Modelling copper-containing proteins

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Chapter 1

INTRODUCTION

Summary

The use of computational techniques to describe the interaction between atoms is being increasingly applied within the field of biophysical chemistry. Besides the prediction of physical properties like the structure of a bio-molecule, it is used to clarify experimental features. Experiments are usually averaged over a relatively long time and over a large ensemble of molecules. Using computational techniques, the behaviour of a single molecule can be analysed over a trajectory in time of which the length can be chosen at will within the limits set by the computational device. The description of the interaction between atoms is modelled with a so-called force field. In this chapter different methods are described to obtain a force field for a transition metal system.

1.1 Copper-containing proteins

This thesis mainly focuses on proteins containing one copper atom. The sites in proteins that are able to incorporate a single copper atom are classified according to their structure and their spectroscopic features like EPR, UV-Vis and resonance Raman (RR) spectra and are distinguished in type-I and type-II Cu-sites.

- Type-I Cu-sites are found in so-called blue copper proteins (or cupredoxins). They owe their name due to their intense absorption around 600 nm arising from π-π^* transition (Chowdhury *et al.*, 2001). These proteins, such as plastocyanin, amicyanin, and stellacyanin are generally involved in mediating electron transfer. In the oxidised state, the site is characterised by an unusually small hyperfine splitting in their EPR spectrum ($<70.10^{-4}$ cm^{-1}) whereas in the reduced state the d-orbital is completely filled and no EPR signal is detected (Coremans *et al.*, 1994; Solomon *et al.*, 1992). The copper atom is usually co-ordinated by a $S\gamma_{Cys}$, two $N\delta_{His}$, often called the equatorial ligands, and a $S\delta_{Met}$ atom, the axial ligand. Occasionally, the latter ligand is replaced by an $O\delta_{Gln}$ atom. Only in the case of azurin a weak fifth ligand, the carbonyl from a glycine residue, is present (Adman, 1991), see Figure 1.1a.
- Type-II Cu-sites often function in enzymes and are colourless. Their EPR spectra are similar to those of simple copper co-ordination compounds (Holm *et al.*, 1996). The copper atom is usually co-ordinated by three histidine residues, see Figure 1.1b. The fourth binding site is meant for the substrate to bind and in absence of a substrate molecule it can be occupied by the solvent, for instance .

In the last few years protein engineering has been applied to these proteins and new sites were obtained. Using Molecular Dynamics (MD) simulations these new sites can be analysed and understood. In some cases the experimental analysis of these new sites showed that they sometimes exhibit unexpected characteristics like their spectroscopy features or mobility. To tackle the arised questions, MD-simulations are used to unravel the behaviour of these proteins.

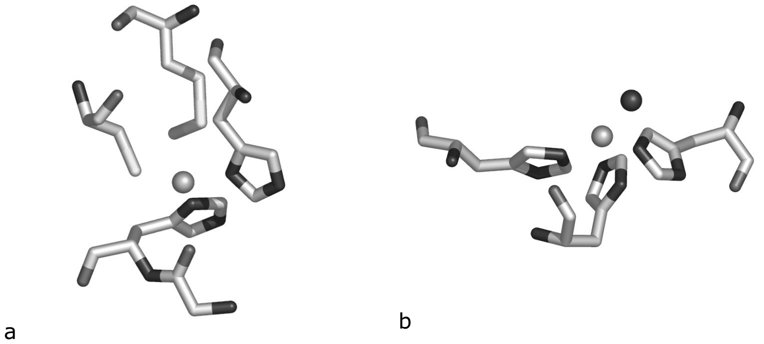

Figure 1.1: The type-I Cu-site of azurin (a) and the type-II Cu-site of nitrite reductase (b). The Cu-atom is shown as a grey sphere, the O_{water}-atom as a black sphere.

