Enzymatic Activity and Excited State Processes in Protochlorophyllide Oxidoreductase

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~ CHAPTER 1 ~

This thesis is devoted to the light-dependent catalytic reaction executed by the enzyme POR and accompanying aspects of Protochlorophyllide excited state photophysics, studied by means of laser spectroscopy. In this chapter a brief introduction to the subject will be given.

1.1. Introduction

The energy for living in most of organisms on Earth is derived from sunlight. The entire energy pyramid, and consequently nutrition pyramid in biosphere is based on a light-dependent process, known as photosynthesis. All photosynthetic (phototrophic) organisms, such as plants, bacteria and algae harvest sunlight for producing chemical energy. Most nonphototrophic organisms consume sunlight energy via food chains. At the molecular level primary utilization of sunlight energy is accomplished in an alliance of *proteins* and *chromophores*.

Chromophores, relatively small organic molecules or molecular residues which possess the physical properties to capture light, are primary light harvesters for many vital processes. Besides photosynthesis, chromophores are involved into processes of vision, phototaxis (movements of organisms towards the light or from the harmful light source), circadian rhythms (24 hours biological clocks), regulation of gene expression, stem growth and seed germination etc. A chromophore absorbs a quantum of light energy and stores it for a short period of time (typically 10^{-12}-10^{-3} s) in form of energy of excited electrons. Light-harvesting and photoreceptor proteins incorporate chromophores into their structures and use initial input signal to trigger further sequences of biochemical reactions.

The molecular signal transduction mechanisms and light-harvesting processes often involve multi-step transfer of energy of the initially excited electron, transfer of charge and/or particles with a very high speed, directionality and efficiency. The transfer reactions of electrons, protons, hydrides and small molecular groups are at the basis of biological activity. These elementary events are necessarily coupled to the dynamics of the surrounding medium of protein, nucleic acids, or solvent, such as making and breaking of hydrogen bonds, small scale conformational changes of amino acids in proteins, the excitation of local vibrations or the excitation of collective phonon modes of the protein. Eventually such local events may give rise to large functional motions on a much longer timescale, like the transport of an electron or proton across a membrane, the completion of an enzymatic reaction or even the structural rearrangement of a photoreceptor. In order for the elementary processes to occur with high efficiency and speed, specific motions of the surrounding protein medium are required.

Proteins are large (hundreds and thousands of atoms) and highly-organized biological molecules. Generally speaking, nearly all functions in living organisms are related to proteins. In bacterial cells, for example, proteins constitute about a half of cell's dry weight. Many proteins are *enzymes*, i.e. biological catalysts which enhance rates of chemical reactions. In enzymatic reactions, the protein binds the initial reactants, usually referred to as *substrate* and *cofactor*, and converts them into different molecules, *products*. The enzyme itself is not consumed during the reaction. Enzymatic activity can be affected by various chemical and physical factors, such as the presence of additional molecules (inhibitors and activators), temperature, concentration of substrates, pH. The presence of light for catalysis is known to be essential only in two enzymes (1): (1) DNA photolyase, the repair protein which under presence of visible light recognizes and removes damages in genes caused by redundant UV radiation, and therefore serves for maintenance of a proper gene code in nearly all living organisms (excepting for placental mammals) (2, 3); (2) and POR (NADPH:protochlorophyllide oxidoreductase, EC 1.3.1.33), also universal enzyme, essential for the regulation of the assembly of entire photosynthetic apparatus. The latter protein is the primary focus of this thesis.

1.2. POR enzyme

Functioning of light-harvesting complexes and reaction centers in photosynthetic organisms depends on efficient biosynthesis of chlorophyll pigments. Chlorophyll biosynthesis involves series of biochemical reactions, where one of the key steps is executed by POR enzyme (4-12) (figure 1.1). POR enzyme belongs to a family of NAD(P)/NAD(H)-dependent dehydrogenase enzymes, which catalyse proton and electron addition and removal reactions with rate enhancements of up to 10^{17} compared to the equivalent reaction in solution (13).

Currently light-independent and light-dependent pathways in chlorophyll biosynthesis have been identified(4, 6-12). Dark chlorophyll biosynthesis is present in cyanobacteria, green algae, mosses, where the reaction is executed by light-independent type of POR enzyme(10). However in all higher plants (also in some lower plants and gymnosperms) light-dependent POR has been found (4, 9). The light-dependent POR binds substrate Protochlorophyllide (Pchlide) and cofactor NADPH in the dark and upon illumination it transforms Pchlide into the product Chlorophyllide (Chlide), which is an immediate chemical precursor of Chlorophyll (figure 1.1). The light-regulated chlorophyll biosynthesis is considered to be an evolutionary more advanced mechanism, and a better adaptation to changing light conditions(4). The development of the entire photosynthetic apparatus upon illumination during germination allows plant to establish self-reliant autotrophic life.

