Studies on the structure and function of the bacteriochlorophyll C antenna complex of the chlorosomes from the phototrophic bacterium Chloroflexus aurantiacus
the pigment-binding function of the 5.7 kDa polypeptide

Author(s):
Lehmann, Rainer Patrick

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STUDIES ON THE STRUCTURE AND FUNCTION OF THE BACTERIOCHLOROPHYLL C ANTENNA COMPLEX OF THE CHLOROSOMES FROM THE PHOTOTROPHIC BACTERIUM CHLOROFLEXUS AURANTIACUS:

The pigment-binding function of the 5.7 kDa polypeptide

A dissertation submitted to the
SWISS FEDERAL INSTITUTE OF TECHNOLOGY
for the degree of
DOCTOR OF NATURAL SCIENCES
presented by
Rainer Patrick Lehmann
Dipl. Biol.
born 25th May 1965
citizen of Germany
accepted on the recommendation of
Prof. Dr. H. Zuber, examiner
Prof. Dr. J. Oelze, co-examiner
1994

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>absorbance</td>
</tr>
<tr>
<td>ALA</td>
<td>5-aminolevulinate</td>
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<tr>
<td>APS</td>
<td>ammonium persulfate</td>
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<tr>
<td>B740</td>
<td>BChl c-antenna complex of the chlorosome core</td>
</tr>
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<td>B796</td>
<td>BChl a-antenna complex of the chlorosome base-plate</td>
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<td>B806-866</td>
<td>intramembrane core antenna complex</td>
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<td>BChl</td>
<td>bacteriochlorophyll</td>
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<td>bacteriopheophytin</td>
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<td>C-terminus</td>
<td>carboxy terminus</td>
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<td>C. aurantiacus</td>
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<tr>
<td>Chl</td>
<td>chlorophyll</td>
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<td>CM</td>
<td>cytoplasmic membrane</td>
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<td>Da</td>
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<tr>
<td>e</td>
<td>extinction coefficient</td>
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<td>g</td>
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<td>high performance liquid chromatography</td>
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**Symbols for amino acids**

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Summary

Green photosynthetic bacteria are characterized by the presence of extramembrane light-harvesting systems, the chlorosomes. The chlorosomes are designed to absorb light in the near infra-red region and to transfer the absorbed energy to other components of the photosynthetic apparatus and ultimately to the photochemical reaction centre. The chlorosomes of the thermophilic gliding bacterium *Chloroflexus aurantiacus* are oblong vesicles of 110 x 30 x 12 nm³ that are attached to the inner side of the cytoplasmic membrane. They comprise the antenna pigment bacteriochlorophyll c (in the range of 10000 BChl c molecules per chlorosome), small amounts of bacteriochlorophyll a (BChl a), three to four major polypeptides, lipids and carotenoids. The polypeptides have apparent molecular weights of 5700, 5800, 11000 and 18000 according to sodium dodecylsulfate polyacrylamide gelelectrophoresis (SDS-PAGE). The 5.7 kDa polypeptide is the predominant protein component, which is thought to be associated with the BChl c in the chlorosomes. It may form a pigment-protein complex with BChl c and thus influence the spectral properties of the pigment, i.e. cause the red shift and the hyperchromicity.

Due to their chemical structure BChl c molecules interact in nonpolar organic solvents, showing strikingly similar spectral properties as BChl c in the hydrophobic environment of the chlorosome interior. Hence, the question arises, whether the characteristic absorption, fluorescence and circular dichroism spectra of the chlorosomes result from chromophore-protein or from chromophore-chromophore interactions.

The present investigation was based on the working hypothesis that BChl c in the chlorosomes of *Chloroflexus aurantiacus* is organized in pigment-protein complexes involving the 5.7 kDa polypeptide. The study aimed at providing experimental evidence for this hypothesis. Above that, it attempted to elucidate the role of the other polypeptides in the chlorosome. To this end, a thorough polypeptide analysis was carried out. The 5.7 kDa polypeptide, whose amino acid sequence was known, and the 11 and 18 kDa polypeptide were isolated in a one-step isolation technique using reverse phase high performance liquid chromatography. The exact molecular masses of the polypeptides were determined by mass spectrometry. For the 5.7 kDa polypeptide the molecular weight deduced from the amino acid sequence was confirmed. Furthermore, several proteins of low abundance were partly sequenced by separation on SDS-PAGE, subsequent electro-blotting onto PVDF membrane and automated Edman degradation.

Structure and function of the protein components were correlated by means of limited proteolysis of chlorosome suspensions and analysis of spectral changes exerted by the enzymatic digestion. It was shown that the proteolytic treatment had only little effect on the absorption spectrum of the chlorosomes, whereas the changes in rotational strength
(CD spectra) were considerable. The susceptibility of the chlorosomes to proteolytic treatment was enhanced by partly dissociating the BChl c antenna with the long-chain alcohol 1-hexanol. Enzymatic digestion under these conditions resulted in a drastic increase in rotational strength indicating strong intermolecular coupling of BChl c molecules. By comparison of the effects of different proteases, namely trypsin and proteinase K, it was shown that the 5.7 kDa polypeptide played a major role in the organization of the pigments. The other proteins appeared not to be associated with BChl c.

In addition, different growth conditions for the culture of *Chloroflexus aurantiacus* were employed. Cells cultivated under low light conditions on complex growth medium possessed chlorosomes with a higher BChl c to protein ratio than cells grown under high light intensity on nutrient-limited medium. Limited proteolysis and spectral analysis were carried out with low light and high light chlorosomes and both were compared. The difference in the intensity of the CD signals and the differing response to proteolytic treatment led to the conclusion that in both types of chlorosomes BChl c aggregates were associated with the 5.7 kDa polypeptide and that the size of the BChl c aggregates was variable.

The data obtained in the course of the present study indicated that the BChl c antenna in the chlorosomes of *Chloroflexus aurantiacus* was constituted of pigment-protein complexes involving the 5.7 kDa polypeptide and a variable amount of BChl c molecules, probably in an oligomeric arrangement. As a consequence the spectral properties of the BChl c antenna pigments (red shift and hyperchromicity) may be due to protein-chromophore as well as chromophore-chromophore interactions.
Zusammenfassung

Die grünen photosynthetischen Bakterien besitzen als gemeinsames Merkmal außerhalb der cytoplasmatischen Membran gelegene Lichtsammelsysteme, sog. Chlorosomen. Die Funktion der Chlorosomen ist die Absorption von Licht im nahen Infrarotbereich, sowie die gerichtete Weiterleitung der Energie auf andere Komponenten des Photosyntheseapparates. Die Energie gelangt letztlich zum photosynthetischen Reaktionszentrum. Die Chlorosomen des thermophilen, gleitenden Bakteriums *Chloroflexus aurantiacus* sind langgestreckte Vesikel mit den Dimensionen 110 x 30 x 12 nm³, die auf der Innenseite der cytoplasmatischen Membran angelagert sind. Sie beinhalten das Antennenpigment Bakteriochlorophyll c (ca. 10000 BChl c Moleküle pro Chlorosom), kleine Mengen von Bakteriochlorophyll a (BChl a), drei bis vier vorherrschende Proteine, Lipide und Karotinoide. Die Polypeptide haben apparente Molekulargewichte von 5700, 5800, 11000 und 18000, bestimmt mittels Natrium Dodecylsulfat Polyacrylamid Gelelektrophorese (SDS-PAGE). Das 5,7 kDa Polypeptid ist das am häufigsten auftretende Protein, von dem angenommen wird, daß es mit BChl c in den Chlorosomen assoziiert ist. Es könnte einen Pigment-Protein Komplex mit BChl c bilden und auf diese Weise die spektralen Eigenschaften des Pigments beeinflussen, d.h. die Rotverschiebung sowie die Hyperchromizität.

Aufgrund ihrer chemischen Struktur sind BChl c Moleküle in der Lage in unpolaren organischen Lösungsmitteln miteinander zu interagieren. Sie zeigen dort erstaunlich ähnliche spektrale Eigenschaften wie BChl c in den Chlorosomen. Daher stellt sich die Frage, ob die charakteristischen Absorptions-, Fluoreszenz- und Zirkular Dichroismus Spektren durch Chromophor-Protein oder Chromophor-Chromophor Wechselwirkungen hervorgerufen werden.

über SDS-PAGE, nachfolgendes Elektro-Blotting auf PVDF-Membran und automatisierten Edman-Abbau.


I. Introduction

I.1. Photosynthesis

1.1. Sunlight

All forms of energy in living organisms and organic matter originate from the electromagnetic energy of the sunlight. In the process of photosynthesis electromagnetic energy is converted into chemical free energy, which can be utilized by all organisms. Electromagnetic energy is determined by the quantum, which is dependent on the wavelength $\lambda$ [nm] and the frequency $\nu$ [s$^{-1}$] of the electromagnetic radiation (Fig. 1). A substance which absorbs a quantum at a certain wavelength (photon) receives the entire energy of the respective quantum. As a consequence, the absorbing substance will either undergo a change in thermal energy or a redistribution of electrons. At very high energy the absorbing molecule or atom will be destroyed.

![Electromagnetic spectrum](image)

Fig. 1. Electromagnetic spectrum. The visible range of electromagnetic radiation comprises wavelengths from 400 nm to about 700 nm. Photosynthetically active radiation extends over a range of 400 nm to more than 1000 nm.
1.2. Definition of photosynthesis

Photosynthesis is the utilization of electromagnetic energy by phototrophic organisms for the synthesis of complex organic molecules (Blankenship, 1992). The ultimate goal of photosynthesis are chemical rearrangements following the general equation

\[
\text{CO}_2 + 2\text{H}_2\text{A} + \text{hv} \rightarrow [\text{CH}_2\text{O}] + \text{H}_2\text{O} + 2\text{A}
\]

where \( \text{H}_2\text{A} \) is \( \text{H}_2\text{O} \) in the case of oxygenic photosynthesis, or reduced sulphur compounds (\( \text{H}_2\text{S}, \text{S}_2\text{O}_3^{2-} \)) and organic substrates for the anoxygenic photosynthesis.

1.2. Primary processes of photosynthesis

The primary processes of photosynthesis can be divided in two phases (Amesz, 1991). Phase one comprises the absorption of light energy and the transfer of excitation energy to the reaction centre (RC). The second phase consists of the charge separation and subsequent electron transport in the RC. Phase one takes place in the photosynthetic antenna systems or light-harvesting systems, which consist of pigment-protein complexes. The evolution of phototrophic life has brought about a variety of different photosynthetic antennas. Some are integrated into the photosynthetic membrane as in higher plants, green algae, purple bacteria and heliobacteria. Others are located on the outside of the membrane as in the case of the phycobilisomes of cyanobacteria and red algae or the chlorosomes in green photosynthetic bacteria (Fig. 2).

The second phase of the primary processes takes place in the RC, which is an integral part of the photosynthetic membrane in all phototrophic organisms.

As a general principle of primary photochemical reactions, photosynthetic pigments (chlorophylls and bacteriochlorophylls) in their protein environment serve the function of primary light reception. Above that, they act cooperatively to transfer energy to the RC. Thirdly, the photosynthetic pigments function as donor and acceotor for the charge separation in the RC: A (bacterio-) chlorophyll is promoted to an excited singlet state, thereby becoming a very strong reductant. The excited singlet state transfers its energy to a nearby acceptor- (bacterio-) chlorophyll or pheophytin leading to a series of ultrafast electron transfer reactions in the RC (Blankenship, 1992).
Fig. 2. Spectral range of antenna systems of oxygenic (plants, algae, cyanobacteria) and anoxygenic (green and purple bacteria) photosynthetic organisms (reproduced from Zuber et al., 1987). Light often represents the growth limiting factor in a certain habitat. As a result, autotrophic organisms have developed a variety of highly efficient antenna systems based on different photosynthetic pigments.
2.1. Photosynthetic reaction centres

The decisive step in the conversion of light into chemical free energy consists in the separation of an electron from a donor molecule and transfer of the electron to an acceptor (charge separation). This occurs within a pigment-protein complex, the reaction centre (RC). Two prototypes of photosynthetic RCs are known. One type is found in the photosystems I of higher plants, of algae and of cyanobacteria, in the green sulphur bacteria (Chlorobiaceae) and in Heliobacteriaceae. It is considered an 'iron-sulphur-type' RC, in which the separated electron is donated to an [Fe-S] cluster. The second type, found in the photosystems II of plants, algae and cyanobacteria, as well as in the purple photosynthetic bacteria, is denoted 'quinone-type', because the separated electron is transferred to a mobile quinone acceptor. It appears that a basic motif for photochemical charge separation is preserved in both types of RCs (Golbeck, 1993). Accordingly, a photochemical RC consists of a dimeric protein core that functions as a scaffold for antenna chlorophylls and a series of bound electron donors and acceptors that serve to stabilize the initial charge separation between primary electron donor and acceptor. The RC can be depicted with the following notation:

\[ \text{PIQ} \; \text{hv} \rightarrow \text{P*IQ} \rightarrow \text{P+I-Q} \rightarrow \text{P+IQ}^- \]

where P is a (bacterio-) chlorophyll primary electron donor, I is a (bacterio-) chlorophyll primary electron acceptor, and Q is a quinone secondary electron acceptor. In this generalized RC, the absorption of a photon results in charge separation between the chlorophyll donor and acceptor molecules. The electron is rapidly transferred to the quinone, which acts to stabilize against the rapid charge recombination between the primary reactants. In the sequence of electron transfer steps the electron is then donated to the mobile quinone in the 'quinone-type' RC and to the \( \text{FA/FB} \; [\text{Fe-S}] \) clusters in the 'iron-sulphur' type RC.

2.2. The excitation of pigment molecules

The initial event of photosynthesis is the absorption of a photon of visible light by a photosynthetic pigment molecule. Upon absorption of a photon, one electron of the pigment molecule is transferred from the ground state to a higher energy level. If the transfer occurs without changing the spin quantum number of the electron, the electron
reaches an excited singlet state. The energy of a photon is thus converted into the increase in orbital electronic energy of the excited state of the molecule (Sauer, 1986). It is a property of photosynthetic pigment molecules to have electronic energy levels closely spaced, so that photons of low energy, i.e. in the visible range, are sufficient to produce excited singlet states. The absorption cross-section of a single pigment molecule being small, it is of advantage for phototrophic organisms to increase the photon capture cross-section. This can be achieved in two principal ways. Firstly, the cross-section for absorption is roughly additive, so there is an increase in proportion to the number of pigment molecules. Secondly, broadening the absorption bands increases the probability of capturing photons of energies (wavelengths) that are not absorbed efficiently by the isolated pigment molecule (Sauer, 1986). As a consequence, phototrophic organisms have evolved large arrays of interacting pigment-protein complexes, which can be regarded as biological antennas for capturing photons and transferring excitation energy to the photosynthetic RC.

2.3. Transfer of excitation energy

For a photosynthetic antenna, which is meant to increase the absorption cross-section associated with each RC, efficient energy transfer is a prerequisite. There are several mechanisms by which this can happen, including the occurrence of delocalized excitons (Pearlstein, 1982), inductive resonance transfer ( Förster, 1948) and perhaps others. A delocalized exciton occurs when a photon is absorbed by a collection of interacting chromophores. The molecular orbitals are distributed over the set of interacting chromophores, rather than being localized on one molecule at a time. Excitation then migrates within the antenna by an inductive resonance ( Förster transfer) mechanism. The occurrence of delocalized excitons broadens the absorption band and in some cases causes it to split into several resolved components as in the example of the BChl α-protein from Prosthecochloris aestuarii (Philipson and Sauer, 1972), see below.
I.3. Structural requirements of the primary processes of photosynthesis

3.1. Photosynthetic pigments

All phototrophic organisms are characterized by the presence of photosynthetic pigments in the form of tetrapyrrol derivatives. The tetrapyrrol molecule can form a cyclic structure, where the four nitrogen atoms of the pyrroli are coordinated to a magnesium atom. This macrocycle is a magnesium porphyrine and forms the basic structure of the chlorophylls and bacteriochlorophylls. Alternatively, the tetrapyrrol can have a linear structure as for the bilins found in the phycobiliproteins. Chlorophylls and bilins are very effective photoreceptors because they contain networks of conjugated double bonds.

Chl a is the principal photosynthetic pigment in the chloroplasts of higher plants. It is a dihydroporphyrine with a methyl group on ring II (Fig. 3). Chl a exhibits absorption maxima at about 420 nm and 660 nm in organic solvents. Chl b in the chloroplasts differs from Chl a in having a formyl group instead of the methyl group on ring II. The absorption maxima of Chl b are at 450 nm and 640 nm in organic solvents. The variation of substituents on the porphyrine system constitutes one possibility to modify the absorption properties of the porphyrine pigment.

BChl a is a tetrahydroporphyrine, derived from dihydroporphyrine by the reduction of one double bond (Fig. 3). The absorption maxima of BChl a are at 375 nm and 770 nm. Reduction of the porphyrine system represents a second possibility of modification for the absorption properties of the pigments.

BChl c is a dihydroporphyrine like Chl a and Chl b but with a 2-(1-hydroxyethyl) group on ring I instead of the vinyl group present in the Chls a and b (Fig. 3). The absorption maxima are at 435 nm and 670 nm. It was shown by Smith et al. (1983) that the hydroxyethyl group of ring I in BChl c can be involved in aggregate formation between BChl c molecules. The aggregate formed upon dilution of BChl c in hexane-methylene chloride (200:1) and exhibited new absorption maxima at 748 nm and 452 nm. Smith et al. (1983) suggested that the BChl c molecules formed dimers or larger aggregates by interaction of the nucleophilic hydroxyl-oxygen of one molecule with the electrophilic magnesium atom of a second molecule as shown in Fig. 4. A
different model involving the C-9 keto group and the Mg in a six-coordinate state was proposed by Bystrova et al. (1979). Aggregate formation (dimers or oligomers) constitutes a third possibility for the modulation of the light-absorbing properties of antenna pigments.

\[
\text{chlorophyll } a \\
\text{R= phytlyl}
\]

\[
\text{bacteriochlorophyll } a \\
\text{R=phytyl or geranylgeranyl}
\]

\[
\text{bacteriochlorophyll } c. \\
\text{R=farnesyl in Chlorobiaceae, stearyl, geranylgeranyl, cetyl, oleyl, phytlyl in } C. \text{ aurantiacus (Fages et al.,1990)}
\]

Fig. 3. legend, see next page.
Fig. 3. Molecular structures of chlorophyll a (Chl a) and the bacteriochlorophylls a and c (BChl a, c). The photosynthetic pigments are large conjugated macrocycles with a variety of side chains attached to the periphery. The constituent rings of the magnesium porphyrine are numbered I-V, as shown for Chl a. The carbon atoms of the macrocycle are numbered according to Fisher, as partly shown for BChl c. BChl c represents the major light-harvesting pigment in the green photosynthetic bacteria. In C. aurantiacus the substituents at positions 4 and 5 are always ethyl and methyl, respectively. BChl c from the Chlorobiaceae can possess a variety of substituents at positions 4 and 5 (ethyl, methyl, n-propyl, isobutyl), which modulate spectral properties of the pigment (Bobe et al., 1990).