1.2 Molecular Dynamics simulations

Newtons equations of motion are used to model the dynamics of the Cu-containing protein. The leap frog algorithm is used for solving these equations. Starting from an initial structure, $\mathbf{r}(0)$, which can be derived by XRD or NMR methods, an initial velocity, $\mathbf{v}(-½)$, is allocated to the individual atoms. A structure at timestep i, is obtained by:

$$\mathbf{r}(i) = \mathbf{r}(i-1) + \mathbf{v}(i-\tfrac{1}{2}).\Delta t \qquad (1.1)$$

The accelaration is obtained with the help of the Newton equations from the potential energy, $V(\mathbf{r})$, of the system:

$$\mathbf{F}(t) = m.\mathbf{a}(t) = -\frac{\partial V(\mathbf{r})}{\partial \mathbf{r}(t)} \qquad (1.2)$$

Consecutively the new velocity is calculated by:

$$\mathbf{v}(i+\tfrac{1}{2}) = \mathbf{v}(i-\tfrac{1}{2}) + \mathbf{a}(i).\Delta t \qquad (1.3)$$

By repeating equations 1.1-1.3 over and over again the dynamics of the protein is obtained.

To describe the interaction between the atoms, interaction functions with concomitant parameter sets, so-called force fields, have to be developed.

1.3 Force field: interaction functions and parameterisation

The energy, V, is basically divided in different terms:

$$V = V_{bond} + V_{anglebend} + V_{impdih} + V_{propdih} + V_{electrostatic} + V_{vanderWaals} \qquad (1.4)$$

where the first four terms represent the bonding interaction and the last two terms describe the interaction between atoms not covalently linked.

Representing the Cu-atom in these functions is not straight-forward since Cu(II) is known for its variation in ligand co-ordination (Deeth and Hitchman, 1986). Its co-ordination complexes display a variety of co-ordination numbers and geometries, not always regular. This high flexibility or ‘plasticity’ arises from the stereochemical properties of the d^9 configuration where the variable orientation of the (highest) half occupied orbital exerts a profound geometrical effect, see Figure 1.2 (Olsson *et al.*, 1998). In the case of blue copper proteins, that transfer electrons, also the Cu(I) variant exists. Cu(I) with completely filled d-orbitals, also exhibits variation in its co-ordination sphere; it prefers a tetrahedral co-ordination sphere but can still adapt to the protein matrix (Guckert *et al.*, 1995; Solomon *et al.*, 1996). To describe the interaction between a metal and its ligands correctly, a quantum approach would be necessary. However, in the case of large molecules such as polymers and proteins or when dynamical features are required, the computational costs of quantum calculations become too high and other, empirical methods have been used to describe the forces within metal sites.

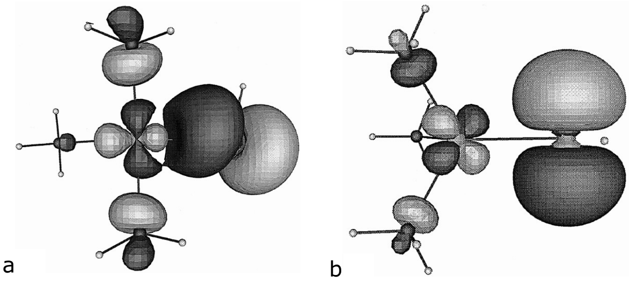

Figure 1.2: The singly occupied orbitals of the tetragonal (a) and trigonal (b) $Cu(SH)(NH_3)_3^+$ complex, (Olsson *et al.*, 1998).

Below, a non-exhaustive overview is given of the different force field terms. The problems encountered when applying these functions to transition–metal systems, are described and the solutions that have been put forward. Furthermore, some parameterisation tools are discussed.

