# Chapter 1
# Introduction

## 1.1 Topics of this thesis

Due to continuous methodological and technical improvements, such as multidimensional heteronuclear NMR and the advent of high-field magnets, structure determination by NMR spectroscopy has become a routine method for water-soluble proteins up to a molecular weight of 25 kDa. Another important technical development lead to the possibility to use pulsed field gradients in high-resolution NMR spectroscopy. However, the optimization of pulsed field gradient sequences in heteronuclear NMR experiments is not a trivial problem. Therefor part of this work was devoted to the development of methods for the analysis and calculation of pulsed field gradients. In chapter 2.3.2 the two programs TRIPLE GRADIENT and Z GRADIENT for signal selection by gradients are introduced. Examples for the use of the programs are given, with special emphasis put on the suppression of artifacts typically occurring in NMR experiments. A practical way to treat rf-inhomogeneities, which are the main source of artifacts, is suggested. It is shown that the calculated gradient sequences achieve very good water suppression in a heteronuclear single quantum coherence (HSQC) experiment. The formalism the programs are based on is extended to include stochastic as well as deterministic translational motion (chapter 2.2). The influence of unrestricted diffusion on the signal amplitudes in any high-resolution experiment using pulsed field gradients of rectangular or sine shapes is solved analytically (chapter 2.4.1). As an example, a heteronuclear quadruple quantum coherence (HQQC) rf-pulse sequence with different sequences of pulsed field gradients is analyzed (chapter 2.4.2).

The second part of this thesis (chapter 3) is focused on methods for structure determination of large systems, which are applied to the integral membrane protein bacteriorhodopsin. To date, only a small number of NMR structures have been reported for proteins larger than 25 kDa. This is largely due to a decrease of the sensitivity of the experiments caused by faster transverse relaxation. When trying to solve *membrane* protein structures, a number of additional problems occur, common to NMR as well as crystallography, namely the low abundance and the instability of the native three dimensional structure outside the membrane. The advantage NMR methods offer is the fact that they work directly on solubilized proteins. Thus the often limiting step of obtaining well-diffracting crystals is absent. However detergent

molecules bound to the protein increase the effective molecular weight, and hence solution NMR was only applied successfully to reveal the structure of segments of one or two α-helices in micelles, so far.

The 26 kDa seven transmembrane-helix protein bacteriorhodopsin is most likely the best understood membrane protein and serves as a model system for membrane transporters. The thermoreversible photo-isomerization of retinal, which is connected via a Schiff base linkage to a lysine side-chain, leads to the pumping of a single proton against an electro-chemical gradient across the membrane, thus producing energy in a form usable for the cell. At rest the so-called dark-adapted state builds up, in which two forms of the protein, one with retinal in all-trans, 15-anti and the other in 13-cis, 15-syn conformation, are in equilibrium.

The native structured form of the protein is stabilized by solubilization in dodecyl-maltoside micelles. This leads to a protein-detergent complex with more than 60 kDa in weight. Standard NMR methods for the assignment of large proteins (see chapter 1.3) based on $^{13}C$, $^{15}N$ and $^{2}H$ labelling were found to be too insensitive for a system of that size due to signal loss by transverse relaxation in the time-intervals needed for polarization transfer from the protons to the heteronuclei. A more effective strategy consists of preparing samples of uniformly deuterated protein containing selectively $^{1}H$ labelled amino acids and to record two-dimensional $^{1}H$ nuclear Overhauser (NOESY) spectra. Thus intervals for polarization transfer are not needed, and hence this technique is comparatively more sensitive. However, these NOESY spectra are also more difficult to assign, because the additional resolution otherwise gained by recording the chemical shift of the heteronucleus in a third dimension is absent. The process of achieving sequence specific assignments under these labelling conditions is described in chapter 3.4. The unique spin system of the retinal is used as a start-off for subsequent assignments of the surrounding amino acids. Since the assignments are obtained from NOESY spectra, distance constraints can be derived immediately. The calculation of chemical shifts (chapter 3.5) and NOEs (chapter 3.6) from crystallographic and preliminary NMR structures is used to verify the assignments and the method for obtaining distance constrains. The experimental distance constraints are then used to calculate (chapter 3.7.1) and compare (chapter 3.7.2) structures of both forms of dark-adapted bacteriorhodopsin, using a high-resolution x-ray structure

of the all-trans form as a starting point. The high accuracy of the approach is demonstrated by a comparison of the NMR all-trans, 15-anti structure to different crystal structures. The newly obtained 13-cis, 15-syn NMR structure is compared to the all-trans form as well as to X-ray structures of two intermediates of the photocycle (chapter 3.7.3).

A more general introduction into the different topics of this thesis will now follow (chapters 1.2-1.4). In the appendices auxiliary data are presented, which characterize the dodecyl-maltoside detergent micelles and the protein/detergent mixed micelles.

