Biophysical Characterization of Carotenoids and Carotenoid Containing Proteins Using Resonance Raman Spectroscopy
This thesis has been reviewed by the following committee:
Prof. dr. R. van Grondelle (Vrije Universiteit, Amsterdam)
Prof. dr. T. Moore (Arizona State University, Tempe, USA)
Prof. dr. L. Valkunas (Vilniaus Universitetas, Vilnius, Lithuania)
Prof. dr. T. Polivka (University of South Bohemia, České Budějovice, Czech Republic)
Biophysical Characterization of Carotenoids and Carotenoid Containing Proteins Using Resonance Raman Spectroscopy

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad Doctor aan de Vrije Universiteit Amsterdam,
op gezag van de rector magnificus prof.dr. V. Subramaniam,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie van de Faculteit der Exacte Wetenschappen
op maandag 29 februari 2016 om 13.45 uur
in de aula van de universiteit,
De Boelelaan 1105

door

Elizabeth Annette Kish

geboren te Amherst, NY, Verenigde Staten

3
promotor: prof.dr. B. Robert
copromotor: dr. A. Gall
Dedicated to all of my friends and family that helped along the way, especially my PhD supervisor Prof. Bruno Robert. To Richard Schultz who died a long time ago, but gave me the confidence and inspiration to follow my dreams; to Jessica LaRusch that encouraged me to pursue a PhD.

“As soon as we adventure on the paths of the physicist, we learn to weigh and to measure, to deal with time and space and mass and their related concepts, and to find more and more our knowledge expressed and our needs satisfied through the concept of number, as in the dreams and visions of Plato and Pythagoras; for modern chemistry would have gladdened the hearts of those great philosophic dreamers. Dreams apart, numerical precision is the very soul of science, and its attainment affords the best, perhaps the only criterion of the truth of theories and the correctness of experiments. So said Sir John Herschel, a hundred years ago; and Kant had said that it was Nature herself, and not the mathematician, who brings mathematics into natural philosophy...

Some lofty concepts, like space and number, involve truths remote from the category of causation; and here we must be content, as Aristotle says, if the mere facts be known. But natural history deals with ephemeral and accidental, not eternal nor universal things; their causes and effects thrust themselves on our curiosity, and become the ultimate relations to which our contemplation extends...

It behoves us always to remember that in physics it has taken great men to discover simple things. They are very great names indeed which we couple with the explanation of the path of a stone, the droop of a chain, the tints of a bubble, the shadows in a cup...

How far even then mathematics will suffice to describe, and physics to explain, the fabric of the body, no man can foresee. It may be that all the laws of energy, and all the properties of matter, and all the chemistry of all the colloids are as powerless to explain the body as they are impotent to comprehend the soul. For my part, I think it is not so. Of how it is that the soul informs the body, physical science teaches me nothing; and that living matter influences and is influenced by mind is a mystery without a clue. Consciousness is not explained to my comprehension by all the nerve-paths and neurons of the physiologist; nor do I ask of physics how goodness shines in one man’s face, and evil betrays itself in another. But of the construction and growth and working of the body, as of all else that is of the earth earthy, physical science is, in my humble opinion, our only teacher and guide.”

-From “On Growth and Form” by D’Arcy Thompson, 1917
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. General Introduction</td>
<td>7</td>
</tr>
<tr>
<td>2. Fermi Resonance a Tool for Probing Peridinin Environment</td>
<td>29</td>
</tr>
<tr>
<td>3. Assignment of IR Bands and Protein-bound Peridinin in its Fundamental and Triplet State by Static FTIR, Time-resolved Step-scan and DFT Calculations</td>
<td>49</td>
</tr>
<tr>
<td>4. Resonance Raman Spectra of Carotenoid Molecules: Influence of Methyl Substitutions</td>
<td>63</td>
</tr>
<tr>
<td>5. Echinonine Vibrational Properties: From Solvents to the Orange Carotenoid Protein</td>
<td>89</td>
</tr>
<tr>
<td>6. Triplet-Triplet Energy Transfer in Dyads</td>
<td>113</td>
</tr>
<tr>
<td>7. Resonance Raman Characterization of Reconstituted Wild-type and Point-mutated LHCII Proteins</td>
<td>131</td>
</tr>
</tbody>
</table>

Conclusion 145

Summary 147

Samenvatting 149

Notes 151
GENERAL INTRODUCTION

1.1. Overview

During the course of my thesis, the general theme of my work has been characterizing the biophysical properties of carotenoids in different environments (e.g. solvents and proteins). Carotenoids are the molecules responsible for many of the colors found in nature, and thus my motivation for pursuing this project came from my general love of the beauty of nature, particularly color, and the curiosity to explore its functional mechanisms. I have used predominately resonance Raman spectroscopy for obtaining information on carotenoid structures, characteristics, and/or environment. In this book, I present my work, starting with this general introduction, which discusses many aspects of carotenoids.

1.2. Carotenoids

1.2.1. General

Carotenoids are a class of molecules with a myriad of biological functions present in a wide range of living organisms on the earth. They are present in bacteria, archaea, and eukarya. The first purified carotene was obtained by Heinrich Wilhelm Ferdinand Wackenroder in 1831. Over the next 70 years, there were over 800 publications in the field and over 600 carotenoids have been discovered since. Carotenoids are divided into two classes: xanthophylls and carotenes. Carotenes contain only carbon and hydrogen atoms whereas xanthophylls, derivatives of carotenes, contain oxygen atoms incorporated into their structure. Xanthophylls were named for their yellow color in 1837 by Jöns Jacob Berzelius (in Latin xanthos means yellow and phyll means leaf) who purified them from autumn leaves. The color of carotenoids, ranging from pale yellow through bright orange to red is directly linked to their molecular structure.
Perhaps the most well-known example is β-carotene that confers carrots their orange color. Carotenoids are one of the molecules that are responsible for the vast coloration of autumn leaves, when the chlorophyll that masks them in green leaves is degraded. They also pigment ripe fruit like tomatoes (lycopene), oranges (violaxanthin), and bananas (β-carotene). Note that the pink color of flamingos, blue color of lobsters, and pink color of salmon can be attributed to carotenoids. Carotenoids cannot be synthesized by animals and must be obtained through the diet, except for the rare arthropod cases such as red pea aphid, spider mite and gall midges, which generally acquired the ability from lateral gene transfer with fungus.

Carotenoids have many different biological functions. In photosynthesis, they are intimately involved in the processes of light-harvesting and photoprotection. Beta-carotene is also called provitamin A, as in animals, the latter is produced by beta-carotene cleavage, leading to retinal, retinol and retinoic acid. Retinal is essential for vision, retinoic acid for fetal development and cell differentiation. Vitamin A deficiency is the largest cause for preventable blindness in the world and indeed much work with carotenoids has been done in this context. It was once popular belief that carotenoids are antioxidants against cancer in humans but after three long-term clinical trials it was observed that they either have no effect or actually promote cancer in heavy smokers. In the human eye, the macula lutea has a yellow coloring due to highly concentrated lutein and zeaxanthin. One study showed that there was a significant effect of β-carotene in reducing age-related macular degeneration (AMD). It is thought that the carotenoids help protect the retina from blue and actinic light. Carotenoids are also found in infant brain, and it is currently proposed that they have a role in brain development and cognitive functions. In the corpus luteum of the ovaries, carotenoids may act as general antioxidants. In animals such as birds, it is believed that carotenoids are an ornamental feature because the vibrancy of
color indicates the quality of health, which is particularly important when choosing a mate\textsuperscript{17}.

Carotenoids are primarily synthesized in photosynthetic organisms and bacteria through a variety of carotenoid biosynthesis pathways\textsuperscript{18}. The intermediate steps of carotenoid synthesis can be regarded as quite complex, involving many enzymes and chemical reactions to achieve many of the final carotenoids that are incorporated into proteins for light harvesting or photoprotection. Briefly, regarding a simplified explanation in higher plants, carotenoid biosynthesis begins through two alternative but interacting pathways that lead to the synthesis of isoprenoid isopentenyl pyrophosphate (IPP). IPP is isomerized by IPP isomerase into the dimethylallyl pyrophosphate (DMAPP). Then, in a process involving geranylgeranyl pyrophosphate (GGPP) synthase, a molecule of 20 carbons named GGPP is generated as DMAPP condenses with three molecules of IPP. Subsequently, phytoene, a symmetrical 40-carbon molecule, is synthesized from two molecules of GGPP by phytoene synthase. Phytoene desaturase synthesizes translycopene from phytoene; translycopene is the first molecule in the pathway that is a chromophore\textsuperscript{19}.

1.2.2. Carotenoid Structure and Electronic Configuration

As stated previously, there are over 600 known carotenoid molecules\textsuperscript{20}. All carotenoids are derived from 8 isoprene molecules, thus they are tetraterpenoids and contain 40 carbon atoms (although there are exceptions to the 40 carbon rule like peridinin which has 39 and fucoxanthin which has 42). Typically, carotenoids are composed of a chain of carbon atoms alternating in single and double bonds; thus forming a conjugated chain where electrons are delocalized, and which is responsible for the carotenoid electronic properties. The longer this conjugated chain, the lower the energy of the carotenoid electronic transition(s) will be.
They can be linear chains or contain cyclical end groups, contain oxygen, possess unique features like a lactone ring in the conjugated chain (peridinin is a well-studied example), be in all trans or cis configuration, etc. Figure 1 shows the structure of some common carotenoids.

![Image of carotenoids](image)

**Figure 1. Examples of some carotenoids**

The electronic state designations of carotenoids are S0 (1\Ag-), S1 (2\Ag-), and S2 (1\Bu+), thus they have approximately C2h symmetry. One unique feature of carotenoids is that the S0 to S1 transition is one photon forbidden because these states share the same symmetry (g/u) and pseudoparity (+/-). Thus, S1 absorption is silent (S1 is also called a “dark” state). One photon transitions from the ground state (S0) are allowed to S2 and confer to carotenoids their color. Figure 2 is a schematic of the excitation and relaxation of a typical carotenoid.
Figure 2. A schematic of carotenoid energy levels, the S₀-S₁ state is forbidden, though the S1 state may be achieved by relaxation from the S₂ state.

The energy of the carotenoid electronic state is firstly sensitive to their structure and configuration, but also to the properties of their environment. Figure 3 shows the absorption spectra of β-carotene dissolved in a range of solvents; the environment around the carotenoid changes the absorption profile of the molecule. There has been extensive comparison on isolated carotenoids dissolved in a range of solvents. Certain factors can affect the energy of electronic states observed for a given molecule such as environmental effects (examples are polarizability or to a lesser extent polarity). Other factors are physical constraints of the molecule that are often found in a protein environment like cis/trans isomerizations. S-cis to trans cycle positions may also be observed; these are partial twists in the bonds that link the chain and the terminal ring. They are neither fully cis nor trans, but somewhere in between.
1.2.3. Photosynthesis

Carotenoids are essential to photosynthesis, and thus were often studied in connection with chlorophyll and/or plants. Photosynthesis is the process in which the energy of the solar photons is captured, converted, and stored by an organism, and the stored energy is used to drive energy-requiring cellular processes. Figure 4 shows a representation of photosynthesis in higher plants. In oxygenic organisms like higher plants, a photon is first absorbed by pigments in the membrane-embedded light harvesting complexes called the light-harvesting chlorophyll a/b binding protein complex II (LHCII)\(^1\). A wide range of different proteins, either soluble or membrane-embedded act as antenna, such as (just to quote algal proteins that I studied during my thesis) the peridinin chlorophyll protein (PCP), fucoxanthin chlorophyll a/c-binding protein (FCP), or violaxanthin-chlorophyll a binding protein (VCP). See figure 5 for structures of LHCII from spinach and reconstituted form PCP from dinoflagellates; there are no crystal structures of FCP or VCP. The pigments of LHCII are chlorophyll a, chlorophyll b,

\(^1\) Note that photons may be absorbed by chromophores that are found in other protein complexes besides LHCII, and this is a simplified explanation of the process of photosynthesis.
lutein, neoxanthin, and xanthophyll-cycle carotenoids; in vivo LHCII form trimers in the membrane\(^{21}\). Light harvesting complexes are found in the thylakoid membrane of chloroplasts; they attach to the photosystem in plants so they can transfer the harvested energy to them. PSII in plants exists in the membranes as dimers and they also contain chromophores, chlorophyll a and \(\beta\) carotene. Supercomplexes between PSII and LHCII, called C2S2 supercomplex, exist in the membrane; each PSII core dimer is bound to two copies of the minor antenna complexes CP29 and CP26 and an LHCII trimer. A C2S2M2 supercomplex is formed when the C2S2 supercomplex binds a further two copies of CP24 and two more LHCII trimers. The scaffolding and arrangement of photosynthetic components is extremely important during photosynthetic regulation, especially in varying environmental light conditions. Plants regulate photosynthesis in real time depending on the type and intensity of light by altering the arrangement of their complexes.

After absorption of photonic energy by pigments in light harvesting complexes, the resulting excitation energy is equilibrated in these complexes on a chlorophyll a molecule. It then migrates to the photosystem II (PSII) protein. A chlorophyll structure denoted P\(_{680}\), long thought to be constituted of two chlorophylls forming an excitonic dimer (and now considered as involving four chlorophylls), is able to perform charge separation when receiving the excitation energy from the antenna pigments. Excitation of P\(_{680}\) results in the rapid donation of an electron to a pheophytin molecule (a chlorophyll that has lost its central magnesium atom) followed by the transmembrane transfer of an electron to a quinone. P\(_{680}\) is reduced by electrons taken from water molecules by a manganese cluster generating molecular oxygen, which is released as waste; simultaneously two protons are pumped into the lumen. Two electron cycles induce the formation of a quinol, which leaves the PSII and reaches the cytochrome b6f complex, while two more protons are pumped into the luminal space. The electrons then migrate to photosystem I (PSI) via plastocyanin, a small
copper electron-transferring protein. There, a second flash, exciting the PSI primary electron donor P$_{700}$, will lead to the production of low potential chemical species such as NADPH, which is a cofactor of many enzymatic reactions. Once the proton gradient has been established, protons will be transferred through ATP synthase, another large protein complex embedded in the membrane; this process will result in the generation of an adenine triphosphate (ATP) molecule, the so-called “energy molecule” of the cell. Also note that NADPH and ATP are the basic requirements for CO$_2$ fixation.


During the water-splitting phase of photosynthesis, there is production of O$_2$. Molecular oxygen formed as a byproduct of photosynthesis is an essential component of the biosphere; however its reactivity is treacherous. The ground state of oxygen is unique- it exists as a triplet where most molecules reside in a singlet state. Triplet chlorophyll may form from intersystem crossing from chlorophyll singlet states; it may also occur when there is excess light absorption, which can lead to radical pair recombination in reaction centers. Occasionally excited state chlorophyll transfers energy to oxygen and though it is a rare event, the excitation of oxygen to the singlet state may occur. When oxygen moves into the singlet state (O$_2^+$) it is extremely reactive. In fact, singlet oxygen is so reactive
that it may interact with virtually anything it encounters in the cell like lipids, proteins, and co-factors, etc. This reaction poses a serious threat to the health of the cell.

1.2.4. Carotenoids in Photosynthesis- Light harvesting and Photoprotection

Carotenoids are found in all light harvesting complexes from oxygen-evolving organisms. Carotenoids have a paradoxical role in photosynthesis. They act as accessory light-harvesting pigments, effectively extending the range of light absorbed by the photosynthetic apparatus because their absorption maxima lie between the two main peaks of chlorophyll absorption. They also perform an essential photoprotective role by quenching triplet state chlorophyll molecules and scavenging singlet oxygen and the other toxic oxygen species formed during stress conditions such as high light within the cell.

![Figure 5. Some examples of light harvesting complexes A. PCP B. LHCII](image)

The protective role of carotenoids was first shown in 1955 when Griffiths et al. made a lethal mutation in the genome of the non-sulfur purple bacterium *Rhodobacter sphaeroides*\(^2\); it was deficient in colored carotenoids and was more photosensitive in the presence of air. In nature, plants are exposed to variable
light intensities. Thus, they must react to these fluctuations. High light conditions can result in damage due to the overreduction of photosynthetic apparatuses and subsequently the production of dangerous reactive oxygen species (ROS). Through a fast process called non-photochemical quenching (NPQ) the energy is dissipated. Many theories have been put forward on the working mechanisms of NPQ. Among them, one was recently shown wherein LHCII in higher plants dissipates the excess energy as heat when a conformational change allows the dark S1 state of lutein to act as a quencher of excited state chlorophyll23. In cyanobacteria, there seems to be an equivalent mechanism of energy dissipation, but at the level of the light harvesting complex, the phycobilisome. A 35 kDa protein, the soluble orange carotenoid protein (OCP), has two forms, a nonactive orange-colored form (OCPo) and an active red-colored form (OCPr) that appears in the presence of intense blue-green light. OCPr has the ability to bind the phycobilisome and shuttle excess energy away in high light conditions24,25 via the echinenone molecule that spans its two domains. In the case of both types of NPQ, the carotenoids are the excess excitation energy quenchers. There are other examples of carotenoids as excess energy quenchers; zeaxanthin is accumulated in large amounts during stress conditions. Also, in certain algae, under stress conditions there is an increase in carotenoid content; high salinity shifts the cycle to accumulate more violaxanthin and high light causes more accumulation of zeaxanthin, though stoichiometrically the total carotenoid content is the same26. During salt stress in tomatoes the accumulation of zeaxanthin has an antioxidant role in the scavenging and quenching of singlet oxygen and/or free radicals in the lipid phase of the membrane27.

1.2.5. Prevention of Singlet Oxygen

The work reported here is focused on carotenoids implicated in two key roles: the harvesting of light in protein complexes and photoprotection due to the quenching or prevention of singlet oxygen. In both major functions, excitation
energy transfer to the carotenoid electronic states is how these roles are accomplished.

![Energy Level Diagram](image)

**Figure 6.** An energy level diagram depicting the possible pathways of energy transfer between oxygen, carotenoids, and chlorophyll. The figure demonstrates how carotenoids compete with oxygen for excitation energy from chlorophyll; this competition is responsible for their prevention of singlet oxygen.

As far as molecular excitation goes, triplet chlorophyll lasts relatively long. Figure 6 shows how carotenoids prevent singlet oxygen species; the lowest excited triplet energy level of carotenoids (having > 8 db) is below that of chlorophyll and the \(^1\)O\(_2\) energy level, which equates to two distinctive protective mechanisms:

1) The T\(_0\) energy of carotenoids lies below that of singlet oxygen. Thus, once energy is transferred from triple chlorophyll to a carotenoid, it cannot be transferred to oxygen; this competition for chlorophyll excitation energy is how carotenoids prevent ROS. The triplet chlorophyll state can be quenched by the carotenoid ground state through triplet-triplet energy transfer (step 1), preventing formation of \(^1\)O\(_2\) (step 2). The triplet carotenoid decays as heat to the ground state (step 3) which does not induce damage to the cell.

2) Singlet oxygen can also be directly quenched by the carotenoid through energy transfer (step 4) which returns the \(^1\)O\(_2\) to its ground state. Again, the triplet carot-
enoid decays to its ground state (step 3) as heat which does not harm the cell.

1.3. Resonance Raman Spectroscopy (RRS)

Raman spectroscopy was the main technique that I used during the course of my PhD, thus I will explain the method and important aspects relative to my work in detail.

1.3.1. Theory

The definition of spectroscopy is the study of the interaction of electromagnetic waves and matter. When monochromatic radiation is incident upon a sample, the light will interact with the sample in some fashion. It can be reflected, absorbed, or scattered; it is the scattering of the radiation that is studied with resonance Raman spectroscopy (RRS).

To speak about RRS, Raman spectroscopy should first be described in detail. Raman spectroscopy was named after Sir C.V. Raman, who reported the phenomenon in 1928. Raman spectroscopy is a technique that usually employs one incident wavelength of electromagnetic radiation from a laser source (normally in the visible, near infrared, or ultraviolet range). The incident photons are scattered by the molecule to be studied; most of the photons will scatter with the same wavelength as the incident photons (elastic scattering - Rayleigh) but some (inelastic scattering) will have a higher (anti-Stokes) or lower (Stokes) frequency. Inelastic scattering is indeed a rare event (approximately only $1 \times 10^{-7}$ of the scattered light is Raman), but with advances in technology the technique began to be highly exploited in the 1980s. The change in frequency of incident photons is due to an energy exchange between the photons and the molecules as they “bounce” off... These changes correspond to the energy of the quantified rotational and vibrational sublevels of the scattering molecules- thus the method yields access to the energy of these sublevels giving access to important
molecular parameters such as configuration, intermolecular interactions, etc. Figure 7 gives a general scheme of Raman spectroscopy.

![Figure 7. A general scheme of Raman spectroscopy.](image)

A particularly exploited Raman technique for biological studies is referred to as “resonance” Raman spectroscopy (RRS) because the incident photon energy used corresponds to an electronic transition of the molecule studied; this means that the incident photon would correspond to a photon that can be absorbed by the molecule. RRS may increase the Raman signal up to $10^6$ orders of magnitude. It is used for its selectivity. Not all vibrational modes are enhanced; only those that are involved in the electronic transition of the molecule and involved in the vibronic coupling. Thus, RRS can be used to probe very specifically the chromophores in complex environments. It is used to obtain spectra of chromophores with exceptional signal/noise ratio, specifically those that absorb in the visible range in the case of carotenoids. An example is an experiment on LHCII; the RRS signal coming at approximately 1520 cm$^{-1}$ when
using an incident wavelength around 500 nm comes exclusively from the carbon
double bond stretching modes of the carotenoids; there are no contributions
from the large protein complex or chlorophyll molecules that are also present.

There are a number of other types of Raman spectroscopy, including surface-enhanced Raman (SERS), tip-enhanced Raman (TERS), transmission Raman, spatially offset Raman, and hyper Raman to name a few.

1.3.2. The Experimental Set-up

There are truly many types of Raman set ups; in our lab most of the
incident laser lines that we use are passed through a monochromator on the way
to the sample. The incident light hits the sample and the scattered light is
collected at a 90° angle from the incident light. The photons are focused by a lens
and then pass through a slit as they enter the spectrometer- it is a two-stage
monochromator (U1000, Jobin Yvon, Longjumeau, France). They are dispersed by
two sets of diffraction gratings which ensure a high dispersion of the light and the
proper rejection of the elastically scattered photons, and are recorded by a CCD
detector (Jobin Yvon, Longjumeau, France). The longer the spectrometer focal
length (the distance between the dispersing gratings and the detector), the higher
the spectral resolution; our machine has a 2 x 1 meter path length. Resolution is
also affected by the groove density of the grating (the higher the groove density
the higher the resolution) and the quality of the detector. In my experiments I
have used 1200 and 1800 gratings, which give approximately 0.5 cm⁻¹ spectral
resolution when using an incident wavelength between 476 and 528nm.

In my Raman experiments, I have performed almost exclusively
resonance Raman spectroscopy. See figure 8 for a simplified schematic of our
setup.
1.3.3. RRS and Carotenoids

Carotenoids have been extensively studied with RRS and many examples can be found in the literature, from fundamental research to industrial applications. Within these studies, the four main bands that are present in most carotenoids when interrogated with RRS have been characterized. Taking as an example a typical RRS spectrum of an all-trans carotenoid (Fig 9), the bands are labelled $v_1$-$v_4$. Generally, we understand the molecular contributions to these peaks, and for many molecules, calculations have also been performed to verify the experimental results.
Figure 9. The RRS spectrum of lycopene showing the four main resonance Raman bands present in all trans carotenoid spectra.

The $v_1$ band, around 1520 cm$^{-1}$, arises from stretching vibrations of C=C double bonds. Its frequency depends on the length of the $\pi$-electron conjugated chain and on the molecular configuration of the carotenoid. The $v_2$ band at 1160 cm$^{-1}$ consists of contributions from stretching vibrations of C-C single bonds coupled with C-H in-plane bending modes, and this region is a fingerprint for the assignment of carotenoid configurations, i.e. isomerization states. The $v_3$ band at 1000 cm$^{-1}$ arises from in-plane rocking vibrations of the methyl groups attached to the conjugated chain, coupled with in-plane bending modes of the adjacent C-H’s. It was recently reported to be a fingerprint of the conjugated end cycle configuration; this hypothesis was recently confirmed by theoretical modelling. Finally, the $v_4$ band around 960 cm$^{-1}$ arises from C-H out-of-plane wagging motions coupled with C=C torsional modes (out-of-plane twists of the carbon backbone). When the carotenoid conjugated system is planar, these out-of-plane modes will not be coupled with the electronic transition, and these bands will not be resonance-enhanced. However, distortions around C-C single bonds will
increase the coupling of these modes with the electronic transition, resulting in an increase in their intensity.

The $\nu_1$ band, found around 1520 cm$^{-1}$, has been well characterized both experimentally and with calculations; some of these calculations were done in my articles that can be seen in Chapters 2, 3, 4, and 5. We have used a series of density functional theory (DFT) calculations to complete geometry optimizations and calculate the vibrational modes of the molecules. Then, we could compare with the experimental spectral bands and characterize where the vibrations specifically come from on the carotenoid molecules. Changes in the position of the $\nu_1$ band can indicate information about the polarizability of the environment around the carotenoid and the apparent conjugation length of the molecule due to $s$-cis or trans conformations.

1.4. My projects

1.4.1. Chapter 2: Fermi resonance as a tool for probing peridinin environment

This work began with the peridinin–chlorophyll $\alpha$-protein (PCP). There are four unique carotenoids in the reconstituted form PCP (rfPCP), therefore we attempted to probe the peridinins separately using different incident wavelengths and RRS. What we found is that it was first necessary to characterize the bands of peridinin in different solvents. When doing these experiments, large shifts of the carbonyl stretching mode, located around 1750 cm$^{-1}$, were observed. Also, it appeared that there were two bands that were not shifting in unison. We concluded that this phenomenon was probably coming from a Fermi resonance between the carbonyl mode and a H stretching mode overtone; we wanted to verify our results and thus completed calculations and a model of the Fermi resonance which corroborated our experimental findings.
1.4.2. Chapter 3: Assignment of IR bands of isolated and protein-bound Peridinin in its fundamental and triplet state by static FTIR, time-resolved step-scan FTIR and DFT calculations

This study represents a significant step towards the full understanding of peridinin (Per) photophysics. We have investigated the vibrational properties of Per in its ground state and in the excited triplet state using static and time-resolved FTIR spectroscopy. We have also completed DFT calculations to validate our experimental findings. In the step-scan FTIR difference spectrum of the peridinin–chlorophyll α-protein (PCP) new peaks and alleged bands belonging to Per or 3Per, where a triplet state is induced by light, have been identified. The nature of this triplet state has been largely debated, mainly on the basis of time-resolved FTIR studies, thus this paper brings us several steps closer to a full understanding of Per photophysics.

1.4.3. Chapter 4: Resonance Raman spectra of carotenoid molecules: influence of methyl substitutions

The nature of the $\nu_1$ stretching mode in the resonance Raman spectrum of a carotenoid, arising from the C=C stretching mode and found around 1520 cm$^{-1}$, can change depending if the molecule is linear or contains β-rings at the ends. We have compared the RRS results of several carotenoid and polyene molecules with the electronic absorption spectra corresponding to the S0-S2 transition and used density functional theory (DFT) to theoretically analyze them. From the calculations we have found that while the effective conjugation length increases in polyene upon s-cis isomerization at the end, it lengthens in conformers of carotenoids containing β-rings. Another noteworthy conclusion is that methyl groups attached to the conjugated chain of carotenoids induce a splitting of the $\nu_1$ band- an observation that is important for understanding the nature of electron delocalization for many other carotenoids and even other types of molecules.
1.4.4. Chapter 5: Echinenone Vibrational Properties: From Solvents to the Orange Carotenoid Protein

The orange carotenoid protein (OCP) contains an echinenone molecule that accepts energy from pigments in the phycobilisome (the light harvesting complex found in cyanobacteria) in instances of high light, thus preventing the over reduction of the plastiquinone pool and production of dangerous oxygen species. We have investigated the OCP using RRS and electronic absorption of the echinenone molecule that spans its N and C terminal domains. There are two forms of the protein, a nonactive (OCPo) and an active (OCPr) form. We have discovered a type of intermediate form in the OCPo that is relatively red-shifted, and have found that the crystal structure that exists of OCPo is probably not the most blue-shifted form of the protein.

1.4.5. Chapter 6: Triplet-Triplet Energy Transfer in Dyads

This chapter, not yet submitted for publication, entails work that was done in conjunction with Arizona State University and Yale University. Here we investigate two artificial photosynthetic dyads that are constituted of the same key components but have different conformations. Each dyad is composed of a porphyrin and a carotenoid that are covalently linked together. We have studied the triplet-triplet energy transfer between these two molecules and found that in the para linked dyad the transfer rate is much slower than in the ortho linked dyad. Using RRS we have investigated the carbon double bond stretching modes of the carotenoid and found that the shift from ground state to triplet state is different in the two dyads; a surprising find since the absorption spectra are almost identical. Calculations on these molecules provide further insight into the cause for the triplet-triplet energy transfer difference and the root of the RRS signal shift.

1.4.6. Chapter 7: Resonance Raman Characterization of Reconstituted wild-type and point-mutated LHCII proteins
This article has not yet been submitted for publication. RRS has been used to evaluate the structure of reconstituted LHCII from wildtype and point-mutated apoproteins. With these mutations the goal was to investigate the chlorophyll b 605 binding site. The RRS shows that the complex is trimerized, which is made apparent by the lutein 2 peak. It is a difficult feat to achieve an LHCII trimer in a reconstitution; often monomers are observed. This manuscript is currently being reworked and further experiments are being completed before its submission, such as low temperature absorption and HPLC.

