Photosynthetic electron transport in purple bacteria: An *in vivo* spectroscopic study

PhD thesis

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Annotation
Electron transport in purple bacteria was studied using combination of absorption spectroscopy and induced bacteriochlorophyll fluorescence in whole cells *in vivo*. Focus is placed on relations between fluorescence yield, the state of the electron transport chain and the membrane potential. A laboratory-built absorption spectrophotometer-fluorimeter is described.

Prohlašuji, že svoji disertační práci jsem vypracoval samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

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Abbreviations

A	absorbance
AFM	atomic force microscopy
ATP	adenosine triphosphate
$B_{A(B)}$	bacteriochlorophyll monomers in the A (B) side of the reaction center
BChl	bacteriochlorophyll
Blc.	*Blastochloris*
BPheo	bacteriopheophytin
CCD	Charge coupled device
Chl	chlorophyll
D1, D2	subunits of the reaction centre of the photosystem II
EM	electron microscopy
FCCP	carbonylcyanide-p-trifluoromethoxyphenyl hydrazone
h	Planck constant (6.626×10^{-34} Js)
$H_{A(B)}$	bacteriopheophytin monomer in the A (B) side of the reaction center
H, L, M	subunits of the bacterial reaction centre
λ	wavelength
LED	light emitting diode
LH	light-harvesting complex of purple bacteria
ν	frequency
NAD	nicotinamide adenine dinucleotide
P_{680}	primary donor of photosystem II
P_{870}	primary donor of purple bacteria
PDA	photodiode array
Pheo	pheophytin
PNB	purple non-sulfur bacteria
PS	photosystem
PSU	photosynthetic unit
Q_A, Q_B	quinone acceptors of the reaction centre
Rb.	*Rhodobacter*
RC	reaction centre
Rps.	*Rhodopseudomonas*
Rsp.	*Rhodospirillum*
Rx.	*Rubrivivax*
T–S	triplet-minus-singlet
UV–VIS–NIR	ultraviolet–visible–near infrared

Contents

I. Introduction

PHOTOSYNTHESIS

Sunlight is the single most important source of energy for the life on Earth. It is through photosynthesis that this energy flow is captured and stored in chemical bonds of organic compounds. Moreover, oxygen is released as a by-product of light-dependent reactions performed by photosynthetic organisms, which makes photosynthesis a significant geochemical factor. Thus understanding of mechanisms of photosynthesis and biology of organisms that perform it are of crucial importance for the understanding of working of the biosphere. It also reveals important principles of energy conversion that may once become of practical use.

As the oxygenic photosynthesis represents the most advanced form of the light-dependent metabolism known, it was most probably preceded by less complex organisation of photosynthetic apparatus. Indeed, among extant microorganisms, there are several groups of phototrophs that make use of photosynthetic molecular machinery that is significantly simpler than that of plants and algae. And while these organisms themselves cannot be counted as the ancestral forms or predecessors of today's oxygenic phototrophs, they provide us with indications of how the original photosynthesisers might have looked like and help us reveal the general principles of the photosynthetic energy conversion.

Two fundamentally distinct modes of phototrophy are known. One is represented by *Halobacterium* of the group Archea. These organisms employ a transmembrane protein called bacteriorhodopsin in which the absorption of light by the carotenoid cofactor (retinal) induces a conformational change that leads to the expulsion of protons into the periplasmic space (Lanyi 2004). The proton motive force is used to drive synthesis of ATP. While haloarchea are the best known studied examples of rhodopsin-based phototrophy, many marine bacteria were found to harbour a similar protein pump, labelled proteorhodopsin. Interestingly, all these proteins are structurally homologous to the sensory rhodopsins of animal retina and the light-activated ion channels of several algae (Spudich 2006).

The other class of phototrophs comprises organisms in which the absorbed radiative energy fuels a sequence of electron transfer steps. These are usually coupled to proton translocation, creating the membrane potential. While in some groups, the light-dependent production of ATP is the only function of photosynthetic apparatus and the electron transfer operates in a cyclic fashion, in others are the electrons ultimately transferred to carbon in a process of CO_2 assimilation. Fundamental role is played by tetrapyrole-based pigments, mainly (bacterio)chlorophylls ((B)Chl) bound to transmembrane pigment-protein complexes. Members of five bacterial phyla are included in this group (Bryant and Frigaard 2006): Proteobacteria (purple bacteria), Chloroflexi, Chlorobi (green sulphur bacteria), Firmicutes (heliobacteria) and Cyanobacteria (including chloroplasts). General overview of fundamental components of photosynthetic energy transforming membranes is presented below. Later, the organisation of light-utilising machinery of purple bacteria will be dealt with in depth.

1. Photosynthetic apparatus - structural aspects

1.1 Reaction centres

At the heart of the tetrapyrole-based photosynthetic apparatus is the reaction centre (RC). It is a dimeric (homo- or hetero-) integral membrane protein with approximate mirror symmetry carrying a system of cofactors that provide an electron transfer pathway. The sequence of electron-transfer steps starts with excitation of molecule of (bacterio)chlorophyll which is part of a dimer lying at one pole of the RC. This is quickly followed by charge separation and transfer of the electron towards the acceptor located at the other side of the RC, across the membrane. The acceptor is either a molecule of quinone or an iron-sulphur cluster. The spatial separation thus achieved reduces the probability of wasteful charge recombination. Redox properties of RC's cofactors and components and nature of electron acceptors separate the RC into two distinct groups: Reaction centres of type 1 are found in cyanobacteria, green sulfur bacteria and heliobacteria. They posses either homodimeric or heterodimeric cores with [4Fe-4S] clusters as their terminal electron acceptors, and operate at low redox potential (about -1200 mV) and thus can produce strong reductants (reduced ferredoxin). Type 2 reaction centres, which are found in cyanobacteria, purple bacteria and filamentous anoxygenic bacteria, have heterodimeric cores with quinones as terminal electron acceptors. They produce strong oxidants (up to 1.2 mV in the case of oxygenic photosystem II) and weak reductants (hydroquinone). The two types of RCs have similar structures and seem to share a common evolutionary origin (Ke 2001).

1.2. Light-harvesting complexes

In order to increase the efficiency of light utilisation, the photosynthetic systems have developed specialised *light-harvesting complexes* (*antennae*) that serve the purpose of capturing the photons and delivering the excitation in an efficient and regulated manner to the reaction centre. The light-harvesting complexes not only increase the absorption cross-section of the photosynthetic apparatus but also expand the range of wavelengths utilisable for photosynthesis. This is accomplished by using non-chlorophyll pigments such as carotenoids and phycobilins to absorb light and transfer the captured energy to chlorophylls. For example in purple bacteria, the greenish bacteriochlorophylls that absorb mainly the radiation below 400 nm and over 750 nm are accompanied in the light-harvesting complexes by carotenoids absorbing in the 400-550 nm range, giving these organisms their typical colour and with it providing broadened useful spectral range.

While there are basically only two types of RCs, the variability of light harvesting complexes fully reflects the variability of photosynthetic organisms. Almost all antenna complexes are pigment-proteins either embedded in the membrane or attached to it. The remarkable exception represent chlorosomes, the high-efficiency light-harvesting structures of green bacteria that are formed from self-assembling pigment-lipid aggregates (Blankenship 2002).

1.3. Cytochromes

The third class of universally present building parts of the photosynthetic apparatus are *cytochromes*. They come as either small soluble heme-binding proteins that act as electron shuttles among the membrane-bound components of the electron transport chain, an example is the cytochrome c_2, or large, multisubunit transmembrane protein complexes with three heme groups and an iron-sulphur cluster-containing subunit (Rieske protein). These are the cytochrome bc_1 complex of anoxygenic bacteria and the cytochrome b_6f complex of oxygenic phototrophs. The purpose of the bc_1 and b_6f complex is the oxidation of membrane quinones coupled to a proton translocation across the membrane. A specific type of as yet unclear function is the cytochrome b_{559} that forms a subunit of the reaction centre of the oxygenic organisms (Blankenship 2002).

2. Brief characteristics of main groups of extant phototrophs

Purple bacteria These are bacteria of the phylum Proteobacteria that produce BChl *a* or *b* under oxic or anoxic conditions. They have type 2 reaction centres and ring forming membrane-intrinsic caroteno-BChl antennae. Many species oxidize sulphide, thiosulphate, or H_2. The carbon fixation proceeds via the reductive pentose-phosphate (Calvin–Benson–Bassham) cycle.

Chloroflexi A bacterial phylum that includes filamentous anoxygenic phototrophs formerly known as the green gliding or green filamentous bacteria. Remarkably, these organisms contain the type 2 RC with the internal antenna of the purple-bacterial type accompanied with the chlorosome as the outer antenna. *Chloroflexus auratiacus* is the typical member of the group. This organism exhibits considerable metabolic diversity and it can grow as an aerobic chemoheterotroph or as an anaerobic photoheterotroph. Using electrons derived from H_2 or H_2S under anoxic or microaerophilic conditions, some strains of Chloroflexus sp. grow photoautotrophically by fixing CO2 through the 3-hydroxypropionate pathway.

Heliobacteria Bacteria belonging to the phylum Firmicutes, closely related to clostridia. They are photoheterotrophs containing type 1 reaction centres and bacteriochlorophyll g.