Fig. 4. Models for the aggregation of BChl c in hexane-methylene chloride (200:1) proposed by Smith et al. (1983).

3.2. Light-harvesting complexes

The early step in the conversion of electromagnetic energy into chemical free energy involves the absorption of a photon by the photosynthetic pigments and the directed migration of energy among the pigments until it is trapped by the RC. This process requires specialized structures, called light-harvesting complexes or antenna complexes. Light-harvesting complexes are specialized in photon absorption and in energy transfer by mobile excitons (Zuber, 1985). Zuber (1985) has compiled general features that occur in the light-harvesting complexes. Several statements, which are directly related to the present studies on the light-harvesting complex of the chlorosome are reported here.

(1) Pigment molecules are specifically bound to proteins and form functionally and spectrally defined pigment-protein complexes.
(2) The polypeptides determine the positions, environments, orientations and spacings of the pigment molecules, i.e. the three-dimensional organization of the pigments for energy transfer.

(3) The specific arrangement of a large number of relatively small polypeptides with pigment molecules is the basis for the three-dimensional structure of the antenna complexes (energy transfer inside the complex).

(4) The structural and functional interaction of several antenna complexes is also based on polypeptide interaction (energy transfer between the complexes).

(5) The combination of various types of pigments with different absorption maxima in the complexes determines the direction of energy transfer (heterogeneous energy transfer).

In summary, it can be postulated, that the light-harvesting pigments have to be associated with proteins in pigment-protein complexes for functional reasons.

3.3. Pigment-protein complexes

The absorption spectrum of any array of chromophores is determined not only by the sum of the characteristic spectra of the isolated chromophores, but by the state of aggregation and by the molecular environment of the members of the array (Olson, 1980). In general, one can observe that complex absorption spectra of photosynthetic membranes revert to the simplified absorption spectra of the chlorophylls or bacteriochlorophylls when the pigments are extracted with organic solvents. In many instances the absorption maxima of the pigment molecules in pigment-protein complexes, found in the various light-harvesting systems, are appreciably red shifted (bathochromic shift) from those in solution. Most noticeable is the in vivo bathochromic shift of the lowest energy (Qy) transition (Table 1). The origin of the bathochromic shift of the Qy transition is still ambiguous (Fisher et al., 1990).
Table 1. Comparison of the absorption maxima of the $Q_y$-transitions of BChls in solution and bound in the pigment-protein complexes.

<table>
<thead>
<tr>
<th>pigment</th>
<th>absorption maximum of $Q_y$-transition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>in vitro</td>
</tr>
<tr>
<td></td>
<td>(acetone/methanol 7:2)</td>
</tr>
<tr>
<td>BChl a</td>
<td>770</td>
</tr>
<tr>
<td>BChl b</td>
<td>790</td>
</tr>
<tr>
<td>BChl c</td>
<td>670</td>
</tr>
<tr>
<td>BChl d</td>
<td>660</td>
</tr>
<tr>
<td>BChl e</td>
<td>648</td>
</tr>
</tbody>
</table>

A large number of pigment-protein complexes have been found in bacteria and plants, which support the idea that the bathochromic shift of the chromophores is partly induced by chromophore-protein interactions. In the following two examples of extensively studied pigment-protein complexes will be presented in order to illustrate the functional role of proteins in light-harvesting antennas.

3.4. Antenna systems of purple bacteria

The antenna complexes of purple photosynthetic bacteria contain different spectral forms of BChl $a$, BChl $b$ and carotenoids. These antenna pigments are non-covalently bound to specific low molecular weight polypeptides, designated $\alpha$ and $\beta$ polypeptides, of 50-70 amino acid residues (Zuber, 1985). The antenna complexes are located in the CM and in invaginations of the latter, called intracytoplasmatic membrane systems. The photosynthetic apparatus of the purple bacteria is composed of the RC surrounded by a core antenna complex and depending on the species and the growth conditions also by peripheral antenna complexes. The core antenna and the peripheral antenna differ in their spectral properties in order to allow a heterogeneous (downhill) energy transfer from the periphery to the core and finally to the RC (Fig. 5).
The peripheral complexes have absorption maxima at around 800 nm and 850 nm (B800-850) or 800 nm and 820 nm (B800-820), whereas the core absorbs between 870 nm and 890 nm (B880). This is achieved by the presence of two main populations of BCHls: one group with absorption bands between 820 nm and 860 nm (pigments of peripheral antenna complexes) or between 870 and 890 nm (pigments of the core antenna complexes) and a second group which absorbs at around 800 nm (Brunisholz and Zuber, 1992). The pigments of the first group are located on the periplasmatic side of the membrane and may possibly form exciton coupled dimers like the special pair pigments in the RC. The second group of pigments, absorbing around 800 nm, is apparently located on the cytoplasmic side. It is, in most cases, a single BCHl molecule, which interacts only weakly with the neighbouring pair of pigments (Brunisholz and Zuber, 1992). The specific antenna polypeptides (α and β) determine the positions, orientations, separations and environments of the pigment molecules. By specific aggregation in a lipid
environment, they combine to produce larger polypeptide formations within the antenna complexes and thus also larger associations of pigment molecules with a high degree of order. This is the structural basis for excitation interactions and excited state energy transfer within the complexes (Zuber, 1985). The amino acid sequences of the polypeptides comprise a hydrophobic stretch, which spans the membrane (about 22 amino acids, equal to about six turns of a putative α-helix). The C- and N-terminal domains contain polar or charged residues and are located on the periplasmic and cytoplasmic sides respectively. Within the transmembrane segment both antenna polypeptides have a conserved histidine residue most probably serving as the fifth ligand to the strongly coupled pair of BCHl molecules (B880, B850). A conserved alanine (small amino acid) is postulated to be a significant structural requirement (Ala-X-X-His) for the B880 and B850 pigments. Further possible interaction sites are aromatic amino acid clusters in the C-terminal part of the transmembrane segment of the antenna polypeptides. Brunisholz et al. (1984) proposed that interaction of BCHl with aromatic amino acid side chains may account for the observed red-shift as well as for the hyperchromic shift of the absorption of the pigments in the antenna complex. In addition, an interesting correlation between the protein environment and the circular dichroistic properties of pigments in the light-harvesting complexes of Rhodospirillaceae was observed (Brunisholz and Zuber, 1988). Core antenna complexes with a strong near infra-red CD signals (Rhodospirillum rubrum, Rhodopseudomonas viridis, Rhodopseudomonas acidophila) are composed of α polypeptides, which contain a phenylalanine adjacent to the conserved histidine, and β polypeptides containing a tryptophan/tyrosine adjacent to a turn in the C-terminal domain. As these structural features are not present in the α and β polypeptides of core complexes with weak near infra-red CD signals, they might have an influence on the circular dichroistic properties of the pigment pair.
3.5. The water-soluble BChl a-protein from *Prosthecochloris aestuarii*

The three-dimensional structure of a water-soluble BChl a-protein from the green phototrophic bacterium Prosthecochloris aestuarii was determined by X-ray crystallography at 2.8 Å resolution by Mathews et al. (1979). The BChl a-protein is a trimer consisting of three identical subunits. Each subunit consists of 7 BChl a molecules, non-covalently bound to a protein (Fig. 6). In five cases the magnesiums of BChl a are liganded to a histidine side-chain, in one case to a polypeptide carbonyl oxygen, and in the other case to a water molecule. The absorption maximum of the water-soluble BChl a-protein is at 809 nm. Hence, the absorption maximum of the BChl a Qy-transition is about 40 nm red shifted in the protein. Optical studies of the BChl a-protein by Philipson and Sauer (1972) revealed that at 77°C the Qy-absorption band showed four resolved components at 789 nm, 804.5 nm, 813.5 nm and 824.5 nm (Fig. 8). The CD band of the BChl a Qy-transition in ether at room temperature was weak and positive (Fig. 7). In the protein the CD band was greater by more than an order of magnitude and consisted of the components 778 nm, 808 nm and 822 nm. At 77°C the CD spectrum exhibited five components at 786 nm, 799.5 nm, 811.5 nm, 817 nm and 823 nm (Fig. 8). Philipson and Sauer (1972) interpreted the multiple components in the absorption (four components) and CD spectra (five components) in terms of an excitation model, i.e. the collective excitation of an assembly of molecules. Several BChl a molecules are resonantly coupled to one another so strongly that it is not possible to excite one molecule individually. Recent spectral simulations of the experimental low-temperature absorption and CD spectra of the water-soluble BChl a-protein by Lu and Pearlstein (1993) showed that BChl 7 served as an excitation energy trap within the protein. Reasonable spectral simulations were only obtained, when the interaction of each of the inequivalent BChl a molecules with the protein environment was taken into account.
Fig. 6. View of the BChl \( \alpha \)-protein subunit from the 3-fold axis, which is horizontal, towards the exterior of the protein. The phytol chains and other bacteriochlorophyll ring substituents have been omitted (Reproduced from Matthews et al., 1979).
Fig. 7. Absorption and CD spectra of BChl a in ether at room temperature, reproduced from Philipson and Sauer (1972).

Fig. 8. Absorption and CD spectra of the Qy-band of the water-soluble BChl a protein at 77°K, reproduced from Philipson and Sauer (1972).
I.4. Green photosynthetic bacteria

The present investigation reports new findings on the main light-harvesting system, the chlorosome, from the green photosynthetic bacterium *Chloroflexus aurantiacus*. Accordingly, this introductory section provides selected information about green bacteria and their light-harvesting systems.

4.1. The phylogeny of the green photosynthetic bacteria

The phylogeny of the eubacteria based on oligonucleotide catalogues of 16S ribosomal RNA digests (Fox et al. 1980) has shown that the photosynthetic bacteria do not form a coherent group. There is wide divergence not only between the green and the purple photosynthetic bacteria, but also the green and purple bacteria themselves are deeply divided. They tend to show closer relatedness to a number of non-photosynthetic eubacteria than to each other, suggesting that photosynthetic energy conversion arose early in evolution and may have been lost by the present aerobic heterotrophs (Woese, 1987). The green photosynthetic bacteria comprise the Chloroflexaceae and the Chlorobiaceae. The Chlorobiaceae (genera: *Chlorobium*, *Chloroherpeton*) form a tight group of only obligate photolithotrophs and their phenotype is generally considered a very primitive one (Gibson et al., 1985). The heterogeneous group of the Chloroflexaceae represents a deep branching in the eubacterial line of descent (Oyaizu et al., 1987). The Chloroflexaceae contain four known members, the thermophilic phototroph *Chloroflexus aurantiacus* (*C. aurantiacus*), two mesophilic gliding species from the genus *Herpetosiphon* (aerobic heterotroph) and the thermophile *Thermomicrobium roseum*. *C. aurantiacus* and the Chlorobiaceae are only distantly related on phylogenetic grounds, because their rRNAs are unrelated (Gibson et al., 1985). Their reaction centres differ greatly (quinone-type in *C. aurantiacus* and [Fe-S]-type in the Chlorobiaceae) and the water-soluble BChl *a*-protein found in *Chlorobium* species was not found in *C. aurantiacus*. Yet, they resemble one another in chlorosome structure and light-harvesting pigment type. On the other hand, the membrane-integrated compounds of the photosynthetic apparatus in *C. aurantiacus* (quinone-type reaction centre, B806-866 complex) show considerable similarity to purple bacteria. Gibson et al. (1985) put forward the possibility that information for chlorosome biosynthesis might have been transferred laterally.
4.2. The photosynthetic apparatus of *Chloroflexus aurantiacus*

BChl c, the major antenna pigment in *C. aurantiacus*, is entirely contained in the chlorosome, an extramembrane antenna complex. BChl c, which absorbs at about 670 nm in solution, is red-shifted to 740 nm in the chlorosome of *C. aurantiacus*. The BChl c antenna of the chlorosome is therefore designated B740. The chlorosome also comprises a minor portion of BChl a. In chlorosomes of *C. aurantiacus* BChl a absorbs at 796 nm and is supposed to be organized in the pigment-protein complex B796. Energy that is captured by the B740 antenna is presumably transferred via the B796 complex downhill to the B806-866 antenna, which is integrated in the CM surrounding the RC. The B806-866 antenna is structurally and functionally homologous to the core antenna complex from Rhodospirillaceae (Wechsler et al., 1991). Excitation energy from the B806-866 complex is finally trapped in the RC bacteriochlorophyll (P870) at 870 nm. The RC is of the 'pheophytin)-quinone type', it contains bacteriopheophytin as an intermediate electron acceptor and two quinones in association with a metal ion (Blankenship, 1992).

4.2.1. Energy transfer in the photosynthetic apparatus of *Chloroflexus aurantiacus*

In the chlorosomes of both *C. aurantiacus* and the *Chlorobiaceae* the organization of BChl c seems to be similar. The Qy-transition moments of BChl c show preferential orientation along the long axis of the chlorosome. In the chlorosomes of *C. aurantiacus* the Qy-transition moments of BChl c were reported to form an angle of about 40° with the long chlorosome axis (Betti et al., 1982) or may be even less than 20° (Van Amerongen et al., 1988). Results obtained by measurements of picosecond polarized fluorescence kinetics in living cells of *C. aurantiacus* (Fetisova et al., 1988) indicated high orientational ordering of the BChl c Qy-transition moments. The energy transfer within the BChl c antenna occurred between chromophores or strongly coupled clusters of chromophores (collective excitation within a cluster) with parallel transition moments. Steady state fluorescence anisotropy of BChl c in isolated chlorosomes reached the value of 0.18 around 750 nm (Miller et al., 1991).
In *C. aurantiacus* steady state fluorescence measurements (Vasmel et al., 1986, Van Dorssen et al., 1986) have shown the overall energy transfer scheme to be

\[ \text{BChl c} \rightarrow \text{BChl a}_{796} \rightarrow \text{[B806-866→P865]} \]

where the brackets indicate intramembrane species. The energy is transferred from the cytoplasmic side of the membrane (chlorosome) to the periplasmic side (special pair of the RC).

The excited state lifetime of BChl c was very short, about 10 ps in isolated chlorosomes (Miller et al. 1991) or 16 ps in whole cells (Causegrove et al., 1990). This time probably reflects energy transfer to the chlorosomal BChl a (Van Grondelle and Sundström, 1988). Three fluorescence bands are resolved in whole cells of *C. aurantiacus* at 750 nm, 810 nm and 880 nm corresponding to emission from BChl c, BChl a_{796} and BChl a_{866}. Energy transfer from BChl a_{806} to BChl a_{866} is very fast, and fluorescence due to BChl a_{806} is not observed (Vasmel et al., 1986). Energy transfer from BChl c to chlorosomal BChl a has an efficiency of 50-60% in isolated chlorosomes. In cells of *C. aurantiacus* this efficiency can approach 100%, although strong variations are observed dependent on the specific conditions (Van Grondelle and Sundström, 1988). At 4 K the overall transfer efficiency from BChl c to the membrane-bound chromophores (B740→B796→B806-866) is low, about 15% (B740→B796 =50%, B796→B806-866 =30%). The excited state lifetime of BChl a_{796} has two components, one about 30 ps the other about 200-300 ps long (Miller et al., 1991). Miller et al. (1991) suggested that the minimum scheme of energy transfer kinetics in isolated chlorosomes could be as follows

\[ \text{BChl c} \ 10 \text{ps} \rightarrow \text{BChl a}_{796} \ 30 \text{ps} \rightarrow \text{BChl a}_{806-866}. \]

### 4.3. The chlorosome

#### 4.3.1. Morphology and composition of the chlorosomes

All members of the families of Chlorobiaceae and Chloroflexaceae are characterized by the presence of chlorosomes (Staehelin et al., 1980). In electron micrographs of thin-sectioned cells chlorosomes appear as ellipsoid structures immediately underlying and...
appressed to the CM. In *C. aurantiacus* the average dimensions of the chlorosomes are 110 nm in length, 30 nm in width and 10 nm thickness (Staehelin et al., 1978). The length and thickness vary, depending on the growth conditions (Golecki and Oelze, 1987). Furthermore, chlorosome dimensions vary from strain to strain (Pierson and Castenholz, 1974). The chlorosome core contains parallel rod elements extending the length of the chlorosome (Fig. 8). The hydrophobic core is surrounded by a 2-3 nm envelope (Staehelin et al., 1980), which is thought to contain protein in a monolayer of lipid. Chlorosomes contain a base-plate composed of a crystalline lattice of particles. Underlying the chlorosome base-plate is the CM attachment site. This region of the CM contains the RCs (Staehelin et al., 1978, 1980) (see Table 2 for more details). Chlorosomes contain antenna BChl c, or d, or e, and also small amounts of BChl a. On a dry weight basis *C. aurantiacus* chlorosomes grown at low light intensity contained 65% protein, 22% BChl c, 11% lipid and 2% carotenoid, mainly β and γ carotene (Schmidt, 1980). Chlorosomes of the green sulphur bacterium *Chlorobium limicola* forma thiosulphatophilum were found to contain 55% protein, 10% lipid, 32% BChl c and 1% carotenoid, mainly chlorobactene (Schmidt, 1980)

---

![Fig. 8. Schematic representation of a chlorosome, generalized.](image-url)
Table 2. Structural parameters of Chlorobium and Chloroflexus chlorosomes

<table>
<thead>
<tr>
<th>Chlorosome</th>
<th>Chlorobium(^a))</th>
<th>Chloroflexus(^b))</th>
</tr>
</thead>
<tbody>
<tr>
<td>length</td>
<td>70-260 nm</td>
<td>106 ± 24 nm</td>
</tr>
<tr>
<td>width</td>
<td>40-100 nm</td>
<td>32 ± 10 nm</td>
</tr>
<tr>
<td>thickness</td>
<td>20-60 nm</td>
<td>12 ± 2 nm</td>
</tr>
<tr>
<td>envelope</td>
<td>3 nm thick</td>
<td>2 nm thick</td>
</tr>
<tr>
<td>core</td>
<td>10-30 rod elements, Ø 10 nm</td>
<td>rod elements, Ø 5.2 nm, 6 nm globular units</td>
</tr>
<tr>
<td>base-plate</td>
<td>5-6 nm thick, lattice: 6 nm periodicity, 40-60° from long axis of chlorosome</td>
<td>lattice(^c)): 6 nm period., 90° from long axis of chlorosome</td>
</tr>
<tr>
<td>membrane attachment site</td>
<td>20-30 large particles (&gt;12 nm)</td>
<td>5 nm particles, 7 nm particles along the perimeter</td>
</tr>
</tbody>
</table>

\(^a\)Staehelin et al. (1980), \(^b\)Staehelin et al. (1978), \(^c\)Sprague et al. (1981)

### 4.3.2. Protein composition of chlorosomes from Chlorobiaceae

The chlorosomes of the two families of green bacteria differ with respect to their polypeptide compositions. Table 3 (on the next page) summarizes chlorosome proteins found by different authors.
Table 3. Proteins found in chlorosomes of Chlorobiaceae