## 1.2 Nuclear magnetic resonance

Nuclear magnetic resonance (NMR) is dealing with the interaction experienced by nuclear spins exposed to magnetic fields. The energy of nuclear spins in a static magnetic field (*Zeeman* interaction) is the product of the field strength, the spin quantum number and the gyromagnetic ratio, which is constant for each type of nucleus. Thus for a nucleus with a spin 1/2, as for example $^{1}H$, $^{13}C$, $^{15}N$, $^{19}F$ or $^{31}P$, there are two energy levels corresponding to the magnetic spin quantum numbers 1/2 and -1/2. If now in addition to the static field a radio-frequency field with a frequency matching the energy difference of the two-level-system is applied, transitions from the lower level to the upper are induced and resonance absorption can be observed. If the spin-bearing nucleus is part of a molecule, a number of additional interactions take place causing a fine structure of the energy levels. The total Hamiltonian consists of operators describing the energy of the complete molecule, including for example electronic and vibrational contributions. A density operator obeying the Liouville-von-Neuman equation describes the time evolution of the whole system. However, the interaction between the spin operators and all other degrees of freedom is generally very small for diamagnetic non-conducting materials. It is therefore possible to introduce a reduced density operator for the spin-system, which is defined as the partial trace of the full density operator over all other degrees of freedom, generally referred to as the *lattice.* Moreover, the spin energy contributes only very little to the total energy of the system. This leads to a description of the spin system evolving irreversibly under the influence of its surrounding, which is always in thermal

equilibrium. We thus get a generalized master equation describing the time evolution of the spin system and its relaxation to thermal equilibrium (Ernst, 1987)

$$\frac{d}{dt}\rho(t) = -i[\mathsf{H}, \rho(t)] - \Gamma\{\rho(t) - \rho_0\} \qquad \textbf{( 1.1 )}$$

where $\rho(t)$ is the reduced density operator, $\rho_0$ is its value at thermal equilibrium, $\mathsf{H}$ is the spin Hamiltonian and $\Gamma$ is the relaxation superoperator, which amounts to the dissipative interactions between the spin system and the lattice. The system is completely characterized by the nuclear spin operators and their coupling to the external field, the radio-frequency field and internal spin interactions. The lattice enters the description only through some global properties like *chemical shifts* and *indirect spin-spin couplings* or *correlation/relaxation times*.

Mathematically, all contributions to the spin Hamiltonian can be described as coupling of two vectors by a Cartesian tensor of rank 2. In general, the interactions are non-isotropic. However, in the liquid state the anisotropies are usually averaged completely by fast random reorientation of the molecules leading to the Hamiltonian (Ernst, 1987)

$$\begin{aligned}\mathsf{H} &= \mathsf{H}_Z + \mathsf{H}_J + \mathsf{H}_{RF} + \mathsf{H}_G \\ &= \sum_i \omega_0^i I_z^i + \sum_{i<j} 2\pi J^{ij} \mathrm{I}^i \mathrm{I}^j + \sum_i \boldsymbol{\omega}_1^i(t)\mathrm{I}^i - \sum_i \mathrm{g}^i(t)\mathrm{r}_i(t)\gamma^i I_z^i\end{aligned} \qquad \textbf{( 1.2 )}$$

with the Zeeman interaction $\mathsf{H}_Z$ including the *chemical shift*, the *indirect* or *scalar spin-spin coupling* $\mathsf{H}_J$ and the external terms describing applied *radio frequency pulses* $\mathsf{H}_{RF}$ and *pulsed field gradients* $\mathsf{H}_G$. The summation extends over all spins in the sample. **I** is the spin angular momentum operator with the component $I_z$ along the quantization axis z, $\gamma_i$ is the gyromagnetic ratio of spin i, $J_{ij}$ is the isotropic part of the scalar coupling constant between spins i and j. The Larmor frequency $\omega_0^i$ is equal to $\gamma^i(1-\sigma^i)B_0$, where $\sigma^i$ is the isotropic part of the chemical shift and the Rabi frequency $\boldsymbol{\omega}_1^i(t)$ is equal to $\gamma^i\mathbf{B}_1(t)$. $B_0$ is the external static magnetic field in z-direction and $B_1(t)$ is the radio-frequency field. **g**=gradB(t) is a time dependent field gradient and $\mathbf{r}_i$ is the position of the nuclear spin i.

The external terms $\mathsf{H}_{RF}$ and $\mathsf{H}_G$ allow the selective excitation and manipulation of coherent superpositions in the spin system. Radio frequency pulse sequences are

designed to extract specific information, like chemical shifts, indirect coupling constants or relaxation rates.

