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INVESTIGATIONS OF CHLOROPHYLL INTERACTIONS IN WATER SOLUBLE CHLOROPHYLL BINDING PROTEIN

Master thesis (30 EAPs)
Applied Measurement Science

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LIST OF ABBREVIATIONS

chl	chlorophyll
FLN	fluorescence line narrowing
FWHM	full width at half maximum
PSB	phonon side band
SHB	spectral hole burn
WSCP	water soluble chlorophyll binding protein
ZPL	zero phonon line

1. INTRODUCTION AND LITERATURE OVERVIEW

Photosynthesis is the process on earth that transforms light energy from the sun into storable energy in form of sugar. This is accomplished by capturing photons in antennae complexes binding chlorophyll (chl) pigment molecules. But these complexes also channel the energy radiationlessly from one chlorophyll to another and, eventually, into the reaction centre, where the light energy can be used.

But the investigation of chlorophyll interactions in naturally abundant light harvesting complexes is an extremely difficult task. There are 42 chl's in trimeric light harvesting complex II which is the most abundant antenna complex of green plants [1]. This complexity makes it extremely difficult to distinguish the contribution of single molecules within the complex spectra. Furthermore, the light harvesting complexes are embedded into the thylakoid membrane and detergents have to be applied to isolate them from the membrane in order to carry out experiments [1]. This adds an uncertainty component in those experiments, whether the complexes are kept in their native state or not.

1.1. Water Soluble Chlorophyll Binding Protein

Experiments on small molecules binding only a few chlorophylls are more favourable for studies of individual pigment-pigment and pigment-protein interactions. One such protein is water soluble chlorophyll binding protein (WSCP). It is most likely a carrier protein responsible for extraction of chlorophylls and delivery to catabolism enzymes [2,3]. This is part of the necessary turn over in biological systems. As the name states, this protein is soluble in water, therefore its natural state is granted in experiments. And it carries 2 to 4 chlorophyll molecules only, which makes interpretation of the obtained data much easier [4] and allows us to use WSCP as a model system to study properties of chls embedded in a protein matrix [5].

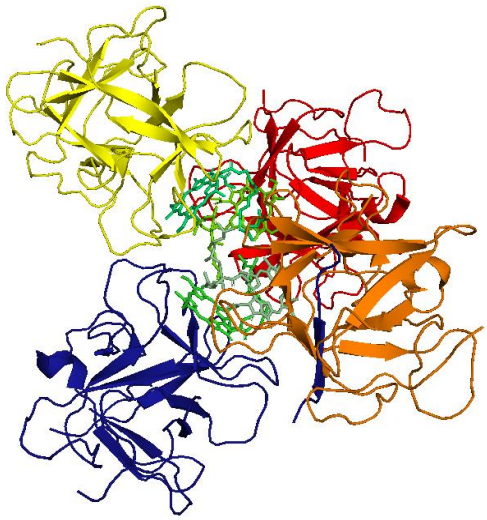

Figure 1: Crystal structure of lepidium virginicum WSCP, showing a homo-tetramer including 4 chls a molecules in the binding pocket [5]

One WSCP subunit has a molecular weight of 24 kDa. But it is only active when bound together as a homo-tetramer which forms a tight binding pocket for 4 chl molecules in total. A single protein can extract a chlorophyll, but the transport takes place only in the associated form. Every type of chlorophyll can be bound, but there are preferences for different species [personal communication with Daniel Palm]. The crystal structure of WSCP from *lepidium virginicum* (Figure 1) was obtained in 2007 using a mixture of chl a and chl b, but the resolution of 2 Å was too low to resolve the difference in the chlorophyll structure. So all chlorophylls were modelled as chl a's [6].

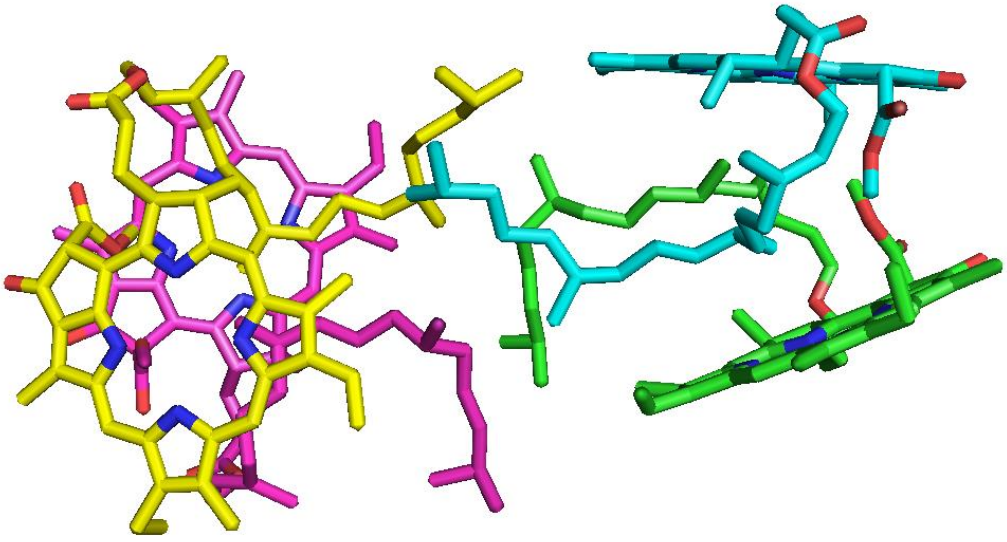

Figure 2:Chlorophyll a orientations in tetrameric lepidium virginicum WSCP [6]

The crystal structure also revealed the orientation of the chlorophylls in the binding pocket. The two closely spaced chls arrange in an open sandwich structure, having an opening angle of 30° and a center-to-center distance of 10 Å between the adjacent chlorophylls. They are opening to the centre of the binding pocket into which the phytyl tails are squeezed. The distance between the less closely spaced chlorophylls is about 20 Å and their orientation plane is twisted by 90° (Figure 2). The binding pocket is quite isolated from water. Only 4 water molecules were resolved each having a coordinating function between the protein and the chlorophyll [6].

More recently the crystal structure of *Brassicaceae* WSCP was published [7]. The paper focused on the deformation of the chl porphyrin ring by the protein alteration and the subsequent change of the absorption spectrum. The orientation features were found to be similar to *lepidium virginicum*.

1.2. Investigating a less complicated system

Unfortunately crystal structures depict a single pigment-protein configuration obtained at low temperature, which is only a snapshot of all possible conformations at physiological temperature. Every atom is nicely coordinated. That does not necessarily correspond to the natural state. In this study, WSCP with 4 chl a's bound was compared to samples only carrying 2 chl a's, from now on called tetramer and dimer, respectively. These are additions to experiments already performed in 2007. In the latter experiments, results were interpreted assuming presence of Chl dimers, while the samples later turned out to bind chl tetramers [2,3]. This differentiation can be made due to improved sample preparation and characterization (see also section 2.1). To determine the differences between both types of WSCP is the aim of this work.

This would allow the characterisation of chlorophyll interaction in a single dimer of closely spaced chl molecules, which is only modified by the protein surrounding it. Also the influence of the additional 2 chl's to the first dimer in a tetramer can be investigated and their contributions analysed.

1.3. Spectroscopy as tool in structure investigations

Crystallography is not the only possibility to gain insight into structural arrangements. Spectral properties of pigments change with excitonic (pigment-pigment) interaction between them, which is mainly defined by their orientation and distance between each other. Also the protein, they are embedded in plays an important role due to pigment-protein interaction. As seen from Figure 4, the absorption of the pigment-protein complex is shifted to the red compared to the pigment in acetone. In addition, the delocalised vibrations of the protein matrix

contribute to the absorbance of the pigment-protein complex, which is referred to as electron-phonon coupling [8].