1.3.1 Covalent bond potential

The covalent bond potential, V_{bond}, can be described using a simple, harmonic function:

$$V_{bond,\,harm} = \tfrac{1}{2}k_{bh}(r_0 - r)^2 \tag{1.5}$$

where k_{bh} is the force constant and r_0 is the equilibrium distance between the two atoms. To prevent a slow square root operation during the simulations to obtain the distance r, other interaction terms have been developed that cost less computational time. A quartic representation, for example, uses the value for r^2 (van Gunsteren *et al.*, 1996):

$$V_{bond,\,quar} = \tfrac{1}{4}k_{bq}(r_0^2 - r^2)^2 = \tfrac{1}{4}k_{bq}(r_0 + r)^2(r_0 - r)^2 \approx k_{bq}r_0^2(r_0 - r)^2 \tag{1.6}$$

At $r \approx r_0$ it describes the same energy well as the harmonic function where $k_{bh}=2k_{bq}r_0^2$. Metal-ligand bonds, however, have a partial bonding character and vary in hybridisation. They are more easily formed and broken than common covalent bonds making a simple harmonic or quartic representation not suitable to describe the dynamics of a transition metal-site. Burton et al., for example, solved this problem by using an anharmonic function like the Morse potential (Burton *et al.*, 1995):

$$V_{bond} = D\left[1 - e^{\beta(r_0 - r)}\right]^2 \tag{1.7}$$

where D is the dissociation energy and β is related to the force constant. It represents metal bonds better when r is significantly different from r_0; at small deviations from r_0 the potential energy increases quadratically while at large distances the energy asymptotically approaches the dissociation energy. Besides the fact that an exponential function takes a relatively large amount of computational time, another drawback of this function is that there are three constants to parameterise for every bond instead of two. It is therefore hardly used to describe bonding interactions between (metal) atoms.

For organic molecules, the specific parameters in the interaction functions have been developed for specific geometries corresponding to a particular hybridization (e.g. sp2 vs. sp3 carbon). It is, however, beyond the scope of standard force fields to parameterise all metal ions in their different redox states with all possible co-ordination structures. Many groups therefore developed force field parameters for the particular metal site they were interested in.

The equilibrium bond lengths for a metal ion and its ligands are usually copied from a crystal structure and sometimes adjusted on a trial and error basis by comparing the results of a structure optimisation with the crystal structure. Also other experimental features like absorption spectra have been used to obtain this parameters (Hush, 1967). The choice of parametrisation for the force constant is often based on the computational technique to be used in order to simulate the property. For a strict optimisation of the structure like in a Molecular Mechanics (MM) study accurate force constants are neccesary. Often a normal mode analysis is performed that provides the vibrational frequencies. The force field parameters, as used in the normal mode analysis, are calibrated to obtain the same frequencies as the experimental frequencies in Resonance Raman (RR) and Infra Red (IR) spectra (Allured *et al.*, 1991; Bol *et al.*, 1998; Gajewski *et al.*, 1998; Kaitner *et al.*, 1999; Merz, 1991; Rappe *et al.*, 1993; Subramanian *et al.*, 1997). Qiu et al. varied the bonding parameters of the Cu-ligand site so that its frequencies matched with the experimental RR frequencies including isotope effects and the relative intensities (Qiu *et al.*, 1998). In the case of MD-simulations, the metal-ligand distances are often constrained using the SHAKE algorithm (Ryckaert *et al.*, 1977) or equivalent procedures (Hess *et al.*, 1997), (Arcangeli *et al.*, 1999; Arcangeli *et al.*, 2001; Ciocchetti *et al.*, 1997; Merz *et al.*, 1991; Wachter and Branchaud, 1996). Costa et al. fixed the metal-ligand distance to the crystallographic distance by introducing arbitrary high force constants (Costa *et al.*, 1998; Costa *et al.*, 1999) while Fields et al. prevent a stiff structure by using arbitrary low force constants (Fields *et al.*, 1991). Accurate force constants for MD-simulations can be obtained by comparing the observed RR frequencies to the frequencies after a Fourier Transform of the autocorrelation of the Cu-ligand distance while varying the force constants (Ungar *et al.*, 1997).