Structural and dynamic information about the molecule is extracted from the coupling constants and the relaxation rates. The main source of relaxation for spin 1/2 is the direct magnetic interaction between the spin dipoles $\mathcal{H}_D$, which is the strongest interaction. Its functional form is similar to the classical interaction energy of two dipoles.

$$\mathcal{H}_D^{ij} = \frac{\mu_0}{4\pi}\frac{\gamma_i\gamma_j\eta}{r_{ij}^3}\left(\mathrm{I}^i\mathrm{I}^j - \frac{3(\mathrm{I}^i\mathrm{r}_{ij})(\mathrm{I}^j\mathrm{r}_{ij})}{r_{ij}^2}\right) \qquad \textbf{( 1.3 )}$$

$\mathcal{H}_D$ depends on the distance $r_{ij}$ and the relative orientation of the dipoles. It averages to zero for molecules in solution and so it does not show up as frequency shift in the spectrum. However, it is the source of a time dependent perturbation causing the relaxation of the spin system towards thermal equilibrium. Different spins do not relax independently. For the populations of the energy levels, the master equation can be derived by formulating classical rate equations connecting the different energy levels and calculating the transition rates quantum-mechanically (Solomon, 1955). The transition rates depend on the type of the interaction and the motional model. In the case of intramolecular dipolar relaxation in liquids, a fixed distance between two spins is usually assumed, which leaves their relative orientation as a source of fluctuation. The simplest model for this reorientation is isotropic rotational diffusion. In that case, the cross-relaxation between the spins is proportional to the inverse sixth power of the distance between the spins (Neuhaus and Williamson, 1989). This fact is used in NOESY spectra (*Nuclear Overhauser Enhanced Spectroscopy*) to obtain distances between spins.

The chemical shift and the indirect spin-spin coupling cause changes in the energy levels of the spin system and can be observed as frequency shifts in the spectra. The dispersion of signals caused by chemical shifts and the possibility of magnetization transfer through indirect spin-spin interaction are the prerequisite for site specific resonance assignments, which are the base for structure determination. A direct structural interpretation of these coupling parameters is however much more difficult than in the case of the NOE. The physical origin of both terms is an electron-nucleus

interaction. In diamagnetic molecules there is no direct magnetic interaction between the nucleus and the electrons.

Chemical shifts arise due to the interaction of the nuclear spin with the electrons, which are perturbed by the static magnetic field. The general theory distinguishes between two effects (Ramsey, 1950): (1) the static magnetic field induces electric currents, which in turn produce an additional magnetic field at the nucleus (*diamagnetic contribution*). (2) The electron cloud is polarized by the magnetic field (*second-order paramagnetic contribution*). The calculation of the second term is particularly difficult, since it requires knowledge of all excited electronic states of the system. To solve this problem, approximate methods from quantum chemistry have to be employed. One problem of the calculation is the choice of a specific gauge. The *exact* solution is gauge-independent, but since the wave functions have to be approximated this need not to be true for the result of such a calculation. More details of computational aspects of chemical shifts are given in recent reviews (Kutzelnigg, 1996; Pulay and Hinton, 1996; Sitkoff and Case, 1998). The *ab initio* methods have generally difficulties modeling solvent or other long range environmental effects on the chemical shifts in an exact manner. A general approach to get around this problem is to use the functional form of a certain type of interaction as it is derived from *ab initio* methods and adjust the parameters of the equations based on a statistical analysis of experimental results. In these so-called semi-empirical models for chemical shift calculations, the long-range or environmental effects are commonly divided into neighbor anisotropy effects (which include effects of ring-currents), electric field effects and close-contact or steric effects. The influence of these long range effects is very important to understand $^{1}H$ or $^{19}F$ chemical shifts, since these atoms are bond to only one other atom and local geometry effects are not as pronounced. This stands in contrast to $^{13}C$ shifts, were usually local geometry terms dominate the chemical shifts.

The physical origin of indirect spin-spin couplings are distortions of the electron shell by the presence of one spin, which in turn changes the magnetic field experienced by a neighbor. The coupling is generally observed between nuclei separated by three bonds or less. The precise calculation requires again the knowledge of all electronically excited states of the molecule, and so the difficulties are much the same as for

chemical shifts. In contrast to chemical shifts, the use of indirect couplings as structural restraints has a much longer history. Mainly the relative simple functional dependence of three-bond-couplings on the dihedral angle is used extensively (Karplus, 1959). The difficulties in deriving quantitative exact coefficients for a Karplus relation *ab-initio* is usually circumvented by the empirical determination of the coefficients in the equation based on a large set of experimental data for molecules with known geometry. The approximate nature of the relation has already been pointed out by Karplus (Karplus, 1963). More recent examinations take the effect of different substituents on the coupling pathway into account, leading to equations with more adjustable parameters (Altona, 1996).

## 1.3 NMR structure determination of proteins in solution

In general there are three steps in the structure determination of a protein (or any other molecule) by NMR:

1. Sequence specific resonance assignments
2. Derivation of structural parameters
3. Structure calculation

The first assignments of protein resonances were obtained from two dimensional proton spectra (Wüthrich, 1986). In this approach, the main problems arise from overlapping signals, which make the assignment process very tedious for proteins above a molecular weight of 10 kDa. Partially, the problem of overlap can be overcome by recording homonuclear 3D spectra (Oschkinat, 1994), however the development of isotope labelling strategies for proteins in the late 1980s with the uniform incorporation of $^{15}N$, $^{13}C$ and more recently $^{2}H$ nuclei (LeMaster, 1994) has triggered the development of a new strategy, which is based on heteronuclear multidimensional NMR spectra. In this approach, the large one-bond coupling constants of neighboring nuclei is used to transfer magnetization efficiently, and a large variety of new multi-pulse, multidimensional experiments for the assignment of resonances and the extraction of structural and dynamic information has been developed for labelled proteins (Cavanagh, 1996; Kay, 1995; Sattler, 1999). In the first step of this approach, all signals of the protein backbone are assigned sequence specifically from a set of the so called backbone experiments, which correlate the amide proton and nitrogen resonances of amino acid i to the backbone carbons of

