy can be performed at high magnetic fields using superconducting magnets, which enhance the Zeeman resolution[18]. Figure 1.6 shows how the separation between the energy levels increases with increasing magnetic field strength, as it occurs moving from conventional X-band (9 GHz) EPR to W- and J-band (95 and 275 GHz, respectively). At these high fields, the spectra of a nitroxide in frozen solution are clearly resolved into three separate regions corresponding to the g-tensor components g_{xx}, g_{yy} and g_{zz} (Figure 1.7). For molecules with **B** parallel to g_x, a resonance, g_{xx}, at the low field side of the spectra (Figure 1.7) is observed and analogously resonances at g_{yy} and g_{zz} for molecules with **B** parallel to g_y and g_z. At the high field side of the spectrum three resonances, split by A_{zz}, are observed.

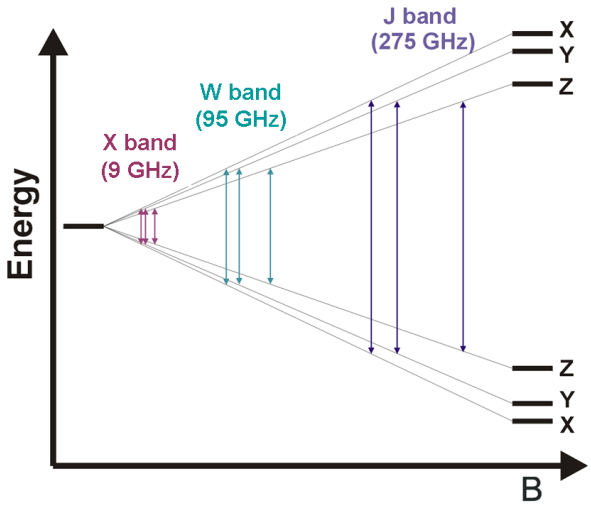

Figure 1.6 Energy levels separation at different magnetic field strength.

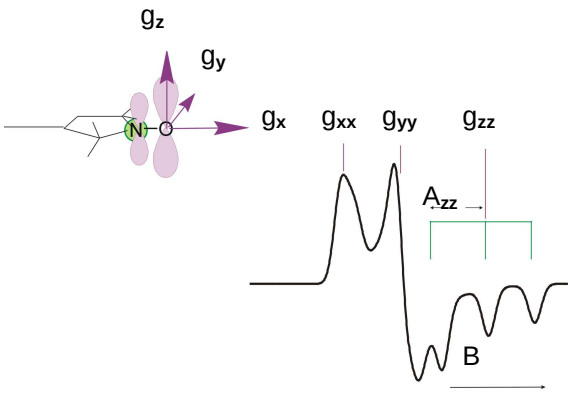

Figure 1.7 High field spectrum of MTSL at low temperature (powder spectrum).

The g-tensor component g_{xx} is particularly affected by hydrogen bonding whereas the A-tensor component A_{zz} is mostly influenced by the polarity of the environment. This is particularly interesting for proteins, for which it is often difficult to determine the local polarity.

In the present thesis, EPR spectroscopy at frequencies up to 275 GHz was performed, using a 275 GHz EPR spectrometer engineered and constructed at Leiden University[19].

Polarity and proticity from High Field EPR on nitroxide spin label

High-field EPR techniques allow to determine properties of the spin label environment such as polarity and proticity. Increasing the field above 95 GHz makes it possible to discriminate

between positions of similar polarity[18], such as those expected for positions at the surface of the protein. The principal g-tensor components and their variation due to solute-solvent interactions can be determined with high precision. The enhanced sensitivity to local structural influences on spin labels has been used to determine changes in the g-tensor as a function of solvent polarity and chemical structure of nitroxides[18,20-23].

The g- and A-tensor values are determined from the experimental spectra. The variation of g_{xx} for different samples is revealed by the shift of the position of the low-field maximum. Generally a protic environment shifts g_{xx} to smaller values, i.e. higher fields. The A_{zz} component of the A-tensor is read off as shown in Figure 1.7. The other components of A, A_{xx} and A_{yy} are too small to be resolved in the spectra.

Recent high-field EPR studies on polarity and proticity

Most studies investigating the properties of the protein environment with spin label EPR so far employ 95 GHz EPR. The polarity differences between different regions of a membrane protein were determined for a transducer protein[24]. Conformational changes of a membrane binding protein and the advantage of EPR at even higher field-frequency combinations are reviewed in Möbius *et al*.[25] The potential of these techniques to improve pH sensing has been explored also in the work of Möbius[25] and Voinov[26].