To investigate these couplings, spectral hole burning (SHB) can be applied. Sample absorptions can be altered by a narrow laser, which excites only resonantly absorbing pigments. Within the excited electronic state of the pigment, the protein environment may undergo a conformational change affecting the pigment's absorption frequency. Once the pigment returns to its ground state, the initial conformation and corresponding absorption frequency can hardly be re-established at low temperature, when conformational protein motions are widely frozen. The difference between absorption spectra obtained before and after this burn process is called SHB-spectrum. To achieve this, absorption spectra are recorded at 4.5 K [8].

Inhomogeneous broadening describes the heterogeneity of the protein environment surrounding the pigment, while homogeneous broadening describes all broadening mechanisms, which are similar for each chemically equivalent pigment molecule bound in a structurally similar protein site [8]. This concerns lifetime broadening and electron-phonon coupling. The latter can be investigated by delta-fluorescence line narrowing spectroscopy (ΔFLN).

Figure 3 shows how an absorption band of a model pigment-protein complex can be understood. A pigment absorbs with a narrow peak at its electronic absorption frequency (blue curve), referred to as the zero phonon line (ZPL), and has an associated vibrational band, called phonon side band (PSB) towards higher energies. The heterogeneity of the protein environment is modelled as a Gaussian inhomogeneous distribution function (dashed line). Convoluting both contributions yields the observed absorption spectrum (red curve) [8].

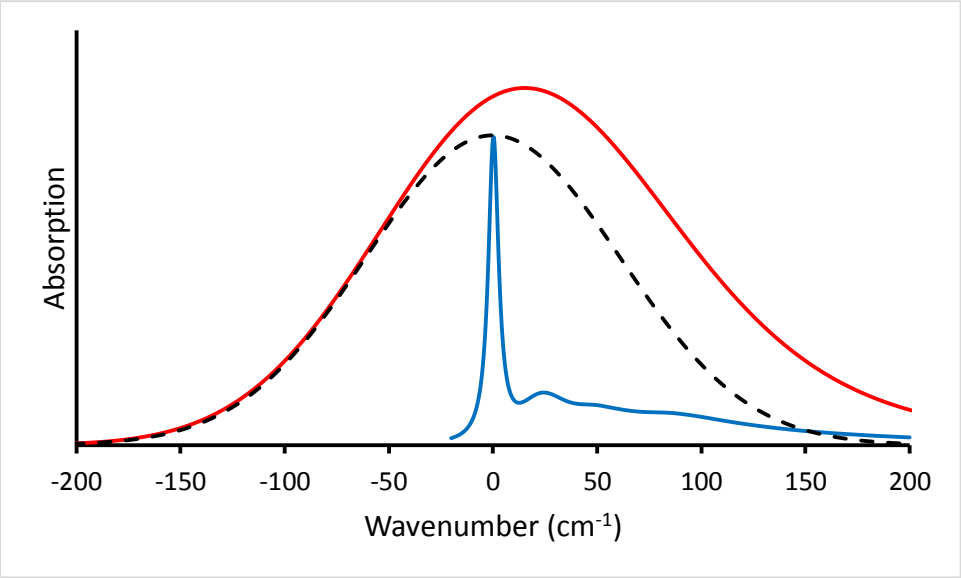

Figure 3: A theoretical curves of homogeneously broadened absorption spectrum (red) for a pigment-protein complex estimated using ΔFLN-spectroscopy (blue). The ΔFLN-spectrum consists of a ZPL at 0 cm^{-1} and an asymmetric PSB towards higher energies. The absorption is a convolution of the pigment absorption and the inhomogeneous curve (dashed, black). The latter can be determined with action spectroscopy.

Investigation of parameters of electron-phonon coupling of a sample by ΔFLN enables us to calculate the absorption spectrum, if the inhomogeneous broadening width is known. Therefore SHB action spectroscopy can be performed. Here the burn efficiencies of the complex at different burn wavelength are tested.

Based on knowledge of the homogeneously and inhomogeneously broadened spectrum of a single pigment determined by SHB and FLN, excitonic calculations can be employed to determine the spectrum of a chl dimer depending on the distance and angle between the pigment molecules, as demonstrated earlier by Hughes et al. [9]. In this work the idea was approached from both ends. Angles and chl-to-chl distances obtained from the crystal structure where used to calculate an absorption spectrum, while fitted parameters from the spectrum were taken to investigate the angles backwards in the native structure. Special emphasis was put to the difference in samples of dimer and tetramer.

Interpretations from experiments in 2011 were drawn with the believe that dimer samples were investigated [2,3]. The additional two chlorophylls may have a profound influence on the absorption of WSCP. Having now the possibility to compare both forms directly will determine their influence.

2. METHODS

2.1. WSCP Preparation

The samples were generously provided by Harald Paulsen and Daniel Palm from the University of Mainz. A new preparation to generate WSCP with two and four chl has been developed by them, but not yet published. The group uses cauliflower (*Brassica oleracea var. botrytis)* WSCP apoprotein, expressed by E. coli. To 100 µM apoprotein chl a is added dropwise, until a final concentration of 500 µM and 25 µM for tetramer and dimer, respectively. Following an incubation of 25 min the tetramer is boiled for 5 min, the dimer is not. Finally, a gelfiltration allows to separate the formed complexes from free apoprotein.

Spectroscopic analysis of the preparations shows a 1:1 ratio of protein to chl a in the tetramer, while the dimer may range from 1:0.4-0.6 [personal communications with Danial Palm]. Also mass spectrometric analysis confirmed a presence of WSCP with two chl a's bound [4].

2.2. Low Temperature Absorption Spectroscopy

Using temperatures of 4.5 K requires liquid helium to cool the sample. A He-bath cryostat (Utreks, Ukraine) was used, where the cuvette was kept just above the liquid helium surface at 4.5 ± 0.2 K. Plastic cuvettes of 10 mm length were used.

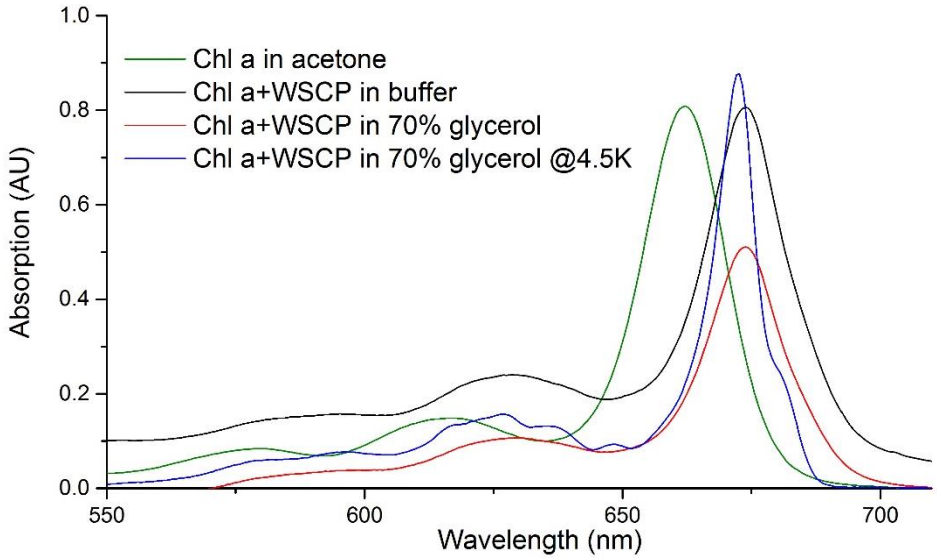

Figure 4: Absorption spectra of chl a in acetone and WSCP, as well as at room temperature and 4.5 K

Water samples however would form crystals while freezing and generate a turbid block, not usable for spectroscopy. Therefore glycerol at 70 % w/v is added to generate a transparent glass at these low temperatures. This again might affect the protein structure. But comparing absorption spectra in buffer and with added glycerol at room temperature, they display the same line shape.