Chlorobi Obligately anaerobic photoautotrophs that (i) oxidize sulfur compounds, H_2 or ferrous iron; (ii) fix carbon by the reverse tricarboxylic acid cycle; (iii) synthesize BChl *c*, *d* or *e* along with BChl *a* and Chl *a*; and (iv) have a photosynthetic apparatus that comprises a type 1 reaction centre, the Fenna-Matthews-Olson (FMO) BChl-*a*-binding protein and chlorosomes that each contain >200 000 BChl *c*, *d* or *e* molecules.

Cyanobacteria This bacterial phylum includes all oxygen-evolving photosynthetic bacteria. They have Chl *a*-containing type 1 and type 2 reaction centres and fix carbon by the reductive pentose-phosphate (Calvin–Benson–Bassham) cycle. Most members of this group have phycobilisomes as light-harvesting antennae. Prochlorophytes are cyanobacteria such as Prochlorococcus spp. that lack phycobilisomes, instead they employ Chl *b*-containing light-harvesting complexes, a sole case among photosynthetic prokaryotes (Blankenship 2002; Bryant and Frigaard 2006). Cyanobacteria gave rise to eukaryotic chloroplasts by the process of endosymbiosis (Margulis 1993 in Blankenship 2002).

2.1. Evolutionary considerations

It is quite safely established that photosynthetic organisms, namely Cyanobacteria, inhabited Earth at least 2.5 billion years ago (Summons et al. 1999). Moreover, there are indications that photosynthetic organisms might have been present on Earth as early as 3.5 billion years ago. Again, these ancient organisms were suggested to be related to Cyanobacteria based on morphological studies of microfossils (Schopf 1993; Schopf and Packer 1987). This would class Cyanobacteria as the earliest documented organisms on Earth. As the cyanobacterial photosynthetic machinery represents the most complex among known phototrophic microorganisms a reasonable assumption is that it was preceded by some simpler organisations of photosynthetic apparatus. Several different scenarios based on different methods exist for the early evolution of photosynthesis. According to some works, Proteobacteria, Heliobacteria or Cyanobacteria aspire at the position of the latest evolving groups while in other analyses the first two groups, along with Chloroflexi, compete for the position of the earliest branches of the evolutionary tree. These proposals assume that the reaction centres of type I and II developed in different organisms and that the presence of both types in Cyanobacteria is a result of fusion. Alternative theory states that (anoxygenic) pro-Cyanobacteria containing both types of reaction centres are the common ancestor of all extant phototrophs which are derived from it by a process of selective loss of one type of reaction centre (Xiong 2007; Mulkidjanian et al. 2006). No consensus on this topic has emerged so far while it becomes widely accepted that evolution of photosynthesis is a very complex process involving multi-layered lateral gene transfer.

The above mentioned theories are concerned with evolutionary relationships of various types of existing photosynthetic organisms but they do not address an equally, or even more, intriguing and intractable problem of the very origin of photosynthesis. Again, to address this topic, many schemes were proposed. These include development of photosynthetic apparatus from phototactic systems or ultraviolet protection systems. Origin of the reaction centre from cytochrome b, which is a ubiquitous membrane protein playing role in electron transfer, was also proposed. There is also evidence for recruitment of enzymes acting in nitrogen fixation into pathways of chlorophyll synthesis (Blankenship 2002 and references therein).

PURPLE BACTERIA

Purple bacteria represent a remarkably metabolically variable group of microorganisms. Generally, they are capable of photoautotrophic and photoheterotrophic growth, as well as aerobic and anaerobic fermentation. They are ubiquitous organisms, although they thrive mainly in anaerobic environments, where the photosynthetic growth is realised. In many species, synthesis of photosynthetic apparatus is suppressed by presence of oxygen. As phototrophs, they are defined by presence of type 2 reaction centre coupled via the quinone/quinol shuttle to the cytochrome bc_1 complex. Unlike oxygenic phototrophs, purple bacterial RC is not coupled to oxygen evolving complex and the potential of oxidised primary donor is much lower (about +500 mV in contrast to ~1.2 V of oxyphototrophs), hence they

are not capable of oxidation of water. Instead, the primary donor is re-reduced by electrons brought from the bc_1 complex, usually by mobile water-soluble c-type cytochromes (Nogi et al. 2005). Thus, purple bacteria make use of a cyclic electron-transfer pathway. The sole product of light-driven reactions is the proton gradient. Reduction of NAD^+ to $NADH+H^+$ needed for carbon fixation is carried out by NADH:ubiquinol-oxidoreductase. Reduced ubiquinone is provided by succinate-dehydrogenase. Organic compounds (such as succinic acid or malic acid), reduced sulphur compounds (thiosulphate or sulphide) or even molecular hydrogen serve as electron donors. Since the reduction of NAD^+ by ubiquinone is thermodynamically unfavourable (E_m for bacterial quinones is at pH 7 in the range 0-100 mV while E_m for NAD^+/NADH pair is about -320 mV), this so called *reverse electron flow* occurs only at the expense of membrane potential (Ormerod 1992; Imhoff 1995)

Carbon assimilation is carried out using Calvin-Bensson-Bassham, thus employing the Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco). Interestingly, both the 8-subunit form (type I), which is present also in oxygenic phototrophs, and the simpler, 2-subunit (prokaryotic) form (type II) of Rubisco is found in purple bacteria. Some species, such as *Rhodobacter (Rb.) sphaeroides* and *Rb. capsulatus* even express both forms (Tabita 1999). Most species of purple bacteria are also capable of nitrogen fixation. Indeed, certain classes of Rhizobia, N_2-fixing symbionts of leguminous plants, are capable of using photosynthetic reactions to supplement energy requirements for nitrogen fixation.

Based on their ability to metabolise reduced sulfur compounds, the purple bacteria split into two main groups, named sulfur and non-sulfur purple bacteria. Unfortunately, the more modern, molecular based taxonomical methods do not support the more intuitive classical system. It was shown that within the class Proteobacteria, the sulfur bacteria do form a single group (within the gamma-subgroup), members of purple non-sulfur bacteria are dispersed among the nonphototrophic microbes within both alpha and beta subgroup of Proteobacteria (Woese 1987).

Purple non-sulfur bacteria represent arguably the best understood and most thoroughly studied group of phototrophs. Several full genomes of have been sequenced (*Rhodopseudomonas palustris, Rhodobacter sphaeroides, Rhodospirillum rubrum, Roseobacter denitrificans, Bradyrhizobium sp. and Roseobacter sp.*), X-ray structures of RC from two species (*Blastochloris (*formerly *Rhodopseudomonas) viridis, Rhodobacter sphaeroides*, both WT and mutant) and also light harvesting complexes (*Rhodopseudomonas acidophila, Rhodospirillum molischianum*) are available (Bryant 2006; McDermott et al. 1995; Papiz et al. 1996; Koepke 1996; Arnoux et al. 1995; Allen et al. 1987a,b, Chang et al. 1986, 1991; Deisenhofer et al. 1985; Chirino et al. 1994; McAuley et al. 2000). This is accompanied by a wealth of spectroscopic data. Also, due the possibility to easily control the electron transfer reactions by light, they also represent model organisms of choice for studies concerning the operation of cytochrome bc_1 complex, X-ray structure of which is also available for the purple bacterium *Rb. capsulatus* (Berry et al. 2004; Crofts et al. 2008)

1. Structure of the photosynthetic unit

1.1. Light harvesting complexes

The photosynthetic unit (PSU) of purple non-sulfur bacteria (PNB) consists of light-harvesting (LH) complexes and the reaction centre. Most species possess two types of LH complexes: LH1 and LH2 that are also denoted B875 and B800-850, respectively, due to the absorption maxima of bound BChl (see also figure I.1 and I.2). However, in species such as *Rsp. rubrum* and *Blc. viridis* only the LH1 complex is present. Depending on the species LH2 is a circular nonamer (octamer) of pairs of small (5-7 kDa) hydrophobic proteins designated α and β. Each αβ pair binds three molecules of BChl *a* that form two groups within the LH complex: i) monomeric BChl molecules situated towards the cytoplasmic side of the complex absorb at 800 nm. Their bacteriochlorin rings are oriented parallel to the membrane; ii) remaining 18 (12) molecules, absorbing at 850 nm, are oriented perpendicular to the membrane near the periplasmic side of the complex. Along with the BChl's, 9 (8) molecules of carotenoids (rhodopin glucoside) with their long axis oriented approximately perpendicular to the membrane plane are found in the complex. (Ke 2001; McDermott et al. 1995; Papiz et al. 1996; Koepke 1996). It was also proposed that peripheral LH antennae may form rings of increasing diameter with variable αβ counts (Kereiche et al. 2008).

For the LH1, the available structures are not so detailed as for LH2. Based on the investigation of 2-D crystals by electron microscopy (EM), the structures of RC-LH1 and LH1 only complexes from *Rsp. rubrum* were determined to 8.5 Å (Karrasch et al. 1995). A crystal structure of RC-LH 1 complex from *Rps. palustris* was solved using the X-ray crystallography to resolution of 4.8 Å (Rozsak et al. 2003). Structure *Rb. sphaeroides* was not determined by crystallographic methods, however, it was modelled by Hu and Schulten (1998) based on structural analogy to projection map of *Rsp. rubrum* LH1. Like the LH2, LH1 forms a circular aggregate, which surrounds the RC. The *Rsp. rubrum* LH1 consists of 16 αβ heterodimers, each of them binding 2 BChl *a*, absorbing at 880 nm, and one carotenoid (spirilloxanthin). The LH1 complex of *Rps. palustris*, consists of 15 pairs of αβ heterodimers and consequently contains only 30 molecules of BChl *a*. Also, structure of this LH1 is rather elliptical than perfectly circular and its continuity is interrupted by a protein designated W. Possible function of this protein as well as its putative counterpart from *Rhodobacter* species, PufX, will be discussed in following sections. Besides LH1 and LH2, in species *Rps. acidophila* another type of light-harvesting complex occurs, designated B800-820 or LH3. This complex seems to be only a spectrally different analogue of LH2 (Ke 2001).

