Structural dynamics of isolated biological and synthetic photoswitches
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Introduction

1.1 Photoactive bio- and synthetic molecules

Light is an ideal in situ chemical and biological manipulation tool that allows for remote and non-invasive control of cell signalling processes. At the same time, part of the electromagnetic radiation that reaches the surface of the earth poses risk to life.\textsuperscript{1-4} Nature thus uses light-sensitive photoreceptor proteins to sense and respond to light. Photoreceptor proteins usually consist of a non-protein chromophore that typically reacts to light via photoisomerization\textsuperscript{5,6} or photoreduction\textsuperscript{7} and it is embedded in the binding pocket of the protein moiety.\textsuperscript{8} These photoreceptors have a key role in the coordination of many growth, regeneration, and protection processes in the life cycle of animals, bacteria, and plants. Essentially, these photoreceptors are crucial for their development.

Well-known examples of photoreceptors in the animal kingdom are opsin proteins (e.g. rhodopsin, melanopsin, and photopsin). It is a light-sensitive photopigment found in the retina of humans and other vertebrates in which the chromophore retinal is bound to opsin. Vision starts with the photoisomerization of retinal upon absorption of visible light.\textsuperscript{5} The light absorbed triggers the $11$-cis-retinal to isomerize within a few picoseconds or less into the all-trans-retinal isomer.\textsuperscript{9,10} The different colours that we perceive depend on the different opsins that interact with the chromophore leading to absorption of light at different wavelengths.\textsuperscript{11,12}

An example of photoreceptor proteins that can be found in a halophilic purple bacterium, \textit{Ectothiorhodospira halophile}, is the Photoactive Yellow Protein.\textsuperscript{13} This protein serves the purpose to trigger blue-light avoiding behaviour of these bacteria\textsuperscript{1} and has become a model system for studying how proteins interact with light and respond to it in the form of a biological signal. The formation of the primary signalling state is characterized by a fast trans-cis photoisomerization of the PYP chromophore, which consists of the \textit{para}-coumaric acid anion covalently linked to the protein backbone via the Cys69 residue and a distal hydrogen-bonding network.\textsuperscript{14} Many studies have focused on the elucidation of the structure and various intermediate states within the photocycle via experimental or computational methods.\textsuperscript{15-20}

In plant systems the conversion of sunlight into useful energy is crucial for their growth and development. This biological conversion process is called photosynthesis. Unlike animals, plants don’t have eyes to sense light instead plants use sophisticated
sensory systems to perceive broad range of light and to regulate optimal exposure to the sunlight. Presently, three different photoreceptor proteins have been identified to be part of these sensory systems, which include red-light sensitive phytochromes\(^{21}\) and, blue-light sensitive cryptochromes and phototropins.\(^{7,22}\) The former proteins play an important role in regulating various events in the life cycle of the plant. They can be in two isomeric forms governed by the conformations of bilin chromophore, in response to red light. The process is reversible by absorption of red light in one direction and far-red light in the other direction.\(^{21}\) Another example are phototropins and in particular flavoproteins that mediate phototropic responses in plants. The structure of phototropins consists of the photosensory part which has the capability to sense Light, Oxygen, or Voltage and is therefore also known as the LOV domain. Like the proteins that have been described earlier which have a chromophore embedded into the protein moiety, protein crystallography has revealed that the LOV domain consists of a flavin mononucleotide blue-light absorbing chromophore, which is bound noncovalently in the interior of the protein via a network of hydrogen bonds and by van der Waals and electrostatic interactions.\(^{23}\) Upon photoexcitation the photoreceptor undergoes autophosphorylation which leads to unfolding of the protein and phototropic signalling. Ultimately, the photoactivation of these proteins triggers a number of events that optimize the photosynthetic machinery of plants and that includes leaf positioning and expansion in the direction of sunlight, as well as regulating the opening of the stomata (pores in the leaf epidermis) for gas exchange.\(^{24}\)

