Modular Switches in Protein Function: 

* A Spectroscopic Approach

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Abstract

Understanding the molecular basis of protein function is a challenging task that lays the foundation for the pharmacological intervention in many diseases originating in altered structural states of the involved proteins. Dissecting a complex functional machinery into modules is a promising approach to protein function. The motivation for this work was to identify minimal requirements for “local” switching processes in the function of multidomain proteins that can adopt a variety of structural substates of different biological activity or representing intermediates of a complex reaction path. For example, modular switches are involved in signal transduction, where receptors respond to ligand-activation by specific conformational changes that are allosterically transmitted to “effector recognition sites” distant from the actual ligand-binding site. Heptahelical receptors have attracted particular attention due to their ubiquitous role in a large variety of pharmacologically relevant processes. Although constituting switches in their own right, it has become clear through mutagenesis and functional studies that receptors exhibit substates of partial active/inactive structure that can explain biological phenotypes of different levels of activity. Here, the notion that microdomains undergo individual switching processes that are integrated in the overall response of structurally regulated proteins is addressed by studies on the molecular basis of proton-dependent (chemical) and force-dependent (mechanical) conformational transitions.

A combination of peptide synthesis, biochemical analysis, and secondary structure sensitive spectroscopy (Infrared, Circular dichroism, Fluorescence) was used to prove the switching capability of putative functional modules derived from three selected proteins, in which conformational transitions determine their function in transmembrane signaling (rhodopsin), transmembrane transport (bacteriorhodopsin) and chemical force generation (kinesin-1). The data are then related to the phenotypes of the corresponding full length-systems. In the first two systems the chemical potential of protons is crucial in linking proton exchange reactions to transmembrane protein conformation. This work addresses the hypothesized involvement of lipid protein interactions in this linkage (1). It is shown here that the lipidic phase is a key player in coupling proton uptake at a highly conserved carboxylic acid (DRY motif located at the C-terminus of helix 3) to
conformation during activation of class-1 G protein coupled receptors (GPCRs) independently from ligand protein interactions and interhelical contacts. The data rationalize how evolutionary diversity underlying ligand-specifity can be reconciled with the conservation of a cytosolic ‘proton switch’, that is adapted to the general physical constraints of a lipidic bilayer described here for the prototypical class-1 GPCR rhodopsin (2).

Whereas the exact sequence of modular switching events is of minor importance for rhodopsin as long as the final overall active conformation is reached, the related heptahelical light-transducing proton pump bacteriorhodopsin (bR), requires the precise relative timing in coupling protonation events to conformational switching at the cytosolic, transmembrane, and extracellular domains to guarantee vectorial proton transport. This study has focused on the cytosolic proton uptake site of this retinal protein whose proton exchange reactions at the cytosolic halfchannel resemble that of rhodopsin. It was a prime task in this work to monitor in real time the allosteric coupling between different protein regions. A novel powerful method based on the correlation of simultaneously recorded infrared absorption and fluorescence emission changes during bR function was established here (3), to study the switching kinetics in the cytosolic proton uptake domain relative to internal proton transfer reactions at the retinal and its counter ion. Using an uptake-impaired bR mutant the data proves the modular nature of domain couplings and shows that the energy barrier of the conformational transition in the cytosolic half but not its detailed structure is under the control of proton transfer reactions at the retinal Schiff base and its counter ion Asp85 (4).

Despite the different functions of the two studied retinal proteins, the protonation is coupled to local switching mechanisms studied here at two levels of complexity, [a] a single carboxylic acid side chain acting as a lipid-dependent proton switch [b] a full-length system, where concerted modular regions orchestrate the functional coupling of proton translocation reactions. Switching on the level of an individual amino acid is shown to rely on localizable chemical properties (charge state, hydrophobicity, rotamer state). In contrast, switching processes involving longer stretches of amino acids are less understood, less generalizable, and can constitute switches of mechanical, rather than chemical nature. This applies particularly to molecular motors, where local structural switching processes are
Abstract

directly involved in force generation. A controversy exists with respect to the structural requirements for the cooperation of many molecular motors attached to a single cargo. The mechanical properties of the Hinge 1 domain of kinesin-1 linking the “neck” and motor domain to the “tail” were addressed here to complement single molecule data on torsional flexibility with secondary structure analysis and thermal stability of peptides derived from Hinge 1 (5). It is shown that the Hinge 1 exhibits an unexpected helix-forming propensity that resists thermal forces but unfolds under load. The data resolve the paradox that the hinge is required for motor cooperation, whereas it is dispensable for single motor processivity, clearly emphasizing the modular function of the holoprotein. However, the secondary-structural data reveal the functional importance of providing high compliance by force-dependent unfolding, i.e. in a fundamentally different way than disordered domains that are flexible but yet do not support cooperativity.

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1. Introduction

The complex organization that distinguishes living organisms from their inanimate surroundings relies upon their ability to execute vectorial processes such as direct movements, ion pumping across membranes, and the assembly of macromolecules and organelle systems. Such phenomena are executed by protein machines that harness chemical energy to drive processes that would be otherwise energetically unfavorable. Not only in this but as the ‘second part of the genetic code’ proteins play a pivotal role in living organisms, facilitating metabolism, communication, transport, and the maintenance of structural integrity. The diversity of functions of proteins is matched only by the diversity of protein structure, each protein being uniquely and exquisitely designed to fulfill its role.

To understand protein function on a molecular basis how function originates in structural features, a variety of techniques and strategies has been applied, ranging from predictions based on the sequence and physico-chemical properties of the constituent amino acids to precise methods for the identification of atoms and the determination of their molecular coordinates. Although the vast increase of the protein crystals reported in the last years has dramatically influenced on how one infers protein signaling, a true understanding of proteins can only be achieved by exploring the relationship that exists between the unique structure adopted by a protein and its function under native-like, i.e. non-crystalline conditions. One of the key challenges faced by today’s protein researchers is to develop appropriate methods that enable them to observe and quantitate structural transitions during protein function at a level of complexity that can still be reliably modeled, but retains the essential features of its ‘real’ counterpart.

Biospectroscopists has successfully borrowed the reductionalist approach from physicist and implemented it in complex biological systems to identify the key players within a macromolecule that are functionally most relevant. Although conceptually a simplification, the dissection of a protein into structural modules carrying specific functions of the holoprotein is strongly supported by analyses of molecular evolution which have shown that structurally homologous and independently folding domains of proteins involved in molecular recognition, enzymatic activity, and others can be found in and exchanged between functionally
diverse systems. This notion is extended here to structurally metastable modules that may act as elementary switches which work in a coordinated fashion in proteins whose functions need to be highly regulated by conformational changes. Coupled reactions, i.e., reactions that mutually affect their chemical and physical properties, play a fundamental role in biological processes and such interactions are regarded as allosteric when binding at one site induces conformational changes which alter the receptivity of a remote site. The stimuli can be pH change, mechanical force, redox potential, electrochemical energy, light, heat etc. Most of the proteins consist of an array of these functional modules whose molecular switching determines their function. The linkage of molecular switches into cross-talking signaling chains generates an intricate network of information flow within a cell. Such networks guarantee the proper differentiation, function and even the initiation of the death of a cell. An ever-increasing body of data suggests that proteins involved in the regulation of cellular events such as signal transduction, the cell cycle, protein trafficking, targeted proteolysis, cytoskeletal organization and gene expression are constructed in a modular fashion from a combination of distant domains.

The motivation of this work was to identify minimal requirements for “local” switching processes in the function of multidomain proteins that can adopt a variety of structural substates of different biological activity or possess intermediate states along a complex reaction path. Proton-dependent structural transitions are of particular interest because they can directly affect the non-covalent intramolecular H-bond networks, thereby providing the most efficient way for allosteric coupling over long distances. On the other hand, these networks are extremely difficult to reveal by experiment and are not deducible from x-ray structures as the latter do not resolve protons. In this work, spectroscopy, specifically infrared (IR) spectroscopy has been employed because it provides atomic resolution for the observation of changes in protein structure during protein function, and is particularly suited for monitoring protonation reactions at acceptor and donor groups. Unlike x-ray crystallography, IR spectroscopy can be applied in the presence of a lipidic phase which poses a critical problem for the crystallization of membrane proteins whose structures are typically free of a planar membrane environment. IR spectroscopy has been used here to elucidate the coupling between protonation and conformation in two related membrane proteins, the bovine visual photoreceptor rhodopsin and the proton pump
bacteriorhodopsin (bR) from *Halobacterium salinarium*. The specific role of the lipidic phase in proton-dependent conformational switching and the kinetic relation between conformational transitions at distant sites, both not evident from crystallography, is addressed.

Whereas these studies exploit the atomic resolution of IR spectroscopy to observe "proton switches" at individual amino acid side chains, conformational switches comprised of larger stretches of amino acids are at the focus of the later part of the work. These switches exhibit mechanical rather than chemical properties and are thus of direct relevance to force generation by molecular motors. Here, the spectroscopic and thermodynamic characterization of a hypothesized mechanically flexible domain required for the cooperation of kinesin-1 from *Drosophila melanogaster* has been performed. It is shown that molecular flexibility is not identical with intrinsic disorder of corresponding domains. Thus, this work has concentrated on switching modules at three levels of complexity, the level of a single amino acid side chain, linking proton exchange reactions to transmembrane conformation through lipid protein interactions, the level of long range proton-dependent conformational coupling in bR, and on the level of structural transitions of amino acid stretches, where chemical force generation is directly linked to conformation. In all systems the spectroscopically determined physical properties of the switching modules are related to the function of the holoproteins.

### 1.1 Peptides as model systems for understanding microswitches

Interactions between proteins and lipids lie at the heart of virtually all membrane processes and are essential for a large variety of cellular processes, including transport, signaling, and membrane biogenesis, but on a molecular level they are very poorly understood, owing to the complexity of re-incarnating them *in vitro* for crystallization. Lipids and protein interact with each other in many ways during their activation. A way to reveal the basic principles of protein-lipid interactions is the use of model systems comprising peptides that mimick transmembrane regions of proteins in synthetic lipid bilayers. Peptides of variable length and hydrophobicity can be designed to answer specific questions related to the full length systems. In this thesis I have designed and utilized peptides to understand the reorientation of side chains near the lipid/water interface under negative
mismatch conditions and could prove that alteration of the peptide charge state can result in conformational changes that increase the effective hydrophobic length of the peptide. Several peptides were derived from the helix 3 of rhodopsin carrying the conserved D(E)RY motif and spectroscopy was called in to study the coupling between conformation and protonation. The $pK_a$ of the conserved carboxyl, its linkage to helical structure, and the effect of protonation on side chain to lipidic head group distance revealed the modular nature of the D(E)RY motif as an autonomous proton switch. In the later part of this work peptides derived from an expected unordered domain from the Hinge 1 region of kinesin-1 were analyzed with respect to secondary structure and stability. The results have resolved the apparent contradiction between data on single molecule processivity, which is independent from the Hinge 1 and the observation of a lack of cooperativity between many motors attached to a single cargo when the Hinge 1 is deleted.

1.2 Chemically driven modular switches

The involvement of the chemical potential of protons in regulating micro-switching of structural transitions in protein activation is widespread. One of the most crucial reactions in biology is actually the proton gradient-driven synthesis of ATP. The ubiquitous ATP synthase (ATPase) takes advantage of the transmembrane proton gradients that are created by energy-transducing systems of which the bacterial light-driven proton pump bR is the most primitive as it generates a proton gradient without any electron transport. Shortly, its mode of action is through a proton wire mechanism including water molecules and protein side-chains. Proton wires are of general importance in membrane biology and have been studied for several transmembrane protein channels such as gramicidin A (1) and the tetrameric channel formed by the M2 protein from human influenza virus (2,3). In bR, however, photoisomerization of the all-trans retinal covalently bound through a protonated Schiff base to a Lys in helix G drives a sequence of reactions resulting in the net transport of a proton to the extracellular side.
1.2.1 Bacteriorhodopsin, a classical biological proton pump

Living cells are in demand of proton transfer across membranes. The simplest form of an energy-transducing proton pump is represented by the bacterial membrane protein bacteriorhodopsin (bR). It was discovered and identified as a light-dependent proton pump in the early 1970s (4). bR, the retinal protein of the halophilic archaea *Halobacterium salinarum*, is the best described system to understand the mechanism of proton translocation due to the existence of high-resolution crystallographic structures (5) including those of several intermediates of its transport cycle (6-9). In contrast to most of the pumps where energy input is from a chemical reaction, bR derives its input to start the pumping from light induced photoisomerization of the *all-trans* retinal to the *13-cis* form. The chromophore retinal is covalently attached to the protein via a protonated Schiff base (PSB).

Upon illumination, retinal isomerizes from all-trans to 13-cis and the key event that follows includes protonation of the Asp85 (Figure 1.1) by deprotonation of the retinal Schiff base which triggers the cascade of hydrogen-bond rearrangements that culminate in release of a proton to the extracellular surface. This coupling is mediated by the side-chain of the positively charged Arg82 residue swinging away from the no longer negatively charged Asp85. Movement of the 13-methyl group of the isomerized retinal initiates, in turn, restructuring of the cytoplasmic region leading to reprotonation of the Schiff base by Asp96, followed by the uptake of a cytosolic proton which restores the protonated state of Asp96. This is accomplished by displacements of the side-chain of Lys216, where the retinal is attached and of the indole ring of Trp182. The ensuing breaking of hydrogen-bonds and repacking of side-chains causes the outward tilt of one of the transmembrane helices (helix F) (10), and allows the intercalation of water in this region. This, in turn, lowers the pK of the proton donor Asp96 and opens a proton transfer pathway towards the retinal Schiff base (11).

Even though with all this wealth of information one of the fundamentally unclear questions regarding the mechanism of proton transport in bR is the structural nature of the ‘switch’ event, that couples the distant domains and ensures the correct timing of the events occurring during bR photocycle. To observe functionally relevant structural transitions in real time and to assign them to specific locations in
the protein structure, this work has developed an Infrared Fluorescence Cross Correlation spectroscopy approach which combines the sensitivity of vibrational and

Figure 1.1 Crystallographic structure of bacteriorhodopsin (PDB code 1C3W) (5) with the cytoplasmic surface facing upward. The amino acids discussed in the text most relevant proton transport, all-trans retinal (purple), and water molecules (green) are shown (11).

fluorescence spectroscopy. Along with mutagenesis this approach has been used to investigate the relation between internal proton transfer reactions at the Schiff base and Asp85 and conformational changes happening in the cytoplasmic side of the protein at helix C, i.e. a location analogous to the conserved carboxylic acid that acts as a proton acceptor in rhodopsin (see below). The information deduced from this novel Cross Correlation approach in conjunction with available crystal structures and published functional studies, provides fresh insights into the understanding of the timing of structural transitions in the bRD96A mutant (Chapter 4).
1.2.2 Rhodopsin, a prototypical G protein-coupled receptor

G-protein coupled receptors have been at the centre of interest for physiologists and pharmacologists long before it was known that they were G-protein-coupled, or even generally accepted that they were physical entities (12). GPCRs or seven transmembrane receptors (7TM) constitute the main family of cell surface receptors for both chemical stimuli (e.g. hormones, neurotransmitters and chemo attractants) and sensory stimuli (e.g. light, odorants and taste molecules). Binding of these extracellular ‘agonist’ ligands triggers receptor activation that couples to and activates signaling proteins such as heterotrimeric GTP-binding proteins (G proteins), which in turn modulate the flow of secondary messengers, such as cAMP, involved in critically important physiological process (such as heart beat). Key initiators of multiple biochemical signaling pathways, GPCRs are also involved in many pathological processes and are targets of many of the available medical drugs used in humans. It is often pointed out that more than 50% of all drugs, with annual world wide sales exceeding $50 billion, regulate GPCR function, and some 30% of these drugs directly target them (13). Determining the molecular mechanisms by which this important family of receptors function is thus of paramount importance and could provide a molecular basis for the development of new therapies for many physiological disorders.

Structurally these receptors are characterized by their seven transmembrane segments (Figure 1.2.), each identified by highly conserved so called fingerprint residues folding up in a characteristic seven helical bundle. Among the three main classes of 7TM receptors, family A (also known as rhodopsin-like receptors) constitutes the quantitatively dominating class, which also is the only one which today could boast of having high-resolution structural information concerning the seven helical bundle (14-19). 7TM receptors transducer transmits extracellular chemical signals into intracellular signal transduction events through a transmembrane allosteric mechanism. Receptors kept in an inactive state until the binding of a ligand ‘agonist’ switches them into an active state within milliseconds. Despite the fact that these receptors are activated by agonists of an incredibly different chemical nature, the overall activation mechanism is believed to be shared among at least the class-1 receptors. The transition between the inactive and active states involves the release of at least two important molecular constraints, known as
the “ionic lock” between charged residues at the cytosolic side of the receptor and the “rotamer toggle switch” in helix 6 (20-22). It is not understood, however, how an extremely large variety of structurally different ligands can operate essentially in the same mechanism.

**Figure 1.2** Topology and common structural features of GPCRs from class A. The characteristic seven transmembrane helices are shown as numbered cylinders TM1-VII. These are connected by intracellular loops ICL-I-III. Potential microswitches of common sequence motif are denoted by white circles (23).

This study has specially concentrated on the physical basis of switching in the bovine photoreceptor, rhodopsin, which provides the molecular basis of vision. It is a prototypical GPCR which allows transducing the absorption of light into a nerve pulse in the retina of the eye. The primary event in vision is the photoisomerization of the covalently bound ligand 11-cis retinal, which evokes structural changes much similar to those induced by the binding of a hormone to hormone receptor. As bR, rhodopsin is a retinal protein with the 11-cis retinal Schiff base linked to an analogous Lys-residue in helix 7. In contrast to bR, it is not a vectorial pump and does not transduce light energy into a chemical potential of protons but acts a single photon sensor. Its heptahelical structure and signal transduction chain renders rhodopsin a prototypical class-1 (rhodopsin-like) GPCR. The more surprising is the involvement of proton transfer reactions in rhodopsin activation reminiscent of those in bR. The current activation model actually consists of two proton switches, an
internal one at the retinal Schiff base chromophore and another "external" at the cytosolic end of helix 3, i.e. analogous to the cytosolic proton acceptor in bR (see above). In this work, I have studied the hypothesized involvement of lipid protein interactions in linking the external activating proton switch to transmembrane conformation.

In addition to the conserved seven transmembrane topological features, GPCRs possess conserved sequence motifs as well. Sequence motifs and post-translational covalent modifications characteristic of class A GPCRs, including chemotaxis receptors, are distributed among the various receptor domains. Some of these features are potentially important to the proper folding and delivery of the receptor to the plasma membrane while others are important for proper structure and activation. In particular, certain side chains are involved in salt bridges and H-bonds that function as structural constraints maintaining the receptor in its active conformation.

Among the conserved regions that could serve as microswitches this study has concentrated on the D(E)RY motif as a proton uptake module that couples to specific lipid protein interactions which stabilize the active receptor conformation but are not deducible from the crystal structure. The Glu/Asp-Arg-Tyr sequence found at the cytoplasmic interface of TM3 (Figure 1.2) is one of the class-defining highly conserved motifs and designated “E/D R Y” according to the generalized numbering scheme for GPCRs (24). It is actually E-R-Y in rhodopsin and D-R-C in the receptor for fMLF. The triad is located at the water-lipid interface, where the alternation of hydrophobic and hydrophilic side chains may be of functional importance for local secondary structure and the transmembrane topology of TM3.

Here, I have investigated by fluorescence and Fourier-transform-infrared (FTIR) spectroscopy the proton-sensitivity of the structure of synthetic peptides derived from this region (Chapter 3). The results evidence that the proton-induced structural change in the D(E)RY motif promotes terminal helicity and affects the lipid head group to TM3-C-terminus distance. The physico-chemical properties of the D(E)RY motif render it an autonomous conformational proton switch that functions independently from ligand-protein interactions. The result will be discussed in the context of rationalizing the conservation of GPCR function despite the high evolutionary diversity with respect to ligand specificity.
1.3 Force driven modular switch in kinesin-1

The most direct influence on a protein can be regarded as its response to external mechanical force. Systems that are subjected to forces in their natural biological switching process are molecular motors. Molecular motors are amazing biological nano-machines that are responsible for most forms of movement in the cellular world. Three types of cytoplasmic motors are known: myosins, which move on actin filaments, and dyenins and kinesins, which use microtubules as tracks. Myosin and dynein were known as early as 1985, but the motor protein responsible for axonal transport was discovered in 1985 by Ron Vale et al. who named the protein “kinesin”, which comes from the Greek *kinein*, meaning to move (25). After this path-breaking discovery the initial years show a flurry of experiments to prove the ability of kinesin to translocate beads or membranous cargo along microtubules at fast speeds. In kinesin and other motors, ATP hydrolysis causes a small conformational change in a globular motor domain that is amplified and translated into movement with the aid of accessory structural motifs. This modular design of motors has given rise to considerable complexity so that each of the three motors comprises a super family whose members may vary appreciably in makeup and function. Today, 18 different classes of myosins, 10 different classes of kinesins and 2 groups of dyenins are identified (26).