Instead of fitting parameters to experimental data, empirical relationships can be used to obtain the parameters. Rappe et al. have developed the Universal Force Field (UFF) completely based on empirical relationships (Rappe *et al.*, 1992; Rappe *et al.*, 1993). In principle the UFF covers all atoms of the periodic table. The bond length between two atoms i and j (r_{ij}) is assumed to be the sum of both single bond radii (r_i) corrected for bond order (n) and electronegativity of the two atoms (x_i and x_j) (O'Keeffe and Brese, 1991):

$$r_{ij} = r_i + r_j + r_{BO} - r_{EN} \tag{1.8}$$

where $r_{BO} = -0.1332(r_i + r_j)\ln(n)$ and $r_{EN} = \frac{r_i r_j(\sqrt{x_i} - \sqrt{x_j})^2}{x_i r_i + x_j r_j}$. The corresponding force constants were based on Badger's rules that assume that the force constant of a bond between two atoms is related to their distance and the row in the periodic Table they belong to (Badger, 1934).

Sometimes quantum chemical methods are used to obtain more information about the system (Carlsson and Zapata, 2001; de Kerpel *et al.*, 1999; Dorigo and Houk, 1988; Gerloch *et al.*, 1981; Ryde, 1995), in combination with experimental features (Whitham *et al.*, 2000). They determined harmonic force constants of the bonds and angles in ^{63}CuOH and ^{107}AgOH by fitting the rotational spectra of ^{65}CuOH, ^{109}AgOH and the deuteratered forms respectively to the Hamiltonian. QM optimisations of small complexes and their frequencies can be used to fit the empirical force field parameters. The parameters can then be applied to larger, for example, polymer structures (Reichert *et al.*, 2001). Together with Remenyi, Comba developed a general parameter set for the Cu-centre of blue copper proteins using a rigid potential energy surface (Comba and Remenyi, 2002). The surface was calculated by moving the position of one ligand systematically while the others were kept fixed. This was done for various Cu-ligand bond lengths, angles and force constants. The obtained potential energy curves were compared to the DFT curve hereby determining the optimal copper force field parameters. Allured et al. performed an equivalent analysis to approximate force constants by fitting harmonic energy potentials along the various co-ordinates to Hartree-Fock calculations (Allured *et al.*, 1991). Seminario introduced a procedure to obtain force constants from Hessians (Seminario, 1996). The 3N component reaction force, δF, due to a displacement δx of the N atoms in a molecular system can be expressed exactly to second order on a Taylor series expansion as:

$$\delta F = -[k]\,\delta x \tag{1.9}$$

where $[k]$, the Hessian, is a tensor of rank 2 and dimension 3N x 3N defined by:

$$[k] = k_{ij} = \frac{\partial^2 E}{\partial x_i \partial x_j} \tag{1.10}$$

The reaction force on atom A due to a displacement of atom B permits an analysis of the nature of the interaction between atoms A and B using the three eigenvalues λ_i^{AB}, eigenvectors $\hat{v}_i^{AB}$ (i=1,2,3) and unit vector $\hat{u}^{AB}$. The force constant of the covalent bond between atoms A and B, k_{AB}, is defined as:

$$k_{AB} = \sum_{i=1}^{3} \lambda_i^{AB} \left| \hat{u}^{AB} \cdot \hat{v}_i^{AB} \right| \tag{1.11}$$

Equivalent procedures were developed to obtain force constants for the angle and dihedral functions. The main advantage of this approach is that it is invariant to the choice of internal co-ordinates.