### 1.5. References


CHAPTER 2

FERMI RESONANCE AS A TOOL FOR PROBING PERIDININ ENVIRONMENT

In the present paper, we provide an extended study of the vibrational signature of a butenolide carotenoid, peridinin, in various solvents by combining resonance Raman spectroscopy (RRS) with theoretical calculations. The presence of a Fermi resonance due to coupling between the lactonic C=O stretching and the overtone of the wagging of the C–H in the lactonic ring provides a spectroscopic way of differentiating between peridinins lying in different environments. This is a significant achievement, given that simultaneous presence of several peridinins (each with a peculiar photophysical role) in different environments occurs in the most important peridinin containing proteins, the peridinin-chlorophyll proteins (PCPs) and the Chl α-c2-peridinin binding proteins. In RRS, small modifications of solvent polarity can give rise to large differences in the intensity and splitting between the two bands, resulting from the Fermi resonance. By changing the polarity, we can tune the frequency of stretching of the C=O and, while the C–H wagging frequency is almost always constant in different solvents, move the system from a perfect resonance condition to off-resonance ones. We have corroborated our spectroscopic findings with a quasi-classical dynamical model of two coupled oscillators, and DFT calculations on peridinin in different solvents; we have also used calculations to complete the peridinin vibrational mode assignments in the 800–1600 cm\(^{-1}\) region of RRS spectra, corresponding to polyene chain motion. Finally, the presence of Fermi resonance has been used to reinterpret previous vibrational spectroscopic experiments in PCPs.

This chapter is based on the following publication:
Introduction

Carotenoids represent one of the most widespread groups of naturally occurring pigments, which are largely responsible for the red, yellow, and orange color of fruits, vegetables, flowers, and a wide range of organisms. Characteristically, carotenoids contain alternating carbon–carbon single and double bonds, from which arise their electronic properties that confer them various functions in biology, ranging from light-harvesting and energy dissipation to protection against singlet oxygen (1).

One carotenoid molecule in particular, peridinin (Per), is found in the light harvesting complexes of dinoflagellates, both in the water-soluble peridinin-chlorophyll-proteins (PCPs) and the membrane-bound Chl a-c2-peridinin binding proteins. (2) PCPs have received considerable attention in the last 15 years (reviewed in ref 3), given the availability of high-resolution structures derived from X-ray crystallography (4), their peculiar photophysical behavior (3,5) their use as a fluorophore in biomedical research (3,6) and as a light-harvesting element in nanostructures for light energy conversion (3,7). Furthermore, artificial PCPs (with simpler structure and/or different chlorophylls) can also be produced by mixing the apoprotein with exogeneous pigments (3-5) making these proteins ideal systems to investigate energy transfer mechanisms.

In contrast to other chlorophyll-protein complexes, soluble PCPs have carotenoid molecules that outnumber the chlorophylls (the normal ratio is 4:1) (2,3). The structure of the PCP complexes consists of two symmetric domains, each with a central chlorophyll (in naturally occurring PCPs, Chl a), usually surrounded by four Per molecules (4). The protein provides distinctive surroundings for the pigment molecules, and in PCPs, each Per displays different absorption properties, suggestive of different functions for these pigments within the protein (3,5).

The molecular structure of Per shows several peculiar features: instead of the C40 structure characteristic of most carotenoids, Per has an unusual C37 skeleton; an allene moiety and a butenolide ring are conjugated with the π system of the carotenoid backbone (3,5). An ester group is located on one β-ring with a tertiary alcohol, whereas an epoxy group with a secondary alcohol is located on the opposite β-ring (the Per structure is shown in Figure 1). The allene and butenolide moieties have been shown to be crucial in determining the peculiar photophysical characteristics of Per, notably the presence of a S0–S1 transition (which is normally forbidden in carotenoids by symmetry), which explains its relatively high fluorescence quantum yield (3,5). The presence in the excited state potential energy surface of a so-called intramolecular charge transfer (ICT) state is stabilized in polar solvents and is further enhanced in protic solvents through hydrogen bonding involving the butenolide C═O of Per as an acceptor (3,5,8-10).
The vibrational band associated with the lactonic C=O stretching has been largely used as a probe to study Per and PCP photophysics (11-17). In particular, the dependence of the position of the carbonyl stretching bands on the environment has been proposed (16) as a rationale to distinguish between the lactonic C=O stretching bands of different Pers in PCPs during singlet–singlet Per-Chl energy transfer (11,13) or upon triplet state formation (12,14,15). The different mechanisms by which the surrounding environment can modulate the position of the lactonic C=O band of Per has recently been investigated by our groups (16) by performing vibrational spectroscopy (Raman and Infrared) experiments on Per in different solvents coupled to QM/MM molecular dynamics simulations, which provide a molecular rationale of the observed behavior. We found that the position of the lactonic C=O stretching band is strongly environment-dependent. Its position can vary between 1735 and 1777 cm$^{-1}$, with two key factors influencing band shape and position: the dielectric constant of the solvent and the involvement of the lactonic C=O in a hydrogen bond (as an acceptor) with the solvent (16). In ref 16, just three solvents were used, following a typical rationale of an apolar solvent (cyclohexane), a polar/aprotic solvent (acetonitrile), and a polar/protic solvent (methanol).

In this work, Per vibrational spectra have been studied in a wider range of solvents for further investigation of the lactonic carbonyl band, using resonance Raman spectroscopy (RRS) and theoretical calculations. RRS is an excellent tool to study carotenoids (18); as a vibrational technique, it yields direct information about the properties of the molecule’s electronic ground state.
Materials and Methods

Sample Preparation

Per was extracted from main-form PCP (MFPCP) from Amphidinium carterae. Pigments of MFPCP were extracted with butanol (19), dried under vacuum, and dissolved in ethanol. Per was separated from the crude pigment extract using a Strata C-18-E (55 μm, 500 mg/6 mL) column (Phenomenex). The sample was applied in 70% of Strata Solution (StS: 50% methanol/50% acetonitrile) followed by a wash step [70% StS for two column volumes (CV)]. Per was eluted by application of 95% StS, dried under vacuum, and shipped at room temperature for further experiments.

Solvents

The solvents used were tetrahydrofuran (THF), hexane, cyclohexane, and acetonitrile (all absolute grade, ≥ 99.5% GC), 1,2-dichloroethane (DCE), methanol (anhydrous, 99.8%), and diethyl ether (≥99.8% GC), all purchased from Sigma-Aldrich (St. Louis, MO). Solutions with Per were freshly prepared and measured immediately; exposure to light and oxygen was avoided.

Spectroscopy

Room temperature UV–vis absorption spectra of Per dissolved in the different solvents were measured in a 1 cm optical path length quartz cuvette using a Varian Cary E5 double-beam scanning spectrophotometer, and optical density was between ∼0.2 and 0.4 OD.

RRS was performed on Per in seven solvents with an excitation wavelength of 514.5 nm; supplementary experiments were carried out for three solvents (cyclohexane, diethyl ether, and acetonitrile) using 488.0, 496.5, and 501.7 nm excitation wavelengths. Excitation was provided by a 24 W Ar+ Sabre laser (Coherent, Palo Alto, California), and spectra were recorded at room temperature with 90° signal collection using a two-stage monochromator (U1000, Jobin Yvon, Longjumeau, France), equipped with a front-illuminated, deep-depleted CCD detector (Jobin Yvon, Longjumeau, France). In order to prevent sample degradation by the absorbed light, low intensity laser power (typically less than 20 mW) reached the sample during recording of the spectra, and sample integrity was systematically assessed by following the evolution of the resonance Raman (RR) spectra during the experiment. Absorption UV–vis spectra were also taken before and after the RRS experiment to ensure there was no sample degradation. Spectra were collected between 800 and 2000 wavenumbers (cm⁻¹).
For each measured spectrum, at least three spectra were collected and averaged; and other treatment was done on the spectra, such as removal of peaks due to cosmic waves or dead detector pixels. A multipoint baseline was taken and spectra were normalized according to the $v_1$ peak. Peak positions were determined, considering second derivatives of the recorded spectra.

The FTIR spectrum of solid Per was recorded on a Bruker Tensor 22 spectrometer equipped with an Attenuated Total Reflectance accessory and a DTGS detector.

DFT Calculations

DFT calculations were performed on the complete Per molecule (Figure 1). The system is relatively large, since it has a C37 carbon skeleton for an overall size of 96 atoms. The DFT calculations were performed using the ORCA package (20,21), with the GGA functional PBE (22) in the spin-restricted Kohn–Sham scheme. The TZVP Ahlrichs basis sets (23) have been employed for all atoms with the SCF convergence defined “TightSCF” (energy change of $1 \times 10^{-8}$; max-density change of $1 \times 10^{-7}$; rms density change of $5 \times 10^{-6}$; and DIIS error of $5 \times 10^{-7}$), and a high precision for the integration grids (“Grid4”) is used. The minimum energy geometry calculation and the vibrational analysis were performed in different solvents using the implicit solvation approach COSMO (24).

Results and Discussion

Figure 2 displays the RR spectrum of Per obtained at 514.5 nm excitation in hexane and acetonitrile (i.e., in solvents of different polarity). Three main groups of bands can be observed, termed $v_1$ to $v_3$. Typical of carotenoids, the $v_1$ band at 1524 cm$^{-1}$ mainly arises from the stretching modes of the C–C double bonds (25). The shoulder of this band at higher frequency (also attributed to a normal mode located on the C–C chain (25)) is slightly more pronounced as solvent polarity decreases (data not shown). Generally in carotenoids, the so-called $v_2$ band comprises a main component and two to three satellites (26). In Per, three bands are observed (at $\sim$1125, $\sim$1140, and $\sim$1180 cm$^{-1}$). We attribute the $v_2$ peak at $\sim$1140 cm$^{-1}$ to coupling between the in-plane rocking vibrations of the methyl groups attached to the conjugated chain with the adjacent C–H in-plane bending modes. A small but significant solvent effect is observed for this band, which shifts from 1140 cm$^{-1}$ (in hexane) to 1145 cm$^{-1}$ (in acetonitrile). DFT calculations actually predict that a band arising from the C–C single bond stretching modes coupled with C–H in-plane bending modes ($v_2$) shifts about 6 cm$^{-1}$ when transferring Per from hexane to acetonitrile (see Table S1 of the Supporting Information). This suggests that the mode becomes a bit more localized on the polar part of the molecule when moving to a more polar solvent. The $v_3$ band in Per appears at around 940 cm$^{-1}$, which corresponds to a lower frequency
value as compared to most carotenoid molecules, for which this band is usually observed around 1000 cm\(^{-1}\). In β-carotene, the \(\nu_3\) band is mainly attributed to rocking modes of the methyl group of the carotenoid chain.\((25)\) At 940 cm\(^{-1}\), β-carotene also has a peak, termed \(\nu_4\), which involves the C–H out-of-plane wagging motions coupled with C=C torsional modes, which are out-of-plane twists of the carbon backbone.\((25)\) This mode is not coupled with the electronic transition for planar molecules and gains intensity upon distortions of the conjugated chain; it is used as a reporter of the carotene planarity.\((26,27)\) Experimentally, a small increase in the frequency value is observed from Per in acetonitrile to hexane/cyclohexane (from 939 to 942 cm\(^{-1}\)). The frequency of the corresponding C–H out-of-plane wagging mode in our DFT calculations (see Table S1 of the Supporting Information) is slightly decreased, when changing from polar to nonpolar solvent. This experimentally observed relatively small effect (3 cm\(^{-1}\) upshift) cannot be therefore described by the static DFT calculations, which also do not take into account the RR intensities.

In the high frequency region of these spectra (around 1750 cm\(^{-1}\)), there is a weaker band (compared to \(\nu_1\), \(\nu_2\), and \(\nu_3\)) arising from the stretching modes of the Per conjugated lactonic C=O group (Figure 3).\((16)\) As expected from the stretching modes of a bond possessing a large intrinsic dipole, these modes are highly sensitive to the polarity of the solvent (Table 1) and display a global downshift in spectra of Per in hydrogen bond-donating solvents like methanol (Figure 3, Table 1), due to the formation of H bond(s) between the

---

Figure 2. Resonance Raman spectra of peridinin in n-hexane and acetonitrile (excitation wavelength 514.5 nm, spectral window 800–2000 cm\(^{-1}\)).

In the high frequency region of these spectra (around 1750 cm\(^{-1}\)), there is a weaker band (compared to \(\nu_1\), \(\nu_2\), and \(\nu_3\)) arising from the stretching modes of the Per conjugated lactonic C=O group (Figure 3).\((16)\) As expected from the stretching modes of a bond possessing a large intrinsic dipole, these modes are highly sensitive to the polarity of the solvent (Table 1) and display a global downshift in spectra of Per in hydrogen bond-donating solvents like methanol (Figure 3, Table 1), due to the formation of H bond(s) between the
methanol OH group (donor) and the Per lactonic carbonyl (acceptor). Surprisingly, two peaks may be observed in every solvent in this spectral region. If we exclude the H-bond-donating solvent methanol, a peak around 1777 cm\(^{-1}\) (hereafter called \(\nu_a\)) is present in every solvent, together with another peak whose position is solvent dependent and varies between 1751.4 and 1767.3 cm\(^{-1}\) (hereafter called \(\nu_b\)) (see Table 1 and Figure 3).

Table 1. Resonance Raman Experimental Values of \(\nu_a\) and \(\nu_b\) peaks, Corresponding to the C=O Stretching Spectral Region for Different Solvents

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Dielectric Constant ((\varepsilon_r))</th>
<th>High Frequency Peak Position (\nu_a)</th>
<th>High Frequency Peak Intensity</th>
<th>Low Frequency Peak Position (\nu_b)</th>
<th>Low Frequency Peak Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>acetonitrile</td>
<td>37.5</td>
<td>1778.0</td>
<td>0.006</td>
<td>1751.7</td>
<td>0.043</td>
</tr>
<tr>
<td>methanol</td>
<td>32.7</td>
<td>1778.0</td>
<td>0.008</td>
<td>1737.7</td>
<td>0.031</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>-----</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>DCE</td>
<td>10.4</td>
<td>1778.0</td>
<td>0.013</td>
<td>1751.4</td>
<td>0.041</td>
</tr>
<tr>
<td>THF</td>
<td>7.6</td>
<td>1776.1</td>
<td>0.020</td>
<td>1757.5</td>
<td>0.039</td>
</tr>
<tr>
<td>diethyl ether</td>
<td>4.3</td>
<td>1775.7</td>
<td>0.032</td>
<td>1759.8</td>
<td>0.031</td>
</tr>
<tr>
<td>cyclohexane</td>
<td>2.0</td>
<td>1776.9</td>
<td>0.043</td>
<td>1767.3</td>
<td>0.029</td>
</tr>
<tr>
<td>hexane</td>
<td>1.9</td>
<td>1776.9</td>
<td>0.035</td>
<td>1766.1</td>
<td>0.015</td>
</tr>
</tbody>
</table>

\(^a\)Frequencies are in wavenumbers (cm\(^{-1}\)), and intensity is in arbitrary units. Note that the reported position of the \(\nu_b\) band in methanol is due to the Per conformer involved as an acceptor in a hydrogen bond with the solvent (see text for further details). Solvent dielectric constants (\(\epsilon_r\)) are also reported.

Per indeed possesses two different carbonyls: the lactonic one and an ester carbonyl. However, the latter is nonconjugated and, hence, is not expected to contribute to the RR spectra. Furthermore, the frequency of the stretching mode of this C\(\equiv\)O, as previously observed (16), is expected at lower wavenumbers.

The two C\(\equiv\)O stretching frequencies (\(\nu_a\) and \(\nu_b\)) could reflect inhomogeneity in our samples. However, the RR spectra we obtained in different solvents allow us to rule out the presence of cis Per isomers in our samples. Carotenoid isomerization, by playing on the molecular symmetry, induces the appearance of additional modes in the RR spectra, which are not observed here (28). Furthermore, DFT calculations predict that, as expected, such cis–trans isomerizations should not affect C\(\equiv\)O frequencies by more than a few wavenumbers (not shown). Finally, even if such a difference in the C\(\equiv\)O stretching value existed, the two C\(\equiv\)O peaks corresponding to two different isomers would shift in parallel, following the solvent polarity.

We cannot hastily rule out other sources of inhomogeneity in our samples, arising either from the Per sample or our solvents. However, if a subpopulation of Per in our sample would differ by its interactions with the solute, it should display a slightly different absorption spectrum (see Figure S1 of the Supporting Information). The Per absorption spectrum
is indeed particularly sensitive to the molecule environment (5,8,9). Thus, we completed a series of experiments using three solvents (cyclohexane, diethyl ether, and acetonitrile), which span the range of dielectric constants, using a range of excitation wavelengths (488.0, 496.5, 501.7, and 514.5 nm). In general, we do not observe any wavelength dependence in the Per RR spectra (Figure 4 displays the lactonic carbonyl region of Per in the different solvents as an example).

Figure 4. Resonance Raman spectra at different wavelengths in the carbonyl region. The solvents are (a) acetonitrile, (b) cyclohexane, and (c) diethyl ether.
In Per, it is now well-established that an ICT state exists in this excited state manifold (3,5,8-10). Presence of this state could induce different resonance conditions, depending on whether or not the excitation wavelength matches with the S0 to S1/ICT transition. However, such change in resonance would be expected to affect primarily the intensity of the C═O stretching mode and not its frequency (which primarily depends on the ground-state electronic structure). Moreover, the absence of large excitation wavelength dependence in our spectra (see Figure 4) indicates either that the resonance with the S1/ICT transition is much weaker than with S2 or that none of the excitation wavelengths used in this study match with the energy of this transition.

We are thus led to conclude that the presence of two stretching frequencies in the Per RR spectra is an intrinsic property of Per itself, and more precisely, of its lactonic C═O. The presence of two peaks associated with the C═O stretching has actually already been described for other unsaturated lactones (29-33), and this effect has been generally interpreted in terms of a Fermi resonance between C═O stretching of the lactone moiety (the “fundamental peak”, red arrow in Figure 1) and the out-of-plane (oop) wagging of the C–H on the lactonic ring (whose overtone lies in the same spectral region as the C═O stretching; the involved C–H is indicated by the blue arrow in Figure 1) (34). The fundamental mode of the lactonic C–H oop wagging should be in the 884–887 cm\(^{-1}\) region, since this is half of the frequency of the signal that we observe (all overtones are double the frequency of the fundamental mode). However, the peak in this range should not be present in our RR spectra, since the lactonic C–H oop wagging mode is not coupled with electronic transitions involving the π-electron conjugated systems, when the molecule is planar. The FTIR spectrum of solid Per shows IR bands between 870 and 900 cm\(^{-1}\) (Figure 5). These data, along with DFT calculations on normal modes of Per strongly support that the lactonic C–H oop wagging lies in, or very close to, the 884–887 cm\(^{-1}\) range. The C═O band splitting observed by the 514.5 nm RRS experiments in different solvents could thus come from intrinsic vibrational properties of the system. The Fermi resonance should be in turn tuned by solvent polarity, which modulates the C═O frequency.
Two different kinds of theoretical calculations were performed to check the validity of this Fermi resonance model, where the resonance is tuned by solvent polarity. First, DFT calculations showed that the lactonic C═O stretching frequency largely decreases as the solvent polarity increases (as could be expected from the simple consideration that polarity reduces the double bond character, see Figure 6a). On the other hand, the lactonic C–H oop wagging modes are nearly constant in all the solvents investigated (see Figure 6b). We can therefore draw the following picture combining DFT with experimental results: for very apolar solvents, the C═O stretching frequency is slightly higher than the lactonic C–H oop wagging overtone. When the solvent polarity increases, the C═O stretching lowers its frequency; for diethyl ether, which has a dielectric constant (εr) of 4, we have perfect resonance (equal intensity between va and vb in RR spectra). By further increasing the solvent polarity, the C═O and the C–H oop wagging overtone become more and more off-resonance and the high frequency peak, va, becomes lower and lower in intensity. (The limit for acetonitrile: εr = 37.5; in this case, va appears just as a weak shoulder).
Figure 6. DFT frequencies (unscaled values) corresponding to C═O stretching (panel a) and C–H wagging (panel b) as a function of solvent dielectric constant, $\varepsilon_r$.

The second theoretical approach we used relies on the usual model to explain Fermi resonance (i.e., the coupling between two harmonic oscillators):

$$V(x_1, x_2) = \frac{1}{2} \omega_1^2 x_1^2 + \frac{1}{2} \omega_2^2 x_2^2 + \frac{1}{2} \gamma x_1 x_2$$

where $x_1$ and $x_2$ are mode positions away from equilibrium (equilibrium is taken as the spectrum from diethyl ether), $\omega_1$ and $\omega_2$ are the corresponding frequencies, and $\gamma$ the coupling. In the case of Per, the first mode corresponds to the high frequency C═O stretching and the second to the low frequency lactonic C–H oop wagging. The coupling form, $\frac{1}{2} \gamma x_1 x_2$, is the same originally proposed by Fermi, discussing for the first time such resonance in the Raman spectrum of CO$_2$ (35). However, in the present case only one low frequency mode has been considered; it is also Raman inactive. The coupling value was obtained from the spectrum of Per in diethyl ether, where the intensities of $\nu_a$ and $\nu_b$ are equal with the incident wavelength of 514.5 nm; from the frequency difference, we can evaluate a coupling value of 15 cm$^{-1}$.

Then, we have generated quasi-classical trajectories where the initial conditions were obtained by a Wigner sampling (36) of the uncoupled Hamiltonian (i.e., the Hamiltonian of two oscillators employing potential of eq 1 where $\gamma = 0$) at 298 K and then propagated by numerically solving the classical Newton equation of motion on the full Hamiltonian, using a standard velocity Verlet algorithm (37). For each system, we have propagated an ensemble of 10000 trajectories for about 100 ps. Then, power spectra were obtained from the Fourier transform of the full velocity–velocity correlation function (vibrational density of states, VDOS). Note that the spectra will be composed by both active and inactive Ra-
man (and/or IR) modes, since our model does not include dipole moments or polarizability. Here we focus our attention on the high-energy mode that corresponds to the C=O vibration and on the position and intensity of the two peaks resulting from the coupling (i.e., the resonance).

On the basis of the picture arising from experiments and DFT calculations, we employ the Fermi resonance model (eq 1) to obtain positions and intensities of the peaks using different values of “uncoupled” C=O, \( v_{0\text{C}=\text{O}} \) (\( \omega_1 \) in eq 1), while the \( \omega_2 \) value was kept constant (\( \omega_2 = 883.5 \text{ cm}^{-1} \), i.e., \( 2\omega_2 = \omega_1 \)). Note that the uncoupled value corresponds to the frequency that would result if no coupling between modes was present. These values were then used as the input parameters for the calculations. The results are shown in Figure 7, where we have assumed, based on experimental results, perfect resonance at 1767 cm\(^{-1}\) (two peaks of the same intensity) and span the uncoupled C=O frequency from 1745 to 1780 cm\(^{-1}\). These C=O frequencies correspond to tuning solvent polarity. On the other hand, as zeroth-order approximation, the coupling was considered unchanged over the whole frequency range. In the same figure, we have added results obtained from experiments. Note that experiments provide the two frequencies (\( \nu_{\text{C}=\text{O}} \)) and intensities for the series of solvents used but not the C=O frequencies (\( v_{0\text{C}=\text{O}} \)) if they were not coupled (we need these values to compare experimental and theoretical results). To obtain the uncoupled \( v_{0\text{C}=\text{O}} \) values from experiments, we have used the simple formula (30), derived from perturbation theory applied to a Fermi resonance coupling between a fundamental frequency and an overtone (eq 1):

\[
v_{a}^{0} = \frac{(v_a + v_b)}{2} + \frac{(v_a - v_b)}{2} \left[ I_a - I_b \right]
\]

where \( v_a \) and \( v_b \) are the experimental peak positions and \( I_a \) and \( I_b \) are the corresponding relative intensities. Experimental and theoretical points are in relatively good agreement concerning intensities, which is surprising due to (1) the simplicity of the model and (2) the fact that the source of intensity in VDOS and RR spectra are very different (VDOS gives purely dynamical information and RRS implies polarizability changes and excited states). Concerning the frequencies of the two peaks, we found a good agreement between experiments and calculations, in particular for the low frequency (\( v_b \)) resonant peaks (black points in Figure 7, panel a). Some differences are obtained in the position of high frequency (\( v_a \)) resonant peaks (red points in Figure 7, panel a), in particular for low frequency off-resonance portion (i.e., points for which \( v_{0\text{C}=\text{O}} \) is lower than 1767 cm\(^{-1}\)). It seems that the experimental data reach a plateau before theoretical points. This can be due to the fact that we have considered the perfect resonance value as 1767 cm\(^{-1}\); shifting it to higher frequencies could recover a similar plateau (note that theoretical calculations show that
va reaches a plateau, just at lower frequencies than experiments). Unfortunately, experimentally the higher frequency corresponds to the less polar solvent available, so we cannot verify the behavior at higher frequencies. We should also keep in mind that the 1767 cm$^{-1}$ value was taken from RR intensities (i.e., from the spectrum from which we have almost identical RR intensities, the one of Per in diethyl ether), while our theoretical model is based on pure vibrational density of states (VDOS). Furthermore, some other approximations, like fixing the coupling value across the frequency range and having considered only one low-frequency mode coupled to the C=O stretching, could be the origin of this slight difference between calculated and experimental values.

![Figure 7](image.png)

*Figure 7. The frequency (upper panel) and intensity (lower panel) of the two peaks obtained from quasi-classical dynamics (full circles and solid lines), employing the Fermi resonance model (eq 1) at different values of the uncoupled C=O stretching frequency ($\nu_{0C=O}$). The high-frequency peak ($\nu_a$) is in red, and the low frequency ($\nu_b$) is in black. In open circles, we show experimental values obtained by using eq 2 on the RRS data.*

Despite these small discrepancies, overall the calculated spectra are in good agreement with the experimental results, thus reinforcing our hypothesis of Fermi resonance.

**Relevance to Biophysical Studies**

The results described above clearly show that the lactonic C=O of Per is extremely sensitive to its surrounding environment (because of the solvent dielectric constant and hydrogen bonding), as suggested before (16). In addition, due to the Fermi resonance effect, even small variations in the dielectric constant of the surrounding environment can be
probed by the lactonic C=O. It has already been pointed out (16) that the lactonic C=O of each Per in MFPCP from *A. carterae* can be “classified” as a C=O in a polar/protic environment (“methanol-like”), in a polar but aprotic environment (“acetonitrile-like”), or in an apolar/aperotic environment (“cyclohexane-like”). With the new data presented in this paper, two new pieces of information can be derived: (a) the Fermi resonance makes it possible to distinguish between Per, whose C=O lie in environments with a difference in ε of 2 or less, in apolar/aperotic environments. Relying just on our previous work (16), such distinction was a priori not possible; as in those cases, the simple effect of the dielectric constant around carbonyls on the position of the lactonic C=O stretching band is not strong enough. With the present results, thanks to the extremely high dependence of the Per Fermi resonance effect on the dielectric constant (particularly evident for apolar environments), these Per carbonyls can now, in principle, be distinguished spectroscopically. It is difficult to propose here a qualitative classification (e.g., “diethyl-ether-like” environment vs “DCE-like” environments) of the same kind of that proposed in ref 16, as in this case the parallelism between the solvent environment in solutions and pigment–protein interactions in protein binding pocket is not as appropriate as in ref 16. Nevertheless, the basic ideas remain: the perfect resonance is for a dielectric constant (around the lactonic C=O) of ~4; for more polar environments, the νa band is more intense than the νb, and for less polar environments, the νb band is more intense than the νa. (b) As a consequence of the Fermi resonance, if two (or more) bands in the lactonic C=O region are observed in a vibrational spectrum of Per-containing proteins (or more broadly speaking, Per-containing systems), this does not necessarily imply that these bands arise from more than one Per. It should be emphasized that, given that a weak Fermi resonance takes place also in quite polar environments, this phenomenon should always be taken into account. In the following, we will analyze some results from the literature.

Bonetti et al. reported (14) in a 3Per/Per step-scan FT-IR difference spectrum in THF, a negative peak for the lactonic C=O of Per at 1761 cm⁻¹; however, their spectrum clearly shows a shoulder at ~1776 cm⁻¹, in agreement with our RR spectrum of Per in THF, shown in Figure 3. Step-scan FT-IR difference spectra at 298 K in MFPCP (15,17) and PCP from *Heterocapsa pygmaea* (14), where 3Per formation is expected, show, among others, several negative bands in the lactonic carbonyl region. For all excitations used, negative bands are observed above 1760 cm⁻¹ (15,17). Two carotenoid triplet states were identified in MFPCP, and both are associated with negative vibrational contributions above 1760 cm⁻¹, which could arise from Fermi resonance. This is, in particular, the case for a negative peak at ~1770 cm⁻¹, associated with the fast-decaying triplet state (14,15,17), whose position matches our RR observations.
Step-scan FT-IR difference spectra at 100 K on MFPCP (12), contrary to the spectra recorded at 298 K, did not show any clear trace of negative bands above 1760 cm\(^{-1}\), which could possibly reflect Fermi resonance for the lactonic C═O of (one or more) Per(s). This, however, is most probably related to a modification of the triplet formation mechanism, as suggested by other spectral features (12). Conversely, these 100 K step-scan FT-IR difference spectra show, beside the expected positive band at 1719 cm\(^{-1}\) (attributed to the lactonic C═O stretching of \(^3\)Per), a positive band at 1766 cm\(^{-1}\). This seems to suggest that a Fermi resonance effect may also be present for the lactonic C═O of (one or more) \(^3\)Per.

Ultrafast IR spectra (11) on MFPCP recorded with a 480 nm excitation also show negative bands at 1770 and 1749 cm\(^{-1}\), which were assigned to Per. The low spectral resolution of the data does not allow for their detailed analysis, but the presence of these two bands most probably reflects a Fermi resonance effect for the lactonic ring of Per. It is worth noting that the ultrafast IR data published on PCP (11) (as well as the data on Per in organic solvents (11,13)) also show two or more positive peaks in the carbonyl region. This seems to suggest that Fermi resonance for the lactonic C═O may also be present when Per is in the S1/SICT state.