<table>
<thead>
<tr>
<th>bacterium</th>
<th>protein (M_r)</th>
<th>pigment</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pelodictyon luteolum</em> 2530</td>
<td>6300&lt;sup&gt;a&lt;/sup&gt;</td>
<td>BChl c</td>
</tr>
<tr>
<td><em>Prosthecochloris aestivalii</em></td>
<td>analogous to the 6300</td>
<td>BChl c</td>
</tr>
<tr>
<td>SK413</td>
<td>protein in <em>Pelodictyon luteolum</em>&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><em>Chlorobium limicola</em> 6230</td>
<td>analogous to the 6300</td>
<td>BChl c</td>
</tr>
<tr>
<td></td>
<td>protein in <em>Pelodictyon luteolum</em>&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><em>Chlorobium phaeovibrioides</em> 2631</td>
<td>analogous to the 6300</td>
<td>BChl e</td>
</tr>
<tr>
<td></td>
<td>protein in <em>Pelodictyon luteolum</em>&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><em>Chlorobium vibrioforme</em></td>
<td>analogous to the 6300</td>
<td>BChl d</td>
</tr>
<tr>
<td><em>f.thiosulfatophilum</em></td>
<td>protein in <em>Pelodictyon luteolum</em>, 6150&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><em>Chlorobium phaeobacteroides</em></td>
<td>34500, 5000-7500&lt;sup&gt;c&lt;/sup&gt;</td>
<td>BChl e</td>
</tr>
<tr>
<td><em>Chlorobium tepidum</em></td>
<td>34500, 5000-7500&lt;sup&gt;c&lt;/sup&gt;</td>
<td>BChl c</td>
</tr>
<tr>
<td></td>
<td>6200, 7700, 8000, 11500,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14500, 23000, 26000,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>27000&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><em>Chlorobium limicola</em></td>
<td>7500&lt;sup&gt;d&lt;/sup&gt;</td>
<td>BChl c</td>
</tr>
<tr>
<td><em>f.thiosulfatophilum</em></td>
<td>6300</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Wagner-Huber et al., 1988, <sup>b</sup>Wagner-Huber et al., 1990, <sup>c</sup>Stolz et al., 1990, <sup>d</sup>Gerola et al., 1988, <sup>e</sup>Chung et al., 1994

The 6.3 kDa polypeptide from *Pelodictyon luteolum* and analogous proteins from other Chlorobiaceae were supposed to have a BChl c-binding function (Wagner-Huber et al., 1988). The 7.5 kDa polypeptide, first isolated from *Chlorobium limicola f. thiosulfatophilum*, was also suggested as a candidate for BChl c-binding by Gerola et al. (1988). Yet, Wagner-Huber et al. (1988) found an analogous protein in whole cells of *Pelodictyon luteolum*, but not in isolated chlorosomes, indicating a different function for this protein. Later, Højrup et al. (1991) favoured the idea that the 7.5 kDa polypeptide...
from *Chlorobium limicola f. thiosulfatophilum*. may bind the chlorosome to the water-soluble BChl \(a\)-protein by salt bridges between Asp residues in the 7.5 kDa polypeptide and Lys or Arg residues in the BChl \(a\)-protein. Immuno-electron microscopy (Wullink et al., 1991) showed that the 7.5 kDa polypeptide was at least partially exposed on the chlorosome envelope. A recent study on the chlorosomes of *Chlorobium tepidum* by Chung et al. (1994) found a total of 8 proteins with molecular weights of 27000, 26000, 23000, 14500, 11500, 8000, 7700 and 6200. The 6.2 kDa polypeptide, which is homologous to the proteins found by Wagner-Huber et al. (1988, 1990), and the 7.7 kDa polypeptide, which is homologous to the 7.5 kDa polypeptide in *Chlorobium limicola f. thiosulfatophilum* (Gerola et al, 1988), were the most abundant proteins.

### 4.3.3. Protein composition of chlorosomes from *Chloroflexus aurantiacus*

The chlorosomes of *C. aurantiacus* contain three major proteins with apparent \(M_r\) of 18000, 11000, 5700 and a minor component of \(M_r\) 5800 (Feick et al., 1982). The 5.8 kDa polypeptide is thought to bind BChl \(a\) in the B790 complex. The 5.7 kDa polypeptide, the amino acid sequence of which is known (Wechsler et al., 1985), seems to be associated with BChl \(c\) in the chlorosome core, whereas the 11 and the 18 kDa polypeptide appear to be localized in the chlorosome periphery (Feick and Fuller, 1984). Information on additional proteins of the photosynthetic apparatus of *C. aurantiacus* was gained by Wechsler et al. (1985 and 1987), who isolated two polypeptides (\(\alpha\) and \(\beta\)) of the B806-866 complex and determined their amino acid sequences (B806-866-\(\alpha\), \(M_r\) 4900, B806-866-\(\beta\), \(M_r\) 6300). Structural similarities of these two proteins with the analogous \(\alpha/\beta\)-light-harvesting polypeptides from purple photosynthetic bacteria clearly indicated the relatedness of the membrane-integrated part of the photosynthetic apparatus of *C. aurantiacus* to the core antenna complex found in purple bacteria. The primary sequence determination of the RC polypeptides of *C. aurantiacus* revealed an L-subunit (\(M_r\) 35014) and an M-subunit (\(M_r\) 34982) (Ovchinnikov et al., 1988 a/b). Here again, the L and M-subunits of the *C. aurantiacus* RC are analogous proteins to the L and M-subunits present in the RC of purple bacteria. Yet, the analogy is incomplete, because the H-subunit found in purple bacteria is missing in the Chloroflexus RC. Furthermore, the genes coding for the L and M-subunits in *C. aurantiacus* are organized in the *pfu* operon, similar to the purple bacteria, but the genes for the \(\alpha\) and \(\beta\) polypeptides of the B806-866
core antenna are not adjacent to the RC polypeptide genes, unlike in purple bacteria (Watanabe et al., 1992). The localization of the different chlorosome proteins according to Feick and Fuller, 1984) is depicted in Fig. 9.

4.3.4. Homologies between chlorosome proteins from Chlorobiaceae and Chloroflexus aurantiacus

The 5.7 kDa polypeptide found in the chlorosomes of C. aurantiacus is 30% homologous (chemical identity) to the smallest and at the same time most abundant protein present in the chlorosomes of all Chlorobiaceae analysed so far. The homologies among the proteins of the Chlorobiaceae is extremely high, in the range of 95-100%
(Wagner-Huber et al., 1990). It was assumed that the *C. aurantiacus* protein plays a major role in the organization of BChl c in the chlorosome (see below) and that the Chlorobiaceae proteins serve the same function (Wagner-Huber et al., 1988, 1990). The sequence homologies are shown in Fig. 10.


### 4.3.5. The function of the 5.7 kDa polypeptide in the chlorosome of *Chloroflexus aurantiacus*

The role of proteins with respect to the organization of BChl c in the chlorosomes of *C. aurantiacus* has been a controversial subject (Wechsler et al., 1985, Griebenow and Holzwarth, 1989, Griebenow et al., 1990, Niedermeyer et al., 1992, Eckardt et al., 1990). The 5.7 kDa polypeptide is the most probable candidate for the BChl c-binding in the chlorosomes of *C. aurantiacus* (Feick and Fuller, 1984, Niedermeyer et al., 1992). It is likely to be buried in the chlorosome and thus it was hardly accessible to proteases such as trypsin and chymotrypsin or hydrophilic photolabel (Feick and Fuller, 1984). Its proteolytic degradation was only possible using long exposures to proteinase K or thermolysin treatment above 45°C (Niedermeyer et al., 1992). Digestion of the 5.7 kDa polypeptide led to a concomitant decrease of the absorption at 740 nm and a drastic increase in rotational strength. Therefore, it seemed reasonable to assume that the 5.7 kDa polypeptide and BChl c form a pigment-protein complex (Feick and Fuller, 1984; Wechsler et al., 1985). As the pigment to protein ratio was considerably higher in the chlorosomes of *C. aurantiacus* than e.g. in antennas of purple bacteria or in
the water-soluble BChl a-protein, BChl c in the B740 complex was assumed to be bound to the 5.7 kDa protein in an aggregated form (Feick and Fuller, 1984; Wechsler et al., 1985). In the binding mode proposed by Wechsler et al. (1985) five glutamine and two asparagine residues, all located on the same side of a hypothetical α-helix, served as possible interaction sites for 7 BChl c molecules. In that way, the BChl c molecules were allowed to interact with one another via hydrogen bonds between the hydroxyethyl group on ring I and the carbonyl oxygen on ring V. Moreover, it was proposed that 12 α-helices formed an aggregate comprising 84 BChl c molecules. The model took into consideration the parallel arrangement of the Qy-transition moments of BChl c along the long chlorosome axis (Betti et al., 1982). It was thought that this complex represented a new type of light-harvesting pigment-protein complex with an oligomeric arrangement of BChl c (Wechsler et al., 1985).

On the basis of the structural properties of the BChl c molecule (see above), which allow the formation of aggregates in non-polar solvents, different authors put forward the hypothesis that the red shift of the BChl c absorption in the chlorosome was caused by chromophore-chromophore interaction rather than chromophore-protein interaction (Smith et al., 1983, Bystrova et al., 1979). Comparison of the absorption properties of BChl c in non-polar solvents (Brune et al., 1987, Olson et al., 1985, Blankenship et al., 1988a) or aqueous solution (aggregated state, Hirota et al., 1992, Miller et al., 1993) with BChl c in the chlorosomes have provided evidence for a chromophore-chromophore interaction in the chlorosomes. All the same, these experiments did not rule out the possibility of a BChl c-binding function of the 5.7 kDa polypeptide.

### 4.4. Objective of the present study

The present study aimed at providing additional evidence for a BChl c-binding function of the 5.7 kDa polypeptide in the chlorosomes of *C. aurantiacus*. In this respect, the model proposed by Wechsler et al. (1985) served as a working hypothesis. Preceding trials to isolate a defined pigment-protein complex using detergents, chaotropic agents or long-chain alcohols proved to be difficult (Eckhardt, 1990). Therefore, the approach chosen in the present study consisted in correlating spectral changes of chlorosomes, induced by limited proteolysis and treatment with long-chain alcohols, to changes on the protein level. In addition, *C. aurantiacus* cells were cultivated under different growth
conditions and the resulting effects on the chlorosome structure were analysed in order to deduce information on the nature of the BChl c antenna.

In the course of the investigations the second most abundant polypeptides in the chlorosome, the 11 and the 18 kDa polypeptide, were further characterized and their possible role in pigment-binding was considered. As all chlorosome proteins were known to be more or less hydrophobic, or of fibrous nature, and therefore difficult to analyse, analytical methods had to be established, which allowed the separation of the entire set of polypeptides.
II. Materials and methods

II.1. Chemicals

All chemicals were of the highest grade available and were purchased from Fluka AG, Buchs, Switzerland, unless mentioned otherwise.

Detergents
Miranol® S2M-SF (caproamphocarboxypropionate) 39% stock solution in distilled water, Miranol inc., South Brunswick, New Jersey.
Sodium dodecyl sulphate (solid) 99%, Fluka AG, Buchs, Switzerland.

Buffers purchased from Sigma
Tris base (Trizma®) tris(hydroxymethyl)-aminomethane
Tricine (N-tris(hydroxymethyl)-methylglycine)

Enzymes of analytical grade were purchased from Boehringer Mannheim GmbH, Germany, unless mentioned otherwise.
chymotrypsin A4 (EC 3.4.21.1), elastase (EC 3.4.21.36), endoproteinase Glu-C (EC 3.4.21.19), proteinase K (EC 3.4.21.14), subtilisin (EC 3.4.21.14), thermolysin (EC 3.4.24.4);
endoproteinase Lys-C (EC 3.4.21.50) from Wako(Cat.No 129-02541), Japan,
trypsin (EC 3.4.21.4) TPCK treated, 7,500 units/mg, Fluka, Buchs, Switzerland.

Protease inhibitor
Pefabloc®SC (4-(2-aminoethyl)-benzenesulfonylfluoride hydrochloride),
Pentapharm AG, Basel, Switzerland.

Resins for chromatography
size-exclusion chromatography: Sephadex LH-60; Sephadex G-50; Sephacryl S-500 superfine, Pharmacia Fine Chemicals, Uppsala, Sweden.
reverse phase chromatography: Nucleosil 1000-7, C4; Nucleosil 300-7, C8, Macherey Nagel AG, Oensingen, Switzerland.
ion exchange resin: mixed bed resin AG 501-X8(D), 20-50 mesh, Bio-Rad Laboratories, Richmond, California.
II.2. Instruments

Conventional chromatography with soft gels was performed using the LKB 2238 Uvicord SII monitor, the LKB 2210 two-channel recorder and the LKB 2070 Ultrorac II fraction collector, LKB Bromma, Pharmacia, Sweden.

High-performance liquid chromatography was performed on an LKB system comprising the LKB 2156 solvent conditioner, two LKB 2150 HPLC pumps, the LKB 2152 LC controller, the 2154 automatic switching valve, a programmable absorbance detector SA 6504 Severn Analytical Limited, Shefford, United Kingdom and a two-channel recorder BD41, Kipp & Zonen, The Netherlands.

RP-HPLC fractions were dried using a speed vac concentrator, Savant Instruments inc., USA. Protein solutions in organic solvents of more than 1ml were concentrated with a rotary evaporator, Rotavap R, Büchi, Flawil, Switzerland.

Centrifuges
Centricon T-2070 ultra centrifuge, Kontron Instruments AG, Switzerland
Sorvall RC-5B centrifuge, Du Pont Instruments, Digitana AG Horgen, Switzerland(CH), distributer.
Sorvall RC3, Digitana AG Horgen, Switzerland, distributer.
Sepatech Biofuge A, Heraeus AG, Switzerland.
BHGHermle Z 230, Hermle GmbH, Gosheim, Germany.

Amino acid analyser
Biotronic LC 3000, Biotronic, Germany.

Protein sequencer
Knauer protein microsequencer 810, Knauer GmbH, Berlin, Germany.

Spectrophotometers
Lambda 5 UV/VIS spectrophotometer, Perkin Elmer, Illinois, USA.
Lambda Array 3840 UV/VIS spectrophotometer, Perkin Elmer.
Jasco J-710 spectropolarimeter, Japan Spectroscopic Co., LTD, Tokyo, Japan.
Fluorolog-2 spectrofluorometer, Spex Industries, Inc., Edison, New Jersey.
II.3. Organism

Chloroflexus aurantiacus J-10-fl (Pierson and Castenholz, 1974a) was obtained from the German Collection of Microorganisms DSM (Braunschweig, FRG). The same strain was also kindly provided by M. Foidl and Prof. J. Oelze, Universität Freiburg, FRG.

3.1. Cultivation under low light conditions

Chloroflexus aurantiacus J-10-fl was grown photoheterotrophically in 1 litre screw-capped bottles. The cultures were illuminated with 150 Wm\(^{-2}\) light intensity of a tungsten lamp. Radiation was measured using a LI-189 quantum/ radiometer/ photometer equipped with a LI-200 SA pyranometer sensor (LI-COR, inc. Lincoln, Nebraska). The cultures were grown at a temperature of 57°C. C. aurantiacus was stored in 30% glycerol in the growth medium at -70°C and pre-cultivated in 100 ml screw-capped bottles before the 1 litre batch cultures were inoculated with 10 ml of the exponentially growing preculture. The 1 litre batch cultures were continuously stirred and harvested after 4 days at high cell density for low-light grown cells.

3.1.1. Composition of medium and solutions

<table>
<thead>
<tr>
<th>Salts (per 10 l medium)</th>
<th>1.0 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>ethylenediaminetetraacetic acid</td>
<td></td>
</tr>
<tr>
<td>CaSO(_4) x 2H(_2)O</td>
<td>0.6 g</td>
</tr>
<tr>
<td>MgSO(_4) x 7H(_2)O</td>
<td>1.0 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.08 g</td>
</tr>
<tr>
<td>Na(_2)HPO(_4)</td>
<td>1.1 g</td>
</tr>
<tr>
<td>KNO(_3)</td>
<td>1.3 g</td>
</tr>
</tbody>
</table>
### Trace Element Solution

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeCl₃ x 6H₂O</td>
<td>0.47 g</td>
</tr>
<tr>
<td>MnSO₄ x H₂O</td>
<td>2.2 g</td>
</tr>
<tr>
<td>ZnSO₄ x 7H₂O</td>
<td>0.5 g</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>0.5 g</td>
</tr>
<tr>
<td>CuSO₄ x 5H₂O</td>
<td>0.025 g</td>
</tr>
<tr>
<td>Na₂MoO₄ x 2H₂O</td>
<td>0.025 g</td>
</tr>
<tr>
<td>CoCl₂ x 6H₂O</td>
<td>0.046 g</td>
</tr>
</tbody>
</table>

The trace elements were dissolved in 10 ml 25% HCl and filled up to 1000 ml with distilled water and then autoclaved for 20 min at 121°C.

### Vitamin Solution

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>folate acid</td>
<td>50 mg</td>
</tr>
<tr>
<td>myo-inositol</td>
<td>250 mg</td>
</tr>
<tr>
<td>pyridoxine hydrochloride (B6)</td>
<td>5 mg</td>
</tr>
<tr>
<td>thiamine hydrochloride (B1)</td>
<td>15 mg</td>
</tr>
<tr>
<td>cyanocobalamin (B12)</td>
<td>50 mg</td>
</tr>
<tr>
<td>biotin</td>
<td>50 mg</td>
</tr>
<tr>
<td>riboflavin (B2)</td>
<td>5 mg</td>
</tr>
<tr>
<td>D-pantothenic acid calcium salt</td>
<td>25 mg</td>
</tr>
<tr>
<td>p-aminobenzoic acid</td>
<td>25 mg</td>
</tr>
<tr>
<td>nicotinic acid</td>
<td>250 mg</td>
</tr>
</tbody>
</table>

The vitamins were dissolved in 500 ml distilled water and the pH was kept constant at 7.0 with NaOH. The solution was filtered with a Millex-GV sterile filter unit with 0.22 µm 0 pores, Millipore S.A., Molsheim, France.

### Additives

<table>
<thead>
<tr>
<th>Additive</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>acid hydrolysed casein hydrolysate</td>
<td>2.5 g/l</td>
</tr>
<tr>
<td>(Merck, Darmstadt, Germany)</td>
<td></td>
</tr>
<tr>
<td>yeast extract</td>
<td>1 g/l</td>
</tr>
<tr>
<td>(Difco Laboratories, Detroit, Michigan, USA)</td>
<td></td>
</tr>
<tr>
<td>glycyl-glycine</td>
<td>0.5 g/l</td>
</tr>
</tbody>
</table>

To prepare 10 litres of growth medium the salts and the additives were dissolved in 10 litres deionized water and adjusted to pH 8.3 with 40-50 ml 1 M NaOH. The medium was autoclaved for 35 min at 121°C in the 1 litre screw-capped bottles used for
cultivation. The medium was complemented with the sterile addition of 1 ml trace element solution and 1 ml vitamin solution per litre growth medium.