These are just a few of the examples of employing high-field EPR to learn about protein structure. The incentive to do such experiments at even higher fields than 95 GHz EPR derives from the presence of multiple components[16,24,25,27] in these spectra. Often the full interpretation of polarity and proticity trends is impeded by overlapping signals in the g_{xx} region of the spectra, which, as shown in Chapter II, can be resolved by EPR at 275 GHz and above[28].

Distance determination by EPR

Distance measurements are used in biological systems for which traditional methods of structure determination do not work well, such as certain peptides, proteins, RNA/DNA complexes or, as in the present thesis, protein-protein complexes. The EPR spectroscopic methods can be used when the biomolecule contains either stable or transient paramagnetic centers, like metal ions or clusters, amino acid radicals, or organic cofactor radicals. If the biomolecule is diamagnetic, it can be spin-labeled with nitroxides.

Both intra- and intermolecular distances between two spin labels may be measured through site-directed spin labelling combined with EPR spectroscopy[9,10,29]. Two types of techniques are normally used: a CW experiment in which, through the analysis of the line broadening caused by the dipolar interaction between two nitroxides, distances in the range of 8-20 Å can be

measured[30,31]. Larger distances (in the 20-70 Å range) can be determined using a pulsed EPR method, double electron-electron resonance (DEER)[15,32-35]as recently reviewed[36,37]. The pulsed techniques allow to measure distance information by producing a spin echo that is modulated at the frequency of the dipolar interaction[38]. The amplitude of the generated spin-echo is analysed and a distance distribution is obtained[34,39].

Model systems that serve as a reference for distance determination are essential for the comparison and the evaluation of the experimental data. Often, rigid molecules possessing two nitroxide groups are used[40]. For structure determination in protein-based systems, the disadvantage of such models is that they do not take into account the flexibility at the spin-label linker. One of the aspects that must be considered is that the linker may have multiple conformations when it is bound to the protein, because rotations over five torsion angles are possible (see spin labeled protein in Figure 1.2). Such mobility affects the distances obtained.

As a model for distance measurements we use azurin, a small protein for which the structure is known from X-ray crystallography[41], with two spin labels introduced by site-directed spin labelling[42].

Paramagnetic NMR for transient protein-protein complexes studies

Weak or transient interactions between proteins occur when the affinity between the proteins is low. Electron-transfer protein complexes are an example of transient complexes and are the result of a compromise between a tight binding, required for the reaction between the two partner proteins to occur, and the need for a fast dissociation, to ensure a high turnover of the complex and rapid electron shuttling. For these reasons, electron-transfer protein complexes are on the border of specific and nonspecific complexes. Several studies provided information on the dynamic nature of transient complexes, offering evidence that differently populated states may contribute to the complex structure. Paramagnetic NMR techniques are effective methods to study the structure of protein complexes and the dynamics of the proteins. Molecules naturally containing a paramagnetic centre (like a metal in metalloproteins), or containing paramagnetic labels specifically attached to them, can affect NMR signals, highlighting dynamics in protein complexes, and providing structural information, even of lowly populated states. The application of paramagnetic NMR, to obtain information about protein structure started already about 40 years ago[43], but has shown rapid progress and increasing popularity in the last ten years. Using paramagnetic tags, different types of NMR methods are employed to investigate structure and dynamics of protein complexes. One of them is the paramagnetic relaxation enhancement technique (PRE), which arises from the large magnetic dipolar interaction that exists between unpaired electrons and nearby nuclear spins. The

PRE effect results in an increase of the relaxation rate of the nucleus, which is manifested as a linewidth change. The rate increase can be used to calculate the distance between the paramagnetic centre and the affected nucleus.

In solution, the correlation time of the dipolar electron-nucleus interaction depends on two factors; the flipping of the electron spin, caused by longitudinal relaxation of the unpaired electrons, and the rotation of the molecule in the magnetic field. The first contribution to the correlation time is characterized by the electronic relaxation time, τ_s, and the second by the rotational correlation time τ_r of the molecule. The effective correlation time, τ_c, is given by $\tau_c^{-1} = \tau_s^{-1} + \tau_r^{-1}$. For metals with a fast electronic relaxation τ_c is dominated by τ_s. For some other metals (Cu^{2+}, Gd^{3+}) as well as nitroxide spin labels, τ_c is determined largely by τ_r. The enhancement of the nuclear relaxation rates is correlated with the distance between the paramagnetic centre and the nucleus, and specifically depends on the inverse of the sixth power of this distance. The relaxation enhancement can be very strong at short range but falls off quickly, yielding distances up to 25-35 Å, depending on the type of paramagnetic tag that is used[44]. This technique, PRE, is used in this thesis to investigate the structure of a transient complex. In a previous work[45] the same protein complex investigated within this thesis has been studied using pseudocontact shifts (PCSs) and chemical shift perturbations (CSPs).