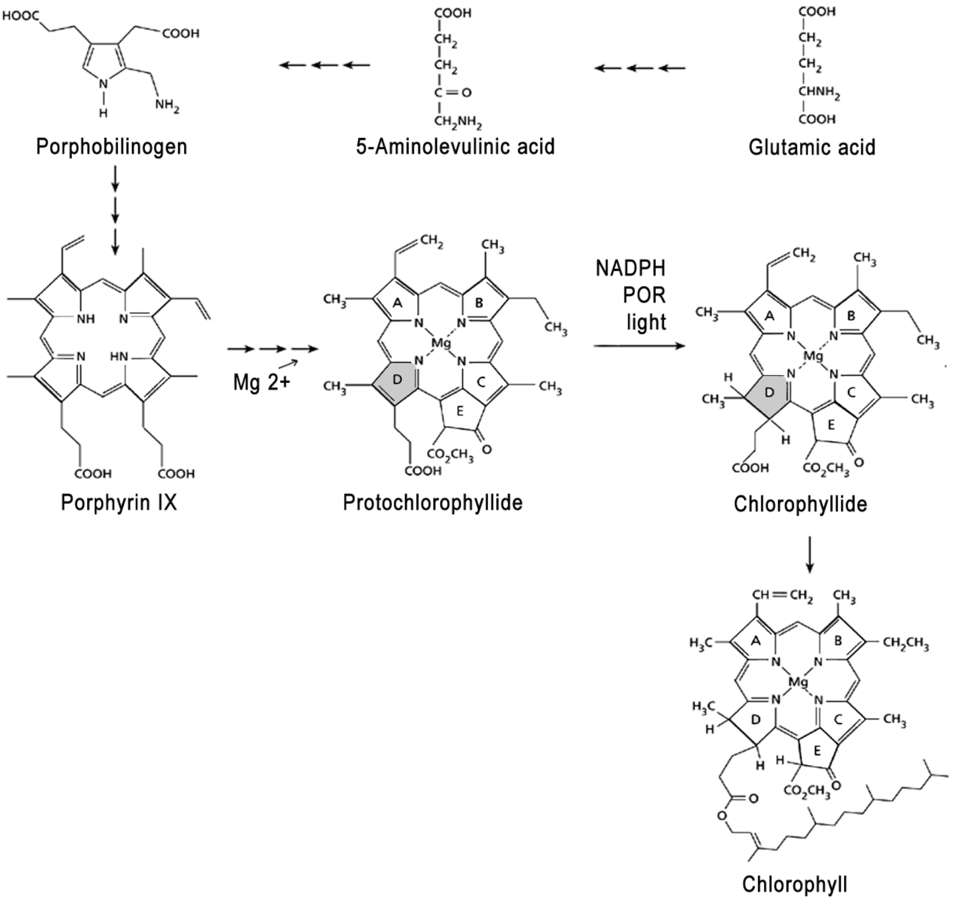

Figure 1.1. The biosynthetic pathway of chlorophyll. The pathway begins with glutamic acid, which is converted to 5-aminolevulinic acid (ALA). Two molecules of ALA are condensed to form porphobilinogen (PBG). Four PBG molecules are linked to form protoporphyrin IX. The magnesium (Mg) is then inserted, and the *light-dependent* reduction of ring D, and the attachment of the phytol tail complete the process. Many steps are omitted in this figure.

An elucidation of the molecular mechanism (14) of the light-dependent enzymatic reaction and major events in the catalytic turnover of POR (5) became possible in reconstituted enzyme-substrate complexes *in vitro*. The POR enzyme has been heterologously overexpressed, first fused with the maltose-binding protein (15) and recently in *E. coli* using a histidine-tagging procedure (16). This yielded sufficient material of the pure enzyme to perform low-temperature fluorescence spectroscopy (17-19) and time-resolved difference absorption measurements (20). As a result the following molecular model of enzymatic reaction has been established. After excitation of Pchlide with a photon of visible light, a tyrosine residue of the enzyme donates a proton (14, 20), and the cofactor NADPH donates a proton and two electrons (hydride) (21, 22) to replace the C=C double bond in protochlorophyllide with the C−C single bond and two new C−H groups to form chlorophyllide (phase III, ring D in figure 1.1). The active site of POR is depicted in figure 1.2.