Outside the domain of PCP photophysics, it should be noted that the sensitivity of the Per C═O vibration band to the surrounding environment may provide a way to investigate the biological role of Per (see, for instance, ref 38) at a molecular level. This role is probably related to the presence, and to the biochemical action, of the unsaturated lactonic ring, as observed for similar butenolide compounds (39).

The observed Fermi resonance between the lactonic C═O and the C–H oop wagging of the lactonic ring is also expected to take place in Per derivatives, such as peridininol, anhydroperidinin, and peridinin-3′ester, given that the lactonic moiety lies in the conjugated chain. In literature, values for the lactonic C═O stretching are also reported for other butenolide carotenoids in solid state (e.g., pyrroxanthin acetate (40)). Also for these molecules, under opportune conditions, Fermi resonance involving the lactonic C═O is expected to take place. Also, as previously mentioned, the occurrence of a Fermi-resonance induced double peak for the C═O stretching has been reported for other simpler butenolide compounds (29-33). This suggests that the role of the polyene chain in the Fermi resonance effect observed for Per is somehow marginal. Indeed, the investigation of vibrational mode localization in Per (16), already pointed out that, despite the conjugation between the polyene chain and the lactonic carbonyl, the normal mode of vibration associated with the lactonic carbonyl stretching is almost exclusively localized on the C═O.
It is therefore very likely that Fermi resonance takes place also in other naturally occurring butenolides. Possibly, this effect can be exploited in the investigation of the interaction of these molecules with their environment to better understand their biological role.

Conclusions

In this paper, we report a detailed characterization of the environmental effects on the vibrational properties of Per, notably on the lactonic C=O stretching band, by using organic solvents as model systems. In particular, we have shown that in aprotic environments, a strong Fermi resonance effect takes place and a clear explanation for its dependency on the dielectric constant of the medium has been presented. The vibrational spectroscopy literature results obtained on PCPs have been re-examined in the light of this new effect, which has so far been underestimated in the analysis of vibrational spectra. In particular, the Fermi resonance may make it possible to distinguish among Pers having their lactonic C=O lying in very similar, but not identical, binding sites in PCPs as well as in other Per-containing proteins. In addition, the same effect has been previously shown to take place also in other butenolide molecules. It is therefore likely that the same approach, the possible appearance of C=O twin bands arising from Fermi resonance, whose effectiveness is modulated by the interaction between the lactonic C=O and its environment, may be used also in biophysical investigations on other biologically relevant butenolides.

Supporting Information

Vibrational frequencies, $\nu_2$ and $\nu_3$, of peridinin as obtained by DFT calculations in different solvents (unscaled frequencies) are shown in Table S1; UV–vis absorption spectra of peridinin in different solvents are reported in Figure S1. This material is available free of charge via the Internet at http://pubs.acs.org.gate1.inist.fr.

Acknowledgment

We thank Dr. Tim Schulte and Dr. E. Hoffman from the Ruhr-Universität Bochum for purifying and sending us peridinin for our experiments. Financial support from European Research Council (Advanced Grant PHOTOPROT n. 267333) is greatly acknowledged. This work was supported by the French Infrastructure for Integrated Structural Biology (FRISBI) ANR-10-INSB-05-01.

References


17. Alexandre, M.; van Grondelle, R. Time-Resolved FTIR Difference Spectroscopy Reveals the Structure and Dynamics of Carotenoid and Chlorophyll Triplets in Photosynthetic Light-Harvesting Com-


34. In ref 33, different from refs 29 and 31, it is suggested that the vibrational mode going in Fermi resonance with the lactonic C=O of butenolide compounds is a symmetric–asymmetric coupling of the C=O and C–C bonds of the O=C–C group. This is possibly also the origin of the Fermi resonance we suggest is taking place in Per. We underline that the basic model used in the present paper, involving a “solvent insensitive” band which undergoes Fermi resonance coupling with a solvent-sensitive C=O band still holds true in such an hypothesis.

The vibrational properties of Peridinin in its fundamental state and in the excited triplet state have been investigated by DFT calculations and static and time-resolved FTIR spectroscopy. The infrared spectrum of Peridinin in its fundamental state has been explored in the whole 2000–600 cm\(^{-1}\) range, and interpreted in term of molecular vibrations. In particular, new infrared bands have been identified and assigned to specific molecular vibrations. Peridinin molecular vibrations have also been investigated by DFT calculations. In addition, putative IR bands belonging to Peridinin and \(^3\)Peridinin have been identified in the step-scan FTIR difference spectrum of the Peridinin–Chlorophyll \(a\)–Protein from *Amphidinium carterae*, where light induce formation of a triplet state localized on one or more Peridinins. The exact nature of the triplet state formed in Peridinin–Chlorophyll \(a\)–Protein from dinoflagellates, in particular the possible involvement in this triplet state of \(^3\)Chlorophyll \(a\), has been largely debated in the last few years (see Carbonera et al., 2014 [3]); time-resolved differential FTIR experiments have played a key role in this debate. Identification of IR marker bands for the main molecule (Peridinin) implicated in this photophysical process is therefore particularly important and makes this study a significant step towards the full understanding of Peridinin–Chlorophyll-\(a\)–Proteins photophysics.
Introduction

Peridinin (Per) is a carbonyl-containing carotenoid found in the light-harvesting complexes of dinoflagellates, both in the water-soluble Peridinin–Chlorophyll–Proteins (PCPs) and in the membrane-bound Chl α–c2 Peridinin binding proteins [1]. In PCPs each Chlorophyll a (Chl α) is surrounded by 3 or 4 Per molecules, located in non-equivalent positions [1]. The presence of Per enables light collection in the spectral region where Chl α poorly absorbs [2]. The excitation is then transferred from $^1$Per$^*$ to $^1$Chl α$^*$ with high efficiency via ultrafast energy transfer. Per also has an important photoprotective role, because it can quickly and efficiently (100%) quench the Chl α triplet state, which could react with molecular oxygen to produce harmful singlet oxygen [3]. The unique photophysical behavior of PCPs has suggested their use for technological applications ( [3] and references therein), and has prompted a large series of spectroscopic investigations on these protein complexes in the last 15 years (see [2] and [3] for recent reviews). Furthermore, the availability of high resolution structures (from X-ray crystallography [4]) for some PCPs has made it possible to deeply interpret the experimental data.

Per has a peculiar molecular structure (see Fig. 1): instead of the C$_{40}$ structure, characteristic of most carotenoids, it has an unusual C$_{37}$ skeleton. A butenolide ring and an allene moiety are conjugated with the π system of the carotenoid backbone. An ester group is located on one β ring with a tertiary alcoholic OH group, whereas an epoxy group with a secondary alcoholic OH is located on the opposite β ring. The allene and the butenolide moieties are crucial in determining Per photophysics, notably the presence of a $S_0$–$S_1$ transition (normally forbidden by symmetry in carotenoids): this explains the relatively high fluorescence quantum yield of Per [2] and [3].

Fig 1. Structure of peridinin

Recently, the role of Per as a $^3$Chl α quencher in PCPs has been the subject of several studies (reviewed in [3]). EPR spectroscopic investigations suggests that, upon excitation of isolated PCP complexes, a triplet state localized on one Per is formed [5]. Conversely, some time-resolved step-scan differential FTIR results [6], [7] and [8] seem to suggest the involvement of Chl α in the triplet state, at least at room temperature. This model is largely based on the proposed identification of several spectral features – present simultaneously in the time-resolved IR difference spectra – for both Per and Chlα.
Vibrational spectroscopy, especially Resonance Raman (RR) and differential IR, can be extremely useful in the investigation of light-harvesting complexes and of photosynthetic reaction centers (see [9], [10] and [11] for reviews), because vibrational bands provide direct structural information. Whereas RR is extremely selective, differential IR can probe almost any constituent of the protein complex; the synergic use of both techniques can therefore be particularly useful (see for instance [12]). Several IR and RR studies have been reported on PCPs [6], [7], [13], [14], [15], [16] and [17]. A crucial point in vibrational spectroscopy is to be able to assign a specific band to a given molecular vibration; this task is particularly critical in differential IR, where contributions from any constituent of the protein are expected. In such a case, band assignment also means being capable of understanding which particular molecule or molecular group is responsible for a given vibration. This represents a difficult step in the analysis of differential IR spectra, but also shows the power of the technique: different molecular events can be monitored and a more detailed insight on the working mechanism of the protein can be obtained. This capability is strongly increased with time-resolved IR difference spectroscopy ([11], [18] and [19] and references therein). Different strategies for band assignment in differential IR spectra have been developed ([3], [9], [20], [21] and [22] and references therein). A powerful and direct method is to compare theoretical calculations with experiments (often in the prototypical environments of organic solvents), a strategy which makes it possible to rationalize the factors that determine the precise position of some bands, e.g. the intermolecular interactions with the surrounding environment (see for instance [23]). We have recently applied QM/MM and DFT calculations associated to IR, non-resonant Raman and Resonance Raman experiments on Per in solution [24] and [25]. The aim was to assign Per vibrational bands in the 1500–2000 cm\(^{-1}\) range, and to elucidate the influence of the environment’s polarity and ability to form hydrogen bonds on these bands, especially on the most relevant (and environmentally sensitive) butenolide C\(\text{O}\) stretching band. These data were then used to interpret differential FTIR spectra obtained on different PCPs under different conditions. In the present work, we have used a different approach: we have focused our attention on the entire 2000–600 cm\(^{-1}\) spectral range of both Per and \(^3\)Per, using DFT calculations, static IR spectroscopy of solid Per, and step-scan differential FTIR on PCP (where, upon excitation, \(^3\)Per is formed). The results are then discussed in the framework of the vibrational spectroscopic studies on Per and on PCPs.

Materials and methods

Sample preparation

PCP from *Amphidinium carterae* (A-PCPs) and Per samples were a kind gift from Dr. R.G. Hiller, Macquarie University, Australia. A-PCPs were purified as described in [26]. A-PCP is a heterodimer made up by two pseudoidentical domains, each with four Pers and a Chl a molecule [4]. Per was isolated from *A. carterae* thylakoids following the method of Martinson and Plumley [27] and purified using reverse-phase HPLC.
Infrared spectroscopy
Static FTIR spectra on solid Per were recorded on a Bruker Vertex 22 spectrometer equipped with an ATR (Attenuated total Reflectance) device and a DTGS detector. Resolution was set to 2 cm⁻¹. Peak position was determined with the 2nd derivative method using the Grams program.
A-PCP samples for step-scan FTIR spectroscopy were centrifuged (110,000 rpm) during 50 min and then concentrated – upon drying with nitrogen – on a CaF₂ disk. A second CaF₂ disk was used to squeeze the sample to reach an absorbance of ~0.6 A.U (amide I region).
Step-scan differential FTIR experiments were performed as previously described [20]. Briefly, a Bruker IFS 88 spectrometer equipped with Opus software and a MCT-A photoconductive detector (Judson type J15D16) was used. The spectral range was set to 1974–1100 cm⁻¹ using appropriate IR filters (LOT-Oriel). A pulse (7 ns, 1.5 mJ) from frequency-doubled Nd:YAG laser (Quantel 780:50) was used to trigger the reaction; the repetition rate was set to 4 Hz. Single-sided interferograms were collected with 4 cm⁻¹ resolution, corresponding to 570 mirror stop positions. The 200 kHz 16-bit analog-to-digital converter of the spectrometer was used, with a first step-scan run in the DC mode in order to obtain the single beam spectrum as well as the phase spectrum. Then an AC-coupled step-scan run was carried out, using the maximum gain possible (128) to increase the dynamic range of the transient signal. 20 Traces were collected and averaged at each mirror position during the AC-session. The duration of such a cycle was ~40 min. Results from 50 cycles obtained on three different samples were used. The reconstructed delta-interferograms were Fourier-transformed using the phase from the DC run and the Blakmann-Harris-3-term apodization function. Absorbance difference spectra with a time resolution of 10 µs were calculated using the formula \[ \Delta A = -\log((S + \Delta S)/S) \] where S stands for the static single beam spectrum (Fourier transform of the DC-coupled interferogram) and \( \Delta S \) is the laser-induced single beam difference spectrum (Fourier transform of the AC-coupled interferogram).
Step-scan differential FTIR experiments were performed a 100 ± 1 K using an Oxford Instruments temperature-controlled cryostat.

Calculations
Per structures were optimized using the B3LYP functional [28] and [29] with the 6-311G(d, p) basis set for both singlet and triplet states. On the minimum energy structures, we performed frequency calculations, obtaining normal modes and IR intensities. A scaling factor of 0.9769 was then applied to better reproduce absolute experimental values [30] and [31]. All calculations were performed with the Gaussian09 package [32].

Results and discussion
In Fig. 2 the spectrum of solid Per in the 1950–600 cm⁻¹ is shown. Peak positions (identified by 2nd derivative analysis) are indicated. The most typical spectral features of Per are
clearly visible at 1929 cm$^{-1}$ (allene stretching), $\sim$1521 cm$^{-1}$ (the so-called $v_1$ band of carotenoids [33]), and around $\sim$1740 cm$^{-1}$ (the C=O stretching of the ester and lactonic carbonyls). The 2nd derivative analysis shows a peculiar feature: four (instead of two) peaks are present (at 1714, 1738, 1754, 1776 cm$^{-1}$) in this C=O stretching region. It is interesting to note that in a previous paper [24], where the IR spectrum of Per was analyzed without using the 2nd derivative method, just three peaks were detected (at $\sim$1717, $\sim$1740, and $\sim$1771 cm$^{-1}$). This new data analysis which has a more precise determination of peak position, was therefore able to separate two spectral contributions (at 1738 and 1754 cm$^{-1}$) in the “central”, intense C=O stretching band. By comparison with DFT calculations and with literature data on peridininol (same structure as Per, but devoid of the ester C=O group) the 1714 cm$^{-1}$ band can be attributed to the ester C=O [24]. The presence of three peaks in the lactonic C=O stretching region is probably related to the presence of non-equivalent Per molecules in the unit cell of the solid state and/or to a Fermi resonance effect [25] which-under specific conditions – can take place between the lactonic C=O and a H wagging mode present on the lactonic ring. The lack of the crystallographic structure of solid Per makes it difficult to deeply analyze this effect.

Other intense IR peaks are visible at 1263, 1247, 1031, 1021, 983 cm$^{-1}$. A complete list of experimental peaks are reported in Table 1 (where IR and Raman peaks on solid Per from previous studies are also reported).

**Table 1. Vibrational spectroscopy studies on Per in solid state.** For comparison, Resonance Raman data from Per in hexane are also shown.
<table>
<thead>
<tr>
<th>IR, ATR (this work)</th>
<th>Raman [34] $\lambda_{\text{exc}} = 1064$ nm</th>
<th>IR, KBr pellet [35]</th>
<th>Raman [36] $\lambda_{\text{exc}} = 785$ nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1929</td>
<td>1928</td>
<td>1929</td>
<td></td>
</tr>
<tr>
<td>1776 (s, sh)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1754 (vs, sh)</td>
<td>1747</td>
<td>1741</td>
<td></td>
</tr>
<tr>
<td>1738 (vs)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1714 (s, sh)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1642 (m)</td>
<td>1642</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1619 (w)</td>
<td>1616</td>
<td></td>
<td>1617</td>
</tr>
<tr>
<td>1593 (sh, vw)</td>
<td>1590</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1542 (m)</td>
<td>1540</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1521 (s)</td>
<td>1522</td>
<td>1521</td>
<td>1524</td>
</tr>
<tr>
<td>1472 (w)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1454 (m)</td>
<td>1448</td>
<td>1456</td>
<td>1450</td>
</tr>
<tr>
<td>1429 (vw)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1379 (m)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1364 (m)</td>
<td>1369</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1350 (m)</td>
<td>1350</td>
<td></td>
<td>1351</td>
</tr>
<tr>
<td>1335 (sh, vw)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1298 (w)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1263 (s)</td>
<td>1253</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1247 (s)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1183 (s)</td>
<td>1181</td>
<td></td>
<td>1181</td>
</tr>
<tr>
<td>1162 (s)</td>
<td>1144</td>
<td>1162</td>
<td>1144</td>
</tr>
<tr>
<td>1123 (s)</td>
<td></td>
<td>1125</td>
<td></td>
</tr>
<tr>
<td>1071 (m)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1047 (s)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1031 (s)</td>
<td>1030</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1021 (s, sh)</td>
<td>1021</td>
<td></td>
<td>1021</td>
</tr>
<tr>
<td>983 (s)</td>
<td>985</td>
<td>985</td>
<td></td>
</tr>
<tr>
<td>956 (w)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>940 (w)</td>
<td>941</td>
<td></td>
<td>940</td>
</tr>
<tr>
<td>912 (w)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>895 (w)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>875 (vw)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>857 (vw)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>850 (vw)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>832 (vw)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>817 (w)</td>
<td>818</td>
<td></td>
<td></td>
</tr>
<tr>
<td>799 (vw)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>767 (w)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>718 (br, vw)</td>
<td>726</td>
<td></td>
<td></td>
</tr>
<tr>
<td>654 (w)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
In order to understand which molecular vibrations are responsible for each IR band, we performed DFT calculations whose results – along with the experimental ones – are reported in Table 2. It is interesting to focus our attention on two of the calculated vibrational modes corresponding to the C=C stretching (unscaled values at 1583, 1565 cm\(^{-1}\)). These modes are quite delocalized over the whole C=C chain. Conversely, the mode at 1692 cm\(^{-1}\) (unscaled value) is localized on the C\(_{21}=C_{22}\) bond (i.e. the C=C double bond between the lactonic ring and the epoxy ring). Most probably this effect is related to the presence of the lactonic ring on the C=C chain.

**Table 2. DFT calculations on Per. Only the vibrational modes with calculated intensity > 50 (a.u.) are reported. The experimental results possibly corresponding to the calculated modes are also shown**

<table>
<thead>
<tr>
<th>Experimental (solid Per)</th>
<th>Tentative assignment, PCP step-scan FTIR difference spectrum</th>
<th>Wavenumber (unscaled)</th>
<th>Wavenumber (scaled)</th>
<th>IR intensity</th>
<th>Mode type</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td></td>
<td>3160</td>
<td>3086</td>
<td>62</td>
<td>C-H stretching (C14-H)</td>
</tr>
<tr>
<td>-</td>
<td></td>
<td>3114</td>
<td>3041</td>
<td>53</td>
<td>C-H stretching (CH3 on ester ring)</td>
</tr>
<tr>
<td>-</td>
<td></td>
<td>3095</td>
<td>3023</td>
<td>100</td>
<td>C-H stretching (CH3 on epoxy ring)</td>
</tr>
<tr>
<td>-</td>
<td></td>
<td>3095</td>
<td>3021</td>
<td>101</td>
<td>C-H stretching (CH3 on ester ring)</td>
</tr>
<tr>
<td>-</td>
<td></td>
<td>3102</td>
<td>3030</td>
<td>60</td>
<td>C-H stretching (CH3 on epoxy ring)</td>
</tr>
<tr>
<td>-</td>
<td></td>
<td>3043</td>
<td>2972</td>
<td>52</td>
<td>C-H stretching (CH3 and CH2 on epoxy ring)</td>
</tr>
<tr>
<td>-</td>
<td></td>
<td>3030</td>
<td>2959</td>
<td>69</td>
<td>C-H stretching (CH3 linked to C9)</td>
</tr>
<tr>
<td>-</td>
<td></td>
<td>3029</td>
<td>2958</td>
<td>64</td>
<td>C-H stretching (CH2 on epoxy ring)</td>
</tr>
<tr>
<td>-</td>
<td></td>
<td>2974</td>
<td>2904</td>
<td>91</td>
<td>C-H stretching (C26-H)</td>
</tr>
<tr>
<td>1929</td>
<td></td>
<td>1999</td>
<td>1952</td>
<td>205</td>
<td>C=C=C stretching</td>
</tr>
<tr>
<td>1776; 1754; 1738</td>
<td></td>
<td>1746</td>
<td>1829</td>
<td>1786</td>
<td>1374</td>
</tr>
<tr>
<td>1714</td>
<td></td>
<td>1809</td>
<td>1767</td>
<td>280</td>
<td>C=O ester stretching</td>
</tr>
<tr>
<td>1642</td>
<td></td>
<td>1692</td>
<td>1653</td>
<td>87</td>
<td>C=C stretch (C21=C22)</td>
</tr>
<tr>
<td>1542</td>
<td></td>
<td>1583</td>
<td>1546</td>
<td>162</td>
<td>C=C stretch (delocalized, C13=C14 and C9=C10)</td>
</tr>
<tr>
<td>1521</td>
<td></td>
<td>1523</td>
<td>1565</td>
<td>1529</td>
<td>510</td>
</tr>
</tbody>
</table>

640 (w) 628 (vw)
| 1379 | 1392 | 1360 | 61 | C-C on ester ring |
| 1364 | 1357 | 1386 | 1354 | 53 | C-C and C-H deformation |
| 1350 | 1384 | 1352 | 172 | C-C and C-H deformation (C17, C19) |
| - | 1365 | 1333 | 65 | C-H deformation (C5) |
| - | 1342 | 1310 | 51 | C-H deformation (C21, C22) |
| 1298 | 1332 | 1301 | 93 | C-H deformation (ester ring) |
| 1263 | 1256 | 1313 | 1282 | 66 | C-H deformation (epoxy ring and polyene chain) |
| - | 1301 | 1272 | 104 | C-H deformation (epoxy ring and polyene chain) |
| 1247 | 1275 | 1245 | 138 | C-H deformation (epoxy ring and polyene chain) |
| - | 1259 | 1230 | 60 | C-H deformation (epoxy ring) |
| - | 1257 | 1228 | 465 | Ester group deformation |
| - | 1255 | 1226 | 251 | C-H deformation (ester ring) |
| 1183 | 1216 | 1188 | 182 | C-H deformation (polyene chain, in plane) |
| - | 1209 | 1180 | 54 | C-H deformation (polyene chain, in plane) |
| - | 1203 | 1175 | 145 | C-H deformation (CH3 of ester ring and polyene chain) |
| 1162 | 1191 | 1163 | 214 | C-H deformation (CH3 of ester ring and polyene chain) |
| 1162 | 1191 | 1163 | 50 | C-H deformation (CH2 and CH3 of epoxy ring) |
| - | 1176 | 1149 | 67 | C-C on ester ring |
| 1123 | 1156 | 1129 | 149 | C-H deformation (ester ring and polyene chain) |
| 1071 | 1140 | 1113 | 107 | C-H deformation (epoxy ring and polyene chain) |
| 1047, 1031 | 1046 | 1022 | 125 | C-H deformation (CH3 of ester ring) |
| 1021 | 1042 | 1018 | 153 | C-H deformation (CH3 of ester ring) |
| - | 1037 | 1013 | 283 | C-H deformation (CH3 of epoxy ring and of polyene chain) |
| - | 1031 | 1007 | 55 | C-H deformation (on epoxy ring) |
| - | 1029 | 1005 | 92 | C-H out-of-plane on polyene chain |
| 983 | 1024 | 1000 | 70 | C-H deformation (ester ring and CH3 linked to C9) |
| 956 | 961 | 939 | 63 | Ester ring breathing |
| 940 | 956 | 934 | 52 | Lactone ring breathing |
| 829 | 810 | 60 | Lactone and epoxy ring breathing |
| 703 | 687 | 57 | Epoxy ring breathing |
| 643 | 628 | 92 | Epoxy ring, lactone ring and polyene chain breathing |
| 357 | 349 | 99 | O-H deformation on ester ring |
| 295 | 288 | 114 | O-H deformation on epoxy ring |
Note: in the DFT analysis, several normal modes (20), with moderate intensity (< 50) are present in the 1522-1473 cm\(^{-1}\) region (unscaled values); the correspondent scaled value range is 1487-1439 cm\(^{-1}\). These modes are due to C-H bending of the CH\(_2\) and CH\(_3\) groups, and they can correspond to the experimentally observed peaks at 1472, 1454 and 1429 cm\(^{-1}\) (which most probably are the result of the superposition of several bands).

Table 3 reports the calculated values for the Per triplet state. In this case, no experimental spectrum of pure \(^3\)Per is available; in Ref. [7], a differential FTIR triplet-minus-singlet spectrum of Per (\(^3\)Per/Per) was obtained from a step-scan FTIR experiment in tetrahydrofuran solution in the presence of an excess of Chl a, which, acting as a sensitizer, induced \(^3\)Per formation. Unfortunately, no precise band position, apart for a positive C=O stretching band at 1733 cm\(^{-1}\), was provided (showing, as previously noticed, a downshift of the C=O stretching band when passing from the fundamental to the \(^3\)Per state); therefore, in Table 3 only the tentative assignment for \(^3\)Per in A-PCP (this work, see below) are reported.

Table 3. DFT calculation on Per triplet. Only the vibrational modes with calculated intensity > 50 (a.u.) are reported.

<table>
<thead>
<tr>
<th>Tentative assignment, PCP step-scan FTIR difference spectrum</th>
<th>Wavenumber (unscaled)</th>
<th>Wavenumber (scaled)</th>
<th>IR Intensity</th>
<th>Mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>3152</td>
<td>3079</td>
<td>56</td>
<td>C-H stretching (C11,C14)</td>
<td></td>
</tr>
<tr>
<td>3101</td>
<td>3029</td>
<td>64</td>
<td>C-H stretching (CH3 on epoxy ring)</td>
<td></td>
</tr>
<tr>
<td>3095</td>
<td>3023</td>
<td>99</td>
<td>C-H stretching (CH3 on epoxy ring)</td>
<td></td>
</tr>
<tr>
<td>3093</td>
<td>3021</td>
<td>102</td>
<td>C-H stretching (CH3 on ester ring)</td>
<td></td>
</tr>
<tr>
<td>3043</td>
<td>2972</td>
<td>53</td>
<td>C-H stretching (CH3 on epoxy ring)</td>
<td></td>
</tr>
<tr>
<td>3029</td>
<td>2958</td>
<td>62</td>
<td>C-H stretching (CH3 on epoxy ring)</td>
<td></td>
</tr>
<tr>
<td>2974</td>
<td>2905</td>
<td>93</td>
<td>C-H stretching (C21)</td>
<td></td>
</tr>
<tr>
<td>1956</td>
<td>1910</td>
<td>199</td>
<td>C=C=C stretching</td>
<td></td>
</tr>
<tr>
<td>1810</td>
<td>1768</td>
<td>268</td>
<td>C=O ester stretching</td>
<td></td>
</tr>
<tr>
<td>1719</td>
<td>1753</td>
<td>715</td>
<td>C=O lactone stretching</td>
<td></td>
</tr>
<tr>
<td>1639</td>
<td>1601</td>
<td>118</td>
<td>C=C stretching (C21=C22)</td>
<td></td>
</tr>
<tr>
<td>1580</td>
<td>1623</td>
<td>108</td>
<td>C=C stretching (C21=C22 &amp; C14=C15)</td>
<td></td>
</tr>
<tr>
<td>1592</td>
<td>1555</td>
<td>117</td>
<td>C=C stretching (C12=C13)</td>
<td></td>
</tr>
<tr>
<td>1534</td>
<td>1584</td>
<td>217</td>
<td>C=C stretching (C16=C17)</td>
<td></td>
</tr>
<tr>
<td>1464</td>
<td>1430</td>
<td>176</td>
<td>C-H deformation (on polyene chain, in plane)</td>
<td></td>
</tr>
<tr>
<td>1408</td>
<td>1375</td>
<td>56</td>
<td>C-H deformation (on polyene chain, in plane)</td>
<td></td>
</tr>
<tr>
<td>1406</td>
<td>1373</td>
<td>64</td>
<td>C-H deformation (epoxy ring)</td>
<td></td>
</tr>
<tr>
<td>1422</td>
<td>1389</td>
<td>47</td>
<td>C-H deformation (on polyene chain)</td>
<td></td>
</tr>
<tr>
<td>1393</td>
<td>1361</td>
<td>77</td>
<td>C-H deformation (on polyene chain and ester ring)</td>
<td></td>
</tr>
<tr>
<td>1371</td>
<td>1339</td>
<td>120</td>
<td>C-H deformation (on polyene chain)</td>
<td></td>
</tr>
<tr>
<td>Wave Number</td>
<td>Intensity</td>
<td>Description</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>-----------</td>
<td>-------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1360</td>
<td>1328</td>
<td>C-H deformation and C17C18C19 motion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1332</td>
<td>1301</td>
<td>C-H deformation (on ester ring)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1271</td>
<td>1241</td>
<td>C-H deformation (epoxy ring and C21C22)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1259</td>
<td>1230</td>
<td>C-H deformation (on epoxy ring)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1257</td>
<td>1228</td>
<td>Ester group deformation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1237</td>
<td>1208</td>
<td>C-H deformation (on polyene chain)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1203</td>
<td>1175</td>
<td>C-H and C-C deformation on ester ring</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1178</td>
<td>1151</td>
<td>C-H deformation (on polyene chain)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1177</td>
<td>1150</td>
<td>C-H and C-C deformation (on ester ring)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1164</td>
<td>1137</td>
<td>C-H and C-C deformation (on ester ring and allene group)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1161</td>
<td>1134</td>
<td>C-H deformation (on polyene chain, C19 in particular)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1140</td>
<td>1113</td>
<td>C-H and C-C deformation on epoxy ring</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1045</td>
<td>1021</td>
<td>C-H deformation (CH3 on ester ring)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1042</td>
<td>1018</td>
<td>C-H deformation (CH3 on ester ring)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1041</td>
<td>1017</td>
<td>C-H deformation (delocalized on CH3 and CH groups)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1021</td>
<td>997</td>
<td>C-H out-of-plane of polyene chain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>961</td>
<td>939</td>
<td>Ester ring breathing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>364</td>
<td>356</td>
<td>O-H deformation on ester ring</td>
<td></td>
<td></td>
</tr>
<tr>
<td>294</td>
<td>287</td>
<td>O-H deformation on epoxy ring</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

It is interesting to note that upon triplet formation the vibrational mode mainly reflecting the stretching of the C21=C22 downshifts, compared to the fundamental singlet state (from 1692 cm⁻¹ of Per to 1639 cm⁻¹ of ³Per; both values are unscaled). This indicates a loosening of the bond order in the triplet state. The other IR active C=C modes of ³Per look much more localized (non-delocalized over the chain) than the C=C stretching modes of Per (see Table 3). This makes it difficult to discuss a downshift or upshift of a given vibrational mode. More broadly speaking, we can say that according to DFT calculations ³Per shows relatively intense peaks at 1623, 1592, 1581 cm⁻¹ (unscaled values).