### 3.2. Cultivation under high light conditions

Low light intensity in batch cultures of *C. aurantiacus* results from self-shading at high cell density. For this reason cultivation under high light intensity of *C. aurantiacus J-10-fl* was done on substrate limited growth medium (see below). The cells were grown photoheterotrophically in 1 litre screw-capped bottles. The cultures were illuminated with 2000 Wm⁻² light intensity of tungsten spot lights. Radiation was measured as before. In order to avoid heating of the highly illuminated cultures the bottles were kept at 57°C in a transparent acrylic glass water bath. The spot lights were installed along the longer sides of the rectangular water bath and shone through the acrylic glass walls (Fig. 1). The 1 litre batch cultures were inoculated with 10 ml of a high-light adapted pregrown culture and continuously stirred during growth. Depending on the experiment the cells were harvested after 22 to 48 h.

Fig. 1. Batch cultures of *C. aurantiacus* grown photoheterotrophically under high light conditions at 57°C.
3.2.1. Composition of medium and solutions

salts without an inorganic nitrogen source (per 10 l medium)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>ethylenediaminetetraacetic acid</td>
<td>1.0 g</td>
</tr>
<tr>
<td>CaSO4 x 2H2O</td>
<td>0.6 g</td>
</tr>
<tr>
<td>MgSO4 x 7H2O</td>
<td>1.0 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.08 g</td>
</tr>
<tr>
<td>Na2HPO4</td>
<td>1.1 g</td>
</tr>
<tr>
<td>CH3COOK</td>
<td>1.3 g</td>
</tr>
</tbody>
</table>

trace element solution as before;

vitamin solution

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>biotin</td>
<td>50 mg</td>
</tr>
</tbody>
</table>

Biotin was dissolved in 500 ml distilled water and the pH was kept constant at 7.0 with NaOH. The solution was filtered with a Millex-GV sterile filter unit (0.22 μm ø pores, Millipore).

additives

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>yeast extract (Difco Laboratories)</td>
<td>0.25 g/l</td>
</tr>
<tr>
<td>glycyl-glycine</td>
<td>0.5 g/l</td>
</tr>
</tbody>
</table>

major carbon source

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-glutamic acid</td>
<td>0.3 g/l</td>
</tr>
</tbody>
</table>

A 0.5 M stock solution of L-glutamic acid was prepared, neutralised with NaOH and autoclaved. Ten litres of growth medium were prepared in analogy to the low light medium. The high light medium was complemented with the sterile addition of 1 ml trace element solution, 1 ml biotin solution and 4 ml of the glutamate stock solution (2 mM glutamate) per litre growth medium.
II.4. Preparative steps

4.1. Preparation of the whole-membrane fraction

All preparative steps for membrane and chlorosome isolation were performed on ice or at 4°C in the case of the centrifugations, unless mentioned otherwise. Frozen cells of *C. aurantiacus* were thawed and resuspended in 50 mM Tris/HCl buffer, pH 8.0, with a motor-driven Potter-Elvehjem homogenizer. The cells were washed with buffer by repeated centrifugation at 8,000 x g for 15 min and resuspension in 100 ml buffer per 10 g wet weight of cells. The cells were disrupted by ultrasonication in the presence of 2 mM Pefabloc® SC, a covalently binding serine protease inhibitor. Alternatively, cells were disrupted with a French pressure cell (Aminco, Urbana, USA) at 138 MPa. Whole cells and large cell debris were sedimented by centrifugation at 27,000 x g for 15 min. The supernatant was centrifuged at 100,000 x g for 1 h and the resulting membrane pellet was washed once by resuspension in 50 mM Tris/HCl buffer at pH 8.0 and centrifugation as before. The membrane pellet contained the cytoplasmic membrane with the chlorosomes attached to it and was designated as the whole-membrane fraction (Feick et al., 1982). The absorbance ratio A740 nm/A866 nm, which reflects the amount of chlorosomes relative to the CM, served as an indicator for chlorosome purity for the subsequent isolation steps (Feick et al., 1982).

4.2. Isolation of chlorosomes

Before the separation of the chlorosomes from the cytoplasmic membrane the whole-membrane fraction was resuspended in buffer with a Potter-Elvehjem homogenizer and adjusted to an OD of 10 at 865 nm. For solubilization 5% w/v Miranol S2M-SF was added and the suspension was stirred at room temperature in the dark for 30 min. Miranol S2M-SF is a zwitterionic detergent and was first used for the solubilization of chloroplast membranes by Markwell et al. (1979). Feick et al. (1982) employed Miranol S2M-SF for the isolation of chlorosomes from *C. aurantiacus*. We used a stock solution of 39% (w/v) of Miranol S2M-SF and prepared different concentrations by dilution. Solutions in Tris/HCl buffer around pH 8.0 were prepared freshly every time, because the diluted detergent became turbid after storage over a few days. The detergent also precipitated during several hours of centrifugation at 4°C in the sucrose density gradient.
To separate the solubilized chlorosomes from the cytoplasmic membrane and liberated pigments the detergent treated suspension was layered onto a four-step sucrose density gradient composed of 45%/30%/20%/10% (w/v) sucrose in 10 mM Tris/HCl, pH 8, 0.02% Miranol. The gradient was centrifuged at 83,500 x g in a swinging bucket rotor (TST 28.38, Kontron) for 12 h. The chlorosome fraction banded at 30% sucrose. It was collected and subsequently diluted 1:5 with buffer and the chlorosomes were concentrated by centrifugation at 100,000 x g for 1 h. The chlorosomes were resuspended in 10 mM Tris/HCl buffer at pH 8.0 and washed twice with the same buffer by sedimentation at 100,000 x g and resuspension.

The same isolation procedure was applied for low light and high light chlorosomes. In the case of high light chlorosomes the resuspended whole-membrane fraction usually had an OD lower than 10 at 865 nm. Therefore, the detergent concentration for the solubilization of the whole-membrane fraction was adjusted to 0.5% w/v Miranol per OD 1 (e.g. 2.5% w/v Miranol at an OD of 5).

Chemical structure of Miranol S2M-SF.

4.3. Extraction of chlorosome polypeptides with organic solvents

Isolated chlorosomes or the whole-membrane fraction were completely dried by lyophilization. Approximately 10 mg of dry weight of chlorosomes or membranes were powdered and dissociated with a mixture of CH2Cl2/CH3OH of 1:1 with 0.1 M ammonium acetate. A 1:1 mixture of CH2Cl2/CH3OH has a dielectric constant of about 20, which corresponds to the dielectric constant of the lipid bilayer. This makes the isolation of membrane-integrated proteins from their lipid environment possible. The presence of 0.1-1 M ammonium acetate (Shiozawa et al., 1980) in a mixture of organic solvents has proven to facilitate the extraction. This effect is due to the ionic strength
introduced by the NH$_4^+$ ion and the interaction of acetate with hydrogen bonds. In order to prevent photooxidation of sensitive amino acid residues such as methionine (oxidation of the thioether group to the sulfoxide and then to the sulfone) or the indole ring of tryptophan, the extraction was performed under dim light with the addition of small amounts of octadecanethiol. The polypeptides were extracted by addition of 1 ml of the extraction solution to the powdered chlorosomes or the whole-membrane fraction and vigorous stirring for 1 min. By this means not only the chlorosome polypeptides were extracted but also the BChls and the carotenoids, which were separated by subsequent size-exclusion chromatography. The extract was centrifuged at 3,000 x g for 5 min and the supernatant was loaded onto a size-exclusion column.

4.4. Chromatographic separation of chlorosome polypeptides

4.4.1. Size-exclusion chromatography on Sephadex LH-60

Chlorosomes polypeptides extracted with organic solvents were further purified by size-exclusion chromatography on Sephadex LH-60, which is a hydroxypropylated dextran.

\[
\text{Sephadex} - \text{O} - \text{CH}_2 - \text{CH} - \text{CH}_3 \\
\text{OH}
\]

Partial structure of Sephadex LH-60

The introduction of hydroxypropyl groups by ether linkages to the glucose units of the dextran does not alter the total number of hydroxyl groups on the matrix but increases the content of alkyl carbon atoms, enhancing the lipophilic character of the gel. Therefore, the gel swells in a mixture of polar and non-polar solvents such as CH$_2$Cl$_2$/CH$_3$OH (1:1). For the separation of the chlorosome polypeptides from the pigments after extraction with organic solvents (see above) a column of 50 cm in length with a radius of 1 cm was used. CH$_2$Cl$_2$/CH$_3$OH (1:1) with 0.1 M ammonium acetate
was used as the eluent at a flow rate of 50 ml/h, which was higher than indicated by the manufacturer, but still effective in separating the polypeptides from the pigments.

4.4.2. Preparative reverse phase chromatography

The polypeptide fractions in CH₂Cl₂/CH₃OH (1:1) with 0.1 M ammonium acetate were concentrated using a rotary evaporator (Rotavap R). To prevent precipitation of the polypeptides during the evaporation of CH₂Cl₂ and CH₃OH, 200 µl of acetic acid was added per 5 ml of polypeptide solution before concentration. The solution was concentrated down to approximately 500 µl. Reverse phase chromatography requires a denatured sample in a solvent that is polar enough to allow hydrophobic interaction of the polypeptide with the reverse phase matrix. This was provided after the evaporation of the CH₂Cl₂ and partly the CH₃OH.

Reverse phase chromatography (RP-HPLC) was performed on HPLC using a normal bore (4 x 125 mm) or a semi-preparative column, Nucleosil 1000-7, C₄ (Macherey Nagel). Eluent A consisted of H₂O containing 0.1% (v/v) TFA and eluent B consisted of acetonitrile containing 0.1% (v/v) trifluoroacetic. The chlorosome polypeptides proved to adsorb to the butyl-substituted reverse phase matrix at a acetonitrile concentration lower than 30%. Consequently, a linear gradient from 20% eluent A to 70% eluent B in 30 min at a flow rate of 1 ml/min was used to elute the polypeptides. The detection wavelength was set to 220 nm.

4.5. Isolation of BChl

For the preparation of BChl c from low-light grown cells of C. aurantiacus pigments were extracted as described by Brune et al. (1987). Different BChl c fractions were separated by RP-HPLC on a self-packed 4 x 125 mm column, Nucleosil 1000-7, C₁₈ (Macherey Nagel). Chromatography was performed in an isocratic mode using 93% methanol/ 7% water at a flow rate of 1ml/min. The samples were injected in 95% methanol (Zapata and Garrido, 1991) in a volume of 40 µl. Before the next injection the column was washed with 20 ml of 100% methanol. All solvents were free of oxygen after flushing with helium or argon. BChls were detected with a programmable, dual wavelength detector, SA 6504 (Severn Analytical Limited) at a wavelength of 668 nm and 280 nm.
4.5.1. Pigment determination

Quantitative BChl a and BChl c measurements were performed as described by Feick et al. (1982) using the extinction coefficients for BChl a of $\varepsilon = 68.6 \text{ mM}^{-1} \times \text{ cm}^{-1}$ (VanDerRest and Gingras, 1974) at 769 nm and for BChl c of $\varepsilon = 74 \text{ mM}^{-1} \times \text{ cm}^{-1}$ at 668 nm.

II.5. Analysis of chlorosomes

5.1. Chromatographic techniques

5.1.1. Size-exclusion chromatography of chlorosomes on Sephacryl S500

Size-exclusion chromatography on Sephacryl S-500 superfine (Pharmacia) with a fractionation range up to $(M_r) 20 \times 10^6$ was employed to compare the size of native chlorosomes with that of 1-hexanol treated chlorosomes and 1-hexanol and proteinase K treated chlorosomes. A column of 50 cm in length with a radius of 1 cm was used at a flow rate of 40 ml/h. Two different eluents were used composed of 100 mM Tris/HCl, pH 8.0, with 0.01% (w/v) Miranol for native chlorosomes or 100 mM Tris/HCl, pH 8.0, 59 mM 1-hexanol and 0.01% (w/v) Miranol in the case of 1-hexanol treated samples. Chlorosome samples were filtered through Millex-GV sterile filter units with 0.22 μm pores (Millipore S.A.) before application to the column. Chromatography was monitored at 280 nm. The elution volume for a small molecule was tested with bromophenol blue and was 320 ml. The void volume corresponded to the elution volume of aggregated chlorosomes and was approximately 52 ml.

5.1.2. Analytical reverse phase chromatography

RP-HPLC was chosen as a one-step isolation technique for the analysis of chlorosome polypeptides, since the hydrophobic parts of the denatured protein provide an important selectivity option not available to methods based on differences in solute size or net
charge. We used a butyl substituted, Nucleosil 1000-7 matrix (Macherey Nagel) in a normal bore, 4 x 125 mm column. Large pore silica ≥ 300 Å have been found to have a more satisfactory retention behaviour than narrow pore silica ≤ 100 Å due to physical entrapment in the latter. Hence, silica with pores of 1000 Å was used. The alkylchain length has been found to have only small influence on the relative retention provided the alkylchain densities are the same. However, it does effect recoveries. Especially for proteins with higher Mr (≥ 2000 Da) longer alkyl chains tend to have low recoveries. Therefore, we preferred a butyl to an octyl or octadecyl phase. We applied a binary mobile phase using acetonitrile and water. Acetonitrile as the organic solvent modifier is a widespread water-mixable organic solvent for RP-HPLC. Its elutropic strength proved to be high enough, especially in the case of the 5.7 kDa polypeptide in the chlorosome. TFA (0.1% v/v) was added as anionic counter ion. A linear gradient elution mode from 20% to 70% acetonitrile in water in 30 min at a flow rate of 1 ml/min was used showing highly reproducible retention times. The detection wavelength was set to 220 nm.

Sample preparation
Chlorosomes in the dry state after lyophilization, or in suspension in 10 mM Tris/HCl, pH 8.0, or in the presence of 59 mM 1-hexanol were dissociated in urea and formic acid. A stock solution of 10 M urea was used, which was deionized by passage through a mixed bed ion exchange resin AG 501-X8(D), 20-50 mesh (Bio-Rad Laboratories). Chlorosomes in the dry state were first dissolved in 8 M urea by vortexing for 1 min and subsequently, 20% formic acid were added. The solution was mixed, centrifuged at 3000 x g for 3 min and applied to the reverse phase column. Chlorosomes in suspension were combined in a ratio of 1:2 with 9 M urea, 10% formic acid to give 6 M urea and 6.7% formic acid. The solution was incubated for 30 min at 55°C in the dark, which led to a complete dissociation of the chlorosomes. The sample was centrifuged for 5 min at 3,000 x g, which led to the formation of a film of precipitated pigments on the surface of the sample. The solution itself had a yellowish colour and was ready to use for RP-HPLC.

5.2. Determination of pigment-protein stoichiometries
The stoichiometry of the 5.7 kDa polypeptide to BChl c in low light chlorosomes of C. aurantiacus was determined with two different methods. In both cases BChl c was quantified, as described before, in an aliquot of a chlorosome suspension. For the
quantification of the 5.7 kDa polypeptide two methods were employed, which involved several of the preparative methods presented before. For the first method (Fig. 2) the chlorosome suspension was lyophilised. The dried chlorosomes were extracted using the organic solvent mixture (CH$_2$Cl$_2$/CH$_3$OH (1:1) with 0.1 M ammonium acetate). Proteins and pigments were separated by size-exclusion chromatography on Sephadex LH 60. The protein fraction was further purified by RP-HPLC and the pure 5.7 kDa polypeptide was finally quantified by amino acid analysis. As depicted in Fig. 2, the extraction with the organic solvent mixture did not completely solubilize the dried chlorosomes. Residual material of more hydrophilic nature formed a colourless pellet which could be solubilized in 6 M urea / 20% formic acid. The solubilized residue was also separated by RP-HPLC and the residual 5.7 kDa polypeptide found therein was quantified by amino acid analysis. The second, more direct approach relied on the one-step isolation technique developed for the analysis of chlorosome polypeptides, which involved the dissociation of native chlorosomes in 6 M urea and 6.7% formic acid, subsequent separation of the polypeptides by RP-HPLC and quantification of the purified 5.7 kDa polypeptide by amino acid analysis.
Fig. 12. Scheme representing the steps followed for the isolation and the quantitative analysis of the 5.7 kDa polypeptide for the determination of the pigment-protein stoichiometry in low light chlorosomes
5.3. SDS-PAGE

The polypeptide pattern of chlorosomes was analysed with discontinuous sodium dodecylsulfate polyacrylamide gel electrophoresis according to Schäger and Jagow (1987). Electrophoresis was performed using the Mini-Protean II dual slab cell system (Bio-Rad Laboratories). The gel size was 7 cm (length) x 8 cm (width) with 0.75 mm thick spacers and a 10 well comb.

Polymerization protocol

The total acrylamide content (T) of the separating gel was 16% and the crosslinking reagent (C) was 3%. In the stacking gel T was 5% and C was 2.7%.

\[ T = \frac{(a+b) \times 100}{V} \text{ [\%]}, \quad C = \frac{b \times 100}{(a+b)} \text{ [\%]} \]

a: acrylamide in grams, b: N,N'-methylene-bis acrylamide in grams, V: volume in millilitres.

The stacking and the separating gel were prepared using the following concentrated stock solutions of acrylamide monomers and buffers.

acrylamide / bisacrylamide

I. 29.2% (w/v) acrylamide / 0.8% (w/v) bisacrylamide
II. 46.08% (w/v) acrylamide / 1.92% (w/v) bisacrylamide

stacking gel buffer
0.5 M Tris/HCl, pH 6.8
0.4% SDS (w/v)

separating gel buffer
3 M Tris/HCl, pH 8.45
0.4% SDS (w/v)

cathode buffer
0.1 M Tris base
0.1 M tricine (N-tris[hydroxymethyl]-methylglycine)
0.1% sodium dodecyl sulphate
anode buffer
200 mM Tris/HCl, pH 8.9

descent buffer
62.5 mM Tris/HCl, pH 6.8
10% glycerol
4% SDS
0.05% bromophenol blue
5% 2-mercaptoethanol

For the preparation of gels the solutions were combined according to the following protocols. Acrylamide polymerization was initiated with ammonium persulfate and N,N,N',N'-tetramethylethlenediamine.

stacking gel composition
acrylamide / bisacrylamide I 260 μl
buffer 400 μl
H2O 910 μl
10% APS 32 μl
TEMED 1 μl

separating gel composition
acrylamide / bisacrylamide II 1.5 ml
buffer 1.5 ml
H2O 1.5 ml
10% APS 22 μl
TEMED 2.2 μl

Sample preparation
Chlorosome samples were precipitated with 10% trichloroacetic acid and extracted twice with cold acetone. Acetone was removed and the samples were dissolved in sample buffer and incubated for 2 min at 100°C. The volume of a sample applied to the gel was 5 μl per slot. The electrophoresis was carried out with a constant current density of 33 mA/cm² at a corresponding voltage of 50-120 V.
5.4. Spectroscopy

5.4.1. Absorption spectroscopy
Absorption spectra from 250 nm to 900 nm were recorded on a Lambda 5 UV/VIS spectrophotometer, Perkin Elmer, with a 1 cm quartz cuvette at room temperature. The slit width was 1 nm and the scan speed 60 nm/min. The response time was set to 0.5 sec. In some cases absorption spectra were recorded on a Lambda Array 3840 UV/VIS spectrophotometer, Perkin Elmer, in the high performance mode using a 1 cm quartz cuvette.