In this study a tungsten light source BPS100 (BWTek, USA) was used for transmission measurements. The spectra were recorded using a 0.3 m spectrograph (Shamrock SR-303i, Andor Technology, UK) with an electrically cooled CCD camera (DV420A-OE, Andor Technology, UK). Resolutions of 0.1 and 0.4 nm were obtained using 1800 and 600 grooves/mm, respectively.

Cooled samples are necessary for the techniques described in the next sections. After either of these techniques, the sample can be heated to about 80 K to reset the sample into its initial state. The sample can be used repeatedly for several techniques.

2.2.1. Spectral Hole Burning

This technique is counted to the group of line narrowing techniques. Using a narrow laser just a small portion of pigments which are resonant at the burn frequency are excited, thereby changing their transition frequencies. In this study a Spectra Physics model 375 dye laser (line width of <0.5 cm-1) pumped by an Ar ion laser (model 171, Spectra Physics, USA) was used.

After a pre-burn absorption spectrum is taken, a laser with a set burn wavelength and stabilized burn intensity “burns” the sample. The effects of the burn process are cumulative, therefore intensifying the laser power or the duration of the burn process result in a stronger alteration of the postburn absorption spectrum. The difference of pre- and postburn absorption yields the SHB spectrum.

At too high fluxes however the sample gets heated and the 4.5 K assumption does not hold anymore. Also irreversible photochemical alterations may occur, which are unwanted.

Spectral holes are alterations of absorption of the sample due to excitation at a narrow burn wavelength [8]. A SHB spectrum consist of the ZPL, at the wavelength of the burning laser (Figure 5). Towards higher energies a real-phonon sideband can be found, corresponding to the PSB in Figure 3. At the red site of the ZPL a pseudo-PSB emerges, which should be ideally symmetric to the real-PSB.

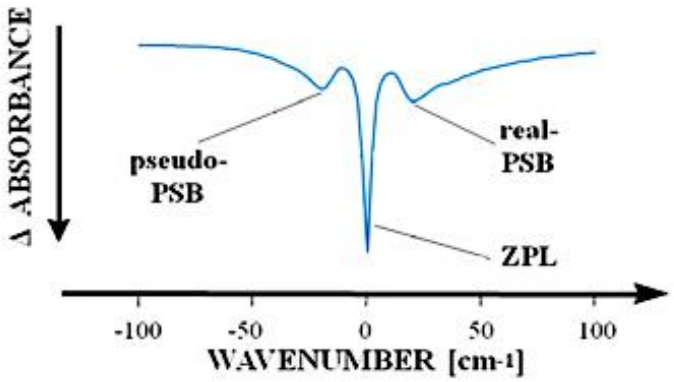

Figure 5: Theoretical SHB-spectra (modified from [8]*)*

2.2.2. Action Spectroscopy

To determine the inhomogeneous distribution function a special form of SHB can be applied, the action spectroscopy. Here are the ZPL of multiple burn wavelengths at a constant fluency are investigated. The resulting points of the ZPL maxima can be approximated by a Gaussian function. The full width at half maximum (FWHM) can be considered the inhomogeneous width.

In this study the burning laser was used at a fluency of 10 mW for 2 min.

2.2.3. Fluorescence Measurement

Using the same burn laser as for SHB, which is directed into the sample perpendicular to the detector, fluorescence line narrowing (FLN) can be achieved. Combining this fluorescence measurement with an intermediate burning process of SHB, generates altered spectra, just like in the absorption counterpart.

The difference of two preburn and postburn FLN spectra is called ΔFLN spectrum and consists of a ZPL, the phonon side band and vibrational lines at lower energies. Although visible in SHB the phonon side band can't be reliably fitted there, due to the anti-hole. In ΔFLN this is not the case and the line shape can be estimated.

In this work the profile of the phonon side band was modelled with a Wolfram Mathematica [10] routine written by Jörg Pieper, already used in another studies [2,11]. It enables to fit the PSB with 3 one-phonon profiles to determine the Huang-Rhys factor, which corresponds to the strength of electron-phonon coupling.

2.3. Calculations

In this study the geometrical properties of the chlorophyll dimer were investigated according to Hughes et.al. [9]. In that paper the angle between one chlorophyll dimer in WSCP was determined from its absorption spectrum, fitted by two Gaussian curves. They reported an angle of 60 °, which was corrected by the crystal structure to 30 ° one year later [6].

Using this theory we created a tool to perform calculations both ways, which is applicable also to other pigment-protein complexes. Crystal structure coordinates were taken to calculate distances and angles between chlorophylls, which would then be used to calculate the excitonic splitting and intensity redistribution between the lower and upper excitonic states of a chl dimer resulting in a stick spectrum. Finally, each excitonic state was modelled as an inhomogeneously broadened transition calculated via Mathematica [10] taking into account the parameters of electron-phonon coupling extracted from ΔFLN spectra.

As two exciton coupled chromophores split their transition, this is determined by dipole-dipole interaction energy J:

$$J = \frac{5.04\mu^2 k}{\varepsilon R_{12}^3} \quad (1)$$

Where J is in cm^{-1}, μ^2 the transition dipole strength equals D in units of $Debye^2$, ε as the dielectric constant, R_{12} is the spatial separation of chl's in nm and k as the orientation factor, given by formula (2):

$$k = cos\theta_{12} - 3sin^2\phi cos\theta \cos(\theta_{12} - \theta) \quad (2)$$

The angles used were determined according to this schematic.

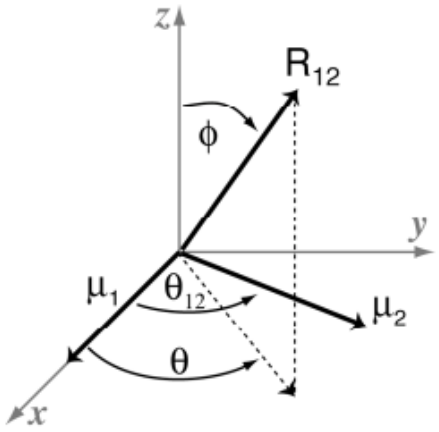

Figure 6: Coordinate system defining the geometric parameters used in the exciton analysis. The origin is put into the centre of the first Chl porphyrin ring, with R_{12} being the separation vector to the second Chl. The transition dipole moments μ_1 and μ_2 are chosen along the x-axis and in the x-y plane, respectively. The angle between them is Θ_{12}, while Θ is the angle from μ_1 to the projection of R_{12} onto the x-y plane. Φ is the azimuthal angle from the z-axis towards R_{12}. (Taken from [9]*)*

The dipole coupling strength D was determined by:

$$D_{\pm} = D_0(1 \pm cos\theta_{12}) \quad (3)$$

using

$$D_0 = 20.2 + 23.6(n - 1) \quad (4)$$

, with n being the reflective index [12].