Structurally, the kinesin-1 molecule of *Drosophila melanogaster* is a tetramer *in vivo* (25) consisting of two heavy chains (Figure 1.3) and two light chains. The two heavy chains of kinesin dimerize into a functional molecule, where each heavy chain consists of a motor domain (1-325aa), neck linker (325-340aa) and neck (coiled-coil 345-378aa). The rest of the tail is mainly a coiled coil with two interruptions (378-440aa and 560-624aa), the second break is also known as Hinge or Kink. The mechanism by which kinesin-1 moves membrane-bounded cargo such as mitochondria and vesicles along microtubules is termed as processive (27). Several works have shown that kinesin motion is processive by giving hundreds of 8 nm steps per microtubule encounter (28-30) or by showing that each encounter resulted in the hydrolysis of ~120 ATP molecules (31). Apart from the processivity kinesin-1 motor’s movement is coordinated between two heads. This was proven experimentally with a kinesin construct with one head (32). The arguments for kinesin processivity and coordination between heads have been reviewed (26,33).
With the development of optical tweezers, it has become possible to measure piconewton forces exerted by single kinesin molecules (29) and the stepping mechanism of this motor (35) is explained by symmetric/asymmetric hand-over-hand mechanism (36) or inch worm model both using 1 ATP molecule for every 8 nm step.

Multiple kinesin motors move single cargo and the cooperativity responsible element in the structure was a matter of debate. It was postulated that Hinge 1 might be the structurally flexible modular domain that provides sufficient compliance during multimotor cargo transport. To test the structural and functional importance of Hinge 1, I employed peptide synthesis (from Hinge 1-derived sequence) followed by their detailed secondary structure and thermal stability analysis. The data derived from these experiments complemented the single molecule observation of motility and torsional compliance of kinesin-1 in which the putative flexible region was removed. It is suggested that the Hinge-1 region is sufficiently structured to resist thermal forces, but that it is disrupted by the forces generated during motility, thereby preventing it from hindering motion in multimotor conditions (Chapter 5).
1.4 Literature Cited


2. Methods

The real-time observation of molecular switching processes in proteins described in the introduction requires structure-sensitive methods that can be applied in native-like conditions such as solutions, vesicles, micelles, hydrated films etc., where these proteins are functional \textit{in vitro}. Another key requirement for the methods that could be used in this type of structure-function research is that they should guarantee a fast data acquisition regime. Spectroscopy fulfills this criterion and hence its different types have been extensively used in this study. The predominant electronic and vibrational spectroscopic techniques employed in this work are described in the following sections. Special emphasis has been placed on the development of the simultaneous combination of infrared and fluorescence measurements to correlate site-specific information with overall structural transitions. This approach has not yet been routinely employed and offers a great potential to study long-range conformational coupling and thus is ideally suited to investigate the coupling between conformations in modular proteins during biologically relevant switching processes (1).

2.1 Fluorescence Spectroscopy

Fluorescence spectroscopy is a widely used technique in the modern day structure-function research. Although, the technique fails to provide direct structural information, it detects changes in the physical environment and dynamic properties of biological macromolecules and requires only small sample concentration. Recent advances in the instrumentation has stretched the reach of this technique which can now be used to carry out studies at many levels ranging from simple but informative steady-state emission for which the instrumentation need is quite modest to advanced sophisticated time-resolved studies (2). The data derived from fluorescence measurements when combined with the structure information gained from IR and CD spectroscopy in this work leads to a detailed insight into the complexity of protein structure-function relations.

In this work, fluorescence spectroscopy has been used to quantitate structural transitions in peptides evoked by physical parameters that are of direct relevance to the function of the proteins from which these peptide sequences have been derived.
As fluorescence is determined by the properties of a defined chromophore and its physical environment, it provides information on a specific site within a larger molecule. This has been used here to observe structural transitions at the water/lipid phase boundary in a transmembrane protein segment (see Chapter 3). This information very well compliments infrared studies which are more informative on secondary structure but often lack the direct relation to the location of an observed structural change within a biopolymer. Likewise, the combination of a local (fluorescence) monitor with a global (circular dichroism) structural monitor has been used to localize flexibility/rigidity in a functional module that links force generation to structural transition in a motor protein (see Chapter 5).

2.1.1 Basic Fluorescence Theory

Fluorescence spectroscopy exploits the electronic transitions of the biomolecules under study. Luminescence is the emission of light which occurs when a molecule is electronically excited. It can be of two types: Fluorescence and phosphorescence (3). In the case of fluorescence, electron de-excitation occurs almost spontaneously and also the emission from a luminescent substance ceases when the exciting source is removed. Generally in fluorescent materials, the excited state has the same spin as the ground state. If A* denotes the excited state of substance A, then fluorescence consist of the emission of a photon:

\[ A^* \rightarrow A + h\nu \]

Where \( h \) is the Planck’s constant and \( \nu \) is the frequency of photon. The absorbed photon is of higher energy than the emitted light (luminescence).

The process of fluorescence is illustrated by the simple electronic-state diagram called Jablonski diagram (Figure. 2.1). The fluorophores can exist in a number of vibrational energy levels, denoted by 0, 1, 2 etc. at singlet ground, first and second electronic state depicted by \( S_0 \), \( S_1 \) and \( S_2 \), respectively. Transitions between states are pointed out in vertical lines to illustrate the instantaneous nature of light absorption expressed by the Born-Oppenheimer approximation, stating that the nuclei don’t move during the electronic transition. There are several important events that precede light emission. A fluorophore is excited to some higher vibrational level of either \( S_1 \) or \( S_2 \) and in most cases rapidly relaxes to the lowest vibrational level of \( S_1 \). This nonradiative process is called internal conversion occurring in \( \sim 10^{-12} \) seconds or less.
Return to the ground state occurs to a higher excited vibrational ground-state level, which then quickly reaches thermal equilibrium. The emission spectrum is typically a mirror image of the absorption spectrum of the $S_0 \rightarrow S_1$ transition. Molecules in the state $S_1$ can also undergo a spin conversion to the first triplet state $T_1$. Emission from $T_1$ is termed phosphorescence and is generally shifted to longer wavelength relative to fluorescence. Conversion of $S_1$ to $T_1$ is called intersystem crossing. Due to the higher quantum yield for fluorescence it is the singlet singlet transition that has been used in this work to monitor tryptophan emission, which however also exhibits long wavelength phosphorescence.

![Jablonski diagram](image)

**Figure 2.1** Jablonski diagram

### 2.1.2 Fluorescence Measurements

Generally fluorescence measurements can be broadly classified into steady state and time-resolved spectroscopy. In this work, measurements were performed mostly in steady state i.e. under constant illumination and observation of emission. Here, the sample is illuminated with a continuous beam of light, and the intensity or emission spectrum is recorded. A sample reaches its steady state immediately after the illumination and in its simplest form the decay of the fluorescent intensity following a short excitation pulse, $I(t)$, is a single exponential process.
\[ I(t) = I_0 e^{-\frac{t}{\tau}} \]

Here, \( I_0 \) is the fluorescence intensity at time \( t = 0 \), and \( \tau \) is the fluorescence life-time. The fluorescence life-time is independent of factors such as excitation intensity, absorption effects, and fading due to photo-bleaching, which on the other hand are sources of error in steady state experiments. Therefore, steady state experiments in this work are all including specific normalization procedures, such that relative changes in the state of a system are obtained. Normalization procedures are described in the respective Chapters 3 and 5.

### 2.1.3 Fluorophores

Fluorescence probe selection is a vital issue in fluorescence spectroscopy. Spectral properties of fluorophores determine the wavelength and time resolution required for the instruments to be used. Fluorophores can be broadly classified into two major groups; intrinsic, which occur naturally and extrinsic which are added to the sample to make it fluorescent. Aromatic amino acids, NADH, flavins, and derivatives of pyridoxal and chlorophyll are some of the intrinsic fluorophores that are widely exploited in spectroscopy. In this work, the intrinsic fluorescence nature of an aromatic amino acid tryptophan present in peptides under study is utilized. These synthetic peptides are derived from the corresponding genomic sequences and the tryptophans are either carried over from their parent sequences or engineered by conservative replacement.

**Intrinsic fluorescence:** Intrinsic fluorescence of proteins in most of the cases is provided by three aromatic amino acids (tryptophan, tyrosine and phenylalanine). The fluorescence of a folded protein is a mixture of the fluorescence from individual aromatic residues. Protein fluorescence is generally excited at 280-295 nm. Most of the emissions are due to excitation of tryptophan residues, with a few emissions due to tyrosine and phenylalanine. These three residues have distinct absorption and emission wavelengths. They differ greatly in their quantum yields and life times. Due to these differences and to resonance energy transfer from proximal phenylalanine to tyrosine and from tyrosine to tryptophan, the fluorescence spectrum of a protein containing the three residues usually resembles that of tryptophan. The table below summarizes the fluorescence characteristics of these three aromatic residues.
Table 2.1 Fluorescence characteristics of three aromatic amino acids (Lifetime and wavelength are shown in nanoseconds and nanometers respectively).

<table>
<thead>
<tr>
<th></th>
<th>Absorption</th>
<th>Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lifetime</td>
<td>Wavelength</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>2.6</td>
<td>280</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.6</td>
<td>274</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>6.4</td>
<td>257</td>
</tr>
</tbody>
</table>

Tryptophan has much stronger fluorescence and higher quantum yield than the other two aromatic amino acids. The intensity, quantum yield, and wavelength of maximum fluorescence emission of tryptophan is microenvironment-dependent in proteins and peptides due to the nonradiative decay pathways available to the indole chromophore (Figure 2.2).

![Figure 2.2] Tryptophan fluorescence intensity depends on its microenvironment

The fluorescence spectrum shifts to shorter wavelength and the intensity of fluorescence increases as the polarity of the solvent surrounding the tryptophan residue decreases. Tryptophan residues which are buried in the hydrophobic core of the proteins can have spectra which are shifted by 10-20 nm compared to tryptophans on the surface of the protein. Variable proximity of excited indole to functional
methods which may participate in nonradiative decay processes, such as proton transfer, electron transfer or exchange, energy transfer and solvent relaxation or quenching causes the lifetime heterogeneity (4).

In this work, the polarity-dependence of tryptophan emission has been used extensively to observe proton-induced structural changes of a transmembrane segment derived from class 1 GPCRs (see Chapter 3) and to follow unfolding of the isolated hinge domain of the motor protein kinesin (see Chapter 5).

2.1.4 Förster Resonance Energy Transfer (FRET)

FRET is a distance-sensitive physical phenomenon where there is a radiationless transmission of energy from one molecule to another. A pair of molecules that interact in such a manner that FRET occurs is often referred to as a donor/acceptor pair. The donor molecule is the dye or chromophore that initially absorbs the energy and the acceptor is the chromophore to which the energy is subsequently transferred. This resonance interaction occurs over greater than interatomic distances, without conversion to thermal energy, and without any molecular collision. The transfer of energy leads to a reduction in the donor’s fluorescence intensity and excited state lifetime, and an increase in the acceptor’s emission intensity.

Some of the primary conditions that need to be met in order for FRET to occur are a) the donor and acceptor molecules must be in close proximity to one another (typically 10-100 Å). b) the absorption or excitation spectrum of the acceptor must overlap the fluorescence emission spectrum of the donor c) the donor and acceptor transition dipole orientations must be approximately parallel. Förster demonstrated that the efficiency of the process (E) depends on the inverse sixth distance between donor and acceptor.

\[
E = \frac{R_o^6}{R_o^6 + r^6}
\]

where \(R_o\) is the Förster distance at which half the energy is transferred and \(r\) is the actual distance between donor and acceptor. The distance at which energy transfer is 50% efficient is referred to as the Förster radius (\(R_o\)) (5).

In this thesis work, 1, 2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(5-dimethylamino-1-naphthalenesulfonyl) has been used as a label in lipid head groups. Due to the overlap of its absorption spectrum with the fluorescence spectrum of
tryptophan the latter can be partially quenched by a neighboring DANSYL group (Figure 2.3) Since this process is distance-dependent it can be exploited to monitor distance changes between a trp-containing peptide segment and the surface of a host vesicle reconstituted with the ‘guest’ peptide (Figure 2.3). This has allowed characterizing proton-dependent changes in the transmembrane topology of a conserved proton-uptake module in class 1 GPCRs (see Chapter 3).

![Figure 2.3 FRET monitored between the Trp and DANSYL in a DANSYL-PE reconstituted peptide reveals the shift of the C-terminus of the peptide to the lipidic head group depending upon the protonation state of a single amino acid side chain.](image)

### 2.2 Circular Dichroism

Circular dichroism (CD) is being increasingly used for examining the structure of biological macromolecules in solution. Among the very many applications CD is widely employed for determining whether a protein / peptide is folded and if so to characterize its secondary structure, to study the conformational stability of proteins in response to temperature, pH, denaturants and to determine whether protein-protein interactions alter the conformation.

In this work, CD-spectroscopy was used as an additional tool to FTIR spectroscopy to access secondary structure of peptides. The advantage of CD is the smaller concentration requirements which makes it applicable under conditions where IR-spectroscopy will be insensitive or promote peptide association/aggregation.
2.2.1 Origin of CD effect

Plane-polarized light consists of 2 circularly polarized components of equal magnitude, one rotating counter-clock wise (left handed, L) and the other clockwise (right handed, R). CD refers to the differential absorption of these 2 components (see Figure 2.4). If, after passage through the sample being examined, the L and R components are not absorbed or are absorbed to equal extents, the recombination of L and would regenerate radiation polarized in the original plane. However, if L and R are absorbed to different extents, the resulting radiation possesses elliptical polarization. A CD signal will be observed when a chromophore is chiral ((6).

![Figure 2.4](image)

*Figure 2.4* The left (L) and right (R) circularly polarized components of plane polarized radiation: (I) the two components have the same amplitude and when combined generate plane polarized radiation; (II) the components are of different magnitude and the resultant (dashed line) is elliptically polarized (6).

2.2.2 CD Spectroscopy on Proteins

CD is a powerful technique that is employed routinely to detect chromophores in proteins such as peptide bond (absorption below 240nm), aromatic amino acid side chains (absorption in the range 260 to 320 nm) and disulphide bonds (weak broad absorption bands centered around 260 nm). The most important type of information that can be deduced from CD studies of protein is the detailed profiling of secondary structure.

**Fractional decomposition of secondary structure:** Peptide bonds give rise to the absorption differences in the region below 240 nm and below in the CD spectra. A characteristic CD spectra in the far UV region consists of contributions from various
regular secondary structures that constitute the proteins (see Figure 2.5). Fractional decomposition of such a CD spectra into its components (helix, sheets, turns etc.) can be accomplished using any of the existing dedicated algorithms which employ the basis data sets comprising the CD spectra of proteins of various folds whose structures have been solved by X-ray crystallography. An online server DICHROWEB, hosted at Birkbeck College, University of London, U.K provides a commonly used online service which allows the parent CD data to be entered in machine units and to be analyzed using various algorithms (SELCON, VARSLE, CDSSTR, K2d and CONTIN) with a choice of data bases. Detailed descriptions of the algorithms and theoretical basis of CD techniques has been reviewed elsewhere (7-9).

2.2.3 CD data presentation

The general practice of representing CD data is in terms of either ellipticity \([\theta]\) degrees or differential absorbance’s (\(\Delta A\)). The Mean Residue Weight (MRW) for the peptide bond is calculated with \(\text{MRW} = \frac{\text{MW}}{N-1}\), where \(M\) is the molecular mass of the polypeptide chain (in Da), and \(N\) is the number of amino acids in the chain; the number of peptide bond is \(N-1\). For most proteins the MRW is 110+/-5 Da (10). The mean residue ellipticity at wavelength \(\lambda\) is given by \(\theta_{\text{mrw}, \lambda} = \text{MRW} \times \theta_{\lambda} / 10 \times d \times c\) where \(\theta_{\lambda}\) is the observed ellipticity (degrees) at wavelength \(\lambda\), \(d\) is the path length (cm) and \(c\) is the concentration (g/ml). Alternatively one can use: \(\theta_{\text{molar}, \lambda} = (100/d \times m) \theta_{\lambda}\), if the molar concentration (\(m\)) is known. In both cases the values are expressed in deg cm\(^2\) dmol\(^{-1}\).

2.2.4 Determination of Sample concentration

As pointed out in the previous section, accurate determination of the concentration of the sample under study in CD spectroscopy is vital in deriving secondary structural information from machine data. In the case of protein samples, the widely used methods to determine the concentration include Lowry method and absorption spectroscopy.
Figure 2.5 Far UV CD spectra depicting various types of secondary structure. Solid line, α-helix; long dashed line, antiparallel β-sheet; dotted line, type-1 β-turn; cross dashed line, extended 3_1 helix or poly (pro) 11 helix; short dashed line, irregular structure (6).

**Absorption spectroscopy**: Absorption spectroscopy involves the comparison of light transmitted through a blank to the light transmitted through an absorbing species. The concentrations of the protein samples containing a single tryptophan were calculated using the Beer-Lambert law: \( A = \varepsilon l m \), where \( A \) is the measured absorbance, \( \varepsilon \) is the extinction coefficient, \( l \) is the path length, and \( m \) is the molar concentration. The molar extinction coefficient was calculated in the ExPASy website with ProtParam Tool ([http://www.expasy.org/tools/protparam.html](http://www.expasy.org/tools/protparam.html) and references therein).
2.3 Fourier Transform Infrared Spectroscopy (FTIR)

Unlike the previous two sections which dealt with techniques working on the principle of electronic transitions this part explains a spectroscopic technique that makes use of vibrational transitions in biomolecules. With the advent of NMR and mass spectrometry in 1970s, infrared was almost an abandoned technique but the last two decades changed the situation dramatically; thanks to the advent of rapid commercial development of Fourier transform infrared spectrometry. Today it has become an established tool in the biophysical laboratories for structural characterization of biomolecules. In contrast to x-ray crystallography FTIR spectroscopy allows to observe structural changes in real time and provides much more freedom in terms of the physical state in which a sample can be studied. Furthermore, FTIR is a non-destructive method (samples can be used for other purposes after the IR spectroscopic measurements), does not imply limits on the size of the molecules that can be studied and is independent of extrinsic probes of protein conformations as it relies on the intrinsic vibrational energy levels of the molecules.

In this work FTIR spectroscopy has been used to access the secondary structure profile of peptides reconstituted in different systems that preserve their functionally relevant properties. Conformational transitions in these peptides mimic key processes occurring in either signaling by GPCRs (Chapter 3) or during motility of kinesin (Chapter 5). Here, FTIR has been particularly used to quantitate the pKₐ of an amino acid side chain which is coupled to the conformational status of transmembrane 3 of rhodopsin. This technique is unique as it provides side chain chemical information (protonation state) and secondary structure information at the same time.

2.3.1 Working Principle

IR spectroscopy is a non-invasive technique providing direct information on bond orders, electrostatic interactions, H-bonding, charge distributions, protonation states, redox states, dynamics and kinetics (11-13). In IR spectroscopy, which is a resonant technique, photons of wavenumbers typically between 10² and 10⁴ cm⁻¹ induce a transition from a vibrational ground level of the molecule under study to an excited state when the energy of the photon equals the energy difference between ground and vibrationally excited state. Since the molecule in the excited level returns
very fast to the ground state by thermal relaxation, this transition will be detected as absorption of photons with energy equal to the energy difference of the involved vibrational levels (Figure 2.6)

![Energy level diagram of IR absorption](image)

**Figure 2.6** Energy level diagram of IR absorption

The infrared spectrum is plotted against the inverse of wavelength, the wavenumber (ν), which is proportional to the transition energy and has the unit cm\(^{-1}\). The horizontal coordinate of the spectrum runs from high wavenumbers to low wavenumbers according to a recommendation of the International Union of Pure and Applied Chemistry (IUPAC). The infrared spectral region is adjacent to the visible spectral region and extends from 0.78 µm to about 1000 µm which can be further subdivided into the near infrared region from 780 nm to 2.5 µm, the mid infrared region from 2.5 µm to 50 µm and the far-infrared region from 50 µm to 1000 µm. The mid-infrared spectral range extending from 2.5 to 50 µm corresponds to 4000 to 200 cm\(^{-1}\) and is of particular interest in protein research (14).

For most molecules the IR spectrum will show several absorption bands which correspond to fundamental vibrations. Fundamental vibrations are those vibrations that do not cause a translation or a rotation of the molecule as a whole and can be excited independently. These vibrations are only visible if the vibration is coupled with a changing dipole moment and if the vibrational wavenumber is identical to the
wavenumber of the incident radiation. Fundamental vibrations can be divided into four types.

A. Stretching vibrations where one or more of the bond lengths change.

B. Planar bending vibrations where one or more bond angles change, while the bond length remains constant.

C. Out-of-plane bending vibrations where one atom oscillates through a plane defined by (at least) three neighboring molecules.