The corresponding scaled values (reported in Table 3), along with direct comparison with the experimental Per IR spectrum, make it possible to propose tentative assignment on the step-scan FTIR difference spectrum (recorded between 10 and 30 μs after the laser flash) obtained on A-PCP, reported in Fig. 3. As described in the introduction, step-scan FTIR difference spectra on isolated PCPs reflect formation of a triplet state, whose nature is still under debate. In fact, whereas there is a general agreement on the fact that the triplet state involves at least one Per molecule (due to the presence in the step-scan FTIR difference spectra of several negative bands corresponding to the spectral position foreseen for Per vibrations), a number of spectral contributions were tentatively attributed to Chl a molecules in strong interactions with Per molecules [6], [7] and [8]. This result how-
ever is in strong contrast with EPR experiments (see [3] and references therein) which suggest the localization of the triplet state just on one Per. In this framework, it is clear that any strategy to assign positive and negative bands in step-scan FTIR difference spectra of PCP can be very helpful to clarify this debated issue.

![Step-scan FTIR difference spectrum recorded between 10 and 30 μs after the flash on PCP from A. carterae (A-PCP) at 100 K in the 1850–1200 cm⁻¹ range. The putative bands for Per (negative peaks) and 3Per (positive peaks) are indicated by an asterisk (*). The FTIR spectrum of solid Per in the same spectral range is also shown.]

Previous kinetic analysis and experiments carried out with different power of the excitation laser pulse [13] have shown that the step-scan FTIR difference spectra on A-PCP, beside the process of triplet formation, reflect also a photothermal effect, particularly strong in the amide II region (peaks at ~1561 cm⁻¹ (negative) and 1530 cm⁻¹ (positive)). These bands therefore do not represent any process related to triplet formation.

Band assignments in the lactonic C=O region (1780–1700 cm⁻¹) have been recently published by our group [3] and [25] and will not be discussed in detail (the reader is referred to the cited literature for further information). It is however interesting to note that the present DFT calculations on Per confirm what has been observed for ³Per in tetrahydrofuran solution [7] and foreseen by DFT calculations on smaller model compounds of Per [13], i.e. a downshift of the butenolide C=O of Per upon triplet formation.

As previously proposed, the 1640, 1538 and 1521 cm⁻¹ negative bands are probably given by C=C vibrational modes of Per. The DFT calculations presented here confirm what was intuitively foreseen, i.e. the shift in position of these IR bands reflecting vibrations on the
C=C chain when passing from Per to $^3$Per. In addition, a further step can be made: putative bands for $^3$Per can be identified in the step-scan FTIR difference spectrum of A-PCP at 1580 and 1533 cm$^{-1}$ (see Fig. 3).

At lower wavenumbers, the negative peaks at 1357 and 1256 cm$^{-1}$ can also be tentatively assigned to Per vibrational modes by direct comparison with the experimental IR spectrum of solid Per. Interestingly, DFT calculations on $^3$Per foresee a shift for both of these modes, explaining why they are observed as negative peaks in the differential spectrum. In this region, identification of $^3$Per in PCP looks more difficult, as there is no direct experimental reference.

It is interesting to note that the present calculations seem to exclude that the positive band visible at 1664 cm$^{-1}$ is due to $^3$Per vibration. This piece of information is interesting because such band, by comparison with literature data, has been tentatively attributed to the 9-keto C=O stretching of $^3$Chl $\alpha$ and taken as a possible evidence of the involvement of $^2$Chl $\alpha$ in the triplet state formed in PCP complexes at room temperature [6], [7] and [8]. The possibility that this spectral feature is due to other molecular groups in the protein (it lie in the amide I region) has however not been ruled out yet.

Conclusion
In this paper, a first detailed assignment of Per vibrational modes were made, by comparison of the IR spectrum of solid state Per, literature-available Raman data of Per in solid state and in solution, and DFT calculations. In addition, DFT calculations for 3Per are also reported. All the obtained data were used to identify new putative marker bands for Per and 3Per in FTIR difference spectra in A-PCP, reflecting formation of a triplet state at low temperature. This point is particularly important as the nature of this triplet state is debated [3], the involvement of Chl $\alpha$ being suggested by the presence of possible IR bands for Chl $\alpha$ and 3Chl $\alpha$ in step-scan FTIR difference spectra obtained in A-PCP at room temperature. It should also be underlined that the energy transfer following light absorption has also been studied by ultrafast IR difference spectroscopy [14] and [15]. Until now, the attention has been mainly focused on the carbonyl stretching region (1800–1650 cm$^{-1}$). The tentative assignment of new IR Per bands in new spectral regions suggest to explore wider spectral ranges to get a more precise picture of the photophysics of A-PCP, as well as of other PCP proteins. This study therefore represents an important step towards the full interpretation of time-resolved difference spectra in PCP proteins, and, more broadly speaking, may prove to be advantageous for spectroscopic vibrational studies of any Per-containing system.

Acknowledgements
We thank Dr. R.G. Hiller for providing us with Per and PCP samples. This work was supported by the French Infrastructure for Integrated Structural Biology (FRISBI) ANR-10-INSB-05-01.

References


CHAPTER 4

RESONANCE RAMAN SPECTRA OF CAROTENOID MOLECULES: INFLUENCE OF METHYL SUBSTITUTIONS

We report here the resonance Raman spectra and the quantum chemical calculations of the Raman spectra for β-carotene and 13,13’-diphenyl-β-carotene. The first aim of this approach was to test the robustness of the method used for modeling β-carotene, and assess whether it could accurately predict the vibrational properties of derivatives in which conjugated substituents had been introduced. DFT calculations, using the B3LYP functional in combination with the 6-311G(d,p) basis set, were able to accurately predict the influence of two phenyl substituents connected to the β-carotene molecule, although these deeply perturb the vibrational modes. This experimentally validated modeling technique leads to a fine understanding of the origin of the carotenoid resonance Raman bands, which are widely used for assessing the properties of these molecules, and in particular in complex media, such as binding sites provided by biological macromolecules.

This chapter is based on the following publication:
Reproduced with permission © American Chemical Society
Introduction

Carotenoids are natural organic molecules built from the assembly of isoprenoid units (Figure 1); they play a number of essential roles in biology (for a comprehensive review of these functions, see the work of Britton et al.(1)). In photosynthesis, they act as light-harvesters (see, e.g., ref 2). They also have a role in photoprotection because of their ability to quench the excited singlet and triplet states of chlorophyll molecules.(1-5) Furthermore, they are able to quench the singlet 1O2 molecule, one of the most harmful reactive oxygen species.(6) Carotenoids are found in a wide range of organisms (algae, animals, plants, etc.) and are often responsible for their bright colors, thus being at the origin of a range of complex signaling reactions in biology. All of the different roles of carotenoids rely on the electronic properties of their linear, conjugated polyene chain. These characteristics give the carotenoid molecule its absorption properties, most often in the blue-green range, which correspond to an electronic transition from the ground to the second electronic state (S0 to S2). However, the whole electronic structure of these molecules turns out to be extremely complex. The existence of a dark, absorption silent, S1 state, lying at energies below the S2 state, was predicted in the 1980s, and experimentally confirmed a few years later.(7, 8) Since then, a number of other low-lying energy states have been suggested by experiments, the nature of which are still not completely clear.(9-11) It became recently apparent that, in polar carotenoids, an intramolecular charge transfer state could underlie or optimize their function.(9) Of course the precise energy position of all of these states highly depends on the carotenoid’s configuration (i.e., whether the molecule is an all-trans or cis isomer), conformation (i.e., whether it is planar or exhibits distortions around the C–C bonds), and the environment.
Figure 1. Chemical structures: (A) 13,13′-diphenyl-β-carotene and β-carotene (atom numbers are used for description of vibrational forms); (B and C) artificially made β-carotene substructures.

In vivo, for instance, the same carotenoid (Car) molecule can display absorption properties differing by more than 2000 cm\(^{-1}\),\(^{12}\) and the ensemble of mechanisms underlying this tuning is far from well-characterized. Recently, smaller absorption variations, observed in photosynthetic proteins, were explained thanks to the combined use of their electronic absorption and resonance Raman spectra.\(^{13}\) Resonance Raman is among the best methods to characterize the structural and electronic properties of Car molecules; as a vibrational technique, it yields direct access to their molecular properties. Resonance Raman scattering occurs when the wavelength of the laser’s excitation is in close proximity to an intense electronic absorption band; resonance Raman signal intensities may display enhancement up to 6 orders of magnitude as compared to normal Raman scattering,\(^{14}\) which is the case for carotenoids. Since Car molecules possess a very large resonance Raman cross-section, their vibrational spectra may be obtained by this method with a very high selectivity even in media as complex as whole organisms. To characterize, for instance, the conformation of Cars in bird feathers,\(^{15}\) or photosynthetic proteins,\(^{4, 16,}\)
17) the variations of Car resonance Raman spectra were used to probe protein conformational changes in vivo. (18)

Resonance Raman spectra of Car contain four main bands termed \(\nu_1\) to \(\nu_4\) (Table 1). The most intense band, \(\nu_1\), located around 1500 cm\(^{-1}\), arises from the stretching modes of the C═C bonds. Thus, it gives access to the structure of the conjugated double bond chain, i.e., the frequency can be considered as a direct measure of the conjugation length of the chain. (19) This band was predicted to be constituted by two modes; (19) however, only one is observed experimentally. The \(\nu_2\) band at 1160 cm\(^{-1}\) arises from a combination of C–C stretching modes and in-plane C–C bending modes. Its structure is highly dependent on the molecular conformation; small changes in this band are observed depending on the chemical structure of the studied molecule. (20) However, thus far, no clear modeling for these fine variations has been provided. The \(\nu_3\) band, at ca. 1000 cm\(^{-1}\), arises from the coupling of the in-plane rocking vibrations of the methyl groups attached to the conjugation chain, with the adjacent C–H in-plane bending. The structure of this band also depends on the chemical nature of Car, (21, 22) but is sometimes split in Cars containing cycles. (18) It was recently proposed that this splitting is a fingerprint of the conjugated end-cycle conformation, (18) but this hypothesis remains tentative. Finally, a weak \(\nu_4\) band at about 950 cm\(^{-1}\) arises from C–H out-of-plane wagging motions coupled with C═C torsional modes. For planar molecules, these modes are expected to be unconjugated with the electronic transition, and, accordingly, this band should be extremely weak. However, it gains intensity when the molecules get distorted around C–C bonds, and is used as a fingerprint of the molecular conformation. (16, 18, 23) This band generally exhibits more than one component, suggesting that the different C–H groups display slightly different frequencies. However, at present, it is still impossible to predict the precise position of the molecule’s distortion from the structure of this band.
Table 1. Four Main Regions of Resonance Raman Spectra of Car with Typical Vibrational Forms

<table>
<thead>
<tr>
<th>Region</th>
<th>Attribution</th>
<th>Frequency, cm(^{-1}) *</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\nu_1)</td>
<td>Stretching C=C modes of the polyene chain</td>
<td>1470 – 1520 (1519 – 1574)</td>
</tr>
<tr>
<td>(\nu_2)</td>
<td>Combination of C-C stretching modes and in plane C-C bending modes of the polyene chain</td>
<td>1090 – 1310 (1124 – 1350)</td>
</tr>
<tr>
<td>(\nu_3)</td>
<td>The coupling of the in-plane rocking vibration of the methyl groups attached to the conjugation chain, with the adjacent C-H in-plane bending</td>
<td>990 – 1020 (1020 – 1050)</td>
</tr>
<tr>
<td>(\nu_4)</td>
<td>C-H out-of-plane wagging motions coupled with C=C torsion modes in the polyene chain</td>
<td>820 – 990 (850 – 1020)</td>
</tr>
</tbody>
</table>

Resonance Raman spectroscopy has proven to be a very sensitive and useful technique for Car studies, but would gain precision if the spectral variations observed in biology could be more finely interpreted. Despite the apparent simplicity of their structure, calculating the vibrational and electronic properties of Car molecules with reasonable precision has only recently become possible through the application of DFT and TD-DFT.(19, 24, 25) However, the robustness of such modeling procedures still needs to be fine-tuned, and the spectra that have been modeled to date have not aided in interpreting all of the spectral variations observed experimentally. Thus, studies of Car derivatives with modified electronic properties are needed. In this paper, we report the resonance Raman of a new, artificial, derivative from \(\beta\)-carotene, namely 13,13′-diphenyl-\(\beta\)-carotene (Figure 1 A), which was used to probe the nature of Car excited states.(26) The spectral properties of this new molecule could be modeled accurately using the same exact method described in ref 19, demonstrating the robustness of this approach. Introduction of the phenyl groups at 13 and 13′ positions induces dramatic perturbations of the resonance Raman spectra of \(\beta\)-carotene. These variations may, in turn, be used to better understand the sensitivity of the \(\beta\)-carotene Raman bands to the environment.

Analysis of computed vibrational spectra may prove to be difficult. To strengthen our conclusions and discover how additional groups connected to the polyene chain effect the Raman spectra, calculations on differently substituted Car molecules have also been performed. Such an “experimental” computational approach was already applied.(19, 27) A series of artificially made structures were constructed, starting from pure polyene, and
progressively CH3 groups were added to the conjugated C=̂C chain (Figure 1B). These molecules will be denoted from m0 (for no methyl) to m3 (where three methyl groups were added to the conjugated chain). A final structure was generated, denoted m13–13', (Figure 1 C), which possesses two CH3 groups at the opposite sides of the polyene chains on carbons 13 and 13'.

Materials and Methods

Samples

β-Carotene (β,β-carotene synthetic type I, C-9750) was from Sigma-Aldrich (St. Louis, MO). 13,13'-Diphenyl-β-carotene was synthesized according to the reported procedure.(26) The solvent used for resonance Raman experiments was n-hexane, absolute grade (≥99.5% GC).

Resonance Raman (Experimental Data)

Resonance Raman spectra were obtained using excitations provided by a 24 W Ar+ Sabre laser (Coherent, Palo Alto, California), and recorded at 77K in a helium-flow cryostat (Air Liquide, Sassenage, France) with 90° signal collection using a two-stage monochromator (U1000, Jobin Yvon, Longjumeau, France), equipped with a front-illuminated, deep-depleted CCD detector (Jobin Yvon, Longjumeau, France). Typically, less than 20 mW reached the sample during the recording of the spectra, and sample integrity was verified by following the evolution of the resonance Raman spectra during the experiment.

Computational

Previously, calculations of the υ1 Raman bands were performed for β-carotene using B3LYP/6-31G, B3LYP/TZVP, B3LYP/6-31G(2df,p), BP86/6-31G(d), BPW91/6-31G(d), B3P86/6-31G(d), B3PW91/6-31G(d), and SVWN/6-31G(d).(19, 28) All methods based on the DFT are able to perform calculations of the vibrational frequency with an overall root-mean-square error of 34–48 cm⁻¹, significantly less than that reported for the MP2 theory (61 cm⁻¹).(29) A scaling factor of 0.96 is used for frequencies calculated by the B3LYP/6-31G(d) method in order to obtain a satisfactory concurrence with the experimental data.(19, 29)

The B3LYP functional in combination with the 6-31G(d,p) basis set is known to provide reasonably good geometries.(30) Previously(26) we performed ground-state β-carotene geometry optimization using the B3LYP functional and a 6-311G(d,p) basis set. We showed that the b3lyp/6-311G(d,p) basis set gives closer equilibrium of the C=̂C bond lengths for
the polyene chain as compared to using cc-pVDZ basis set and b3lyp/6-31G(d) method.(19) Accordingly, we chose using the B3LYP/6-311G(d,p) method of the present study.

The polar environment can cause the shift of Raman frequencies,(19) and this should be true for protein environments. However, as it was shown using the PCM method, that solvent effect is limited on the positions of the Raman frequencies.(28, 19) Moreover, the dependence of the shift of the Raman peaks between calculated in vacuum and experimental ones is linear over all spectra.(19) Thus, all calculations were performed in vacuum.

The calculations were performed using the B3LYP/6-311G(d,p) method available in the Gaussian 09 package (Rev D.01).(31) The geometry parameters of all structures were optimized first using this method. The same level of the DFT calculations was used for estimating the Raman activity of the bands. In this paper we mainly focused on the ground state Raman spectra and how frequencies in spectral regions from \( \nu_1 \) to \( \nu_4 \) are influenced by structural differences between \( \beta \)-carotene and 13,13'-diphenyl-\( \beta \)-carotene. The resonance Raman intensities were only estimated for \( \beta \)-carotene by the method presented in refs 24 and 37. However, overall, we mainly paid attention to the relative intensities of the Raman peaks in a given Raman spectrum as follows from calculations using Gaussian 09.(31) The normal modes of vibrational frequencies and equilibrium geometries of the ground and first active excited electronic state were calculated by the GAMESS-US code using the DFT and TD-DFT methods at the 6-311G(d,p) level.(33)

**Results**

Resonance Raman (Experimental Data)

Figure 2 displays the experimental resonance Raman spectra of 13,13'-diphenyl-\( \beta \)-carotene and \( \beta \)-carotene. The spectra are largely different, confirming the expected influence of the presence of the phenyl rings on the vibrational modes of carotene. In the \( \nu_1 \) region, while a single band is observed in the spectra of \( \beta \)-carotene, three bands are present in the spectra of 13,13'-diphenyl-\( \beta \)-carotene at 1487, 1507, and 1526 cm\(^{-1}\). The structure of the \( \nu_2 \) band is also significantly affected by the presence of the phenyl ring. In \( \beta \)-carotene, it comprises an intense band at 1158 cm\(^{-1}\), accompanied by three weaker satellites at 1173, 1190, and 1210 cm\(^{-1}\). In 13,13'-diphenyl-\( \beta \)-carotene, the main band is split into two components displaying similar intensity at 1151 and 1158 cm\(^{-1}\); in addition to the weak satellites at higher frequency 1176 and 1182 cm\(^{-1}\), a new band appears at 1104 cm\(^{-1}\). The \( \nu_3 \) band is shifted to higher frequencies at 1014 cm\(^{-1}\) (1003 cm\(^{-1}\) for \( \beta \)-carotene). Finally, in 13,13'-diphenyl-\( \beta \)-carotene, very significant contributions are observed in the \( \nu_4 \) region at 956, 967, and 976 cm\(^{-1}\), suggesting that, indeed, the presence of the phenyl
influences the relaxed structure, also resulting in a new active Raman mode at about 915 cm\(^{-1}\).

![Figure 2. Resonance Raman of \(\beta\)-carotene (top) and 13,13′-diphenyl-\(\beta\)-carotene (bottom) obtained at 77 K (excitation wavelength 476.5 nm).](image)

Simulation of the Spectra

It is evident that the C2h symmetry of \(\beta\)-carotene cannot be preserved because of the end \(\beta\)-rings.\(^{(34)}\) However, \(\beta\)-carotene can still have the C2 symmetry, and the corresponding vibrational modes may be characterized as symmetric or antisymmetric according to the C2 symmetry operation. Only one conformer of \(\beta\)-carotene possesses C2 symmetry. When phenyl rings are attached nonsymmetrically with respect to the polyene chain, the C2 symmetry of \(\beta\)-carotene is destroyed. Our calculations show that each phenyl group in 13,13′-diphenyl-\(\beta\)-carotene has two stable positions forming from different dihedral angles (about 30°) with the polyene chain. The total energy difference between the three possible conformers is less than 0.0008 eV. However, most of the vibrational modes concerned with the polyene chain still behave as almost symmetric/antisymmetric according to the C2 symmetry axis of the polyene chain.
As reported before,(19) in order to match the experimental frequencies with the calculated ones, a factor of about 0.97 has to be applied to the calculated frequencies (Table 2). We will give the scaled vibrational frequencies unless stated otherwise.

Table 2. Most Active Modes of Calculated Raman Spectra and Experimentally Obtained Resonance Raman from β-Carotene and 13,13'-Diphenyl-β-carotene

<table>
<thead>
<tr>
<th>Structure</th>
<th>vibrational mode</th>
<th>Calculated frequencies*, cm⁻¹</th>
<th>Calculated Relative intensity **</th>
<th>Attribution ***</th>
<th>Experimental frequency, cm⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-carotene</td>
<td>$v_{1}$</td>
<td>1524.17 (1571.31)</td>
<td>W</td>
<td>Symmetric two phase C=C stretch +15-15'; -13-14; -13'-14'; +11-12; +11'-12'; -9-10; -9'-10'; +7-8; +7'-8'; no β-rings.</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>$v_{21}$</td>
<td>1183.43 (1220.03)</td>
<td>S</td>
<td>C-C stretch +15-15'; symmetric C-C stretch -12-13; -12'-13'; -8-9; -8'-9'; +6-7; +6'-7'; -1-2; -4-5; -1'-2'; -4'-5' from β-rings.</td>
<td>1170</td>
</tr>
<tr>
<td></td>
<td>$v_{22}$</td>
<td>1159.91 (1195.78)</td>
<td>S</td>
<td>C-C stretch +10-11; +10'-11'; +6'-7'; C-C stretch and deformation of (5'-6') β-ring.</td>
<td>1150</td>
</tr>
<tr>
<td></td>
<td>$v_{22}$</td>
<td>1156.32 (1192.08)</td>
<td>S</td>
<td>C-C stretch: +10-11; +10'-11'; +6-7; C-C stretch and deformation in (5-6) β-ring.</td>
<td>1150</td>
</tr>
<tr>
<td></td>
<td>$v_{23}$</td>
<td>1153.37 (1189.04)</td>
<td>VS</td>
<td>Symmetric C-C stretch +14-15; +14'-15'; +10-11; +10'-11'; -1-6 and -1'-6' from β-rings.</td>
<td>1156</td>
</tr>
<tr>
<td></td>
<td>$v_{24}$</td>
<td>1136.00 (1171.13)</td>
<td>W</td>
<td>Symmetric C-C stretch +14-15; +14'-15'; -12-13; -12'-13'; -10-11; -10'-11'; -10'-9'; +8-9; +8'-9'; -6-7; -6'-7'; +1-6 and +1'-6'</td>
<td>1156</td>
</tr>
<tr>
<td>( \nu_3 )</td>
<td>991.82 ( (1022.49) )</td>
<td>W</td>
<td>Symmetric CH(_3) “rocking” at the chain and in the ( \beta )-rings; small C-C bonds deformations in all polyene chain and ( \beta )-rings deformations.</td>
<td>1003</td>
<td></td>
</tr>
<tr>
<td>( \nu_{4,1} )</td>
<td>974.46 ( (1004.6) )</td>
<td>W</td>
<td>Symmetric C-H and C atoms out of chain plane wagging with small ( \beta )-rings deformations.</td>
<td>970</td>
<td></td>
</tr>
<tr>
<td>( \nu_{4,2} )</td>
<td>969.37 ( (999.35) )</td>
<td>W</td>
<td>Symmetric C-H and C atoms out of chain plane wagging (mainly localized at the ends and in the middle of the chain) with small ( \beta )-rings deformations.</td>
<td>960</td>
<td></td>
</tr>
<tr>
<td>( \nu_{4,3} )</td>
<td>956.20 ( (985.77) )</td>
<td>VW</td>
<td>Symmetric C-H and C atoms out of chain plane wagging (mainly localized at the ends and in the middle of the chain) with small one ( \beta )-ring deformations and wagging of CH(_3) groups at that ( \beta )-ring.</td>
<td>960</td>
<td></td>
</tr>
</tbody>
</table>

<p>| 13-13’-diphenyl-( \beta )-carotene | ( \nu_{5,1} ) | 1519.67 ( (1566.67) ) | VS | Symmetric two phase C=C stretch+(15-15’); -(13-14); -(13’-14’); +(11-12); -(11’-12’); -(9-10); +(7-8); +(7’-8’); no ( \beta )-rings. | 1526 |
| ( \nu_{5,2} ) | 1492.83 ( (1539) ) | VS | Symmetric one phase C=C stretch+(13-14); +(13’-14’); +(11-12); +(11’-12’); +(9-10); +(9’-10’);phenyl rings in plane valence stretching and deformation vibrations (in opposite phase with vibrations of 13 and 13’ atoms and changing of the lengths of C13-C16 and C13’-C16’ bonds). | 1508 |
| ( \nu_{5,2}’ ) | 1473.43 | VS | Symmetric one phase C=C stretch +(13-14); +(13’-14’); +(11-12); | 1485 |
| $\nu_{2,1}$ | 1177.25 | S   | C-C stretch $+(15'-15')$; symmetric C-C stretch $-(12-13)$; $-12'-13'$; $+10'-11'$; $+(-8'-9')$; $+6'-7'$; $+(-1'-2')$, $-4'-5'$ from $\beta$-rings; in phase phenyl rings and the chain deformations. | 1177 |
| $\nu_{2,2'}$ | 1158.84 | VS  | Symmetric C-C stretch $+(14'-15')$; $+(12-13)$; $+(10-11)$; $+6'-7'$; $5'$-$6$' $\beta$-ring and very small phenyl deformation. | 1150 |
| $\nu_{2,2''}$ | 1155.87 | W   | Symmetric one phase C-C stretch $+(14-15)$; $+(14'-15')$; $+(10-11)$; $+10'-11'$; $\beta$-rings and very small phenyl deformations. | 1115 |
| $\nu_{2,3'}$ | 1155.05 | W   | Symmetric C-C stretch $+(14'-15')$; $5'$-$6'$ $\beta$-ring and very small phenyl deformation. | 1115 |
| $\nu_{2,3''}$ | 1141.00 | W   | C-C stretch: $-(14-15)$; $+(14'-15')$; $-10'-11'$; $-6'-7'$; deformations of $5'$-$6'$ $\beta$-ring; in plane deformations of 16' phenyl ring. | 1140 |
| $\nu_{2,4}$  | 1094.72 | W   | Symmetric C-C stretch: $+(12-13)$; $+(12'-13')$; symmetric in phase vibration of phenyl rings with atoms 13 and 13'; no $\beta$-rings. | 1104 |</p>
<table>
<thead>
<tr>
<th>ν₃</th>
<th>1011.33</th>
<th>W</th>
<th>Symmetric CH₃ “rocking” at the chain and in the ending rings; small symmetric C-C stretch: +(8-9); -(8’-9’); -(6-7); -(6’-7’); small β-rings deformations.</th>
<th>1014</th>
</tr>
</thead>
<tbody>
<tr>
<td>ν₄₁</td>
<td>972.13</td>
<td>VW</td>
<td>Symmetric C-H out of chain plane vibration at (5′-6′) β-ring, (5-6) β-ring deformations and C-H wagging out of phenyl ring plane.</td>
<td>975</td>
</tr>
<tr>
<td>ν₄₂</td>
<td>971.69</td>
<td>W</td>
<td>Symmetric C-H out of chain plane vibration with along all the chain; small β-rings and phenyl rings deformation.</td>
<td>970</td>
</tr>
<tr>
<td>ν₄₃</td>
<td>964.43</td>
<td>W</td>
<td>Symmetric C-H out of chain plane vibration with (5-6) β-ring deformations.</td>
<td>960</td>
</tr>
<tr>
<td>ν₄₄</td>
<td>960.92</td>
<td>W</td>
<td>C-H out of chain plane vibration with (5-6) β-ring deformations.</td>
<td>960</td>
</tr>
<tr>
<td>ν₄₅</td>
<td>910.41; 910.58; 938.57; 938.74</td>
<td>W</td>
<td>Two practically degenerated modes of C-H out of chain plane and phenyl ring out of phenyl plane wagging; very small in plane chain deformations.</td>
<td>915</td>
</tr>
</tbody>
</table>

* scaling factor 0.97. Unscaled calculated frequencies in parentheses
** VW – very weak, W – weak, S – strong, VS – very strong.
*** (i-j) denotes the change of bond length between i and j atoms (Fig. 1); (j-i-k) – the change of valence angle; "+" and "-" denotes opposite phases of the change of corresponding internal coordinates.

Figure 3 displays the calculated Raman spectra of β-carotene and 13,13′-diphenyl-β-carotene, generated using B3LYP/6-311G(d,p). We investigated four major Raman spectral regions (Table 1) from ν₁ to ν₄, where the highest active Raman frequency values are attributed to the ν₁ region, and the lowest Raman frequency values are at the ν₄ region. The attribution of a particular vibrational frequency to the appropriate region from ν₁ to ν₄ was based on the analysis of the calculated vibrational form as described in the Introduction. With respect to β-carotene, we observe in the calculated spectra of 13,13′-diphenyl-β-carotene:

(i) the splitting of the ν₁ peak into three components,
(ii) the splitting of the most intense component of the ν₂ region,
(iii) the upshift of the most intense component of the $\nu_3$ region, and
(iv) the appearance of bands with non-negligible intensity in the $\nu_4$ region.

Figure 3. Calculated Raman spectra of $\beta$-carotene and 13,13'-diphenyl-$\beta$-carotene. Calculations were performed using B3LYP/6-311G** (the scaling factor is 0.97).

