Summary

To understand in detail how proteins can generate interpretable signals about the physical conditions in our environment is a formidable challenge. In the natural sciences, photoactive proteins have generally been accepted as the most suitable model systems for this challenge. Photoactive proteins contain pigments (colour–absorbing molecules or chromophores) embedded in a protein matrix. The chromophores absorb light, and translate it by means of a certain mechanism into biological activity. The main experimental tool used to study this mechanism is picosecond visible pump–mid–infrared probe spectroscopy. This technique allows for the detection of structural changes in molecules, such as isomerization of a chromophore, or changes in hydrogen bond network interactions. For example, the change in the vibrational frequency of one single atom bond due to a changed interaction with its environment can be detected. It is the perfect tool to investigate biological systems such as proteins, and in this work it was used to study fundamental biological processes such as proton transfer, isomerization, hydrogen bond dynamics and ligand dissociation. In this work, three different proteins are studied on an ultrafast timescale (from picoseconds to nanoseconds): Green Fluorescent Protein (GFP), Photoactive Yellow Protein (PYP), and FixL. In addition, quantum chemical calculations on the chromophore of PYP are presented to obtain a better understanding of the occurring photo–induced processes.

In chapter 2 the conducted ultrafast time–resolved experiments on GFP from the jellyfish Aequorea vicotoria are described. Our results show unambiguously that the proton transfer process in GFP does not occur according to the generally accepted model. It is widely known that the colour of the fluorescent light emitted by GFP depends on a light–induced proton transfer reaction that occurs in a ‘proton–wire’, formed by the chromophore, a water molecule (W22), S205 and E222. Freshly synthesized GFP in the ground state contains a protonated chromophore (i.e. neutral), absorbs in the near UV and emits blue fluorescence, whereas upon excited state (ES) formation the emitted fluorescence shifts to the green part of the spectrum.

We have studied GFP’s ES dynamics using ultrafast visible/mid–infrared pump–probe spectroscopy to elucidate the dynamics of proton transfer in the proton–wire. Model calculations based on available crystal structures proposed pathways starting from the chromophore or starting from the end of the wire, i.e. from the deprotonated E222. Our data show that, following optical excitation, a rapid (3 ps) protonation of E222 occurs, prior to chromophore deprotonation. The chain of proton transfer reactions starts therefore at the acceptor end of the wire. The anionic chromophore appears bi–exponentially with time constants of 8 and 165 ps. Our
conclusions corroborate recent electronic structure calculations done by other groups, who found that ‘pulling’ a proton over the wire, initiated by proton transfer from the serine to the glutamate, is energetically most favourable. Structures similar to GFP’s proton–wire may have a wider role in Nature than only in (green) fluorescent proteins, as successful pathways are generally conserved.

The largest part of this thesis is focussed on PYP (chapters 3, 4, 6 and 7). Picosecond infrared spectroscopy is used to study the native wild type protein and the E46Q PYP mutant (chapters 3 and 4). PYP is a protein found in the swimming bacterium *Halorhodospira halophila*. The bacterium requires light to perform photosynthesis and to survive, but too much (blue) light is harmful. The bacterium is negatively phototactic, which means that it is able to sense the intensity of light and, according to its needs, swim towards or away from it. PYP is thought to be the mediator between the environment and an interpretable signal for the bacterium. It follows a photocycle with distinct intermediate steps, each with a different conformation and its own spectroscopic properties. The negatively charged chromophore is embedded in a hydrogen bonding network with Glu46, Tyr42 (both donating a H–bond to the phenol) and Cys69 (donating a H–bond to the chromophore’s C=O).