5.4.2. Circular dichroism
Circular dichroism spectra from 300 nm to 800 nm were recorded on a Jasco J-710 spectropolarimeter with cuvettes of 1 mm to 5 mm pathlength at 22°C. The slit width was 2 nm and the scan speed 20 nm/min. The response time was set to 1 sec or 2 sec. The spectra were smoothed using the noise reduction option of the J-700 system software. The measurement of the instrument θ, in millidegree, was converted to ΔA (ΔA = θ /32.96).

5.4.3. Steady state fluorescence spectroscopy
Fluorescence emission spectra were recorded on a Fluorolog-2 spectrofluorometer (Spex Industries, Inc., Edison, New Jersey), equipped with a near infrared photomultiplier R406 Hamamatsu, Photonics K.K., Japan. Chlorosome samples were adjusted to an OD of 0.1 at 740 nm and measured with 1 cm x 1 cm cuvette.

5.4.4. Fluorescence polarization measurements
The polarization of the fluorescence was measured on the Fluorolog-2 spectrofluorometer with polarizers in the excitation light beam and in front of the emission monochromator. The polarization of the fluorescence (p) is defined as the ratio of the difference between the intensities of the parallel (I||) and the perpendicular (I⊥) components of the fluorescence and the sum of these intensities.
Although fluorescence polarization is most frequently found in the literature, fluorescence anisotropy (a), defined as

\[ a = \frac{I_{||} - I_{\perp}}{I_{||} + 2I_{\perp}} \]

is physically more correct, because it takes into account the two perpendicular directions of the emitted fluorescence relative to the plane of the polarized excitation light. Hence, we determined the anisotropy of the fluorescence. In theory the calculation of the anisotropy involves only two measurements, with the excitation polarizer oriented vertically and the emission polarizer alternately oriented vertically and horizontally. These measurements can be designated \( I_{vv} \) and \( I_{vh} \), respectively; the first subscript indicating the position of the excitation polarizer, the second that of the emission polarizer. In practice, however, instrumental artefact make it necessary to acquire four measurements: \( I_{vv}, I_{vh}, I_{hv} \) and \( I_{hh} \). The equation can be expressed as

\[ a = \frac{I_{vv} - GI_{vh}}{I_{vv} + 2GI_{vh}} \]

where \( G \) is the grating factor, \( I_{hv} / I_{hh} \). \( G \) is primarily a function of the wavelength of the emission spectrometer and was determined at the different wavelengths used for anisotropy measurements.

### 5.5. Electron microscopy

For electron microscopy chlorosomes were adsorbed to glow discharged carbon film and negatively stained with 2% uranyl acetate. The preparations were examined with a Philips EM 301 electron microscope. The micrographs were taken by Dr. E. Wehrli, ETH Zürich. Alternatively, chlorosomes adsorbed to glow discharged carbon film were freeze dried at -80°C for 2 h. Chlorosomes were unidirectionally shadowed with Pt/C (1.5 nm) at an elevation angle of 45°. Micrographs were taken by Dr. A. Staehelin, Boulder, Colorado, on a Philips CM 12 electron microscope, operated at 100 kV.
II.6. Modifications of chlorosomes

6.1. Proteolytic treatment of chlorosomes

A suspension of chlorosomes in buffer was adjusted to an OD of 2-4.5 at 740 nm. 1μg of proteinase K or trypsin per 1 ml of chlorosome suspension was added for proteolytic treatment. The samples were incubated at 37°C in the dark for 1 h. The digestion was stopped by adding 5 mM Pefabloc® SC, a covalently binding serine protease inhibitor, and the absorption and CD spectra were recorded immediately afterwards. Aliquots for analyses on reverse phase chromatography or SDS-PAGE were taken out of the samples and denatured immediately after the spectral measurements.

6.2. 1-hexanol treatment of chlorosomes

Buffer saturated with 1-hexanol (59 mM 1-hexanol at 25°C, Kinoshita et al., 1958) was prepared at room temperature by adding 0.8% v/v of 1-hexanol to 10 mM Tris/HCl, pH 8.0 in a separating funnel. After shaking, the solution was allowed to stand for at least 1 h before separation of the clear 1-hexanol saturated buffer. Chlorosomes were treated with 1-hexanol saturated buffer by adding the buffer to a small volume of a highly concentrated chlorosome suspension (Matsuura and Olson 1990).

6.3. SDS treatment of chlorosomes

Chlorosomes were treated with SDS and subsequently fractionated by size-exclusion chromatography on Sephacryl S-500 superfine (Pharmacia). SDS treated chlorosomes were prepared by diluting chlorosomes in 10 mM Tris/HCl, pH 8.0, and adding 3%(w/v) SDS to give a final SDS concentration of 0.3% and an absorption of 70 at 740 nm. The chlorosomes were stirred at room temperature in the dark for 20 min. A 1 ml sample was loaded onto the Sephacryl S-500 column described before, equilibrated with 10 mM Tris/HCl, pH 8.0, 100 mM NaCl and 0.3% SDS. The flow rate was 40 ml/h and the detection wavelength was set to 280 nm.
II.7. Polypeptide analysis

7.1. Solubilization of the 5.7 kDa polypeptide

Before attempting the solubilization of the 5.7 kDa polypeptide it was necessary to consider an assay of solubility. As the amino acid sequence of the 5.7 kDa polypeptide contains three tryptophans and one tyrosine (Wechsler et al., 1985) the extinction of the polypeptide at 280 nm was chosen as a criterion for solubility. According to a statistical determination by Mach et al. (1992) the extinction coefficients for tryptophan and tyrosine in native proteins are 5540 M⁻¹cm⁻¹ and 1480 M⁻¹cm⁻¹, respectively. The overall extinction coefficient for the 5.7 kDa polypeptide is the sum of the extinction coefficients of three tryptophans and one tyrosine equal to 18100 M⁻¹cm⁻¹. Accordingly, 1 µM of protein has an extinction at 280 nm of 0.018 cm⁻¹. The solubilization trials were performed with the 5.7 kDa polypeptide after purification by RP-HPLC in the dry state. Solubilization was usually attempted with 800 µl of different solvents and 2 nmol of protein in micro test tubes (Eppendorf®, Hamburg, Germany). The samples were vigorously stirred and incubated for 1-24 h at room temperature and the extinction at 280 nm was measured after centrifugation at 3,000 x g. At extinction values below 0.01 the chosen solvent was considered to be inadequate to achieve complete solubilization. As the dry state of the 5.7 kDa polypeptide seemed to imply almost total insolubility a second method consisted in dialysing the polypeptide in 70% acetonitrile, 0.1% TFA against different buffers.

7.2. Electro-blotting of polypeptides

For sequence analyses of chlorosome polypeptides protein bands from SDS-PAGE were electro-blotted onto polyvinylidene fluoride (PVDF). Electro-blotting was performed using the blotting apparatus and the transfer conditions described by Frank et al. (1993) with minor modifications according to Frank (personal communication).
The following was modified:

**transfer buffer:**
- 7 mM Tris
- 50 mM glycine
- no methanol

**staining with amidoblack:**
- 0.1% w/v amidoblack
- 40% v/v methanol
- 10% acetic acid

The bands of blotted polypeptides were excised and the strips were directly introduced into the automated Edman sequencer, provided the proteins were not blocked on the N-terminus (see Internal sequence analysis, below). In the case of the B806-866-α and β polypeptides, which are both blocked by a formyl-methionine on the N-terminus, the N-terminus had to be deformylated. This was achieved by incubating the PVDF with the polypeptide in an oxygen-free reaction vial with 10 µl of 2.5% HCl in methanol on the bottom for 1 h. The PVDF strips were not soaked but only incubated in the gas phase to avoid losses of protein.

**7.3. Amino acid sequence determination**

**7.3.1. N-terminal sequence determination**

N-terminal amino acid sequences of polypeptides electro-blotted onto PVDF membrane were determined by automated Edman degradation on a Knauer Model 810 protein microsequencer (Knauer GmbH, Berlin). The phenylthiohydantoin amino acid derivatives were identified by reverse phase HPLC as described by Frank et al. (1989).

**7.3.2. Internal sequence determination**

**Solution 1:**
- 1%(w/v) polyvinylpyrrolidone-40 (PVP-40, Aldrich) in 100 mM acetic acid, 10%(v/v) methanol

**digestion buffer:**
- 100 mM sodium phosphate buffer at pH 8.0

**extraction mixture:**
- 5% TFA, 70% isopropanol in water

For internal sequence analysis applied to the 11 kDa polypeptide blocked on the N-terminus the polypeptide was digested with endoproteinase Lys-C on the PVDF
membrane after electrophoresis and electro-blotting. For that purpose the amidoblack stained protein was cut out of the PVDF membrane and the small membrane strip was transferred into a Waters injection tube ("Wisp tube"). The dry membrane was wetted with 5 μl of methanol. 500 μl of solution 1 was added and incubated for 2 h at 37°C (saturating the glass vial and the PVDF). The solution was removed and the PVDF membrane was rinsed with Milli-Q water three times. The PVDF membrane was rinsed once more with digestion buffer. 25 μl of digestion buffer containing 1 μg endoproteinase Lys-C was added and the digestion was allowed to take place at room temperature over night. Following the digestion 28 mg of guanidine was added to the digestion mixture in the Wisp tube resulting in a final volume of 50 μl. The sample was reduced by adding 10 mM dithiothreitol and incubation for 2 h at 37°C. The probe was then alkylated by adding 25 mM iodoacetamide and incubation for 30 min at room temperature. The PVDF membrane was transferred into a PVP-40 coated Eppendorf tube and the Wisp tube containing the guanidine solution was kept on the side. The peptides were extracted from the PVDF by adding 25 μl of extraction mixture and sonication for a few minutes. The probe was further incubated overnight at room temperature under gentle agitation. The extraction mixture was transferred into the Wisp tube containing the guanidine solution. The PVDF membrane was washed twice with 60 μl of 0.1% TFA and the washings were transferred into the Wisp tube. The final volume was about 190 μl. The PVDF was discarded. The peptides of the endoproteinase Lys-C digested 11 kDa polypeptide were separated by RP-HPLC and two fragments were subsequently sequenced.

7.4. Amino acid analysis

For amino acid analysis polypeptide samples were hydrolysed in constantly boiling 6 N HCl for 24 h at 110°C in vacuo and analysed on a Biotronic LC 6000E analyser.

7.5. Mass spectrometry

Polypeptides were solubilized in 1,1,1,3,3,3-hexafluoro-2-propanol and analysed by electro-spray ionisation mass spectrometry using a TSQ 710 triple quadrupole mass spectrometer, Finnegan MAT, San José, California. Spectra were accumulated in profile mode scanning from 400-2000 M/Z in 4 sec. The spectra were recorded by Dr. P. James, ETH Zürich.
III. Results

III.1. Analysis of polypeptides isolated from low light chlorosomes

In this section I will describe two separation techniques for chlorosome proteins together with some findings about the purified polypeptides. Both methods, RP-HPLC and SDS-PAGE, were employed for the analysis of the polypeptide pattern of the chlorosomes. Above that, the separation techniques were used to prepare small amounts of polypeptides for further characterization as, e.g., determination of molecular mass, amino acid sequence determination or solubilization trials.

1.1. A one-step technique for the isolation of the three major chlorosome polypeptides

The isolation of the 5.7 kDa polypeptide, which constitutes the predominant protein in the chlorosomes of *Chloroflexus aurantiacus*, was first achieved by Wechsler et al. (1985). Wechsler et al. (1985) extracted the polypeptides from lyophilized chlorosomes in CHCl₃/CH₃OH (1:1), containing 0.1 M ammonium acetate and 20% acetic acid. The extract was chromatographed on Sephadex LH60 yielding 4 polypeptide fractions. Three of them contained the 11 and 18 kDa polypeptide, and the fourth fraction, which showed the strongest absorption at 280 nm, contained pure 5.7 kDa polypeptide. This isolation procedure allowed the purification of preparative amounts of the smallest chlorosome polypeptide, but it required large amounts of isolated chlorosomes. Furthermore, the size-exclusion chromatography on a 3 x 150 cm Sephadex LH60 column made this procedure too time-consuming to use it as a convenient, one-step isolation protocol for the analysis of the chlorosome polypeptides. Feick (1985) published preliminary investigations aimed at establishing a rapid isolation protocol for the chlorosome polypeptides from *Chloroflexus aurantiacus* membranes using RP-HPLC with a C-3 substituted silica column (pore size 300 Å) and a ternary elution gradient from water to acetonitrile/propanol. Although the chromatography resulted in acceptable resolution
separating four different polypeptides (apparent Mr 3700, 5300, 11000 and 18000), the fact that the 5.7 kDa polypeptide was eluted in multiple peaks remained unsatisfactory. Multiple peaks of the same polypeptide are caused by its inhomogeniety, which is possibly due to the oxidation of methionine and tryptophan residues of the polypeptide and the formation of protein aggregates. Most certainly, this was induced by the sample preparation, which involved the precipitation of a membrane suspension in 10% TFA and the solubilization of the precipitate in 67% formic acid and 33% isopropanol. In our laboratory too, excessive use of formic acid as a solvent for chlorosome polypeptides in connection with organic solvents produced multiple peaks of the same polypeptide on RPC. To minimize this effect the dissociation of chlorosomes, whether in the dry state or in suspension, was performed using high concentrations (6-8 M) of urea with the addition of 10-20% formic acid. Figure 13 shows the chromatography on RP-HPLC of chlorosomes solubilized from the dry state in 8 M urea with the subsequent addition of 20% formic acid. The 5.7 kDa polypeptide was eluted, in essence, as one fraction (peak 2) with two very small adjacent elution peaks. The preceding peak (1) tended to increase at the expense of the main peak upon longer incubation in the dissociating solvent, so that an oxidation reaction could be the origin of such inhomogeniety (see mass spectrometric analysis below). Fraction 3 also contained the 5.7 kDa polypeptide according to N-terminal sequence analysis and amino acid analysis. The fractions 4 and 5 contained the 11 and 18 kDa polypeptide, respectively. The chromatogram revealed that these two polypeptides, which were more hydrophobic than the 5.7 kDa polypeptide were eluted in comparatively low yields. The staining intensities obtained with Coomassie blue on SDS-PAGE predicted smaller amounts for the 11 and the 18 kDa polypeptide compared to the 5.7 kDa polypeptide (Fig. 16). But, as the detection wavelength of the chromatography was set to 220 nm (absorption of the peptide bonds), meaning that higher molecular weights have higher molar extinctions, one should expect more absorption for the fractions of the 11 and 18 kDa polypeptides. It is possible that these two polypeptides were solubilized in urea and formic acid but precipitated during chromatography. Indeed, it was frequently observed that both the 11 and the 18 kDa polypeptide precipitated as soon as they were eluted from the column. Consequently, they did not give rise to extinctions proportional to their effective amounts.
1.2. Characterization of the 11 and 18 kDa polypeptide

1.2.1. Sequence determination

At the beginning of the present investigation little was known about the two chlorosome polypeptides with apparent molecular weights of 11,000 and 18,000 (Feick et al., 1982). Feick and Fuller (1984) found out that these two polypeptides formed heterodimers upon crosslinking with DTBP (dimethyl 3,3'-dithiobis [propionimidate]), which indicated a close proximity between the two polypeptide molecules. The fact that they were susceptible to photolabelling with 3-azido-2,7-naphthalenedisulfonate and to proteolytic
cleavage led to the conclusion that the 11 and the 18 kDa polypeptide were part of the chlorosome envelope layer. In the meantime, Niedermeyer and Feick have cloned and sequenced the genes of the two polypeptides (presented on occasion of the EMBO workshop (1993) 'Green Bacteria and Heliobacteria', held in Nyborg, Denmark).

As mentioned above the purification of the 11 and 18 kDa polypeptide is a difficult task. Due to their insolubility in water the protein chemical analysis requires appropriate handling of the polypeptide molecules. One-dimensional PAGE proved to be sufficient to obtain pure polypeptides in small amounts. Subsequent electro-blotting onto PVDF opened the possibility for amino acid sequence determination. In this way, an N-terminal fragment of about thirty residues was determined for the 18 kDa polypeptide. In the case of the 11 kDa polypeptide, which was blocked on the N-terminus, internal sequence determination, following endoproteinase Lys-C cleavage, allowed the determination of only one small fragment. In addition to that, internal sequence information of this polypeptide was gained after proteolytic treatment of chlorosomes with trypsin and sequencing from PVDF as before. The obtained amino acid sequences for the two polypeptides are depicted below.

the N-terminus of the 18 kDa polypeptide
SNETTNERDGFLMAAGFVGATRIGLVA...

Lys-C fragment of the 11 kDa polypeptide (upper fragment)
...
[ELMYAFASLP-DF-EI--K...]
...ANATKERL...

possible overlap with a cyanogen bromide fragment that was sequenced by Eckardt (1990) (lower fragment).

tryptic fragment of the 11 kDa polypeptide
...
[RNDSFVXSSXXXFGEIVRLGFSXF...
1.2.2. Molecular mass determination using mass spectrometry

The determination of the molecular masses of the isolated 11 and 18 kDa polypeptide was of value for the confirmation of the amino acid composition deduced from the nucleotide sequence. Niedermeyer and Feick calculated molecular weights of 10,820 and 15,545 for the so-called 11 and 18 kDa polypeptide.

As shown in Fig. 14 A,B the mass spectra for both molecules were very broad and comprised several species of different masses ranging from 10,602 to 10,696 Da in the one case and from 15,419 to 15,524 Da for the second molecule. The observed molecular mass for the main species of the 11 kDa polypeptide was MH\(^+\)\(_{\text{obs.}}\) = 10658 Da and for the 18 kDa polypeptide MH\(^+\)\(_{\text{obs.}}\) = 15438 Da. The broadness of the signals resulted from inhomogeneity in the samples probably due to oxidation reactions during preparation. Although the observed range of values is close to the deduced values by Niedermeyer and Feick the above results are not accurate enough to confirm or to contradict the deduced molecular masses, i.e. the determined nucleotide sequences.

Fig. 14. (A) Mass spectrum of the 11 kDa polypeptide (electro-spray ionisation).
1.3. The 5.7 kDa polypeptide

1.3.1. Molecular mass by mass spectrometry

The amino acid sequence of the 5.7 kDa polypeptide reported by Wechsler et al. (1985) was 51 amino acids long with arginine at position 51. The nucleotide sequence encoding the 5.7 kDa polypeptide revealed that, in fact, there was a serine at position 51 and arginine formed the C-terminus resulting in a polypeptide of 52 amino acids (Theroux et al., 1990). The exact molecular mass of the 5.7 kDa polypeptide isolated by the one-step isolation procedure was verified using mass spectrometry. The observed molecular mass was $M_{\text{H}^+}\text{obs.} = 5678.1 \pm 0.4$ units (Fig. 15 A). Based on the primary structure the calculated mass was $M_{\text{H}^+} = 5680.25$ units (5679.25 units without the proton). This value takes into account the natural distribution of the isotopes for C, N, O, S. This means that there was a difference of $2 \pm 0.4$ units between the observed mass and the theoretical value. The divergence exceeded the instrumental error for unknown reasons.
Nevertheless, the mass of the isolated polypeptide corresponded almost perfectly to what had been expected, indicating the absence of possible post-translational modifications. In addition, the mass determination of the minor fraction of the 5.7 kDa polypeptide, eluting in peak 1 in Fig. 13, yielded a mass that was 16 units higher (Fig. 15 B). It was concluded that this species of the polypeptide had undergone oxidation with the addition of an oxygen molecule.