By generating an absorption spectrum with these parameters it would be possible to optimize the fit and gain angles observed from spectra, contrary to the fixed alignment in a crystal structure. This tool could be beneficial in future studies of temperature dependent behaviour of WSCP and the possible change in opening angle within the dimer during that process.

3. RESULTS AND DISCUSSION

3.1. Dimer and tetramer absorption and fluorescence

The absorption spectrum of the sample investigated in 2011 matches the tetramer sample [2,3]. Therefore it can be noted, that indeed the assumption of a dimer, was incorrect.

Dimer and tetramer samples are overall similar, with the absorption maximum at 672 nm. But if the spectra are normalized at this maximum a small difference can be found. The shoulder in the diminishing side of the maximum at 681 nm contains a shoulder which is more prominent in the dimer.

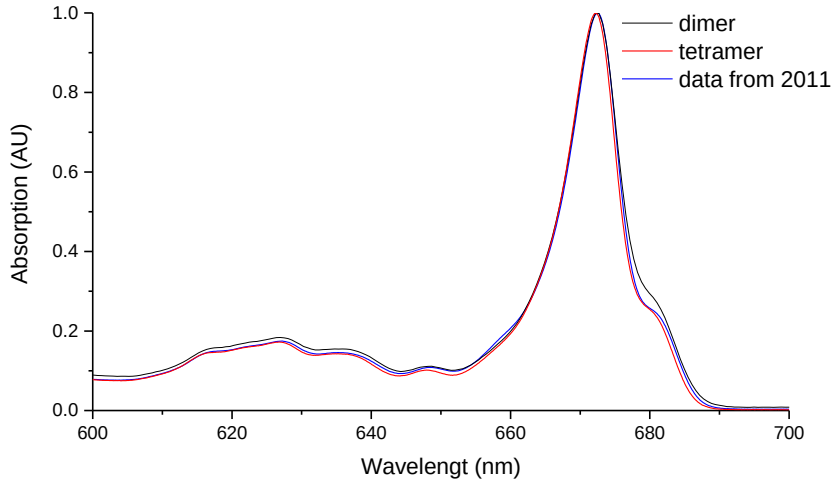

Figure 7: Absorption spectra of dimer and tetramer normalized to 1. Also displayed is an absorption spectrum taken in 2011

The fluorescence spectra on the other hand are almost identical. Here again a normalisation was performed. The strongest deviation is visible at 696 nm, right next to the main peak.

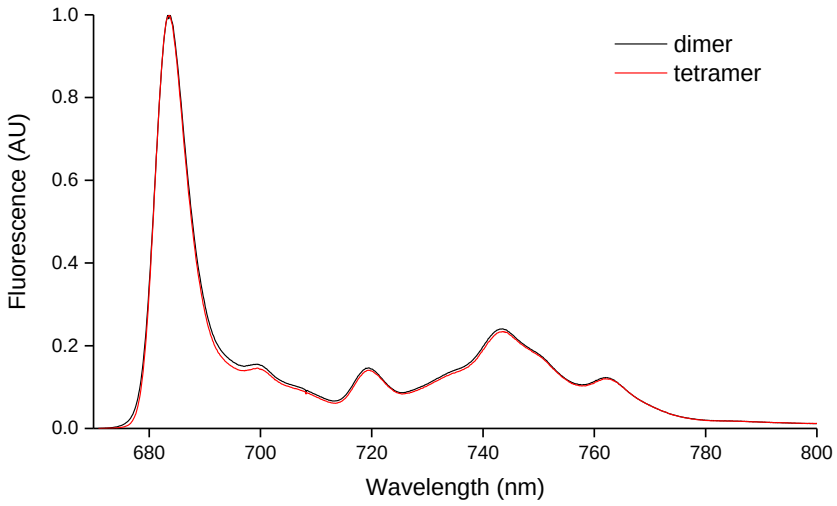

Figure 8: Fluorescence spectra of dimer and tetramer at different concentrations

3.2. Spectral hole burning artefact

The samples were stored at 4 °C, as recommended by the providing group and analysed over several weeks. Due to different concentrations of the samples, the tetramer was diluted 4 times before glycerol was added. For the spectral hole burning measurements a wavelength adjustable dye laser was used.

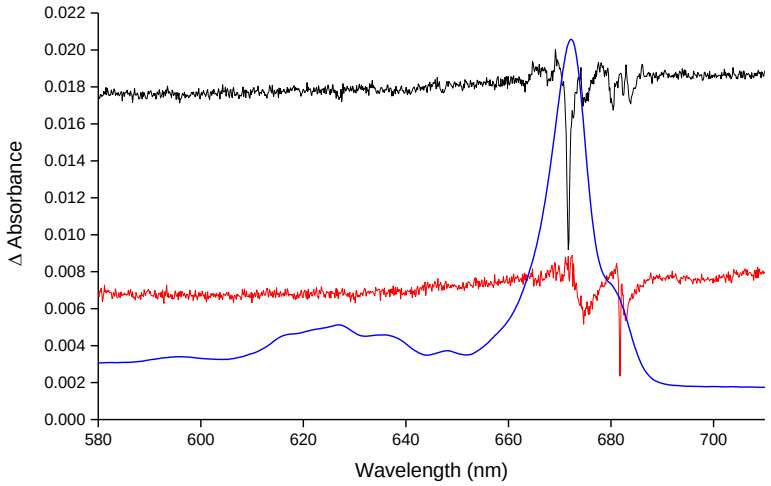

Figure 9: Absorption (blue) and hole burning spectra at 672 (black) and 682 nm (red) of the dimer with burn fluences of 12 and 3.6 J/cm², respectively

In the first experiment both samples were tested for the wavelength of 672 nm and 682 nm, which correspond to the maximal absorption and the shoulder of the absorption. The respective absorption spectra were include in the figures to illustrate position of the holes.

The burn at 672 nm displays in the dimer a strong ZPL and the corresponding PSB (Figure 9). At 682 nm an additional hole can be observed, disrupted by vibrational bands. Burning at 682 nm shows a similar ZPL and PBS structure and a hole at 675 nm.

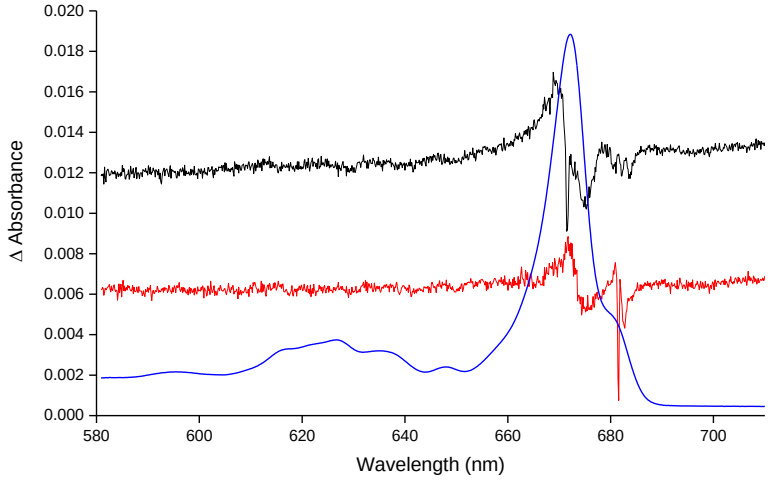

Figure 10: Absorption (blue) and hole burning spectra at 672 (black) and 682 nm (red) of the tetramer with fluences of 15 and 6 J/cm², respectively

For the tetramer the SHB spectra are without any significant difference. Unfortunately in this first experiments no consistent fluences were applied, since it was just a test on the new samples. The results however are in contrast with experiments done 4 month later.