residue i and i+1. The backbone assignments are then the starting point to identify the side chain resonances. Problems occur for larger proteins due to faster transverse relaxation, which lowers the efficiency of magnetization transfer and therefore the sensitivity of the experiments. The size limit of about 30 kDa for uniformly $^{15}N$, $^{13}C$ labelled proteins can be increased by additional incorporation of $^{2}H$. The magnetic dipole moment of $^{2}H$ is a factor of 6.5 lower than that of $^{1}H$, which reduces the dipolar contribution to the relaxation of directly attached carbons by approximately the same factor. The optimal $^{13}C$ linewidhts are achieved in 100% deuterated proteins. Assignments of all backbone resonances (amide $^{15}N$ and $^{1}HN$, $^{13}C\alpha$, $^{13}CO$ and eventually $^{13}C\beta$) can be made in such samples after replacing the exchangeable amide deuterium by protons.

It is to be expected that in the near future the tractable size limit is going to increase further by using transverse relaxation optimized spectroscopy (TROSY) at very high magnetic field strengths (Wüthrich, 1998). TROSY experiments (Pervushin, 1997) make use of the fact that apart from the dipolar interaction, the anisotropy of the chemical shielding tensor is a source of relaxation. The relative orientation of the tensors describing chemical shielding and dipolar interactions is fixed in the molecular coordinate frame. The fluctuations of the interactions, caused by rapid reorientations of the molecule in solution, are therefore correlated. The TROSY experiment selects only transitions for which the transverse relaxation rate is given by the difference of the dipolar and chemical shielding anisotropy terms. The orientation and size of the chemical shift tensor relative to the dipolar interaction tensor is particularly favorable for the amide proton/nitrogen two-spin system. In a 100% 13C, 15N, 2H (non-exchanging hydrogen atoms) labelled sample, the advantage of TROSY is made use of most efficiently, since the above mechanisms are dominating the relaxation of the $^{1}H$-$^{15}N$ moiety. The chemical shielding relaxation increases relative to the dipolar contribution with increasing external field strength leading to complete cancellation of the two mechanisms at a proton frequency slightly above 1 GHz.

While the concept of complete deuteration of all non-exchanging hydrogen is preferential for backbone resonance assignment, this approach has a significant disadvantage. Traditionally the most important structural distance constraints are short proton-proton distances (smaller than 5-6 Å), which are derived from NOESY spectra

(Clore and Gronenborn, 1989; Wüthrich, 1986). Replacing protons by deuterium therefore either reduces the number of distance constraints in site-specifically deuterated samples or reduces the volume of proton-proton NOEs in the case of random fractional deuteration (LeMaster, 1994). The reduction of the NOE volume is partially compensated by the more favorable linewidth of the signals, which leads to a higher maximal peak intensity ($I_{max}$ is proportional to the inverse of the transverse relaxation time $T_2$). However, even if complete assignments might be obtained for large proteins, it will be more and more difficult to get enough distance constraints for the calculation of a high-resolution NMR structure. Additional structural restraints are therefore highly desirable.

A second class of structural restraints widely used in current protocols for structure determination are dihedral angle restraint. Traditionally, they are derived from measurements of vicinal coupling constants, which are translated to dihedral angles by a Karplus relation. In a newer approach, dihedral angle constraints are obtained from an analysis of cross-correlated relaxation (Reif, 1997), either between two pairs of spins which relax mainly due to their dipolar interaction, or between a dipolar relaxing pair of spins and a single spin, the relaxation of which is dominated by chemical shift anisotropy. An overview of current experiments used to obtain dihedral angle constraints is given in (Griesinger, 1999).

The direct interpretation of chemical shifts was used widely during the first years of protein NMR. In the 1D-proton spectra measured at that time, only a few strongly ring-current shifted signals could be resolved and identified. With increasing resolution and the advent of multidimensional NMR, the chemical shifts were no longer used as structural parameter because of the lack of accurate relations between structure and chemical shift. This situation did change only very recently (Case, 1998; Oldfield, 1995; Szilagyi, 1995; Wishart and Sykes, 1994b), for mainly two reasons: (1) with a growing number of NMR protein structures, the database of chemical shifts is growing, which allows the statistical analysis of correlations between local geometry and chemical shifts. (2) The availability of fast computers improves the performance of ab initio quantum chemical methods for chemical shift calculations. The current size limit for routine calculations is reported to be 15-20 non-hydrogen atoms (Case, 1998). While ab initio calculations are very useful to establish a

functional dependence of the chemical shifts on local structural parameters (dihedral angles etc.), they have generally more problems to model environmental effects on the chemical shifts (solvent interaction, steric effects etc.). Nonlocal electrostatic terms are an example for a poorly understood contribution. Empirical estimates of the expected magnitude have been proposed (Buckingham, 1960), but application to proteins are plagued by well-known problems of understanding electrostatic interactions at a microscopic level (Sharp and Honig, 1990; Warshel and Russell, 1984). One of the better-understood contributions to chemical shifts is the ring-current effect of conjugated groups (Haigh and Mallion, 1980). For protons bound to side-chain carbons, these theories are particularly successful in explaining the observed large structural shifts.