1.2. The reaction centre

The RC of PNB consists of three protein subunits, designated H, L and M with respective molecular masses of approx. 28, 31 and 35. L and M subunits bind the cofactors: primary donor (P), formed by a dimer of BChl (denoted D_A, D_B), two monomeric BChl (B_A, B_B), two molecules of bacteriopheophytin (BPheo) (H_A, H_B) and two quinones (Q_A and Q_B). These molecules are arranged on an approximate twofold symmetry axis. Subscripts A and B denote cofactors bound on L and M respectively. The overall symmetry is violated by the presence

of carotenoid (spheroidene in *Rb. sphaeroides*, 1,2-dihydroneurosporene in *Blc. viridis*) bound to M-subunit near the B_B. An atom of iron (in some cases manganese) is situated between the Q_A and Q_B molecules.

The H-subunit does not bind any cofactors, and its removal from the RC does not affect the photochemical activity of the LM-complex. In its absence Q_A can accept two electrons, with the electron transfer to Q_B being inhibited. In *Blc. viridis* and *Rubrivivax (Rx.) gelatinosus* a four-heme c-type cytochrome subunit is bound on the periplasmatic side of the RC (Ke 2001).

Comparison with the oxygenic RC

There is a large degree of structural and functional homology between the purple-bacterial reaction centre (BRC) and the RC of oxygenic phototrophs (PSIIRC). The functional cores of both RCs are organised as approximately symmetrical dimers that operate as the light-driven quinone reductases with only one branch serving for electron transfer under standard conditions. The inner part of the PSIIRC is composed of two transmembrane subunits, D1 and D2, that correspond to the L and M subunits of BRC, respectively. Cofactors bound by D1 and D2 are six molecules of Chl *a*, two pheophytins, two beta-carotenes, two plastoquinones and an atom of iron. Two additional chlorophylls in comparison to BRC are located on the periphery of the D1/D2 complex. They do not form the part of the standard electron transfer pathway but probably play a role in the alternative electron transfer pathways that serve a photoprotective role (Blankeship 2002).

There are however slight differences between the BRC and PSIIRC in spatial organisation and binding of cofactors that produce difference in their physical properties. Namely, in BRC, the primary donor is the special pair of BChl and the accessory BChls are spectrally well distinguished, in PSIIRC, the excitation is delocalised among the four molecules of Chl. On the other hand, the dimeric nature of BRC primary donor is preserved in the oxidised state, whereas in the PSIIRC the cation appears to posses more monomeric character (Lendzian et al. 1993; Dekker and van Grondelle 2000). Differences in cofactor-protein binding probably account also for the remarkable difference in the redox potential of the oxidised primary donors. This difference is about 500 mV and its impact is immense, as it allows oxidation of water by the PSIIRC.

This process is carried out by the oxygen evolving complex (Blankeship 2002). At the heart of this remarkable structure is a cluster of four manganese and one calcium atoms (Loll et al. 2005). The manganese atoms are ligated mainly by amino acid residues of the D1 subunit. The structure of the cluster is further supported by the 33 kDa peripheral membrane protein (PsbO). In eukaryots, two more proteins (22 and 17 kDa) support the structure of the oxygen evolving complex. The overall reaction catalysed by the oxygen evolving complex can be summed up as:

$$2H_2O \rightarrow O_2 + 4H^+ + 4e^-.$$

The other main part of PSIIRC that lack its bacterial analogue is the cytochrome b_{559}. This two-subunit structure is bound close to the stromal face of the thylakoid membrane. Its function remains to be definitely established.

While the D1/D2/cyt b_{559} complex of the PSIIRC was shown to be able to carry out

primary photochemistry, although not the oxygen evolution (Nanba and Satoh 1987) native PSIIRCs are known to contain several more, mostly small, protein subunits. Of the larger components that form the core of native PSII, we shall mention the CP43 and CP47 subunits that act as the inner antennae of type that is fundamentally different from the symmetrical light harvesting complexes of bacteria. This complexity of the native structure also distinguishes the PSII from its bacterial counterpart which operates *in vivo* as the three-subunit LMH complex. For a more extensive comparison of PSIIRC and BRC see Kalman et al. 2008).

2. Operation of the photosynthetic unit

The energy harvesting by the bacterial PSU has been a subject of extensive experimental research for a long time. With an advancement of knowledge about the structure of the RC and LH complexes there came also the possibility of modelling of the physical processes occurring in the PSU.

The B800 → B850 energy transfer rate is about 1/(700 fs) (Shreve et al. 1991) the LH2 → LH1 excitation transfer rate is about 1/(3.3 ps) (Hess et al. 1994). When the excitation starts at the carotenoid molecule in LH2, the rate constant for the energy transfer to BChl *a* is 1/(61 fs) (Cogdell et al. 2003). In the work of Ritz et al. (2001), results of quantum calculations of the excitation energy transfer are presented which agree reasonably well with the experimental results. Also, these results suggest that the light harvesting system does not act as a funnel to the RC, rather it is better described as a reservoir. The rationale for the reservoir picture becomes clear when one considers that RC's exist in two spectral forms. In the "open" form, the RC special pair is neutral and can utilise excitation toward an electron transfer. After the electron transfer, the RC is in the "closed" form with the special pair BChl being in a cation state and unable to utilise further excitation, until it is reduced by the uptake of an electron. To prevent overheating of the special pair from dissipation of the excess energy arriving while the RC is closed, the energy of the RC is lifted above that of the B875 BChl aggregate in LH1. Consequently, the back-transfer rate from the RC to LH1 becomes faster than the forward transfer rate from LH1 to the RC. If the RC is closed, excitation is returned to the LH1 complex, and thus, dissipation can be spread over a much larger area. The dissipation is even over larger area due to a transfer from LH1 to LH2 complexes and subsequent transfer between LH2 complexes. For the energy transfer between LH1 and RC, the rate constants available in literature vary between 1/35 to 1/50 ps (Bernhardt and Trissl 2000; Amesz and Neerken 2002).

The excitation of P in the RC leads to a sequence of electron transfer steps, initiated by formation of the P^+B^- state within about 3 ps, followed in about 1 ps by the P^+H^- state (back reaction time 3-6 ns). Within next 200 ps the electron is transferred to Q_A, while the $Q_A^- \rightarrow Q_B$ electron transfer occurs within hundreds of microseconds. The primary step is remarkably efficient with quantum yield near to unity (Wraight and Clayton 1974; Cohen Stuart and van Grondelle 2009 and references therein).

The sequence of reactions occurring in the RC is well established, although the exact role of monomeric BChl as well as the exact nature of the excited states involved is still a

matted of intensive research. Also, other possible charge separation pathways, not requiring the P*, have been discovered (van Brederode et al. 1999). Moreover, by introducing specific mutations of aminoacids in the vicinity of P and B the L and M subunits, the energy of P^+B^-states can be changed and substantial formation of $P^+H_B^-$ observed (Kirmaier et al. 2003 and references therein).

While the electron transfer in the RC proceeds through the cofactors situated in the L-polypeptide of the RC, with the exception, of Q_B, the M-dominated branch serves probably the photoprotective role. When the electron transfer is inhibited beyond H_A, then the radical pair decays to the ground state through internal conversion, fluorescence or intersystem crossing. The latter process leads to formation of the triplet state of primary donor. The danger of triplet states of (bacterio-)chlorophylls stems from the fact, that the molecules in triplet state may react with O_2, which is also a triplet in its ground state, producing highly reactive singlet oxygen, which in turn may damage the photosynthetic apparatus. The carotenoids are able to quench the (B)Chl triplet at much faster rate than triplet oxygen, thus preventing the cell structures from oxidative damage (Ke 2001).

3. Cytochromes

The cytochrome bc_1 complex is a large, three-subunit membrane protein. It consists of cytochrome b, cytochrome c_1 and the Rieske protein.

Cytochrome *b* is an eight-helix protein of molecular mass of 40-45 kDa. It noncovalently binds two heme cofactors, denoted cytochrome b_H and b_L, respectively. The cyt b_H (for "high") is located near the cytoplasmic side of the cell membrane and its redox potential is about +50 mV. The other cytochrome is located near the periplasmic side and its redox potential is about -100 mV, thus L for "low". Near to each hems there are sites for ubiquinone binding. The site labelled Q_i (i for "inside") is nearer to the high-potential heme. Here, the reduction of quinone occurs. The other site denoted Q_o ("oxidising" or "outer") lies closer to the low-potential heme and it is the place of ubiquinone oxidation.

The Rieske protein contains a 2F-2S iron-sulphur cluster located on the periplasmic side of the membrane with redox potential about +280 mV. Remarkably, this subunit is known to undergo a large scale motions during its catalytic operation, which is to reduce the cytochrome c_1.

Cytochrome c_1 is also located mainly at the periplasmic face of the membrane in which it is anchored to it by a single transmembrane helix. It receives electrons from Rieske centre and transfers it to the mobile cytochrome, c_2.