Fig. 15. (A) Mass spectrum of the 5.7 kDa polypeptide, which was eluted as the main fraction on the RP-column (peak 2, Fig. 13).
Fig. 15. (B) Mass spectrum of the 5.7 kDa polypeptide, most probably an oxidized species, (peak 1, Fig. 13). The mass is 16 units higher compared to the observed mass for the main species.

1.3.2. Solubility

The solubility of the 5.7 kDa polypeptide was tested in order to find suitable conditions for a reconstitution of the polypeptide with BChl c. In Table 4 a list of solvents and conditions of solubilization are presented, which aimed at solubilizing the 5.7 kDa polypeptide from the dry state. Table 5 summarizes the results of several attempts to dialyse the 5.7 kDa polypeptide in 40% acetonitrile, i.e. after isolation by RP-HPLC, against different buffers.
Table 4.
Solubilization from the dry state

<table>
<thead>
<tr>
<th>solvent</th>
<th>subsequent addition of</th>
<th>observed solubility</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% hexafluoroisopropanol</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>100% ethylacetate</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>100% CH3CN</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>CH3CN, 0.1%TFA</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>CH3CN, 0.1%TFA</td>
<td>60% H2O</td>
<td>-</td>
</tr>
<tr>
<td>CH2Cl2/CHOH 1:1, 0.5 M ammonium acetate</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>CH2Cl2/CHOH 1:1, 0.5 M ammonium acetate, 20% acetate</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>100% 1-hexanol</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>10 mM Tris/HCl, pH 8 saturated with 1-hexanol</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>TFE</td>
<td>40% CH3CN</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 5.
Dialysis of the 5.7 kDa polypeptide in 40% CH3CN, 0.1%TFA against different buffers

<table>
<thead>
<tr>
<th>buffer</th>
<th>pH</th>
<th>observed solubility</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM Tris/HCl, saturated with 1-hexanol</td>
<td>7</td>
<td>-</td>
</tr>
<tr>
<td>20 mM potassium phosphate</td>
<td>7</td>
<td>-</td>
</tr>
<tr>
<td>100 mM potassium phosphate</td>
<td>7</td>
<td>-</td>
</tr>
<tr>
<td>300 mM sodium acetate</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>6 M urea, potassium phosphate</td>
<td>7</td>
<td>(+)</td>
</tr>
<tr>
<td>8 M urea, potassium phosphate</td>
<td>7</td>
<td>++</td>
</tr>
</tbody>
</table>

The dry state proved to very unfavourable for the solubilization of the 5.7 kDa polypeptide. Only 1,1,1,3,3,3-hexafluoro 2-propanol was capable of dissolving the dry polypeptide. According to the results obtained for the solubility trials involving dialysis, low pH seemed to be of advantage for the solubilization since the 5.7 kDa polypeptide precipitated upon dialysing against pH 5-7. Precipitation did not occur during dialysis against 8 M urea.
1.4. Polypeptide pattern of low light chlorosomes on SDS-PAGE

SDS-PAGE represents a powerful analytical tool for the analysis of chlorosome polypeptides. As the polypeptide pattern of proteolytically treated chlorosomes can be analysed in comparison with native chlorosomes, SDS-PAGE has provided essential information for the assignment of spectral changes caused by limited proteolysis (Feick and Fuller, 1984; Niedermeyer et al., 1992). Sample preparation is of crucial importance for separating the chlorosome polypeptides on a gel. The best results were obtained, when the samples applied to electrophoresis were free of pigments and lipids. This was achieved by extracting the lipophilic substances from precipitated chlorosomes with acetone. Niedermeyer et al. (1992) proved with radioactive label that no protein was lost during the acetone washings. The polypeptide pattern of native chlorosomes on SDS-PAGE stained with Coomassie brilliant blue is depicted in Fig. 16. The pattern showed three main polypeptides with apparent molecular weights of 5,700, 11,000 and 16,000, representing the chlorosome proteins designated as 5.7 kDa, 11 kDa and 18 kDa polypeptide. The 5.7 kDa band was the predominant band. Additionally, a number of up to ten faintly staining bands were visible on the gel. Several of these bands could partly be sequenced and the information gained about the unknown proteins was summarized in Table 6.

![Fig. 16. SDS-Polyacrylamide gelelectrophoresis showing the polypeptide pattern of native low light chlorosomes. Lane 1: Low molecular weight standard (Sigma), lane 2: Chlorosome polypeptides. Coomassie blue staining.](image-url)
Table 6.
Chlorosome polypeptides on SDS-PAGE.

<table>
<thead>
<tr>
<th>no</th>
<th>apparent Mr on SDS-PAGE</th>
<th>N-terminal sequence</th>
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</thead>
<tbody>
<tr>
<td>1a*)</td>
<td>70,000</td>
<td>ES-GRSAGGKL-P-NL...</td>
</tr>
<tr>
<td>1b*)</td>
<td>70,000</td>
<td>ES-GRSAGGKL-P-NL...</td>
</tr>
<tr>
<td>2</td>
<td>42,000</td>
<td>blocked</td>
</tr>
<tr>
<td>3</td>
<td>18,000</td>
<td>MIVQEQRVSINAPPATVERYLTDQLMME-P...</td>
</tr>
<tr>
<td>4</td>
<td>16,000</td>
<td>SNETT... (18 kDa polypeptide)</td>
</tr>
<tr>
<td>5</td>
<td>11,000</td>
<td>blocked (11 kDa polypeptide)</td>
</tr>
<tr>
<td>6</td>
<td>8,000</td>
<td>SVKESGGIFGMFREVGFWEQV-NWAE...</td>
</tr>
<tr>
<td>7</td>
<td>7,500</td>
<td>MRDDDLV... B808-866-8</td>
</tr>
<tr>
<td>8</td>
<td>5,700</td>
<td>ATRGWFS... (5.7 kDa polypeptide)</td>
</tr>
</tbody>
</table>

*) 1a and 1b correspond to two different bands with apparent Mr of about 70000. Both bands had identical N-termini.

### III.2. Characterization of low light chlorosomes

#### 2.1. Isolation

There are basically two methods for the isolation of chlorosomes. One involves the zwitterionic detergent Miranol S2M-SF (Feick et al., 1982; Feick and Fuller, 1984) and was developed for *Chloroflexus aurantiacus* chlorosomes and the other uses the chaotropic reagent NaSCN and was introduced by Gerola and Olson (1986) for the isolation of chlorosomes from *Chlorobium limicola*. There is no genuine standard protocol for the isolation of chlorosomes from *C. aurantiacus* and every new publication introduces a modified procedure. Fortunately, different isolation methods applied to *Chloroflexus* chlorosomes result in similar preparations with comparable appearances on electron micrographs, identical polypeptide patterns and absorption and fluorescence spectra. The CD properties of *Chloroflexus* chlorosomes, however, are not consistent
throughout the literature. But it is questionable, whether different isolation methods for chlorosomes are the origin for the observed differences (Brune et al., 1990).

In my experiments chlorosomes were isolated essentially according to Feick and Fuller (1984) using Miranol S2M-SF as the detergent for the detachment of the chlorosomes from the cytoplasmic membrane. The most important observations, especially with respect to differences between chlorosomes isolated from low-light grown cells and high-light grown cells will be outlined in the following paragraphs.

Cells were stored frozen at -20°C and thawed just before the preparation of the whole-membrane fraction (WMF). Freezing and thawing, whether of whole cells or of isolated chlorosomes, had no influence on the quality of the preparation. The breakage of the cells was usually performed by ultrasonication. For comparison, cells were disrupted by passage through a French pressure cell, but the spectral properties of chlorosomes isolated from the two cell batches, especially the CD spectra, were exactly the same for both preparations. Cells were broken in the presence of 2 mM Pefabloc SC, a water-soluble serine protease inhibitor. The inhibitor added in this step was washed out in the subsequent steps, so that it did not interfere with the protease experiments. The absorption spectrum of the WMF prepared from low-light grown cells is shown in Fig. 17.
The spectrum comprises the chlorosome specific absorption bands at 796 nm, 741 nm, 463 nm and 325 nm as well as the absorption bands of the B806-866 complex. The absorbance ratio $A_{740\text{nm}}/A_{866\text{nm}}$, which served as an indicator for the purity of chlorosomes (Feick et al., 1982), was 22-25. The molar ratio of BChl c/BChl a was about ten. After the treatment of the WMF with Miranol S2M-SF and sucrose density gradient centrifugation on a gradient composed of 10%/20%/30%/45% sucrose in 10 mM Tris/HCl, pH 8.0, 0.2% Miranol S2M-SF, the chlorosomes banded in the 30% layer (Fig. 18, band 3 and Fig. 19, solid line). The B806-866 complex was mainly found in the 20% layer together with lighter chlorosomes or chlorosome fragments, which gave rise to an absorption band at 740 nm (Fig. 18, band 2 and Fig. 19, dashed line). Liberated BChl c and carotenoids, which were not associated with the
chlorosomes, remained in the upper layer of the gradient (Fig. 18, band 1 and Fig. 19, dotted/dashed line). After three subsequent centrifugations and resuspending in 10 mM Tris/HCl, pH 8.0, the chlorosomes were essentially free of sucrose and detergent and served as the starting material for further analyses (chlorosomes in the native state).

![Schematic representation of the sucrose density gradient. Numbers one to three indicate the layers, which were analysed by absorption spectroscopy. The corresponding absorption spectra are shown in Fig. 19.](image)

**Fig. 18.** Schematic representation of the sucrose density gradient. Numbers one to three indicate the layers, which were analysed by absorption spectroscopy. The corresponding absorption spectra are shown in Fig. 19.

![Absorption spectra of the different layers in the sucrose density gradient.](image)

**Fig. 19.** Absorption spectra of the different layers in the sucrose density gradient.
2.2. Spectroscopic properties of isolated chlorosomes in the native state

2.2.1. Absorption properties

The absorption spectrum of chlorosomes in the native state is shown in Fig. 20. The strong, near infra-red absorption band at 740 nm represents the Q_y-transition of BChl c in the antenna complex. Compared to the absorption of BChl c in acetone/methanol (7:2), the Q_y-transition is red shifted by more than 70 nm (668 nm → 740 nm). A small fraction of liberated BChl c is present in the preparation and causes an absorption maximum at 668 nm. The strong absorption band at 462 nm represents the Soret-band of the BChl c-complex, which is 27 nm red shifted compared to BChl c in solution (435 nm). The carotenoids in the chlorosomes add to the broad absorption of the Soret-band. The maximum at 326 nm is also caused by a transition of BChl c.

The absorption band at 796 nm is due to the chlorosome-specific BChl a complex (B790), which acts as a transmitter to the B806-866 complex in the cytoplasmic membrane. The absorbance ratio A_{740 nm}/A_{796 nm} was 15 and the molar ratio of BChl c /BChl a was 18. The broad absorption at 866 nm was due to BChl a, which is not associated with the B790 complex. This absorption was caused by a minor fraction of the B806-866 antenna complex, which had not completely been separated from the chlorosomes during purification. Interestingly, this absorption tended to increase slightly upon longer storage of the chlorosomes.
2.2.2. Circular dichroism

The CD spectrum of chlorosomes in the native state showed a conservative, S-shaped CD signal with a negative maximum at 723 nm and a positive maximum at 750 nm (Fig. 21). The zero-crossing was at 740 nm. In addition, there was an S-shaped signal centred around 325 nm and a shoulder around 780 nm was observed in some samples. Approximately the same spectrum was observed for the whole-membrane fraction of *C. aurantiacus* cells, which consists of the cytoplasmic membrane with the chlorosomes attached to it (Fig. 22).
Fig. 21. CD spectrum of native chlorosomes in 10 mM Tris/HCl, pH 8.0. The absorbance at 740 nm was adjusted to 2.5 and the pathlength of the cuvette was 5 mm.

Fig. 22. CD spectrum of the whole-membrane fraction of low-light grown cells in 10 mM Tris/HCl, pH 8.0. The absorbance at 740 nm was 1.1 and the pathlength of the cuvette 2 mm.
2.2.3. Fluorescence

The fluorescence emission spectrum of low light chlorosomes in the native state showed two maxima at 750 nm and 806 nm, when chlorosomes were excited in the Soret-band of the BChl $c$ aggregate at 460 nm (Fig. 23). The fluorescence at 750 nm stemmed from BChl $c$ absorbing at 740 nm and that at 806 nm from BChl $a$ absorbing at 796 nm. Both emission bands were enhanced after reduction of the chlorosome suspension by the addition of a small amount of sodium dithionite.

![Fluorescence emission spectrum](image)

Fig. 23. Fluorescence emission spectrum of low light chlorosomes in the native state in 10 mM Tris/HCl, pH 8.0 (solid line). The same sample after the addition of a small amount of sodium dithionite (dashed line). The excitation wavelength was set to 464 nm.
2.3. Electron microscopy

Negative staining electron micrographs of the chlorosome preparation depicted ellipsoidal bodies with typical dimensions of 110 nm in length, 30 nm in width and 10 nm thickness (Staehelin et al., 1978). Single chlorosomes were hardly observed, instead, chlorosomes stuck together. Occasionally, patches of cytoplasmic membrane were associated with chlorosome aggregates (Fig. 24 a,b).

Fig. 24. (a) Negative staining electron micrographs (2% uranyl acetate) of native chlorosomes. Magnified 128,000 times (by Staehelin).
Fig. 24. (b) Negative staining electron micrographs (2% uranyl acetate) of native chlorosomes associated with a cytoplasmic membrane patch. Magnified 128,000 times (by Staehelin).

The metal-coated specimen revealed most interesting substructures (Fig. 25a,b): Chlorosomes were partly covered with globular particles of about 5 nm diameter. The particles appeared to be arranged in two or three rows of ten particles parallel to the long chlorosome axis. As the chlorosome preparations did not seem to contain reaction centres judging from their absorption spectra, the particles might not represent reaction centres. It is conceivable that the BChl $\alpha$-protein complex of the baseplate (B796) constitutes visible structures, which correspond to the number of reaction centres. In addition to these readily visible structures some of the chlorosomes showed very faint striations at right angles to the longest chlorosome axis.
Fig. 25. (a) Native chlorosomes. Unidirectionally shadowed specimen, Pt/C, 45°. Magnified 171,000 times (by Staehelin).
2.4. Pigment-protein stoichiometry in low light chlorosomes

The stoichiometry between the 5.7 kDa polypeptide and BChl c was determined using two different methods. For the first method the starting material was lyophilized chlorosomes, whereas the second method was performed with a chlorosome suspension. In both cases, the 5.7 kDa polypeptide was quantified by amino acid analysis. For the interpretation of the amino acid compositions the number of phenylalanine residues was
set to two residues per polypeptide molecule, because this resulted in a reasonable fit for most of the other amino acids compared with the amino acid composition deduced from the sequence of the 5.7 kDa polypeptide. The first set of data was obtained with the 5.7 kDa protein isolated from three different lyophilized chlorosome preparations (Table 7). The second set of data was obtained with protein isolated from four different chlorosome suspensions (Table 8).

Table 7.
Amino acid compositions of the 5.7 kDa polypeptide isolated from three different lyophilized chlorosome preparations (I,II,III). Phenylalanine was set to two residues per polypeptide molecule.

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<th>III</th>
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<td>5.9</td>
<td>5.7</td>
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Table 8. Amino acid compositions of the 5.7 kDa polypeptide isolated from four different chlorosome suspensions (I,II,III,IV). Phenylalanine was set to two residues per polypeptide molecule.

<table>
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<th>amino acid</th>
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<th>III</th>
<th>IV</th>
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<td>1.4</td>
<td>1.3</td>
<td>1.0</td>
<td>1.4</td>
</tr>
</tbody>
</table>

The data of the amino acid compositions revealed that, on the whole, the values for histidine were too high by a factor of 2, glycine was too high by a factor of 1.4 and proline by a factor of 1.5. The values for methionine, on the other hand, were too low by a factor of 2.5. The divergencies between the measured amino acid compositions and the composition deduced from the amino acid sequence originated from systematic errors of the amino acid analysis. The value for phenylalanine, however, was considered to represent the true amount of hydrolysed 5.7 kDa polypeptide and was used for the calculation of the stoichiometries. Using the first method the molar ratio of the 5.7 kDa polypeptide to BChl c was equal to 1:21.5 ±2.8 (n=3). This value was corrected for the residual material that was not solubilized in the organic solvent extraction. The residual protein was equal to 5%-10% of the total amount of 5.7 kDa polypeptide found in the lyophilized chlorosomes. Using the second method the molar ratio of the 5.7 kDa polypeptide to BChl c was 1:19.1 ±3.3 (n=4).
2.5. Bacteriochlorophyll composition

The BChl c composition of *C. aurantiacus* consists of only the 4-Et,5-Me homologue, which is esterified predominantly with stearyl alcohol (Gloe and Risch, 1978). The separation of the BChl extract from whole cells of *C. aurantiacus* yielded six BChl c-containing fractions (Fig. 26). According to Fages et al. (1990) the main fractions of BChl c in *C. aurantiacus* were the stearyl and the geranylgeranyl esters. Moreover, the cetyl, the oleyl and the phytol esters were found by Fages et al. (1990). The fractions 1, 3, 5 and 6 contained almost pure BChl c with an absorption maximum at 668 nm in 93% methanol. In all the fractions, however, a broad, but small absorption between 750 and 770 was also present. Fraction 2 was clearly composed of a mixture of BChl c and BChl a with maxima at 668 nm and 767 nm. Fraction 4 contained BChl d absorbing at 658 nm and 430 nm in 93% methanol. BChl d might be a biosynthetic precursor of BChl c and is more frequently found in high-light grown *C. aurantiacus* cells (Brune et al., 1987).

![Fig. 26. RP-HPLC on Nucleosil 1000-7, C18, of a bacteriochlorophyll extract of low-light grown cells of *C. aurantiacus*. The fractions 1, 3, 5 and 6 contained almost pure BChl c. Fraction 2 was a mixture of BChl c and BChl a. Fraction 4 was found to contain BChl d.](image-url)
III.3. Modification of low light chlorosomes

3.1. General experimental scheme

The following experiments aimed at acquiring data on the BChl c-binding function of the 5.7 kDa polypeptide in the chlorosome of *C. aurantiacus*. For that purpose chlorosomes were modified by means of proteolytic treatment, treatment with 1-hexanol or SDS. Spectral changes, brought about by the different treatments of the chlorosomes, were monitored using absorption spectroscopy, circular dichroism and fluorescence spectroscopy. The spectral changes were correlated to alterations of the polypeptide pattern observed on SDS-PAGE and by RP-HPLC. In addition, modified chlorosomes were analysed using negative staining electron microscopy.