1.3.2 Angle bend interaction

Angle bend potentials can be modelled using a harmonic function like:

$$V_{anglebend} = \tfrac{1}{2} k_{\varphi} (\varphi_0 - \varphi)^2 \tag{1.12}$$

A problem with this type of function is that at an angles of 180° the function takes the shape of a cusp (unless ϕ_0=180°) (Norrby and Brandt, 2001). In reality the 180° angle should represent a stationary point. Therefore other representations of the angle bend interaction like a cosine function have been developed (Mayo *et al.*, 1990; van Gunsteren *et al.*, 1996):

$$V_{anglebend} = \tfrac{1}{2} k_{\varphi} (\cos(\varphi) - \cos(\varphi_0))^2 \tag{1.13}$$

Most organic force fields were initially developed for only one equilibrium angle which is sufficient for most organic molecules. In metal sites, however, the ligands may be arranged in different co-ordination geometries so that more than one equilibrium value should be defined (e.g. in octahedral co-ordination ϕ_0= 90, 180, 270°). This is called the unique-labelling problem. It becomes even more complicated when taking into account that one and the same metal ion can exhibit more than one ligand conformation, e.g. tetragonal vs trigonal or four- vs six-co-ordination. Furthermore, a large variation often occurs for L-M-L bond angles. Distortions of more than 30° have been observed indicating a soft angular deformation potential. A standard harmonic function like (1.12) is not able to describe this properly. To avoid these problems the angular term is often disregarded in MD-simulations of a metal structure. Instead, the ligand-ligand interactions are modelled on a non-bonded basis. In MM-calculations in general more effort is invested to obtain a detailed force field. Many different representations and parameterisation tools have been developed to obtain proper descriptions.

Allured et al. introduced an elegant way to treat angular potentials like the d^8 complexes of Rh(I), Pd(II) and Pt(II): $Rh(PH_3)_4^+$, $Pd(NH_3)_2Cl_2$ and $Pt(NH_3)_2Cl_2$, by developing the SHAPES force field (Allured *et al.*, 1991). In this force field they avoid the unique labelling problem by using the following potential energy function for the angular interaction term:

$$V_{anglebend} = k_\varphi(1 + \cos(n\varphi + \varphi_0)) \tag{1.14}$$

where n is the multiplicity and φ_0 the phase shift. Furthermore, they described the geometry using spherical internal co-ordinates. Instead of the common L-M-L angles, the polar angle with the z-axis and the azimuthal angle in the xy-plane were used in the force field.

The UFF is equiped with (Rappe *et al.*, 1993):

$$V_{anglebend} = k_\varphi \sum_{n=0}^{m} C_n \cos(n\varphi) \quad \text{where} \quad C_n = f(\varphi_0) \tag{1.15}$$

Halgren developed an expression to calculated the force constant for the angle φ defined by the atoms i, j and k:

$$k_\varphi = 1.75 * Z_i * C_j * Z_k (r_{ij} + r_{jk})^{-1} {\varphi_0}^{-2} \exp(-2D) \tag{1.16}$$

where $D = \frac{(r_{ij} - r_{jk})^2}{(r_{ij} + r_{jk})^2}$, hereby incorporating atomic constants Z and C, the distance r, the angle φ_0 and the asymmetry factor ($\exp(-2D)$) (Halgren, 1990).

Melnikov and Pletnev used a Gillespie-Kepert model to improve the calculated co-ordination geometry of the metal chromophore using MM (Melnikov and Pletnev, 1997). The model assumes that the deformation of the bond angles around the metal ion is caused by mutual repulsion of the ligands. An extra term is added to the force field:

$$E_{GK} = A_{ij}(r_{eff,i}{}^2 + r_{eff,j}{}^2 - 2r_{eff,i}r_{eff,j}\cos(\varphi_{iMj}))^{-3} \tag{1.17}$$

where $r_{eff,i} = d_i r_i$. d_i is an arbitrary constant between zero and one belonging to ligand i which differs for different bonds like aromatic or amino bonds.

Burton and Deeth avoid the unique labelling problem by modelling the interactions between the ligands on a non-bonded basis (Burton and Deeth, 1995). They state that it automatically treats the Jahn-Teller effect in six-co-ordinated species and it models different Cu-N interactions and co-ordination numbers using a single set of force field parameters and without recourse to any external constraints, while in standard MM force fields, different parameters are needed to describe different Cu-N bond lengths. Instead, they incorporated an additional electronic energy term in the MM force field that incorporates the d-orbital energy (Burton *et al.*, 1995). Hereby, it takes the geometric effects of the stereochemistry of the d-electrons into account for any co-ordination number, molecular symmetry and ligand type.