At this time SHB were taken from 620 and 670 nm (Figure 11). The dimer burn spectrum at 620 nm does not contain a ZPL since the absorption of the complex at that wavelength is rather minimal. Still, those states which are excitonicly coupled in the burning process and generate holes. In this particular case holes at 675 and 682 nm can be observed like previously. But another, very broad hole, appears at 698 nm. For the tetramer the obtained spectra look again very similar (annex A).

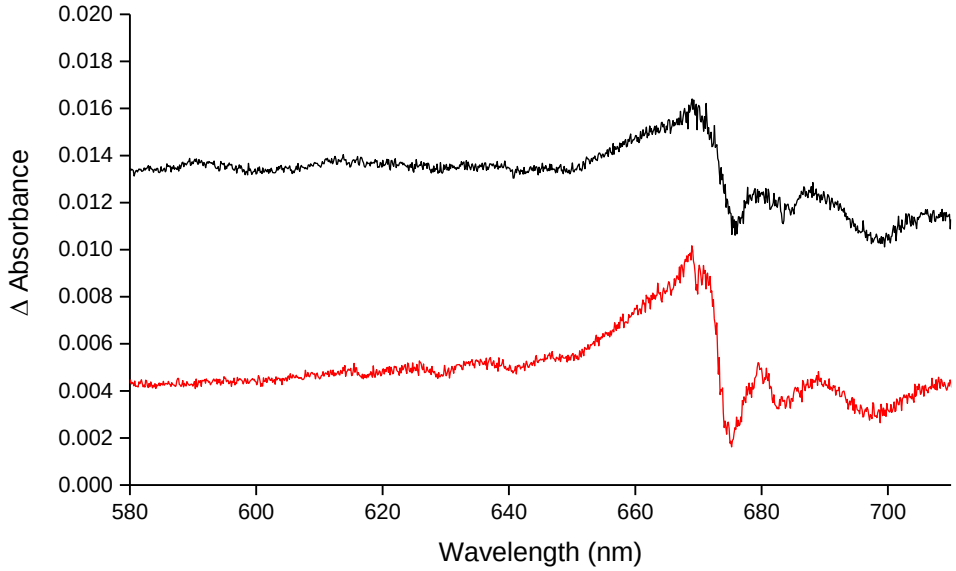

Figure 11: SHB spectra of the dimer burned at 620 nm (black) and 670 nm (red)

At 698 nm WSCP absorption is minimal. Yet the burned hole is quite intensive and therefore the increase in absorption rather significant. Burning at 698 nm however, could not generate a SHB spectrum.

A similar behaviour to light harvesting complexes II aggregates were drawn [13]. Inquiries at the provider of the samples, Daniel Palm in Mainz, showed aggregates in gel-electrophoreses (annex B). In which way the formation of higher order complexes may occur to add a whole state is not clear. Since both samples are displaying an equal effect (see below) the effect has to be due to a change in the dimer surrounding.

3.3. Comparison of line narrowing spectroscopic data

3.3.1. Spectral hole burning

In other regards the results of SHB spectroscopy showed consistent similarities between both samples. An overlay of both hole burning spectra shows how alike they are (Figure 12).

Placement, depth and width of the observed holes are nearly identical. The storage induced lowest energy state is consistent in appearance and burn-efficacy in both samples.

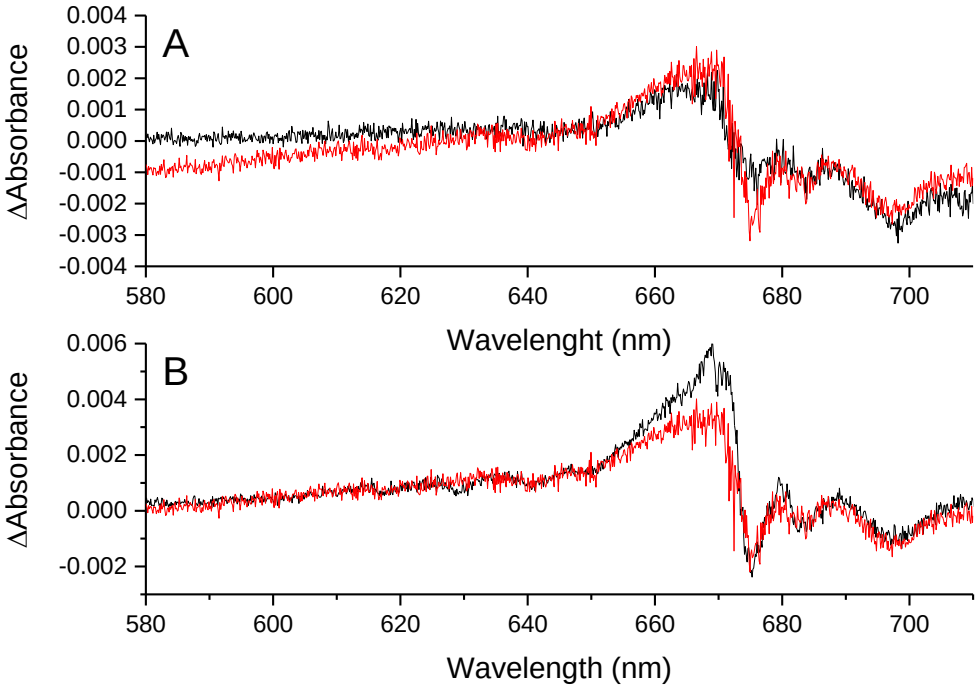

Figure 12: Overlaid SHB spectra of dimer (black) and tetramer (red) at 6 J/cm² burned at 620 nm (A) and 670 nm (B).

In further investigations of WSCP it might be to focus on this lowest state and how it is formed. By doing so insights into artificial broadening of absorption could be found and applied in similar projects described by Braun et al. [14].

3.3.2. Action spectroscopy

Burning with the same laser intensity for a fixed time period at different wavelengths generates an action spectrum. The ZPL positions and intensities are displayed and fitted by a Gaussian function, which provides the inhomogeneous line width as its FWHM.

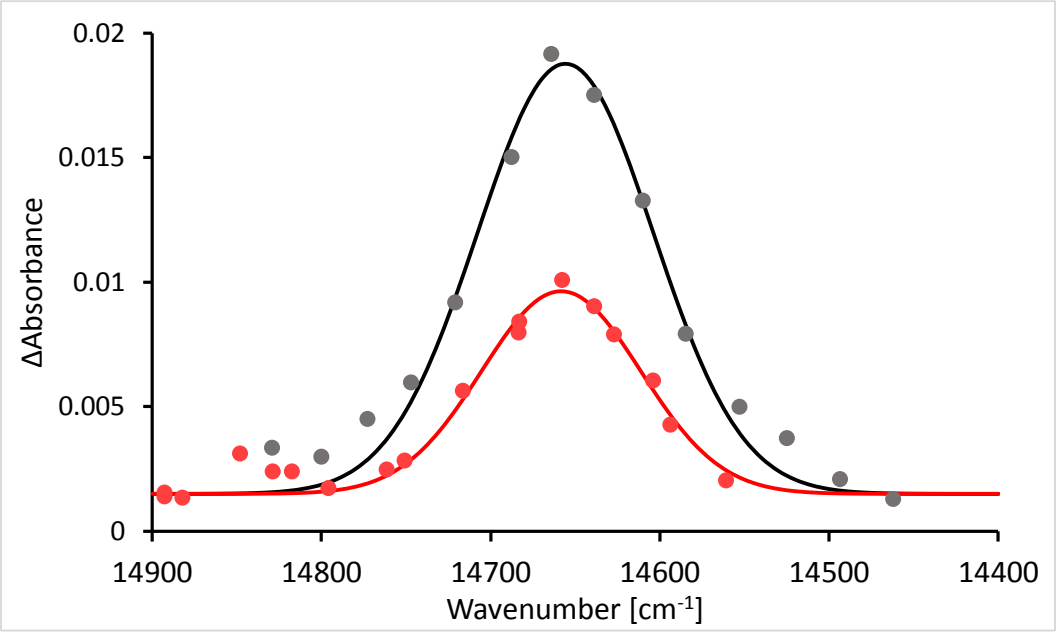

Figure 13: action spectra obtained from dimer (black) and tetramer (red). Dots symbolize the ZPL maxima, while the lines are Gaussian fits

In Figure 13 the two recorded action spectra and their fits are shown. The fits applied had a common baseline, which resulted in a poorer fit on the flanks for the dimer, but made them more comparable (Table 1). The width was determined as 122.5 cm^{-1} for the dimer and 110.7 cm^{-1} for the tetramer. This difference is rather small and can very well be explained by the methods inaccuracy.