Apart from maintaining the circadian rhythm and regulating growth via large molecular systems such as the photoreceptor proteins, other protection systems in the form of relatively simple biomolecules are found in plants and humans. In fact, some of these simple molecules form natural protection system against harmful radiation. In the area of horticultural research, these biomolecules are known as natural antioxidants that are produced endogenously and have proven to protect the photosynthetic apparatus when a plant is subjected to stress.\(^{25}\) The abundance of naturally occurring antioxidants found in plants can be overwhelming and is a subject in its own right. Within the context of the present thesis, however, two categories of antioxidants (or biomolecules) are of particular interest: those with the backbone of cinnamic acid or stilbene. Derivatives of cinnamic acid can be found in a wide variety of edible plants including cinnamon, tomatoes, grapes, apples, and coffee beans that exude a variety of phenolic compounds like coumaric acid, chlorogenic acid, caffeic acid, and ferulic acid.\(^{25}\) Various studies have found that phenolic compounds that are produced in plants limit the growth of particular kinds of microbes and insects. In fact, some fruits like apples have peels that contain higher levels of antioxidants and can therefore have a longer storage life.\(^{26}\) It is also known that antioxidants are crucial for our health as they deactivate free radicals that are harmful and damaging to our cells. Hence, the necessity to add fruits and vegetables that are rich in antioxidants to our daily diet, which also validate the expression “an apple a day keeps the doctor away”.

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Antioxidants come in many forms and varieties. The other form that we are interested in has a stilbene backbone. An example like resveratrol (3,5,4’-trihydroxystilbene) is commonly found in the skin of red grapes (Vitis vinifera) leading to a noticeable presence of resveratrol in many wines, and particularly in red wines. This may very well explain the “French paradox” which suggests that French people have less coronary heart diseases in spite of their diet which usually contains high levels of fat and cholesterol. We notice, however, that it has also been suggested that this should be attributed to other polyphenols in red wine that would offer a larger degree of protection to human blood vessel cells.

Like the E. Halorhodospira bacterium that exhibits a blue-light avoiding behaviour, humans also have natural defence mechanism to protect themselves from harmful UV radiation emitted from the sun, for example, DNA bases that are already optimized to protect themselves from UV radiation by harmlessly dissipate the damaging UV energy. In order to survive in the sun-drenched planet earth, humans have photoprotective system in a form of biological compounds. Some of these compounds that are found in human skin are urocanic acid, melanin and eumelanin. These natural substances, which are embedded in the skin, are able to dissipate absorbed harmful energy into harmless heat. In fact, these natural photoprotectants are capable of dissipating more than 99.9% of the absorbed harmful UV radiation in a non-harmful way.

All the biomolecules and chromophores described earlier exhibit properties that have also attracted considerable interest from a medical point of view. Some have proven to have positive effects -attributed to their radical scavenging activities and antioxidant properties- in the treatment of cancer, diabetes, and cardiovascular diseases. In addition, some of them are efficient UV-absorbers and have found their way as ingredients of sunscreens. The things we have learned from nature on the machinery of chromophores in proteins, and especially on the benefits of phytochemicals in plants, are not by all means coincidently. In fact the discovery dates back to ancient times as illustrated for example in the ancient text of Hippocrates.

“In like manner, with respect to all the others, such as barley-water, the drinks made from green shoots, those from raisins, and the skins of grapes and wheat, and bastard saffron, and myrtles, pomegranates, and the others, when the proper time for using them is come, they will be treated of along with the disease in question, in like manner as the other compound medicines” (Hippocrates 400 B.C.E)

Nowadays, these biomolecules are the basis for novel synthetic molecules that aim to mimic and improve on their benefits as they have come forward from nature. At the same, the studies of these molecules extend our knowledge on how nature works. Within the context of this thesis, some of the synthetic molecules being studied include (i) cinnamate derivatives of the PYP chromophore, (ii) cinnamate-based UV-
Chapter 1

filters that have been chemically adapted with an octyl side-chain, and (iii) azobenzene. The latter system is similar to stilbene, but has a N=N double bond instead of a C=C double bond. The common property of these synthetic molecules is their ability to respond to electromagnetic radiation, converting photon energy either into mechanical action or dissipating the energy in a harmless manner. The functionalities that these molecules possess have huge potential to be used as basic building blocks for other extended functionalities in the areas of molecular motors\textsuperscript{36}, molecular switches\textsuperscript{37}, photolithography\textsuperscript{38}, pharmaceuticals\textsuperscript{39}, and medical and neurological applications.\textsuperscript{40,41}