The overall catalytic reaction of the cytochrome bc_1 complex can be summed up as follows:

$$2UQH_2 + 2cyt_2^{ox} + UQ + 2H+ \rightarrow 2UQ + 4H+ + 2cyt_2^{red} + UQH_2$$

That is, in total two ubiquitous are oxidised, one molecule of ubiquinone reduced, along with two cytochromes c_2. Protons released by the ubiquinole oxidation are expelled to the periplasmic side and two are taken up from the cytoplasmic side during the reduction of ubiquinone (Crofts and Berry 1998; Crofts et al. 2008).

Cytochrome c_2 is a small soluble protein that covalently binds a single heme. Its purpose in photosynthetic reaction is to shuttle electrons between the cytochrome bc_1 complex

and the reaction centre. In addition to cytochrome c_2, variety of small mobile cytochromes is present in different species of purple bacteria (Nogi et al. 2005).

In some species of purple bacteria, another distinct class of c-type cytochromes is found. It forms a tightly bound subunit of the reaction centre. This subunit covalently binds four hemes that form a row with the lowest redox potential heme lying closest to the RC. This heme group can be oxidised by the primary donor within hundreds of nanoseconds and is in turn reduced by interheme transfers on microsecond timescale. The tetraheme cytochrome receives electrons from the mobile cytochromes, such as c_2. Bacteria that contain this subunit of the reaction centre are represented by e.g. *Blc. viridis* and *Rx. Gelatinosus* (Nogi et al. 2005).

4. Supramolecular organisation

In vivo, the protein complexes described above are embedded in the plasmatic membrane which usually forms invagination in the form of lamellae, vesicles or tubes. These are usually denoted the *intracytoplasmic membranes*. Upon disruption of the cells, these membranes fragment and form inside-out vesicles called *chromatophores*.

In last fifteen years, the focus has been shifting from single protein structures toward the *in vivo* organisation of the PSU, with the atomic force microscopy (AFM) studies playing a fundamental role. One of the obvious questions arising from the knowledge about operation of the electron transport chain of PNB is how does the Q_BH_2 pass through the LH1 towards the cytochrome bc_1 complex. It appears that the block of the electron transport lies between the ubiquinone and cytochrome bc_1 complex and the deletion of LH1 complex restores the capability for the photosynthetic growth (McGlynn et al. 1994; Pugh et al. 1998). In *Rb. sphaeroides* and a closely related species *Rb. capsulatus*, a gene called *pufX* was identified (Recchia et al. 1998), product of which is required for the photosynthetic growth (Lilburn and Beatty 1992; Lilburn et al. 1992). It has been proposed that PufX protein incorporates in the LH1 and permits the ubiquinone exchange (Barz et al. 1995a,b). The EM images of the negatively stained photosynthetic membranes of *Rb. sphaeroides* suggested that the two RC-LH1 complexes form a dimer in which the LH1 forms an open, horseshoe-shaped aggregate instead of circular (Jungas et al. 1999). The pictures also indicated existence of RC-LH1-cytochrome bc_1 supercomplex. It was suggested that the PufX plays a key role in this supercomplex formation and the general organisation of photosynthetic membrane (Frese et al. 2000). The existence of supercomplexes was also proposed before, based of the analysis of the flash-induced changes of the redox state of P and cytochromes (Joliot et al. 1989).

Study on wild type *Rb. sphaeroides* (Bahatyrova et al. 2004) showed that the RC-LH1 complexes indeed form dimers in this species. Moreover, these dimers seem to be arranged in linear arrays separated from each other by a row of LH2 complexes. Also, significant heterogeneity of organisation of photosynthetic membrane was observed. In some parts of the membrane samples of the above-described organisation was replaced with large LH2-only areas without RC-LH1 complexes. Interestingly, what was not revealed by these AFM images is the position of cytochrome bc_1 complex. Anyway, it seems rather obvious that it does not form the complex with the RC-LH1 dimer.

Interestingly, *pufX* has been found only in *Rb. sphaeroides* and *Rb. capsulatus*, however, it was proposed, that the W protein in *Rps. palustris* may be a PufX analogue. In contrast to results obtained by AFM, single-molecule spectroscopy studies on both the LH1 and RC-LH1 complexes from *Rsp. rubrum* strongly indicate that the LH1 is almost perfectly circular; moreover, the regularity of its shape is enhanced by presence of RC (Gerken et al. 2003).

In conclusion, function of PufX and its potential analogues remain a matter of debate while it became obvious that the "large scale" organisation may differ significantly even among closely related species that contain the same set of components of photosynthetic apparatus, also within single cell there are domains that differ in arrangement of protein complexes (Holden-Dye et al. 2008; Sturgis and Niederman 2008).

SELECTED ASPECTS OF OPTICAL SPECTROSCOPY

Methods of optical spectroscopy are of crucial importance in photosynthetic research. This section presents an introduction into the absorption spectroscopy and kinetics of fluorescence yield. The aim is specifically to facilitate understanding of chapters 2 to 5 of this work[*].

1. Basics of absorption spectroscopy

Potential energy of electrons bound in a molecule can assume only discrete levels. In order that the molecule can absorb incoming radiation, the amount of energy carried by photons must correspond to a difference between the energy levels, formally:

$$\Delta E_{2,1} = h\nu,$$

where ν is the frequency of the radiation and h denotes the Planck constant (6.626×10^{-34} Js). Upon absorption, the radiant energy is transformed into the electric potential energy of the valence electrons – the excited state is formed. General term for the inverse process when the electron moves from higher to lower energy level with accompanying emission of a photon is *luminescence*. Transition between energy levels is accompanied by changes of the spatial distribution of electrons which manifests itself as a transition dipole. Magnitude of this induced dipole determines the intensity of the absorption band. If the dipole cannot arise due to the symmetry reasons, the energy transition is forbidden and the light is not absorbed even if the condition of the energy difference is met.

Atoms in molecules are in perpetual periodic motion. Energies pertinent to these vibrational motions are also quantized with energy differences among these levels corresponding generally to photons of wavelengths lying in the micrometer regions. Non-radiational transition among vibrational energy levels is called *internal conversion*.

Absorptive properties of a substance are usually quantified using *absorbance* which is defined as:

$$A(\lambda) = -\log(I / I_o),$$

where I/I_o is the ratio of transmitted to incident intensity, or *transmittance*. The plot of

[*] This chapter is based mostly on texbooks of Hoppe et al. 1983, Prosser 1989 and Blankenship 2002. When cited, original research articles are referenced in text as appropriate.

absorbance versus the wavelength represents the absorption spectrum of the sample. According to the Beer-Lambert law, absorbance is proportional to the concentration of the absorbing substance (c) and the path length of the light through the sample (l):

$$A(\lambda) = \varepsilon(\lambda)cl,$$

where ε represents the molar *extinction coefficient*, which is the wavelength-dependent quantity that characterises the probability of electronic transition. Integral of extinction coefficient is proportional to the square of the transition dipole mentioned above.

Any factor that affects the electronic structure of molecules, be it a change of the redox state, chemical bonding or presence of external electric or magnetic fields is reflected in the changes of the absorption spectra. Absorption spectroscopy is thus a powerful tool for study of processes that occur on molecular level and spectroscopic methods have been crucial in photosynthesis research.

1.1. Practical aspects

Instrumentation

A spectrometer for measurement of absorbance in the ultraviolet to near-infrared region consists of a measuring light source, a monochromator for selection of light of given wavelength, sample compartment and a detector. Usually, liquid samples placed in transparent cuvettes are analysed.

Since 1950's, measurement and analysis of difference spectra has been a staple approach in studies concerning the function of photosynthetic apparatus. The difference spectra are obtained by subtracting the absorbance spectra of the same sample acquired before and after a treatment. Such spectrum thus contains only contribution of molecules that have underwent some kind of change as result of the perturbation. Naturally, in photosynthesis research, study of light-induced changes is the most common approach.

Several requirements must be met by an instrument to be used in research into photosynthetic reactions. The first condition is high sensitivity. Samples of photosynthetic material contain a large amount of pigments, while only a small fraction actually undergoes a change – e.g. only the chlorophyll of the primary donor of the reaction centre is oxidised by light while to each reaction centre there may be thousands of pigment molecules in the light harvesting complexes. Thus the overall change of absorbance can easily be as low as 0.01 % even if all reaction centres are oxidised. Further requirement concerns the temporal resolution. The early electron transfer steps in the reaction centre occur on the picosecond timescale. The excitation transfer processes are even faster (femtoseconds). Such processes can be resolved using pulsed lasers as actinic and measuring light sources. Thirdly, since photosynthetic machinery is naturally selected for high efficiency of light utilisation, the intensity of the measuring beam has to be kept as low as possible not to disturb the sample during recording of the spectra. Also, isolated components of photosynthetic apparatus are very sensitive and low light exposure is necessary to avoid production of artefacts due to photodamage.

There are two concepts of spectrometer construction that address these problems. Either the spectra are recorded using monochromic light in a sequential manner, or a pulse of

polychromic light is applied to the sample and the spectral analysis is carried out after passing of the measuring pulse through the sample. The advantage of the former approach lies in the low exposure and simpler detection. The drawback of this approach is the necessity of stepwise recording of the spectrum which is time consuming and brings about the danger of time-dependent drifts introducing errors in the spectra. The latter approach reduces this problem, while it requires higher intensity of measuring light and more complex, multichannel detectors. The experimental setup presented in the chapter 2 is of the multichannel type. Also, a more detailed discussion of the spectrometer construction is given in chapter 2.