3.2. Proteolytic treatment

3.2.1. Screening of proteases

A first step for the application of proteases to chlorosomes of *C. aurantiacus* was the screening of several proteases. Six different enzymes were tested with respect to their effects on the polypeptide pattern of the chlorosome. The serine proteases chymotrypsin, elastase, proteinase K, subtilisin and trypsin and the Zn-metalloprotease thermolysin were chosen. The susceptibility of chlorosomes towards the enzymes was tested in 10 mM Tris/HCl, pH 8.0 at 37°C for 1 h, and in the same buffer saturated with 1-hexanol. Tris buffer saturated with 1-hexanol was used for the modification of chlorosomes in subsequent experiments and the effect of 1-hexanol on the chlorosomes will be described in the respective section. The effect of the proteolytic activity of the different proteases on the polypeptide pattern of the chlorosomes on SDS-PAGE is depicted in Fig. 27. The lanes 2-7 show chlorosomes treated with thermolysin (2), chymotrypsin (3), elastase (4), proteinase K (5), subtilisin (6) and trypsin (7) in 10 mM Tris/HCl, pH 8.0. The lanes 10-14 show chlorosomes treated with the same enzymes (in the same sequence) in 10 mM Tris/HCl, pH 8.0, saturated with 1-hexanol. The lanes 15 and 16 represent the blanks without an enzyme, i.e. chlorosomes in 10 mM Tris/HCl, pH 8.0 (15) and in buffer saturated with 1-hexanol (16). As can be seen on the gels the 18 kDa polypeptide was cleaved by every enzyme except from elastase in 1-hexanol.
saturated buffer. Similarly, the 11 kDa polypeptide was cleaved by nearly every enzyme except elastase under both conditions. Nevertheless, it is also possible that a band with an apparent Mr of 11000 was a fragment of the 18 kDa polypeptide. The 5.7 kDa polypeptide resisted proteolytic cleavage by all enzymes tested in 10 mM Tris/HCl, pH 8.0. In 1-hexanol saturated buffer, however, it was totally degraded by proteinase K and subtilisin. For subsequent experiments, proteinase K was chosen, because it was able of totally degrading the 5.7 kDa polypeptide in 1-hexanol saturated buffer, which led to changes of the spectral properties of chlorosomes. Trypsin, on the other hand, cleaved only the 11 and the 18 kDa polypeptide, but not the 5.7 kDa polypeptide. Trypsin was selected with the intention to compare its effects on the spectral properties of chlorosomes to those induced by proteinase K.

![Polypeptide pattern of chlorosomes digested with thermolysin (lane 2), chymotrypsin (3), elastase (4), proteinase K (5), subtilisin (6), trypsin (7) in 10 mM Tris/HCl, pH 8.0. In 1-hexanol saturated buffer, however, it was totally degraded by proteinase K and subtilisin. For subsequent experiments, proteinase K was chosen, because it was able of totally degrading the 5.7 kDa polypeptide in 1-hexanol saturated buffer, which led to changes of the spectral properties of chlorosomes. Trypsin, on the other hand, cleaved only the 11 and the 18 kDa polypeptide, but not the 5.7 kDa polypeptide. Trypsin was selected with the intention to compare its effects on the spectral properties of chlorosomes to those induced by proteinase K.](image-url)
10 mM Tris/HCl, pH 8.0 and with thermolysin (8), chymotrypsin (10), elastase (11), proteinase K (12), subtilisin (13), trypsin (14) in 10 mM Tris/HCl, pH 8.0, saturated with 1-hexanol. Lane 15: Chlorosomes in 10 mM Tris/HCl, pH 8.0, lane 16: Chlorosomes in 10 mM Tris/HCl, pH 8.0, saturated with 1-hexanol. Lanes 1 and 9: Low molecular weight standard (Sigma).

### 3.2.2. Proteolytic treatment with trypsin

Native low light chlorosomes were treated proteolytically using trypsin in 10 mM Tris/HCl, pH 8.0, at 37°C for 1 h. The absorption spectrum of low light chlorosomes in the native state shown in Fig. 20 was not changed significantly by the treatment. The absorption intensity at 740 nm remained stable and only the absorption band at 796 nm decreased slightly. In the same way the CD spectrum remained almost unchanged apart from a very slight increase in the rotational strength of the S-shaped signal at 740 nm and the disappearance of the shoulder at 780 nm (Fig. 28).

*Fig. 28. CD spectrum of native chlorosomes in 10 mM Tris/HCl, pH 8.0 (solid line) and trypsin treated chlorosomes (dotted line). The absorbance at 740 nm was adjusted to 2.5 and the pathlength of the cuvette was 5 mm.*
The polypeptide pattern of trypsin treated low light chlorosomes on SDS-PAGE revealed that the 11 and 18 kDa polypeptide were cleaved and gave rise to new bands in the gel (Fig. 28, lane 2). The largest fragment with an apparent Mr of 11000 was identified as an N-terminal fragment of the 18 kDa polypeptide (SNETT...) by amino acid sequence analysis. Underneath the N-terminal fragment of the 18 kDa polypeptide there were three distinct bands. The first band with a Mr around 10000 started with the sequence SVPVR-ND... and could not be assigned to a known protein. The second band with the sequence RNDSFV... was a fragment of the 11 kDa polypeptide. The third band underneath, which was also present in undigested chlorosomes, was N-terminally blocked. This indicated that it was the uncleaved B806-866-β polypeptide. In contrast to the 11 and the 18 kDa polypeptide, the 5.7 kDa polypeptide was not affected by trypsin. The staining intensity of the band at Mr 5700 remained unchanged. This finding was substantiated by analysis of the polypeptide pattern on RP-HPLC.

![Fig. 29. Comparison of trypsin and proteinase K treated chlorosomes on SDS-PAGE. Lanes 1 and 4: Untreated chlorosomes, lane 2: Chlorosomes digested with trypsin, 1 h at 37°C, lanes 3 and 6: Low molecular weight standard (Sigma), lane 5: Chlorosomes digested with proteinase K, 1 h at 37°C.](image)
3.2.3. Proteolytic treatment with proteinase K

When native low light chlorosomes were treated using proteinase K in 10 mM Tris/HCl, pH 8.0, at 37°C for 1 h, the absorption spectrum changed slightly, as can be seen in Fig. 30. The 796 nm band went down almost quantitatively. The absorption maximum at 740 nm decreased by about 10% accompanied by an increase of the absorption band of liberated BChl c at 668 nm. The decrease was much less pronounced, only 2%-4%, upon treatment with proteinase K at 30 °C or below. In the CD spectrum (Fig. 31) there was a twofold increase in the rotational strength of the S-shaped signal at 740 nm compared to native chlorosomes. In addition, a new positive feature appeared at 462 nm. The polypeptide pattern of proteinase K treated chlorosomes on SDS-PAGE is depicted in Fig. 29, lane 5. The 11 and the 18 kDa polypeptide were degraded and only small amounts of fragments appeared in the range of 7000-11000 (Mr). Unlike the trypsin treatment, the treatment with proteinase K affected the 5.7 kDa polypeptide, which was obvious from the smaller staining intensity of the band at 5700 (Mr). The analysis of the proteinase K treated sample on RP-HPLC confirmed the partial degradation of the 5.7 kDa polypeptide. Quantification of the remaining 5.7 kDa protein by amino acid analysis (Table 9) revealed that approximately 60% had been degraded.
Fig. 30. Absorption spectrum of low light chlorosomes treated with proteinase K at 37°C for 1 h (dashed line) and control without proteinase K (solid line).
Fig. 31. CD spectrum of native chlorosomes in 10 mM Tris/HCl, pH 8.0 (solid line) and proteinase K treated chlorosomes (dotted line). The absorbance at 740 nm was adjusted to 2.0 and the pathlength of the cuvette was 5 mm.
Table 9.
Quantification of the 5.7 kDa polypeptide after treatment of chlorosomes with proteinase K and subsequent isolation of remaining protein.

<table>
<thead>
<tr>
<th>amino acid</th>
<th>amount [nmol]</th>
<th>%</th>
<th>amount [nmol]</th>
<th>%</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASP</td>
<td>2.43</td>
<td>11.7</td>
<td>0.74</td>
<td>9.6</td>
<td>12.2</td>
</tr>
<tr>
<td>THR</td>
<td>1.11</td>
<td>5.3</td>
<td>0.43</td>
<td>5.6</td>
<td>4.1</td>
</tr>
<tr>
<td>SER</td>
<td>1.69</td>
<td>8.1</td>
<td>0.84</td>
<td>10.9</td>
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</tr>
<tr>
<td>GLU</td>
<td>2.62</td>
<td>12.6</td>
<td>0.98</td>
<td>12.8</td>
<td>12.2</td>
</tr>
<tr>
<td>GLY</td>
<td>1.87</td>
<td>9.0</td>
<td>0.87</td>
<td>11.3</td>
<td>8.2</td>
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<tr>
<td>ALA</td>
<td>3.31</td>
<td>16.0</td>
<td>0.94</td>
<td>12.2</td>
<td>18.4</td>
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<tr>
<td>VAL</td>
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<td>7.9</td>
<td>0.46</td>
<td>6.0</td>
<td>8.2</td>
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<tr>
<td>MET</td>
<td>0.00</td>
<td>0.0</td>
<td>0.00</td>
<td>0.0</td>
<td>2.0</td>
</tr>
<tr>
<td>ILE</td>
<td>1.10</td>
<td>5.3</td>
<td>0.32</td>
<td>4.2</td>
<td>6.1</td>
</tr>
<tr>
<td>LEU</td>
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<td>2.5</td>
<td>0.30</td>
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<tr>
<td>TYR</td>
<td>0.33</td>
<td>1.6</td>
<td>0.00</td>
<td>0.0</td>
<td>2.0</td>
</tr>
<tr>
<td>PHE</td>
<td>0.74</td>
<td>3.6</td>
<td>0.19</td>
<td>2.5</td>
<td>4.1</td>
</tr>
<tr>
<td>HIS</td>
<td>1.59</td>
<td>7.7</td>
<td>1.19</td>
<td>15.5</td>
<td>2.0</td>
</tr>
<tr>
<td>LYS</td>
<td>0.00</td>
<td>0.0</td>
<td>0.00</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>ARG</td>
<td>1.36</td>
<td>6.6</td>
<td>0.42</td>
<td>5.5</td>
<td>6.1</td>
</tr>
<tr>
<td>PRO</td>
<td>0.44</td>
<td>2.1</td>
<td>0.00</td>
<td>0.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Due to a systematic error of the amino acid analysis the values for histidine were far too high. The fact that methionine was totally missing may have resulted from oxidation during hydrolysis. Nevertheless, the total amount of amino acids served for the quantification of the remaining 5.7 kDa protein.
3.3. Reversible conversion of chlorosomes with 1-hexanol

When chlorosomes were suspended in 10 mM Tris HCl, pH 8.0, saturated with 1-hexanol at 25°C (59 mM 1-hexanol) the absorption band at 740 nm disappeared and a new band appeared at 668 nm (Fig. 32).

![Absorption spectrum of chlorosomes](image)

**Fig. 32.** Absorption spectrum of chlorosomes in 10 mM Tris/HCl, pH 8.0, saturated with 1-hexanol (59 mM) at 25°C. The 740 nm band disappeared and a new band appeared at 668 nm.

The blue shift of the Q_y transition of BChl c in the chlorosomes was strongly temperature dependent and did not go to completion at temperatures near 0°C. Fig. 33 shows the conversion of a chlorosome suspension in buffer saturated with 1-hexanol on ice upon heating to 20°C. The absorption band at 668 nm increased at the expense of the band at 740 nm. During the course of the conversion isosbestic points appeared at
720 nm and 460 nm. Provided there was no molecular species involved that had no absorption at the respective wavelength, it can be concluded that there were only two molecular species involved in the conversion.

![Graph showing conversion of chlorosomes in 59 mM 1-hexanol upon heating from 0°C to 20°C.](image)

Fig. 33. Conversion of chlorosomes in 59 mM 1-hexanol upon heating from 0°C to 20°C.

The blue shift was reversible when the chlorosomes were diluted with 10 mM Tris HCl, pH 8.0, to half the saturating 1-hexanol concentration (29.5 mM). Fig. 34 shows the absorption spectrum of chlorosomes treated with 59 mM 1-hexanol after dilution to 29.5 mM 1-hexanol compared with the original spectrum of native chlorosomes. As can be seen, the 740 nm band was not completely restored but attained, at the most, 80% of the original absorption intensity. Furthermore, the restored absorption band did not reach 740 nm, but a 2-15 nm shorter wavelength. It was frequently observed that the diluted chlorosomes in 29.5 mM 1-hexanol had the absorption maximum at 727 nm.
Fig. 34. Absorption spectrum of low light chlorosomes in the native state in 10 mM Tris/HCl, pH 8.0 (solid line) compared with chlorosomes diluted to 29.5 mM 1-hexanol after treatment with 59 mM 1-hexanol (dashed line).

The CD spectra of chlorosomes in 59 mM 1-hexanol and after dilution to 29.5 mM 1-hexanol are shown in Fig. 35. The S-shaped signal of native chlorosomes with a zero-crossing at 740 nm was converted to a signal with a positive maximum at 740 nm in 59 mM 1-hexanol. The rotational strength of the new feature was significantly smaller than that of native chlorosomes. The magnitude of the converted signal depended on how complete the conversion to the 668 nm absorbing form had been. It is noteworthy that all the samples in 59 mM 1-hexanol still showed a weak CD signal around 740 nm, even when the blue shift of the 740 nm band appeared to be complete in the absorption spectrum. After dilution to 29.5 mM 1-hexanol the S-shaped signal centred at 740 nm was restored to 90% of the original intensity (Fig. 35). This indicated an almost complete restoration to the original intermolecular dipole coupling among the pigments.
Fig. 35. CD spectrum of low light chlorosomes in 59 mM 1-hexanol, 10 mM Tris/HCl, pH 8.0 (solid line) and after dilution to 29.5 mM 1-hexanol (dotted line). The concentration of chlorosomes corresponded to an absorbance of 2.0 at 740 nm in the native state.

The fluorescence emission of chlorosomes in 59 mM 1-hexanol excited at 665 nm, i.e. in the Qy-band of monomeric BChl c, showed a very weak but broad emission around 720 nm (not shown). A much stronger emission band, however, was located around 800 nm. Providing this band stemmed from BChl a, the energy transfer from BChl c to BChl a was still working in 59 mM 1-hexanol. Surprisingly, the emission at 800 nm became much weaker compared to the BChl c emission at 676 nm, when chlorosomes in 59 mM 1-hexanol were excited at 438 nm, the Soret-band of monomeric BChl c. The fluorescence emission spectra in Fig. 36 show the dominating BChl c emission upon excitation at 438 nm. As revealed by the spectra run at different temperatures, the intensity of the emission was strongly temperature dependent.
Figure 36. Fluorescence emission spectra of chlorosomes in 59 mM 1-hexanol, 10 mM Tris/HCl, pH 8.0, upon excitation at 438 nm. The spectra were run at 21°C (dotted line), 24°C (dashed line) and 27°C (solid line).

3.4. Proteolytic treatment in 59 mM 1-hexanol

In order to investigate the role of the chlorosome proteins for the pigment organization we used the reversible conversion of chlorosomes with 1-hexanol in combination with proteolytic treatment. After conversion to the 668 nm absorbing state in buffer saturated with 1-hexanol the chlorosomes were treated with either proteinase K or trypsin and the rotational strength as well as the absorption of the pigments was measured. There were no significant changes in the absorption spectrum, apart from a 10 nm shift of the B790 band, when blue shifted chlorosomes in 59 mM 1-hexanol were treated with proteinase K. Unlike the absorption spectra, the CD spectra of chlorosomes in 59 mM 1-hexanol before and after the treatment with proteinase K differed significantly (Fig. 37).
Fig. 37. CD spectra showing the irreversible proteolytic modification of chlorosomes in 1-hexanol. Solid line: chlorosomes treated with proteinase K in 59 mM 1-hexanol, dotted line: control without proteinase K. The concentration of the chlorosomes corresponded to an absorbance of 4.5 at 740 nm in the native state and the pathlength of the cuvette was 5 mm.

The samples treated with proteinase K in 59 mM 1-hexanol gave rise to a giant positive signal with a maximum at 735 nm. The formation of the giant signal was concentration dependent and occurred only at BChl c concentrations above 20 μM. A different effect of the proteinase K treatment was observed in samples with low BChl c concentrations. There, the weak CD signal of chlorosomes in 59 mM 1-hexanol disappeared. In contrast to the remarkable effect of proteinase K, the treatment of chlorosomes in 59 mM 1-hexanol with trypsin had almost no effect on the CD spectrum, independent of the BChl c concentration.
Dilution of the proteinase K treated sample to 29.5 mM 1-hexanol did not restore the CD spectrum of the native chlorosome, but led to a strong, S-shaped signal centred around 730 nm shown in Fig. 38. The trypsin treated sample could not be restored to the original spectrum, either. The spectrum of trypsin treated chlorosomes after dilution to 29.5 mM 1-hexanol resembled the weak signal of converted chlorosomes in 59 mM 1-hexanol.

![Graph showing CD spectrum](image)

Fig. 38. CD spectrum of the proteinase K treated sample after dilution to 29.5 mM 1-hexanol.

The analysis of the samples on SDS-PAGE (Fig. 39) revealed that the chlorosome proteins were totally degraded by proteinase K in 59 mM 1-hexanol (lane 3). With trypsin, however, only the 11 and 18 kDa polypeptides were degraded and the 5.7 kDa polypeptide was not affected (not shown on SDS-PAGE). Analysis of the samples by RP-HPLC also clearly proved that the 5.7 kDa polypeptide was degraded by proteinase K in 59 mM 1-hexanol but not affected by trypsin (Fig. 40).
Fig. 39. SDS-PAGE showing the effect of proteinase K on the chlorosome proteins 59 mM 1-hexanol. Lane 1: Untreated chlorosomes; 2: Chlorosomes in 59 mM 1-hexanol; 3: Chlorosomes digested with proteinase K in 59 mM 1-hexanol; 4: Low molecular weight standard (Sigma). As can be seen in lane 2, the chlorosome polypeptides, especially the 18 kDa polypeptide, formed aggregates in 59 mM 1-hexanol and yielded weaker bands in the gel compared with the control.

Fig. 40. Comparison of the trypsin and the proteinase K treatment of hexanol-dissociated chlorosomes analysed by RP-HPLC (see figure on the next page). Reverse phase chromatography of chlorosome proteins as described in materials and methods. The linear gradient from 20% to 70% eluent B (acetonitrile, 0.1% trifluoroacetic acid) is drawn in and the chlorosome proteins are numbered: Peak 1: 5.7 kDa polypeptide, peak 2: 11 kDa, peak 3: 18 kDa. (A) Chlorosomes after incubation in 59 mM 1-hexanol. (B) Chlorosomes treated with trypsin in 59 mM 1-hexanol. (C) Chlorosomes treated with proteinase K in 59 mM 1-hexanol. As can be seen in panel B, the 18 kDa polypeptide was not totally degraded by trypsin. The remaining peak may be due to a large fragment with the same retention time.
3.4.1. Steady state fluorescence anisotropy of modified chlorosomes

The steady state fluorescence anisotropy of native chlorosomes was compared to that of proteinase K treated chlorosomes, chlorosomes in 59 mM 1-hexanol, chlorosomes treated with proteinase K in 59 mM 1-hexanol, BChl c in a monomeric form in buffer saturated with 1-hexanol and as aggregates in half the saturating 1-hexanol concentration. The results were summarized in Table 10.