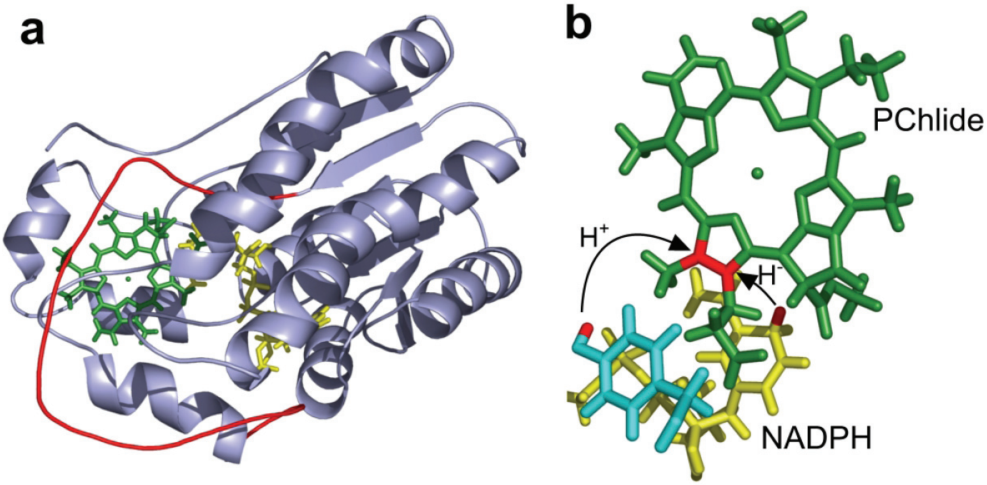

Figure 1.2. Homology model of POR from Synechocystis(12). **(a)** The structure consists of a central parallel β-sheet comprising seven β-strands, surrounded by nine α-helices. The 33-residue insertion (red) is unique to POR, and is proposed to be involved in Pchlide (green) binding. **(b)** Three-dimensional model of the POR-catalysed reaction based on the structural homology model of POR(12) and the proposed mechanism of hydride and proton transfers(14). The proton at the C18 position of Pchlide is derived from Tyr 189 (numbering in Synechocystis POR, cyan) and the hydride transferred to the C17 position is derived from the proS face of NADPH (yellow).

1.3. Structural flexibility of proteins

Biological organisms depend on the optimal functioning of enzymes and proteins under varying environmental conditions. Enzymes need to efficiently adapt to certain conditions in order to remain (or become) fully functional. In the vision of current enzymology there are several models to explain the huge catalytic power of enzymes. For example, according to the theory of transition state, catalysis is enabled by lowering of the activation energy barrier, or rising of the ground state energy of bound substrates (23, 24). Enzymes achieve it either by providing an electrostatic interactions resulting in electronic rearrangement of substrates, i.e. their polarizability, or by direct distortion of substrates by stretching bonds or/and altering bond angles, bringing substrates closer to each other or reorienting them.

Also enzyme can create an alternative pathway for the reaction by temporarily reacting with substrates so forming an enzyme-substrate intermediate complex.

Performing these functions, as well as initial binding of substrate and if needed, a cofactor, and later release of products, requires efficient switching between different states and structural flexibility. Several enzymes (such as ubiquitin, calmodulin cyclophilin A, lipase, cholesterol oxidase) have been reported to have an "off" and an "on" configuration and the dynamic interconversion between different conformational states has been shown to be important for catalysis (25-27).

Therefore the role of protein motions in explaining the power of enzymes is currently one of the most challenging questions in biology (13, 28-33). In addition, whether and which conformational changes occur during enzyme turnover or protein adaptation and what exactly triggers them, are important questions to resolve in order to obtain a better understanding of how enzymes and proteins function.

1.4. The aim of this thesis

The primary aim of this work is to answer whether conformational changes take place in the enzymatic reaction executed by POR; to characterize the sequence of elementary events that occurs; and to clarify if the process of Pchlide reduction is coupled to specific protein dynamics.

Why is POR the system of choice? The unique requirement of light for initiation of catalysis in POR enzyme, and the fact that enzyme-substrate complex is formed in the dark implies that the catalysis can be initiated by a short pulse of light. It makes the POR:Pchlide:NADPH complex a suitable model system for studying the mechanisms and timescales of enzymatic proton and hydride transfers, as well as their relation to conformational changes of the protein, since it eliminates diffusion from the catalytic reaction. The POR catalysis, involving the transfer of two protons and two electrons, might be also representative for a larger group of enzymes, the alcohol dehydrogenases, and probably serve as a generic model system for understanding of the mechanism of more complicated enzymatic events.

The involvement of the Pchlide excited state in the catalytic reaction suggests that some of the proton transfer events may occur on the picosecond timescale during the Pchlide excited state lifetime. In general, reports of ultrafast (femto- and picoseconds) proton transfer in biological systems are limited. On one hand, this is because the rate of many biological reactions is restricted to a relatively slow diffusion-dependent binding of substrates, and on the other hand, because proton transfer does not always lead to a clear spectroscopic change as for example an electron transfer reaction does. Most biological studies are therefore limited to the microsecond or millisecond time scales, and inherent proton transfer reaction properties are deduced from isotope effects, pH dependences etc. In POR, the formation of the enzyme-substrate complex in the dark prior to illumination is a doorway to follow fast proton and electron transfer processes in real-time and a way to remove slow diffusion limited binding and release events from the experiment. It is also interesting to elucidate the mutual influence of enzyme and chromophore, i.e. whether the local excitation of a Pchlide chromophore is coupled or assist to any conformational change of the protein, or how the protein can assist to proton and electron transfer to happen.