Problems

Several possible pitfalls have to be kept in mind while recording spectra of photosynthetic material. The Beer-Lambert law presented above assumes that the sample consists of homogeneously distributed of noninteracting absorbing particles dispersed in medium that does not cause light scattering. Moreover, the intensity of incident radiation is assumed to be low enough not to disturb the energy equilibrium of the sample. While the latter requirement is quite easily met by using a measuring radiation of low intensity and this condition is practically violated only in cases where laser beams are used, meeting the first two points in real samples of photosynthetic material posses a more complicated task, especially in the case of measurements performed on whole cells.

Samples of algae and bacteria in fact consist of small particles that contain a high concentration of pigments that are dispersed within a medium of low absorbance rather than being homogeneous solutions of pigments. The results of such inhomogeneity is that a part of the measuring beam passes through the medium missing the cells while the remaining part that hits the particles is very strongly absorbed. This is known as the *sieve effect*. Its consequence is the apparent flattening of the peaks in the absorption spectrum. Thorough discussion of this phenomenon is given in the work of Duysens (1956).

Scattering prevents light from reaching the detector, thus increasing apparent absorbance. It typically increases at shorter wavelengths, although the exact relation between the wavelength and intensity of scattering depends on the size of the particles. Also, in chloroplasts and bacteria, a time dependent, light-induced changes of light scattering have been observed (see chapter 4). In a standard absorption spectrometer, the approach to reduce scattering is to place the sample as close to the detector as possible. The

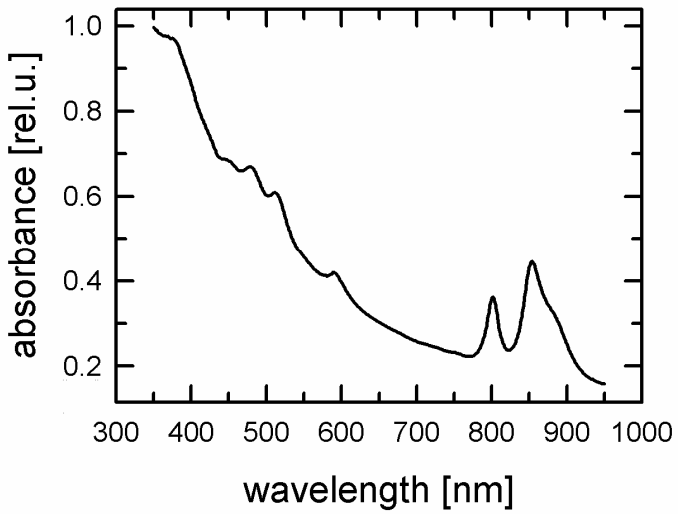

Fig. I.1 Absorption spectrum of whole cells of purple bacterium *Rhodobacter sphaeroides*

effect of light scattering is illustrated on the sample of suspension of bacterial cells shown in Figure I.1; compare this figure with the spectra of isolated membranes presented in Figure I.2b, that are practically free from the scattering.

Moreover, samples containing photosynthetic pigments are usually fluorescent. When the fluorescence reaches the detector, it causes apparent lowering of absorbance. Since the fluorescence emission is homogenously distributed into all directions in non-oriented samples, its negative effect can be lowered by increasing the distance between sample and detector in an apparent trade off with the elimination of influence of light-scattering. The more practical way is to obtain the recording of the stray fluorescence in separate measurement and subtract it from the final recordings. This is especially important in experiments involving intense actinic light sources such as the gas discharge flash lamps.

1.2 Origins of absorbance features common in photosynthetic material

Types of absorbance changes commonly observed in photosynthetic samples will now be reviewed specifically with respect to following chapters. Unless indicated, sample spectra come from our measurements. For comparison, reader is referred to monographs, such as Ke (2001).

Intermolecular interactions Intermolecular interactions determine to a great extent the absorbing properties of photosynthetic samples. Purple bacteria offer a striking example of this effect since only one type of tetrapyrol pigment (BChl *a* in the case of *Rb. sphaeroides*) is used here for both light harvesting and as the primary donor in RC. As shown in Figure I.2, while the redmost maximum of isolated BChl *a* lies around 770 nm in acetone, the non-covalent binding to protein and pigment intermolecular inter-actions give rise to several absorption bands in the infrared region. It has been shown that bands of BChl *a* bound to pigment-protein complexes may lie beyond 950 nm (Permentier et al. 2001).

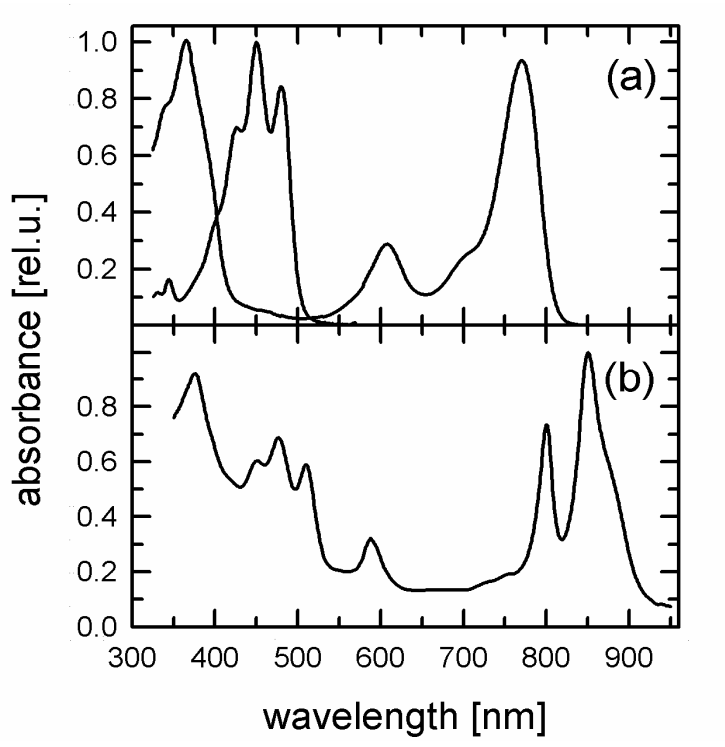

Fig. I.2 a Absorption spectra of bacteriochlorophyll *a* and spheroidene in solution. **b** Spectrum of chromatophores isolated from *Rhodobacter sphaeroides*

Redox reactions These form the basis of operation of the photosynthetic apparatus. Conversion of pigment molecules to ions is manifested by decrease (bleaching) of their absorption bands and appearance of peaks pertinent to energy transitions of molecular ions. In Figure I.3, we show the light-induced

difference spectrum of purple-bacterial RC.

Measurement was performed on RC without additions thus the figure shows the difference between the spectrum of light-induced $P870^{+}Q_A^{-}$ state and the $P870^{0}Q_A^{0}$ state. Originally the spectrum consists of three bands: BPheo at about 750 nm, accessory BChl *a* around 800 nm and the primary donor BChl *a* around 870 nm. Upon excitation, primary donor is oxidised, consequently its band bleaches, which manifests as negative band in difference spectrum. Other changes visible in the spectrum are due to presence of electric charges affecting absorption of accessory BChl and BPheo. These will be explained later.

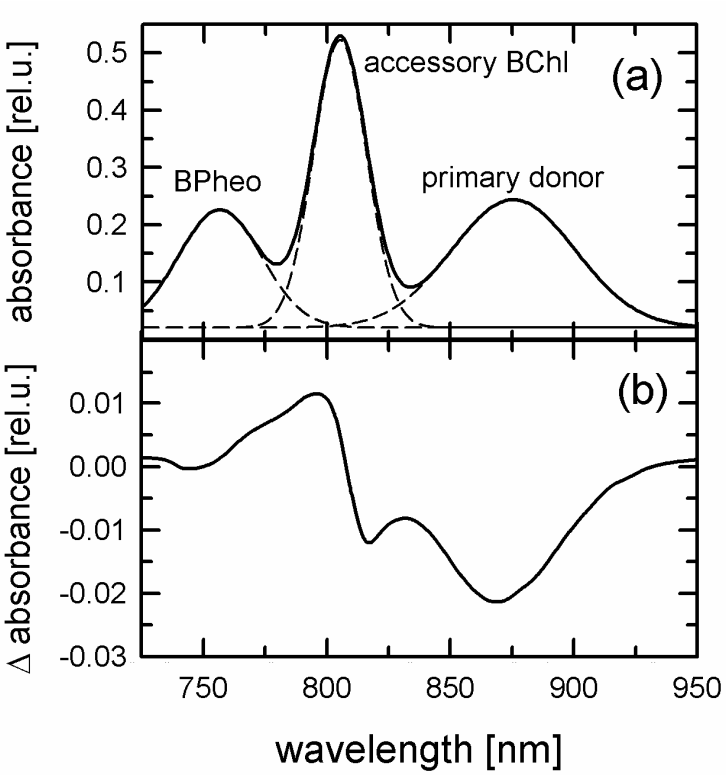

Fig. I.3 a Absorption spectra of the reaction centre of *Rhodobacter sphareoides* with indication of components. **b** Light-induced difference spectrum of the reaction centre

Redox reactions of cytochromes can also be monitored using bleaching of heme absorption bands. This is illustrated in Figure I.4 that shows oxidised minus reduced spectrum of both *b*- and *c*-type cytochromes.