D. Torsion vibrations where a dihedral angle (the angle between two planes, which have one bond in common) is changed.

The above vibration types are illustrated in Figure 2.7

The bending vibrations are subdivided into rocking, twisting and wagging bands. In general, the absorption bands due to any of these vibrations can be assigned to different wavenumber regions as detailed(15). The vibrations can also be classified by symmetry, where symmetrical vibrations retain the symmetry of the group and asymmetrical vibrations disturb one or more of the symmetry elements of the molecule.

Biomolecules contain one or many functional/structural groups which possess vibrational wavenumbers that are nearly independent of the rest of the molecule. To take an example, the stretching vibration of the carbonyl group is always observed in the range of 1650-1740 cm⁻¹. These wavenumbers that characterize the functional or structural group present in the sample are termed group frequencies. The presence of various group vibrations in the IR spectrum is of great importance in identifying the absorbing molecules. For many groups involving only two atoms, the approximate
wavenumber of the fundamental vibration can be calculated from a simple harmonic oscillator model with

\[ \nu = \frac{\sqrt{k}}{2\pi\sqrt{\mu}} \]

Where \( k \) is the force constant of the bond and \( \mu \) is the reduced mass of the two atoms. From the equation it is clear that fundamental vibrations for groups with higher reduced mass appear at lower wavenumbers. Characteristic wavenumbers of vibrations for most groups of interest are in the 800-4000 cm\(^{-1}\) region. Two interesting regions can be distinguished; the absorption bands of the CH stretching vibrations which are visible around 3000 cm\(^{-1}\) and many other vibrations are visible in the fingerprint region from 800 to 1800 cm\(^{-1}\).

2.3.2 FTIR spectrometer

Fourier transform infrared techniques small amount of sample (in the order of 2-5 µl). The spectrometer is based on a Michelson interferometer (Figure 2.8.) which consists of a ‘globar’ source (a silicon carbide rod heated to about 1000 K), a beam splitter, two mirrors and a detector. One of the perpendicular mirrors can move and the other is fixed. Between the fixed and moving mirror is a beam splitter, where the light from the IR source can be partially reflected to the fixed mirror and partially transmitted to the movable mirror. The spectrometer gives the best performance when the beam splitter has a reflectance and transmittance of 50% for the wavenumber region of interest. The beam splitter for the mid-infrared spectral range is made up of a thin layer of Ge or Si, having high refractive index, deposited on materials with lower refractive index like KBr or CaF\(_2\). A polychromatic beam of radiation emitted from the ‘globar’ source is divided into two ‘halves’ (one ‘half’ is transmitted to a moving mirror and the other to the fixed mirror) by the beam splitter which are recombined after a path length difference has been introduced. The intensity variations of the resulting beam are measured as a function of the path length difference. The recombined beam is focused on to the sample and then reaches the detector. The detector monitors changes in intensity and is made of a photoconductive film that is an alloy of mercury, cadmium and tellurium (MCT) deposited on an inert support. These detectors operate at liquid N\(_2\) temperature with enhanced sensitivity.
Because of the effect of interference, the intensity of the beam measured at the
detector depends on the difference in path length in the two arms of the
interferometer. The optical path difference, called the retardation ($\delta$), is twice the
path difference between the two arms. The intensity for a specific wave number $\nu$ at
the detector can be thus described as

$$I(\delta) = \frac{1}{2} I^0(\nu) \left[ 1 + \cos 2\pi \nu \delta \right]$$

where $I^0(\nu)$ is the light intensity of the source.

Only the varying part of the measured intensity is of interest for FT-IR
spectroscopy, this signal is called the interferogram, $I(\delta)$. The light intensity of the
source, $I^0$, has additional wavenumber dependence because of instrumental
characteristics such as beam splitter efficiency, mirrors or detector response and the
characteristics of the system that is studied. The interferogram is then written as:

$$I(\delta) = B(\nu) \cos 2\pi \nu \delta$$

where $B(\nu)$ gives the single beam intensity or spectrum which is the intensity of the
source at the wavenumber $\nu$ modified by the system. The response of the system
that is studied is part of this spectrum and can be resolved with a suitable reference
measurement that corrects the measured spectrum for the wavenumber dependent
intensity of the source and instrumental characteristics.

$I(\delta)$ can be seen as the cosine Fourier transform of the spectrum $B(\nu)$. The
spectrum is calculated from the interferogram $I(\delta)$ by computing the cosine Fourier
transform of $I(\delta)$. When the spectrum of the light source is continuous, the
interferogram can be represented by the integral

$$I(\delta) = \int_{-\infty}^{\infty} \left\{ B(\nu) \cos 2\pi \nu \delta \right\} d\nu$$

and

$$B(\nu) = 2 \int_{0}^{\infty} \left\{ I(\delta) \cos 2\pi \nu \delta \right\} d\delta$$

Since the interferogram is a function of the moving mirror position, it is very
crucial to precisely determine the mirror position. For this purpose a monochromatic
beam from a He-Ne laser of wavelength 632.8 nm is directed together and coaxial
with the infrared beam in order to monitor the position of the mirror and also the
digitization of the infrared interferogram, which is required for the mathematical process of the discrete Fourier transformation.

Atmospheric water vapor and carbon dioxide have strong contributions to infrared spectrum. Therefore, the sample chamber of the interferometer is continuously purged with dry air or N₂ and a background spectrum is recorded before the sample measurement. In order to eliminate the atmospheric or environmental contributions, the sample single beam spectrum is normalized against the background spectrum. Consequently, the infrared absorbance of a sample is calculated as

\[ A = \log \frac{I(\nu)}{I_0(\nu)} \]

where \(I(\nu)\) and \(I_0(\nu)\) are the single beam spectra of the sample and the background respectively. The resolution of an FTIR spectrometer is related to how far the moving mirror travels in one scan. While the mirror moves from the ‘+d’ to ‘-d’ position, a spectrum of approximately ‘1/d’ resolution is obtained. The signal to noise ratio (S/N) of a spectrum is increased by collecting and averaging multiple interferometer scans of the sample before the Fourier transform. In this work typically, 256 scans were averaged for each spectrum with 2cm⁻¹ resolution.

![Figure 2.8 The Michelson interferometer](image)
2.3.3 FTIR Spectroscopic Techniques

The study of biological samples under physiological conditions implies the preparation of samples in aqueous solution which in turn imposes major difficulties for infrared spectroscopy because of the strong water absorbance in the interesting regions of the spectrum. A quick fix for this problem is the usage of the short path length and higher sample concentration to minimize the water absorbance. Another, now widely used technique to circumvent this problem is attenuated total reflection (ATR).

**Attenuated total reflection (ATR) spectroscopy**: Unlike in the transmission mode, water is not a limiting factor in the ATR technique. As shown in the Figure 2.9, an infrared transmissive crystal is used to create total internal reflections of the measuring infrared beam. At the interface to the outer medium (e.g. protein or water) an evanescent wave is formed that probes the sample and therefore can be used to record an absorbance spectrum of the outer medium. The depth of penetration of the evanescent wave depends on the wavelength of the light beam, the ratio of indices of reflection of the used materials, and the angle of incidence. As a result, the amount of water overlaying the crystal does not influence the water absorption in the measured spectrum as the penetration depth is in the order of the wavelength, i.e. ~1µm. Correspondingly, the exchange of the solution with simultaneous observation of induced changes is possible. Thus, this method has the advantage that the overlaid buffer on a dried film sample can be exchanged without disturbing the sample. For the samples in solution it is helpful to separate the sample compartment close to the ATR crystal from a buffer reservoir by a dialysis membrane which makes it possible to alter the reservoir contents without disturbing the sample (16).

**Use in pKₐ determination**: The experimental method of attenuated total reflectance (ATR) FTIR enables the spectroscopic studies of proteins in water, thus allowing precise control of external parameters such as ionic strength, pH and temperature. Using ATR in combination with FTIR difference spectroscopy, small changes in the characteristic vibrations of different groups in proteins can be measured accurately. Since the acidity of internal groups is influenced by the local environment, changes in the vicinity of such groups lead to shifts in pKₐ. It has been shown that the amplitudes of characteristic vibrations and their centre frequencies vary with the pKₐ of the corresponding group, so that changes in the pH of the external solution lead to
changes in the ATR FTIR spectrum. Numerical fitting of the pH dependent spectral amplitudes or frequency shifts to Henderson-Hasselbach equation provides the pK_a values of the individual groups in proteins. Likewise internal pH monitoring is possible by evaluating the IR absorption bands of suitable buffers. Both approaches have been used in this work (see Chapter 3).

**Figure 2.9** Out-of-compartment arrangement of Attenuated total reflection spectroscopy

**Time-resolved infrared spectroscopy:** Today’s commercially available FTIR spectrometers are equipped with instrumentation that allows the measurements to be performed in a time-dependent fashion. The most common time-resolved infrared techniques that are in use are rapid-scan, the step-scan and single wavelength measurements as reviewed.

**Time-resolved infrared difference spectroscopy:** Even a protein of relatively small size ~20 KD has about $10^4$ vibrational modes. Hence, the infrared absorption spectrum, dominated by amide I (C=O stretch) and amide II (NH bend coupled with C-N stretch bands) to which every amino acid present contributes, fails to provide information on individual bands but only on the global features of the protein. Time-resolved FTIR difference spectroscopy resolves this problem and has been first introduced in the study of light-driven proton pump bacteriorhodopsin (bR). For an FTIR difference spectrum induced of a reaction A to B, one calculates the absorbance spectrum of B minus the absorbance spectrum of A. Thus, the vibrations from groups that are not changed during the reaction cancel each other, and only the
changes during the reaction are seen allowing the resolution of individual absorptions (17). This technique provides information that is complementary to X-ray structure analysis, including information on H-bonding, the protonation state, the charge distribution, and time dependence of the protein reactions.

**The rapid-scan technique**: The principle of the rapid-scan FTIR mode of time-resolved spectroscopy is simple: after taking a reference spectrum of the protein in its ground state, one induces a reaction in the protein (e.g. by a laser flash) and records interferograms in shorter times than the half-lives of the reactions. Here, the movable interferometer mirror is moved at a maximum speed of 10cm/s. From one complete forward and backward movement of the mirror, up to 4 spectra can be obtained which results in a maximum time resolution of about 10 ms at 12 cm\(^{-1}\) optical resolution. A single experiment can yield a full series of time-resolved spectra. In this work the rapid scan technique has been used to study a key intermediate of the bacteriorhodopsin photo-cycle (Chapter 4).

2.3.4 FTIR Finger prints of Proteins

FTIR spectroscopy has emerged as a useful tool for the characterization of protein secondary structure with a precision lying between that of purely predictive and the molecular coordinate approaches. The extent of this application of FTIR spectroscopy is vast as the number of proteins to be characterized is increasing on a routine basis. There has been a recent initiative to compile an IR data bank for proteins which involves world wide consortium of laboratories. The extraction of information from FTIR spectrum is a task that requires the assignment of the different regions to individual molecular groups of the protein under study. The infrared spectra of proteins provide a wealth of information on structure and environment of the protein backbone and of amino acid side chains.

**Protein backbone vibrations**: The FTIR spectrum of a protein yields information about the structure of a protein through the protein backbone vibrations, which show characteristic vibrational frequencies upon attaining different secondary structure profiles. The absorption signals from individual secondary structure elements originating from a specific vibration superpose in the amide modes, which are amide I-to-V and amide A.
Amide I (1600-1700 cm\(^{-1}\)) arises mainly from the C=O stretching vibration and is the most commonly used spectral region for the secondary structure analysis of proteins. Minor contributions from the out-of-phase CN stretching vibration, the CCN deformation and NH in-plane bend also constitute the amide I vibration. Amide II (1480-1580 cm\(^{-1}\)) is mainly from the C-N stretching vibrations and in-plane N-H bending and lesser contributors are C-C stretching vibrations (18,19). An N-H stretching vibrations is classified as amide A (3325-3280 cm\(^{-1}\)) and is sensitive to the local environment of the protein.

The amide I band of polypeptides and proteins is most sensitive to the protein structure and has been extensively used in deriving secondary structure information from spectra. The amide I band is intrinsically broad which renders difficult the identification of different secondary-structural elements. The typical IR signatures of α-helix, unordered structure and β-sheet are shown in Figure 2.10. α-helix and random coil structures overlap due to broad bands located at close wavenumbers in H\(_2\)O buffer. However, there are methods and software available which provides an efficient solution to this problem.

**Figure 2.10** Secondary structure motifs in the amide I region of IR spectrum (14)

Second-derivative is one of the most commonly used method for resolving overlapping bands and can be performed with the software (OPUS) equipped with Bruker FTIR spectrometers. Local maxima are reflected as minima in the second derivative profile of a protein spectrum and is especially useful for protein analysis in the amide I region. Fourier Self Deconvolution (FSD) is also used to dissect the component band positions in an overlapping band. FSD works by decreasing the width of all lines contributing the spectral region under consideration. As the lines...
get narrower, peaks are separated increasing the visibility of underlying peaks. The spectrum is deconvolved by multiplying the corresponding region of the interferogram with a function to amplify the signal amplitude and taking the Fourier transform again to get the deconvolved spectrum. The line shape function is the product of an increasing exponential function and decreasing triangular apodisation function. In most cases, FSD and second derivative are used together to get information about all the component bands. Curve Fitting also reveals the component bands from the absorption spectrum and is performed by stepwise iterative adjustment towards a minimum root-mean-square error of the different parameters determining the shape and position of the absorption peaks.

**Amino acid side chain vibrations:** Since amino acid side chains are often at the heart of the molecular mechanism of macromolecules their infrared absorption provides very valuable information on the site specificity of protein function. FTIR allows following in a single experiment the fate of single/several individual groups involved in the structural transition of the protein under study together with the observation of protein backbone and thus allows comparing the kinetics of backbone structural changes with those of amino acid side chain signals. In these experiments the general route followed is to induce a transition in the sample (change in pH, trigger by light etc.) and identify the responsible residues by repeating the experiment with selected mutants/peptides. In amino acid side chains, protonation state, coordination of cations, and hydrogen bonding are the dominant factors that determine the band position of the group frequencies. FTIR spectroscopy thus being one of the very few techniques that is able to determine the protonation state of the side chains which has important consequences for the electrostatic interactions in proteins has been successfully utilized in probing the protonation state of Asp and Glu residues during proton pumping by bR (20-23). Their observation is crucial for the investigation of allosteric couplings of proton movements and protein conformations as shown in Chapter 3 and 4.

**Side chains with carboxyl groups- Asp, Glu:** Carboxyl groups are unique monitors to follow proton pathways by vibrational spectroscopy in proteins. The bands of interest are the ν(C=O) vibration of the protonated carboxyl group (1710-1760 cm⁻¹) whose spectral region is generally free from the absorption of other amino acids. The deprotonated carboxylate group shows two strong bands near 1400 and 1570 cm⁻¹ for
the symmetric and the antisymmetric vibrations, respectively. The infrared marker bands for the carboxyl group containing amino acids are extensively reviewed in (24).

2.4. Fluorescence- Infrared-Cross-Correlation spectroscopy

A technically challenging part of this thesis was the actual integration of the site specific information from the extremely sensitive fluorescence spectroscopy with global secondary structure information gained from infrared spectroscopy. This goal has been accomplished by the development of a generalized multidimensional spectroscopic approach that could facilitate the investigation of long range conformational coupling in proteins. This method works by the integration of fluorescence emission and infrared absorption data recorded simultaneously from the same protein sample that undergoes conformational transitions in response to an external perturbation. A program was written using OPUS (measuring software provided by Bruker) that allowed the automated acquisition of IR and FL data and also the activation of the sample by external perturbation. This method has been successfully employed here to understand the relation between internal proton transfer reactions and proton uptake through the cytoplasmic half channel of a proton pump, bacteriorhodopsin (Chapter 4).

Unlike any other conventional spectroscopic approach, this method provides information on H-bonding, protonation states, secondary structure (deduced from IR data) and site-specific hydrophobicity, mobility, electrostatics (deduced from fluorescence data) simultaneously in a single shot experiment (Figure 2.11). IR signals from the site of labeling (e.g. aa1) and from the residues that are more distant but allosterically coupled to the labeled site (aa4) will respond highly correlated to a functional transition or an external perturbation whereas uncoupled transitions (aa2, aa3) can exhibit uncorrelated kinetics relative to the time-resolved signal from the site-specific label. These data are then evaluated by a cross-correlation technique as described in the later sections.
Figure 2.11 Information from intrinsic IR absorbers at multiple sites combined with fluorescence monitor at a specific site. Binding of a ligand (red oval) will affect amino acid 4 (aa₄) and its IR-signal will be highly correlated with that of amino acid 1 (aa₁) and with fluorescence changes from its vicinity when allosteric coupling to the ligand-binding exists.

2.4.1 Instrumentation

Using attenuated total reflectance (ATR) Fourier-transform infrared (FTIR) difference spectroscopy, additional channels for excitation and detection of fluorescence where established by light guides positioned above the sample on the ATR crystal.

Using 2D-cross-correlation techniques, the kinetic asynchronicity of the emission from natural or artificial site-specific fluorophores relative to the secondary structure-sensitive IR-absorption bands can be determined (for schematics of experimental set up refer Figure 2.12) Thereby, IR absorptions can be assigned in a model-free and unbiased way to secondary structural elements that either react in synchronicity with fluorescence signal and thus are likely to be allosterically linked or react in a disrelated fashion, indicating their kinetic independence.
2.4.2 2D-Correlation

Two-dimensional (2D) correlation spectroscopy is a versatile technique for the in-depth analysis of various spectral data. Lately, this method has been used in analyzing vibrational spectral data where the spectral intensity is plotted as a function of two independent spectral variables. Most of the 2D correlation spectroscopy experiment relies on an external perturbation (for a general experimental scheme see Figure 2.13). The 2D technique is suited for measurements of systems that undergo a reversible process on a set of 2D-IR correlation spectra. These dynamic spectra were found particularly useful in emphasizing special features that are not observable in conventional one-dimensional spectra. In this work, light is used as an external perturbation agent to induce the photocycle in bacteriorhodopsin. Temperature, in a similar fashion can be used as an external perturbation and we for example have used it in experiments with the cytoskeletal protein actin in complex with flavanoids. The results from these experiments demonstrate the correlation of the loss of ligand-dependent static quenching of intrinsic tryptophan emission during thermal unfolding with the loss of structure as monitored by FTIR spectroscopy (*Manuscript under preparation*).
Methods

Figure 2.13 General scheme for obtaining 2D correlation spectra.

In this method, an external perturbation (pH, temperature) of the sample causes changes in its state, order or surroundings which results in changes in the measured spectrum. The spectral variation induced by the applied perturbation is known as a dynamic spectrum. The practical way of doing such an experiment will be to collect a series of perturbation-induced dynamic spectra in a systematic way and then to transform into a set of 2D correlation spectra by cross correlation analysis.

Math behind 2D Correlation: The formalization of 2D correlation has been described in detail (25). The dynamic spectrum $y(v, t)$ of a sample induced by external perturbation can be represented as

$$
\tilde{y}(v, t) = \begin{cases} 
  y(v, t) - \overline{y}(v) & \text{for } T_{\text{min}} \leq t \leq T_{\text{max}} \\
  0 & \text{otherwise}
\end{cases}
$$

where $\overline{y}(v)$ is the reference spectrum of the system. The fundamental idea governing 2D correlation spectroscopy is a quantitative comparison of the patterns of spectral intensity variations observed at two different spectral variables over some finite observation interval between $T_{\text{min}}$ and $T_{\text{max}}$. The correlation function can be expressed as follows.

$$
X(v_1, v_2) = \langle \tilde{y}(v_1, t) \cdot \tilde{y}(v_2, t') \rangle
$$
The 2D correlation intensity $X(\nu_1,\nu_2)$ represents the measure of synchronicity of the spectral intensity variations $\tilde{y}(\nu,t)$ measured at two different spectral variables, $\nu_1$ and $\nu_2$ during a fixed interval of the external variable $t$. The symbol $\langle \rangle$ denotes the scalar product.

2.4.3 Hetero-spectral correlation

"Hetero" correlation analysis is a unique and powerful feature of the generalized 2D correlation technique and can be classified as hetero-spectral, hetero-sample and hetero-perturbation. In this work, I have used hetero-spectral correlation, where two different spectral probes (infrared and fluorescence) are used simultaneously for the same sample under the influence of a common perturbation (light). Here, we have determined the synchronicity between the temporal behavior of the IR-absorption difference bands evoked by a photoreaction in bacteriorhodopsin and the simultaneously measured change in fluorescence of an introduced fluorescein label at a defined cytosolic site. This analysis allows detecting in a model-free manner even small differences in conformational relaxation at different sites in a macromolecule. It has allowed demonstrating how internal proton movement can precede or lag behind the movement of helix 3 in the cytoplasmic half channel of BRD96A (Chapter 4).