In order to cast some light on these aspects, spectroscopic measurements on the reconstructed POR:Pchlide:NADPH complex were done, as well as the measurements on the isolated Pchlide chromophore. Various modern spectroscopic tools were used, mainly (i) ultrafast visible pump–probe spectroscopy, (ii) ultrafast visible pump – mid-IR probe spectroscopy, (iii) time-resolved fluorescence measurements with sincroscan streak camera, and (iii) a range of additional spectroscopic instrumentation, such as FTIR (Fourier transform infrared), FLN (fluorescence line narrowing) and Raman spectroscopy.

1.5. Synopsis

The thesis can be divided into two major parts. The first part, comprised of chapter 2, 3 and 4, is devoted to a study of the POR enzymatic reaction with ultrafast visible pump-probe and FTIR spectroscopy.

The main results of transient absorption and FTIR experiments are presented in **chapter 2** in a *condensed* form. It is deduced from the kinetic modeling of the transient absorption data that that the first catalytic turnover of POR enzyme requires two photons. The POR:Pchlide:NADPH complexes, which have never been illuminated prior to the experiment, are in an inactive conformation, and after absorption of the first photon the inactive state is converted into an active conformation. After absorption of the second photon the signal of the first catalytic intermediate I675* appears on the picosecond timescale. The active state of the enzyme is a long-lived and persists on a timescale of at least 24 hours; it also survives at least one turnover. Direct experimental evidence for protein conformational changes, followed by product formation, is obtained in the FTIR experiment.

Chapter 3 is thoroughly focused on a quantitative analysis of series of transient absorption measurements using a sequential and branched kinetic modeling. The refined intrinsic rates of $(10\ \mathrm{ps})^{-1}$ and $(360\ \mathrm{ps})^{-1}$ for the I675* formation are derived from a systematic analysis of many datasets, recorded in different sample preparations and experimental sessions. In addition, the reaction and activation dynamics in thermophilic and mesophilic enzyme is analyzed at room temperature and a reduced speed of product accumulation for the thermophilic enzyme is found. The comparison of rates of the I675* formation in the protiated and deuterated samples confirms that the first catalytic intermediate I675* is either a protiated and/or a strongly hydrogen-bonded complex.

In **chapter 4** a comprehensive analysis of the FTIR spectra and assignment of IR signatures of the light-induced protein rearrangements and product formation are discussed in detail. The FLN technique is used to disentangle vibrational modes of bound chromophore and protein signals. The results of the FLN experiment indicate that binding of Pchlide to POR active site creates a very strong hydrogen bond between the C=O keto group of the chromophore and a protein residue. The visible pump-mid-IR probe experiment clearly demonstrate a correlation between formation of the first catalytic intermediate I675* and the dynamics of the C=O keto group of the chromophore.

The second part of this thesis is devoted to the aspects of the exited state photophysics of Pchlide. Since the excited state of Pchlide is directly involved into the enzymatic reaction, the measurements on the isolated chromophore in different solvents were done to obtain a better understanding of the catalytic events taking place in POR.

In **chapter 5** we describe the results of the time-resolved fluorescence, visible and infrared pump-probe measurements on Pchlide in a range of solvents (methanol, THF, buffer solution, neat water). We observe the effects of extensive multiphasic emission quenching during the Pchlide excited state lifetime and assign it to the solvation of the excited state. In methanol solvation process occurs in conjunction with a strengthening of a hydrogen bond(s) to the Pchlide C=O keto group. During the excited state lifetime (2.5-4.5 ns) a triplet state is populated with a maximal yield of ~23%.

In **chapter 6** rather “abnormal” quenching of the Pchlide emission in aqueous solution is demonstrated. The optical properties and excited state dynamics of Pchlide in aqueous solution are shown to be determined by formation of aggregates consisted of four Pchlide monomers. A comprehensive kinetic target analysis of a set of the power-dependent transient absorption spectra reveals spectral signatures and lifetimes of one-exciton and high-exciton states.

In **chapter 7** we present theoretical calculation of difference absorption spectra of one-, two-, three and four-exciton states for linear aggregate consisted of 4 monomers by using standard exciton theory with phenomenological lineshape. The developed model is applied to simulate a series of power-dependent kinetics of Pchlide in aqueous solution. The relaxation of the high-exciton states into the one-exciton manifold occurs via cascading exciton-exciton annihilation process with total rate of ~10 ps. This extremely slow annihilation is explained by assuming couplings of exciton states with charge-transfer states.