Table 1: Parameters of the Gaussian fits used for the action spectra

	dimer	tetramer
x_{centre} [cm^{-1}]	14656	14658
x_{centre} [nm]	682.32	682.20
y_0 [ΔAU]	0.00151	0.00151
y_{centre} [ΔAU]	0.0188	0.0096
FWHM [cm^{-1}]	122.5	110.7

3.3.3. Delta fluorescence line narrowing

This technique yields a straight forward approach to quantify the electron-phonon coupling. Due to the narrow excitation the emitted fluorescence reflects the specific interactions of that state. Hence the Huang-Rhys factor, describing the phonon coupling constant, can be determined by relating the PSB to the ZPL. Furthermore related vibrational states emit light.

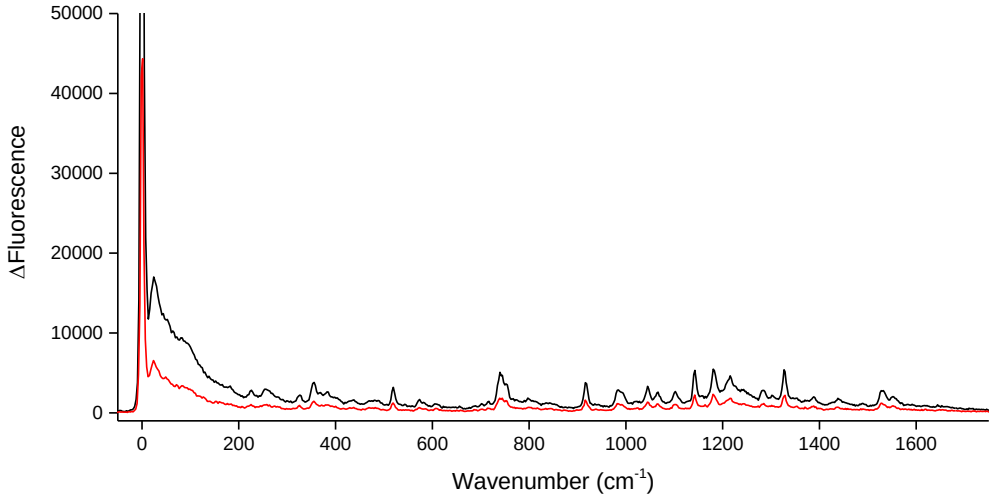

Figure 14: ΔFLN spectra of dimer (black) and tetramer (red) with their vibrational states

Despite the lower concentration used for the tetramer the vibrational states are well formed and are displayed in Figure 14. Their positions match those of the dimer (Table 2) with the biggest deviation of 5 cm^{-1}. They also correspond to those reported in 2011, only fewer were determined [2].

Table 2: vibrational bands observed in ΔFLN

ν_j (cm^{-1})		ν_j (cm^{-1})	
dimer	tetramer	dimer	tetramer
225	225	1044	1046
257	258	1065	1065
325	328	1101	1101
354	355	1142	1141
381	382	1179	1182
397	395	1215	1215
432	432	1238	1233
482	482	1282	1284
519	519	1299	1303
573	574	1326	1326
607	610	1386	1388
740	740	1436	1438
795	795	1528	1530
917	917	1532	1532
983	983		

The PSB was fitted using a routine written by Jörg Pieper. It is capable of describing 3 one-phonon-profiles of the PSB with a Gaussian rising and a Lorentzian conclusion for each. Their combined relative intensity, compared to the ZPL, is the Huang-Rhys factor S. In Figure 15 the

calculated fits of the PSB are displayed. The ZPL was set to be a Lorentzian curve and are not displayed to focus on the PSB.

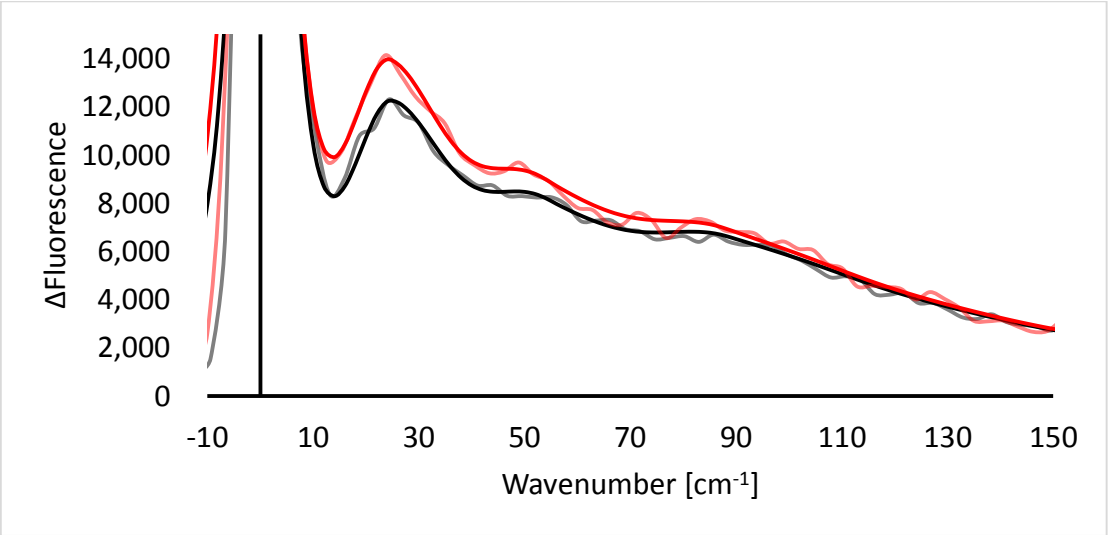

Figure 15: PSB fits for dimer (black) and tetramer (red). 3 side band wings were fitted. The tetramer fluorescence was normalized to that of the dimer

Judging from Figure 15 the tetramer seems to have a greater S-value. But its ZPL width is increased compared to the dimer, intensifying the PSB as well. The parameters extracted were very similar for both samples. The S_{total} determined in 2011 was 0.81 [2], which is comparable to the factor 0.77 of the tetramer (Table 3). The dimer has a marginally increased coupling constant of 0.8.