1.2 High-resolution laser spectroscopy

The advent of the laser has revolutionized spectroscopy. It has become a powerful tool which initially covered the deep UV to the IR range of the electromagnetic spectrum, but with the introduction of the Free Electron Laser is now rapidly expanding to shorter as well as longer wavelengths.\textsuperscript{42,43} Spectroscopic techniques employ light to interact with matter that provide us in this way with valuable information on the molecular structure, intrinsic and intermolecular properties, and on the functionality of matter, regardless whether the matter is gas, liquid or solid, and from small molecules to large complex molecules. The information is presented to us in various types of spectra using techniques that are constantly improved and extended as to serve specific research targets in biology, physics and chemistry. By detecting a measurable variable as a function of wavelength, a set of spectral lines is determined that can be considered as a highly unique fingerprint of structure and dynamics. These spectra can thus be used to detect, identify and quantify physical and chemical properties of atoms and molecules. Laser systems with small spectral linewidths enable us in principle to study in great detail the plethora of transitions associated with the electronic, vibrational and rotational energy levels of a molecule. However, in order to make optimum use of this linewidth a number of considerations need to be taken into account. This concerns first of all the phase (gas, solution, or solid) in which we study the molecule of interest. Under solution and solid conditions, molecules interact with each other and/or with molecules of the solvent. As a result, each molecule experiences a slightly different environment, resulting in slightly different transitions that ultimately blend and erase the potential resolution offered by the laser bandwidth. Obviously, the gas phase offers in this respect a much better environment.

Nevertheless, as the complexity of the molecules increases other problems arise. For small molecules vibrational and rotational lines can usually be well resolved in the gas phase due to the limited internal degrees of freedom. However, as molecules become larger, the number of degrees of freedom increases and concurrently the density of quantum states. As a result of the high density of states, many more states
are initially occupied and thus many more transitions can occur. This leads to very complex spectra and a high spectral congestion that even under high-resolution conditions result in broad unresolved spectra.\(^{44}\) In order to retain as much as possible the advantages of the gas phase for high-resolution spectroscopy, the main task is to reduce the internal energy of the molecules as much as possible. Boltzmann predicts that the population of states in a system is reduced as the temperature of the system drops. The way to go forward is therefore to reduce the temperature, and bring all molecules in the same electronic, vibrational, and rotational quantum state. Another aspect to be considered is that during the time of the experiment the molecule should be isolated, that is, it should not have any interaction with other molecules that could affect its transitions. In a more pictorial way, one could say that a high-resolution spectroscopist aims to perform a single-molecule experiment on many molecules at the same time.

1.3 Molecular beam spectroscopy

Molecular beam sources are the most established way to obtain cold, isolated molecules. Dunnoyer (1911) first demonstrated such beams and showed that atoms move in a long straight line inside an evacuated chamber. Since its discovery, it has become an important research instrument for gas-phase spectroscopists. There are two types of molecular beam sources and both work on the same principle of allowing molecules to escape from a high pressure source through a small orifice into a vacuum chamber. The earliest source of a molecular beam was effusive and had a hole diameter that was smaller than the mean free path of the gas particles in the reservoir. As a result, molecules every now and then escape from the reservoir through the hole and are not subject to collisions during the expansion process. In such a configuration the velocity components of the molecules are conserved. The velocity distribution and the internal degrees of freedom of the molecules in the source are therefore more or less maintained in the molecular beam.

In supersonic sources the mean free path becomes smaller than the orifice. This can be achieved by increasing the pressure in the source or the diameter of the hole.\(^ {45}\) Molecules and atoms that escape through the hole collide frequently. Upon exiting the orifice, collisions have converted the total available energy of the molecules in the reservoir - consisting of random translational energy and internal rotational and vibrational energy - into directed kinetic energy along the beam axis. As the collisions take place in the expansion region, the translational cold bath acts as a refrigerant to cool the rotational and vibrational degrees of freedom, and the molecules thus become internally cold. The low temperatures in a supersonic molecular beam are ideal for spectroscopic studies, since the reduced number of occupied energy levels leads to a simplification of the recorded spectrum as compared to that of room temperature molecules. Moreover, an added advantage of the technique is that in experiments on
cold molecules produced from the supersonic expansion, much less fragmentation is observed upon photon absorption because of the reduced amount of internal energy.