2.5 Literature Cited

3. Modular Proton Switching in GPCR Function

3.1 Introduction

On a molecular level, what does molecular switching mean in the context of a protein structure? In principle, the protein structure responds by conformational changes to external mechanical forces or to inter- or intramolecular forces. The latter are particularly important in signaling cascades of GPCRs where they provide the primary signal. GPCRs consist of seven membrane spanning α-helical segments and constitute the main family of cell surface receptors for both chemical stimuli (e.g. hormones, neurotransmitters and chemoattractants) and sensory stimuli (e.g. light, odorants and taste molecules). In the super family of class-1 GPCRs, the transmission of the primary signal from the extracellular ligand binding site to the cytosolic phase is conserved despite the large diversity in ligand recognition. One of the widely accepted reasons for this conserved functional mechanism is the presence of many identical structural features at the cytosolic G protein interacting site.

Current models converge towards a picture in which “microdomains” act as conformation switches that are coupled to different degrees to the primary activation process. The important goal of this work is the identification of a putatively conserved conformational switch at the cytosolic phase of rhodopsin, a prototypical class-1 GPCR. It will be shown how a specific membrane environment plays a key role in allowing a putatively conserved proton-dependent switching process in a highly modular manner, i.e. in the absence of the complex helical interactions of the full length system. The identified module provides a common coupling mechanism between pH and receptor conformation in class-1 GPCRs allowing a receptor to use the proton chemical potential as an additional source of free energy to attain the active conformation thus exceeding the free energy that originates in the immediate ligand-receptor interactions.

The keystone in the overall process of activating these receptors is the structural rearrangement of several transmembrane helices in particular helices 3 and 6 triggered by presumably conserved switches. The widely accepted view of rhodopsin activation focuses on two activating ‘proton switches’ which are as follows: breakage of an intramolecular salt bridge (1) by transfer of the Schiff base proton to its counter ion Glu-113 (2), followed by movement of helix-6 (H6) (3-4) in
the metarhodopsin IIₐ (MIIₐ) to MIIₐ transition. The MIIₐ state takes up a proton at Glu-134 (5) in the class-conserved D(E)RY motif at the C-terminal end of helix-3 (H3) (Figure 3.1) leading to the MIIₐH⁺ intermediate (6,7) which activates transducin (Gt), the G protein of the photoreceptor cell. Glu-134 regulates the pH-sensitivity of receptor signalling (8) in membranes as reviewed in (9) and in complex with Gt the protonated state of the carboxyl group becomes stabilized (10). This charge alteration is linked to the release of an "ionic lock", originally described for the β₂-adrenergic receptor (11) which also in rhodopsin stabilizes the inactive state (7) through interactions between the cytosolic ends of H3 and H6 (12). In the absence of a lipidic bilayer, proton uptake and H6 movement become uncoupled (6). Lipidic composition affects MII formation, rhodopsin structure, and oligomerization e.g. ref. (13-15) and differs at the rhodopsin membrane interface from the bulk lipidic phase (16). Vice versa, MII formation specifically affects lipid structure (17). Although of fundamental importance for GPCR activation, the potential implication of lipid protein interactions in "proton switching" is not clear. A functional role of Glu-134 in lipid interactions has been originally derived from IR-spectra where E134Q replacement abolished changes of lipid head group vibrations in the MIIGt complex (10). Computational approaches emphasized the “strategic” location of the D(E)RY motif (18) and the Glu-134 carboxyl pKₐ may critically depend on the lipid protein interface (19). However, the implications for proton switching are not evident and the theoretical interest is contrasted by the lack of experimental data addressing the effect of the lipidic phase on side chain protonation, secondary structure, and membrane topology of the D(E)RY motif.

Here, the coupling between conformation and protonation in single transmembrane segments derived from H3 of bovine rhodopsin was studied. The suspected "modular" function of the D(E)RY motif was specifically addressed by determining parameters not evident from the crystal structures, i.e. the pKₐ of the conserved carboxyl, its linkage to helical structure, and the effect of protonation on side chain to lipid head group distance. We show that the D(E)RY motif encodes an autonomous "proton switch" controlling side chain exposure and helix formation in the low dielectric of a lipidic phase. The data ascribe a functional role to lipid protein interactions which couple the chemical potential of protons to an activity promoting conformation in a ligand-independent manner.
Modular proton switching in GPCR function

Figure 3.1 View of a part of the rhodopsin structure (1l9H) featuring the conserved D(E)RY motif (color-code: white, hydrophobic; blue, basic; red, acidic; green, hydrophilic). Location of Glu134 in the water-accessible site of the phase boundary is essential for its participation in lipid-protein interactions. Helices 1 and 4 have been removed for clarity.

3.2 Experimental Procedures

Lipids and detergents. 1, 2-dipalmitoyl-sn-glycero-3-phospho-L-serine (PS), L-α-phosphatidylcholine (PC), cholesterol (CH), n-octyl β-D-glucopyranoside (OG), and n-dodecyl-β-D-maltoside (DM) were from Sigma (Taufkirchen, Germany). 1, 2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(5-dimethylamino-1-naphtalene-sulfonyl) (DANSYL-PE) was from Avanti Polar Lipids (Alabaster, USA).

Fourier transform IR spectroscopy and circular dichroism. Fourier transform IR (FTIR) spectra were obtained with peptides (~10 mg/ml) in 5 % DM, 100 mM sodium phosphate buffer, using a vector 22 spectrometer (Bruker, Ettlingen, Germany) at 2 cm⁻¹ resolution. 30 µl of the sample were transferred into a Bio-ATR-II cell (Bruker, Ettlingen, Germany) and the pH changed from 8.8 to 3 by dialysis (100 mM sodium phosphate). Pure buffer spectra were used as spectral references to
calibrate the pH-dependent absorption strength and frequency of the PO stretching modes. Thereby, a very sensitive real time monitor of pH changes could be implemented. Circular dichroism (CD) spectra were recorded at room temperature with a J-815 instrument (JASCO, Gross-Umstadt, Germany) at ~4 l/min N₂-flow rate from 200 to 260 nm in 0.1 cm cuvettes on DM-solubilized peptides (0.2-0.3 mg/ml). Ellipticity θ was recorded in mdeg.

**Fluorescence spectroscopy.** Fluorescence measurements were performed with a Perkin–Elmer LS55 spectrometer (Perkin-Elmer, Rodgau-Jügesheim, Germany) in 200 µl cuvettes at 285 nm for Trp excitation and emission recorded from 290 to 450 nm (slit width of 5 nm). Peptide-containing micelles were prepared by solubilization of 1-2 mg peptide in ~200 µl of 5 % DM followed by 30-fold dilution in 100 mM phosphate buffer of different pH. "Lipid-doped" peptide-containing micelles were prepared by dissolving ~2 mg peptide in 5 % DM (~600 µl) and mixing with the appropriate vacuum-dried amounts of DANSYL-PE and PS in a molar ratio of 1:2.5:1, respectively. 20 µl aliquots were diluted 30-fold in a series of phosphate buffers (100 mM) allowing duplicate recording of emission spectra at each pH. Peptide-containing vesicles were prepared by mixing 1-2 mg of peptide solubilized in ~ 800 µl of 5 % OG with PS, DANSYL-PE, and PC in the ratio 1:2.5:2.5, respectively, with a final total lipid:peptide ratio of 50-80. The detergent was removed by 15 hrs of flow cell dialysis, the suspension was sonicated and freeze-thawed 10 times, and diluted in 100 mM phosphate buffers. Cholesterol-containing vesicles were prepared in parallel from the same stock by supplementing an aliquot of the OG-solubilized mixture with cholesterol to a final total lipid:cholesterol ratio of 1:0:2.

**Synthetic peptides.** Peptides were synthesized and HPLC-purified (free of trifluoroacetate) by ThermoFisher (Ulm, Germany) with carboxyl- and amino-termini amidated and acetylated, respectively. Peptides of the following sequences were derived from amino acids 108-138 in H3 of rhodopsin. 

H₃E: TGC NLA(E) GFF ATL GGA(E) IAL WSL VVL AIE RYV V; H₃E₂₇Q: TGC NLA(E) GFF ATL GGA(E) IAL WSL VVL AIQ(E) RYV V; H₃E₂₇D: TGC NLA(E) GFF ATL GGA(E) IAL FSL VVL AID RYV W; H₃W₁₉F/V₃₁W: TGC NLA(E) GFF ATL GGA(E) IAL F(W)SL VVL AIE RYV W(V); H₃W₁₉F/V₃₁W/E₂₇Q: TGC NLA(E) GFF ATL GGA(E) IAL F(W)SL VVL AIQ(E) RYV W(V); native amino acids in
brackets were replaced by the preceding ones. The helicity of the peptides was assessed by infrared spectroscopy and circular dichroism measurements.

As shown in Figure 3.2 the degree of helicity depends strongly on even very subtle changes in the sequence. In the context of the most native-like sequence in peptide H3E the additional shift of the tryptophan from position 126 to 138 as well as the further replacement of the Glu134-homologue by glutamine does not interfere with a predominantly helical secondary structure. Spectral decomposition of the integral IR absorption in the amide I range into gaussian/lorentzian curves shows 70-80 % α-helical structure for H3E and H3W19F/V31W/E27Q with peak frequencies at 1654-1656 cm⁻¹. The CD spectra of the latter peptides (Figure 3.2, inset) were converted to mean residue ellipticity (deg · dmol⁻¹ · cm²) \( \Theta_{\text{res}} = 100 \cdot \Theta / (c \cdot l \cdot n) \), where c is the molar concentration of the peptide, l optical path length (0.1 cm) and n the number of amino acids in the peptide (here 31). The fraction p of helical structure was calculated as \( p = -\Theta_{\text{res}222} / [39500 \cdot (1-2.7/n)] \) as described (20). Consistency requires that the average number of peptide bonds in helical conformation complies with \( v/31 = p \) which was obtained for \( y=22 \) and \( p = 0.71 \). Thus, ~22 out of the total of 31 amino acids in each of the two peptides are on average in a helical conformation which is on the lower side of the range estimated by FTIR spectroscopy and suggests that 4-5 disordered amino acids form the C- and N-terminal helical ends. In contrast to the largely helical peptides H3W19F/V31W and H3W19F/V31W/E27Q the related pair of sequences H3E and H3E27Q shows helicity only for H3E. Three independent synthesis rounds using different technologies failed to produce peptides H3E27Q and H3E27D with helical structures upon detergent solubilization. The CD spectra of these peptides are rather featureless (Figure 3.2, inset) and their IR-absorption shows only a small fraction of helical structure (1656 cm⁻¹) relative to a predominating amide I mode at low frequency indicative of aggregation. Thus, under the conditions of the FTIR and CD experiments, the replacement of Glu-27 by Gln allows helix formation only in the presence of the additional W19F replacement. Therefore, the proton-induced conformational switch was studied by FTIR in the least modified sequence, i.e. H3E, whereas the negative control was provided by H3W19F/V31W/E27Q.
Figure 3.2 secondary structure of H3-derived peptides. The infrared spectrum of solubilized H3E shows a predominant helical secondary structure exhibiting an amide I absorption at 1655 cm$^{-1}$ in agreement with previous data of its lipid-reconstituted form. Replacement of E27hotocycle scheme depicting K by either Asp or Gln leads to mis-folding of the corresponding peptides H3E27D and H3E27Q evident from weak amide absorption at 1652-1656 (two lower traces) and featureless CD spectra (inset). However, the E27Q replacement is not detrimental to helix formation in the background of the additional W19F replacement, resulting in the peptide H3$^{W19F/V31W/E27Q}$ with a clear spectral signature of helical secondary structure in both IR and CD data. Helical content is 75-80% (from spectral decomposition of IR-absorption) and 70% (from CD, see Experimental Procedures).
ICL2: AIE RYV W(V)VC KPM SNF RFG; this peptide was derived from amino acids 132-149, containing the C-terminal end of H3 and its cytosolic extension.

3.3 Results

3.3.1 Coupling of protonation and conformation by the D(E)RY motif of rhodopsin

The linkage between conformation and carboxyl protonation in the D(E)RY motif was studied in the peptide H3E corresponding to aa 108 to 138 of rhodopsin. The native residues Glu-113 and Glu-122 were replaced by alanines, rendering the carboxylate in the Glu-134 homologue (i.e. Glu-27 in the peptide) the only carboxyl in H3E. The symmetric carboxylate stretching mode of Glu-27 (1401 cm\(^{-1}\)) and the narrow amide I absorption at 1656 cm\(^{-1}\) (typical of the helical secondary structure of the peptide backbone) were used to monitor by FTIR spectroscopy the side chain protonation and secondary structure, respectively. Figure 3.3(A) shows the absolute IR absorption and the pH-induced difference spectra, obtained by dialysis-coupled ATR-FTIR difference spectroscopy (21). Spectra recorded at different pH were subtracted from a reference spectrum acquired at pH 8.8, where the Glu-134 homologue is fully ionized (maximal absorption at 1401 cm\(^{-1}\)). The stretching vibrations of the buffer phosphates between 1200 and 800 cm\(^{-1}\) served as a pH monitor. The macroscopic pK\(_a\) of Glu-27 was identified by the spectrum with half-maximal intensity of the 1401 cm\(^{-1}\) band. The later was obtained at pH 5.9, i.e. 1-2 pH units above the pK of a glutamate side chain in aqueous solution. Also the peptide backbone exhibited pH-dependent transitions, giving rise to changes of the amide II band around 1550 cm\(^{-1}\) (overlapping with the antisymmetric carboxylate stretching mode at \(~\)1560 cm\(^{-1}\)) and the amide I absorption at 1656 cm\(^{-1}\). The structural change was evaluated using the titration spectra in Figure 3.3(B). The inset shows that the 1656 / 1638 cm\(^{-1}\) absorption change correlates linearly with the carboxylate absorption at 1401 cm\(^{-1}\). The secondary structural change is coupled to side chain protonation with a common pK\(_a\) of 5.9, with a more helical structure favoured at acidic pH. The integral intensity change at 1656 cm\(^{-1}\) corresponds to 3-5% of the total amide I absorption of H3E (Figure 3.3(A)), indicating that side chain protonation extends the helical structure by an average of 1-2 peptide bonds.
Figure 3.3 pH-dependency of the infrared absorption of H3-derived peptides. A) Amide absorption and pH-induced absorption changes of H3E. i) The narrow amide I mode at 1656 cm$^{-1}$ evidences the predominant helical structure of H3E (5 % DM, 100 mM phosphate buffer, pH 8.8). ii-vii) Difference spectra generated by subtraction of the absorption at pH 3 (Glu-27 side chain protonated) from the spectra recorded during acidification (colour-coded...
blue to red) show the decreasing intensity of the symmetric COO⁻ stretching vibration of Glu-27 at 1401 cm⁻¹. Absorption changes of the buffer phosphates (1200-800 cm⁻¹) provide an internal pH reference. Traces in blue were generated by subtracting the "pH 8.8 minus pH 5.5" IR-difference spectrum of calibration buffers from the dialysis-induced difference bands in the peptide sample. Inflection of the residual phosphate bands in v) and vi) shows that half maximal absorption at 1401 cm⁻¹ is obtained by a change from pH 8.8 to pH >5.5. B) Linear relation between carboxyl side chain protonation and helix extension. Acidification causes reduction of the carboxylate absorptions at 1401 cm⁻¹ and 1560 cm⁻¹ (antisymmetric) and an increase of α-helical structure (negative band at 1656 cm⁻¹). This pH sensitivity is lost upon E27Q replacement in H3W19F/V31W/E27Q (black line, scaled to the same amount of peptide) showing essentially only the residual pH-dependent absorption by the micellar solution. Inset: the plot of the absorption at 1656/1638 cm⁻¹ versus the integral 1401 cm⁻¹ band reveals a common pKₐ of 5.9 for side chain protonation and helix formation (vertical and horizontal lines). Upper and right axis show the fraction of protonated peptide and the corresponding pH, respectively, related by the sigmoidal curve (gray) in a four state model (see text and Figure 3.6 A).

This process was abolished upon E27Q replacement in the peptide H3W19F/V31W/E27Q (readily adopting a helical structure in contrast to the alternative negative control sequence H3E27Q, see Experimental Procedures) which did not exhibit the pH-sensitive 1656/1638 cm⁻¹ difference band (Figure 3.3(B)). Thus, the H3 secondary structure is controlled by the protonation state of the carboxyl in the D(E)RY motif.

The functionality of the proton-dependent rearrangement of the C-terminal structure in the H3W19F/V31W background containing the Glu-134 homologue is shown below and proves its independence from the altered location of the Trp.

3.3.2 Protonation of the D(E)RY motif alters the C-terminal structure

The up shift of the pKₐ relative to that of a glutamate in water indicates that side chain protonation at the H3 C-terminus is stabilized by the concomitant helix extension. To prove the localization of the structural transition, a Trp was C-terminally attached, replacing the Val¹³⁸ homologue of rhodopsin and, for the purpose of specificity, Trp-19 (corresponding to the native Trp-126) was replaced by Phe, resulting in the construct H3W₁₉F/V₃₁W which shows a pH-sensitive emission in the 300 to 400 nm range (Figure 3.4). Trp-31 is two residues away from the tyrosine of the D(E)RY motif. It acts as an energy acceptor for the excited state of tyrosine, which shows appreciable emission only in the absence of Trp-31 or in the presence of the more distant Trp-19 (Figure 3.4, inset A).
Figure 3.4 pH sensitivity of the tyrosine / tryptophan emission of peptides derived from H3 of rhodopsin and its cytoplasmic extension. Emission of H3_{W19F/V31W} at the indicated pH is subtracted from a pH 8.8 reference emission spectrum. Acidification (negative lobe) induces a blue shift of the emission (set of coloured traces). The pH sensitivity of the Tyr-29/Trp-31 emission is caused by Glu-27 and disappears upon E27Q replacement in H3_{W19F/V31W/E27Q} (thin lines), i.e. the identical peptide used as the control for FTIR spectra in Fig. 3.3(B). The shift of the Trp-emission maximum is also seen in the lipid-reconstituted peptide (black trace, pH8 vs. pH 4). Inset A: emission spectra of the H3-derived peptides: i) replacement of Trp-19 (Trp-126 in rhodopsin) in H3_{W19F/E27Q} reveals the unquenched emission from Tyr-29 with negligible intrinsic pH-sensitivity (48). ii) In H3_{E}, tyrosine emission is superimposed with that of Trp-19. iii) H3_{W19F/V31W}, substitution of a Trp for the C-terminal Val-31 provides a fluorescence "reporter" at the phase boundary and quenches tyrosine emission. iv) Additional E27Q replacement in H3_{W19F/V31W/E27Q} has little effect on the Tyr-29/Trp-31 emission (gray line). Inset B: pH-dependent change of peak to peak values (from upper panel) of H3_{W19F/V31W} in DM (filled circles) and of the water-soluble peptide ICL2 (open circles).

The Trp-dominated emission in H3_{W19F/V31W} provides a sensitive fluorescence monitor of structural transitions in the D(E)RY motif due to both the geometrical constraints for FRET and the hydrophobicity dependence of Trp emission itself. The pH-induced emission difference spectra show that in the ionized state of Glu-27 (positive lobe at 365 nm) the emission of Trp-31 is shifted to longer wavelengths.
versus the protonated state (negative lobe at 320 nm). This could reflect reduced quenching of tyrosine as well as exposure of Trp to a more hydrophobic environment at pH < 6. The half-maximal emission change from the D(E)RYVW sequence is interpolated to pH 5.6 and is lost upon E27Q replacement. This parallels the infrared results and supports the localization of the detected secondary structure formation within the direct vicinity of the D(E)RY motif. The protonation-induced blue-shift of the Trp emission maximum is also observed with H3W19F/V31W in DOPC vesicles (Figure 3.4), although with a ~50% reduced amplitude which is probably caused by the presence of zwitterionic (i.e. more hydrophilic) head groups as compared to the neutral phase boundary of the DM micelle. Due to the strong light-scatter in the near UV caused by the vesicles also the signal to noise ratio is ~5-fold reduced. In contrast to the systematic pH-dependence of Trp emission shown for the TM3-derived peptides, an unsystematic pH-dependence was obtained from the peptide ICL2 (Figure 3.4, inset B) containing the D(E)RY motif and the ensuing second cytosolic loop of rhodopsin (Ala-132 to Gly-149 with V138W replacement).