These spectra can thus be used to understand in detail the origin of the resonance Raman bands that are characteristic to these molecules.

The $\nu_1$ region in the Raman spectra ranges from about 1470 to 1520 cm$^{-1}$ (without the scaling factor it is between 1519 and 1574 cm$^{-1}$). Calculations give 11 frequencies that can be attributed to the $\nu_1$ band in $\beta$-carotene and 12 frequencies in 13,13'-diphenyl-$\beta$-carotene. As calculated previously, two modes ($\nu_{1-1}$ and $\nu_{1-2}$) contribute in the $\nu_1$ region of $\beta$-carotene (Table 2); both correspond to symmetric C=C stretching modes of the polyene chain. One very strong mode we denominate $\nu_{1-2}$ (1514.97 cm$^{-1}$), and the other, weaker line, to $\nu_{1-1}$ (1524.17 cm$^{-1}$; see Table 2 and Figure 4). It is worthwhile to mention that C=C stretching from the terminal $\beta$-rings does not participate in these vibrations. These modes have been previously denominated $\nu_{1a}$ and $\nu_{1b}$, or $\nu_{1a}$ and $\nu_{1b}$, or $\nu_1$ and $\nu_{1split}$, or $\nu_{67}$ and $\nu_{66}$. (19, 34-36) However, although calculations predict about 10 cm$^{-1}$ difference between
the latter two modes, they are not distinguishable in the experimental spectra (see Figure 2). A closer look suggests that the presence of a weak high frequency band in this region distorts the shape of the ν₁ band (arrow in Figure 4). Calculations give three intense peaks in the spectrum of 13,13'-diphenyl-β-carotene, a result that actually matches with the experimental observation. The first two, ν₁–2′ (1473.73 cm⁻¹) and ν₁–2″ (1492.83 cm⁻¹) may be regarded as a split ν₁–1 from β-carotene due to interactions between the symmetric stretching vibration of the polyene chain and vibrations of the phenyl rings. The third frequency, ν₁–1 (1519.67 cm⁻¹), is a pure symmetrical C=C stretching vibration of the polyene chain, similar to the one in β-carotene. Clearly, the larger difference between ν₁–1 and ν₁–2′ or ν₁–2″ in 13,13'-diphenyl-β-carotene (26.83 and 46.23 cm⁻¹) than between ν₁–1 and ν₁–2 in β-carotene (9.2 cm⁻¹) is the result of interactions of stretching vibrations in the chain with vibrations in the phenyl rings of 13,13'-diphenyl-β-carotene (Figure 4 and Table 2).

![Figure 4. Raman shift in the ν₁ range: (A) calculated spectra (the values are without the scaling factor); (B) experimental data spectra (arrow: distortion in the shape of the ν₁ band).](image)

The presence of ν₁–1 and ν₁–2 type modes in a carotene-derived molecular structure is strongly dependent on the presence of substituents on the conjugated polyene chain. The structures containing methyl groups on the same polyene chain side (β-carotene-m0, β-carotene-m1, and β-carotene-m2) do not exhibit splitting of the ν₁ Raman band into ν₁–1 and ν₁–2 (Table 3). The 13,13'-diphenyl-β-carotene, β-carotene and, β-carotene-m13–13′
have ν_{1-1} and ν_{1-2}, which differ by the vibration phase. When only one ν1 Raman band (ν_{1-2}) is present, it corresponds to the one phase C=C vibrations over all of the polyene chain (Table 3); the analysis of β-carotene-m0 to m2 shows that the frequency of the peak depends on the presence of additional groups connected to the polyene chain (Table 3). The more CH₃ groups present, the higher its frequency. However, the presence of the ν_{1-1} Raman mode tends to decrease the ν_{1-2} frequency (Table 3). We note here that the weak bands at lower frequencies (at about 1445 cm⁻¹) in 13,13'-diphenyl-β-carotene and β-carotene arise from CH₃ vibrations (Figure 3). The 13,13'-diphenyl-β-carotene actually exhibits an additional mode between the ν_{1-2'} and CH₃ bands at 1473 cm⁻¹ (ν_{1-2'}), which arises from the phenyl rings (Table 2, Figure 3). The phenyl ring vibrations are coupled with one phase vibrations of C=C in the polyene chain (Table 3). So the 13,13'-diphenyl-β-carotene has two one-phase vibrations (ν_{1-2'} and ν_{1-2}, Table 2) in the ν₁ region, one with and one without vibrations of the phenyl rings.

**Table 3. Calculated Raman Active Modes in the ν₁ Region of the Artificially Made Structures of the β-Carotene**

<table>
<thead>
<tr>
<th>Structure</th>
<th>ν_{1-1'} cm⁻¹</th>
<th>ν_{1-1} attribution**</th>
<th>ν_{1-2'} cm⁻¹</th>
<th>ν_{1-2} attribution**</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-carotene</td>
<td>1524.17 (1571.31)</td>
<td>Symmetric two phase C=C stretch +(15-15'); -(13-14); -(13'-14'); +(11-12); +(11'-12'); - (9-10); -(9'-10'); +(7-8); +(7'-8'); no β-rings.</td>
<td>1514.97 (1561.82)</td>
<td>Symmetric one phase C=C stretch +(15-15'); +(13-14); +(13'-14'); +(11-12); +(11'-12'); +(9-10); +(9'-10'); no β-rings.</td>
</tr>
<tr>
<td>13-13'-diphenyl-β-carotene</td>
<td>1519.67 (1566.67)</td>
<td>Symmetric two phase C=C stretch+(15-15'); -(13-14); -(13'-14'); +(11-12); +(11'-12'); -(9-10); -(9'-10'); +(7-8); +(7'-8'); no β-rings.</td>
<td>1492.83 (1539)</td>
<td>Symmetric one phase C=C stretch+(13-14); +(13'-14'); +(11-12); +(11'-12'); +(9-10); +(9'-10'); phenyl rings in plane valence stretching and deformation vibrations (in opposite phase with vibrations of 13 and 13' atoms and changing of the lengths of C13-C16 and C13'-C16' bonds).</td>
</tr>
<tr>
<td>β-carotene-m0</td>
<td>-</td>
<td>-</td>
<td>1565.48</td>
<td>Symmetric one phase C=C stretch+(15-15'); +(13-14); +(13'-14'); +(11-12); +(11'-12'); +(9-10); +(9'-10'); +(7-8); +(7'-8'); no β-rings.</td>
</tr>
<tr>
<td>β-carotene-m1</td>
<td>-</td>
<td>-</td>
<td>1568.63</td>
<td>The same as in β-carotene-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
</tr>
<tr>
<td>β-carotene-m2</td>
<td>-</td>
<td>-</td>
<td>1570.60</td>
<td>The same as in β-carotene-m0</td>
</tr>
<tr>
<td>β-carotene-m3</td>
<td>-</td>
<td>-</td>
<td>1569.21</td>
<td>The same as in β-carotene-m0</td>
</tr>
<tr>
<td>β-carotene-m13-13'</td>
<td>1574.55</td>
<td>Symmetric two phase C=C stretch +(15-15'); -(13-14); -(13'-14'); +(11-12); +(11'-12'); - (9-10); -(9'-10'); +(7-8); +(7'-8'); no β-rings.</td>
<td>1563.07</td>
<td>The same as in β-carotene-m0</td>
</tr>
</tbody>
</table>

*scaling factor 0.97. Unscaled calculated frequencies in parentheses

** (i-j) denotes the change of bond length between i and j atoms (Fig. 1); (j-i-k) – the change of valence angle; "+" and "+" denotes opposite phases of the change of corresponding internal coordinates.

The ν2 region ranges from 1090 to 1310 cm\(^{-1}\) (in the calculations, unscaled frequencies range from 1124 to 1350 cm\(^{-1}\)); there are three and five clear peaks in the β-carotene and 13,13'-diphenyl-β-carotene, respectively (Table 2). Such an increase in complexity in this region is actually observed in the experimental spectra (Figure 2). The highest frequency peaks are at 1183.43 cm\(^{-1}\) for β-carotene and 1177.25 cm\(^{-1}\) for 13,13'-diphenyl-β-carotene (ν2-1). They arise from the C–C stretching modes of the polyene chain without any participation of the β-rings or the phenyl rings. Three modes participate to the band observed at about 1160 cm\(^{-1}\), observed at 1159.91, 1156.32, and 1153.37 cm\(^{-1}\) for the β-carotene and 1158.84, 1155.87, and 1155.05 cm\(^{-1}\) for the 13,13'-diphenyl-β-carotene (ν2-2). They arise from the C–C stretching modes together with deformation of the β-rings (not involving phenyl rings). The most intense mode, at about 1140 cm\(^{-1}\), is split into two components at about (1141 and 1140.83 cm\(^{-1}\)) for 13,13'-diphenyl-β-carotene both in experimental spectra and in calculations. These vibrations arise from C–C stretching modes of the polyene chain together with one phenyl ring and one β-ring (ν2-2' (ν2-3'), Table 2). In β-carotene this band arises from one symmetric mode with frequency 1136.00 cm\(^{-1}\) (ν2-3), including similar nuclear motions as well as vibrations of both end rings. An additional mode for 13,13'-diphenyl-β-carotene is found both in experimental spectra and in calculations, at 1094.72 cm\(^{-1}\) (ν2-4). It arises from symmetric carbon single bond stretching modes of the chain near the phenyl rings, together with symmetric in-plane deformation of the C–C bonds of the phenyls. More vibrations in this region with negligible intensities are obtained from theoretically calculated Raman spectra. They involve pure vibrations of phenyl-rings or β-rings that are not coupled to the conjugated chain. It is of note that the main mode in this region, at 1141 cm\(^{-1}\) (ν2-3), has a much larger Raman intensity for 13,13'-diphenyl-β-carotene with respect to β-carotene.
The bands in the ($\nu_3$ region (the range of 990–1020 cm$^{-1}$, Table 1) have been attributed to modes arising from the coupling of the in-plane rocking vibrations of the methyl groups attached to the conjugation chain and the adjacent C–H in-plane bending modes. Three modes are obtained from calculations in this region for β-carotene, and 13,13’-diphenyl-β-carotene spectra exhibit at 992, 999, and 1004 cm$^{-1}$, and at 995, 998, and 1011 cm$^{-1}$, respectively (Figure 5). They are absent in pure polyenes (m0 molecule in Figure 5). As observed in the experimental spectra, the frequency of these modes is upshifted in 13,13’-diphenyl-β-carotene spectra (Figure 2). The first two modes (at approximately 992 cm$^{-1}$ in β-carotene) and the last two modes (at approximately 1011 cm$^{-1}$ in 13,13’-diphenyl-β-carotene) are symmetric and antisymmetric rocking vibrations of all CH3 groups in the chain and in the ending β-rings (Table 2). The symmetric modes of 991 cm$^{-1}$ in β-carotene and 1011 cm$^{-1}$ in 13,13’-diphenyl-β-carotene are the most intense in the calculated Raman spectra. Calculations show that the most intense vibrational frequency mode in the ($\nu_3$ region in 13,13’-diphenyl-β-carotene is shifted by about +20 cm$^{-1}$ with respect to the most intense mode of the same type in β-carotene. The other modes in this region in both compounds arise from vibrations of CH3 groups in one side of the chain and in the corresponding ending β-ring only (an example of these modes is shown in Figure 6). It is worth noting that the phenyl rings do not have any influence on the ($\nu_3$ regional modes.

(Figure 5. N3 Raman spectra calculated for β-carotene, 13,13’-diphenyl-β-carotene, and artificially made β-carotene substructures. Calculations were performed using B3LYP/6-311G** (the values are without scaling factor).
Considering calculations, the final region of the resonance Raman spectra in the range of 820–990 cm\(^{-1}\) is termed \(\nu_4\) (it is between 850 and 1020 cm\(^{-1}\) in the calculations without the scaling factor). In this region of the spectra, there are many resonance Raman inactive vibrations from the ending \(\beta\)-rings (deformation of rings and "rocking" of CH2 and CH3 groups). There are three weak Raman vibrations in this region for \(\beta\)-carotene: 969.37 cm\(^{-1}\), 974.46 cm\(^{-1}\), and one very weak vibration at 956.20 cm\(^{-1}\). All of them correspond to almost pure typical \(\nu_4\) region vibrations in slightly different parts of the polyene chain (i.e., C–H out-of-the plane wagging vibrations) together with slight deformations of \(\beta\)-rings (see Figure 7 as an example). 13,13′-Diphenyl-\(\beta\)-carotene has three weak Raman frequencies in this region: 960.92, 964.43, and 971.68 cm\(^{-1}\); the first two correspond to \(\nu_4\) vibrations mixed with deformation of one of the ending rings. The third very weak vibration at 972.13 cm\(^{-1}\) in addition to characteristic typical \(\nu_4\) region vibrations includes deformations of both \(\beta\)-rings and phenyl rings (C–H wagging out-of-plane phenyl vibrations). In the experimental spectra, it could be attributed to the weak peak at the right side shoulder of 13,13′-diphenyl-\(\beta\)-carotene at about 975 cm\(^{-1}\) (Figure 2). In this molecule an additional weak band consisting of two lines of almost the same frequency at 910 cm\(^{-1}\) can also be distinguished (Figure 2). It is composed of pure out-of-plane C–H wagging, C16, C16′, and phenyl ring wagging modes with very small in-plane deformations of the chain. During this wagging motion of C16 and C16′ atoms, very small in-plane deformations of the polyene chain are initiated. In the case of symmetric phenol rings with respect to the polyene chain, contribution of both phenyl rings together with small vibrations of the chain are expected at this frequency. In the case of nonsymmetrical conformers, two vibrations with practically the same frequency, made from contributions of one ring only, together with its corresponding half of the polyene chain, can be resolved.
Figure 7. Vibrational forms corresponding to the 990.64 cm\(^{-1}\) calculated mode of 13,13’-diphenyl-\(\beta\)-carotene compared to the value experimentally observed at 950 cm\(^{-1}\) (see Table 2), shown as one example of the three main modes calculated in the \(v_4\) region of this molecule. The same is the case for the \(\beta\)-carotene. Nuclear vibrations: (A) 13,13’-diphenyl-\(\beta\)-carotene at 990.64 cm\(^{-1}\); (B) \(\beta\)-carotene at 985.77 cm\(^{-1}\) (the values are without the scaling factor).

For estimations of resonance Raman intensities of \(\beta\)-carotene in \(v_1-v_4\) regions, the calculation methods described in refs 32 and 37 was used; i.e., only influences of valence C–C vibrations of the polyene chain on resonance Raman intensities were accounted for. For this purpose, the difference in equilibrium bond lengths in the polyene chain of corresponding internal vibrational C–C coordinates were evaluated from the geometry optimization of \(\beta\)-carotene in the ground and first optically allowed excited states. Thus, only the part from normal coordinates corresponding to polyene valence C–C vibrations was taken into account. The resonance Raman intensities in the \(v_1-v_4\) regions should be enhanced due to the \(\pi\pi^*\) electronic transition in the polyene chain. The quite quantitative results of the calculated resonance Raman intensities show that all most intense contributions in the calculated Raman spectra (Table 2) correspond to those observed in the experimental resonance Raman spectra. In addition, we performed the same calculation for the lower frequency contributions in the RR spectra, and obtained similar agreement between calculated and experimental spectra (data not shown).
Discussion

Four major spectral ranges, labeled as $\nu_1$, $\nu_2$, $\nu_3$, and $\nu_4$, have been determined in the resonance Raman spectra of Cars, each of them have been extensively used to characterize the properties of these molecules in vivo and in vitro. Here we compared the modes participating in each of these regions in experimental and calculated spectra for $\beta$-carotene and for 13,13'‐diphenyl-$\beta$-carotene, in which two methyl groups were substituted for phenyl rings. The presence of these conjugated phenyl groups has a strong influence on all four major regions of the Raman spectra.

The presence of the phenyl induces splitting of the $\nu_1$ region into three components as a result of the interaction of phenyl vibrations with valence vibrations of the polyene chain. Similarly, the main band of the $\nu_2$ region also splits into two components for 13,13'‐diphenyl-$\beta$-carotene. The bands in the $\nu_3$ region are upshifted by the presence of the phenyl groups, and bands with non‐negligible intensity in the $\nu_4$ region are also distinguishable. For all these observations, an excellent agreement is obtained between the experimental and simulated data. This led us to perform calculations on additional, artificial structures, in order to fully understand the influence of phenyl rings and methyl substitutions on conjugated chains of Cars.

As reported before,(19) in order to match the frequencies obtained experimentally with the calculated ones, a factor of 0.97 has to be applied to the calculated frequencies (Table 2). With the scaling factor, the agreement between the calculated spectra and the spectra observed experimentally is remarkable in all spectral regions (Figures 2 and 3).

Between all investigated molecules only $\beta$-carotene possesses C2 symmetry. However, most vibrations of the polyene chain can still be characterized as antisymmetric or symmetric vibrations in analogy with $\beta$-carotene. Symmetric vibrations are naturally the most active in calculated Raman spectra. Both $\nu_1$ and $\nu_2$ regions contain additional peaks in the Raman spectra of 13,13'‐diphenyl-$\beta$-carotene with respect to $\beta$-carotene. Analysis of differently substituted molecules shows that the most active v1 band always splits into two parts when substituted groups (either methyl or phenyl) are present at opposite sides of the polyene chain (Table 3): $\nu_{1-1}$ and $\nu_{1-2}$. The $\nu_{1-2}$ mode is split into two modes $\nu_{1-2'}$ and $\nu_{1-2''}$ in 13,13'‐diphenyl-$\beta$-carotene. The $\nu_{1-1}$ and $\nu_{1-2}$ bands are very close to each other for $\beta$-carotene, actually resulting in a single band in the experimental spectra. However, these modes are clearly distinguishable in 13,13'‐diphenyl-$\beta$-carotene as the splitting is much larger in the presence of the phenyl rings (Figure 4). The vibrational modes of $\nu_{1-2'}$ and $\nu_{1-2''}$ in 13,13'‐diphenyl-$\beta$-carotene arise from in‐phase and out‐of‐phase phenyl group vibrations with $\text{C} \equiv \text{C}$ stretching of the polyene chain (Table 3). The vibrational modes of
ν₁₋₁ (1519 cm⁻¹ for 13,13′-diphenyl-β-carotene and 1524 cm⁻¹ for β-carotene) correspond to pure one phase valence C=\(\text{C}\) vibrations in the polyene chain and are not influenced by phenyl groups.

As compared to β-carotene, there are two additional peaks in the ν₂ region of 13,13′-diphenyl-β-carotene, which are related to phenyl groups: at about 1141 and 1095 cm⁻¹. The peak at 1141 cm⁻¹ consists of a split Raman mode (at 1140.83 and 1141.45 cm⁻¹) where vibrations correspond to C–C stretching modes of half of the polyene chain together with deformation of one phenyl ring and one β-ring. This peak may be regarded as a split mode of 1136.00 cm⁻¹ in β-carotene. The splitting is influenced by phenyl rings non-symmetrically positioned in respect to the polyene chain. The appearance of the other additional peak in 13,13′-diphenyl-β-carotene at 1097 cm⁻¹ (ν₂₋₄ in Table 2) is fully determined by the influence of the phenyl rings. This mode may be regarded as a new mode of small vibrations of the polyene chain induced by valence vibrations in the phenyl rings.

Regarding ν₃ and ν₄, two additional remarks may be worth noting from the calculations:

(i) The ν₃ band of β-carotene arises from modes largely involving the nuclei of the conjugated end cycles. The frequency of this mode will thus be sensitive to the overall conformation of these rings, and the fine structure of this region may actually be used, as was already proposed in ref 18 to determine changes in the end ring configurations in vivo. The presence of phenyl rings in 13,13′-diphenyl-β-carotene induces a localization of the vibrations at the ends of the polyene chain and the involvement of C atoms in the middle of the chain become negligible.

(ii) In the ν₄ region, the observed modes are highly delocalized over the molecule. The presence of additional modes, as observed in the fucoxanthin and/or peridinin molecules,(21, 25) likely translates to the existence of methyl groups in different chemical environments, which gives rise to the presence of an additional collective mode. However, additional bands observed cannot be attributed to a specific methyl, as was proposed earlier.(21) The small peak in the experimental spectrum of 13,13′-diphenyl-β-carotene at about 975 cm⁻¹ observed in calculations is a vibration characteristic to the ν₄ region, but in addition includes deformations of both β-rings and deformations in phenyl rings. One additional very small peak in this region is found in the calculated spectra of 13,13′-diphenyl-β-carotene at about 910 cm⁻¹. Actually there are two vibrations (910.41 and 910.58 cm⁻¹), which are constituted of the different halves of Car; they can be regarded as out-of-plane phenyl C atoms and C–H bond wagging vibrations in phenyl rings. These phenyl ring vibrations seem to initiate the ν₄ region type vibrations and very small C–C deformations in the polyene chain. In the low frequency range, the presence of phenyl substit-
uents on the Car conjugated chain results in lower intensity in the 950–990 cm\(^{-1}\) region as compared to the intensity of the \(v_3\) (1003 cm\(^{-1}\)) band (see Figures 2 and 3). In the calculations, these modes appear, as proposed before, to involve out-of-plane wagging modes of the C–H bonds. In the calculated spectra, a series of three modes is observed, with close frequencies around 950 cm\(^{-1}\) (990 in the calculations without the scaling factor), which involve all H atoms of the conjugated C═C chain, methyl rocking motions, and the coordinates of the nuclei of the conjugated end cycles (see Figure 7 as an example). This region has been widely used to determine distorted conformations of Car molecules in proteins,(18, 21, 22) since these modes gain intensity when the molecule partially loses its planar symmetry. However, as they appear delocalized over the whole conjugated part of Car (Figure 7), it is unlikely that the appearance of such a frequency can be easily related to a distortion around a specific C–C bond.

Conclusions

The results obtained in this paper allowed us to test the robustness of present day calculations performed on vibrational properties of carotenoid molecules. The ensemble of alterations of the vibrational spectra of β-carotene upon introduction of conjugated phenyl groups at positions 13 and 13’ are precisely predicted by the method used. It is thus clear that our approach is highly predictive, and opens the way for performing even more sophisticated carotenoid modeling in the near future. Likewise, comparison of the vibrational properties of β-carotene and phenyl-substituted molecules led to a more comprehensive understanding of all of the regions used as fingerprints for studying these molecules, which will be extremely useful for characterizing the structure and properties of carotenoids in complex media, such as the binding sites provided by proteins in living organisms.

Acknowledgment

The public access supercomputer from the High Performance Computing Center (HPCC) of the Lithuanian National Center of Physical and Technology Sciences (NCPTS) at the Physics Faculty of Vilnius University was used. The study was partly funded by the Lithuanian–Latvian–Taiwan project TAP-LLT-12-003 (M M. and L V.) and from the Social Foundation of the European Community under Grant Agreement No. VP1-3.1-ŠMM-08-K-01-004/KS-120000-1756 (J.S.). Financial support from European Research Council (Advanced Grant PHOTPROT no. 267333) is greatly acknowledged. This work was supported by the French Infrastructure for Integrated Structural Biology (FRISBI) ANR-10-INSB-05-01 and by Basic Science Research Program (Grant No. 2012R1A2A2A01045344) through the National Research Foundation of Korea.
References


CHAPTER 5

ECHINENONE VIBRATIONAL PROPERTIES: FROM SOLVENTS TO THE ORANGE CAROTENOID PROTEIN

Orange carotenoid protein (OCP) is a cyanobacterial photoactive protein which binds echinenone as a chromophore; it is involved in photoprotection of these photosynthetic organisms against intense illumination. In its resting state, OCP appears orange (OCPo), and turns into a red form (OCPr) when exposed to blue-green light. Here we have combined resonance Raman spectroscopy and molecular modeling to investigate the mechanisms underlying the electronic absorption properties of the different forms of OCP. Our results show that there are at least two carotenoid configurations in the OCPo, suggesting that it is quite flexible, and that the OCPo to OCPr transition must involve an increase of the apparent conjugation length of the bound echinenone. Resonance Raman indicates that this chromophore must be in an all-trans configuration in OCPo. Density functional theory (DFT) calculations, in agreement with the Raman spectra of both OCP forms, show that the OCPo to OCPr transition must involve either an echinenone s-cis to s-trans isomerization which would affect the position of its conjugated end-chain rings, or a bending of the echinenone rings which would bring them from out of the plane of the C=C conjugated plane in the OCPo form into the C=C plane in the OCPr form.

This chapter is based on the following publication:
Introduction

Recently, the so-called Orange carotenoid protein (OCP) was implicated in photosynthetic photoprotection of cyanobacteria \(^{29-32}\), a group of photosynthetic bacteria in which phycobilisomes (PBS) are often the main light harvesting complexes. Phycobilisomes are huge soluble protein architectures; they contain hundreds of phycobilin chromophores that harvest light and pass energy to chlorophylls in the photosystem for use in photosynthesis. It was proposed that OCP can bind to the PBS in high light situations and shuttle energy away, thus preventing over-excitation of the photosystems \(^{29,30,33}\). When purified, OCP appears orange (OCPo) in color; its color is due to a ketocarotenoid molecule, 3’hydroxyechinenone (hECN) that spans its N-terminal and C-terminal domains \(^{34}\). After illumination with intense blue-green light, the protein appears red in color (OCPr) due to spatial configuration changes in hECN\(^{29}\). OCP has thus been described as a light-powered molecular switch \(^{34,35}\) which, once photoactivated, exists in a metastable form able to bind to PBS and to accept excitation energy from the bilins.

The x-ray structure of OCPo from Arthrospira maxima and Synechocystis PCC 6803 have been determined to 2.1Å and 1.65Å resolution, respectively \(^{34,35}\), but no structure exists for the red form of the protein. The mechanisms underlying the changes in electronic properties of the OCP-bound echinenone during the orange to red transition are still a matter of debate. Thus, the aim of this work is to describe these mechanisms as precisely as possible, i.e. what happens to echinenone during this transition. One technique of choice for studying protein-bound carotenoid molecules is resonance Raman spectroscopy (RRS). Resonance Raman, as a vibrational technique, yields detailed information about the molecules that scatter photons. Moreover, because there is a large signal amplification when Raman excitation comes near an electronic transition (i.e. in the absorption range), this technique allows selective observation of chromophores and has been highly exploited in the field of carotenoids \(^{17,36-38}\). Typically, carotenoids display four spectral regions, termed \(v_1-v_4\), which provide information on the molecular configuration of the scattering molecules \(^{39,40}\), on their effective conjugation length \(^{38}\), and on the distortion experienced by these molecules around their C-C bonds \(^{37}\). Recent advances have made it possible to conduct room temperature experiments on carotenoid proteins without much loss of information \(^{40}\), which allow more straightforward comparison with in vivo situations. Finally the progress in molecular physics of conjugated molecules now permits the modelling of the carotenoid resonance Raman spectra \(^{41,42}\).

Although resonance Raman has already been applied to OCP \(^{29,43}\), echinenone is a carotenoid containing a conjugated carbonyl group, and previously no systematic vibrational studies of such carotenoids were performed. It is known that the electronic properties of carotenoids may be highly disturbed by the presence of such groups; for example, they may give rise to additional electronic states such as an intramolecular charge transfer state (ICT), which could be crucial for the in vivo functionality of these molecules \(^{44,45}\). For now, two hypotheses have been drawn: from time-resolved measurements and low temperature resonance Raman it was concluded that the OCPo to
OCPr transition corresponds to an increase in the echinenone conjugation length. From structural and Raman studies on a related protein, it was proposed that echinenone undergoes a transient trans to cis conformational change.

Since a trans/cis isomerization generally results in shortening of the conjugation chain, at least for carotenoids devoid of conjugated carbonyl groups, in the following work we have investigated experimentally and computationally the vibrational properties of echinenone and other carbonyl-containing carotenoid molecules in solvents, and compared them to those obtained for OCPo and OCPr at room temperature in order to investigate the configuration and conformation of echinenone in the different spectroscopic forms of OCP.

Materials and Methods

Carotenoids and Solvents. (rac.)-3-Hydroxyechinenone ((3RS)-3-hydroxy-β,β-carotene-4-one, synth., cryst.) (hereafter 3-hydroxyechinenone) was obtained from CaroteNature, canthaxanthin (β,β-carotene-4,4′-dione) was obtained from Roche, lycopene ((Ψ,Ψ-carotene 1-9879, from tomato) and β-carotene (β,β-carotene synthetic type I, C-9750) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Lutein (β,ε-carotene-3,3′-diol) was isolated and purified as previously described. All carotenoids studied were in all-trans (all-E) geometric isomeric configurations.

The solvents used in this study were tetrahydrofuran (THF), n-hexane, cyclohexane and acetonitrile (all absolute grade, ≥ 99.5 % GC), 1,2-dichloroethane (DCE), toluene, pyridine and methanol (anhydrous, 99.8 %), chloroform and carbon disulfide (CS₂) (anhydrous, ≥ 99 %), diethyl ether (≥ 99.8 % GC), and dimethyl sulphoxide (DMSO) (≥ 99.5 % GC) all purchased from Sigma-Aldrich (St. Louis, MO, USA).

OCP Sample preparation. OCP was prepared as described in ref. Briefly, OCP was isolated from Kₘ and Sp-resistant his-tagged OCP mutants (ΔcrtR, OCP overexpressing; in Synechocystis PCC 6803). The cells (1 mg chl.ml⁻¹) were broken in mild light conditions in 0.1M Tris·HCl buffer pH=8 using a French Press. Membranes were pelleted and the supernatant was loaded onto a Ni-ProBond resin column. The OCP was further purified on a Whatman DE-52 cellulose column. After column elution, the samples were dialyzed against the final buffer. Such OCP, obtained in overexpressing ΔcrtR Synechocystis strain in which zeaxanthin and hECN are absent, contains an echinenone (ECN) molecule instead of hECN. The final optical density was approximately 10 OD at 500 nm. Samples were left in darkness; these were used directly for the OCPo measurements without dilution. To photoconvert the protein to the OCPr form a Flexilux 150 HL Universal lamp was used. Figure 5 displays the absorption spectra (and their derivatives) of the two spectroscopic forms of purified OCP used for the experiment described in this article, OCPo and OCPr.