1.3.3 Dihedral angle interaction

Improper dihedral angles are defined to keep groups of atoms like tetrahedral centres near a specified spatial configuration using:

$$V_{impdih} = \tfrac{1}{2}k_{\xi}(\xi_o - \xi)^2 \tag{1.18}$$

Proper or trigonometric dihedral angles are usually described by:

$$V_{propdih} = k_{\theta}(1 + \cos(n\theta - \theta_0)) \tag{1.19}$$

where n is the multiplicity and θ_0 is 0° or 180°.

Only a few groups have included this interaction term in the force field of the metal they want to describe. Sometimes the same parameters for a proper dihedral like the Cα-Cβ-Sγ-Cu dihedral and the improper dihedral Nδ-Cγ-Cε-Cu were copied from the Cα-Cβ-Sγ-H and Nδ-Cγ-Cε-H dihedral force field (Ungar *et al.*, 1997). In the DREIDING force field, the force constants of the dihedrals are determined by the rotational barrier (Mayo *et al.*, 1990).

1.3.4 Electrostatic interactions

In Cu-chelates, the formal charge of the metal plus its chelating agent is often zero. In these cases electrostatic interactions are often disregarded since they only have a minor effect on the structure of the complex. In the case of proteins, however, electrostatic interactions are very important for folding, stability and structure and cannot be neglected. The electrostatic or Coulomb interaction is commonly described by:

$$V_{electrostatic} = \frac{q_i q_j}{4\pi\varepsilon r} \qquad (1.20)$$

where ε is the dielectric constant, q is the charge on atoms i and j and r the distance between the two atoms.

The environment has a large influence on the charge distribution within a molecule which varies in time. Also the charge distribution within a transition metal site may vary and can be influenced by electronic effects in- and outside the complex. Polarisable force fields have been developed that give the opportunity to vary the dipole moment of a set of atoms during the simulations (Caldwell and Kollman, 1995; Rick, 2001; Yu *et al.*, 2003). Unfortunately, these force field are not optimal as of yet. Until they are, one charge distribution is used during an MD-simulation that describes the average electrostatic interactions of the chromophore.

Quantum approaches, like ab initio, DFT or semi-empirical methods, appear to be the most accurate solution to model the charge distribution within the ligand (Comba and Remenyi, 2002; de Kerpel *et al.*, 1999; Konecny *et al.*, 1998; Libeu *et al.*, 1997; Popovic *et al.*, 2001; Rizzuti *et al.*, 2004).

Since quantum approaches are computationally expensive, others have chosen for a (semi-) empirical approach. Ungar et al. based the total charge of the Cu and of its ligands (a cysteine, two histidine and a methionine residue) on a semi-empirical calculation (Ungar *et al.*, 1997). The distribution within the histidine and cysteine residues was based on an interpolation between the CHARMM charge distributions in protonated and unprotonated state of the residues. Others have estimated the charge backdonation from the cysteine residue to the Cu-ion using ENDOR experiments supported by self-consistent-field X_α scattered wave molecular orbital calculations (Libeu *et al.*, 1997; Werst *et al.*, 1991).

Developing the Universal Force Field, Rappe and Goddard used the so-called charge equilibration approach to obtain the charge distribution of the atoms (Rappe and Goddard, 1991). It predicts the charge distribution in molecules for use in MD-simulations based on the idea that the chemical potential (χ) of all bonded atoms should be equal (Sanders, 1983):

$$\chi_1 = \chi_2 = .. = \chi_N \tag{1.21}$$

where

$$\chi_i = \left(\frac{\partial V}{\partial q}\right)_i = \tfrac{1}{2}(IP_i - EA_i) + \sum_j \frac{q_j}{4\pi\varepsilon r_{ij}} \tag{1.22}$$

where IP is the ionisation potential and EA the electron affinity of atom i.

