STUDY OF BACTERIORHODOPSIN AND COENZYMES USING FEMTOSECOND TIME-RESOLVED SPECTROSCOPY

Summary of Dissertation

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I. INTRODUCTION

A new dawn of optics has started in 1960, with the development of the first laser. In 1964, invention of mode locking allowed the generation of ultrashort pulses and 20 years later the pulse duration reached 27 fs with the use of prism compressors. Since, the continuous evolution of lasers has contributed to a lot of scientific discoveries in almost all research areas of natural sciences. However, in many applications one needs much more energy than that an oscillator can provide. From the 70's, this demand of high-energy ultrashort pulses led to the intensive research of short-pulse amplification. By using amplified pulses of 10^{10} W/cm^2 intensity we are able to investigate the theoretically foreseen nonlinear effects of light-matter interactions. In the last 15-20 years a lot of new results have emerged in the interdisciplinary fields of femtochemistry and femtobiology and the pioneering work of Ahmed H. Zewail was awarded with the Nobel-prize in chemistry. Nowadays, the pump-probe technique developed by him is a very effective tool in the hands of researchers investigating ultrafast biological and chemical processes.

This thesis deals with the spectroscopic applications of ultrashort laser pulses. In my experiments, either femtosecond pulses of nJ energy or amplified pulses of several mJ were used, depending on the requirements of the corresponding experiment.

The thesis is separated into two parts. First, a possible application of the membrane protein bacteriorhodopsin (bR) in optical communication is demonstrated. In the second part, two biologically important coenzymes, *flavin adenine dinucleotide* (FAD) and the reduced form of *nicotinamide adenine dinucleotide* (NADH) are investigated by means of femtosecond time-resolved spectroscopy. These coenzymes play a role in the energy producing processes of the cells by converting chemical energy to useful metabolic energy (in the electron transport chain), which provides information about the metabolic state of the cells.

II. SCIENTIFIC OBJECTS AND GOALS

One of the objects of my biophysical investigations is *bacteriorhodopsin*, a light energy converting protein found in the cell membrane of *Halobacterium salinarum*, which has been one of the most significant molecules of the biological and biophysical research of the past 30 years. Upon light absorption, the protein pumps a proton across the membrane from the cytoplasm to the extracellular moiety and the generated electrochemical gradient is further used for ATP synthesis. During the proton translocation process the protein undergoes a

cyclic change of conformational states with well distinguishable absorption spectra. The consequent light-induced refractive index change offers the possibility of a wide range of applications in photonics, nonlinear optics, and bioelectronics. In spite of bR being used in several optical devices and applications, experimental data on its concentration-dependent refractive index and optical dispersion are not available. Therefore, one of my aims was **(1) to measure the concentration-dependent refractive index of bacteriorhodopsin suspensions and to determine its Sellmeier-coefficients in the visible range of the spectrum.**

The speed and bandwidth of today's telecommunication components are continuously growing, the desired 1 Tbit/s trafficking rate is thought to be achieved by using all-optical data processing devices. One of the biggest challenges in integrated optics (IO) is to find new materials of proper nonlinear optical (NLO) properties which can play an active, controlling role in integrated optical circuits. Besides inorganic crystals, materials of biological origin are also considered for use as active elements in IO devices, among which the *bacteriorhodopsin* has generated the most interest. Due to its favorable NLO properties and mechanical stability, bR is a promising candidate as an active material in future photonic applications. **(2) In order to demonstrate the feasibility of a protein-based integrated optical device, I investigated the subpicosecond (BR→I) transition of the protein photocycle by means of ultrafast pump-probe techniques and a waveguide-based photonic structure.**

An important part of my research activities conducted at the Institute of Biophysics was the development of a fluorescence kinetic measuring system based on optical frequency up-conversion, by which the fluorescence of different natural and artificial chromophores can be monitored with a good time-resolution in a wide spectral range. Among the most important autofluorescent molecules are the coenzymes FAD and NADH, which play a key role in the redox processes of the molecular respiratory chain. The *in vivo* fluorescence decay rates of these molecules are often used to characterize healthy and diseased cells and tissues. However, we have very few experimental data about how the individual components (e.g. polarity of solvent, pH, etc.) of the cell's micro-environment affect these characteristic rate constants. Comparison of the experimental findings concerning the effects of these parameters with the *in vivo* results can be important in future medical applications. **(3) My aim was to measure the effect of different micro-environments on the fluorescence lifetime of coenzyme FAD on the 100 fs – 10 ns timescale. The molecule was investigated both in its free forms in different solvents and when bound to a protein.**