Spectroscopy. Absorption spectra were collected using a Varian Cary E5 Double-beam scanning spectrophotometer. RR spectra were recorded with a 90° signal collection using
a two-stage monochromator (U1000, Jobin Yvon, Longjumeau, France) equipped with a front-illuminated, deep-depleted CCD detector (Jobin Yvon, Longjumeau, France). Excitation wavelengths were provided by a 24 W Sabre Argon laser (Coherent, Palo Alto, California), except for 647 nm, which was provided by an Innova 90 Krypton laser (Coherent, Palo Alto, California). Unless otherwise indicated, less than 2 mW reached the sample (power values given in the following sections refer to the laser wattage, not the power arriving at the sample), and the sample integrity was verified by following RR spectral evolution during the experiment. The error is + or – 0.5 cm–1 for the RRS experiments. All experiments were done at 4°C unless stated otherwise.

Sample conversion from OCPo to OCPr can be a problem during RRS experiments due to laser exposure. Also, spontaneous conversion from OCPr to OCPo will occur in darkness. Conversion in both directions is extremely reliant on temperature. To keep the samples as pure OCPo or OCPr, the experiments were done at 4°C in a 1mm path length cuvette that contained a stir bar mixing the sample during the course of the experiments. Purity of OCPo and OCPr can be tracked by the position of the v1 peak, so the OCP form was monitored during the course of the experiments.

Computational details. We have considered different isomers of echinenone and canthaxanthin that are shown in Figure 1. We have considered echinenone in different isomers and not 3-hydroxyechinenone because in the present experiments in protein the pigment is echinenone and furthermore the two molecules show identical spectroscopic properties. In addition to that we have performed calculations of two isomers of 3′-hydroxoechinenone that show v1, v2 frequencies and S0-S2 transitions almost identical to the corresponding echinenone values (see table S1).
Figure 1: Molecular structures, showing the different isomers of echinenone we considered in this work: (a) T(trans)C(is), (b) CC, (c) TT, (d) CT and the three isomers of canthaxanthin: (e) TT, (f) CT, (g) CC.
All structures were first optimized using the B3LYP functional with a relatively small basis set, 6-31G(d), and then were re-optimized with an extended basis set, 6-311G(d,p). For these geometries we have performed frequency calculations, obtaining normal modes and Raman intensities. A scaling factor of 0.9769 was used to better reproduce the absolute experimental values.

On the same optimized geometries, we have performed TD-DFT calculations to obtain $S_0 \rightarrow S_2$ transition energies, with the B3LYP functional and 6-311G(d,p) basis set (TD-B3LYP). Also the Tamm-Dancoff approximation was used in TD-DFT calculations (TDA-B3LYP), since Vaswani et al. have reported that TDA-DFT was better able to reproduce results on another carotenoid (peridinin) than normal TD-DFT. The transition to the $1^1B_u^+$ bright excited state, corresponding to what observed experimentally, has mainly a HOMO$\rightarrow$LUMO character and thus TD-DFT is able to characterize it correctly.

To locate bands that are enhanced in the resonance Raman effect, we have identified normal modes located on the polyene chain corresponding to the part of the molecule in which there is the largest change in molecular orbital upon electronic transition. Exactly the same procedure was recently used by Macernis et al. on a large series of carotenoids and was able to correctly reproduce experimental data.

All calculations were performed with the Gaussian09 package.

Results

Vibrational Properties of Echinenone and Canthaxanthin:

Figure 2 displays the room temperature resonance Raman spectra of two carotenoid molecules containing conjugated carbonyl groups in hexane at room temperature, 3-hydroxyechinenone (top) and canthaxanthin (bottom). These spectra contain four main groups of bands, denoted $\nu_1$ to $\nu_4$, typical of carotenoid molecules. The $\nu_1$ band, around 1520 cm$^{-1}$, arises from stretching vibrations of C=C double bonds. Its frequency depends on the length of the $\pi$-electron conjugated chain and on the molecular configuration of the carotenoid. The $\nu_2$ band at 1160 cm$^{-1}$ consists of contributions from stretching vibrations of C-C single bonds coupled with C-H in-plane bending modes, and this region is a fingerprint for the assignment of carotenoid configurations, i.e. isomerization states. The $\nu_3$ band at 1000 cm$^{-1}$ arises from in-plane rocking vibrations of the methyl groups attached to the conjugated chain, coupled with in-plane bending modes of the adjacent C-H’s. It was recently reported to be a fingerprint of the conjugated end cycle configuration; this hypothesis was recently confirmed by theoretical modelling. Finally, the $\nu_4$ band around 960 cm$^{-1}$ arises from C-H out-of-plane wagging motions coupled with C=C torsional modes (out-of-plane twists of the carbon backbone). When the carotenoid conjugated system is planar, these out-of-plane modes will not be coupled with the electronic transition, and these bands will not be resonance-enhanced. However, distortions around C-C single bonds will increase the coupling of these modes with the electronic transition, resulting in an increase in their intensity. Altogether, these spectra are quite
similar to those of \(\beta\)-carotene or of any carotenoid devoid of conjugated carbonyl groups. Here, it is worth noting that previously the presence of a 3'-hydroxy on the echinenone was expected to result in no effect on either the electronic or the vibrational properties of the molecule, and according to our experiments and calculations it does not (see supplementary material figures S1, S2, and table S1), thus 3-hydroxyechinenone can be described as echinenone as far as these parameters go.

\[
\begin{align*}
\text{Intensity (a.u.)} & \quad \text{Raman Shift (cm}^{-1}) \\
\text{3-hydroxyechinenone} & \quad \text{canthaxanthin}
\end{align*}
\]

![Figure 2. Resonance Raman spectra of echinenone (top) and canthaxanthin (bottom) in hexane obtained at room temperature. Excitation wavelength: 514 nm. Spectral range: 900-1700 cm}^{-1}.

The presence of a carbonyl group is the only chemical difference between echinenone and \(\beta\)-carotene. It results in an upshift of the electronic absorption properties of this molecule, as well as a downshift of the resonance Raman \(\nu_1\) band of about 5 cm}^{-1}, indicating that this carbonyl group influences the structure of the ground-state of echinenone and its electronic properties. Note, echinenone resonance Raman spectra contain no band which can be attributed to the stretching modes of the conjugated C=O, although it is conjugated with the C=C chain (figure 2). It was recently reported that the \(\nu_1\) frequency of carotenoid molecules exhibits a slight, but significant sensitivity to the polarizability of the solvent surrounding the molecules \(38\). This is also the case for echinenone, and the dependence observed is similar to that of previously studied carotenoid molecules. Canthaxanthin possesses one carbonyl group on each of its conjugated cycles, thus one more carbonyl group than echinenone. However, while the addition of a carbonyl to \(\beta\)-carotene makes a significant change to the electronic transition and resonance Raman \(\nu_1\) band, these two carotenoid molecules display frequencies for both at nearly exactly the same positions.
Models of canthaxanthin and echinenone spectra (see below) show that, for these carotenoid molecules, the $\nu_1$ band corresponds to a C=C stretching delocalized over the whole conjugated chain, but with larger participation of central bonds, which explains why both spectra are almost identical.

4°C comparison of echinenone in the red and orange forms of OCP with echinenone in solvents

Resonance Raman spectra were measured for both forms of OCP using different excitation wavelengths. Figure 3 shows the resonance Raman spectra of OCPo and OCPr, excited at 514.5 nm, as compared to that of 3-hydroxyechineone obtained in similar experimental conditions (the solvent is hexane which is relatively apolar for an organic solvent, similar to a hydrophobic protein environment). OCP transition from orange to red at 4°C (similar to room temperature because the sample is unfrozen) induces modifications of the resonance Raman spectra strikingly similar to those which have been reported at low temperature. As was noted in ref 29, OCP transition from orange to red induces three main changes in the resonance Raman spectra, namely i) a downshift of the frequency of the $\nu_1$ band, ii) a decrease of the intensity of the $\nu_4$ band, and iii) small changes in the structure of the $\nu_2$ and $\nu_3$ bands. The resonance Raman spectrum of the OCPo appears very similar to that of isolated 3-hydroxyechineone in n-hexane, suggesting that echinenone, in OCPo, is close to the relaxed form that it adopts in solvents. However, the frequency of $\nu_1$ band is observed at 1520 cm$^{-1}$ for 3-hydroxyechinenone in hexane and 1516 cm$^{-1}$ for the OCPo. Closer examination of the $\nu_2$ band (figure 3 insert) shows a single peak for echinenone in solvent at 1004 cm$^{-1}$ while this band is clearly broadened in the spectrum of the OCPo. An additional component is present in this region in the spectrum of OCPo, downshifted at 1002.5 cm$^{-1}$. Also striking is the fact that the $\nu_1$ band splits into two clear components (figure 3) in OCPo, one at 1518 and one at 1527 cm$^{-1}$, values which we took from the second derivative of the spectrum.
Does resonance Raman excitation promote OCP transition?

When analyzing a photoactive protein with resonance Raman spectroscopy, the excitation wavelength, which matches with the chromophore’s transition, may trigger the protein activity. To reduce this phenomenon, very low excitation power was used in the experiments described above. However, the discovery of two $v_1$ bands for OCPo necessitates careful examination as to whether those are natively present in the protein, or the result of its photocycle. The first control consists in progressively increasing the power of the excitation laser used in the previous experiments by an order of magnitude. If one component of the $v_1$ arises from the OCP photoactivity, a change in the structure of the $v_1$ should be observed according to the laser power. Figure 4a shows spectra obtained at 514.5 nm with laser powers on the sample ranging from 400 µW to 3 mW at 4°C. It is clear that the $v_1$ structure does not change according to the laser power at this temperature. Resonance Raman spectra of the orange form were also measured using excitation wavelengths in pre-resonance conditions, i.e. with excitations located outside of the absorption range of echinenone in OCP. In these conditions, the resonance Raman analysis is not expected to induce OCP photoactivity. Figure 4b compares resonance Raman of the orange form of OCP measured using 488.0, 514.5, 528.9 and 647.1 nm. The structure of the $v_1$ is identical in the three latter spectra, although 647.1 nm is an excitation which is not...
able to trigger OCP conversion activity. We must thus conclude that the echinenone in OCPo displays at least two configurations, which result in the two $\nu_1$ frequencies observed in the resonance Raman spectra. These experiments do not directly yield information about the electronic properties of these conformations. However, the relative contributions of the two different $\nu_1$ bands varies when scanning the excitation wavelength in the blue green range (see the 488.0 nm excitation wavelength in figure 4b, chosen as an example where the high frequency $\nu_1$ contribution is clearly more intense than when exciting at 514.5 nm). If the two echinenone conformations had exactly the same absorption properties, one should expect the ratio between the two $\nu_1$ bands to stay the same for each excitation wavelength. We can thus conclude that each of these conformations has different absorption properties, although we cannot precisely determine their position. It is clear however, that a major form in OCPo contributes at 500 nm (figure 5a). As this position is downshifted as compared to that of OCPr, we will attribute to the upper frequency $\nu_1$ to this echinenone form. The lower $\nu_1$ frequency should correspond to echinenone absorbing further in the red. A similar idea was previously suggested in ref $^{63}$, where deconvolution of the absorption spectra showed that there should be a mixture of species in OCPo at ambient temperature, which was attributed to a mixture of OCPo and OCPr. However, if our results confirm the presence of at least two different carotenoid species in OCPo at room temperature, the frequencies observed in resonance Raman clearly indicate that the second species present in OCPo is not proper OCPr. It is worth noting at this stage that the resonance Raman of OCPr, when converted properly, does not indicate any sign of heterogeneity at any probed excitation wavelength, indicating that the ensemble of OCPo configurations are able to convert into the same red form of the protein.
Figure 4: A. $v_1$ region of OCPo taken at different intensities of incident laser power. Excitation wavelength: 514 nm. B. RRS spectra of the $v_1$ peak of OCPo taken at different incident laser excitation powers: 488.0 nm, 514.5 nm, 528.9 nm, and 647.1 nm.
Figure 5: A. Absorption spectra of the OCP protein (containing echinenone) in its orange and red forms at room temperature. B. Second derivative spectra of the OCP absorption spectra.
Configuration of echinenone in the orange and red forms of OCP

As stated above, the 4°C resonance Raman spectra of the orange and red forms of echinenone are globally similar to the spectra of isolated 3-hydroxyechineone in solvent. In particular, only a very small additional component in the $\nu_2$ region can be observed in the OCPo spectra (figure 3). Resonance Raman spectra are exquisitely sensitive to the molecular configuration of carotenoid molecules. In particular, most of the cis-trans isomerizations result in the presence of additional satellite bands in the $\nu_2$ region; the absence of additional component with substantial intensity allows to discard these configurations. However, when isomerization occurs at the end of the electron delocalized chain (7-cis), the $\nu_2$ region can undergo little to no change. However, the extent of the changes in the $\nu_1$ frequency upon 7-cis isomerization is limited to 1-2 cm$^{-1}$ again, the presence of such configuration cannot explain the differences between the resonance Raman spectra of OCPo and OCPr, as the frequency of the $\nu_1$ shifts by 11 cm$^{-1}$, i.e. by more than an order of magnitude than what is observed upon 7-cis isomerization. As the $\nu_1$ band represents a direct measurement of the effective conjugation length of the C=C chain of the carotenoid molecules, we are thus left with three echinenone configurations (two in the orange form and one in the red form) which display different C=C chain conjugation lengths, which cannot be explained by a cis-trans isomerization.

Previously, we have shown that comparing the 0-0 transition to the $\nu_1$ peak position of carotenoids in solvents has been a useful way to show that solvent polarizability affects the effective conjugation chain length. In this study, several solvents have been used: THF, n-hexane, cyclohexane, acetonitrile, DCE, toluene, pyridine, methanol, chloroform, carbon disulfide, diethyl ether, and DMSO. A series of carotenoids (lutein, $\beta$ carotene, 3-hydroxyechinenone, canthaxanthin, and lycopene) were dissolved in each solvent and plotted on the graph as a function of the 0-0 transition (taken from the absorption spectrum) and the $\nu_1$ peak frequency position (figure 6). As the polarizability of the solvent increases, the $\nu_1$ peak frequency and 0-0 transition red shift, and a larger effective conjugation length is observed. The $\nu_1$ and 0-0 transition of OCPo and OCPr have been plotted for comparison (figure 6, OCP: orange and red dots; echinenone: blue squares). In OCPr, the echinenone configuration displays a similar effective conjugation length to isolated 3-hydroxyechinenone (thus planar, all-trans), in a highly polarizable environment. In OCPo, one form has a slightly shorter conjugation length than echinenone in OCPr, but we have no information on its absorption properties so it has not been plotted. The other’s 0-0 transition is found at 500 nm (figure 5), and its effective conjugation length is much shorter than in isolated echinenone, although other features of resonance Raman indicate it is still all-trans.
Carotenoid configuration is also determined by the torsions around the C-C bonds (s-cis isomerizations); the change observed in the $v_4$ region actually indicates that the configuration of echinenone is highly distorted in OCPo. In a recent study, it was shown that $\beta$-carotene, and more generally carotenoids containing conjugated end-cycles (such as lutein and $\beta$-carotene derivatives with different C=C chain lengths) actually exhibit shorter effective conjugation in solvents than expected. For instance, $\beta$-carotene displays an effective conjugation length of 9.6, whereas it formally contains 11 conjugated C=C double bonds in its linear form. This was attributed to the out-of-plane and/or s-cis trans-cis configuration of the end conjugated cycles. In LHCII, the two bound lutein molecules display an absorption shift of about 20 nm, as do the two $\beta$-carotene molecules bound to the photosystem II reaction center. In these cases, all concerned carotenoid molecules are clearly all-trans, however, the red-absorbing $\beta$-carotene and lutein molecules actually display effective conjugation lengths longer than in solvents, both from an electronic (absorption) and Raman ($v_1$ band frequency) point of view. It was concluded that this was achieved through protein-carotenoid interactions that hold the conjugated end-ring in the
plane of the C-C conjugated chain, thus increasing the participation of the cyclical C=C bonds to the overall conjugation length of the molecule. The echinenone absorption shift between the orange and red form is about 30 nm, and could thus be accounted for by a similar mechanism (figure 5). Both in LHCII and in PSII reaction centers, the C=C conjugated chain of the end-cycle ring coming into plane is accompanied by a downshift of the $\nu_3$ band frequency, which involves the nuclear coordinates of the conjugated end-cycles $^{62}$, or by the presence of a new, lower frequency component. It is striking that similar differences can be observed between Raman spectra of the echinenone in the OCPo and OCPr, and that in this case as well, the red-absorbing echinenone is associated with the presence of a lower frequency component in the $\nu_3$ region.

*Modeling electronic and Raman properties of echinenone*

Resonance Raman data allow to discard the presence in OCP of isomers with rotations around C=C bonds. We have thus modelled four isomers of echinenone, differing in the rotation around the C6-C7 and C6’-C7’ bonds (Figure 1). These isomers are denoted CC, CT, TC, and TT where C represents s-cis and T represents s-trans on either side of the chain at the C6-C7 and C6’-C7’ positions, respectively. As stated above, isomerization around these bonds is not expected to have a large effect on the $\nu_2$ band structure in the RRS spectra, but may influence the frequency of the $\nu_1$ band. Coordinates of all optimized geometries with corresponding relative energies are reported in the supporting information (tables S2 and S3). We note that the CC isomer is slightly more stable in energy, similarly to what was obtained by Liu et al. on $\beta$-carotene $^{64}$. $S_0 \rightarrow S_2$ transition energies obtained from TDA-B3LYP/6-311G(d,p) calculations are shown in Figure S3 of supplementary materials, where we also show the HOMO$\rightarrow$LUMO orbitals that are involved in the bright electronic transition. All values are also reported in table S4 of supporting information. The order of transition energies from higher to lower is CC > CT $\sim$ TC > TT. A red shift is thus progressively obtained when rotating the terminal group from cis to trans configuration, reflecting the fact that the effective conjugation of the chain is increased. TDA-B3LYP results are in better agreement with experiments than TD-B3LYP, but the trend observed is the same. Note that this is not surprising since Vaswani et al. $^{53}$ have already reported that the Tamm-Dancoff approximation (TDA) improves the reliability of TD-DFT methods in the case of carotenoids. Here and hereafter we use TDA-B3LYP results.

The different normal modes composing the $\nu_1$ and $\nu_2$ bands are reported in table S5 of the supporting information. These s-cis isomerizations do not affect the structure or the position of the $\nu_2$ band; however they have a clear effect on the $\nu_1$ frequency. Figure S4 displays the $\nu_1$ bands of different isomers as obtained by calculations where Raman intensities are considered. The C=C stretching normal modes composing the $\nu_1$ band for these different isomers are displayed in tables S6 and S7. The most intense C=C stretching band (and also the lowest frequency C=C stretching mode) for all isomers corresponds to the $\nu_{11}$ band in the experimental spectra. These results are similar to what was obtained for peridinin by both static and dynamic calculations, as well as for $\beta$-carotene $^{65}$. For $\nu_{13}$, similarly to what was obtained for the $S_0$-$S_2$ transition, the molecules with s-trans bonds
are red-shifted as compared to those with s-cis bonds at the C6-C7 and C6'-C7' positions. In order to summarize the shifts and for comparison with experimental data, the values of the S0-S2 absorption and the ν1 position of different isomers are plotted in Figure 7. In this figure it is clear that when moving from CC to TT isomers, through TC and CT (that behave almost similarly), the red shifts of both the ν1 position and the S0-S2 absorption are of the same order of magnitude as those observed when OCP undergo from its orange to its red form. Note that these trends hold true for other carotenoid molecules with end cycles containing conjugated C=O; we have performed similar calculations on canthaxanthin (figure 1). Three possible isomers along C6-C7/C6'-C7' isomerization of canthaxanthin were considered (figure 1), and the results are fully consistent with those obtained for echinenone both theoretically (figure 7) and experimentally (see figure 6, and tables S4-S7).

![Figure 7: ν1 peak position vs S0→S2 transition energy for echinenone and canthaxanthin isomers as obtained in B3LYP/6-311G(d,p) calculations (TDA for excited states). We show scaled vibrational frequencies.](image)

The extensive modeling of carbonyl-containing carotenoid molecules reported in this section indicates that the presence of the oxygen atom does not fundamentally affect the behavior of the vibrational modes of carotenoid molecules upon isomerization. In particular, as for in β-carotene, moving the in-plane echinenone cycle to an s-trans configuration results in a ν1 frequency downshift. This suggests that echinenone isomerization, from s-cis to s-trans around the C6-C7 and/or C6'-C7' bonds after light absorption could account for the OCPo vs OCPr differences observed in the S0-S2. In the frame of this hypothesis, calculations suggest that the echinenone in OCPo is in CC configuration (or at least the fraction of echinenone with the most upshifted (in energy) electronic absorption transition), since the ν1 position of the CC isomer is similar to that
obtained for OCPo (figure 3). In OCPr it would thus isomerizes providing the TC, CT or TT isomer. The extent of the frequency change of the $\nu_1$ resonance Raman band suggests that the OCPr forms should rather be TT. To summarize, a first hypothesis consistent with both the vibrational data and calculations would be that OCPo to OCPr transition would correspond to a CC to TT conversion of the bound echinenone.

On the other hand, calculations give information why the resonance Raman band arising from the C=O stretching modes is not present in the spectra. This is due to two effects: (1) their Raman intensity is low; (2) the changes in molecular orbitals corresponding to S0-S2 transition only marginally concerns this part of the molecule, different from other carbonyl containing carotenoids like peridinin where C=O stretching is visible in RRS and can be used to characterize the differences in environment.

Discussion and Conclusions

The set of resonance Raman experiments described in this paper first show that two configurations of echinenone exist in the orange form of OCP, and only one in the red form. Calculations show that red-absorbing echinenones correspond to molecules with longer conjugated chains, and consequently display $\nu_1$ Raman bands with lower frequencies. In the OCPo, two frequencies are observed in the $\nu_1$ Raman band, one at 1518 and one at 1527 cm$^{-1}$. The latter must thus correspond to the echinenone with the highest energy electronic transition, which contributes at 500 nm in the OCP absorption spectra. The exact position of the electronic absorption of the other echinenone form is unknown, thus first we will discuss the molecular mechanisms underlying the photoinduced changes between the 500 nm-absorbing OCPo and OCPr. Vibrational spectra show that this conversion cannot be attributed to a cis-trans (or trans-cis) isomerization around C=C bonds. Theoretical calculations show that the presence of a C6-C7/C6'-C7'di-s-cis echinenone isomer could account for the properties of 500 nm-absorbing echinenone in OCPo. Conversion to the echinenone red form would then correspond to the rotation of both end cycles to an s-trans configuration. However, electronic energy and vibrational frequency calculations can be only performed on stable carotenoid configurations, i.e. on structures that correspond to a (local) energy minimum. In proteins, binding sites may induce steric hindrances that stabilize a configuration not corresponding to energy minima for isolated echinenone. The OCPo to OCPr transition may also correspond to a carotenoid configuration change not accessible by the present set of calculations. For instance, neighboring amino acids may push the echinenone end-cycles out of the C=C conjugated plane. This will decrease the effective conjugation length of the molecule eventually even more efficiently than s-cis isomerization (where the cycle at the end is back into the C=C plane). In the crystal structure of the OCPo, the plane of both end cycles largely deviate from that of the C=C conjugated plane (close to 34° out-of-plane for the cycle with C=O, and 53° for the other (figure 8)). Such a configuration would thus correspond to all-trans echinenone, with large uncoupling of both end-cycles, and is compatible with the vibrational data and theoretical calculations. From there, any configuration change of the molecule allowing the ring(s) to enter into the C=C conjugation plane would in turn
increase the effective conjugation length of the molecule, inducing both the absorption redshift, and the $\nu_1$ and $\nu_3$ band frequency downshift, as experimentally observed. It is to be noted that such a configuration change is likely to reduce the $\nu_4$ band intensity, by ensuring that the molecule's symmetry is close to planar. Also, the inclusion of a C=O in conjugation with the C=C conjugated chain is likely to scramble the fine structure of the electronic absorption, as extensively discussed in $^{67}$, which is observed during the orange to red transition.

Figure 8: Electron density map of echinenone in the OCP protein (PDB ID: 3MG1). The software used was Coot$^{68}$. We would thus conclude that in the OCP, both cycles have a configuration close to in-plane. This is in line with the fact that in OCP, the echinenone configuration displays a similar effective conjugation length to isolated echinenone, and possibly even slightly longer. Indeed the 0-0 absorption transition of OCP, as determined by the second derivative of the absorption spectrum (figure 5b), is at 531 nm; the $\nu_1$ band is at 1516 cm$^{-1}$. In solvents the absorption of echinenone ranges from 487 to 535 nm, and the frequency of its $\nu_1$ band is between 1519 and 1521 cm$^{-1}$ (table 1, figure 6). It has been suggested by De Re et al. that during ultrafast experiments the echinenone in the OCP has an inhomogeneous nature; their hypothesis that the protein opens upon conversion to OCP and lets some solvent molecules surround the carotenoid. $^{69}$ Our results show that OCP is spectroscopically homogeneous, in the sense that all of the echinenones in OCP are in the same conformation in a highly polarizable environment. The properties of the echinenone environment, as deduced from RRS, are consistent with echinenone’s accessibility to water molecule(s).
In the OCPo at room temperature, on the other hand, we observe two \( \nu_1 \) frequencies, one at 1527 cm\(^{-1}\), and one at 1518 cm\(^{-1}\). From the frequency of the associated \( \nu_1 \), we must conclude that it is red-shifted as compared to the 500 nm-absorbing echinenone in OCPo, but likely to be slightly blue-shifted relative to echinenone in OCPr. As already mentioned, such an additional form in OCPo had been predicted from the analysis of the electronic absorption of OCPo \(^{63}\), however, it was then postulated to be a contamination by OCPr, while our results suggest it is not. At 77K, which is close to the temperature where the crystal structure of OCP was solved, only one high-frequency \( \nu_1 \) is observed (1) for OCPo, at 1520 cm\(^{-1}\). Taking into account that the frequency of the \( \nu_1 \) modes of carotenoids downshift by about 8 cm\(^{-1}\) upon cooling from room temperature to 77K \(^{70}\), the echinenone form stabilized at 77K must correspond to the farthest blue observed at room temperature, and thus is the form which possesses end-cycles out of the C=C plane, as observed in the crystal. The crystal structure actually suggests a mechanism for driving the end-cycles from out-of to in the C=C plane. Around the carotenoid cofactor, there is an area where the atomic structure does not perfectly fit the electron density map (see in figure 8 areas of red and green indicating poorly placed and missing atoms). The carotenoid chain may thus have some room to move, while the rings at the terminal ends are locked solidly in place; a ‘jump rope’ motif of echinenone in OCP, allowed by the crystal structure, could lead to the OCPo to OCPr molecular transition as deduced from our RRS experimental results.

It is worth noting that 1527 cm\(^{-1}\) corresponds almost exactly to the frequency observed for

Table 1: RRS experimental values of echinenone and canthaxanthin in different solvents are shown. For absorption experiments, the 2\(^{\text{nd}}\) derivative was taken of the spectrum to determine the value of the 0-0 transition. The \( \nu_1 \) peak frequency was taken from resonance Raman spectroscopy experiments. All values are in cm\(^{-1}\).
carotenoid molecules with an effective conjugation length of 9, *i.e.* a molecule of echinenone in which none of the cycles would influence the frequency of the $v_1$. In the absence associated electronic absorption spectrum, we cannot conclude that this form is unique, or corresponds to a broad distribution of echinenone configurations. As they all convert to OCPPr in the presence of green light, it is the most likely that this echinenone configuration or distribution of configuration corresponds to the presence of dynamic disorder into the OCPO, which allows echinenone end-cycles to adopt a range of geometries (among which only one survives at low temperature or in the crystal). This picture is actually consistent with the fact that conversion of OCPO to OCPPr involves a large protein conformational change, which is triggered by a change in echinenone configuration associated with a small free energy change. For this process to occur, even with the observed low probability, OCP must be close to a saddle point between its OCPO and OCPPr conformations, and is thus likely to permanently partially interconvert at room temperature.

Acknowledgements

Thank you to Daniel Picot for discussion about crystallography data and help with Coot software. This work was supported by the ERC funding agency (PHOTPROT project), the National Research Agency (ANR, Cyanoprotect Project), and The French Infrastructure for Integrated Structural Biology (FRISBI).

The abbreviations used are: DFT, density functional theory; hECN, 3’hydroxyechinenone; HOMO, highest occupied molecular orbital; ICT, internal charge transfer state; LHCII, light harvesting complex II; LUMO, lowest unoccupied molecular orbital; OCP, orange carotenoid protein; PBS, phycobilisome; PSII, photosystem II; RRS, resonance Raman spectroscopy.

References


CHAPTER 6

TRIPLET-TRIPLET ENERGY TRANSFER IN DYADS

Two artificial photosynthetic dyads, both constituted of the same building blocks but with different conformations due to the position of the linkage, were studied and their triplet-triplet (T-T) energy transfer rates and spectroscopic features of their triplet states compared to those of natural systems. In the para carotenoporphyrin (dyad 1) a slow T-T energy transfer between the two chromophores occurs, while in the ortho one (dyad 2) the T-T energy transfer between the chromophores is ultrafast.