The first proposals for supersonic jet expansions of gases date back to 1951. They produced a high beam density\textsuperscript{46,47} that offered tremendous advantages over conventional effusive sources. However, to avoid scattering the design had to take nozzle apertures and adequate pumping speed into consideration. The early years of supersonic molecular beam development therefore faced stringent requirements related to available pumping speeds. The development of pulsed nozzles relieved these restrictions considerably. Molecular beams that are operated in the pulsed mode require less severe pumping requirements. The orifice can be opened repeatedly for a short duration of time and excessive gas flow into the vacuum chamber is therefore limited while spectroscopic measurements can still be performed adequately with pulsed lasers. Molecular beam techniques have thus been revolutionized from 1970 onwards by the combination of the advancement of nozzle design, be it solenoid-driven\textsuperscript{48,49} or piezo-driven\textsuperscript{50,51}, the availability of efficient vacuum pumps, and the development of lasers which started in the 60s. The cold molecules prepared by a supersonic expansion also enabled the studies of weakly bound molecules such as van der Waals molecules, and weakly bonded species that are difficult, if not impossible, to study in any other way. These van der Waals molecules and molecular clusters are formed from clusters of the target molecule with other molecules like water,\textsuperscript{52,53} methanol,\textsuperscript{54,55} helium,\textsuperscript{56} argon,\textsuperscript{57} and many others that can be seeded easily into the molecular beam. One of the spin-offs of the possibility to generate such clusters is that it enables researchers to link isolated molecular properties to properties under solvated conditions using strategies like microsolvation.\textsuperscript{58–60}

1.4 Resonance Enhanced MultiPhoton Ionization

Cold molecules may have revolutionized high-resolution spectroscopy but they would not have had such an impact without the development of experimental techniques that provide new routes to probe them. One of these techniques, which is actually at the basis of the experimental studies in this thesis, is multiphoton ionization (MPI) which very well complements molecular beam spectroscopy.\textsuperscript{61,62} In order to ionize a molecule, photons are needed with an energy that is at least larger than the adiabatic ionization potential of the molecule, though Franck-Condon considerations indicate that for efficient ionization even larger energies, given by the vertical ionization potential, are needed. It is well known that molecules composed of carbon and hydrogen can be subcategorized into aromatic compounds and aliphatic compounds. The former molecules, which include benzene and aromatic rings, normally have ionization potentials on the order of 7-9 eV, while the ionization potentials of the latter occur at higher energies, typically in the range of 9-10eV.\textsuperscript{63,64} Such energies can be obtained by electron impact ionization, but for the present
purposes we are interested in optical means to ionize the molecule. The ionization potentials mentioned above imply that one-photon ionization requires light in the vacuum ultraviolet (VUV) region. Such light can, albeit not trivially, be produced by table-top lasers, for example by using rare gases to triple the frequency of 355 nm output of a Nd:YAG radiation to 118 nm (10.5eV). These methods are usually unselective and, most importantly, do not provide information on electronically excited states.

In contrast to these non-selective methods, Resonance Enhanced MultiPhoton ionization (REMPI) offers a powerful approach to map out the molecular vibrational and electronic manifold. Moreover, it provides the possibility to perform species-selective ionization and detection by combining REMPI with mass-selective detection methods such as time-of-flight mass spectroscopy, which enables one to filter out signals associated with other species than the one of interest. With this method molecules are ionized by simultaneous or sequential absorption of two or more photons of an intense pulsed laser beam. The condition for ionization is that the sum of the absorbed photon energies must exceed the ionization potential of the molecule. The simplest form of multiphoton ionization is the one-color version in which ionization is provided by two equal photons from a single laser pulse. Such a direct two-photon absorption via a virtual state is possible and can be observed, but is clearly much less efficient than a sequential (1+1) absorption in which the molecule has a real intermediate state that is in resonance with the photon energy. Subsequently, the absorption of the second photon leads to ionization if the intermediate level is located above half of the energy between the ground state and the ionization potential.