The atoms can be assigned the formal charge, for example +2e for the oxidised Cu atom and -0.5e for the co-ordinating cysteine residue. Occasionally, the distribution is adjusted to obtain the proper experimental feature (Arcangeli *et al.*, 1999; Arcangeli *et al.*, 2001; Ciocchetti *et al.*, 1997; Falconi *et al.*, 2001; Fields *et al.*, 1991; Gross and Pearson, 2003; Pappalardo *et al.*, 2003; Qiu *et al.*, 1998; Wang *et al.*, 1994). Only in rare cases, the charge of the metal site is completely neglected (Wachter and Branchaud, 1996).

Several groups have studied the effect of the charge distribution on the property of interest. Rizutti et al., for example, performed several MD-runs using different charge distributions (GROMOS96 vs DFT) and different numbers of Cu-ligand constraints (Rizzuti *et al.*, 2004). They showed that when all Cu-ligand constraints are defined, the applied charge distribution does not affect the optimised structure significantly. De Rienzo et al. also tested several charge distributions from literature based on DFT {De Kerpel, 1999 #386; Ullmann, 1998 #659} and concluded that the charge distribution within the metal site did not affect the electrostatic field {De Rienzo, 2000 #133}. Shen et al., however, compared the structure of the Cu-Zn active site of superoxide dismutase after an optimisation and a short, 15ps, MD-run where charge distributions were obtained by *ab initio* studies (various system sizes), using the electronic equalisation method and applying the formal charge of the GROMOS96 force field (Shen *et al.*, 1990). In this study, where no covalent bonds or constraints involving the metal were included in the force field, the metal site showed significant structural differences for the various charge distributions.

1.3.5 Van der Waals interaction

The van der Waals interaction is often modelled by a Lennard Jones (LJ) term:

$$V_{\mathrm{vander\,Waals}} = \frac{C_{12}(i,j)}{r^{12}} - \frac{C_6(i,j)}{r^6} \tag{1.23}$$

where C_{12} is the repulsive steric interaction and C_6 is the attractive non-bonding interaction arising from induced dipole - induced dipole interactions.

Occasionally, the LJ-values for Cu-ion have been copied from the Zn atom (Ungar *et al.*, 1997) or be taken from the experimental value in the gas state (Barrow and Aziz, 1988; Hogervorst, 1971). Other experimental features like the radial distribution in the solution, relative or absolute free energy of solvation, experimental lattice constants and lattice energies (Aqvist, 1990; Lybrand *et al.*, 1985; Lybrand and Kollman, 1985; Marrone and Merz, 1992; Marrone and Merz, 1993; Peng *et al.*, 1997) and *ab initio* calculations of ion solvent complexes (Hashimoto and Morokuma, 1994; Kisternmacher *et al.*, 1973; Straatsma and Berendsen, 1988; Toth, 1996) are used to fit the van der Waals parameters. Bartolotti et al. calculated the attractive Lennard-Jones parameter, C_6 from (1.23), for Cu from the London dispersion energy:

$$C_6 = \frac{3}{2}\sum_i \sum_j \frac{f_i f_j}{\omega_i \omega_j (\omega_i + \omega_j)} \tag{1.24}$$

where f is the dipolar oscillator strength and ω the dipolar transition frequency (Bartolotti *et al.*, 1991).

1.4 Conclusions

In the absence of computational time to use quantum mechanics, many empirical force fields have been developed that describe the average interaction between the atoms of the copper co-ordination site of a protein.

In small Cu-complexes the bonding properties are very important compared to large protein structures. The complexes have a relatively high flexibility and do not form a predefined structure like in proteins where the Cu-binding site is preformed. Furthermore, the interest in small Cu-complexes often relates to their structure. In these cases the charge of the system is usually disregarded and bonding parameters like equilibrium values and force constants are very important to obtain an optimised structure.