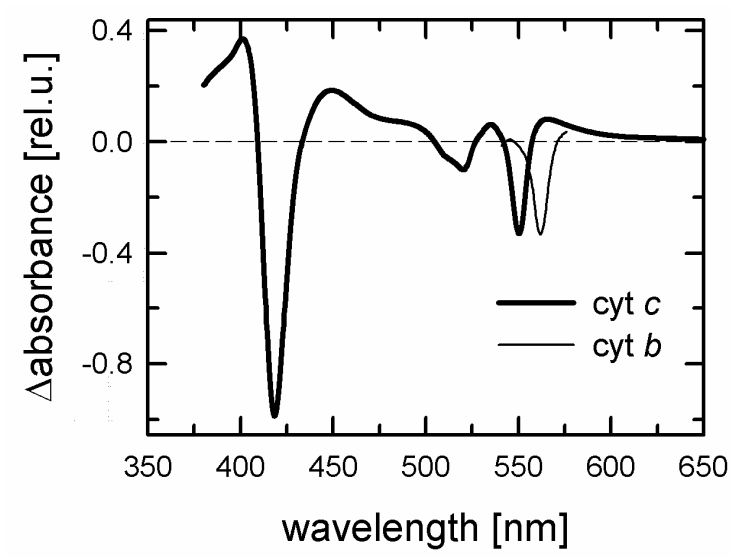

Fig. I.4 Oxidised minus reduced difference spectra of cytochromes. Adapted from http://www.medphys.ucl.ac.uk/research/borg/research/NIR_topics/spectra/spectra.htm

Electrochromism Another important type of absorbance changes occurring in photosynthetic samples arise due to the effect of electric fields produced by the electron transfer reactions and proton translocation. When a molecule is placed in electric field, its energy levels are changed, which results in shift of the absorption bands. This is known as the *Stark effect*. In photosynthetic apparatus,

pigment molecules are mostly bound to proteins that are embedded in membrane and the transfer of charges proceeds in fixed direction with respect to the components of the photosynthetic machinery, e.g. protons are transferred from cytoplasmic to periplasmic side of the membrane, thus parallel to the axis of subunits of light-harvesting complexes. Such situation gives rise to typical derivative-shaped features in the absorbance spectra that are due to parallel (electrochromic) shift of absorption bands of number of pigments. Extent of the shift, usually expressed as the difference between the peak and trough of the electrochromic bands, can be used to quantify the magnitude of the electric charge. This use of the absorbance changes is simplified by the fact that for many pigment-protein complexes, the electrochromic shift is linearly proportional to the electric field that induced it. This property is quite unexpected since for nonpolar molecules, such as carotenoids, the theory predicts only quadratic dependence of the shift of absorption bands on the intensity of the electric field. Indeed, this is the case of isolated pigments. It was proposed that large local electrostatic charges polarise the pigments and allow the linear electrochromic shifts (Reich and Sewe 1977; Sewe and Reich 1977).

Electrochromic shift to the blue of the accessory BChl *a* in the reaction centre caused by the presence of the oxidised primary donor can be observed in Figure I.3. This is the derivative-shaped feature centred at 800 nm. The bands around 780 nm appear due to the electroch-romic effect of Q_A^- upon BPheo (Tiede et al. 1996).

In Figure I.5, we present possibly the most famous example of electrochromic signal in photosynthetic samples: carotenoid bandshift observed in purple bacteria. This effect arises as a gradual shift of absorption bands of a subset of antenna-bound carotenoids which is proportional to the magnitude of the membrane potential. (Jackson and Crofts 1969; de Grooth and Amesz 1977; Hellingwerf et al. 1988). The first derivative of the absorption spectrum of the bacterial photosynthetic membrane is presented for comparison.

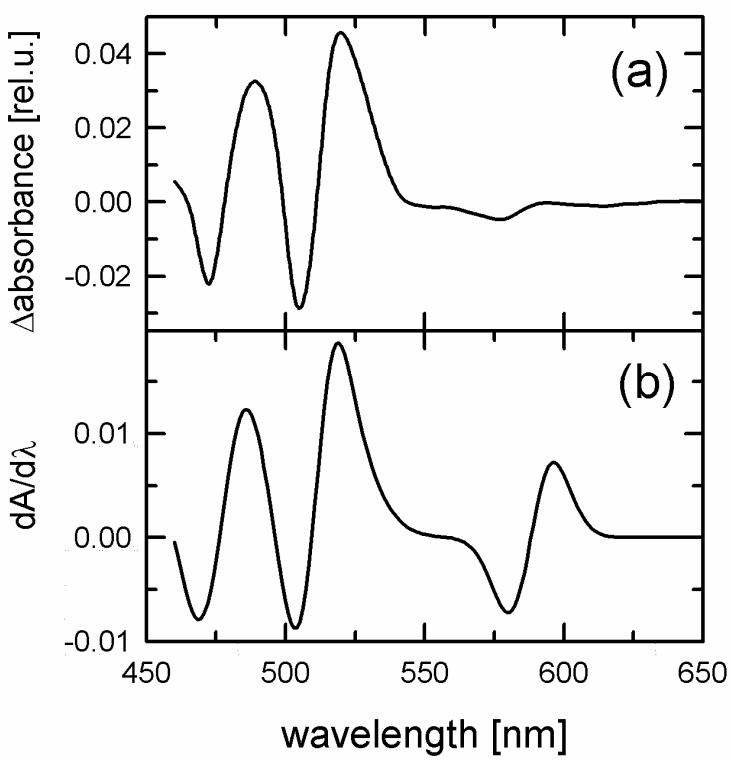

Fig. I.5 a Electrochromic shift of carotenoids in chromatophores of *Rhodobacter sphaeroides*. Note also the electrochromic shift of BChl *a* around 600 nm. **b** First derivative of the 450-750 nm region of the chromatophore absorption spectrum from the figure I.2b.

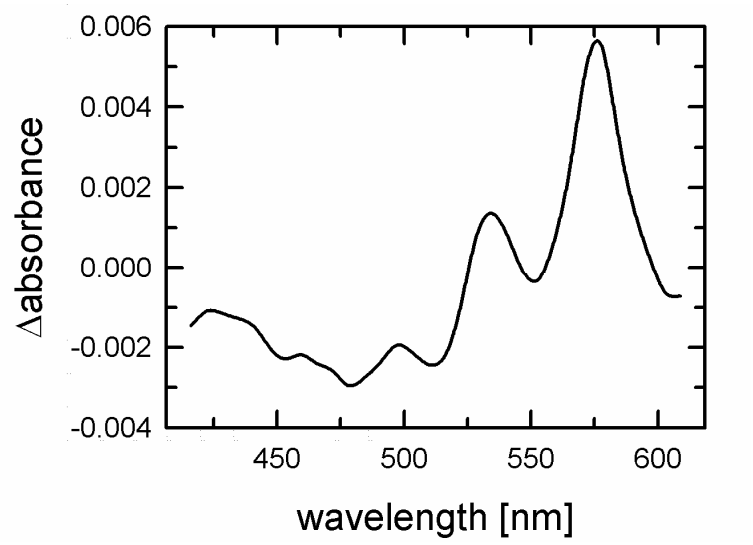

Fig. I.6 Triplet minus singlet difference absorption spectra of carotenoid in the LH2 antenna of purple sulfur bacterium (*Thiocapsa sp*)

Triplet states According to *Pauli exclusion principle*, two electrons occupying the same orbital must have *spins* (thus their magnetic moments) of opposite directions. Such state is denoted *singlet*. However, once one of the electrons is propelled into the excited state, the particles no longer share the same orbital and their spins are free to change to the same direction. This gives rise to a so called *triplet* state. Formation of triplet states is accompanied by bleaching of the bands corresponding to the singlet-singlet transitions and appearance of bands that correspond to transitions between triplet state energy levels. This is illustrated in the Figure I.6 that shows the spectroscopic signature of carotenoid triplet state formation in antenna of purple bacteria (a triplet minus singlet spectrum). It shows the bleaching of the carotenoid triple peak pertinent to the singlet-singlet transition, and appearance of a new, band at its red side (around 570 nm), which is due to triplet-triplet transition (cf Monger et al. 1976).

2. Fluorescence

Measurements of absorption of electromagnetic radiation do not exhaust the range of spectroscopic techniques. Another set of powerful methods represent those based on analysis of radiation emitted by molecules.

Among the processes that allow the excited molecule to release the energy acquired by the absorption and return to the ground state are also those that involve emission of photon. The emission which accompanies the transition between singlet states is called *fluorescence*.

Spectral properties of fluorescence are mainly determined by the fact, that in most cases, internal conversion proceeds much faster that the emission, thus part of the absorbed energy is quickly converted to vibration energy. Consequently, the energy content of photons emitted is lower that photons absorbed and the maximum of the emission band is red-shifted with respect to the absorption band, this difference is known as the *Stokes shift*. This is also the case when the molecule is excited into the higher energy states. For example, in chlorophylls, both absorption of red (1st excited state) and blue (2nd excited state) light results in the same red fluorescence emission.

There are two basic approaches to spectrally resolved fluorescence measurements. In the first case, the emission is measured at a fixed wavelength. The wavelength of the exciting beam is changed resulting in a plot of intensity of emission versus the excitation wavelength – the excitation spectrum. Such spectra are instrumental in analysis of energy transfer between pigments. Without energy transfer, the excitation spectrum corresponds to the absorption spectrum. However, when some other pigments are present which absorb and transfer energy to the one whose emission is detected, their absorption bands appear in the excitation spectrum and the efficiency of the transfer can be quantified. The other experimental approach, based on scanning the emission wavelength with the excitation fixed, yields the emission spectrum. Such spectra are useful e.g. for identification of chemical composition of samples.