Table 3: PSB fit parameter

		dimer			tetramer		
FWHM of ZPL	Γ_Z [cm^{-1}]	6.2			7		
S_{total}		0.8			0.77		
		profile			profile		
		I	II	III	I	II	III
Huang-Rhys factor		0.41	0.23	0.16	0.41	0.22	0.14
peak phonon frequency	ω_m [cm^{-1}]	25	54	88	25	54	88
FWHM of Gaussian wing	Γ_g [cm^{-1}]	18	23	27	18	23	27
FWHM of Lorentzian wing	Γ_L [cm^{-1}]	34	62	70	34	62	70

Except for the difference in the shoulder of the absorption spectrum, WSCP dimer and tetramer behave identical. This leads to the conclusion that the two dimers in the tetramer are too separated and interact only marginally with each other. This finding corresponds to their distance of approximately 20 Å [6], the maximal possible range of chlorophyll interaction. Furthermore they are twisted out of sync for their π-electron system.

Confirming these similarities suggests that tetrameric samples, which are easier to generate, may be interpreted as two identical chlorophyll dimers, simplifying their analysis.

3.4. Structural analysis of Dimeric chl a WSCP

A further aim of this project was to build a tool for future WSCP investigations, focusing on the opening angle between dimer chlorophylls. In 2006 Hughes, et al. formulated an easy way to examine an absorption spectrum to conclude the opening angle [9]. The intention here was it to generate a matrix to work in two ways. Using the crystal structure of a complex to calculate the angle and generate a theoretical absorption spectrum. Comparing this spectrum to an observed one and adjusting parameters to fit both would enable to quantify the parameters influence on the pigment-protein complex. Our approach has the addition of modelling the inhomogeneous broadened transition in contrast to Gaussian functions used by Hughes.

Coordinates of the chlorophyll nitrogen atoms were extracted from the pdb-file (2DRE) and angles according to Figure 6 determined. The distance was measured between the magnesium ions in nm.

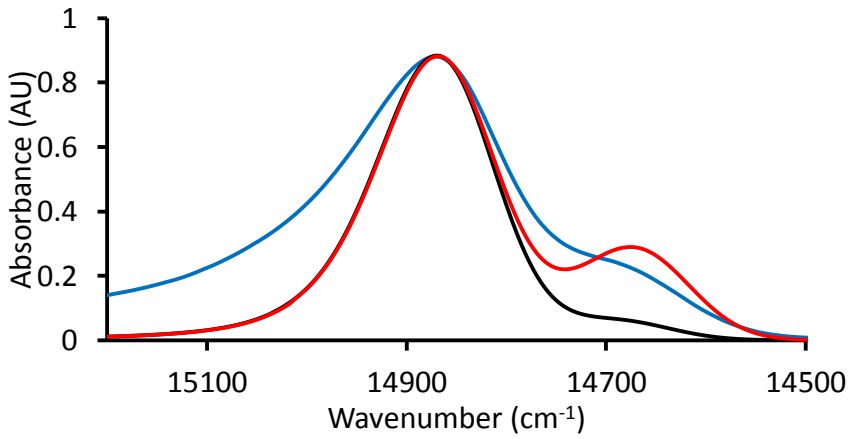

Figure 16: Absorption spectrum of dimer (blue) with calculated spectra using 30° (black) and 60° (red) as parameters

Using the PSB profile obtained in ΔFLN an inhomogeneous broadened transition was generated, with the corresponding width determined by action spectroscopy. This transition was then duplicated and split by the dipole-dipole energy J (Equation 1). The respective intensity distribution was defined as the transition dipole strength D_- and D_+ (Equation 3), which is only dependent on the angle.

Figure 16 depicts the initial stage of generating an absorption spectrum from the crystal structure derived angle of 30° (black). The striking feature is the lack of intensity in the shoulder. If on the other hand an angle is set to 60° (red) intensity shifts up significantly. Hughes

reported an angle of 60° using this technique. A year later the crystal structure corrected that value to 30° [6] and was since verified multiple times [15–17].

This discrepancy might be due to the simplistic calculation of the transition dipole strength D (Equations 3). By applying 60°, as used in the initial form of this approach, a more reasonable fit is achievable. Therefore our conclusion is that the calculation is incomplete. However, we were not able to find an improvement to that formula and discontinued the work on this tool.

3.5. Outlook

Many aspects of WSCP molecule with only 2 chlorophyll molecules are fascinating. There is no doubt the research on that field will go on rapidly. Similar analysis as described here using chlorophyll b are already planned, further chlorophyll variant might follow.

To gather information on the natural state at room temperature, temperature dependent analysis are reasonable, to see for example how the opening angle of the dimer changes. Although the applied approach of calculating the opening angle was unsuccessful, other, less basic approaches were effective [16].

Also the structural differences of a dimer and a tetramer complexes are interesting. How is the binding pocket missing chlorophylls arranged? Is it maybe collapsed? One method to investigate these features is neutron scattering, which provides a silhouette of the complex. It would also be applicable to look into the formation of higher order complexes, generating the 698 nm state, visible in SHB spectra.

WSCP will continue to be an excellent study object for chlorophyll and pigment-protein interactions. The behaviour of dimer and tetramer found here will provide base of interpretation for further investigations.

SUMMARY

For the first time Water soluble chlorophyll binding protein (WSCP) containing only two chlorophyll a molecules (dimer) was subjected to spectroscopic analysis. It was compared to so far known WSCP with 4 chlorophylls (tetramer) using line narrowing spectroscopy techniques.

WSCP is a favourable molecule to analyse chlorophyll interaction. Its low count of chlorophylls enable basic interpretation of spectroscopic data, in contrast to complexes like light harvesting complexes. Yet so far it was not sufficiently established, whether a tetrameric or a dimeric spectroscopic character was primarily observed.

Both samples turned out to be overall very similar with certain minor differences. The low temperature absorption spectrum of the dimer displays a more prominent shoulder at 682 nm. The inhomogeneous width, determined by action spectroscopy, is with 122 cm^{-1} marginal bigger in the dimer, than in the tetramer with 110 cm^{-1}. However this is not a significant difference, since the estimated error is too big to be certain. The phonon side band of the dimer was found to be coupled slightly stronger than the tetramer, displaying Huang-Rhys factor values of S_{dimer}=0.8 and $S_{tetramer}$=0.77.

Overall the dimer behaves very much like the complex carrying 4 chlorophylls. That leads to the conclusion, that the tetramer's chlorophyll interaction resemble that of two independent dimers, with minimal inter-dimer influences. Previously discussed mechanisms, assuming dimeric character of WSCP, can therefore still be regarded valid.

An attempt was made to build a tool to analyse the opening angle between the dimeric chlorophylls. This tool could be used in further studies, for example temperature depending examinations. The completion of the tool was unsuccessful, due to a flaw in the used theory, which could not be overcome to this point.

Another interesting feature of the investigated samples was found after storage. During spectral hole burning a previous unobserved lowest energy state was found at 698 nm. This state is most likely induced by polymerisation of WSCP. This finding might be applicable in creation of artificial photosynthesis with broader absorption.

KOKKUVÕTE

Selles uurimistöös analüüsiti esimest korda spektroskoopiliselt dimeerset veeslahustuvat klorofülli siduvat proteiini (water soluble clorophyll binding protein ehk WSCP) ning võrreldi seda seni tuntud WSCP tetrameerse molekuliga, kasutades line narrowing spektroskoopia meetodeid.

WSCP on klorofülli uurimiseks eelistatud molekul. Selle madal klorofüllisisaldus võimaldab teha spektroskoopiliste andmete baasanalüüsi vastupidiselt klorofüllikompleksidele nagu valgust püüdvad kompleksid (light harvesting complexes). Sellegipoolest ei olnud siiani täpselt teada, kas spektroskoopilistes uuringutes oli käsitletud tetrameerset või dimeerset molekuli.