The work performed in this thesis has led to a nearly complete picture of the ultrafast start of the photocycle, from photoexcitation up to 3 ns. Upon (blue) light excitation, the negative charge on the phenolic ring of the chromophore moves towards the carbonyl. The charge translocation rearranges the π–conjugation, rendering the C=O of the chromophore more single bond (*i.e.* C–O; chapter 3, 4 and 6). The rearrangement may facilitate the picosecond trans to cis isomerization (characterized by the appearance and disappearance of distinct normal modes; chapter 7). At the same time, the rearrangement also increases the hydrogen bond strength between the chromophore’s C=O (now more C–O) and Cys69, possibly hampering the isomerization. The charge translocation and the isomerization process are also reflected upon changes in hydrogen bond interaction between the phenol and Glu46, but the hydrogen bond remains intact throughout the probed time scale in our experiments (3 ns; chapter 3 and 4). Once the *p*-coumaric acid chromophore is isomerized, and the hydrogen bond between the carbonyl of the chromophore and the protein backbone (*i.e.* Cys69) is lost, the biologically productive *I₀* state will be formed (chapter 4). The formation of *I₁* from *I₀* is characterized by a 90–100% yield and we therefore conclude that *I₀* is a stable long–living cis ground state configuration, which structurally relaxes on the nanosecond time scale to form *I₁* (chapter 3). However, if the hydrogen bond between Cys69 and the chromophore’s C=O
fails to break, a cis ground state intermediate is formed in a few picoseconds (chapter 4). The breakage of the hydrogen bond is therefore an independent process of the isomerization mechanism. A biologically productive state is only formed in about 30% of the photocycle attempts, while about 60% forms the ground state intermediate (the remaining 10% thermally decays into the ground state from ES). The failed photocycle attempts then reform the ground state, and are ready to give it another try.

The combination of charge translocation and isomerization upon light absorption seems to be a common theme in photosensors. The protein appears to play an active role in combining the two, directing the photocycle, since it not only stabilizes the negative charge on the phenolic oxygen in the ground state by an extensive hydrogen bond network, but also the chromophore’s conformation (by restraining the chromophore’s carbonyl). A detailed understanding of the chemical kinetic mechanism of a protein, along with a chemical basis for its efficiency, is important for the understanding of protein function, and leads to a better understanding of human metabolism.

In chapter 5, the heme–binding protein FixL is studied. We have focussed on BjFixL, the oxygen sensor from the bacterium Bradyrhizobium japonicum. The heme has a central iron atom, where a diatomic molecule (the ligand) can bind, and is therefore similar to myoglobin and hemoglobin, the oxygen carriers for the cells in the human body. BjFixL consists of two separate domains, a heme–PAS domain and a signalling kinase domain. Upon binding of oxygen, conformational changes occur in the heme–binding domain, which in turn forms a biological signal for the kinase domain. The ligand photodissociation properties of the heme binding protein bjFixLH are studied with picosecond visible pump–mid–infrared probe spectroscopy. The diatom CO is used as a ligand, which upon photoexcitation with a visible laser pulse is released from the heme. The infrared frequency of the CO molecule is very sensitive to interactions with the surrounding protein, and acts therefore as a probe for the chromophore–binding pocket dynamics. The infrared difference spectra indicate that the escape of photolyzed CO to solvent is preceded by transient docking within the protein in a manner similar to globins. Two orientations of the CO appear to be involved in this docking, influenced by the energy of the pump laser pulse. On a picosecond timescale, protein rearrangements may have decreased the rotational constrainedness of the CO molecule. On a nanosecond timescale, further protein relaxation may have caused the CO molecule to migrate to a new site, changed its rotational orientation, or changed its pocket conformation. Due to its similarity with the globins, but having a completely different protein (PAS–)fold, this mechanism might therefore comprise of a general motif in heme–binding
The results discussed in this thesis have increased our understanding of GFP, PYP and FixL. These proteins are used by Nature to perceive a signal from a changing environment, and ultimately generate an interpretable signal to the cell or bacterium. Signal transduction in the studied proteins is initiated by ultrafast mechanisms common in Nature, such as proton transfer, isomerization, changes in hydrogen bonding, and ligand dissociation. The understanding of protein function is important as it is related to health and disease, and could potentially lead to improved medicine.