Apart from the spectra, an important quantity that characterises fluorescence emission is its quantum yield which equals the ratio of photons emitted to absorbed. The yield can also be computed using the rate constants of all processes that compete for the relaxation of excited state. For the pigments found in the photosynthetic apparatus, the main pathways are photochemistry (P), energy transfer (ET), internal conversion (IC), which converts the excited state energy into vibrational energy of the molecule, and intersystem crossing (ISC), which gives rise to triplet states, and fluorescence (F). Thus for fluorescence yield one can write:

$$F = \frac{k_F}{k_P + k_{ET} + k_{IC} + k_{ISC} + k_F}$$

From the physiological point of view, fluorescence is irrelevant for photosynthetic organisms and its yield is only a few percents. On the other hand, practical importance of fluorescence as a probe into photosynthetic machinery is immense. Indeed, time-resolved measurements of fluorescence yield have become one of the staple methods in plant physiology and biophysics. Principles of these methods will be explained in the following sections.

It has been long known that when a photosynthetic organism is suddenly illuminated, changes in the (bacterio-)chlorophyll fluorescence yield are observed. Time course of these changes is known as the fluorescence induction curve or Kautsky curve (e.g. Govindjee 1995). During the induction curve fluorescence yield starts at low level, denoted F_o and gradually, in several intermediate steps, rises towards maximal level, F_M. Under normal conditions, this maximum is usually followed by a multi-step decrease towards a steady state level. The low, F_o level represents the state in which the photosynthetic apparatus is ready to utilise the incoming energy with maximal efficiency. This means that the primary donors of the reaction centres are reduced and primary quinone acceptors are oxidised. Such RC's are labelled *open*.

Under illumination the electron transport starts to operate, which brings about transient oxidation of primary donors and reduction of acceptors. The RC, in which either the primary donor was oxidised and/or the primary acceptor reduced, so that the energy delivered from the light-harvesting complexes cannot be utilised for the charge separation, is labelled *closed*. In such RC the excitation returns to the antenna and thus the probability of it being emitted as

fluorescence is increased. When all available reaction centres have been closed, the maximal fluorescence yield F_M is reached.

Hence in the first approximation, the changes in fluorescence yield monitor changes in the proportion of open and closed reaction centres. Values F_o and F_M together allow estimation of the maximal yield of photochemistry.

In reality, however, other processes take place in the photosynthetic apparatus, which change the energy flows and thus change the yield of fluorescence. On one hand there are regulatory mechanisms that balance the thermal dissipation in the light-harvesting complexes with the flow of energy to the reaction centres to protect these from the damage from excess energy. On the other hand there also processes that result from the damage of the photosynthetic apparatus. Both types of these processes increase the thermal dissipation and lower the fluorescence yield, thus they are denoted as *nonphotochemical quenching*. Extent of effect of these processes upon the energy conversion can also be quantified using fluorescence yield measurements (e.g. Roháček and Barták 1999).

2.1. Practical considerations

Experimental approaches to the fluorescence measurements usually utilise combination of pulsed as well as continuous illumination to achieve defined state of the sample, which is crucial for the interpretation of the obtained values of fluorescence yield. Usually, weak pulsed measuring radiation is applied to measure the fluorescence yield and the actinic illumination, in the form of continuous light or intense light flashes is used to drive photosynthetic reactions. The logic behind this approach is following. The reaction centre acts as a two electron gate and has to receive two excitations in order to initiate the electron transport, before charge recombination occurs and the centre returns to the open state. If the radiation that strikes the sample is delivered in short pulses of low intensity, the probability that any reaction centre carries out second charge separation quickly enough to produce the doubly reduced quinone acceptor is very low. Consequently, the long term effect of the measuring radiation on the photosynthetic apparatus is negligible. Since the intensity of the pulses is kept constant, the intensity of fluorescence induced by measuring pulses is proportional to F_o. When a strong actinic illumination is applied along with measuring pulses, photosynthetic reactions take place and the intensity of fluorescence emitted from the sample increases. The stray fluorescence due to the actinic light is subtracted and the signal actually recorded is only the response to constant measuring pulses. Consequently, this value remains proportional to the fluorescence yield.

Strictly speaking, measurement of the fluorescence yield would require that the overall emission of the sample is detected in all directions. This is however difficult to achieve, especially in the case of in vivo measurement, with for example the emission directed into the leaf being completely out of the reach of the detector. Hence only ratios, such as F_o/F_M are usually analysed, rather than absolute values. Also, the absorption of the sample remains constant during the course of measurement.

When compared to absorption measurements, measurements of fluorescence yield represent a significantly simpler approach to the investigation into workings of the

photosynthetic apparatus. Firstly, transparency of the sample is not required as fluorescence can be detected from the surface. This allows remote sensing and also spatially-resolved measurements of photosynthetic efficiency of whole plants or even the whole canopy.

Moreover, light scattering does not pose such problem as in absorption spectroscopy and spectral analysis is not required, provided that appropriate filters are applied to select the fluorescence wavelength. At the same time, the fluorescence yield, can be easily measured in times spanning several orders of magnitude, while it contains information on the processes ranging from the primary excitation transfer and charge separation efficiency to the operation of the regulatory processes within the membranes. This relative simplicity of the fluorescence measurements accompanied with the richness of information it carries is naturally one of the factors behind continuing appeal of the method for researches in the fields ranging from biophysics to plant ecology.

The other side of the coin is that all processes within the photosynthetic machinery are encoded into a two-dimensional signal, which brings along substantial difficulties for the interpretation of the fluorescence yield measurements. Often other methods, such as gasometry and – if possible – absorption spectroscopy are still to be applied in order to simplify interpretation of fluorescence signal and extract valuable information from it. In recent years, instruments that offer the possibility of parallel measurement of fluorescence and specific absorbance changes, although not of whole spectra, have become available commercially (see www.bio-logic.info, www.walz.com).

OUTLINE OF THE THESIS

Chapter 2 is concerned with a novel instrument that allows simultaneous measurement of absorbance changes and fluorescence yield. The device was conceived and constructed by Dr. Pavel Šiffel at the Department of Photosynthesis of the Institute of Plant Molecular Biology, Academy of Sciences of the Czech Republic. A description of the device is given and examples of its possible applications in studies of bacterial and plant systems are reviewed. Following chapter presents a study of the nonphotochemical fluorescence quenching in reaction centres isolated from plants in relation to the reduction of pheophytine. It fully illustrates the advantages of combined analysis of simultaneously acquired absorbance and fluorescence signals.

This experimental approach is further extended from isolated complexes toward the scale of whole organism in chapters 4 and 5 that present study of whole cell of purple bacteria. Although functioning of the electron transport chain in these organisms has been thoroughly studied using both absorbance and fluorescence (e.g. Joliot et al. 1989; 2005; Mascle-Allemand et al. 2008) and to some extent also mathematical modelling (Geyer et al. 2006; Klamt et al. 2008), most of the studies focus either on isolated membranes or whole cells under strict experimental conditions. In contrast, we aimed at obtaining data that would characterise functioning of the photosynthetic machinery in its native state. Chapter 4 contains a detailed study of both the absorbance changes associated with the *in vivo* operation of the electron transfer chain in *Rhodobacter sphaeroides* in the second to 1-minute time

range. Moreover, analysis of the factors that determine the energy conversion is provided based on the measurements of fluorescence yield. A simple model describing the relation between the state of the electron transport chain and the fluorescence yield is introduced. Chapter 5 supplements the results presented in chapter 4 with characterisation of the early events in the absorbance and fluorescence transients on the millisecond time scale.

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II. New multichannel kinetic spectrophotometer-fluorimeter with pulsed measuring beam for photosynthesis research

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ABSTRACT

A multichannel kinetic spectrophotometer-fluorimeter with pulsed measuring beam and differential optics has been constructed for measurements of light-induced absorbance and fluorescence yield changes in isolated chlorophyll-proteins, thylakoids and intact cells including algae and photosynthetic bacteria. The measuring beam, provided by a short (2 μs) pulse from a xenon flash lamp, is divided into a sample and reference channel by a broad band beam splitter. The spectrum in each channel is analyzed separately by a photodiode array. The use of flash measuring beam and differential detection yields high signal-to-noise ratio (noise level of 2×10^{-4} in absorbance units per single flash) with negligible actinic effect. The instrument covers a spectral range between 300 and 1050 nm with a spectral resolution of 2.1, 6.4 or 12.8 nm dependent on a type of grating used. The optical design of the instrument enables measuring the difference spectra during an actinic irradiation of samples with continuous light and/or saturation flashes. The time resolution of the spectrophotometer is limited by the length of Xe flash lamp pulses to 2 μs.

Překlad abstraktu:

Zkonstruovali jsme mnohokanálový kinetický spektrofotometr-fluorimetr s pulsním měřicím svazkem a diferenciální optikou pro měření světlem indukovaných změn absorpce a fluorescence v izolovaných chlorofylových proteinech, tylakoidech a intaktních buňkách včetně řas a fotosyntetických bakterií. Měřicí svazek, poskytovaný krátkým (2 μs) zábleskem xenonové výbojky, je rozdělen širokopásmovým děličem svazků do vzorkového a referenčního kanálu. Spektrum každého kanálu je samostatně analyzováno řadou fotodiod. Použitím pulzního měřicího svazku a diferenciální detekce je zajištěn vysoký poměr signál/šum (šum činí 2×10^{-4} absorpčních jednotek na jeden záblesk) se zanedbatelným aktinickým efektem. Přístroj pokrývá spektrální oblast 300 – 1050 nm se spektrálním rozlišením 2.1, 6.4 nebo 12.8 nm v závislosti na použité difrakční mřížce. Konstrukce přístroje umožňuje měření rozdílových spekter v průběhu aktinického ozáření vzorků prostřednictvím kontinuálního nebo pulsního světla. Časové rozlišení spektrofotometru je limitováno délkou záblesků xenonové lampy na 2 μs.