3.3.3 Protonation-dependent repositioning of the D(E)RY motif

Side chain neutralization and helix extension in the vicinity of the D(E)RY motif is expected to alter the hydrophobic length of the H3 segment. To test whether these coupled processes affect the distance between the H3 C-terminus and the phase boundary in a micellar or lipidic environment the C-terminal Trp of the peptide H3W19F/V31W was employed as a donor fluorophor whose emission is quenched by a 5-dimethylamino-1-naphthalenesulfonyl (DANSYL) group (via FRET) in measurements including DANSYL-labeled phosphatidyl ethanolamine (DANSYL-PE). Figure 3.5 (inset) shows that excitation at 285 nm of "DANSYL-PE-doped" peptide-containing micelles evokes emission from Trp and DANSYL at 345 nm and 524 nm, respectively. Trp emission was typically 10-30% of that in the absence of DANSYL-PE. The 524 nm emission increased upon acidification, whereas Trp emission decreased, indicating a more efficient energy transfer when Glu-27 is protonated. DANSYL absorption and emission is intrinsically pH-independent in the pH 4-10 range (22,23).
Figure 3.5 pH sensitivity of lipid peptide interactions. Reconstitution of H3_{W19F/V31W} in DANSYL-PE-containing vesicles quenches the excited state of Trp-31. The residual emission of Trp-31 at 345 nm (normalized to the DANSYL emission upon direct excitation by 336 nm light) is plotted versus pH and monitors alterations of the C-terminal peptide geometry relative to the lipid head groups. Squares: PS:DANSYL-PE:PC:CH (molar ratio of 1:2.5:2.5:1.5); triangles: PS:DANSYL-PE:PC (molar ratio of 1:2.5:2.5); circles: "lipid-doped" DM-micelle containing PS:DANSYL-PE (molar ratio of 1:2.5). Lines are fits to a Henderson-Hasselbach equation. Inset: Representative spectra of H3_{W19F/V31W} in vesicles showing Trp-31 and DANSYL emission at 345 nm and 524 nm, respectively, and the excitation spectra of DANSYL-PE (λ_{em}=524 nm). Red: excitation and emission spectra at pH 4.3; black: excitation and emission spectra at pH 8.8. At lower pH, the emission of DANSYL at 524 nm increases, whereas that of Trp-31 at 345 nm decreases due to more efficient FRET. The pH-sensitivity is shown in the range, where DANSYL absorption is independent of pH (22, 23).

To correct for the different amounts of DANSYL-PE-labeled micelles or vesicles in the different samples, however, the residual Trp emission was normalized with respect to the fluorescence efficiency upon direct excitation of DANSYL-PE at 336 nm in these systems. Thereby, the residual Trp emission from samples derived from the same stock of a given lipidic composition but transferred to different buffers
is scaled to the same total concentration of vesicles in each buffer. The evaluation is restricted to the quenching of the donor fluorescence, i.e. to the residual Trp emission at 345 nm. This allows comparing the pH sensitivity of DANSYL-mediated quenching of Trp-31 emission in micelles and vesicles consisting of PC, PS, and cholesterol at different pH (Figure 3.5) and independently of the determination of absolute FRET efficiency. For better comparison of the apparent $pK_a$ values obtained from fits to Henderson-Hasselbach curves, the traces (and original data) where scaled to the fitted signal level at pH 9 for each sample. The quenching of Trp-31 exhibited an apparent $pK_a$ of 6 ± 0.3 which was independent of the assessed lipid composition and of the presence of cholesterol within the accuracy of the experiment. Trp fluorescence was more efficiently quenched at acidic pH indicating that upon neutralization of Glu-27 the C-terminus moves from an aqueous to a more hydrophobic environment near the lipid head groups. This agrees with the slight blue shift of the Trp emission (causing a negligible change of the overlap integral between DANSYL absorption and the Trp emission of < 0.5 %) and would favour the deactivation of the excited state of Trp-31 upon shortening of the averaged donor to acceptor distance.

Alternatively, the C-terminal Trp may become rotationally constrained if it gets partially immersed in the lipidic phase. This could also reduce Trp emission if a more favourable alignment with the DANSYL electronic transition moment is achieved. The same mechanistic conclusion would apply: the H3W19F/V31W C-terminus moves from an exposed to a more lipid-immersed state with Glu-27 protonated, whereas the opposite transition contradicts the data. No attempt was made to determine and compare absolute FRET efficiencies in the different lipidic compositions by further evaluation of the DANSYL emission at 524 nm in relation to Trp quenching. The unknown effect on the rotational freedom of both the C-terminal Trp and the DANSYL group in the different lipidic compositions would render a more detailed analysis ambiguous.

### 3.3.4 Hydrophobicity links protonation to conformation

The high $pK_a$ of carboxyl protonation in micelles and vesicles implies that the protonated state of the Glu-134 couples to a free enthalpy-delivering reaction. The coupling depends on the $\alpha$-helix preceding the D(E)RY motif in a hydrophobic phase.
as it is not seen in ICL2 (Figure 3.4). The low dielectric constant in vesicle membranes and in the interior of a micelle could promote both stabilization of the protonated side chain and of helical secondary structure. Therefore, we have asked whether a hydrophobicity-mediated "proton switch" is consistent with thermodynamic estimates of helix stability and charge-dependent partitioning of a carboxyl side chain at a phase boundary. The coupling of conformation to protonation is described by the microscopic equilibria defined in Figure 3.6(A). Peptide conformations with an exposed (E) or buried (B) state of the ionized carboxylate of Glu-27 interconvert with the equilibrium constant $K_C$ and their protonated counterparts (EH and BH) with the equilibrium constant $K_{CH}$. State function properties require that the free enthalpy difference for the conformational transition in the protonated vs. the ionized state (i.e. $ΔΔG = -RT \ln K_{CH}/K_C$) equals that of transferring a neutral versus an ionized glutamic acid side chain from an aqueous to a hydrophobic medium (i.e. $ΔΔG = -RT \ln K_B/K_E$). Free enthalpies $ΔG^{tr}$ have been determined for water to octanol transfers (24).

The reported $ΔΔG$ of 14.7 kJ (3.52 kcal) for glutamic acid provides an estimate of the enthalpy available to link protonation to conformation by a purely hydrophobicity-driven mechanism. These transfer enthalpies do indeed reproduce the measured $pK_a$ of 5.9 for protonation / helix extension with a $ΔG_c$ of 6 kJ for the transition of the ionized state from the exposed to the buried conformation. The resulting free energy surface of the system is shown in Figure 3.6(B). Upon acidification, the system moves along the "valley" from less than 10 % protonation with > 80 % of the carboxylates exposed to the aqueous phase at pH 7 (microscopic $pK_B$ of the buried state) to more than 90 % protonation with > 80 % of the neutral side chains buried at pH 4.4 (microscopic $pK_E$ of the exposed state). The equilibrium states trace out a trajectory that runs roughly diagonally through the conformation / protonation plane reproducing the linearity between the measured amide I and carboxylate absorption changes.

3.4 Discussion

Breakage of the "ionic lock" at the cytosolic H3/H6 interface in the transition to the $G_t$ -activating state of rhodopsin is linked to protonation of Glu-134 in the class-conserved D(E)RY motif in H3. This constitutes one of the two "proton
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switches" that control rhodopsin activation (25) and is likely to operate in other class-I GPCRs (11); (26-29). Here, we have shown that the D(E)RY motif represents an autonomous "proton switch" that is not per se dependent on interhelical contacts. Instead, local lipid protein interactions alter the pKₐ of the side chain carboxylate by providing a medium of low dielectricity that stabilizes the protonated state, thereby, coupling protonation to transmembrane positioning and helix extension. Qualitatively, the switching mechanism can be understood on the basis that carboxyl side chains are the most potent residues in defining transmembrane helix terminations (30). Quantitatively, switching in the D(E)RY motif is surprisingly efficient exhibiting a large and "symmetric" pH modulation of the conformation which is ideally realized when ∆Gₖ = ½ ∆ΔG. Switching originates in the protonation-induced drop of the free enthalpy of carboxyl transfer by ∆ΔG = 14.7 kJ. In the coupled system (Figure 3.6 (A)), the measured pKₐ correlates with a ∆Gₖ of 6 kJ which is indeed close to the above condition. However, ∆Gₖ is different from ∆G°ₖ of 15.2 kJ (24) for the hydrophobic burial of an ionized glutamate. Therefore, the E -> B transition cannot consist of the mere "swing out" of the carboxylate into the hydrophobic phase. The demonstrated formation of ~2 helical peptide bonds fully accounts for the reduction by 8-9 kJ due to the stabilizing effect of additional intramolecular H-bond formation (31). "Proton switching" and reorganization of the lipid peptide interface, shown by the "tryptophan to lipid" FRET measurements, are thus in excellent agreement with the energetics of helix stability and side chain partitioning. The relation of ∆Gₖ ~ ½ ∆ΔG is a robust feature of the IR signature of the D(E)RY motif for coupling enthalpies that shift the microscopic pK of the buried glutamate to 6.7 - 8.0 (in our evaluation pK = 7), i.e. close to the pK estimated for Glu-134 in MIIₚ (25).

How can a lipid-mediated coupling mechanism be understood in the context of crystal structures of rhodopsin? The intrinsic properties of the D(E)RY motif favour the equilibration of substates such that 1) the Glu-134 side chain becomes protonated in the hydrophobic phase, 2) the helical propensity of the C-terminal end of H3 increases, 3) the H3 C-terminal end approaches the membrane. Figure 3.6 (D) shows the H3 structure in dark rhodopsin and opsin, where the "ionic lock" is broken (32). In contrast to the dark state structure (Figure 3.6(C)), Tyr-136 of the D(E)RY-motif in opsin aligns with the other cytosolic tyrosines (not shown) indicative of a net shift
of the H3 C-terminus towards the membrane surface similar to the protonation response of the isolated H3\textsubscript{E} segment. The most striking feature, however, is the transition from a $\chi^2$ gauche state in dark rhodopsin to an extended rotamer of the Glu-134 side chain in opsin also seen in the "partially active" $\beta_2$-adrenergic structure (27); (33). A lipid bilayer imposes further hydrophobic constraints not present in the crystals. Although the C\textalpha-atom of Glu-134 points to the putatively lipid-facing side already in the dark (34). The "ionic lock" prevents it from "partitioning" between exposed and buried states. However, once "liberated" after activation, it can be stabilized in the protonated, activity-promoting extended conformation by hydrophobic burial. In the absence of a lipidic phase, packing of Glu-134 against the helix-2 helix-4 interface in the crystal (32) appears to provide the low dielectric environment accommodating the (putatively neutral) side chain.

The crystal structure supports such a hydrophobicity-controlled rotamer state as there are no specific interactions that favour the extended conformation (32). The partially active state-like features in opsin suggest that upon receptor activation in a bilayer, H3 becomes liberated from interhelical constraints such that it can insert the C-terminal end slightly more into the membrane. However this occurs efficiently only upon protonation and lipidic burial of the extended Glu-134 side chain, i.e. by forming the MII\textsubscript{b}H\textsuperscript{+} species. Thereby, hydrophobic matching is achieved for the MII\textsubscript{b} transmembrane configuration of H3, for which the partially active opsin structure could serve as a model. In the native receptor, the cytosolic chemical potential of protons thus overcomes the geometrical constraints on transmembrane position imposed on the MII\textsubscript{a} to MII\textsubscript{b} transition by the planar bilayer. The latter disfavours the burial of the strongly hydrophilic patch placed by the D(E)RY motif between the strongly hydrophobic flanking regions (Figure 3.6(D)) but stabilizes it in the MII\textsubscript{b}H\textsuperscript{+} state.

Also in the absence of a planar lipidic phase, the same coupling of protonation to local carboxyl burial and helix stabilization occurs, as shown here in micelles. For the full length delipidated receptor, however, the flexible non-planar micellar environment can easily adjust to the hydrophobic profile of the protein, irrespective of protonation-dependent increase of hydrophobicity of the D(E)RY motif. After the release of dark state constraints, the repositioning of H3 relative to the rest of the hepta-helical bundle can therefore proceed without the need of the protonation-
induced formation of a more contiguous hydrophobic profile across the interspersed D(E)RY motif. The micelle adapts to the MIIₜ conformation without requiring MIIₜH⁺ formation. The latter occurs as an uncoupled local pH-dependent transition similar to that seen with the synthetic peptides which are "uncoupled" from the holoprotein. The reason for this is the lack of a defined hydrophobic thickness relative to which side chain rotamers or surface protonation of the receptor would be constrained in the detergent micelle. Therefore, a lipid-mediated proton switch is in full agreement with the pH-insensitivity of the total amount of MII (i.e. the sum of states carrying an unprotonated Schiff Base) formed and with the uncoupling of H6 movement from proton uptake in detergent-solubilized rhodopsin (6). The intrinsic properties of the D(E)RY motif are thus sufficient to explain key features of the cytosolic "proton switch" in membranes and in delipidated rhodopsin.

The proposed autonomous local proton-dependent equilibration between activity-promoting and activity-impeding conformations of the D(E)RY motif at the phase boundary independent of specific ligand interactions further agrees with recent MD simulations of the β₂-adrenergic receptor (35). Here, the ionic lock appears to fluctuate between the open and closed state without corresponding "global" conformational switching of the entire inactive-like receptor structure to an active conformation. Although proton exchange reactions were not modelled in the MD calculations, our data support the notion that receptor activation originates in the shift of locally defined structural equilibria. Fluctuations of the "ionic lock" imply a continuous "exploration" of its physical environment. In rhodopsin, this may lead to an efficient shift of the local conformational equilibrium once dark state constraints are lifted in MII or opsin. In rhodopsin, however, proper function relies on efficient minimization of dark noise and there is no indication that protonation couples to conformation already in the dark (36). Such a stringent selection for noise reduction has not occurred for the β₂-adrenergic receptor. Although the frequent formation of the "ionic lock" appears to be a typical trait of the inactive receptor, sporadic breakage of the ionic lock even in the presence of inverse agonists parallels the biochemical phenotype of a basal activity in β-adrenergic receptors (27,37) not found in dark rhodopsin.
Figure 3.6 Protonation occurs in conformations with the H3 glutamic acid side chain either exposed to water (E) or buried in the hydrophobic phase of a membrane or micelle (B). The free enthalpies of protonation in these two states define the association constants $K_E$ and $K_B$ and differ by 14.7 kJ, i.e. the difference in water to octanol transfer enthalpies (33). The same $\Delta G$ applies for the exposed to buried conformational transitions (equilibrium constants $K_C$ and $K_{CH}$, with $\Delta G$ values of 6.0 and -8.7 kJ, respectively).

B) The free enthalpy $G_{sys}$ of the coupled system. $G_{sys}$ describes a potential surface as a function of the proton saturation $f_H$ and the fraction $f_E$ of exposed carboxyl side chains. $G_{sys}$ is the minimized sum of the individual chemical potentials $\mu_i = \mu_i^\circ + RT \ln c_i$ of all states at a given proton saturation (exposed ionized state chosen as reference, i.e. $\mu_E^\circ = 0$ kJ) and plotted from 5% to 95% saturation along both coordinates. The trajectory of minimal enthalpy (gray line) reproduces the observed linear relation between carboxyl and amide absorption (squares). The arrow delineates the amplitude of the conformational transition induced by a pH change from 4.4 (microscopic $pK_E$ of the exposed state) to 7.0 (microscopic $pK_B$ of the buried state).

C) Rhodopsin dark state structure (Protein Data Bank ID code 1LH9). The dotted line defines the putative membrane border. Tyr-136 of the D(E)RY motif is "displaced" towards the cytosol. Colour code: red and blue, formal negative and positive, respectively; green, neutral; white, hydrophobic.

D) Structural interpretation of the four states of the D(E)RY motif. The Glu-134 side chain switches from a gauche in E to a trans rotamer in B and Tyr-136 aligns with the other cytosolic tyrosines (not shown) in opsin (Protein Data Bank ID code 3CAP)
by sliding about one peptide bond deeper into the bilayer. Similar to H3E, helicity extends further to the C-terminal end in opsin (symbolized by the ribbon thickness). In the realm of the late rhodopsin intermediates described by (6) the E state corresponds to dark rhodopsin, and we assign the B and BH states to the MII_E and MII_EH intermediates, respectively. The EH state is probably not accessible by dark rhodopsin (44), where interhelical contacts prevent exploration of active-like substates and suppress dark noise to a much larger extent than in other GPCRs. Structure graphics were created with VMD (version 1.8.3).

Importantly, however, the conformational shift, whether in the inactive (β2-adrenergic receptor) or the active receptor state (rhodopsin), does not require a corresponding switch in the ligand-binding site and is shown here to operate as an isolated proton switch module even in the absence of the hepta-helical bundle. It is rather the stabilization of one of the substates (in rhodopsin the membrane-buried protonated state of the Glu-134 side chain) that adds a ΔG to the population of the other accessible substates, thereby shifting the equilibrium to an activity-promoting ensemble of conformations. Here, we have shown that the lipidic phase contributes to the energetics of the functionally relevant conformational substates.

In a broader sense, the regulation of the cytosolic GPCR structure by re-equilibration of lipid protein interactions after removal of dark state constraints appears to originate in small stretches of class-conserved amino acids. Their physical properties are preserved in corresponding peptides demonstrated here for the isolated transmembrane segment H3 as a part of the "ionic lock" and shown previously for the amphipathic helix-8 of rhodopsin (38) which participates in signal transfer to the cytosolic face along the transmembrane H-bond network of rhodopsin (39). Similar to recent results on lipid-dependent secondary structure in rhodopsin (15) our data show that the lipidic phase constrain the structure of the D(E)RY motif, rather than adopting to it. We attribute a functional relevance to these constraints in linking protonation to GPCR conformation via a proton-driven structural re-equilibration at the protein lipid interface which is independent of ligand-specific interactions. The evolutionary diversity underlying ligand-specificity can thus be reconciled with the conservation of a cytosolic ‘proton switch’ that is adapted to the general physical constraints of a lipidic bilayer.
3.5 Literature Cited

4. Concerted Modular Switches in Bacteriorhodopsin

4.1 Introduction

In the preceding chapter it has been shown that protonation is coupled to conformation though a conserved switching mechanism in class 1 GPCRs. The proton uptake contributes to the energetics of conformation equilibria in the G protein receptor rhodopsin and is not involved in a vectorial transport mechanism as in the case of well characterized proton pumps. However, the similarity of a transient cytosolic proton uptake in rhodopsin and possibly other class I GPCRs to another heptahelical retinal protein, the classical proton pump bacteriorhodopsin (bR), is intriguing since proton exchange reactions in rhodopsin resemble those occurring in the cytosolic halfchannel of bR (for the detailed bR photocycle refer to Figure 4.1).

Figure 4.1 Photocycle scheme depicting K, L, M, N, and O states distinguished by their spectral properties in the visible, infrared, Raman, and NMR, and available crystal structures. The substates of M and N are identified from kinetic or spectral data or by their structural differences (1). The initial state carries a protonated all-trans retinal Schiff base (PSB) which undergoes photoisomerization to 13-cis PSB as the primary and only light-dependent step.
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Deprotonation of retinal Schiff base in the M state is the hallmark of the bR photocycle. The spectroscopic changes widely observed in the visual rhodopsins of higher organisms triggered by light-activated retinal isomerization also involve changes in protonation of the Schiff base formed between retinal and the lysine residue in the seventh transmembrane helix (2). The analogy between bacteriorhodopsin, rhodopsin, and G-protein-coupled receptors is more interesting if one considers that rotation of the C13-C14 or C11-C12 retinal double bond is an intense, local perturbation that spreads in discrete steps to the rest of the protein and drives functionally relevant conformational changes (3), much like ligand binding does in the receptors. Despite of all these stunning similarities in structure and activation kinetics there is a marked dissimilarity in the event of proton release in the case of bR and rhodopsin. In bR, proton release is not to the same side as the uptake but occurs to the extracellular side by a regulated timing of water accessibility and internal proton transfer reactions at the Schiff base and the extracellular and cytoplasmic half channels. Also this process can be considered as a modular coupling of proton switches which operate in a concerted fashion, thereby allowing vectorial proton transport across the cell membrane. As is typical of modules, some of the switches can be knocked out by mutation without blocking others. In combination with trapping of intermediate states in crystals this has led to a number of high resolution structures that can be correlated with a variety of successive intermediates in the native systems (4).

In spite of all these developments, controversy exists as to large scale conformational changes which allow the entry of water into the cytoplasmic halfchannel to provide a proton translocation route during the M to N transition in bR. The crystal structures of the M (11) and N’ (5) states, before and after N, showed either disorder at the ends of helices F and G, or no significant large-scale structural changes, respectively. Since it is well known that large-scale structural changes can be hindered by the three-dimensional (3D) crystal lattice, these results do not rule out any of the large conformational changes in the M and N states derived from different kinds of spectroscopy that have been suggested to drive the entry of water into the cytoplasmic half channel. This controversy emphasizes the importance of real time observations of structural transitions and their assignments to specific locations in the
protein structure. In such cases, spectroscopy is the method of choice and recently EPR spectroscopy has addressed the issue of opening and closure of cytoplasmic halfchannel of bacteriorhodopsin (6).

In this work, we have used the in house-developed Infrared Fluorescence Cross Correlation Spectroscopy approach to understand the conformational coupling between the cytoplasmic half of the protein and the internal proton transfer, i.e. reprotonation of the retinal Schiff base and reformation of the charged state of the counter ion during the photocycle of the proton pump. The main objective of this study was to correlate structural changes from a specific region of the cytoplasmic half sensed by an introduced fluorescence label, fluorescein attached at an engineered cysteine (position 101) at the end of helix C with the infrared absorption changes collected from the entire protein. Thereby, the IR signature of conformational transitions that are not directly linked to the labeled site can be distinguished from those that participate in the transitions at the labeled site. This approach was used to investigate the relation between the internal proton transfer reactions. Furthermore, the influence of azide on the correlation of dynamic changes happening in these two distant domains has been addressed as azide is known to enter the cytoplasmic channel and provides increased proton conductivity in other D96-mutants. This Cross Correlation approach coupled with mutagenesis will provide more insights to the understanding of the timing of structural transitions in the bRD96A mutant.