The coenzyme NADH has two chromophores (*nicotinamide* and *adenine*) between which an efficient FRET (fluorescence resonance energy transfer) process can occur. The efficiency of the energy transfer is strongly dependent on the conformational state of the molecule. **(4) In order to deeply understand the intramolecular processes following excitation, I used ultrafast transient absorption measurement techniques to investigate the open and closed conformations of the coenzyme.**

III. MATERIALS AND METHODS

The experiments were conducted using femtosecond light sources including Ti:sapphire oscillators and chirped pulse amplifiers (CPAs). In the following subsections, I describe the experimental methods and setups used in my work together with the preparation of biological samples.

1. The sample used in the measurement of the concentration dependent refractive index of bR was obtained in the form of suspension from the Institute of Biophysics, Biological Research Centre of HAS. The concentrations between 0 and 80 μM were set using 10 mM, pH 7.0 Tris(hydroxymethyl)-aminomethane buffer (Sigma-Aldrich Co. LLC).

To determine the refractive indices of the liquid samples, we have chosen a classical yet robust refractometry method based on the measurement of the angle of minimal deviation. The sample suspensions were filled into a hollow prism, which was then inserted into a goniometer. The eyepiece of the goniometer was replaced with a camera sensitive in the near IR. In order to cover the selected wavelength range, two types of light sources were used. In the wavelength range of 390–780 nm, the light of a Xe lamp with a power of 1 kW was collimated by achromatic lenses. The required wavelength was selected by an SPM-2 monochromator having a bandwidth of 0.4 nm, so that the peak spectral power at the sample never exceeded 60 μW within the 390–780 nm range. Hence, the effect of photobleaching could be neglected. For the wavelength range of 780–880 nm, femtosecond laser pulses from a 70 MHz Ti:Sa laser oscillator were used. A quasi-monochromatic component of the 100 nm bandwidth (FWHM) pulses was selected by a Jobin Yvon H20UV monochromator and then was imaged onto the surface of the hollow prism.

These measurements were carried out using the Ti:sapphire oscillator and a high pressure Xe lamp at the TeWaTi Laboratory, Department of Optics and Quantum Electronics, University of Szeged, under the supervision of Dr. Karoly Osvay.

2. The feasibility study of ultrafast optical switching was conducted in the pump-probe scheme utilizing the 10 Hz, 800 nm amplified pulses of a TW-class laser system. The beam was split into two parts. By injecting the more intense beam into an optical parametric amplifier followed by a sum frequency mixer and a compressor, 150 fs, 80 μJ green pump pulses were generated. These pulses excited the absorption band of bR at a center wavelength of 530 nm, starting the photocycle of the membrane protein. The probe pulses were obtained from the less intense beam by utilizing a 1800 1/mm line density gold grating, a slit, and a Fabry-Perot interferometer (finesse ≈ 50) to narrow the spectrum yielding 1 μJ pulse energy and a bandwidth of 0.2 nm near 790 nm. The transform limited pulse duration was 3 ps. After propagating through a delay line, the probe pulses were directed onto a plane, grating coupled waveguide coated with a bR film. The change in the light intensity coupled into the waveguide under an appropriate angle of incidence was measured with a fast photodiode (Thorlabs, DET10A) and a multichannel oscilloscope (LeCroy WaveRunner 6100A).

These measurements were carried out using the hybrid laser- and optical parametric chirped pulse amplifier system at the TeWaTi Laboratory, Department of Optics and Quantum Electronics, University of Szeged, under the supervision of Dr. Karoly Osvay and Dr. Andras Der.