The PCS is a consequence of the time-averaged anisotropic component of the unpaired electron spin. The pseudocontact effect is described by the magnetic susceptibility tensor $\Delta\chi$, and can provide long-range restraints for structure determination, with an r^{-3} dependence, where r is the distance between the metal and the nucleus. The PCS also provides angular information, because the size of the PCS contribution depends both on the orientation of the protein nuclei relative to the magnetic susceptibility tensor and on the distance from the paramagnetic centre. When intermolecular PCS are measured, from the metal in one protein to the nuclei of another, information about the orientation of one protein relative to the other can be obtained. Figure 1.8 shows the geometric parameters that are used in PRE and PCS technique.

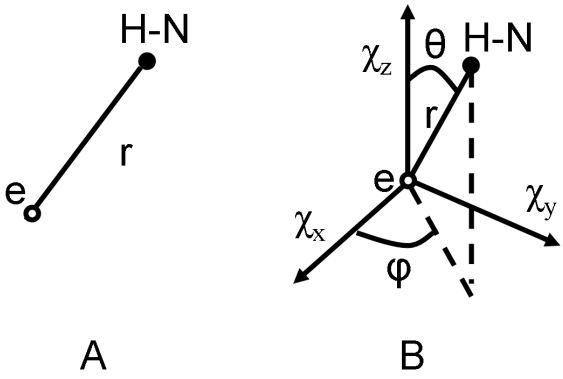

Figure 1.8 Schematic representations of the geometric dependence of the paramagnetic effects in paramagnetic relaxation enhancement (PRE) (A) and pseudocontact shifts (PCSs) (B)[46]. The unpaired electron is represented by 'e' and the observed nuclei, in this case an amide group, by 'H-N'. The axes labeled with 'χ' represent the orientation of the magnetic susceptibility tensor. The idea and the layout of the figure were taken from ref. 47 in modified form.

The CSP analysis is generally used to define a binding site and to calculate the dissociation constant of the protein complexes. In practice, CSP are found by comparison of the spectra during the titration of one protein with another protein with which it forms a complex. Well-defined complexes yield large and localized CSP. Conversely, if CSP arises from a time average of the relative orientation between the proteins due to the dynamic nature of the complex, as it happens in transient complexes, the changes are small and spread over a large area of the protein.

Thesis outline

This thesis is organised as follows. In chapters II and III the EPR experiments are described. Chapter II focuses on the polarity/proticity of the environment of the spin labels.

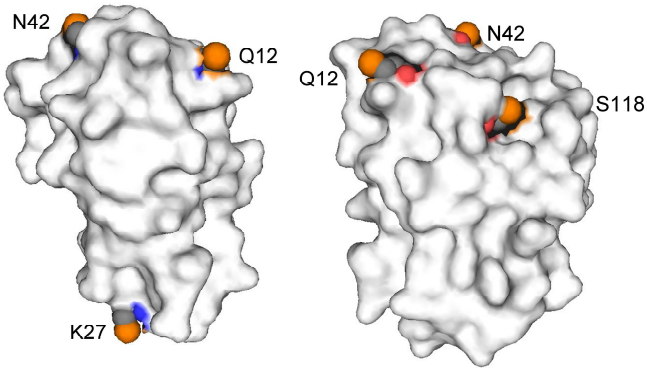

Figure 1.9 Azurin is depicted in surface representation (grey), while the Cys residues are shown with the sulphur in orange. The right view is rotated by 90° around the vertical axis relative to the left one.

Four single mutants of azurin were prepared by site directed mutagenesis. Figure 1.9 shows the location of the mutated residues. The properties of these sites are investigated by EPR at 95 GHz and 275 GHz.

Chapter III illustrates distance measurements by a pulsed, two frequencies EPR technique (DEER). Two double mutants are described: the first one, in which Q12 and K27 have each been replaced by a cysteine (Cys) (Q12C/K27C); and the second one in which K27 and N42 were replaced by Cys (K27C/N42C). The singly labeled mutant protein K27C was used as reference. It was shown that distances in the 4 nm region can be measured with high accuracy.

In Chapter IV the dynamics in the complex of *Nostoc* sp. PCC 7119 cytochrome *f* – plastocyanin (Cyt *f*-Pc) investigated by NMR is described. The PREs from five spin labels on Cyt *f* were used as distance restraints in docking calculations. A previous study on the same complex indicated that the proteins spend most of the time in a well-defined, single-orientation structure. Here we suggest instead that the complex is more dynamic. These two apparently contrasting results can actually coexist in an encounter complex model.

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