4.2 Experimental Procedures

**bR sample preparation:** The preparation and expression of bR mutant protein D96A/V101C in *H. salinarium*, in which alanine replaces aspartic acid 96 and cysteine replaces valine 101, was performed according to the procedures described (7,8). Labeling of bR with 5-(iodoacetamido)-fluorescein (IAF, Molecular probes) and determination of labeling stoichiometry were performed as described in (7,9). The absorption spectra of the fluorescein bound bR-D96A/V101C was measured which indicated an absorption maximum at $\lambda_{\text{max}} = 492$ nm. The expression and labeling of bR mutant was carried out at the lab of Ulrike Alexiev, Frei Universitat, Berlin.
**ATR-FTIR spectroscopy:** 1 nmol of bR mutant sample reconstituted in acidic buffer (HCl-H$_2$O pH 4.9, 1:100, protein: buffer) with or without azide (5µl of 10mM Na azide +40µl of sample) was dried on the diamond crystal of an ATR cell (Resultec). The photocycle was induced by sample illumination for 10 s with a 100 W projector through a light guide using a 575 nm long pass filter (Schott, Mainz). FTIR measurements on M formation and decay under ambient humidity were performed with a vector 22 FTIR spectrophotometer (Bruker, Karlsruhe). The time-dependent spectra were obtained at 2 cm$^{-1}$ spectral time resolution and 30 s as the initial acquisition time (corresponding to 64 scans for dark state recording) followed by the 10 s activation and then subsequent recording of interferograms. Between IR-data acquisitions, a brief closing of an automated shutter in front of the PMT served as an event marker to allow proper selection of the continuously recorded fluorescence data for signal averaging, such that a single integral emission intensity could be assigned to the IR-difference spectra of each time-slice (controlled by a automated shutter in the path of photon counter) covering a total of ~30 minutes.

**Fluorescence measurement:** The simultaneous observation of IR spectral changes and fluorescence intensity was accomplished by coupling a branched light guide to the ATR-FTIR cell. One arm was used for exciting the horizontally positioned sample on the ATR crystal from top with a laser diode emitting at 488 nm (Ocean optics). Emission was collected through the other arm transporting the light to a photomultiplier tube equipped with a 540 nm long pass filter. The fluorescence signal was averaged for each time interval during which interferograms were accumulated for individual IR difference spectra resulting in 14 time-dependent difference spectra and the corresponding averaged fluorescence intensities.

**Heterospectral cross-correlation:** The synchronous spectrum is obtained as (equal to the real part of $X(v_1,v_2)$)

$$\Phi_{syn}(v,I) = \sum_{i=1}^{14} A_{v_i} I_i$$

where $A_{v_i}$ is the amplitude of an IR-absorption band at wavenumber $v$ and time $i$, $I_i$ is the integrated fluorescence intensity at this time. Plotting $\phi(v)$ versus $v$ yields the synchronous spectrum which assigns amplitudes to each wavenumber such that the root mean square deviation between the measured absorption decay at this wave-
number over time and the fluorescence decay scaled with $\phi(v,I)$ is minimal. The residual deviations are represented by the disrelation spectrum

$$
\Phi_{di} (v, I) = +\sqrt{\Phi_{syn} (v, v) \Phi_{syn} (I, I) - \Phi_{syn}^2 (v, I)}.
$$

The origin of the residual disrelation was analyzed by subtracting the $\Phi_{syn} (v, I)$ scaled to the initial amplitude at the wavenumber of interest ($v$) from each measured subsequent difference spectrum in proportion to the fluorescence signal $I$ at the corresponding time $i$. Bands that vanish in each of the resulting subtractions are fully synchronized with the change at the fluorescence label. Bands that vanish in the first subtraction ($i=1$) but get inverted at later times ($i > 1$) decay faster than the fluorescence from the label. Bands that keep their signs as in the originally measured difference spectra decay slower than the fluorescence signal.

### 4.3 Results

Bacteriorhodopsin is a light-driven proton pump capable of capturing and transporting protons from the inner side to the outer side of the bacterial cell membrane. In structural terms, bacteriorhodopsin is a seven-transmembrane helix protein consisting of a retinal chromophore that lies near the center of the membrane attached to Lys216 through a protonated Schiff base ($-\text{HC}_{15} = \text{NH}^+$) which stays in all-trans conformation in the non-activated state of the photocycle. The Schiff base is at the heart of the proton pumping mechanism and the structures involved in the proton release lie on its extracellular side while those involved in the proton uptake reside on the cytoplasmic side. The photoisomerization of the retinal from all-trans to 13-cis sets off a cyclic sequence of reactions that drive the transport of a proton across the cell membrane, while others have light-sensing function, analogous to bacterial chemotaxis receptors. The numerous intermediate states in the reaction cycles, with transiently changed retinal and protein conformations, as well as changed protonation or hydrogen-bonding states of buried residues have been studied extensively by spectroscopy (10), site-specific mutagenesis (11), and by comparisons across many species (12). Together, these studies have established the connections between the spectroscopically observed intermediates BR-K-L-M-N-O-BR. The
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The photocycle begins when the retinal chromophore absorbs a photon, resulting in the isomerization of the C13=C14 bond, converting the all trans retinal into its 13-cis form. This is followed by a sequence of changes in the protein which ultimately returns to the ground state in ~10 ms. During the photocycle one proton gets transported across the membrane and bR accomplishes this uphill translocation by changing the proton access gateway to the Schiff base from the extracellular side to the cytoplasmic side and back. Asp96 is the key residue in reprotonating the Schiff base from the cytosol (coincident with formation of N intermediate) after its proton has been transferred to Asp 85 in the extracellular channel (coincident with formation of the M state).

The Mutant bRD96A/V101C was studied here, where the proton donating group to the Schiff base was replaced by alanine and a single cysteine carrying a fluorescence label, fluorescein was introduced instead of valine 101 at the C-terminal end of helix C (see Figure 4.2). This position is analogous to that of the conserved proton uptake site in class-1 GPCRs. In this system, the late M is trapped where the counter ion Asp85 is protonated giving rise to a ~1765 cm⁻¹ band and the Schiff base is unprotonated as revealed by the negative chromophore bands from 1100-1300 cm⁻¹ and by the strong negative ethylene stretching mode at 1526 cm⁻¹ wavenumber corresponding to the depletion of the protonated Schiff base ground state and the lack of IR activity in the unprotonated M state (Figure 4.2). This state decays without the accumulation of any other intermediates on the time scale of our measurements. Since the late M intermediate is trapped, the cytoplasmic channel is open. However re-protonation of the Schiff base, i.e. N state formation, is slowed down for two reasons. 1) the proton donor Asp-96 has been removed, 2) Invasion of water into the channel is reduced by low ambient humidity. This allows the prolonged observation of the M decay and thus enhances signal averaging such that a highly sensitive correlation between the integral fluorescence signal and the IR absorption changes is facilitated.
Figure 4.2 Sketch of the bacteriorhodopsin structure. This representation emphasizes (2I1X.pdb) the location of covalently bound 5-(iodoacetamido)-fluorescein at helix C and the position of protonated Asp85 which is used as the infrared marker band for cross correlation analysis. The Schiff base is unprotonated in this mutant during the time course of our measurements.

4.3.1 Internal proton transfer precedes cytoplasmic halfchannel closure

Our experiments were designed to investigate the coupling of domains in the internal proton transfer site of bR mutant to the changes happening in the cytoplasmic half especially the changes in the environment of fluorescein-labeled helix C. Light-induced FTIR difference spectra generated at defined time intervals contain the M state which decays slower than the wild type bR evident from the decrease of all the initially formed difference bands (Figure 4.3 (red)). Simultaneously, the increased fluorescence of the fluorescein in the M state also relaxes back to its lower initial integral intensity (Figure 4.3, inset). Three
representative raw data traces are shown. White filled circles show the average of seven independent experiments and correspond to the data integration over the time intervals of IR spectra acquisition. These data forms the basis of cross-correlation with the IR spectra.

**Figure 4.3** 14 time-dependent IR difference spectra covering 30 minutes of M decay of Cys101 fluorescein-labeled bRD96A, azide free sample (red trace is the first in the decay series and blue the last). *Inset:* logarithmic plot of simultaneously measured decrease of fluorescence from the label at Cys-101. Integration intervals for fluorescence averaging are delimited by signal deflections from a shutter closure between interferogram recordings.

**Cross-correlation of IR changes with cytoplasmic half channel kinetics:** Heterospectral cross-correlation was carried out on the raw data as described (see section 4.2). The analysis shows that most of the spectral changes in the IR are highly correlated with the kinetics of the cytoplasmic half channel monitored by the fluorescence as is evident from the large synchronous amplitude (Figure 4.4 (black trace)) relative to the disrelasion spectrum (red trace). Nevertheless, the data acquisition and analysis is sensitive enough to pick up the small meaningful kinetic
differences that exist between these two processes as revealed by the non-vanishing disrelation spectrum.

![Graph showing cross-correlational analysis of IR absorption changes with fluorescence emission from fluorescein at Cys101 of bRD96A azide free sample. The synchronous amplitude (black) is much higher in comparison with disrelation amplitude (red). Inset: spectral deviations of the measured spectra at time points 1, 5 and 9 (red, blue, black respectively) from the synchronous correlation scaled to the initial amount of M (there by the 1763 cm$^{-1}$ marker band and protonated state of Asp 85 vanishes in the first difference). Band inversion over time shows that Asp 85 deprotonates faster than closure of the cytoplasmic channel.](image)

**Figure 4.4** Cross-correlational analysis of IR absorption changes with fluorescence emission from fluorescein at Cys101 of bRD96A azide free sample. The synchronous amplitude (black) is much higher in comparison with disrelation amplitude (red). Inset: spectral deviations of the measured spectra at time points 1, 5 and 9 (red, blue, black respectively) from the synchronous correlation scaled to the initial amount of M (there by the 1763 cm$^{-1}$ marker band and protonated state of Asp 85 vanishes in the first difference). Band inversion over time shows that Asp 85 deprotonates faster than closure of the cytoplasmic channel.

The crucial question is the relative timing of internal proton transfer monitored by the absorption at 1761 cm$^{-1}$ and movement in the cytoplasmic half channel sensed by the fluorescence label. It is not obvious from the disrelation spectrum, which by definition has a positive sign, irrespective of which of the correlated IR and fluorescence signals is the faster or slower. However, this information can be determined by subtracting the synchronous spectrum $\phi_{\text{syn}}(\nu, I)$ from the original difference spectra, such that the initial 1761 cm$^{-1}$ absorption band vanishes for the first difference spectrum (Figure 4.4, inset-red trace). Subtracting $\phi_{\text{syn}}(\nu, I)$ from the
subsequent measured difference spectra in direct proportion to the fluorescence signal (i.e. in full synchronicity) would cancel out all difference bands that are fully synchronized with the fluorescence signal. Instead, the subtraction leads to the inversion of the 1761 cm⁻¹ band and all other difference bands. This is exemplified for the subtraction from the fifth (Figure 4.4, blue trace) measured difference spectra and for the ninth (Figure 4.4, black trace), where the band inversion is found to be the largest. This demonstrates that the fluorescence signal, caused by closure of the cytoplasmic half channel, decays slower than the infrared difference spectra, leading to an ‘over subtraction’ of the difference bands and thus to their inversions. The correlation results clearly show that the cytoplasmic channel closure lags behind the internal proton transfer. This indicates that the barrier for channel closure is affected by the internal proton transfer such that the release of the proton from the Asp85 to the extracellular side facilitates channel closure. ‘Once the proton goes the channel closes’. The ‘individual kinetics’ exhibited by these two regions again proves the notion of the concerted action of two modular switches in this part of the bR photocycle. They make up a system of four substates, rather than a stringent switch of the global protein structure between only two states.

4.3.2 Cytoplasmic channel kinetics in the presence of azide

Studying Asp 96 mutants has tremendously helped in the better understanding of the Schiff base reprotonation occurring between M-N transitions in bR photocycle. In these mutants deprived of internal proton donor, protons to the Schiff base is supplied from the cytoplasmic bulk phase. The proton conduction pathways in these mutants however depend heavily on the replacement residues for Asp 96 which is exemplified by the fact that the decay kinetics of the M state are different in D96A, D96G and D96N. Azide and other anions of small weak acids are known to modify the reactions in the photocycle and in many cases are shown to repair the kinetic defects in the M decay of Asp 96 mutants depending on the nature of the substituted residue in the mutation. Azide fulfills the role of an alternative proton donor to the Schiff base by penetrating the protein from the intracellular side and thereby donating the proton needed to complete the photocycle. In the case of bRD96A mutant, azide has little effect on the protonation rate of the Schiff base contrary to the D96N phenotype. All this points to the fact that the nature of the side
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Chain of residue 96 has an influence on the distribution of water molecules in the cytoplasmic region of bR.

**Figure 4.5** 14 time dependent IR difference spectra covering 30 minutes of M decay of Cys101 fluorescein-labeled bRD96A, azide containing sample (red trace is the first in the decay series and blue the last). *Inset*: logarithmic plot of simultaneously measured decrease of fluorescence from the label at Cys-101. Integration intervals for fluorescence quenching are delimited by signal deflections from a shutter closure between interferogram recordings.

The infrared-fluorescence combined measurements used in this study generated the light-induced M state of bRD96A/V101-C (in the presence of azide) which decays almost at the same rate as in the azide-free sample, in accordance with the previous published results. This is evident from the comparison of decay curves of both, the initially formed IR-difference bands and the fluorescence signal (Figure 4.3 and Figure 4.5). Similar to the azide-free sample, here also the elevated fluorescence signal of fluorescein in the M state relaxes back to its lower initial integral intensity (Figure 4.5, *inset*) in tandem with IR changes. Three representative raw data traces are shown. White filled circles show the average of seven independent experiments.
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and correspond to the data integration over the time intervals of IR spectra acquisition. These data forms the basis of cross-correlation with the IR spectra.

**Cross-correlation reveals that azide lowers the energetics for cytoplasmic helix movement:** Hetero-spectral cross-correlation was carried out on the raw data as described (see section 4.2). The analysis shows that most of the spectral changes in the IR are highly correlated with the kinetics of the cytoplasmic half channel monitored by the fluorescence as is evident from the large synchronous amplitude (Figure 4.6 (black trace)) relative to the disrelation spectrum (red trace). Nevertheless, the data acquisition and analysis is sensitive enough to pick up the small residual differences in internal proton transfer monitored by the decay of the 1761 cm\(^{-1}\) band (deprotonation of Asp85) and of the chromophore absorptions (1000-1300 cm\(^{-1}\)) linked to reprotonation of the retinal Schiff base. The disrelation spectrum in the presence of azide (Figure 4.6 red trace) very much resembles that in its absence (Figure 4.4, red trace). However, applying the same subtraction procedure as above clearly leads to a different result. Again the synchronous spectrum was subtracted from the first difference spectrum to abolish the 1761 cm\(^{-1}\) band (Figure 4.6, *inset*- red trace) but instead of the band inversion in the subtraction at the later times, Figure 4.6 (*inset*- black trace) shows that the sign is conserved throughout the measured spectral range. Thus, the subtraction of the synchronous difference-spectra in strict proportion to the fluorescence signal does not lead to complete cancellation of the IR-absorption changes. This shows that in the presence of azide, the fluorescence signal decreases faster than the measured IR-absorption changes. The closure of the cytoplasmic half channel, in particular the movement of helix F as the main contributor to cytosolic conformation in the bRD96A mutant can thus proceed faster than the internal proton transfer. Whereas the cytosolic conformation is rather strongly dependent on internal proton transfer in the absence of azide, the energy barrier for helix movement is reduced by azide without an obvious effect on internal proton transfer rates. These results are again in conjunction with the modular nature of concerted switching mechanisms in bR photocycle as it clearly shows partial kinetic independence of the modules.
Figure 4.6 Cross-correlational analysis of IR absorption changes with fluorescence emission from fluorescein at Cys101 of bRD96A azide-containing sample. The synchronous amplitude (black) is much higher in comparison with disrelation amplitude (red). Inset: spectral deviations of the measured spectra at time points 1, 5 and 9 (red, blue, black respectively) from the synchronous correlation scaled to the initial amount of M (thereby the 1763 cm⁻¹ marker band of the protonated state of Asp 85 vanishes in the first difference). Conservation of the sign of the retinal absorption bands over time shows that Asp 85 deprotonates slower than closure of cytoplasmic half channel.

4.4 Discussion

The bRD96A mutant has a partial M-like structure already in the dark (13). This concerns the more ‘open arrangements’ of the helices C, B, and G. Therefore, the light induced conformational changes are expected to be largely related to the movement of helix E and F which has been shown to be a hallmark of the opening of the cytoplasmic half channel in native M formation. Their concerted outward movement has been shown to start in M and culminate in the N state to provide the proton pathway from Asp96 to the retinal Schiff base including the rearrangement and additional uptake of water molecules in the cytoplasmic channel (6,14-16). Generation of this pathway is largely blocked in D96A despite it being a more open cytoplasmic conformation. This is due to the specific effect of substituting alanine at
position 96 which creates a hydrophobic cavity that does not take up water readily as compared to other mutations at this site.

The cross-correlation data directly address the linkage between the repositioning of helixes E and F on one hand monitored by the label in the helix C facing the cytosolic ends of helixes E and F at about equal distances (6) and the internal proton movements at Asp85 and the retinal Schiff base on the other hand. The data show that channel closure is strongly coupled to Schiff base reprotonation shown here to occur slightly faster. However, in the presence of azide, the relaxation of helices E and F can precede Schiff base reprotonation. This is remarkable since in this mutant azide does not restore proton conductivity as it does in other D96 mutants. Therefore, the data indicate that H-bond mediated interactions in the cytoplasmic half channel normally occurring at Asp96 are restored by azide and facilitate channel closure independently of proton conductivity and of internal proton transfer reactions. This hints at a function of internal water and/or Asp96 in providing both a pathway for reprotonation of the Schiff base and a site for H-bond restructuring that allows efficient reformation of the ground state interactions between helices E, F, and C. Whereas the first function cannot be restored by azide in bRD96A, the H-bond network restructuring is facilitated and thus not as strongly dependent on the preceding internal proton transfer anymore. The cross correlation shows that channel dynamics are actually not increased by enforcing the linkage to internal proton transfer in the presence of azide which would have led to full synchronicity, rather than to the observed acceleration of cytosolic transitions over the internal proton transfer steps. In fact, the operation of an additional mechanism, i.e. the proposed effect on cytosolic H-bond linkages, in promoting channel closure is in full agreement with literature, as azide does not operate on the internal proton transfer.

The data gained from these experiments allows addressing the question of the IR signature that could be assigned to the conformational change monitored by the fluorophore (fluorescein). By definition, the corresponding IR difference band has to exhibit a minimal peak in both the disrelation spectra of the azide-free and the azide-containing measurements. The highest degree of synchronicity in these experiments is observed at 1690 cm$^{-1}$. Therefore we relate, this band to helix F movement which appears to affect a high frequency carbonyl stretching vibration. The later is typical
of antiparallel β–sheet-structure. bR possesses a single antiparallel β sheet at the extracellular side which occludes the retinal from the aqueous phase.

Figure 4.7 Bacteriorhodopsin structure showing the van der Waals contacts existing between residues Val101/Phe171 and also between residues Glu194 (helix F) and Ile78 (helix C). The later contact renders them the role of a hinge for helix F movement.

The sheet forms the extracellular loop between helices B and C (Figure 4.7). Remarkably helix F contacts the helix C at the exact transition from the β-sheet to the start of α-helical structure at the extracellular side of helix C. A close look of the protein structure reveals that van der Waals contact is formed between residues Ile 78 at the beginning of helix C and the backbone of Glu194 at the end helix F.

It is reasonable to expect that cross correlation will identify structural transitions in helix F if they affect the relative position of the C-terminus of helix C. The above mentioned van der Waals contact is ideally positioned as a pivoting point for helix F outward movement and may thus transmit the helix F movement to helix C. Therefore, we assign the 1690 cm$^{-1}$ vibration to helix F-C interface rather than to
a peptide group on the direct vicinity of the fluorescence, as there is no $\beta$-sheet structure present than that at the F-C interface. However, the direct effect of helix F movement (with the Glu194/Ile78 pair acting as a hinge) on the fluorescence of the label can be fully rationalized by the structure. The labeled residue at position 101 is in direct proximity to the N-terminus of helix F and thus an efficient sensor (opposing phenylalanine) of the helix F-helix C contact. The rigid body movement of helix F will thus generate the observed high synchronicity between the IR signal from the ‘hinge’ in the $\beta$-sheet and the fluorescence change upon the approach of the cytosolic ends of helices C and F. Fluorescence infrared cross correlation can thus very specifically reveal long range conformational coupling and relate them directly to structural details.