3. The FAD sample was purchased from Sigma-Aldrich Co. LLC. For my measurements, I prepared a 3 mM stock solution in 50 mM, pH 7.0 phosphate buffer, which was diluted in 1:1 ratio with tri-distilled water, dioxane, ethanol, and DMSO solutions to keep the FAD concentration at a constant value of 1.5 mM. The flavocytochrome-bound FAD sample was obtained from Dr. Csaba Bagyinka and Dr. Gabor Rakhely.

For time-resolved fluorescence measurements, we have recently constructed a Ti:sapphire laser oscillator based apparatus capable of measuring time-resolved fluorescence both with the technique of frequency up-conversion and time correlated single photon counting (TCSPC). The output beam of the laser oscillator was split into two beams providing the optical pump and the gate pulses, respectively. The pulses traveling in the pump beamline were focused into a beta-barium borate (BBO) crystal in order to generate their second harmonic. The fluorescence emitted as a result of exciting the sample by the so obtained pump beam was collected and focused by a pair of large aperture off-axis parabolic mirrors onto a second BBO crystal constituting the frequency up-conversion unit, where it was sum frequency mixed with the gate pulses. The generated frequency up-converted beam was then focused onto the entrance slit of a monochromator. A liquid nitrogen cooled

1024x256 pixel CCD was mounted on one of the exit ports of the monochromator making it possible to detect all the spectral components simultaneously. A 300 line/mm optical diffraction grating was used to spectrally disperse the beam. In the case of TCSPC, the fluorescence was directly imaged onto the entrance slit of the monochromator instead of traveling through the up-conversion unit. The second output port of the monochromator had an avalanche photodiode (IDQuantique, ID-100) mounted onto it, which was connected to a single photon counting electronic unit (Becker & Hickl GmbH).

These measurements were carried out using a femtosecond Ti:sapphire oscillator at the Laboratory for Femtobiology of the Institute of Biophysics, Biological Research Centre of HAS (Szeged, Hungary) under the supervision of Dr. Geza Groma.

4. In the case of measurements of absorption kinetics, a 3.0×10^{-4} M NADH solution was prepared freshly in 0.1 M PIPES (1,4-Piperazine-diethanesulfonic acid) buffer in pH = 7.0 and was kept at 24 ˚C during the measurements. This buffer was proven optimal in avoiding thermal NADH degradation. The equilibrium ratio of the folded and unfolded conformational states was controlled by the methanol concentration of the sample. Time-dependent differential absorption spectra upon excitation of the adenine group were measured at 0, 50, 70 and 80 % methanol concentrations.

Femtosecond absorption kinetics measurements were carried out by the standard pump-probe method. The excitation pulses were obtained at 266 nm by third harmonic generation of an amplified Ti:sapphire laser system (5 kHz, 40 fs, 500 μJ). A white light continuum pulse train was generated by focusing a small portion of the fundamental beam into a piece of bulk material and the 330-630 nm range was selected by spectral filters. The pulses were then split into two beams before reaching the sample: one serving as the probe beam, while the other provided a reference for determining the accurate absorption changes. After the sample, the probe and reference pulses were directed into a monochromator and the spectra were detected by a cooled CCD camera. The time resolution of the kinetic measurements was ~100 fs. A 0.5 mm diameter square-aperture quartz cuvette was used as sample holder where the liquid samples were circulated using a peristaltic pump. Data analysis and evaluation was performed using Matlab codes including time- and spectral calibration results, background correction data and SVD filtering. The data were then corrected for the solvent response, pump-probe two-photon absorption and solvated electron signals.

These measurements were carried out using an amplified Ti:sapphire laser system at the laboratory of the CNRS-IPCMS DON (Department of Optics and Nanophotonics, Strasbourg, France) under the guidance of Prof. Stefan Haacke.

IV. NEW SCIENTIFIC RESULTS

My scientific results are summarized in the following thesis statements:

1. From the measurements of the angle of minimum deviation, I have calculated the refractive index values of bR taking into account the temperature and pressure dependent Sellmeier equation of air. First, I have determined the refractive index of TRIS buffer, then the refractive indices of bR suspensions with different concentration in the wavelength range between 390 and 880 nm. For the first time I have published the Sellmeier coefficients of TRIS buffer and bR suspensions [J1, C1-C4, K1-K3].