While many compounds can be ionized by two photons of the same energy, many larger molecules have excited states with an energy that is less than half of the ionization energy. For such compounds a one-color two-photon ionization approach clearly does not work. In these cases a one-color (2+1)-MPI scheme may be useful, as has been demonstrated for compounds like camphor and hydrogen sulfide, while in other compounds different schemes are required. Generally, however, one has to revert to two-color resonance enhanced two-photon ionization where the excitation step and ionization step are performed with two laser pulses of different laser wavelengths. Utilizing a two-color ionization scheme has the added advantage that the fluence of the laser pulses for excitation and ionization can be controlled independently, which is important when the transition probabilities of the two processes are significantly different. A scenario that is often encountered is one in which excited states are subject to fast nonradiative decay processes. In these systems the excited state is difficult to ionize by one-color REMPI without saturation of the excitation step. Two-color ionization can overcome this problem. Similarly, when excited states are studied that can decay to lower-lying excited states, ionization of the ‘hot’ intermediate state usually requires laser radiation with large photon energies for the ionization step (for example, a 193 nm ArF excimer laser). Finally, two-color
ionization scheme offers the possibility to perform studies on the photodynamics of excited states since the delay between excitation and ionization can be controlled externally.

Molecules can often adopt more than one stable conformation. This severely impedes the analysis of a REMPI excitation spectrum because in such cases this spectrum is composed of the overlapping excitation spectra of the various conformations. To disentangle such spectra and obtain conformation-selective excitation spectra double-resonance techniques like UV-UV depletion spectroscopy can be used. The basis of these techniques is a probe signal that is generated using a one- or two-color two-photon ionization scheme. A pump beam that precedes the probe is then introduced to the sample with a constant time delay. In the UV-UV hole burn configuration the pump wavelength is fixed to one particular transition in the excitation spectrum and the probe wavelength is scanned, while in the UV-UV depletion configuration the reverse occurs, that is, the probe wavelength is fixed to one particular transition and the wavelength of the pump laser is scanned. When the pump beam is on resonance, it efficiently removes population from the ground state of one particular conformer. If the probe beam is on resonance with a transition from the same conformer, this removal will show up as a dip in the ion signal since pump and probe have a common ground state. In contrast, if the pump beam is on a resonance of a different conformation, the probe channel is not affected since population has been removed from a different ground state, thus the ion yield remains unchanged. By changing the beam with the fixed frequency to a different band in the excitation spectrum and repeating the wavelength scan of the other beam one is able to assign bands to one particular conformation, and record conformation-specific excitation spectra.

The same concept can be employed to obtain IR absorption spectra of the ground state. Such information is important as it can provide an unambiguous assignment of the conformation when experimental spectra are coupled with ab initio predictions. In that case the probe beam is fixed to a particular electronic transition, but the pump beam is now replaced with an infrared (IR) laser that scans the infrared region. Some of the double-resonance experiments described in the present thesis require access to IR regions that cannot easily, or not at all, be produced by table-top lasers. These experiments have been performed at the Free Electron Laser for Infrared eXperiments (FELIX) facility where a wide spectral range between 100-4000 cm\(^{-1}\) can be accessed with a macro pulse duration of about 5\(\mu\)s and pulse energies around 100mJ.

1.5 Outline of the thesis

In the present thesis, we have employed one-, two-, and three-laser high-resolution molecular beam spectroscopy utilizing the previously described REMPI
techniques to study the spectroscopy and excited-state dynamics of molecular systems that form the chromophoric part of the Photoactive Yellow Protein and determine the initial phase of the PYP photocycle. We study these properties under isolated conditions, but also under conditions that mimic important aspects of the biological environment of the chromophore in PYP. To this purpose Chapters 2 to 5 report on studies of methyl-4-hydroxycinnamate (OMpCA), methyl-4-hydroxycinnamate thio ester (TMpCA), and 5-hydroxy indan-(1E)-ylidene)acetic acid thio-methyl para-coumaric acid, a derivative of TMpCA in which the single bond adjacent to the phenyl ring is locked (RL-TMpCA).

Key information on the intrinsic properties of the electronically excited states of the active form of the chromophore of PYP has been provided by studies on OMpCA. Chapter 2 addresses the question to what extent the excited-state dynamics of this compound are affected by hydrogen bonds as solution-phase studies and quantumchemical calculations indicate that they play an important role in tuning the isomerization dynamics. To simulate such interactions we employ clusters of OMpCA with water. Spectroscopic studies in the OH stretch region identify the binding site of the water molecule. A careful analysis of line widths and a detailed comparison with the excited-state dynamics observed in phenol enables us to assess the relative importance of possible decay channels.