Introduction

During the first steps of the photosynthetic process, the absorption of photons by antenna pigment-protein complexes and the subsequent transfer of the excitation energy to the reaction centers are both intimately linked with the potential production of dangerous oxidative species. Although its yield is low, production of (bacterio)chlorophyll ((B)Chl) triplet excited states by intersystem crossing from (B)Chls excited singlet states is a major source of singlet oxygen in photosynthetic organisms, one of the most dangerous chemical species for living organisms (Foote, 1976). In photosynthetic complexes, this sensitization reaction is precluded by transfer of the triplet excited state from (B)Chls to carotenoid molecules, which feature a triplet state energy below that of singlet oxygen. This quenching reaction reduces the lifetime of the (B)Chl triplet state by many orders of magnitudes.

In the light-harvesting (LH) proteins from most (anoxygenic) purple bacteria, the T-T transfer from BChl to carotenoid molecules occurs in the nanosecond range. By contrast, in light-harvesting complexes (LHC) from oxygenic organisms, we recently showed that this transfer is ultrafast, the chlorophyll triplet state decaying faster than it is formed. The mechanisms underlying this ultrafast T-T transfer, which was proposed to represent an adaptation of oxygenic photosynthetic organisms to their oxygen-rich environment, are not yet fully understood. Some relevant observations are that when ultrafast T-T transfer occurs between chlorophyll and carotenoid molecules, the presence of the triplet state on the carotenoid has an unusually strong influence on the chlorophyll Q transition, and the decay of this perturbation decays with the carotenoid lifetime. Moreover, the carotenoid triplet state exhibits an anomalous resonance Raman signature, suggesting a partial loss of its typical triplet state character. From these observations, it was tentatively proposed that the triplet state could be delocalized over the carotenoid/chlorophyll couple, and thereby shared between these molecules. Understanding in detail the relationship between photoprotective T-T energy transfer mechanisms and the exposure of the photosynthetic organism to oxygen is essential in order to extend those mechanisms to reengineered photosynthetic systems where the production of oxygen and therefore the steady state level of oxygen in the membranes would be much higher than current levels.
Over the last three decades, a large numbers of carotenoid/tetrapyrrole synthetic dyads have been designed and synthesized, in which the absorption of a photon is followed by the formation of a triplet state by intersystem crossing at the level of the tetrapyrrole, and subsequent transfer of the resulting triplet energy from the tetrapyrrole to the carotenoid\textsuperscript{7-10}. In these dyads, depending on their precise chemical properties and on the way the tetrapyrrole and the carotenoid molecules are linked, the T-T transfer kinetics range from tens of microseconds to the sub-nanosecond range in which the actual T-T energy transfer rate was not determined because intersystem crossing in the tetrapyrrole is the rate limiting step\textsuperscript{7,8,11}. In this work, we have studied two closely related dyads, exhibiting slow and fast T-T energy transfer using a combination of vibrational and transient absorption spectroscopic methods, and density functional theory (DFT) calculations. These dyads, which mimic the dynamics and spectroscopic signatures characteristic of the natural systems, open the way for disentangling the molecular mechanisms underlying the kinetics of triplet-triplet transfer \textit{in vivo}.

\textbf{Methods}

\textit{Experimental procedures}

The para- and ortho carotenoid porphyrin dyads (see structures in figure 1A) were synthesized as described in Refs 12,13. Steady-state absorption spectra were measured on a Shimadzu UV-3101PC spectrometer. The nanosecond-millisecond transient absorption measurements were performed using a 4-5 ns pulse of an optical parametric oscillator (Ekspla NT342B) with a repetition rate of 10 Hz, and a Proteus spectrometer (Proteus) from Ultrafast Systems. The instrument response function was 5 ns. Global analysis (van Stokkum et al, 2004) of the transient absorption data analysis was carried out using ASUFIT developed in a MATLAB environment (Mathworks Inc.). Resonance Raman spectra were obtained using a 24 W Sabre laser (Coherent, Palo Alto, California), and were recorded at 77K with 90° signal collection using a two stage monochromator (U1000, Jobin Yvon, Longjumeau, France), equipped with a front-illuminated, deep-depleted CCD detector (Jobin Yvon, Longjumeau, France). Low temperature of the sample was ensured using a Helium flow cryostat (Air Liquide, Sassenage, France). Resonance Raman spectra of the ground and triplet state of the carotenoid moiety of the dyads were recorded as extensively described in (Gall et al., 2011).

\textit{Theoretical Calculations}

All electronic structure calculations were performed using the Gaussian 09 program (revision D01)\textsuperscript{14} and Q-CHEM programs. The ground state singlet and excited state triplet geometries of both dyads were optimized at the B3LYP,\textsuperscript{15} CAM-B3LYP,\textsuperscript{16} CAM-B3LYP-D3,\textsuperscript{16,17} \textomega B97XD\textsuperscript{18} and M06-2X\textsuperscript{19} density functional theory methods in conjunction with the 6-31G(d) basis set. Solvent effects (THF) were simulated using the SMD solvation model.\textsuperscript{20} The Raman spectra and relative intensities of Raman peaks were simulated at the same levels of theory on corresponding optimized geometries. This approach was successfully
applied in previous studies to model substituent effects on the resonance Raman spectra of carotenoid molecules.\textsuperscript{21,22} The ultrafine grid keyword was specified for all DFT calculations and the Raman and Wiberg bond order calculations\textsuperscript{23} were carried out as implemented in Gaussian 09. The fragment spin difference (FSD) scheme\textsuperscript{24,25} was used to quantify the triplet energy transfer couplings, and these were evaluated at the CIS/6-31G(d) level of theory using the Q-CHEM program (version 4.0.1).\textsuperscript{26} In the FSD calculation, the boundary between donor and acceptor domains of each dyad is defined to be at the ester oxygen-methylene carbon bond.

Hybrid quantum mechanics/molecular mechanics (QM/MM) calculations were carried performed for the natural systems using the ONIOM formalism\textsuperscript{27} as implemented in Gaussian09. The starting structures for the LHC2 and LH2 complexes were obtained from their x-ray structures (PDB ID: 1RWT\textsuperscript{28} and 1KZU\textsuperscript{29} respectively). The TAO package of Tao and Schlegel\textsuperscript{30} was used to set up the ONIOM calculations. Full details are provided in the Supporting Information. In the QM/MM models, the QM layer is composed of the chlorophyll, carotenoid (lutein for LHC2 and rhodopsin glucoside for LH2) as well as all amino acid residues that are within 3.5 angstrom (based on x-ray coordinates). The rest of the system is modeled using the AMBER96 force field.\textsuperscript{31} The AMBER parameters for the (bacterio)chlorophyll were obtained from Vasil’ev and Bruce\textsuperscript{32} whereas the parameters for lutein and rhodopsin glucoside were obtained using the general amber force field (GAFF). The atomic charges for LUT and RG1 were obtained at the HF/6-31G* level using the antechamber program.\textsuperscript{33,34} Thus, all QM/MM geometries and Raman calculations were carried out at the ONIOM(\omega B97XD/6-31G(d):AMBER) level of theory.
Figure 1. **A**: Molecular structure of the two dyads and carotenoid examined in this study (1) ortho-ester carotenoporphyrin, (2) para-ester carotene porphyrin **B**: Room temperature electronic absorptions of para and ortho carotenoporphyrin in methyl-THF. Black line: ortho-ester carotenoporphyrin, red line: para-ester carotenoporphyrin, blue line: porphyrin.
Results

Both ortho- and para- ester carotene porphyrin dyads comprise a carotenoid having 9 conjugated double bonds, linked to a porphyrin by an ester bond (see figure 1A), and the only difference between these two dyads is the linker point of attachment on the meso-phenyl group of the porphyrin. The electronic absorption spectra of these dyads are nearly identical (figure 1B). In these spectra, porphyrins which exhibit the same symmetry as hemes, display intense contributions at 419 nm, and relatively weak transitions in the visible range at 516, 552, 591, and 647 nm. In both dyads, excitation of the porphyrin at XXX nm is followed by intersystem crossing over the nanosecond porphyrin singlet lifetime to yield the porphyrin triplet state, which decays by T-T energy transfer to the linked carotenoid. Kinetic traces measured both in the porphyrin ground state and carotenoid triplet absorption range evidence dramatic porphyrin triplet lifetime differences between the two dyads (figure 2 and 3), as already reported in Bensasson et al, 1981. In the ortho-carotenoporphyrin dyad no intermediate species can be observed between the porphyrin excitation and the appearance of the carotenoid triplet state (figure 3A). This indicates that the rise of porphyrin triplet state through intersystem crossing is slower than the triplet-triplet transfer to the carotenoid molecule, thus in the subnanosecond range. On the contrary, in the para-carotenoporphyrin dyad, a porphyrin triplet is clearly observed, which is transferred into 3.5 µs to the carotenoid molecule.
Figure 2: Kinetic traces measured at 425 nm (A) and 530 nm (B) after ortho- carotenoporphyrin dyad excitation, and at 460 nm (C) and 530 nm (D) after para- carotenoporphyrin dyad excitation. Both dyads were dissolved in argon saturated benzonitrile.

Resonance Raman Spectra of both dyads were recorded using a 514 nm excitation. As this excitation line match the position of the $S_0/S_2$ electronic transition of the carotenoid in the dyads, it is expected to enhance the contribution of this part of the molecule only. Raman spectra of carotenoid molecules generally contain the four groups of bands namely: i) the $\nu_1$, observed around 1530 cm$^{-1}$ arises from the C=C stretching modes, the $\nu_2$, at 1160 cm$^{-1}$, which arises from a combination of C–C stretching modes and in-plane C-bending modes, the $\nu_3$ at about 1000 cm$^{-1}$ from in-plane rocking vibrations of the methyl groups attached to the conjugated chain and the weak $\nu_4$ band, at about 950 cm$^{-1}$, from C–H out-of-plane wagging motions coupled with C=C torsional modes. The frequency of $\nu_1$ and the structure of $\nu_2$ are exquisitely sensitive to the carotenoid configuration$^{35}$. 
Both these bands indicate that the carotenoid in both dyads are in all-trans configuration (data not shown), and, altogether, the resonance Raman of both dyads are nearly identical (data not shown). These spectra do not change after dyad illumination at 77K or at room temperature (data not shown), indicating that no light-induced cis-trans isomerization occurs, although the carotenoid triplet state is populated at the end of the excitation energy decay cascade. With increasing laser power, using an excitation at 514 nm, located close to the T1/Tn transition of the carotene, a number of small bands appear in the spectrum. As discussed extensively in ref 4, the observed power dependence of the appearance of these features is consistent with progressive, dynamic accumulation of a transient state, and the bands observed at higher laser intensity are characteristic of the resonance Raman spectra of carotenoid molecules in their triplet states. Figure 4 displays the ν1 region of the resonance Raman spectra of both dyads obtained with a 514.5 nm excitation at low and high power (5 and 25 µW laser power reaching the sample, respectively). For both dyads, increasing the intensity of the excitation line results in the appearance of a strong signal in the ν1 region, down-shifted relative to the position of the carotenoid ground-state ν1, typical from the ν1 of carotenoid triplet state. In the para carotenoid porphyrin dyad, this band occurs at 1498 cm⁻¹, i.e. downshifted by 26 cm⁻¹ as compared to the frequency of the carotenoid ground-state ν1, while it occurs at 1508 cm⁻¹ in the ortho carotenoporphyrin dyad, downshifted by only16 cm⁻¹ relative to the ground-state ν1. As discussed in ref 4, the build-up of a ‘normal’ triplet state of a carotenoid induces a 24 cm⁻¹ downshift of that band, due to the transition of one electron from the bonding highest occupied molecular orbital (HOMO) to an antibonding orbital. The build-up of the carotenoid triplet state in the para dyad is thus similar to that observed for isolated carotenoid molecules, while in the ortho dyad, it induces a smaller downshift of this mode.
Figure 4. Resonance Raman spectra (exc. wavelength 514.5 nm, T 77K) of para (left) and ortho (right) carotenoporphyrin dyads. Top spectrum: high intensity (triplet); middle spectrum: low intensity; bottom spectrum: computed difference between spectra obtained at high and low intensity.

Computational Calculations

Computational calculations were carried out to simulate the Raman spectra for both dyads in THF to better understand the origin of the anomalous resonance Raman shift (ARRS). Normal mode, bond order and spin population analyses were also carried out to explain the spectral features in both dyads.

Figure 5. Optimized global minimum geometries of dyads 1 (top) and 2 (bottom) in the singlet ground state in THF.
Geometries

The ωB97XD/6-31G(d) optimized geometries of the lowest energy structures of the ortho (1) and para (2) dyads are depicted in Figure 5. In dyad 1, the porphyrin moiety is stacked approximately 3 Å above the aromatic ring of the carotenoid. By comparison, dyad 2 adopts an open configuration where the porphyrin is considerably distant from the carotenoid. The stacked conformation of 1 is supported by experimental 1H NMR data where very large upfield shifts of about 3 ppm were observed for the carotenoid aryl ring, which is indicative of strong shielding by the porphyrin π system.12 For 2, the carotenoid resonances have frequencies that are all within 0.2 ppm of the resonance positions for the corresponding protons of the carotenol benzoate,12 suggesting that there is negligible interaction between the porphyrin and carotenoid moieties. It is worth mentioning that the optimized geometries are sensitive to the choice of DFT method. Notably, only the CAM-B3LYP-D3,16,17 ωB97XD18 and M06-2X methods led to a stacked conformation for the ortho dyad, signifying the importance of dispersion corrections in modeling the structures of compounds of these types. As discussed in more details below, the calculated Raman spectra also depend intimately on geometry and this will dictate our choice of DFT method for simulating the Raman spectra.

Figure 6. Calculated ωB97XD/6-31G(d) Raman spectra of the ground (black line) and triplet (red line) states of the ortho (TOP) and para (BOTTOM) dyads. The frequencies are unscaled, and the normal modes have been fitted to a Lorentzian (FWHM 20 cm⁻¹).

Raman Spectra

The Raman spectra of the ground state singlet and excited state triplet of each dyad are shown in Figure 6. In the triplet state, the v1 band corresponding to the C=C carotenoid stretch in the ground state is split into two bands that are denoted by v1a and v1b. Notably, the v1a band of 2 is blue shifted relative to the v1 band by 26 cm⁻¹, whereas the corresponding Δν is only 16 cm⁻¹ for the ortho-dyad. Calculations based on a model carotenoid 3 depicted in Figure 1 shows a blue shift that is very similar to 2. The calculated blue
shift compares favorably with the experiment (ca. 10 cm\(^{-1}\) in Figure 4). It is worth pointing out that when the calculations were carried out on geometries optimized using DFT methods without dispersion corrections (B3LYP and CAM-B3LYP), there were no discernible differences in Raman spectra for 1 and 2. As noted before, dispersion corrected DFT methods predict a folded conformation for 1 where the porphyrin ring is stacked approximately 3 Å above the aromatic ring of the carotenoid. The anomalous resonance Raman shift (ARRS) in 1 must therefore stem from the π-π interactions between the porphyrin ring and carotenoid.

We have further analyzed the normal mode composition of the \(\nu1\), \(\nu1a\) and \(\nu1b\) bands. It is important to note that there is a complete reversal of bond order of the innermost carbon bonds of the carotenoid in going from the ground state to triplet excited state. Following the double bonding numbering scheme in Figure 1, the C=C bond 6 in the singlet state becomes C–C bond in the triplet state, and C–C bond 6′ becomes C=C bond in the triplet state. Normal mode simulations indicate that both \(\nu1a\) and \(\nu1b\) bands are comprised of C=C stretches of different groups of double bonds in the carotenoid. Consistent with previous studies,\(^{36,41,42}\) the \(\nu1b\) band corresponds to a relatively localized C=C stretch of the innermost double bonds 5′ and 6′ as depicted in Figure 1, whereas the \(\nu1a\) band at ca. 1505 cm\(^{-1}\) is associated with the C=C stretch of the peripheral double bonds. The nature of these normal modes is also presented graphically in Figure 7 displaying the magnitude of the displacement of individual CC bonds associated with each normal mode. As shown, the dominant normal mode in the \(\nu1\) band is relatively delocalized across the carotenoid (with a node around CC bond 6), whereas the corresponding normal mode for the \(\nu1b\) band is localized around the inner 6, 6′ CC bonds. Furthermore, it is also apparent that dyad 2 features a normal mode displacement profile that is almost identical to that of the model carotenoid 3. In contrast, the normal mode corresponding to \(\nu1a\) band in dyad 1 is significantly skewed towards the aromatic ring of the carotenoid that is in close proximity to the porphyrin.
Figure 7. The magnitude of displacement of individual CC bonds (Å) for the individual normal modes associated with the three Raman bands for ortho dyad, para dyad and model carotenoid.
Figure 8. Top: Cumulative spin density in the carotenoid for ortho and para dyads. Bottom: Calculated changes in bond order of individual CC bonds in the carotenoid for ortho and para dyads.

**Bond order and spin population analyses**

Analysis of the changes in bond order of the C=C and C-C bonds in these systems between the singlet and triplet states provides further insights into the origin of ARRS (Figure 8 top). The bond order change of a particular bond is defined by the bond order of that bond in the triplet excited state minus that in the singlet ground state. Accordingly, a positive bond order change indicates a gain in double bond character upon excitation to the triplet state and vice versa. The numbering of double bonds follows that shown in Figure 1. In 1, we found that the C=C bonds (8, 9 and 10) in the carotenoid moiety loses more
double bond character compared to 2. This effect is also mirrored in terms of a larger gain in double bond character of the C-C bonds in the triplet state (7' to 10'). By comparison, the bond order change in 2 is identical to that of 3 (data not shown). As noted in Figure 7, the v1a normal mode has increased contributions from the CC bonds in the porphyrin end of the carotenoid (relative to the para dyad and model carotenoid), and the gain in double bond character of these C-C bonds are therefore consistent with a v1a band that is more blue-shifted for dyad 1.

Table 1. Calculated αB97XD/6-31G(d) C=C stretching frequencies (cm^{-1}).

<table>
<thead>
<tr>
<th>Dyad</th>
<th>ν1 a</th>
<th>ν1a b</th>
<th>Δν c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ortho-dyad</td>
<td>1524</td>
<td>1510</td>
<td>14</td>
</tr>
<tr>
<td>Para-dyad</td>
<td>1524</td>
<td>1498</td>
<td>26</td>
</tr>
<tr>
<td>Carotenoid d</td>
<td>1529</td>
<td>1503</td>
<td>25</td>
</tr>
<tr>
<td>LHCII Lutein620</td>
<td>1530</td>
<td>1503</td>
<td>27</td>
</tr>
<tr>
<td>LH2 Rhodopin glucoside</td>
<td>1526</td>
<td>1497</td>
<td>29</td>
</tr>
<tr>
<td>Lutein</td>
<td>1527</td>
<td>1496</td>
<td>31</td>
</tr>
<tr>
<td>Rhodopin glucoside</td>
<td>1528</td>
<td>1498</td>
<td>30</td>
</tr>
</tbody>
</table>

a Scaled by 0.9018. b Scaled by 0.9292. c Δν = ν1 - ν1a. d Model carotenoid depicted in Figure 1.

These results indicate that there must be a higher spin population close to the porphyrin end of the carotenoid in 1 relative to 2. Indeed, the cumulative spin population plot in Figure 8 shows that the spin sums up to 2.0 (for a triplet) at a faster rate for dyad 2 compared to 1. In a related vein, it has been postulated in several earlier studies^4 that the origin of the ARRS is due to delocalization of the carotenoid triplet to the proximal porphyrin. However, the calculations indicate that the spin population in the porphyrin of 1 in its triplet state is very small (ca. 0.35 %). Interestingly, when the model carotenoid 3 is constrained to be in the same geometry as that in the triplet state of the ortho ester 1, the calculations predict a very similar Δν of ca. 15 cm\(^{-1}\) (Table 1). Since the spin population in the model carotenoid must be localized in the carotenoid, the results strongly suggest that the observed APPΣ for 1 is in fact due to perturbation of the carotenoid geometry as a
result of \(\pi-\pi\) interactions with the porphyrin. These conclusions are also in accord with recent electron paramagnetic resonance measurements\(^{43}\) which indicated the absence of spin population in the porphyrin for related natural systems.

**Natural systems**

As noted in the introduction, the ARRS is also observed in natural systems such as LHCI\(I\) from higher plants but not in anoxygenic systems such as LH2. It is therefore of interest to ask if the origin of the presence/absence of ARRS in these systems are the same as that for the synthetic dyads. To this end, QM/MM calculations were performed for the LHCI\(I\) and LH2 complexes which may be considered as the natural system analogues of the Ortho and Para dyads respectively. In the LHCI\(I\) and LH2 complexes, there are several (bacterio)chlorophylls that are in the vicinity of the carotenoid as depicted in Figure 9. Since it is impractical to include all the (bacterio)chlorophylls in the QM layer of the QM/MM model, only the chlorophyll that couples most strongly to the carotenoid is included in the QM layer. For LHCI\(I\), calculations indicate that the chlorophyll near the center of lutein couples most strongly, whereas in LH2, it is the bacteriochlorophyll closest to the 2’ end of rhodopsin glucoside. Accordingly, the QM layer is composed of the (bacterio)chlorophyll, the carotenoid and all amino acid residues that are within 3.5 angstrom of the carotenoid (Figure 9).

![Figure 9. The QM layer in the QM/MM model for LHCI\(I\) and LH2. The remainder of the system is modelled by the AMBER force field (not shown).](image)

Applying the same methodology used for the dyads, theory predicts \(\Delta \nu\) to of about 27 cm\(^{-1}\) and 31 cm\(^{-1}\) for lutein in LHCI\(I\) and in the gas phase respectively. On the other hand, \(\Delta \nu\) is estimated to be 29 cm\(^{-1}\) and 30 cm\(^{-1}\) for rhodopsin glucoside in LH2 and in the gas phase respectively (Table 1). It is worth mentioning that the absolute values of \(\Delta \nu\) do not compare particularly well with experiment (cf. 18 cm\(^{-1}\) and 24 cm\(^{-1}\) for LHCI\(I\) and LH2), but this is presumably because the scale factors optimized for the dyads might not be appropriate for the carotenoids in the natural system. Notably, the conjugation length of the carotenoids in the synthetic dyads and natural system carotenoids are different. Nonetheless, comparison of \(\Delta \Delta \nu\) values reveals an ARRS for lutein in LHCI\(I\). The normal mode (Figure 10), bond order and spin population analyses (Figure 11) on the two natural systems show a strong similarity to the synthetic dyads. Notably, in LHCI\(I\) we see that the dominant nor-
nal mode ν1a band has increased contributions from CC stretches closer to the 10’ end (see numbering scheme in Figure 1) compared to the corresponding mode for lutein in THF. The cumulative spin population and bond order analyses similarly indicate that there is increased spin population at the 10’ end of lutein 620 in LHCII. On the other hand, the corresponding analyses for LH2 shows negligible differences for rhodopin glucoside in the protein and in the gas phase. Accordingly, the origin for ARRS in LHCII appears to be identical to that in Ortho dyad 1, where the close proximity between the carotenoid and (bacterio)chlorophyll breaks the symmetry of the spin population in the triplet carotenoid and increase the bond order of the CC stretches associated with the ν1a normal mode.

Figure 10. The magnitude of displacement of individual CC bonds (Å) for the individual normal modes associated with the three Raman bands for LUT in LHCII and gas phase, and RG in LH2 and gas phase.

Correlation with TET rates

The triplet energy transfer (TET) coupling for 1 and 2 as well as for LHCII and LH2 (based on their QM/MM optimized geometries) using the fragment spin difference scheme of Hsu et al.24,25 Consistent with the experimentally observed triplet-triplet transfer rates, the coupling is approximately three orders of magnitude higher in 1 (ca. 32 cm\(^{-1}\)) compared to 2 (0.024 cm\(^{-1}\)). The natural system calculations are in qualitative agreement where the coupling is approximately 25 cm\(^{-1}\) and 6 cm\(^{-1}\) for LHCII and LH2. It is therefore
reasonable to conclude that both ARRS and fast TET are intimately related to how strongly the carotenoid interacts with the chromophore. As such, the combined-experimental analyses show that ARRS provides a useful spectroscopic signature for identifying systems that undergo fast triplet energy transfer.

Figure 11. Top left: Cumulative spin density in the carotenoid for LUT in LHCII and gas phase. Bottom left: Calculated changes in bond order of individual CC bonds in the carotenoid LUT in LHCII and gas phase. Top right: Cumulative spin density in the carotenoid for RG in LH2 and gas phase. Bottom right: Calculated changes in bond order of individual CC bonds in the carotenoid for RG in LH2 and gas phase.

Acknowledgements

This work was supported by the ERC funding agency (PHOTPROT project), and by the National Research Agency (ANR, Cyanoprotect Project). This work was supported by the French Infrastructure for Integrated Structural Biology (FRISBI) ANR-10-INSB-05-01. The synthesis, computational calculations, and transient absorption spectroscopy were funded by the US Department of Energy under contract DE-FG02-03ER15393 and the Human Frontiers Research Project.
References


CHAPTER 7

RESONANCE RAMAN CHARACTERIZATION OF RECONSTITUTED WILD-TYPE AND POINT-MUTATED LHCII PROTEINS

Resonance Raman spectroscopy was used to evaluate the structure of reconstituted light-harvesting chlorophyll (Chl) a/b complexes of photosystem II (LHCII), obtained from wild-type (WT) or point-mutated (S123 exchanged to P (S123P) or to G (S123G)) apoproteins. In all the reconstituted proteins, lutein 2 displayed the same distorted conformation observed in purified LHCII trimers. Reconstituted WT and S123G also exhibited the same conformation for bound neoxanthin (Nx) molecules, so the structure of all carotenoid binding sites is identical for these protein complexes. The Chl b interactions for these reconstituted proteins are observed to be slightly different from purified LHCII, indicating that a small number of Chl b binding sites are unable to provide the natural H-bonding partner to their formyl carbonyl. By contrast, the S123P mutation induces dramatic changes in the intermolecular interactions involving bound Chl b molecules, in addition to a change in Nx conformation. Hence this point mutation, located in the linker segment between helices B and C of LHCII, induces a perturbation of the whole interface between these helices, where Nx and most Chls b are located. Such structural reorganization may explain the observed differences in the photochemistry of this complex.

Introduction

Photosynthesis in higher plants begins with the absorption of solar photons by specialized pigments located in the highly complex light harvesting system of these organisms. The excitation energy is efficiently transferred to reaction centers of photosystem (PS) I or II where it is then converted into chemical potential energy. In PSII, light collection is achieved by a highly complex antenna system, involving many partner proteins - the major and minor light-harvesting chlorophyll (Chl) a/b complexes (LHCII and CP24, CP26 & CP29, respectively), and the inner antenna proteins (CP43 and CP47). LHCII is the most important complex involved in the process of light absorption, not only because it binds half of the Chls in the photosynthetic membrane, but also because it is involved in a number of regulatory mechanisms that ensure plant fitness under ever-changing environmental light conditions. It is responsible for the partition of energy transfer between the two PSs according to the light quality through the so-called transition states, and is capable of dissipating the excess excitation energy under high light via the fast phase of non-photochemical quenching (NPQ). This membrane protein is naturally present as a trimer of subunits encoded by a family of highly homologous nuclear genes (lhcb1-3). The LHCII structure has been resolved to better than 3 angstrom resolution. Each LHCII subunit possesses three transmembrane helices, and binds 14 Chls (8 Chls a and 6 Chls b) as well as four carotenoid molecules - two luteins, one neoxanthin (Nx), and a xanthophyll cycle carotenoid. This structure provided an essential framework to model the electronic properties of LHCII, as well as the cascade of events following the photon absorption in
this protein (see e.g. 76). However, it does not allow a fine understanding of the events occurring in LHCII upon state transition or upon qE build-up. The former requires LHCII phosphorylation, and the latter was proposed to be associated with a LHCII conformational change, which brings it from a state where it efficiently harvests solar photons to a state where it efficiently dissipates the excitation energy 71,73,23.

Generally, the engineering of a series of site-selected mutants designed to impair or modify the function of a given protein is of great help in understanding the molecular mechanisms underlying its activity, as well as testing the different models for these mechanisms. In the case of LHCII, this task is particularly difficult, partially because of the degeneracy of the nuclear genes encoding its subunits. An alternative approach, pioneered by the work of Plumley and Schmidt 77 and continued by Paulsen et al 78, involves expressing the LHCII gene(s) in Escherichia coli, and reconstituting the protein by mixing the resulting apoproteins maintained in solution in the presence of detergent with the proper proportion of photosynthetic pigments. This approach is obviously complex, taking into account the large number of cofactors which must find their proper place in the rapidly refolding protein, but it has been quite successful and found a large number of applications. Perhaps the most spectacular purpose is the mechanistic analysis of LHC refolding in vivo and the determination of the Nx binding site in CP29 prior to any information from X-ray analysis of crystal structures 79.

However, most of these studies concerned either monomeric minor LHC antenna, such as CP29, or reconstituted LHCII monomers. In its native form, LHCII is a trimer, and obtaining close-to-native LHCII trimer is a particularly daunting task 80; although the overall shape of the monomer corresponds to that of the native protein, it exhibits spectroscopic properties which slightly but significantly differ from the trimer 81. In particular, the low temperature linear dichroism suggests that these reconstituted trimers seem to bind slightly less Chl b than the native LHCII. Recently, we reported LHCII trimer reconstitution for either WT or apoproteins mutated at the locus S123, a site chosen for its strategic position at the junction between the helix C and lumenal loop of LHCII 82. It was shown in particular that mutations at this locus affect the Nx binding to the trimers. Also, the yield of formation of lutein 1 and lutein 2 triplet states, which result from the quenching of Chl triplets 83, is affected by these mutations 82. This suggests that the cascade of energy transfer after photon absorption is affected in these mutants. In order to interpret the effect of single amino acid exchanges on the LHCII function produced by reconstitution of modified apoproteins, the quality of the reconstituted complexes and the structural impact of the mutation should be checked by the wider range of techniques available. Among these techniques, a method of choice is resonance Raman spectroscopy (RRS), which has already been extensively applied to LHCII studies 61,84. RRS provides selective vibrational information on the different types of pigments bound to this protein (Chl a, b and carotenoid molecules), according to the excitation wavelength used to produce the resonance effect 36. As a vibrational method, it yields precise information on the molecular configuration of the LHCII-bound carotenoid molecules, and provides an exquisitely sensitive description of the interactions between the conjugated carbonyl groups of Chl molecules and the neigh-
boring amino acids. Moreover, by progressively scanning the carotenoid absorption band with the Raman excitation, it was shown that this method may selectively provide structural information on the LHCII-bound Nα and on the red-absorbing lutein in these complexes. This method can thus be used (and was already used in the case of CP29), to evaluate the structure of reconstituted LHCII trimers, whether they are obtained from WT or point mutated apoproteins. We report in the present paper resonance Raman studies of reconstituted WT and the point-mutated S123P and S123G.