An alternative way to approach the correlation between secondary and tertiary structure and chemical shift changes in proteins is a pure statistical analysis of chemical shifts in proteins of known structure. The approach is based on the analysis of so called secondary shifts, which are defined as the difference between chemical shift in a protein and the random coil shift of the corresponding nucleus. The random coil values are measured in short peptides, which are assumed to be unstructured (Wishart, 1995; Wüthrich, 1986). Over the last years, a number of investigations explored the dependence of secondary shifts of $^{1}$H (Asakura, 1995; Ösapay and A., 1991; Ösapay and A., 1994; Williamson and Asakura, 1993) $^{13}$C (Iwadate, 1999; Spera and A., 1991) and $^{15}$N (Le and Oldfield, 1994) on local structure. Secondary chemical shifts have been applied to determine the secondary structure of proteins (Pastore and Saudek, 1990; Wishart, 1992; Wishart and Sykes, 1994a; Wishart and Sykes, 1994b), to guide structure refinement (Beger and Bolton, 1997; Celda, 1995; Kuszewski, 1995; Luginbühl, 1995; Ösapay and A., 1994; Pearson, 1997, Cornilescu, 1999) and to judge the quality of structures (Williamson, 1995).

Another relatively recently developed approach for the determination of protein structures uses the orientational constraints from residual dipolar couplings (Prestegard, 1998; Tjandra, 1997; Tolman, 1995). The time average of the dipolar interaction (Eq. 1.3) is only zero in cases of isotropic tumbling of the molecule. If molecules have preferential orientations in the presence of a magnetic field, the assumption of isotropic reorientation does not hold any longer, resulting in a non-zero

average contribution of the dipolar interaction. The additional term in the spin Hamiltonian is like the indirect coupling proportional to $I_z^i I_z^j$ and causes a multiplet splitting of resonances which depends on the relative orientation $\Theta$ of the line connecting the two spins and the magnetic field $B_0$. Thus the obtained angular constraints are relative to the laboratory frame. Knowing $\Theta$ for many bonded pairs of spins, such as all backbone amide $^{15}N$ and $^{1}H$ in a protein, provides valuable information about the 3-dimensional structure. This information can be used to increase the precision of a protein structure or more importantly give unique information in cases where NOEs are hard to obtain, as for example in bigger proteins or protein complexes.

## 1.4 Bacteriorhodopsin

Archeabacteria are evolutionary so different from other prokaryotes, that they are classified into a "third kingdom of life" next to the eukaryotes and the "classical" prokaryotes or eubacteria. The main difference between this rather heterogeneous group of organisms and the eubacteria is the plasma membrane composition. Archae lack peptidoglycan also called murein, a baglike molecule which consists of a framework of covalently linked polysaccharide and polypeptide chains which otherwise build the rigid eubacterial cell wall. The family of haloarchae populates habitats with salt concentrations exceeding four molar. Their cell membrane contains a special class of retinal proteins with photosensoric (*sensory rhodopsins I* and *II*) and photosynthetic functions (*bacteriorhodopsin* and *halorhodopsin*). These intrinsic membrane proteins with a molecular weight of approximately 26 kDa share a common "seven transmembrane helix topology" arranged in two arcs. A transmembrane pore is formed mainly between the four helices, B, C, F and G. The retinal which is bound to a lysine in helix G interrupts the channel and separates an extracellular from a cytoplasmic part (Haupts, 1999; Oesterhelt, 1998)

Bacteriorhodopsin found in *Halobacterium salinarum* is the most extensively characterized member of this protein family. Its function is to pump protons from the inside to the outside of the cell. The electrochemical gradient established across the membrane is used for ATP-synthesis. The cell wall of *Halobacterium salinarum* consists of small patches, which are highly ordered in a 2D-hexagonal lattice,

containing Bacteriorhodopsin as the only protein. The protein forms a trimer in this so-called "purple membrane" and is surrounded by 10 lipids (5-6 phospholipids, 2-3 glucolipids and 0-1 sulpholipid) which are highly immobilized. The structure of bacteriorhodopsin has been determined by electron crystallography and more recently by x-ray studies on different crystal forms up to a resolution of 1.55 Å (Luecke, 1999b). The similarity between these structures determined by different approaches is very high with the main differences showing up in three of the surface loops (Subramaniam, 1999).

Retinal, the aldehyd of vitamin A, is the functional group of many different light absorbing proteins, the most prominent of which is rhodopsin contained in the human retina. The response of such proteins is triggered by the absorption of a photon. A general concept to classify the catalytic cycles of the retinal in halobacterial proteins is the IST model (Haupts, 1997). It postulates the necessity for five key events following the mandatory first step of photoisomerization (I*): Two steps of ion transfer (T), twice a change in the accessibility of the active site (switch S) and the thermal back-isomerization (I) of the retinal to its ground state. It can be shown that only four out of 720 possible combinations of these processes show vectoriality, meaning that only in those four cases as a net result ions are being transported across the membrane. The ion specificity and the vectoriality of the different retinal proteins can be changed by altering the physico-chemical conditions or by point mutations (Haupts 1999). The structural explanation for these experimental results remains a big challenge.