The main difference in force fields for proteins compared to small Cu-chelates is the charge distribution. In Cu-chelates the charge of the atoms is usually ignored. In proteins a large variety of charge distributions is applied. The charge distribution can be derived from quantum mechanical calculations but also simple formal charges are used, +2e on the Cu-atom for instance.

A clear difference in force field development is observed in the goal of the research. When using MM-calculations to obtain structural features covalent bonds and sometimes angles are defined in the force field. Experimental features or quantum chemical calculations are used to obtain accurate parameters. In the case of MD-simulations the metal-ligand distances are often constrained to the crystallographic distances. Only in a few cases where the specific mobility of the metal-site is important, non-bonded force fields are used.

1.5 Scope of this thesis

The knowledge of metalloproteins is growing fast and thereby the applications of this knowledge as well. During recent years when experiments were performed on copper-containing proteins, new questions came up regarding the dynamics and the spectroscopic features of the *wt* and mutated proteins. Small variations in the primary structure sometimes appeared to have a great impact on spectroscopic features. The research described in this thesis addresses some of these questions and tries to explain the differences in experimental features using MD-simulations.

Chapter 2 describes the development of a universal force field for type-I Cu-sites. Since the use of Cu-ligand bonds in the force field restricts the dynamics of the Cu-site and prohibits the influence of its environment, the developed force field is on a non-bonded basis. The optimal charge distribution of the Cu-site was verified by systematically varying the amount of back donation of the equatorial ligands, two histidine residues and an unprotonated cysteine residue, and the polarisation of the axial ligands, a methionine residue and a glycine residue, of the Cu-atom in the protein azurin. By comparing the optimal modelled structures using these charge distributions with the experimental structure, optimal parameters were obtained. Using this force field, it was investigated how the Cu-site behaves in a range of proteins and how the different environments in the proteins affect the Cu-site. Next, it was checked whether the force field is capable to predict the structure and stability of mutated type-I as well as type-II Cu-sites.

Chapter 3 describes how the force field is used to model the structure of an azurin variant of which the structure could not be determined experimentally. Resonance Raman spectroscopy has revealed that a mutated cysteine residue on the outside of the protein might co-ordinate the Cu-atom together with the original co-ordinating cysteine residue inside the protein. In this research a molecular dynamics simulation is performed where these residues are pulled together slowly in different ways, thereby obtaining three structures. The non-bonded force field as developed in chapter 2 was then used to verify the stability of the structures.

In **chapter 4** the dynamic differences as obtained from fluorescent and phosphorescent spectroscopy between *wt* azurin and two variants are analysed. The substitution of a large phenylalanine residue by a small serine residue nearby a fluorescent and phosphorescent tryptophane residue has led to an unexpected larger phosphorescent lifetime. This indicates a more stable tryptophan environment. In contrast to this, the tryptophan residue appeared to be more accessible to external ligands, indicating a larger void and a higher fluidity of its environment. The dynamical features of the several azurin variants containing mutations close to the tryptophan residue as well as the solvent accessibility were extensively analysed.

In **chapter 5** the redox properties of azurin and some mutants are evaluated. Since the non-bonding force field does not distinguish between different redox states a force field based on quantum chemical calculations is used. Using DFT, the charge distribution is obtained for the oxidised and reduced state of azurin. Cu-ligand bonds are constrained to their crystallographic distances. By performing free energy calculations the redox potential of the proteins is calculated.

Finally, a problem related to enzyme dynamics is addressed in **chapter 6**. In a type-II Cu-containing protein, quercetinase, the substrate appeared to have a large influence on the dynamical properties of a loop that may close the substrate cavity. Using MD-simulations with a DFT-based charge distribution and with constrained Cu-ligand bonds, the effect of substrate removal on the dynamics of the loop is analysed.