![Absorption spectra](image)

*Figure 1: Room temperature absorption spectra of (from bottom to top) purified native LHCII, LHCII reconstituted from wt lhcb1 apoprotein, S123P Lhcb1 apoprotein, and S123G Lhcb1 apoprotein.*

**Experimental procedures**

*Construction of the native or reconstituted LHCII.* Protein expression, site-directed mutagenesis and in vitro protein reconstitution were performed for obtaining the WT and point mutated recombinant LHCII. The methods for site-directed mutagenesis, pea Lhcb1 apoprotein expression and LHCII reconstitution were extensively described in Liu et al., 2008. Briefly, the apoprotein of different Lhcb1 species were overexpressed and isolated with the method described by Paulsen et al. (1990). LHCII was reconstituted according to the
method described in using total pigment thylakoid extract. Absorption spectra of these reconstituted LHCIIIs were similar to that reported in, and are displayed in figure 1. Prior to resonance Raman measurements, reconstituted LHCII were concentrated using 100 kDa centricon devices (Millipore) down to an OD of about 5 at 675 nm. For comparison, the native LHCII were purified from spinach according to.

**Spectroscopy.** Absorption spectra were collected using a Varian Cary E5 Double-beam scanning spectrophotometer. Resonance Raman spectra were recorded with 90° signal collection using a two-stage monochromator (U1000, Jobin Yvon, Longjumeau, France) equipped with a front-illuminated, deep-depleted CCD detector (Jobin Yvon, Longjumeau, France). Excitation wavelengths were provided by a 24 W Sabre laser (Coherent, Palo Alto, California); typically, less than 20 mW reached the sample. Measurements were performed at low temperature (77 K) using a nitrogen-flow cryostat (Air Liquide, Sassenage, France). Sample integrity was verified by following RR spectral evolution during the experiment, as well as measuring the fluorescence properties at the very spot producing the Raman signal, using the Raman spectrometer as a fluorimeter.

**Results**

*Lutein 2 in reconstituted LHCII.* As stated above, resonance Raman provides detailed information on the molecular configuration of carotenoid molecules. Carotenoid resonance Raman spectra display four main groups of bands, denoted ν₁ to ν₄, which can be satisfactorily modelled from DFT calculations. The ν₁ band, of highest frequency, above 1500 cm⁻¹, arises from stretching vibrations of C=C double bonds. Its position depends on the length of the π-electron conjugated chain and on the molecular configuration of the carotenoid. The ν₂ band at 1160 cm⁻¹ contains contributions from stretching vibrations of C-C single bonds coupled with C-H in-plane bending modes, and this region is a fingerprint for the assignment of carotenoid isomerization states. The ν₃ band at 1000 cm⁻¹ arises from in-plane rocking vibrations of the methyl groups attached to the conjugated chain, coupled with in-plane bending modes of the adjacent C-H’s. It was recently reported to be a fingerprint of the conjugated end cycle configuration; this hypothesis was recently confirmed by theoretical modelling. Finally, the ν₄ band around 960 cm⁻¹ arises from C-H out-of-plane wagging motions coupled with C=C torsional modes (out-of-plane twists of the carbon backbone). When the carotenoid conjugated system is planar, these out-of-plane modes will not be coupled with the electronic transition, and these bands will not be resonance-enhanced. However, distortions around C-C single bonds will increase the coupling of these modes with the electronic transition, resulting in an increase in their intensity see e.g.
Figure 2: Resonance Raman spectra (514 nm excitation, leading to lutein 2 contributions) of (from bottom to top) purified LHCII from spinach, LHCII reconstituted from WT Lhcb1 apoprotein from pea, LHCII reconstituted from S123P Lhcb1 apoprotein, and LHCII reconstituted with S123G Lhcb1 apoprotein. Spectral range displayed: 920-1600 cm\(^{-1}\).

In LHCII, trimerization induces a redshift of the absorption position of one of the luteins, namely lutein 2, bringing the lower energy component of the absorption transition of this molecule to 507 nm \(^{86}\). It was shown that the 514.5 nm line excitation of these complexes results in near-to-selective excitation of this molecule \(^{86}\). Figure 2 displays the resonance Raman of native LHCII or LHCII reconstituted with WT and modified Lhcb1 apoprotein. It is clear that these spectra are nearly identical. For LHCII purified from thylakoid membranes, the transition from trimer to monomer induces first a downshift of the \(\nu_1\) band by 4 cm\(^{-1}\) to 1526 cm\(^{-1}\). This was recently interpreted as resulting from steric hindrances induced by the protein binding site in the trimeric form, which push the conjugated end-cycles of the lutein into its conjugated plane \(^{40}\). LHCII monomerization also induces the disappearance of the structure of the \(\nu_4\) band \(^{86}\). In the trimeric form, this band is composed of two clear components at 955 and 965 cm\(^{-1}\), which are actually typical for native LHCII trimers. Such band structure results from slight distortions of the lutein induced by the reorganization of its protein binding site upon trimerization. The fact that these features are observed in the reconstituted LHCII trimers indicates that, in the complexes reconstituted from WT and
mutant Lhcb1 apoprotein, trimerization induces exactly the same constraints on the lutein 2 binding site, while inducing a redshift of its absorption transition.

![Figure 3: Resonance Raman spectra (488.0 nm excitation, leading essentially to neoxanthin contributions) of (from bottom to top) purified LHCII from spinach, LHCII reconstituted from WT Lhcb1 apoprotein from pea, LHCII reconstituted from S123P Lhcb1 apoprotein, and LHCII reconstituted with S123G Lhcb1 apoprotein. Spectral range displayed: 930-1600 cm$^{-1}$.](image)

At 488 nm, it was proposed that the major contribution in the Raman spectra arises from the LHCII-bound 9-cis Nx$^{86}$. Because of its configuration, resonance Raman spectra of this molecule are slightly different from those of all-trans lutein, and these changes are accompanied by a small downshift of the $v_1$ band. This proposal was fully confirmed by the study of LHCII from the npq2 mutant of Arabidopsis thaliana, which is unable to synthesize Nx. In the 488 nm-excited resonance Raman spectra of these LHCII, the disappearance of the bands which were attributed to Nx was observed$^{93}$. In all the spectra displayed in figure 3, the small additional bands indicating the contribution from 9-cis carotenoid (indicated by arrows) are equally present. The $v_1$ contributes at higher frequency in all these spectra (1531 cm$^{-1}$ for the purified LHCII, upshifted by 1 cm$^{-1}$ for the reconstituted LHCII).
Figure 4: $\nu_4$ region of Resonance Raman spectra (488.0 nm excitation) of (from bottom to top) purified LHCII from spinach, LHCII reconstituted from WT Lhcb1 apoprotein from pea, LHCII reconstituted from S123P Lhcb1 apoprotein, and LHCII reconstituted with S123G Lhcb1 apoprotein. Spectral range displayed: 930-990 cm$^{-1}$.

It was shown that the $\nu_4$ band of Nx, as excited at 488 nm, is particularly sensitive to LHCII conformation, and gains intensity upon protein aggregation and/or detergent removal in vitro, and upon build-up of qE in vivo. Figure 4 displays this spectral region for the purified and reconstituted LHCIIIs. While it is clear that the spectral components in the $\nu_4$ are identical for the purified LHCII and the LHCII reconstituted from wt and S123G Lhcb1, the introduction of the point mutation S123P induces a clear change of the spectra. In wild-type LHCII, three components appear at 954, 964 and 974 cm$^{-1}$, with relative intensities of 0.5, 1.0, and 0.3. In this mutant, the component at 954 cm$^{-1}$ is more intense and broader, possibly containing an additional band at lower frequencies, and the highest frequency component is also higher, and is shifted to 971 cm$^{-1}$. As this region corresponds to modes sensitive to Nx distortions, it may be concluded that the introduction of a proline at position S123 has an effect on Nx configuration, and as the latter is induced by the structure of its protein binding site, we must conclude that the introduction of this residue modifies the protein structure around the Nx binding site.

Chlorophyll b molecules. Resonance Raman spectra of Chl molecules contain bands which arise from those vibrational modes coupled with the electronic transition used to produce the resonance (most often the highest energy Soret band to avoid interference with the Chl intrinsic fluorescence$^{94}$). As extensively documented (see e.g.$^{95,96}$) these modes generally arise from vibrations highly delocalized on the Chl macrocycle, some in the low fre-
quency range (around 300 cm\(^{-1}\)) which involve the central Mg atom, some in the high frequency range (above 1630 cm\(^{-1}\)) which arise almost purely from the C=O stretching mode \(^{97}\). The modes involving the Mg atom are sensitive to the coordination state of this atom. A series of modes in the mid-frequency range has also been determined to be sensitive to the Chl macrocycle conformation \(^{98,99}\). However, none of these modes including the low frequency modes, undergoes frequency shifts large enough that they can be used for conclusive analysis of pigment protein complexes containing as many Chls as LHCII, even if Chl \(a\) and Chl \(b\) can be selectively excited because of the different positions of their respective Soret transitions. In the high frequency range the stretching modes of the conjugated C=O groups of Chl contribute, namely formyl and keto for Chl \(b\), and keto for Chl \(a\) \(^{100}\). The keto stretching modes of Chl \(a\) and \(b\) contribute at ca 1700 cm\(^{-1}\) when free-from-interactions, and this frequency may downshift to 1660 cm\(^{-1}\) upon intermolecular interactions. Formyl carbonyl vibrates at 1660 cm\(^{-1}\) when free from interactions, and may down-shift to 1620 cm\(^{-1}\) upon binding with surrounding partners. Because of the presence of 8 Chl \(a\) in LHCII, their keto carbonyl stretching region is very congested and not easy to employ to compare different complexes. On the contrary, the six Chls \(b\), with their formyl and keto carbonyl vibrating in two different frequency ranges, can provide detailed information on the structural changes experienced by LHCII \(^{36,73}\). In order to evaluate the reconstituted WT LHCII and mutated ones, we compared the resonance Raman spectra of these complexes, excited at 441.6 nm, where Chl \(b\) contributions are dominating the spectra, in the carbonyl frequency region (figure 5). Spectra of LHCII reconstituted from the WT apoprotein and the S123G mutant apoproteins are nearly identical, indicating that the interaction states of the Chls \(b\) are similar in these complexes. These spectra differ from those obtained from the purified LHCII. The carbonyl stretching region of purified LHCII displays two main contributions at 1630 and 1640 cm\(^{-1}\), which can be readily attributed to formyl carbonyl in interactions with neighboring partners. A small contribution at 1656 cm\(^{-1}\) could correspond either to an additional free-from-interaction formyl carbonyl or to strongly bound keto ones. At 1675 cm\(^{-1}\) a band arising from interacting C=O carbonyl groups contribute, and, at higher frequencies, between 1686 and 1705 cm\(^{-1}\) free-from-interaction keto carbonyl contribute, in a different electrostatic environment \(^{97}\). In the reconstituted complexes, the intensity of the 1640 cm\(^{-1}\) band is decreased by 20%, while the intensity of the 1656 cm\(^{-1}\) contribution increases. This indicates that a number of Chl \(b\) molecules in the reconstituted complexes are present in sites unable to provide them their natural formyl partner. It must be noted that no decrease of the band at 1630 cm\(^{-1}\) corresponding to the most strongly bound formyl is observed, indicating that such an interaction is likely able to perform optimized recruitment of Chl \(b\) at these sites during the reconstitution procedure. Concerning the keto carbonyl, the contributions corresponding to the free-from-interaction ones are very similar between the purified and reconstituted complexes. A slight profile change is observed in the 1670 cm\(^{-1}\) region, where the main contribution is slightly shifted in the reconstituted complexes, indicating the presence of slightly stronger bound keto carbonyl groups.
Figure 5: Resonance Raman spectra (441.6 nm excitation, leading to Chl b contributions) of (from bottom to top) purified LHCII from spinach, LHCII reconstituted from WT Lhcb1 apoprotein from pea, LHCII reconstituted from S123P Lhcb1 apoprotein, and LHCII reconstituted with S123G Lhcb1 apoprotein. Spectral artefacts (due to high fluorescent background of the sample) in the top spectrum are denoted by arrows. Spectral range displayed: 1500-1730 cm⁻¹.

It is worth noting that this profile is slightly different between the LHCII reconstituted from WT and S123G apoproteins, which probably indicates very small changes in the positioning of a limited number of Chls b in the S123G mutant. The resonance Raman spectra obtained at similar excitation wavelength of LHCII reconstituted from S123P mutated apoproteins exhibit dramatic differences as compared to these complexes. The band at 1660 cm⁻¹ is completely missing, while the 1630 cm⁻¹ band becomes much broader and gains intensity. This suggests that the free-from-interactions formyl carbonyl of Chl b in this reconstituted complex have become all bound. The analysis of the keto carbonyl stretching modes is more difficult, partly because of the low quality of the resonance Raman spectra of this complex. However, we cannot exclude that the 1660 cm⁻¹ band slightly gains in intensity, which could indicate that some of the keto carbonyl groups, strongly bound in purified LHCII, have become less strongly bound or free-from-interactions. In all these cases, the experiments showed that the S123P mutation induced an important reorganization of a number of Chl b sites in the reconstituted complexes.
Discussion

Resonance Raman analysis of the reconstituted LHCII trimers yields a number of conclusions about the carotenoid conformation of these complexes. When the reconstitution is performed using either WT or S123G apoprotein, the conformation of the lutein 2 and Nx molecules is exactly the same as those in purified LHCII, indicating that the structure of the carotenoid binding sites is identical in these three complexes. For Nx, this is quite surprising, as previous studies have convincingly shown that the amount of Nx bound to the S123G mutant is lower than in the WT. This may just indicate that when Nx is bound to the LHCII, it binds to its native binding site in the same way as in the WT. As the binding site of lutein 2 is deeply embedded in the protein, at the level of helices A and B, and runs from one side to the other, while Nx is in close contact with C, this indicates that the folding of the reconstituted LHCII is identical to that of the native one. As resonance Raman is extremely sensitive to slight C=C conjugated chain distortions, this actually indicates that the detailed structure of these reconstituted complexes is very similar, if not identical to that of the purified ones. In the reconstituted WT LHCII and S123G, the Chl b interactions are observed to be slightly different from the purified ones. The main conclusion is that a small number of Chls b are located in sites unable to provide the natural partner of their formyl carbonyl. The exact number of mislocated Chls b is not easy to determine, as many factors may influence the intensity of the carbonyl stretching modes, as their geometry or their binding state. However, considering the overall intensity changes in the carbonyl stretching frequency region, these changes should affect between 10 and 20% of the LHCII-bound Chls b. In CP29, it is well documented that a number of Chl binding sites may accommodate both Chl a and Chl b in reconstituted complexes, while most of the sites have been reported to be selectively occupied by Chl a or Chl b in the crystal structure. As Chl a has no formyl carbonyl, a limited number of Chls b (located in the Chl a sites) where no partner for the formyl carbonyl of Chl b is present, would fully explain our present resonance Raman data. We may further conclude that the exchange of Chl b for Chl a occurs only in these sites where the interactions with the formyl carbonyl results in a 1640 cm$^{-1}$ stretching mode, i.e. those providing the less strong H-bond to the formyl carbonyl. Additionally, the profile of the free-from-interaction keto carbonyl stretching mode is slightly different in the S123G mutant. Although it may represent only very small alterations of the Chl b positioning, this may, together with the decrease of Nx in these complexes, participate in the alteration of the circular dichroism signal observed in this mutant.

The situation is quite different for the S123P. In this mutant, the lutein 2 binding site is untouched by the mutation, again indicating that the fold and structure of LHCII at the level of helix A and B are identical to the native one. However, the conformation of the Nx binding site is perturbed by this mutation. The Nx molecule is in close contact with helix C, and, as noted in the Liu’s seminal paper, five of the six Chls b are located in its immediate neighborhood (Fig 6). Our Raman results show dramatic changes in at least half of the Chl b binding sites. They strongly suggest that, while the overall fold of LHCII is untouched by the mutation at the level of helix A and B, an important reorganization occurs at the
level of helix C. The positioning of the whole helix C itself might actually be affected by the mutation. The 123 locus lies in the loop between helix B and helix C, and the presence of a proline is likely to affect the conformation and flexibility of this loop, probably inducing an overall motion of helix C relative to the two other helices.

![Diagram of helices](image)

**Figure 6: Reconstituted LHCII. Here we see five of the six Chls b are located near the Nx niche, which is effected by mutation of S123 (S123 is indicated by the red arrow).**

The structural results, obtained from resonance Raman characterization of reconstituted LHCII complexes described in this paper yield a quite accurate picture of the effect of the mutation at the 123 locus. Replacement of the native serine by a glycine, has limited structural effects, though inducing both a lower affinity for Nx and slightly disturbing the positioning of the Chl b molecules. Inserting a proline at this site probably disturbs the interactions between helix C and helix A and B, modifying both the Nx conformation and the environment of a large fraction of Chl b molecules. In both cases, the difference in triplet yield observed in the LHCII reconstituted from the mutated apoprotein is likely to be explained by a different equilibration of the excitation energy induced by the perturbation of Chl b molecules. However, it is of note that the large structural perturbation induced by the insertion of a proline at position 123 has no large effect on the electronic properties of the LHCII complexes, although it is quite clear from a number of studies that these complexes may become highly quenched upon changes in their environment. This suggests that the LHCII structure, although able to quickly respond to external conditions, is also built to maintain its function over a large fraction of its accessible conformational space.
References


Acknowledgements
This work was supported by the National Basic Research Program of China (Grant No. 2011CBA00904), the Key Research Program of the Chinese Academy of Sciences Grant (KSZD-EW-Z-018), and by the ERC funding agency (PHOTPROT project).
CONCLUSION

Carotenoids are abundant in nature and play a crucial role in many processes. Their functions are vast; here we have characterized them biophysically in several systems using mainly resonance Raman spectroscopy with the aim to understand them both in the studied system and in general to progress our collective knowledge on these important molecules. These days, a new type of energy production is sought and advancement has been made in both algae farms and in new types of solar cell designs. Carotenoids are exploited for both applications.

Our work is novel because we have characterized carotenoids in complicated systems, namely protein environments, whose complexity make it difficult to interpret RRS spectra. Here, we have succeeded, which is demonstrated by the consistency of DFT calculations with our experimental data. One major accomplishment of our work regarding the DFT calculations (which are present in five of the chapters) was running so many different data sets on these large molecules, and determining which ones were the best for reproducing the experimental vibrational spectra. Around the same time that we were doing this work, many other groups were performing similar calculations and found that the same data sets worked for them. This verification from completely different groups was important, and certainly advances the technique to the next level of development. We aim to make the experiment obsolete; ideally the calculations would suffice to show a given RRS spectrum. As the development improves, we get closer and closer to this goal (see chapter 4).

Our work with peridinin is a benefit not only to those working on the unique light harvesting complex of dinoflagellates, peridinin chlorophyll protein, but also to the research being done on similar molecules. Indeed, when presenting this work at conferences one of the most asked questions was if a Fermi resonance is present
in other butenolides. The answer is yes, a Fermi resonance can be seen in most butenolides and we have presented a thorough literature review in our 2014 article. The work with peridinin is a good starting point to understand this phenomenon for other molecules and gives some insight into how the signals should behave experimentally as far as the mode mixing.

The work that was done on orange carotenoid protein was indeed interesting and will likely be useful in the not-distant future, as this protein is relatively new and is picking up great interest by groups around the world. Understanding the role of the carotenoid that is located spanning the two protein domains is perhaps the most important aspect of this protein, since it is likely the mechanism of energy dissipation for NPQ in certain cyanobacteria. Here we were the first to investigate it so thoroughly with RRS, and deduce the actual twisting and bending of the carotenoid. Our experiments also show that there are two forms of non-active OCP, a fact that may be critical in understanding the working mechanism of OCP in vivo. More work should be done on this interesting and complex protein; here we have advanced the knowledge of the biophysics of the protein.

Regarding the entire thesis, this kind of fundamental work is the base of applied and other fundamental research. Broad work of this sort is at the interface of biology, chemistry and physics; thus there are implications for many types of investigations. The aim is that it will be a benefit to as many areas of research as possible, may they be industry working on new forms of energy production, research being done on photosynthetic organisms, general carotenoid and similar molecule studies, resonance Raman investigation, and to all that can find this kind of work an asset.
Summary

Carotenoids are important molecules in nature. In addition to endowing color to many plant and animal species, they are involved in cellular process such as light harvesting and photoprotection. The work of this thesis was to study the vibrational properties of specific carotenoid molecules using resonance Raman spectroscopy; carotenoids were investigated in solvents and in purified protein complexes to deduce information ranging from their conformational structure to insight into the way the function in vivo. Specifically, we have looked at different carotenoids in light harvesting complexes from dinoflagellate, cyanobacteria, and plants, work that was done in collaboration with groups around Europe and Asia. Chapter one is a general introduction to carotenoids, resonance Raman spectroscopy, photosynthesis, and my projects.

Chapter 2 is work that began with the peridinin–chlorophyll a-protein (PCP). We realized that a preliminary study of peridinin in solvents would be necessary to understand the carotenoid in vivo. Thus, this publication came from this preliminary fundamental work on peridinin in solvents. We discovered a Fermi resonance in the carbonyl region of the RRS spectra. Our experimental results were corroborated with calculated spectra and a model done with our collaborators.

Chapter 3 is an extension of the work done in Chapter 2. This study looks at peridinin photophysics and investigates its vibrational ground state properties. Again, calculations were done to validate the experimental findings. In the step-scan FTIR spectrum of PCP new peaks have been identified belonging to a triplet state induced by light. The nature of this triplet state has been largely debated, mainly on the basis of time-resolved FTIR studies, thus this paper brings us several steps closer to a full understanding of peridinin photophysics.

Chapter 4 investigates the vibrational properties of carotenoid molecules that contain substitutions of chain methyl groups. The stretching mode in the resonance Raman spectrum of a carotenoid arises from C=C stretching modes and is normally found around 1520 cm\(^{-1}\). Density functional theory (DFT) was used to theoretically analyze this peak. From the calculations we have found that while the effective conjugation length of carotenoids increases in linear polyenes upon
s-cis isomerization towards the end of the chain, it lengthens in conformers of carotenoids containing β-rings. It was also found that methyl groups attached to the conjugated chain of carotenoids induce a splitting of the $\pi 1$ band.

Chapter 5 examines the orange carotenoid protein (OCP). This 35 kDa soluble protein has been implied in photoprotection in certain cyanobacteria. It contains an echinenone molecule spanning its N and C terminal domains that gives the protein its distinct orange color. Upon subjugation to high light intensities, the purified protein appears red. Our work was to investigate the vibrational differences of the echinenone molecule in the two OCP forms. We have discovered a type of intermediate form of echinenone in OCP that is relatively red-shifted, and have found that the crystal structure that exists of OCP is probably not the most blue-shifted form of the protein.

Chapter 6 has not been published yet. It is an investigation on the triplet-triplet energy transfer in artificially made carotenoporphyrin dyads. These molecules are used as models for the carotenoid and chlorophyll molecules that exist in light harvesting complexes found in higher plants. We have studied the triplet-triplet energy transfer between these two molecules and found that in the para linked dyad the transfer rate is much slower than in the ortho linked dyad. Using RRS we have investigated the carbon double bond stretching modes of the dyads. The triplet state peak is different in the two dyads; a surprising find since the absorption spectra are almost identical. Calculations on these molecules suggest that there is an effect of the triplet state porphyrin on the ground state of the carotenoid in the ortho dyad.

Chapter 7 is nearly ready for submission. It is a study of wild-type and point-mutated light harvesting complex II (LHCII) proteins. Spectra from RRS showed that the complex was trimerized, made apparent by the lutein 2 peak. The chlorophyll b 605 binding site was also investigated. Notably, it is difficult to obtain LHCII trimer in a reconstitution; often monomers are observed. Further experiments are being completed before submission of this work, such as low temperature absorption and HPLC.
Samenvatting
Biofysische karakterisering van carotenen en caroteen bevattende eiwitten met Resonantie Raman Spectroscopie

Carotenen zijn belangrijke moleculen in de natuur. Naast het kleur geven aan veel planten en diersoorten zijn carotenen betrokken bij cellulaire processen zoals licht oogsten en fotoprotectie. Het werk in dit proefschrift was het bestuderen van de vibrationele eigenschappen van specifieke caroteen moleculen met gebruik van resonantie Raman spectroscopie; carotenen werden bestudeerd in oplosmiddelen en in gezuiverd eiwitcomplexen om informatie te vinden van de conformationele structuur tot en met het functioneren in vivo. We hebben specifiek gekeken naar verschillende carotenen in antennecomplexen uit dinoflagellaten, cyanobacteriën en planten. Het werk is gedaan in samenwerking met groepen in Europa en Azië. Hoofdstuk één is een algemene introductie van carotenen, resonantie Raman spectroscopie, fotosynthese en mijn projecten.

Hoofdstuk 2 is werk dat begon met het peridinine-chlorofyl a-eiwit (PCP). We realiseerden dat een voorbereidend onderzoek naar peridinine in oplosmiddelen nodig was om het caroteen in vivo te begrijpen. De publicatie is voortgekomen uit dit voorbereidende fundamentele werk over peridinine in oplosmiddelen. We ontdekten een Fermi resonantie in de carbonylregio van de RSS spectra. Onze experimentele resultaten werden door collaborateurs ondersteund met berekende spectra en een model.

Hoofdstuk 3 is een vervolg van het werk in hoofdstuk 2. Dit onderzoek kijkt naar de fotofysica van peridinine en de vibrationele eigenschappen in de grondtoestand. Opnieuw zijn er berekeningen gedaan om de experimentele resultaten te valideren. In het step-scan FTIR spectrum van PCP zijn nieuwe pieken geïdentificeerd, deze pieken horen bij een door licht geïnduceerde triplet toestand. De aard van deze triplet toestand is uitgebreid bediscussieerd, vooral op basis van tijdsopgeloste FTIR studies, daarmee brengt het artikel ons stappen dichter bij een volledig begrip van de fotofysica van peridinine.

Hoofdstuk 4 onderzoekt de vibrationele eigenschappen van caroteenmoleculen die substituties van de keten-methylgroepen bevatten. De $\nu_1$ strekmodus in het resonantie Raman spectrum van een caroteen komt voort uit de C=C strekkingen.
en wordt normaliter gevonden rond 1520 cm\(^{-1}\). Dichtheidsfunctionaaltheorie (DFT) werd gebruikt om deze piek theoretisch te analyseren. Uit de berekeningen bleek dat hoewel de effectieve conjugatie lengte van carotenen toeneemt in lineaire polyenen bij s-cis isomerisatie aan het einde van de keten, de lengte afneemt bij conformaties van carotenen die een β-ring bevatten.

Hoofdstuk 5 onderzoekt het orange carotenoid protein (OCP). Dit 35 kDa oplosbare eiwit wordt in verband gebracht met fotoprotectie in bepaalde cyanobacteriën. Het bevat een echinenone molecule dat zich uitstrekt langs de N en C terminus en het eiwit de karakteristieke oranje kleur geeft. Bij blootstelling aan hoge lichtintensiteit kleurt het eiwit rood. Ons werk was het onderzoeken van de vibrationele verschillen van het echinenone molecule in de twee OCP vormen. We hebben een nieuwe tussenvorm ontdekt van echinenone in OCP die relatief rood-verschoven is. Ook vonden we dat de bestaande kristalstructuur van OCP waarschijnlijk niet de meest blauw-verschoven vorm van het eiwit is.

Hoofdstuk 6 is nog niet gepubliceerd. Het is een onderzoek naar de triplet-triplet energieoverdracht in een kunstmatige caroteenporfyrine moleculen. Deze moleculen worden gebruikt als model voor caroteen en chlorofyl in antennecomplexen van hogere planten. We hebben triplet-triplet energie overdracht bestudeerd tussen de twee gelinkte moleculen en vonden dat in de overdrachtssnelheid in de para gelinkte variant veel langzamer is dan in de ortho gelinkte variant. We hebben de strekmodus van de carbon dubbele bindingen in de moleculen bestudeerd. De piek van de triplet toestand verschilt in de twee varianten; een verrassing want de absorptie spectra zijn bijna identiek. Berekeningen aan deze moleculen suggereren dat de triplet toestand van porfyrine effect heeft op de grondtoestand van caroteen in de ortho variant.

Hoofdstuk 7 is bijna klaar om ingediend te worden voor publicatie. Het is een studie van wild-type en punt-gemuteerd light harvesting complex II (LHCI\(_II\) eiwitten. RRS spectra laten zien dat het complex getrimeriseerd is, dit is herkenbaar aan de luteïne 2 piek. De chlorofyl b 605 bindingslocatie is ook bestudeerd. Het is moeilijk om LHCI\(_II\) trigemer in een reconstitutie te verkrijgen; vaak worden monomer gevonden. Verdere experimenten worden afgereond voordat dit werk wordt ingediend, bijvoorbeeld lage temperatuur absorptie en HPLC.