In bacteriorhodopsin, photon absorption changes the conformation of the retinal from all-trans, 15-anit to 13-cis, 15-anti. After relaxation, the Schiff-base proton is transferred to aspartic acid 85 in the extracellular channel, which in turn, through cooperative rearrangements and coupling of pK values, causes a proton to be released at the exit of the channel. The key players in this process are arginine 82, glutamic acids 194 and 204 and several water molecules. After the deprotonation of the Schiff base, two key events occur in the M intermediate: (1) the change in proton accessibility from the extracellular to the cytoplasmic side and (2) major structural rearrangements of the protein in helix F and G (Luecke, 1999a). The mechanism of these changes during the M state are still unknown. The decay of M is accomplished by proton transfer from D96, which in turn gets reprotonated by D38 on the entrance

of the cytoplasmic channel. Single point mutations D96N and D38R both slow down the M decay, indicating cooperativity of the events in the cytoplasmic channel probably involving water molecules as well (Haupts, 1999; Lanyi, 1997; Oesterhelt, 1998).

The initial (light-adapted) state $BR_{568}$ (all-trans, 15-anti configuration) equilibrates in the dark with a second species ($BR_{548}$) of 13-cis, 15-syn geometry, which is not taking part in the photocycle. In the so-called dark-adapted state of bacteriorhodopsin, which is observed in all spectra throughout this thesis, these two forms occur in a mixture with ratios between 1:1 and 1:2.

## 1.5 References


Altona, C. (1996) Vicinal coupling constants & conformation of biomolecules, in *Encyclopedia of nuclear magnetic resonance* [D. M. Grant and R. K. Harris (eds.)], John Wiley, London, pp. 4909-4923.

Asakura, T., Taoka, K., Demura, M. and Williamson, M.P. (1995) The relationship between amide proton chemical shifts and secondary structure in proteins. *J. Biomol. NMR*, **6**, 227-236.

Beger, R.D. and Bolton, P.H. (1997) Protein Φ and Ψ dihedral restraints determined from multidimensional hypersurface correlations of backbone chemical shifts and their use in the determination of protein tertiary structures. *J. Biomol. NMR*, **10**, 129-142.

Buckingham, A.D. (1960) *Can. J. Chem.*, **38**, 300-307.

Case, D.A. (1998) The use of chemical shifts and their anisotropies in biomolecular structure determination. *Curr. Op. Struct. Biol.*, **8**, 624-630.

Cavanagh, J., Fairbrother, W. J., Palmer III, A. G. and Skelton, N. J. (1996) *Protein NMR spectroscopy - principles and practice*. Academic Press, San Diego.

Celda, B., Biamonti, C., Aernau, M.J., Tejero, R. and Montelione, G.T. (1995) Combined use of $^{13}C$ chemical shifts and $^{1}H\alpha$-$^{13}C\alpha$ heteronuclear NOE data in monitoring a protein NMR structure refinement. *J. Biomol. NMR*, **5**, 161-172.

Clore, G.M. and Gronenborn, A.M. (1989) Determination of three-dimensional structures of proteins and nucleic acids in solution by nuclear magnetic resonance spectroscopy. *Crit. Rev. Biochem. Mol. Biol.*, **24**, 479-564.

Cornilescu, G., Delaglio, F. and Bax, A. (1999). Protein backbone angle restraints from searching a database for chemical shift and sequence homology. *J. Biomol. NMR* **13**: 289-302.

Ernst, R.R., Bodenhausen, G. and Wokaun, A. (1987) *Principles of Nuclear Magnetic Resonance in One and Two Dimensions*. Clarendon Press, Oxford.

Essen, L., Siegert, R., Lehmann, W.D. and Oesterhelt, D. (1998) Lipid patches in membrane protein oligomers: crystal structure of the bacteriorhodopsin-lipid complex. *Proc Natl Acad Sci U S A*, **95**, 11673-8.

Griesinger, C., Hennig, M., Marino, J.P., Reif, B., Richter, C. and Schwalbe, H. (1999) Methods for the determination of torsion angle restraints in biomacromolecules, in *Biological magnetic resonance, Volume 16: Modern Techniques in protein NMR* [Krishna and Berliner (eds.)], Kluwer Academic / Plenum Press.

Gryk, M.R., Abseher, R., Simon, B., Nilges, M. and Oschkinat, H. (1998) Heteronuclear relaxation study of the PH domain of β-spectrin: Restriction of loop motions upon binding inositol trisphosphate. *J. Mol. Biol.*, **280**, 879-896.

Haigh, C.W. and Mallion, R.B. (1980) *Prog. NMR Spectrosc.*, **13**, 303-344.