4.5 Literature Cited

5. Force Driven Modular Switch in Kinesin

5.1 Introduction

In the preceding chapters, the role of an individual amino acid side chain in modular switching of GPCRs at the C-term of helix 3 (Chapter 3) and the correlation of internal proton transfer reactions with external proton dislocation in the corresponding helix 3 of bacteriorhodopsin has been discussed, respectively (Chapter 4). In both cases the switches are operated by the chemical potential of protons. The underlying similarity has been shown to originate in alternative side chain partitioning at a membrane border for the D(E)RY motif in GPCRs, where as in bR the surface conformational changes strongly depend on the existence of an H-bond-network in the cytoplasmic channel around Asp 96. In the absence of this residue, azide can substitute for the H-bond-interaction and thereby accelerate channel closure over internal proton transfer reactions. In the following section of this work we have asked whether conformational switching may be linked also to direct mechanical forces rather than chemical free energies. The biological systems that are subjected to forces in their natural biological switching process are classified in general as ‘Molecular motors’. In the cases of proteins belonging to the class of molecular motors the modular function is well established as different functions such as force generation, cargo binding, force transmission and binding to other proteins or cargo are assigned to different domains of these proteins. A key question concerns the hypothesized flexibility/extendibility of a motor protein that allows the cooperation of non-synchronized molecular motors, here kinesin-1 from Drosophila melanogaster, when they are attached to the same cargo and thus prone to mutual interference that reduces the speed of movement.

In this work the hypothesis of a functionally relevant partial unfolding event triggered by the force generated in the motor domain will be addressed. It will be shown how sequence-encoded secondary structure propensity may enable a functional module to couple structural flexibility in a predefined region to the ATPase cycle. The physiological role of the identified and spectroscopically characterized module lies in allowing cooperativity of motors attached to a single cargo. The modular switching processes in the mentioned systems imply that a sequence-encoded local physical property can be recruited for the concerted
switching process in larger multidomain systems such as motors or receptors. It is a great challenge to identify these sequence dependent physical parameters and prove their capability to undergo conformational switching independently of full length structure constraints. Only if this independence is maintained a true modular function is proved. Correspondingly, the approach taken here employs peptide synthesis to generate segments derived from the native proteins that preserve their sequence-encoded properties which are related to the function in full length systems.

Kinesin-1, an important member in the kinesin family mediates the transport of membrane-bound organelles along actin filaments or microtubules and the fashion of their movements is termed processive. As a two-headed motor Kinesin-1 remains attached to the filament all the time and works in tandem with its counterparts without causing impedance to each other and moves large organelles such as mitochondria in a hand-over-mechanism (1-4). The amazing coordination among these motors and lack of impedance either in vivo or in vitro (5) has been accounted to the flexibility (high torsional compliance) of a domain within the kinesin molecule. Hunt and Howard (6) suggested that the compliant region within kinesin-1 might correspond to a ~45-65 amino-acid region near the motor domain.

This region termed Hinge 1, lies between the dimerization domain (also called the ‘neck,’ amino acid (aa) 341-376 in Drosophila), a coiled coil just carboxyl-terminal to the motor domain that is seen in the three-dimensional structure of the kinesin-1 dimer (7), and Coil 1 (aa 439-560), a coiled coil that forms part of the elongated kinesin tail domain (8) (Figure 5.1). There are three reasons for thinking that Hinge1 (aa 377-438) is flexible. First, it contains prolines not predicted to form a coiled coil (9,10) (Figure 5.1 C), a protein domain that has high torsional rigidity. Second, high torsional flexibility is still evident in kinesins truncated after aa 430. Third, removal of Hinge 1 from Neurospora crassa kinesin-1 drastically reduces the microtubule gliding speed at high densities (from 2.6 to 0.7 mm/s) (11).

To test the structural and functional importance of Hinge 1, we measured the motility and torsional compliance of kinesin-1 in which the putative flexible region was removed (work done in collaboration with Alvaro Crevenna, Joe Howard, MPICBG, Dresden).
**Figure 5.1** Domain organization of Hinge 1 region of Drosophila kinesin-1. (A) Amino-acid sequence alignment made with clustalW of wild-type kinesin-1 (Dm, Drosophila melanogaster; Hs, Homo sapiens; Mm, Mus musculus; Nc, Neurospora crassa). Letters ‘a’ and ‘d’ denote mainly hydrophobic positions in heptad repeats. Horizontal lines represent sequences sampled by mode peptides 1-5 (left to right). (B) Structures of corresponding regions from x-ray crystallography (7), circular dichroism (12) and NMR (13). (C) Coiled coil likelihood calculated from PairCoil (14) and Coils (9) programs using a 21-aa window. The x axis represents amino-acid position. (D) Predicted probability of secondary-structure formation given by PSIPRED (15) and predict protein (PP) (16). Predicted secondary structure is presented below each plot; the subset (from PP) with probability higher than 80% is shown. (E) Secondary structural organization of Hinge 1, based on this study. Interstrand interactions of central part of Hinge 1 are hypothetical (see discussion section for details).

The results from the motility assays and video-microscopy demonstrate that Hinge 1 is necessary for high speed at high kinesin density, but is not required for high speed at low kinesin density, consistent with the necessity of Hinge 1 for efficient many-motor activity. But the compliance of this region was found not to be high and to explain this unexpected behavior which could not yet be rationalized by
other functional assays we employed peptide synthesis (five 30aa-polypeptides that cover Hinge 1) followed by their detailed secondary structure analysis by spectroscopy. This approach is similar to that used by Tripet et al (17) to study the role of the neck region in processivity. We found evidence for secondary structure that leads us to hypothesize that the Hinge 1 region is sufficiently structured to resist thermal forces, but that it is disrupted by the forces generated during motility, thereby preventing it from hindering motion in many-motor conditions.

5.2 Experimental Procedures

Model peptides derived from the Hinge 1 sequence. The Hinge 1 sequence was cut into five 30aa long overlapping pieces (Figure.5.1). Each peptide was synthesized, HPLC-purified, and characterized by mass-spectroscopy by ThermoElectron (Ulm, Germany). Trifluoroacetic acid was removed and the peptides were used without further purification. The N-terminal peptide (peptide 1) and C-terminal peptide (peptide 5) contain at least 2 heptads of the flanking coiled coil regions. One set of five peptides contained a Trp (W) residue at the same relative position (aa 11) to monitor by fluorescence the thermal unfolding via changes in the Trp environment. Trp at this position in peptide 1 is natural in Dm kinesin-1, while the Trp in peptide 2 is naturally found in kinesin-1’s from other species (N. crassa, U. maydis, C. elegans and S. racemosum) replacing the Gln (18) at this position in Dm kinesin. Sequences of the other peptides where chosen such that the Trp also replaces a Gln in each case, motivated by the naturally occurring Gln to Trp replacement. Additionally, the non-fluorescent native Dm sequences corresponding to the structurally most different peptides 2 and 3 (shown in this study) and the homologous native (non-fluorescent) sequences of kinesin-1 from Homo sapiens were studied for comparison. The sequences of peptide 2 and 3 derived from the Hinge 1 of human kinesin-1 are: RNGETVPIDEQFDKEKANLEAVTVDKDITL (3478 Da) and FTVDKIDITLTNDKPATAIGVIGNFTDAERR (3321 Da), respectively.

FTIR spectroscopy. FTIR spectra were recorded with a Vector 22 (Bruker, Rheinstetten, Germany) equipped with a diamond ATR-cell (Resultec, Illerkirchberg, Germany). The peptide concentrations used were 6-8 mg/ml in phosphate buffer (100 mM KCl, 50mM Na₂HPO₄, pH 8.6). For one spectrum 256 interferograms were
recorded with a resolution of 2 cm\(^{-1}\) at 20\(^\circ\)C and referenced against pure buffer spectra under identical conditions. Absorption spectra were evaluated by fitting the amide I envelope with five Gaussian/Lorentzian curves. Bandwidths were restricted to 30 cm\(^{-1}\) initially. Band frequencies, shapes, and intensities were free to vary in the final fit using the PeakFit software (version 4.12, Seasolve, San Jose, CA) and stayed below 30 cm\(^{-1}\).

**CD spectroscopy.** A CD Spectroscope Jasco J-810 15OS (Jasco, Gross-Umstadt, Germany) was used with the following settings: Nitrogen (N\(_2\)) flow at 5 l/min, scan speed at 100 nm/min, bandwidth and data pitch 1 nm, continuous scanning mode (20 scans averaged) and 1 mm cuvette path length. Wavelength measured from 260 to 190 nm. CD buffer (10 mM KCl, 5 mM Na\(_2\)HPO\(_4\) in ddH\(_2\)O at pH 8.69). The ellipticity at wavelength \(\lambda\), the percentage of secondary structure present for each spectroscopic CD signal and the concentration of tryptophan-containing peptides were calculated according to the procedures described in Chapter 2 of this thesis.

Peptide concentrations ranged from 0.2 to 0.4 mg/ml.

**Fluorescence spectroscopy.** The intrinsic fluorescence of Trp was used to monitor temperature-induced structural transitions in the model peptides. Emission of the peptides was excited at 280 nm and measured from 295 to 400 nm at 2 nm slit width using a PerkinElmer LS 55 instrument (PerkinElmer, Vaudreuil, Quebec, Canada), equipped with a temperature-controlled cuvette connected to a water bath thermostat (Hake, Frankfurt, Germany). The temperature dependence of Trp emission, however, is an intrinsic property of this residue and not per se an indication of a structure-dependent change in the Trp environment within a polypeptide chain. Therefore, we have analysed the structure-sensitive contribution by normalizing the emission of the peptides relative to the corresponding intensity recorded under identical conditions from free Trp in aqueous solution. The emission of the single Trp present in the model peptides was and measured in buffer (100 mM KCl, 50 mM phosphate, pH 8.6) at temperatures between 23 and 70 \(^\circ\)C. The fluorescence of free Trp was measured at identical temperatures in the same buffer. The ratio of the emission at 345 nm from the peptide (\(E_{\text{pep345}}\)) relative to that of free Trp in aqueous solution \(E_{\text{W345}}\) was determined as:

\[
\Phi_{\text{pep}}(T) = \frac{E_{\text{pep345}}(T)}{E_{\text{W345}}(T)} \quad \text{Eq. 1}
\]
In the same way, the emission of free Trp in ethanol/water mixtures was scaled to that in water, yielding the ratio:

$$\Phi_{\text{EtOH}}(T) = \frac{E_{\text{EtOH-W345}}(T)}{E_{W345}(T)}$$  \hspace{1cm} \text{Eq. 2}

$\Phi_{\text{EtOH}}(T)$ depends exponentially on $T$, such that

$$\log[\Phi_{\text{EtOH}}(T)] = pT + \text{const}$$  \hspace{1cm} \text{Eq. 3}

This is shown in Figure 5.3A (Inset), which also evidences that the slope $p$ increases with increasing admixture of ethanol, i.e. with increasing hydrophobicity of the Trp environment. We have used this dependency to determine $\Delta H$ and $\Delta S$ for unfolding of the peptides in a two state model, assuming different hydrophobicity in the initial native (concentration $N$) and thermally unfolded state (concentration $U$), whose fractional concentration is defined as:

$$U_r(T) = \frac{1}{1 + K}; \quad K = e^{-\Delta G/RT}$$  \hspace{1cm} \text{Eq. 4}

where $K$ is the equilibrium constant of the unfolded and native state $U/N$ (reversibility was found for all peptides in the assessed range of 23-70 °C). The measured temperature dependence of $\log \Phi_{\text{pep}}(T)$ was reconstructed by assigning temperature-independent slopes $p_N$ and $p_U$ to the native and unfolded peptide states, respectively, in the plot of $\log \Phi_{\text{pep}}(T)$ versus $T$:

$$p_N = \frac{d \log[\Phi_{\text{pep}}(T)]}{dT} \quad \text{and} \quad p_U = \frac{d \log[\Phi_{\text{pep}}(T)]}{dT}$$  \hspace{1cm} \text{Eq. 5}

Thus, the temperature dependence in the measured trace was fitted by

$$d \log \Phi_{\text{pep}}(T)/dT = p_U U_r(T) + p_N (1 - U_r(T)) = \Delta p U_r(T) + p_N$$  \hspace{1cm} \text{Eq. 6}

and the pair of $\Delta H$ and $\Delta S$ values was determined that gave the least quadratic deviation from the data (with $\Delta p = p_U - p_N$, where $p_N$ was the initial slope of the plot at room temperature and $p_U$ a fit parameter equal or larger than the slope at 70° C).

**Gel filtration.** A Superdex Peptide 10/300 GL column (Amersham Biosciences, GE HealthCare, Uppsala, Sweden) was used. The running buffer (100 mM KCl and 100 mM Na$_2$HPO$_4$ at pH 8.6) and all liquids were filtered with a 0.22-µm filter and degassed in bottles rinsed 3 times with filtered water. As standards we used: a) Ribonuclease, (MW 13700), b) Aprotinin (MW 6512), c) Insulin chain A (MW 2531.64) and d) Insulin chain B (mw 3495.89). Before samples were run the column
was cleaned (at 0.25 ml/min) with 4 column volumes (CV) of water, 2CV of EtOH, 2CV of water and finally equilibrated with 2CV of running buffer. Each sample was filtered with a 0.22-µm filter before loading. One hundred microliters of sample (1 mg/ml) were loaded for each peptide and run at 0.25 ml/min.

5.3 Results

5.3.1 Hinge 1 is necessary for microtubule speed at high motor densities

Motility assays carried out at different densities of the motors were performed at saturating ATP concentrations (1mM). At high as well as reduced motor densities the microtubule gliding speed were found to be similar for both full length and truncated kinesin-1 (the truncated construct lack Hinge 2 and the C-terminal domains). Evidently, as in the full-length construct, these truncated motors, which lack Hinge 2 and the C-terminal domains, can cooperate at high densities to move microtubules at high speeds. In contrast, the deletion construct that lacked Hinge 1 moved significantly more slowly at high densities than at low densities. These results imply that Hinge 1 domain is important for preventing motors from interfering with each other at high densities.

5.3.2 Torsional flexibility of Hinge 1

Since Hinge 1 corresponds to a clear break between two coiled coil domains, the working hypothesis was that it might be an unstructured domain, and therefore have high flexibility. Such flexibility could allow many kinesins to operate simultaneously without interfering with each other. Loss of this domain could therefore provide a structural basis for its low gliding speed at high density. This hypothesis was tested by measuring the torsional flexibility of individual kinesin molecules by analyzing the pivoting motions of microtubules gliding over surfaces coated with kinesin at low densities. In these assays ATP was present at 5µM which is a concentration that is high enough to observe motor operation, but low enough to allow a sufficiently long observation time so that the rotational fluctuations of moving microtubules could be measured. The results were in general agreement with the expectation that the construct (ΔΤΔΗ) lacking the Hinge 1 domain would be less flexible than full-length and other truncated constructs (ΔΤ) used in this study.
Furthermore, the data from the torsional flexibility assays on $\Delta T\Delta H$ construct differ from those of full-length and $\Delta T$ construct suggesting that it corresponds to a long-lived stiff state. Thus, deletion of Hinge 1 specifically abolishes slow and infrequent large-angle angular transitions. The absence of slow transitions in the $\Delta T\Delta H$ demonstrates that these transitions are in general not attributable to the unbinding and rebinding of kinesin to the anti-His antibody used for immobilization, or to the unbinding of one head from the microtubule and rebinding with a twist. If either of these phenomena had occurred, large fluctuations would have also been observed in $\Delta T\Delta H$, which binds to antibody and microtubule in the same ways as $\Delta T$.

### 5.3.3 Torsional elasticity of kinesin constructs

To determine whether the small-amplitude angular fluctuations of microtubules could be accounted for by thermal fluctuations of a linear torsionally elastic element within the kinesin molecule, we performed Fourier analysis on recordings made of the long-lived stiff state. The results indicate that the rotational fluctuations of the long-lived ‘small amplitude states’ were similar in $\Delta T$ and $\Delta T\Delta H$.

For a detailed description of the methodology and results of the sections 5.3.1, 5.3.2, 5.3.3 see our published work (19).

### 5.3.4 Secondary structural features of the Hinge 1

How is the sporadic large amplitude fluctuations related to the secondary structure of Hinge 1? In order to assess the secondary structure-forming propensity of the Hinge 1 sequence we designed five overlapping 30mer peptides that spanned the hinge region (60aa). The first peptide started at the penultimate heptad repeat of the neck coiled coil and the fifth peptide ended after the second heptad repeat of Coil-1. The peptides overlapped by at least 10aa and contained an introduced tryptophan in position 11, as shown in Fig. 5.2A. These peptides were analyzed by CD, fluorescence, and FTIR spectroscopy. Qualitatively, only peptides 3 and 4 exhibited CD spectra that are indicative of partial $\alpha$-helix formation at room temperature resulting in the characteristic $\alpha$-helical signature at 222 nm (20), in addition to the contribution from unordered conformations showing negative CD below 205 nm (Figure 5.2B). Helicity of peptides 3 becomes further enhanced by cooling to 2 °C and is lost upon heating to 60°C (Figure 5.2B, Inset). Peptide 4
behaved almost identically (not shown). This is clearly contrasted by peptides 1, 5, and 2 which exhibit only marginal ellipticity at 222 nm already at room temperature. Helix-forming peptides 3 and 4 were additionally analyzed by FTIR spectroscopy (Figure 5.2C). The structure-sensitive amide I absorption band (i.e. the peptide carbonyl stretching vibration) was decomposed into five different spectral features at 1622 cm$^{-1}$, 1643 cm$^{-1}$, 1655 cm$^{-1}$, 1670 cm$^{-1}$, 1684 cm$^{-1}$ for both peptides. The peaks around 1655 cm$^{-1}$ are indicative of the peptide carbonyl absorption of $\alpha$-helices. Absorptions at 1684 and 1622 cm$^{-1}$ are typically associated with $\beta$-sheet structure (reviewed in (21,22), whereas the 1670 cm$^{-1}$ absorption can be associated with turns. For comparison, and in agreement with CD results, the broadening of the amide I and II absorptions of peptide 2 (Figure 5.2C, bottom) and its lower amide I frequency confirm the virtual absence of secondary structure as opposed to peptides 3 and 4. However, the corrected (23) relative integral intensity in the 1652-1656 cm$^{-1}$ range (15-20 % at 23 °C) for peptides 3 and 4 is only ~60 % of that predicted from CD (30-35 %), and the main IR absorption lies in the 1640-1645 cm$^{-1}$ range usually assigned to random structure. Similar relations between CD and FTIR spectra have been observed with other peptides and proteins and have been considered indicative of coiled coil formation (24). Almost identical results were obtained for peptide 4 (Figure 5.2C, bottom). Although the distinct helix-forming potential of peptide 3 and 4 is obvious from CD, both peptides are only partially folded at any of the conditions tested. Therefore, the FTIR data may indicate helix-helix interactions in peptides 3 and 4 under the high-concentration conditions of the FTIR experiment (6-8 mg/ml) but should not be regarded as proof of tertiary structure, particularly coiled coil formation. In the lower concentration regime used in CD and fluorescence (below) helix interactions are also not suggested by the data. The more general distinction between secondary structure-forming potential in peptides 3 and 4 versus the largely unstructured peptide 2 is consistent with the FTIR results.
Figure 5.2 UV circular dichroism and infrared spectra of model peptides. (A) Amino acid sequences of the synthesized 30-aa-peptides. Each peptide (1-5, left to right, with molecular weights of 3602, 3375, 3071, 3070, and 3526, respectively) contains a tryptophan residue (W) in position 11. (B) Far UV circular dichroism spectra of peptides 1-5 at 20°C in mean
residue molar ellipticity. Circles correspond to fits from CDSSTR used for ranking helical propensity shown in Table 5.1. Inset Change of CD spectral signature during denaturation of peptide 3 with temperature (bottom to top: 2, 10, 20, 30, 40, 50, 60 °C). (C) Infrared absorption spectra of peptide 3 (upper panel), and peptides 2 and 4 (lower panel). Major peaks in the spectra are indicated showing that peptides 3 and 4 can be fitted with identical spectral components. The higher absorption at 1643 cm\(^{-1}\) relative to 1656 cm\(^{-1}\) may indicate helix-helix interactions, see text for details in peptides 3 and 4. For comparison, the broad amide absorptions of the unstructured peptide 2 are superimposed in the lower panel. (D) Overlaid CD spectra of peptide 2 (lower traces) and peptide 3 (upper traces) with either the native sequences (black) or with a tryptophan substitution at position 11 (gray, see also Figure 5.1A). (Inset) Thermal unfolding and refolding of peptide 3 carrying the native kinesin-1 sequence. After having been heated to 90 °C the CD spectrum at 20 °C (gray) reproduces the initial trace measured at 20 °C (black). (E) Comparison of the induction of secondary structure by TFE at 2 °C for peptides 2 and 3 derived from Hinge 1 of \(Dm\) (upper traces) and \(Hs\) (lower traces). Thin lines without TFE, thick lines with 32 % TFE. Circles show the fit by CDSSTR resulting in 70 % helicity for the \(Dm\) peptide 2.