UV-UV double resonance experiments demonstrate that under the employed molecular beam conditions on OMpCA four different species can be detected with distinct electronic excitation spectra. However, such experiments do not provide an unambiguous assignment in terms of which conformer should be assigned to which species. In order to come to such an assignment Chapter 3 reports IR absorption spectra in the fingerprint region and compares them with spectra predicted by high-level quantumchemical calculations. Further information on the role of the environment is obtained by comparison of the IR spectra of the isolated conformers with spectra obtained for OMpCA-H2O clusters and for OMpCA in solution.

Chapter 2 and 3 employ one-color ionization techniques. Chapters 4 and 5, on the other hand, employ techniques in which excitation and ionization can be performed independently, and demonstrate that such techniques can significantly augment our knowledge on the excited states of PYP chromophores. Comparison of multiphoton ionization and laser induced fluorescence excitation spectra in Chapter 4 leads to the assignment of the hitherto elusive excitation spectrum of the V(ππ*) state. Pump-probe experiments put the relaxation pathways of the lower electronically excited states of OMpCA in a completely different perspective. The studies show that excitation to the “bright” ππ* state is followed by internal conversion to a lower-lying “dark” nπ* state, which so far had been out of reach to spectroscopists due to its almost zero oscillator strength. Importantly, this Chapter shows that the ordering of these excited states is very susceptible to environmental conditions, an asset that later
in the thesis is employed in the application of similar cinnamate-based compound in sunscreens.

In the protein, the PYP chromophore is present in the form of a thioester. Although this may appear to be only a slight difference with the oxyester form -as also assumed by many others in the past- Chapter 5 shows that the opposite is actually true. Studies on TMpCA and RL-TMpCA show spectra that give evidence for much faster excited-state decay dynamics than observed for OMpCA. This can partly be attributed to the theoretically predicted reversed ordering of the $V'(\pi\pi^*)$ and $V(\pi\pi^*)$. At the same time, however, one has to conclude that interactions with the environment play an important role. Also, the internal conversion pathway to the nπ* state is completely suppressed, an observation that is rationalized in terms of the geometry changes that occur upon excitation to the various excited states. The same Chapter addresses the role of single-bond ($\alpha$-twist) and double bond rotation ($\beta$-twist) in photoisomerization by comparison of excited-state properties of TMpCA and RL-TMpCA.

Photoisomerization of the PYP chromophore is the basis for initiating the photocycle of PYP that ultimately leads to signalling. The dynamics beautifully illustrate how nature uses the chromophore to efficiently convert photon energy into harmless heat, but at the same time use this conversion pathway to induce a mechanical response. Due to the strong UV absorption property of the cinnamate-based molecule, it has also been formulated into sunscreens that are used by us to protect ourselves from the harmful effects of UV radiation. In Chapter 6 the excited state dynamics of one of the most commonly used UV-B filters, ethylhexyl methoxycinnamate (EHMC), and a simplified derivative, methyl-4-methoxycinnamate (MMC), are studied under molecular beam conditions. Delayed ionization experiments reveal that the efficiency of the EHMC and MMC to dissipate absorbed energy is compromised by the “dark” nπ* state. Our results suggest that embedding of the active ingredient into a polar environment could very well diminish undesired photoinduced side reactions.

The experiments on the sulphur-containing chromophores show unresolved spectra that appear to contradict the photodynamics observed for the same chromophores in solution. To study to what extent fast photodynamics can still be observed with nanosecond spectroscopy, we have chosen to study in Chapter 7 trans-azobenzene. Azobenzene is a molecule whose functionality derives from the same trans-cis photoisomerization process that is responsible for the photofunctionality of the chromophore of PYP, and that has become a very popular building block for molecular switches and molecular machinery applications. Chapter 7 presents the first high-resolution excitation spectra of the $S_1(n\pi^*)$ and $S_2(\pi\pi^*)$ states. These spectra enable us to determine the relevant features of the potential energy surfaces of these two states, the time scales on which dynamical processes occur, and make a convincing case for the structural pathway along which photoisomerization occurs.
1.6 References

Chapter 1