Haupts, U., Tittor, J., Bamberg, E. and Oesterhelt, D. (1997) General concept for ion translocation by halobacterial retinal proteins: the isomerization/switch/transfer (IST) model. *Biochemistry*, **36**, 2-7.

Haupts, U., Tittor, J. and Oesterhelt, D. (1999) Closing in on bacteriorhodopsin: progress in understanding the molecule. *Annu. Rev. Biophys. Biomol. Struct.*, **28**, 367-399.

Iwadate, M., Asakura, T. and Williamson, M.P. (1999) $C^{\alpha}$ and $C^{\beta}$ caron-13 chemical shifts in proteins form an empirical database. *J. Biomol. NMR*, **13**, 199-211.

Karplus, M. (1959) *J. Chem. Phys.*, **30**, 11.

Karplus, M. (1963) Vicinal proton coupling in nuclear magnetic resonance. *J. Am. Chem. Soc.*, **85**, 2870-2871.

Kay, L.E. (1995) Pulsed field gradient multi-dimensional NMR methods for the study of protein structure and dynamics in solution. *Prog. Biophys. Mol. Biol.*, **63**, 277-99.

Kuszewski, J., Quin, J., Gronenborn, A.M. and Clore, G.M. (1995) The impact of direct refinement against 13Ca and 13Cb chemical shifts on protein structure determination by NMR. *J. Magn. Reson. Ser. B*, **106**, 92-96.

Kutzelnigg, W., Fleischer, U. and van Wüllen, C. (1996) Shielding calculations: IGLO method, in *Encyclopedia of nuclear magnetic resonance* [D. M. Grant and R. K. Harris (eds.)], John Wiley, London, pp. 4284-4291.

Lanyi, J.K. (1997) Mechanism of ion transport across membranes. Bacteriorhodopsin as a prototype for proton pumps. *J. Biol. Chem.*, **272**, 31209-31212.

Le, H. and Oldfield, E. (1994) Correlation between $^{15}N$ NMR chemical shifts in proteins and secondary structure. *J. Biomol. NMR*, **4**, 341-348.

LeMaster, D.M. (1994) Isotope labeling in solution protein assignment and structural analysis. *Prog. Nucl. Magn. Reson. Spectr.*, **26**, 371-419.

Luecke, H., Schobert, B., Richter, H.T., Cartailler, J.P. and Lanyi, J.K. (1999a) Structural changes in bacteriorhodopsin during ion transport at 2 Angstrom resolution. *Science*, **286**, 255-260.

Luecke, H., Schobert, B., Richter, H.T., Cartailler, J.P. and Lanyi, J.K. (1999b) Structure of Bacteriorhodopsin at 1.55 Å Resolution. *J Mol Biol*, **291**, 899-911.

Luginbühl, P., Szyperski, T. and Wüthrich, K. (1995) Statistical basis for the use of $^{13}C\alpha$ chemical shifts in protein structure determination. *J. Magn. Reson. Ser. B*, **109**, 229-233.

Mitschang, L., Ponstingl, H., Grindrod, D. and Oschkinat, H. (1994) Geometrical representation of coherence transfer selection by pulsed field gradients in high-resolution nuclear magnetic resonance. *J. Chem. Phys.*, **102**, 3089-3098.

Mitschang, L. (1999) Signal Selection in High-Resolution NMR by Pulsed Field Gradients: I. Geometrical Analysis. *J. Magn. Reson.*

Neuhaus, D. and Williamson, M. (1989) *The nuclear Overhauser effect in structural and conformational analysis*. VCH, New York.

Oesterhelt, D. (1998) The structure and mechanism of the family of retinal proteins from halophilic archaea. *Curr. Opinion Struct. Biol.*, **8**, 489-500.

Oldfield, E. (1995) Chemical shifts and three-dimensional protein structures. *J. Biomol. NMR*, **5**, 217-225.

Ösapay, K. and Case, D.A. (1991) A new analysis of proton chemical shifts in proteins. *J. Am. Chem. Soc.*, **113**, 9436-9444.

Ösapay, K. and Case, D.A. (1994) Analysis of proton chemical shifts in regular secondary structure of proteins. *J. Biomol. NMR*, **4**, 215-230.

Oschkinat, H., Müller, T. and Dieckmann, T. (1994) Protein structure determination with three- and four-dimensional NMR spectroscopy. *Angew. Chem. Int. Ed. Engl.*, **33**, 277-293.

Pastore, A. and Saudek, V. (1990) The relationship between chemical shift and secondary structure in proteins. *J. Magn. Reson.*, **90**, 165.

Patzelt, H., Ulrich, A. S., Egbringhoff, H., Düx, P. Ashurst, J., Simon, B., Oschkinat, H. and Oesterhelt, D. (1997) Towards structural investigations on isotope labelled native bacteriorhodopsin in detergent micelles by solution-state NMR spectroscopy. *J. Biomol. NMR*, **10**, 95-106.

Pearson, J.G., Le, H., Sanders, L.K., Godbout, N., Havlin, R.H. and Oldfield, E. (1997) Predicting chemical shifts in proteins: structure refinement of valine residues by using ab initio and empirical geometry optimizations. *J. Am. Chem. Soc.*, **119**, 11941-11950.