Uurimistöö käigus selgus, et mõlemad uuringuproovid on üsna sarnased kindlate väikeste erisustega. Dimeersel molekulil on madalal temperatuuril läbi viidud spektromeetrias 682 nm lainepikkuse korral näha prominentsem õlg. Aktsioonispektroskoopia käigus mõõdetud inhomogeenne laius on dimeeril tetrameeriga võrreldes marginaalselt suurem. Leitud erinevus ei ole aga statistiliselt oluline. Uuringu käigus selgus, et dimeeri fonooni külgriba on ühendatud veidi tugevamalt kui tetrameeril, vastavate Huang-Rhys faktori väärtustega Sdimeer=0.8 ja Stetrameer=0.77.

Kokkuvõttes käitub dimeer oma parameetritelt väga sarnaselt kompleksiga, mis kannab 4 klorofülli molekuli. Siit järeldub, et tetrameerne kompleks sarnaneb uuringutes kahe iseseisva dimeeriga ning interdimeersed mõjutused on minimaalsed. Varasemalt teoreetiliselt arutatud mehhanismid, mis on eeldanud WSCP kompleksi dimeerset iseloomu, võib seega lugeda teaduslikult põhjendatuks.

Prooviti leida võimalust dimeersete klorofüllide vahelise avatud nurga analüüsimiseks. Seda arvutust saaks kasutada tulevastes, näiteks temperatuurist sõltuvates uuringutes. Tööriista loomine ei olnud aga edukas kasutatud teoorias olnud vea tõttu, mida ei suudetud parandada.

Uuritud proovidel leiti pärast nende hoiustamist veel üks huvitav omadus. Spectral hole burning meetodit kasutades leiti 698 nm lainepikkuse juures varasemalt avastamata jäänud WSCP madalaima energiatasemega vorm, mis tekib kõige tõenäolisemalt WSCP polümerisatsiooni tõttu. See leid võib olla rakendatav laiema absorptsiooniga kunstliku fotosünteesi loomisel.

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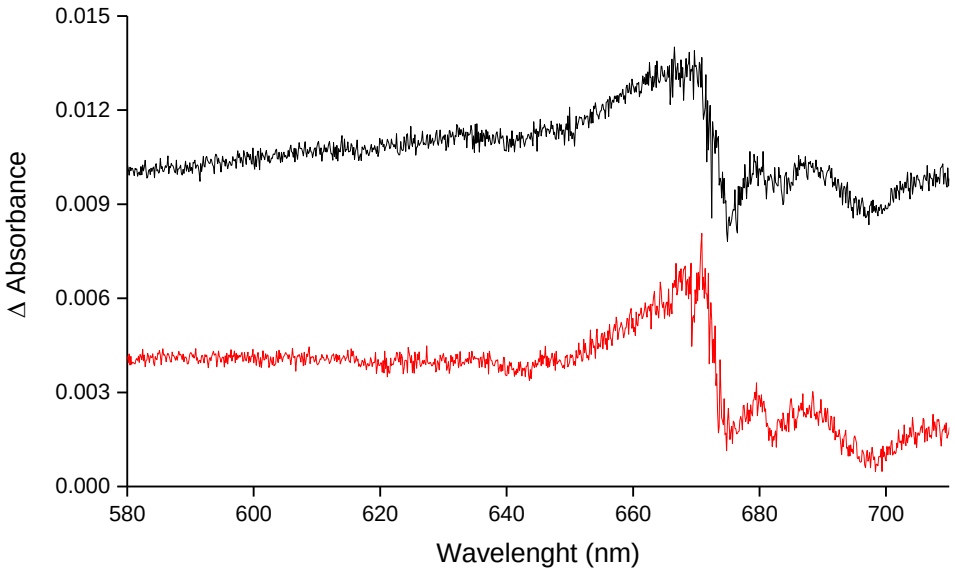
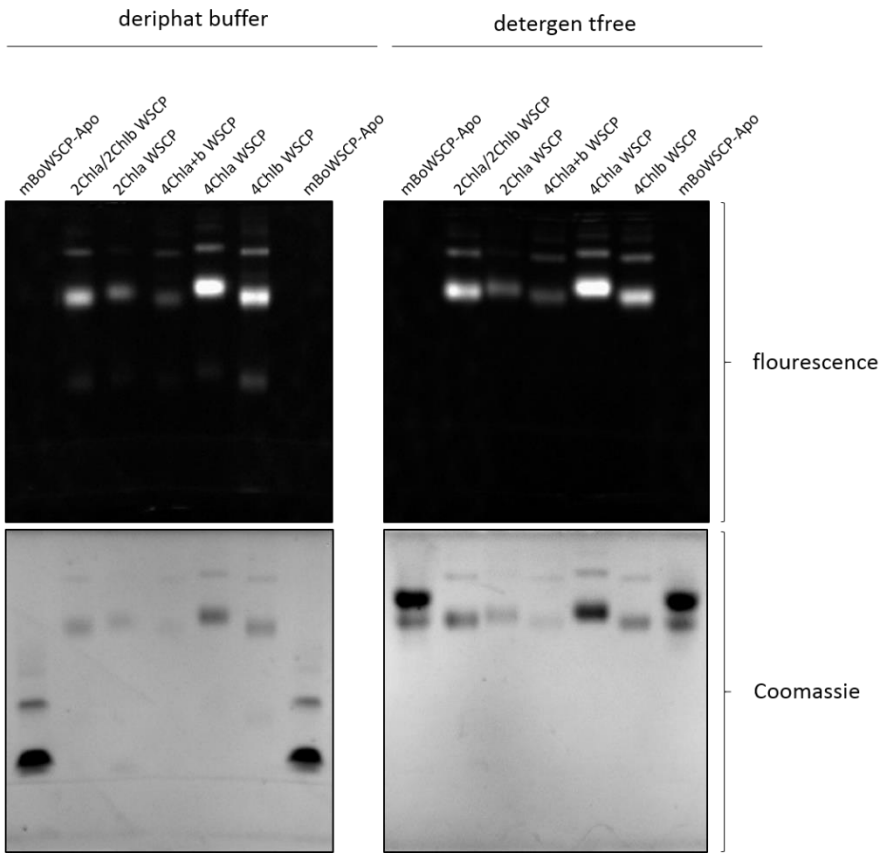
ANNEX A



Annex A: SHB spectra of tetramer at 620 nm (black) and 670 nm (red) burned with 6 J/cm² after 4 month of 4 °C storage

ANNEX B



Annex B: Gel electrophoreses of several chl-variant substituted WSCP, verifying formation of higher order complexes

INFORMATION SHEET

Klorofülli molekulide interaktsioonide uurimine veeslahustuvas klorofülli siduvas proteiinis

Line narrowing spektroskoopia meetodeid rakendati vaid kaht klorofülli molekuli sisaldava veeslahustuva klorofülli siduva proteiini (water soluble clorophyll binding protein ehk WSCP) uurimiseks. Tulemust võrreldi naturaalselt esineva, 4 klorofülli molekuli sisaldava proteiiniga. Statistiliselt olulisi erinevusi kahe kompleksi vahel ei leitud.

Märksõnad: klorofüll, madala temperatuuri spektroskoopia, line narrowing spektroskoopia meetodid

Investigations of chlorophyll interactions in Water Soluble Chlorophyll Binding Protein

Line narrowing spectroscopy was applied to water soluble chlorophyll binding protein (WSCP) containing only 2 chlorophylls. It was compared to natural occurring 4 chlorophyll WSCP. No significant difference were found.

Keywords: chlorophyll, low temperature spectroscopy, line narrowing techniques

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