Autorský podíl:

David Bína má na publikaci podíl 30 %.

III. Conformational changes and their role in non-radiative energy dissipation in photosystem II reaction centres

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ABSTRACT

Accumulation of reduced pheophytin in photosystem II under illumination at low redox potential is known to be accompanied by a pronounced decrease of a chlorophyll fluorescence yield. Simultaneous measurement of this fluorescence quenching and absorbance changes in photosystem II reaction centres, in the presence of dithionite, showed each event to have a different temperature dependence. While fluorescence quenching was suppressed more than 20 times when measured at 77 K, pheophytin accumulation decreased only 5 times. At 77 K, the fluorescence was quenched considerably, but only in those reaction centres where reduced pheophytin had been accumulated at room temperature before sample freezing. This showed that the accumulation of reduced pheophytin above 240 K was accompanied by an additional, most probably conformational, change in the reaction centre that substantially enhanced non-radiative dissipation of excitation energy.

Překlad abstraktu:

Je známo, že akumulace redukovaného feofytinu ve fotosystému II osvětleném za nízkého redoxního potenciálu je provázena významným poklesem výtěžku fluorescence chlorofylu. Simultánní měření tohoto zhášení fluorescence a absorpčních změn v reakčních centrech fotosystému II v přítomnosti dithionitu ukázala rozdílnou teplotní závislost těchto dvou jevů. Zatímco zhášení fluorescence bylo při 77K potlačeno více než dvacetkrát, akumulace feofytinu poklesla pouze pětkrát. Při 77K byla fluorescence významně zhášena pouze v reakčních centrech, kde byl redukovaný feofytin akumulován při pokojové teplotě před zmrazením vzorku. To ukazuje, že akumulace redukovaného feofytinu při teplotě přes 240K je provázeno další, pravděpodobně konformační, změnou v reakčním centru, která významně zvyšuje míru nezářivé disipace excitační energie.

Autorský podíl:

David Bína je druhým autorem článku, jeho podíl tvoří 20 %.

IV. Kinetics of *in vivo* bacteriochlorophyll fluorescence yield and the state of photosynthetic apparatus of purple bacteria

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ABSTRACT

The light-induced electron transport in purple bacterium *Rhodobacter sphaeroides* was studied *in vivo* by means of kinetic difference absorption spectroscopy and kinetics of bacteriochlorophyll fluorescence yield. Measurements of redox state of the oxidised primary donor and cytochrome *c* and the membrane potential revealed a complex pattern of changes of the electron flow. Effects of the membrane potential on the fluorescence yield were also analysed and a model for the fluorescence induction curve is presented. The data indicate substantial positive effect of the membrane potential on the fluorescence emission *in vivo*. Moreover, light-induced changes in light scattering were observed, which suggests occurrence of structural changes on the level of the photosynthetic membrane.

Překlad abstraktu:

Pomocí diferenční absorpční spektroskopie a kinetiky fluorescence bakteriochlorofylu jsme studovali elektronový transport in vivo u purpurové bakterie *Rhodobacter sphareoides*. Měření redoxního stavu primárního donoru a cytochromu *c* naznačují, že během ozáření dochází k významným změnám v rychlostech toku elektronů. Také jsme analyzovali vliv membránového potenciálu na výtěžek fluorescence a předkládáme matematický model fluorescenční indukční křivky. Získaná data ukazují významný kladný vliv membránového potenciálu na výtěžek fluorescence. Dále jsme pozorovali změny rozpytlu světla vyvolané aktinickým zářením, což naznačuje že ve vzorku dochází ke strukturálním změnám na úrovni fotosyntetické membrány.

Autorský podíl:

David Bína je prvním autorem této publikace, jeho podíl tvoří 80 %.

V. Absorbance changes accompanying the fast fluorescence induction in the purple bacterium *Rhodo-bacter sphaeroides*.

this chapter has not been published before

ABSTRACT

We present a study of the fluorescence and absorbance transients occurring in the whole cells of purple nonsulfur bacterium *Rhodobacter sphaeroides* on the millisecond timescale under pulsed actinic illumination. The fluorescence induction curve is interpreted in terms of combination of effects of redox changes in the reaction centre and the membrane potential. Present results support the earlier observations of the positive effect of the membrane potential on the fluorescence yield. Advantages of the pulsed actinic illumination for study of the operation of the electron transport chain *in vivo* are discussed.

Překlad abstraktu:

Predložená práce studuje přechodové jevy ve fluorescenci a absorpčních změnách u purpurové nesirné bakterie *Rhodobacter sphareoides*, měřené na milisekundové časové škále s využitím pulsního aktinického záření. Průběh fluorescenční indukční křivky je vysvětlen kombinací vlivu redoxních změn v reakčním centru a membránového potenciálu. Tyto výsledky podporuijí předchozí pozorování pozitivního účinku membránového potenciálu na výtěžek fluorescence. Také předkládáme diskusi výhod pulsního aktinického záření pro studium fungování řetězce prenosu elektronů *in vivo*.

Tato kapitola nebyla dosud publikována

VI. Summary

In photosynthetic apparatus, light is used to drive a sequence of electron transfer steps. These may terminate by reduction of coenzymes used for synthetic reactions, such as assimilation of CO_2, and are also coupled to proton translocation across membranes. This generates membrane potential used to fuel synthesis of ATP which covers the energy requirements of the cell metabolism. At the heart of the molecular machinery performing the electron transport lie reaction centres. These are pigment protein complexes that perform the light-induced charge separation whereby the electron is rapidly transferred across the distance comparable to the thickness of the membrane from a primary donor (bacterio)chlorophyll molecule to an acceptor molecule. In the reaction centres found in purple bacteria and oxygenic organisms, the stable acceptor in the reaction centre is a molecule of quinone. In these reaction centres, changes of the redox state of the molecules involved in the electron transport are accompanied by changes of the intensity of the induced (bacterio)chlorophyll fluorescence. The yield of the emission is directly related to the efficiency of the energy transformation in the reaction centre. Hence, measurement of the (bacterio)chlorophyll fluorescence yield presents us with a tool for investigation of the functioning of the electron transfer in photosynthetic organisms.

Moreover, changes in redox state of molecules involved in photosynthetic electron transport are accompanied with characteristic changes in their absorption spectra. Thus, measurement of time-dependent changes of absorbance provides another possibility for studying the kinetics of electron transfer. Consequently, both absorption and emission spectroscopy have been staple methods in photosynthesis research.

This thesis presents a description of a laboratory-built instrument that provides its user with the possibility to measure both absorbance changes and fluorescence yield on transparent samples of photosynthetic material with the temporal resolution of microseconds and spectral resolution of nanometres while covering the visible to near infrared range of wavelengths. The sensitivity of the instrument in the units of absorbance is in the order of 10^{-4} even in the single shot mode, where the data are not obtained by accumulation and subsequent averaging.

To illustrate the utility of the combined fluorescence and absorbance measurements as well as the capability of the experimental setup, a study of fluorescence quenching in the isolated reaction centres is presented. Here, the analysis of changes of fluorescence yield with respect to the extent of the reduction of pheophytine at cryogenic temperatures revealed an effect of conformational changes on the energy dissipation in the reaction centre.

The second part (chapters 4 and 5) of this thesis presents studies concerning the electron transfer in whole, living cells of purple bacterium *Rhodobacter sphaeroides*.

Photosynthetic apparatus of purple bacteria is significantly simpler as compared to oxygenic organisms. In oxy-phototrophs, two types of reaction centres operate in series to perform both reduction of coenzymes for synthetic reactions and the generation of the membrane potential. Electrons for reductions are extracted from water. In contrast, in purple

bacteria we find only one type of reaction centre, which in cooperation with the cytochrome *bc* complex performs a cyclic electron transport. Mobile quinones in membrane carry electrons from the reaction centre toward the cytochrome bc_1 and mobile cytochromes c_2 in the periplasmic space bring them back to the photo-oxidised bacteriochlorophyll in the reaction centre. Final product of this process is solely the membrane potential utilised to synthesise ATP.

This thesis presents a comprehensive study of changes of absorbance and fluorescence yield that accompany the operation of the bacterial electron transport chain under constant and pulsed actinic illumination. Induction curves of redox changes of reaction centre, c-type cytochromes as well as the build-up of the membrane potential are presented and analysed. The time domain covered ranges form millisecond to minutes. This represents a novel insight into the operation of bacterial photosynthetic apparatus *in vivo*. The study of kinetics is accompanied by detailed analysis of factors influencing the fluorescence emission. The data support the hypothesis that fluorescence yield in purple bacteria is under partial control by the membrane potential, a fact that has to be taken into account when the fluorescence is used as a sole source of information on the electron transport.

In addition to that, data measured on bacteria further illustrate the capabilities of the experimental setup presented in the chapter 2. The data show the possibility of extending its application from homogeneous samples of suspensions of isolated pigment-protein complexes to the whole living cells of photosynthetic organisms, that is, samples that exhibit considerable light scattering properties as well as quite unfavourable ratio of photoactive pigments to total pigment content.