2. Employing pump-probe measurements on a thin bR film dried onto the surface of an integrated optical waveguide, I have demonstrated that the peak of resonance of the mode coupled into the waveguide shifts after excitation on a sub-picosecond timescale due to the index change induced by the absorption of bR. Based on the comparison of my measurement results with the time constants of the photocycle of bR, I have confirmed that the amplitude of the measured signal is controlled by the index change during the BR→I transition. The results suggest that the BR→I transition of the photocycle of bR can be exploited for ultrafast switching based purely on optical principles [J2, C5-C9, C11-C16, C20, K4, K5].

3. I have participated in the construction and calibration of a complex apparatus for the measurement of femtosecond time-resolved fluorescence kinetics. I have measured the fluorescence kinetics of FAD in a wide time- and spectral range in water, water–dioxane, water–ethanol, water–DMSO solutions. The fluorescence kinetics was also investigated when the FAD molecule was bound to flavocytochrome, which helps to understand the intra- and extramolecular interactions of the coenzyme. Employing a new evaluation algorithm for the first time (Basis Pursuit Denoising), I have determined that the fluorescence of FAD is characterized by four decay components, which can be attributed to one open and three closed conformations. In addition, a 500-fs time constant was also identified and linked to salvation dynamics in water. FCC fluorescence kinetics, which was studied for the first time in this work, showed quenching larger than in all other environments, which suggests a strong interaction between the chromophore and the amino acid side chains. The experiments

conducted in a wide temporal and spectral range and the data analyzed using new and very efficient methods resulted in a model of the fluorescence mechanisms of FAD, which is more thorough and complex than the earlier models in the literature [J3, J4, C10, C17, C19, C21, K6].

4. I have measured the transient absorption of NADH using a femtosecond pump-probe technique at 0%, 50%, 70%, and 80% methanol concentrations, which provide information about the different conformational states of the molecule. The global analysis of the measured data yielded the time constants of 250 fs, 3 ps, and 350 ps. The 250-fs time constant can be attributed to the excited state absorption of the adenine group. The other two decay components have large amplitude values in methanol-free samples. These observations suggest that the energy transfer in water from adenine to nicotinamide is characterized by a 3-ps time constant, while the nicotinamide group returns to the ground state in 250 ps. The population of the closed conformational state, which facilitates energy transfer, gradually decreases with increasing methanol concentration [J5, C18], which leads to a decrease of the weight of the above slow components.

V. PEER REVIEWED PUBLICATIONS RELATED TO THE THESIS:

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C4. **Z. Heiner**, K. Osvay
Measurement of the Concentration Dependent Refractive Index of Dark-Adapted Wild-type Bacteriorhodopsin in 390-880 nm
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C5. L. Fábián, M. Mero, **Z. Heiner**, K. Osvay, A. Dér
Ultrafast Integrated Optical Switching Based on the Protein Bacteriorhodopsin
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C6. L. Fábián, M. Mero, **Z. Heiner**, M. Kiss, K. Osvay, A. Dér
Ultrafast Integrated Optical Switching Based on the Protein Bacteriorhodopsin
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C7. **Z. Heiner**, L. Fábián, M. Mero, M. Kiss, K. Osvay, A. Dér
Ultrafast, Protein-Based All-Optical Switching
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C10. **Z. Heiner**, A. Makai, G. I. Groma
Ultrafast fluorescence kinetics measurement based on up-conversion method
COST Meeting & Training School, Nonlinear Nanophotonics 2010, Visegrád, Hungary

C11. Pal Ormos, L. Fabian, **Z. Heiner**, M. Mero, M. Kiss, E. Wolff, K. Osvay, A. Der
Subpicosecond photonic switching based on bacteriorhodopsin
55th Annual Meeting of Biophysical Society 2011, Baltimore, USA, 2634-Pos/B620

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C17. **Z. Heiner**, A. Makai, F. Sarlós, Cs. Bagyinka, A. Tóth, G. Rákhely,G. I. Groma
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