To assess possible structural consequences of the Trp-substitution (used for fluorescence monitoring of unfolding; see below), CD spectra of the structurally most different peptides 2 and 3 were compared with spectra of the native \(Dm\) sequences. The pair-wise superposition of the CD spectra of peptides (Figure 5.2D) demonstrates that the Gln to Trp replacement causes a slight reduction in the helical content of peptide 3 (reduction of the 222 nm band), rather than acting as a helix-inducing residue. The preceding unsubstituted peptide 2 is again disordered (virtual absence of ellipticity at 222 nm). Thus, the distinct gradient of structure-forming potential along the region sampled by peptides 2 and 3 is a robust intrinsic property of Hinge 1. The helix-forming propensity of peptide 3 is further underscored by its high refolding potential: the Inset in Fig. 5D shows the full recovery of the CD-signature at 20 °C of the unsubstituted sequence after heating to 90 °C. Furthermore, the native sequence can adopt up to 70 % helicity, whereas even in the presence of 32 % TFE and at 2 °C the helicity of peptide 2 does not exceed 30 % (Figure 5E upper traces). Given the overlap between the peptides, their distinct differences in helical propensity reveal a abrupt transition in the structure-forming potential between the central and the flanking region of Hinge 1. This suggests that the functionally required flexibility of the Hinge 1 does not originate in an intrinsically disordered domain.

We have asked whether a sharp transition in the structure-forming potential exists in the Hinge 1 domain of kinesin from other species. Figure 5E (lower traces) show CD spectra of the homologous peptides 2 and 3 derived from the Hinge 1 of
kinesin-1 from *Homo sapiens* (*Hs*, without Trp substitution). Whereas the human peptide 3 does not exceed 8 % helicity even in the presence of 32 % TFE at 2 °C, the preceding human peptide 2 sequence adopts 33 % helicity under these conditions. Although less pronounced than in *Dm*, a distinct difference of helical propensity within a 15 amino acid window also resides in the human Hinge 1. The presence of a proline in the center of the human peptide 3 is probably the reason why its helicity is low, whereas a proline is situated in the more marginal position 7 in the more helical human peptide 2.

**5.3.5 Thermal stability of Hinge 1 model peptides**

In the context of the proposed function of the Hinge 1 as a flexibility-providing segment, we addressed the energetics of structural transitions in the model peptides using thermal unfolding experiments in which the fluorescence of the introduced Trp-residues at position 11 was monitored (see Experimental Procedures). As shown above, the structure of both the most helical and the least helical peptide is affected by the introduced Trp. This allows the description of unfolding by a two-state transition where the fluorescence-based assessment of thermal stability is more appropriate than in CD because unfolding has to proceed at least up to position 11 to become observable. A more distinct temperature dependence and a more stringent definition of the unfolded state is thus achieved as partially (C- and N-terminally) unfolded states are not monitored which otherwise broaden the temperature profiles in CD leading to ambiguous results for peptides that never adopt a 100 % helical structure.

All Trp-containing peptides exhibited a temperature-induced decrease of Trp emission measured at 340 nm. However, the normalized emission $\Phi(T)$ relative to that of free Trp in aqueous solution increased strongly with temperature ($T$) for all peptides (Figure 5.3A) such that a plot of $\log\Phi_p(T)$ versus $T$ (see Experimental procedure) deviated from linearity by a distinct upward curvature. In contrast, at a given degree of hydrophobicity of the Trp environment adjusted by water: ethanol mixtures, $\log\Phi_{\text{EtOH}}(T)$ increased linearly with $T$ (Figure 5.3A, *Inset*). The steepness of the linear relation depended on solvent polarity and becomes larger at higher hydrophobicity.
Thermal denaturation and oligomerization of peptides. (A) Tryptophan fluorescence intensity plotted against temperature for five Dm peptides. Solid lines are fits to a model in which equilibration occurs between a folded and unfolded state (see Experimental Procedures). Plots obtained with the most stable peptides 3 and 4 (black lines) exhibit strongest curvature (i.e. largest change in $U_r = U/(N+U)$, according to Eq.6) at higher temperatures than the curves of less stable peptides (gray lines). Corresponding thermodynamic parameters used for the fits are shown in Table 5.1 (Inset) Normalized fluorescence intensity of free Trp in 0%, 10%, 20% and 50% ethanol in water mixtures shows an increase of temperature sensitivity with increasing solvent hydrophobicity. Temperature-dependence of Trp emission from the peptides corresponds to an increasingly hydrophobic environment, as mimicked by high ethanol content. (B) Elution profiles of five peptides (1mg/mL) from a gel-filtration column. Peptides 3 and 4 elute in monomeric and dimeric fractions. We interpret the elution profiles of peptides 1, 2, and 5 as unfolded proteins (see text). Upper arrows show locations of standards ribonuclease A (A), dimeric insulin A chain (B), aprotinin (C), and dimeric insulin B chain (D).

Thus, we assigned the upward curvature in the $\log \Phi_p(T)$ plot of the peptides to the transition of Trp from a more aqueous to a more hydrophobic environment formed upon denaturation / aggregation of the peptides. We modelled the $T$-dependence of this fluorescence change by a corresponding free energy of unfolding which causes the transition between the two Trp environments. The $\Delta H$ and $\Delta S$ values were determined such that the observed curvature of the $\log \Phi (T)$ plot was reproduced (Experimental procedures). Peptide 3 showed the highest enthalpy for unfolding and the largest gain in entropy (Figure 5.3A and Table 5.1). Peptide 1 showed the smallest values for both $\Delta H$ and $\Delta S$, whereas peptides 2, 4, and 5 fall in an intermediate range. Thus, the ranking of the peptides by thermal stability parallels their degree of helix-forming potential as summarized in Table I. Based on these
data, we propose that Hinge 1 exhibits a central region with a large helical propensity (mainly sampled by peptide 3) which is flanked by less structured and less stable regions (mainly sampled by peptides 1, 2, and 5). The $\Delta H$ and $\Delta S$ values for unfolding of the most stable and most helical stretch in the Hinge 1 correspond to an equilibrium concentration of the unfolded state of $\sim 5\%$ at room temperature. Interestingly, a probability in the low percentage range also describes the occurrence of the large amplitude fluctuations in $\Delta T$ and full-length kinesin-1, suggesting that there may be a correlation between thermal unfolding of the Hinge 1 and the appearance of high compliance non-Brownian fluctuations.

5.3.6 Homo-oligomerization of the model peptides

The structure and stability profile in Hinge 1 may support specific tertiary contacts in native kinesin where the corresponding sequences are aligned next to each other. We have addressed this question by gel filtration assays to determine the oligomerization state of the model peptides. Peptide 3 and 4 exhibit similar elution peaks (Figure 5.3B). The right-hand peak ($\sim 12 \text{ ml}$) is the first elution, which corresponds to the monomeric state. The next peak ($\sim 11 \text{ ml}$) elutes close to aprotinin and the dimeric insulin chains, suggesting the dimeric state of the peptide. Dimerization for peptides 3 and 4 corresponds roughly to 47% and 57%, respectively. Peptides 1, 2, and 5 elute earlier, as expected for unfolded peptides, which typically elute with a volume larger than that expected from the nominal peptide mass. The results from gel filtration are consistent with the CD spectroscopic evidence that peptides 3 and 4 are $\alpha$-helices, whereas peptides 1, 2, and 5 lack a defined structure.

5.4 Discussion

This work has studied the functional role and structural properties of the Hinge 1 region adjacent to the kinesin neck. The data presented here support the function of the Hinge 1 as a modular domain that provides flexibility/extendibility under the influence of external force and in away that is different from the behaviour of disordered flexible protein domains. The deletion of Hinge 1 has no effect on the speed of microtubules driven by single kinesin molecules, indicating that it is not essential for the hand-over-hand motility, at least under the low force conditions of
our gliding assays. However, deletion of Hinge 1 impairs microtubule motility at high kinesin densities. This suggests that Hinge 1 is required to provide sufficient flexibility to compensate for strain generated when many motors are attached to and moving the same microtubule. Surprisingly, however, the compliance measurements on single kinesin molecules do not fit simply with this picture. Instead, all the motor constructs spent most of their time in a low-flexibility state whose torsional rigidity was almost unaffected by the deletion of Hinge 1. The torsional stiffness of this state, $8 k_B T$, based on the angular variance of 7 degrees, is similar to that expected of a coiled coil (assuming a persistence length of 100 nm and a coiled coil length of 10 nm ((25)). Rather than exhibiting a continuous high compliance, the presence of Hinge 1 is associated with rare non-Brownian angular fluctuations of large amplitude occurring on a time scale of seconds. CD spectra show that the central part of the Hinge 1 sequence exhibits a high $\alpha$-helix-forming potential flanked by less ordered segments. The ensemble of these data demonstrate that Hinge 1 is required for motor cooperativity but neither its structural, nor its mechanical properties are consistent with a disordered domain.

How can this be related to a structure-based understanding of the measured torsion elasticity? The presence of Hinge 1 allows transitions between two different torsional states. The predominant long-lived low-amplitude Brownian fluctuations in the Hinge 1-containing proteins indicate that the hinge domains have low compliance. Such low compliance is consistent with the formation of secondary structure, which reduces flexibility within Hinge 1. The spectroscopic data show that a strong helical propensity resides in the central region of Hinge 1 giving rise to low compliance due to stabilizing intrastrand interactions. We interpret the occurrence of an additional state with high compliance as evidence for rare unfolding events, leading to the formation of a more flexible disordered domain that mechanically separates the coiled coil domain of the neck from that of the stalk. This non-Brownian response is specific to Hinge 1 because it is abolished in the Hinge 1 deletion construct $\Delta T \Delta H$. Hence, the flexibility of Hinge 1 required for maximal speed at high motor density is not directly reflected in its torsion elasticity. Instead, "rare" unfolding events appear to generate a state of high compliance that is observed in single-molecule experiments as an infrequent deviation from the overall coiled coil-like behaviour. Our spectroscopic results provide a reasonable structural basis to
reconcile the unexpected existence of secondary structure in the Hinge 1 with the requirement of flexibility for which a disordered domain intuitively appears to be the better candidate. The data provide evidence for a distinct profile of secondary structure-forming propensity in the Hinge 1 sequence, exhibiting a central part with a high tendency for helix formation and a large enthalpy of unfolding (peptides 3 and 4) approaching reported mean H-bond enthalpies of ~4 kJ per residue. The N- and C-terminal extensions from the central region of Hinge 1 are essentially structure-less which is again paralleled by the reduced unfolding entropies. Thus, the mechanical, structural, and thermodynamic data are in agreement. CD and fluorescence data clearly show the formation of secondary structure and the presence of intrastrand stabilizing enthalpic interactions, respectively, but do not indicate coiled coil formation. In full-length kinesin and in ΔT, however, the two kinesin heavy chains are in close proximity and favourably preoriented by the dimeric heavy chains. This may promote secondary structure formation and dimerization of the central ~30 aa long α-helical stretch within Hinge 1. Although interstrand interactions in the central hinge region cannot be proven or disproven based on the present peptide studies alone, such interactions would agree with the observed high-rigidity state of full-length kinesin-1 and ΔT. This hypothetical coiled coil-like arrangement is sketched in Fig 5.1E. If such a dimeric segment exists within Hinge 1 it is probably less stable than "canonical" coiled coil regions and, importantly, it lacks additional stabilization from the unstructured flanking domains. Therefore, we ascribe the less frequent high compliance state of full-length kinesin and the ΔT construct to the unfolded state of Hinge 1 even in the case that the rather low dimerizing potential seen with the isolated peptides becomes enhanced upon dimerization of the full-length kinesin-1 heavy chains.

Previous work on full-length bovine-brain kinesin carried out in the absence of ATP measured a higher compliance than in the present work (6). A possible explanation for this discrepancy is that the earlier measurements were made over much longer times (tens of minutes rather than tens of seconds in the present work) such that the unfolded state(s) may have been sampled more frequently. On the other hand, Hua and Gelles have also used non-zero ATP (5 and 400 nM) concentrations and measured a rms angle of 20-30˚C (26), some three-fold larger than the rms we observed for the low-compliance state. The larger rms angle may also be due to
greater sampling of the high-compliance state in their measurements that were made at lower ATP concentrations over longer times.

Based on our functional and structural studies, we suggest that at high motor densities the strain exerted by the force-generating motor domain on Hinge 1 induces the transition of the structured regions from helical to disordered stretches. We propose that with increasing torque and strain exerted during multiple-motor motion, $\alpha$-helical segments adjacent to the most stable central sequence will increasingly unfold. Finally, the entire Hinge 1 may adopt an entropic spring-like behaviour, once the secondary structure has fully unfolded at larger torques as is the case at high motor density. In this model, the Hinge 1 region can be thought of as a "flexibility reservoir" that is accessed by the gradual growth of flanking disordered domains in a strain-dependent unfolding process. Thereby, reorientation of misaligned motor heads into a more favourable position for microtubule attachment can be accomplished during motor co-operativity in a strain-dependent manner as sketched in Figure 5.4. The function-dependent increase in flexibility causes an overall reduction of drag during motor co-operation as the infrequently occurring high compliance state seen in the single-molecule experiment becomes populated, thereby, preventing motor interference. In contrast, in the absence of strain exerted by other motors, there is no functional requirement of either flexibility or secondary structure in the hinge for single molecule microtubule attachment and processivity.

Due to the divergence of the Hinge 1 sequence it is difficult to predict whether (i) helical stretches are interspersed in the corresponding regions of other kinesins and (ii) whether similar flanking sequences exist that would support unfolding of the encompassed helical structures during function. Remarkably, the homologous sequence of Hinge 1 from human kinesin-1 sampled by peptides 2 and 3 also exhibits a clear secondary structure-forming potential adjacent to an essentially unordered region (Table 5.1). The presence of a proline in the center of the human peptide 3 is probably the reason why its helicity is low, whereas a proline is situated in the more marginal position 7 in the more helical human peptide 2. In both organisms, the functionally required flexibility of Hinge 1 appears to originate in local alterations of helical propensity in a 15 to 30 amino acid window rather than being the consequence of a contiguous intrinsically disordered domain.
Table 5.1 Thermodynamic and structural parameters of Hinge 1 model peptides

<table>
<thead>
<tr>
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<th>Peptide 1</th>
<th>Peptide 2</th>
<th>Peptide 3</th>
<th>Peptide 4</th>
<th>Peptide 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta H$ (kJ)</td>
<td>52.6</td>
<td>78.6</td>
<td>115.5</td>
<td>75.0</td>
<td>80.5</td>
</tr>
<tr>
<td>$\Delta S$ (JK$^{-1}$)</td>
<td>141</td>
<td>244</td>
<td>367</td>
<td>221</td>
<td>254</td>
</tr>
<tr>
<td>helical propensity</td>
<td>m</td>
<td>l (m)</td>
<td>h (l)</td>
<td>h</td>
<td>l</td>
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Unfolding enthalpies and entropies of D$_{m}$ peptides were derived from the temperature sensitivity of fluorescence emission of tryptophan (see Eq. 4 and Eq.6 in Experimental Procedures, and Figure. 5.3. Helicity was determined by evaluation of CD spectra (Figure. 5.2). The ranking of helical propensity is expressed as follows. High (h), helicity >35% without or >65% with TFE (32%) at 2°C; medium (m), helicity >15% at 20°C (no TFE) or >30% with TFE (32%) at 2°C; and low (l), helicity <15% under all conditions. Helical propensity of the HS peptides is given in brackets.

Our hypothesis that force generated by the motor domain could lead to partial unfolding of a protein sequence may apply in other situations. For example, studies on peptides from the dimerization domains of kinesin-1 provided evidence that the dimerization domain (also called the neck) might unfold during motility (17). Such unfolding would make it easier for the two heads in a kinesin dimer to span the 8 nm between adjacent tubulin dimers in the microtubule.

However, later work showed that single kinesin-1 molecules could still move processively when dimerization was strengthened by using more stable coiled coils (27) or by cysteine-mediated cross linking (28); This suggested that unfolding is not necessary for motility. However, if motor forces are capable of unwinding protein domains, then it is possible that partial unfolding of the neck sustains normal processivity under load (29). Similarly, the model proposed here explains why the lack of the Hinge 1 in $\Delta T\Delta H$ does not affect processivity of an individual motor but causes speed reduction when many motors attach to the same microtubule but can no longer generate compliance in response to an increasing torque.
Figure 5.4 Model of the functional role of strain-dependent unfolding of the Hinge 1 region during motor co-operation. (A) Under single-molecule conditions a kinesin can bind and perform its mechanochemical cycle without any mechanical hindrance. (B) In a multi-motor situation, a kinesin molecule bound to a surface or cargo has a given orientation which does not allow following the microtubule protofilament axis (α-tubulin in light grey and β-tubulin in dark grey). (C) If wild type or ΔT binds with one head to the microtubule, the force exerted by the other motors through the cargo unfolds the Hinge 1, thereby reducing drag. This will allow forward stepping due to the neck and both head domains working independently of any other motor bound to the same surface or cargo. In contrast, if ΔTΔH binds to the microtubule, an attached but misaligned motor cannot step forward and it will exert a force (F) against the motor ensemble mediated by the common cargo. Therefore, a reduction of speed (v), i.e. an increase of drag, will be observed. Symbols: φ, nucleotide-free; D, ADP; and DP, ADP-Pi).
5.5 Literature Cited

6. Conclusions

Protein function is a never-ending enigma and I have explored in this thesis the metastable nature of small regions in proteins whose structural transitions may constitute modular switches that are of direct importance in their function. To experimentally prove this notion, this work used a combination of peptide synthesis with spectroscopy that allowed following structural transitions relevant to molecular switching processes in vitro. The results from this thesis have proven that carefully tailored peptides could serve as realistic model systems which could be used to understand the micro-switching events that could be triggered even in the absence of full-length coordinates of the whole protein. Two conserved modular switches driven by chemical potential of protons have been investigated here a) the evolutionary conserved D(E)RY triad whose protonation state regulates the activation kinetics of rhodopsin, a key member of class-1 GPCRs, in the presence of a native-like lipidic environment b) an analogous site was studied in bacteriorhodopsin, the classical proton pump, where conformational changes at distant sites are coupled to internal proton transfer reaction events during the photocycle. Both systems belong to the class of light-transducing heptahelical membrane proteins where the chemical potential of protons links proton exchange reaction to transmembrane conformation.

The data derived from the biochemical analysis and secondary structure sensitive spectroscopy (Infrared, Circular dichroism, Fluorescence) of rhodopsin-derived peptides shows convincingly that the lipidic phase is a key player in coupling proton uptake at a highly conserved carboxylic acid of the D(E)RY motif to transmembrane conformation during the receptor activation independently from ligand protein interactions. Unlike the case of rhodopsin, where exact timing of modular switching events is of little importance, bacteriorhodopsin achieves vectorial proton transport by the precise relative timing in coupling protonation events to conformational switching at distant domains. The interdependence of microswitches in these events cannot be mimicked with peptides and in this work a specially designed transport deficient bR mutant (bRD96A/V101-C) carrying a fluorescence label at the cytoplasmic end was used. To achieve the goal of observing the subtle structural changes happening at distant domains of these interdependent microswitches a novel methodology of two dimensional spectroscopy involving the
powerful combination of global secondary structure information derived from IR spectroscopy with the specific local changes gathered from fluorescence monitoring was developed. The cross correlation of the data from these experiments proves the modular nature of domain couplings and shows that the energy barrier of the conformational transition in the cytosolic half but not its detailed structure is under the control of proton transfer reactions at the retinal Schiff base and its counter ion Asp85.

In the above discussed protonation related microswitches the switching behavior could be ascribed to the local side chain chemistry. In contrast to these short chemical switches, large modular switches composed of stretches of amino acids are involved in the case of molecular motors where local switching processes are directly related to force generation. This work has studied the mechanical properties of the Hinge 1 domain of kinesin-1 to understand the structural requirements behind the cooperativity exhibited by multimotors in cargo transport. Secondary structure and thermal stability data from the Hinge 1-derived peptides complement single molecule observations on torsional flexibility in multimotor cases and shows convincingly that the Hinge 1 domain exhibits an unexpected helix-forming propensity that resist thermal forces but unfolds under load.

This spectroscopic work has unraveled many of the important factors regulating protein function such as pKa, structural changes, lipidic environment, unfolding stability. These processes are normally ‘frozen’ in crystals, or a priorily not observable, such as H-bonds and protonation states. Thus, the data gained from the three distinct functional modules of two important classes of proteins provides crucial insights towards linking the functionality of proteins to their structure. Despite the fact that the number of protein crystals reported are on a phenomenal rise over the last years one still needs this kind of spectroscopic research as it is the only way of following vital structural transitions (resulting from altered pKa, unfolding conditions etc.) that in conjunction with the atomic resolution of crystals could finally break the riddle of mystery wrapping the protein function.
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Erklärung

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Die Dissertation wurde von PD Dr. Karim Fahmy, Division of Biophysics, Institute of Radiochemistry, Forschungszentrum Dresden-Rossendorf betreut.

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06.10.2009 Sineej Madathil