Pervushin, K., Riek, R., Wider, G. and Wüthrich, K. (1997) Attenuated $T_2$ relaxation by mutual cancellation of dipole-dipole coupling and chemical shift anisotropy indicates an avenue to NMR structures of very large biological macromolecules in solution. *Proc. Natl. Acad. Sci. USA*, **94**, 12366-12371.

Prestegard, J.H. (1998) New techniques in structural NMR - anisotorpic interactions. *Nature Struct. Biol.*, **5**, 517-522.

Pulay, P. and Hinton, J.F. (1996) Shielding theory: GIAO method, in *Encyclopedia of nuclear magnetic resonance* [D. M. Grant and R. K. Harris (eds.)], John Wiley, London, pp. 4334-4339.

Ramsey, F.N. (1950) Magnetic shielding of nuclei in molecules. *Phys. Rev.*, **78**, 699-703.

Reif, B., Hennig, M. and Griesinger, C. (1997) Direct measurement of angles between bond vectors in high-resolution NMR. *Science*, **276**, 1230.

Sattler, M., Schleucher, J. and Griesinger, C. (1999) Heteronuclear multidimensional NMR experiments for the structure determination of proteins in solution employing pulsed field gradients. .

Sharp, K.A. and Honig, B. (1990) Electrostatic interactions in macromolecules: theory and applications. *Annu. Rev. Biophys. Chem.*, **19**, 301-332.

Sitkoff, D. and Case, D.A. (1998) Theories of chemical shift anisotropies in proteins and nucleic acids. *Prog. NMR Spectros.*, **32**, 165-190.

Solomon, I. (1955) Relaxation processes in a system of two spins. *Phys. Rev.*, **99**, 559-565.

Spera, S. and A., B. (1991) An empirical correlation between protein backbone conformation and Ca and Cb chemical shifts. *J. Am. Chem. Soc.*, **113**, 1406.

Stepisnik, J. (1981) Analysis of NMR Self-Diffusion Measurements by a Density Matrix Calculation. *Physica*, **104B**, 350-364.

Stepisnik, J. (1985) Measuring and Imaging of Flow by NMR. *Progr. NMR Spectr.*, **17**, 187-209.

Subramaniam, S. (1999) The structure of bacteriorhodopsin: an emerging consensus. *Curr Opin Struct Biol*, **9**, 462-468.

Szilagyi, L. (1995) Chemical shifts in proteins come of age. *Prog. NMR Spectros.*, **27**, 325-443.

Thomas, D.J., Mitschang, L., Simon, B. and Oschkinat, H. (1999) Signal Selection in High-Resolution NMR by Pulsed Field Gradients: II. The Design of Gradient Pulse Sequences. *J. Magn. Reson.*

Tjandra, N., Omichinski, J.G., Gronenborn, A.M., Clore, G.M. and Bax, A. (1997) Use of dipolar 1H-15N and 1H-13C couplings in the structure determination of magnetically oriented macromolecules in solution. *Nature Struct. Biol.*, **4**, 732-738.

Tolman, J.R., Flanagan, J.M., Kennedy, M.A. and Prestegard, J.H. (1995) Nuclear magnetic dipole interactions in field-oriented proteins: Information for structure determination in solution. *Proc. Natl. Acad. Sci. U.S.A.*, **92**, 9279-9283.

Warshel, A. and Russell, S.T. (1984) Calculation of electrostatic interactions in biological systems and in solutions. *Quart. Rev. Biophys.*, **17**, 283-422.

Williamson, M.P. and Asakura, T. (1993) Empirical comparison of models for chemical-shift calculation in proteins. *J. Magn. Reson. Ser. B*, **101**, 63-71.

Williamson, M.P., Kikuchi, J. and Asakura, T. (1995) Application of 1H NMR chemical shifts to measure the quality of protein structures. *J. Mol. Biol.*, **247**, 541-546.

Wishart, D.S., Bigam, C.G., Holm, A., Hodges, R.S. and Sykes, B.D. (1995) $^{1}H$,$^{13}C$ and $^{15}N$ random coil NMR chemical shifts of the common amino acids. I. Investigations of nearest-neighbor effects. *J. Biomol. NMR*, **5**, 67-81.

Wishart, D.S., Richards, F.M. and Sykes, B.D. (1992) The chemical shift index: a fast and simple method for the assignment of protein secondary structure through NMR spectroscopy. *Biochemistry*, **31**, 1647-1651.

Wishart, D.S. and Sykes, B.D. (1994a) The $^{13}C$ chemical shift index: a simple method for the identification of protein secondary structure using $^{13}C$ chemical-shift data. *J. Biomol. NMR*, **4**, 171-180.

Wishart, D.S. and Sykes, B.D. (1994b) Chemical shifts as a tool for structure determination, in *Methods in Enzymology* [N. J. O. a. T. L. James (ed.) Academic Press, San Diego, Vol. 239, pp. 363-392.

Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids*. Wiley-Interscience, New York.

Wüthrich, K. (1998) The second decade - into the third millenium. *Nature Struct. Biol.*, **5**, 492-495.