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## Enzymatic Activity and Excited State Processes in Protochlorophyllide Oxidoreductase

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## *Summary*

The research presented in this thesis is devoted to unraveling the molecular mechanisms of the catalytic reaction in the protochlorophyllide oxidoreductase (POR) enzyme. The ternary photoactive complex consisting of the POR enzyme, substrate protochlorophyllide (Pchlde) and cofactor NADPH can be formed in the dark, and upon absorption of a photon by Pchlde, a proton and a hydride are transferred to Pchlde from a tyrosine residue of the enzyme and NADPH, respectively. In this way, the double C=C bond in Pchlde is replaced with a single C-C bond, and Pchlde is efficiently reduced into chlorophyllide (Chlide), an immediate chemical precursor of chlorophyll. Since the reaction is light-dependent it is an important regulatory step in the development of the entire light-harvesting apparatus in all photosynthetic organisms. The unique requirement of light by POR for catalysis allows the reaction to be initiated by a short laser pulse and the fast enzymatic events to be monitored in real time via associated spectroscopic changes.

The dark photoactive complex and the product state have distinct absorption bands. The spectral changes associated with the conversion from the dark state to the product state have been measured (i) as a function of time and (ii) as a function of illumination history. The application of carefully designed optical experiments along with comprehensive methods of analysis have allowed for a highly quantitative characterization of the reaction kinetics over a timescale from a few femtoseconds ( $10^{-12}$  s) to a few nanoseconds ( $10^{-9}$  s), and the accumulation of the catalytic product under various illumination conditions for the first time. The results are presented in **chapter 2, 3 and 4** and comprise the first part of the thesis.

In chapter 2 and 3 is described that the amount of product Chlide in the sample depends non-linearly on the illumination received by the sample. By measuring the response of the sample on a first, and subsequent second, and third photon (etc), on a picosecond time scale, we find that absorption of a first photon transforms the initially inactive enzymes into active enzymes, and, after absorption of a second photon, the catalytic conversion of Pchlde into Chlide takes place. The spectral changes in the mid-IR region, associated with the initial step, contain mainly protein signals and imply that some rearrangements in the protein structure are initiated during the activation phase. The absorption changes associated with the accumulation of catalytic products can be induced only by continued illumination. The activation process appears to be more efficient in mesophilic enzyme than in thermophilic enzyme, in measurements performed under room temperature conditions. These findings demonstrate that the efficiency of the activation of the enzyme largely depends on the structural flexibility and the conformational state of the protein. The observation of shifts in the vibrational frequency of the Pchlde C=O group in bound and unbound states reveals that the active site of POR forms strong hydrogen bonds with the substrate, which is possibly an additional essential factor for catalysis. The catalysis in active enzymes starts with the bi-phasic formation of an intermediate in the excited state, I675\*, with effective rates of  $\sim (4 \text{ ps})^{-1}$  and  $(180 \text{ ps})^{-1}$ , which is further converted into the final product Chlide with  $\sim 30\%$  efficiency. The formation of I675\* shows a solvent kinetic isotope effect for both rate constants, implying the involvement of proton movement in this reaction.

To obtain a better understanding of the Pchlde dynamics in the enzyme, we studied the intrinsic Pchlde excited state dynamics in several solvents. The dilution of the chromophore in solvents with different electron and proton donating properties, and of H-bonding interaction strength, is a mimic of the protein environment. Measurements in THF, methanol, buffered and neat water solutions using ultrafast visible and mid-IR difference absorption spectroscopy and fluorescence emission decay clarify how the local environment influences the excited state dynamics of Pchlde. The results comprise the second part of the thesis and presented in **chapter 5, 6, 7**.

The various experiments reveal that the optical spectra and dynamics of Pchlde in solution depend on it being in either the monomer or aggregated state. The aspects of the Pchlde photophysics in the monomer state are elucidated in **chapter 5**. In organic solvents the Pchlde electronic excited state has a nanosecond lifetime

and decays to the triplet state with a quantum yield of ~23%. During the excited state lifetime, the stimulated emission is quenched, which we attribute to the solvation of the excited state, which has a mixed internal charge-transfer character<sup>(41)</sup>, into a state with stronger charge-transfer character. Pchlde in methanol shows spectral signatures and dynamics similar to observed in the inactive POR complex. Hence, for the POR complex the responses characteristic only for substrate can be separated from the spectral features and dynamics originating from the catalytic reaction and products. The formation of the state I675\* occurs not in solution and is characteristic for the enzyme dynamics only. Initially, we proposed the nature of the I675\* intermediate to be a strongly hydrogen-bonded state (in chapter 2). Linking the transient absorptions in isolated Pchlde and in the POR complex and the observation of the isotopic dependence in the intrinsic rates of the I675\* formation (kinetic isotope effect), we now suggest that the formation of the I675\* state corresponds to the proton transfer reaction from the Tyrosine to Pchlde (chapter 3).

**Chapter 6** and **7** provide a detailed description of spectroscopic signatures and dynamics of Pchlde in aqueous solution. From the non-linear power-dependent saturation of the transient absorption follows that Pchlde in water forms aggregates with an average size of four, and consequently excitonic interactions dominate the optical properties. In chapter 7 a theoretical modeling of the changing spectral properties as the excitation density is increased is given in terms of population of multi-exciton manifolds of an excitonically coupled linear chain of parallel oriented Pchlde molecules. A quantitative simulation of the data is provided, that shows that the exciton states are populated up to the 4-exciton state. The decay of the multi-exciton manifold is found to be relatively slow (in the order of 10 ps), therefore it is suggested that the exciton states in the Pchlde aggregates are mixed/coupled with charge-transfer states (CTS), similar to the results found in chapter 5. The formation of a CTS is an intrinsic property of Pchlde, which in protic solvents occurs in conjunction with strengthening of the site-specific H-bonding interaction. So far it is clear that especially the C=O keto group in Pchlde is an important element for creating H-bond with solvent and protein, and that the CTS formation and H-bonding dynamics at the keto site are essentially correlated phenomena. In different parts of the thesis it is discussed that the local excitation of the Pchlde chromophore and CTS are perhaps also important factors for triggering protein conformational changes, and for creating an electronic configuration that favours subsequent proton and electron transfer reactions.

In conclusion, the joint analysis of the various spectroscopic data collected on the photoactive POR complex and the chromophore in solution, has allowed for a better understanding of the molecular mechanism of the enzymatic photoreduction taking place on the picosecond time scale, and in particular, to elucidate the special role of the protein in catalysis. The results presented in this thesis create a solid base for future works, which will lead to a full identification of the reaction pathway and intermediate state(s) involved at room temperature, together with the identification of the structural changes that lie at the origin of the activation process. The demonstration of the unique requirement of light for both activation of the protein and for catalysis makes POR an important model system for studying the role of protein conformational changes on activity. The novel results obtained on the Pchlde aggregates are highly relevant for understanding of the optical properties and dynamics observed in experiments on many natural photosynthetic complexes.