Table 10.
Fluorescence anisotropy of chlorosomes and BChl c aggregates.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Emission wavelength</th>
<th>Anisotropy</th>
</tr>
</thead>
<tbody>
<tr>
<td>native chlorosomes</td>
<td>754 nm</td>
<td>0.25</td>
</tr>
<tr>
<td>proteinase K treated chlorosomes</td>
<td>749 nm</td>
<td>0.26</td>
</tr>
<tr>
<td>chlorosomes in 59 mM 1-hexanol</td>
<td>676 nm</td>
<td>0.013</td>
</tr>
<tr>
<td>chlorosomes in 59 mM 1-hexanol, proteinase K</td>
<td>676 nm</td>
<td>0.027</td>
</tr>
<tr>
<td>BChl c in 59mM 1-hexanol</td>
<td>676 nm</td>
<td>0.05</td>
</tr>
<tr>
<td>BChl c in 29.5mM 1-hexanol</td>
<td>745 nm</td>
<td>0.27</td>
</tr>
</tbody>
</table>

The fluorescence anisotropy was high for chlorosomes in the native state and after proteinase K treatment as well as for BChl c aggregates in 29.5 mM 1-hexanol. Anisotropy was low for all samples in 59 mM 1-hexanol and almost no difference was observed for untreated chlorosomes in 1-hexanol saturated buffer and the proteinase K treated sample.

3.4.2. Electron microscopic analysis

Electron micrographs of native chlorosomes, trypsin treated chlorosomes and chlorosomes in 59 mM 1-hexanol, before and after the treatment with proteinase K are shown in Fig. 41. The electron micrographs revealed no significant difference between the native chlorosomes and the trypsin treated chlorosomes. Both samples showed ellipsoid bodies of an average length of 110 nm and an average width of 30 nm. In
59 mM 1-hexanol the chlorosomes increased in length and width to about 160 nm and 50 nm, respectively. After the treatment with proteinase K in 59 mM 1-hexanol the sample showed much larger bodies of different size and shape. Most of the aggregates were ellipsoid, but there were also round particles with an average diameter of 30-50 nm (Table 11).

Table 11.
Sizes of chlorosomes in electron micrographs after treatment with proteases and 1-hexanol. The values are averages of more than 30 chlorosomes measured on three different micrographs for every single treatment.

<table>
<thead>
<tr>
<th>chlorosome treatment</th>
<th>chlorosome size in two dimensions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>length [nm]</td>
</tr>
<tr>
<td>native state</td>
<td>110 ±21</td>
</tr>
<tr>
<td>trypsin in 10 mM Tris/HCl, pH 8</td>
<td>110 ±18</td>
</tr>
<tr>
<td>59 mM 1-hexanol</td>
<td>160 ±34</td>
</tr>
<tr>
<td>59 mM 1-hexanol, proteinase K</td>
<td>220 ±70</td>
</tr>
</tbody>
</table>

Fig. 41. (a-d) (next page) Negative staining electron micrographs of various chlorosome samples. Native chlorosomes (a), trypsin treated chlorosomes (b), chlorosomes in 59 mM 1-hexanol (c) and chlorosomes treated with proteinase K in 59 mM 1-hexanol (d) (by Wehrli).
3.4.3. *Size-exclusion chromatography analysis of modified chlorosomes*

Upon filtration using a Millex filter (0.22 μm) isolated low light chlorosomes in 100 mM Tris/HCl, pH 8.0, were retained on the membrane. A single chlorosome with the dimensions 110 x 30 x 10 nm$^3$, however, should easily pass through the pores of the membrane. It appeared that chlorosomes sticked together, which was also evident from the electron micrographs. When isolated chlorosomes were suspended in 100 mM Tris/HCl, pH 8.0, in the presence of 0.1% (w/v) Miranol S2M-SF, they passed through the Millex filter unit. As a result, size-exclusion chromatography of native chlorosomes on Sephacryl S 500 was also performed in 100 mM Tris/HCl, pH 8.0, 0.1% (w/v) Miranol S2M-SF. Chlorosomes treated with 59 mM 1-hexanol were run in the same buffer saturated with 1-hexanol. The elution profiles of native chlorosomes, 1-hexanol treated chlorosomes and 1-hexanol and proteinase K treated chlorosomes are presented in Fig. 42. Native chlorosomes were eluted in two fractions. One part in the void volume and the other shortly afterwards. From electron microscopic studies it was clear that chlorosomes size was, to some extent, inhomogeneous ranging from 90 x 26 x 10 nm$^3$ to 130 x 34 x 10 nm$^3$ (see Table 11). In addition, the inhomogeneity in chlorosome size observed in the SEC experiment was probably due to the fact that the detergent treatment did not separate all chlorosomes from one another. The absorption spectra of the two fractions collected from the SEC revealed that the second fraction contained chlorosomes with stronger absorption from the B806-866 complex. This indicated that chlorosomes devoid of CM had a stronger tendency to stick together and thus, they were eluted in the void volume. Chlorosomes in 100 mM Tris/HCl, pH 8.0, 0.1% (w/v) Miranol S2M-SF, 59 mM 1-hexanol, were eluted as one fraction in the void volume. Chlorosomes treated with proteinase K in buffer saturated with 1-hexanol were also eluted as one fraction in the void volume, but the total absorption at 280 nm corresponded to only 1/3 of that observed for the untreated hexanol chlorosomes.
3.5. SDS treatment

When low light chlorosomes were treated with 0.3% SDS in 10 mM Tris/HCl, pH 8.0, the absorption intensity at 740 nm increased by about 20%. At the same time the CD spectrum changed significantly (Fig. 32). The relative intensity of the S-shaped signal at 740 nm (ΔA/A740 nm) increased by a factor of 5-10, the zero-crossing was shifted to 738 nm and a positive maximum appeared at 462 nm, reminiscent of the changes upon treatment with proteinase K.
Fig. 43. CD spectrum of chlorosomes treated with 0.3% SDS. The absorbance at 740 nm corresponded to 1.6 in the native state. The pathlength of the cuvette was 2 mm.

Size-exclusion chromatography of the SDS-treated chlorosomes on Sephacryl S500 led to the separation of two fractions. Fraction 1 was eluted in the void volume and contained the totality of BChls and no protein, as was revealed by SDS-PAGE. The CD spectrum of that fraction was identical to the spectrum of the sample before chromatography. Fraction 2 contained all the chlorosome proteins, which were not separated from one another because of the wide separation range of the size-exclusion matrix. In addition, this fraction also comprised carotenoids.
III.4. Influence of growth conditions on the composition of the photosynthetic apparatus of *Chloroflexus aurantiacus* cells

4.1. Whole cells

*Chloroflexus aurantiacus* cells adapted to different growth conditions by variation of the pigment composition. Changes in pigmentation reflected differences in the composition of the photosynthetic apparatus. Cultures of *C. aurantiacus*, which were grown phototrophically under different conditions, designated as low light and high light conditions, could readily be distinguished. High-light grown cultures were orange, whereas low-light grown cultures were dark green (Fig. 44). The absorption spectra of whole cells in 60% glycerol are shown in Fig. 45 (A),(B). Relative to the absorption intensity at 464 nm low light cells had a much stronger absorption band in the 740 nm region compared to high light cells. High light cultures were grown for different periods of time, namely 22 h, 30 h and 48 h, in order to observe changes in the pigment composition caused by self-shading. This experiment showed that the 740 nm band increased relative to the 464 nm band upon longer cultivation (22 h→48 h).

Fig. 44. Batch cultures of *C. aurantiacus* grown under high light (left) and low light conditions (right) in srew cap bottles.
Fig. 45. Absorption spectra of whole cells of *C. aurantiacus* in 60% glycerol. (A) Low-light grown cells. (B) High-light grown cells after 22 h of growth (solid line) and after 48 h of growth (dotted line).

### 4.2. Characterization of high light chlorosomes and comparison with low light chlorosomes

#### 4.2.1. Isolation of high light chlorosomes

The isolation of chlorosomes from high-light grown cells was performed analogously with the isolation of low light chlorosomes. The absorption spectrum of the whole-membrane fraction is represented in Fig. 46.
Fig. 46. Absorption spectrum of the whole-membrane fraction in 10 mM Tris/HCl, pH 8.0, prepared from cells grown under high light conditions for 22 h.

It exhibited absorption bands at 865 nm, 803 nm, 739 nm and 465 nm, similar to the low light WMF, but with completely different ratios. The carotenoid absorption in the 425-495 nm range was extremely high compared to the BChl c band at 739 nm. The 739 nm absorption, on the other hand, was weak compared to the extinction in the 280 nm region. Compared to the WMF of low light cells the absorption ratio A740 nm/A866 nm in the WMF isolated from cells grown under high light conditions for 22 h was ten times lower. Equally, the BChl c /BChl a ratio was about 1:1 in high light membranes compared to 11:1 in low light membranes. After solubilization of the WMF with Miranol S2M-SF and sucrose density gradient centrifugation, three bands were obtained as depicted in Fig. 47. The absorption spectra of the respective bands are shown in Fig. 48. Band 1 contained highly enriched B806-866 complex with a strong
absorption of carotenoids. Band 2, in the 30% layer, contained all the 740 nm absorbing material together with a strong 796 nm absorption and a very high carotenoid portion. The third band, at high density, was found to consist mainly of carotenoids. The second band was collected and further purified by several washings in 10 mM Tris/HCl, pH 8.0. The purified material, which showed the absorption spectra represented in Fig. 49, was designated as high light chlorosomes in the native state.

Fig. 47. Schematic representation of the sucrose density gradient. Numbers one to three indicate the layers, which were analysed by absorption spectroscopy. The corresponding absorption spectra are shown in Fig. 48.
Fig. 48. Absorption spectra of the different layers in the sucrose density gradient.
Fig. 49. Absorption spectra of high light chlorosomes in 10 mM Tris/HCl, pH 8.0. Solid line: Chlorosomes isolated from 22 h high-light grown cells, dashed line: Chlorosomes isolated from 48 h high-light grown cells.

4.2.2. Spectroscopic properties

The effects of low light and high light conditions on the pigment composition of the whole-membrane fraction and the isolated chlorosomes as well as the influence of the length of growth on the high light cultures were obvious in the absorption spectra (Figs. 17, 20, 46 and 49) and were summarized in Table 12. Under low light conditions the ratio of BChl c to BChl a in the isolated chlorosomes was ten times higher compared to the chlorosomes from cells grown under high light conditions for
22 h. Prolonged growth under high light conditions led to an increase of the BChl c to BChl a ratio. Based on the absorption intensity at 268 nm the absorption at 740 nm was about 5 times higher in low light chlorosomes.

Table 12.
Effect of light intensity and duration of growth on the pigment composition of the whole-membrane fraction and the isolated chlorosomes

<table>
<thead>
<tr>
<th>growth conditions</th>
<th>whole-membrane fraction</th>
<th>isolated chlorosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A740/ A866</td>
<td>BChl c/ BChl a [mol/mol]</td>
</tr>
<tr>
<td>low light</td>
<td>25</td>
<td>11</td>
</tr>
<tr>
<td>high light 22 h</td>
<td>2.5</td>
<td>0.9</td>
</tr>
<tr>
<td>high light 30 h</td>
<td>3.3</td>
<td>1.4</td>
</tr>
<tr>
<td>high light 48 h</td>
<td>3.4</td>
<td>1.6</td>
</tr>
</tbody>
</table>

The CD spectra of chlorosomes isolated from cells grown under low light and high light conditions showed different intensities of the near infra-red signal. A typical CD spectrum for low light chlorosomes, as shown in Fig. 21, had a strong, S-shaped signal centred at 740 nm. Based on the OD of the 740 nm band (ΔA/ΔA740 nm) the high light chlorosomes exhibited a six fold weaker CD effect in the 740 nm region (Fig. 50) than low light chlorosomes. The relative intensities of the CD signals at 740 nm of chlorosomes isolated from high light cultures increased by a factor of two upon prolonged growth. The near infra-red CD signals of the different chlorosome preparations were about the same with respect to the position of the zero-crossing (740 nm), the positive maximum (755 nm) and the negative maximum (725 nm).
4.2.3. Response to proteolytic treatment

Low light and high light chlorosomes were affected to different extents by proteolytic treatment in 10 mM Tris/HCl, pH 8.0, at 30°C. As can be seen in Table 13, low light chlorosomes hardly responded to proteolytic treatment. The degradation of the 11 and 18 kDa polypeptides with trypsin did not change the absorption intensity of the 740 nm band at all. Treatment with proteinase K, which partly degraded the 5.7 kDa polypeptide in addition to the 11 and 18 kDa polypeptide, led only to a very slight decrease of the 740 nm band. In high light chlorosomes, however, trypsin brought about a 9% decrease of the absorption intensity at 740 nm and proteinase K caused a 60% decrease. At the same time a 3 nm blue shift of the 740 nm band could be observed and the 668 nm absorption of liberated BChl c came up in the spectrum. Analysis on SDS-PAGE revealed that the 5.7 kDa polypeptide was completely degraded by proteinase K in high light chlorosomes (Fig. 51).
Table 13.
Remaining absorption intensity and blue shift of the 740 nm absorption band after proteolytic treatment with trypsin or proteinase K.

<table>
<thead>
<tr>
<th></th>
<th>low light chlorosomes</th>
<th>high light chlorosomes&lt;sup&gt;a)&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>remaining intensity [%]</td>
<td>blue shift [nm]</td>
</tr>
<tr>
<td>control</td>
<td>97</td>
<td>0</td>
</tr>
<tr>
<td>trypsin</td>
<td>97</td>
<td>0</td>
</tr>
<tr>
<td>proteinase K</td>
<td>95</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a)</sup> Chlorosomes isolated from cells after 22 h growth under high light conditions.

Fig. 51. SDS-PAGE comparing the degradation of the chlorosome proteins in high light chlorosomes upon digestion with trypsin and with proteinase K in 10 mM Tris/HCl, pH 8.0 at 30°C for 1 h. Lane 1: Low molecular weight standard (Sigma), lane 2: 22 h high light chlorosomes in the native state, trypsin treated sample, lane 4: proteinase K treated sample.

The relative CD intensity (ΔA/A740 nm) of low light chlorosomes doubled upon digestion with proteinase K but it did not change after treatment with trypsin (section III.3.2.2. and 3.2.3.). In the case of the 48h high light chlorosomes the relative intensity increased fivefold after the degradation of the 5.7 kDa polypeptide with proteinase K and remained constant with trypsin (Fig. 52).
Fig. 52. CD spectra showing the proteolytic treatment of 48 h high light chlorosomes, solid line: 48 h high light chlorosomes with an absorbance of 3.7 at 740 nm, dashed line: sample treated with trypsin for 60 min at 30°C, dotted line: sample treated with proteinase K for 60 min at 30°C. The pathlength of the cuvette was 1 mm.

4.2.4. Pigment-protein stoichiometries

The determination of the pigment-protein stoichiometries for high light chlorosomes was attempted by one of the methods employed for low light chlorosomes using chlorosome suspensions as the starting material. However, the RP-HPLC separation used for the preparation of the 5.7 kDa polypeptide from low light chlorosomes proved to be inapplicable to high light chlorosomes. The sample preparation, which involved the dissociation of suspended chlorosomes in urea and formic acid, did not allow a separation of the three main chlorosome polypeptides from high light chlorosomes by chromatography. Instead, the 5.7 kDa polypeptide was eluted in two broad peaks and the 11 and the 18 kDa polypeptide were not eluted at all. It seemed probable that the extraction of the proteins from their lipid environment was hindered by the higher carotenoid content of the high light chlorosomes. Consequently, the correct stoichiometry
between the 5.7 kDa polypeptide and BChl c could not be determined. Therefore, the ratio of 5.7 kDa polypeptide to BChl c in high light chlorosomes was roughly estimated based on both the stoichiometries measured in low light chlorosomes (1:21.5 ±2.8 and 1:19.1 ±3.3) and the absorption ratios A740 nm/A268 nm observed in the different chlorosome preparations. This method implied, that the absorption at 268 nm was mainly caused by the 5.7 kDa polypeptide, although the 11 and the 18 kDa polypeptide also contributed to this extinction. The absorption ratios A740 nm/A268 nm for high light chlorosomes were lower by factors of 5.2, 2.8 and 2.4 for 22 h, 30 h and 48 h high light chlorosomes compared to the absorption ratio of low light chlorosomes. The stoichiometries of the 5.7 kDa polypeptide to BChl c for high light chlorosomes were obtained by multiplication of the average stoichiometry of low light chlorosomes of 1:20 by the above factors. The stoichiometries amounted to 1:4, 1:7 and 1:8 for chlorosomes isolated from cells grown under high light conditions for 22 h, 30 h and 48 h, respectively. Additional information about the different pigment-protein stoichiometries in high light and low light chlorosomes came from analysis on SDS-PAGE. It was obvious that, based on the same amounts of BChl c, the high light chlorosomes contained several times more of the 3 major chlorosome polypeptides 5.7 kDa, 11 kDa and 18 kDa (Fig. 53).

Fig. 53. SDS-PAGE showing the polypeptide patterns and the difference in the pigment-protein stoichiometries of low light and high light chlorosomes. Lane 1: Low molecular weight standard (Sigma). Lane 2: Low light chlorosomes. Lane 3: High light chlorosomes. Both chlorosome samples contained the same amount of BChl c.
IV. Discussion

IV.1. Chlorosome polypeptides from *Chloroflexus aurantiacus*

1.1. Structural aspects of the presumable BChl c-binding polypeptide

Wechsler et al. (1985) put forward the hypothesis of a pigment-protein complex for the BChl c antenna in the chlorosome involving the 5.7 kDa polypeptide and 7 BChl c molecules. The authors supposed that the 5.7 kDa polypeptide would preferentially form an α-helix between Trp 5 and Ile 42 (38 residues, helix length 57 Å). As was remarked by Gerola et al. (1988), the secondary structure prediction according to Chou and Fasman (1978) does not necessarily support an α-helical arrangement over the whole length of 38 residues. Using the Chou and Fasman prediction with a window size of 7 residues (Fig. 54) there could well be a short α-helix between Ser 7 and Ala 14 and a second helix between Ala 31 and Asn 41. The structure in between would be β-sheet. The prediction, however, is based on X-ray data of water-soluble globular proteins and it is questionable, whether it can be of value for the 5.7 kDa polypeptide, which is located in a lipid environment and may bind a number of BChl molecules.

The fact that histidine residues play a major role in pigment-binding in different light-harvesting complexes as well as the homology between the chlorosome polypeptides from different Chlorobiaceae species and *C. aurantiacus* (Wagner-Huber et al., 1988, 1990) suggest that His 24 in the 5.7 kDa polypeptide is a possible binding site for BChl c. Interesting, in this respect, is the observation that the amino acid sequence of the 5.7 kDa polypeptide exhibits a remarkable symmetry around His 24 (Brunisholz, personal communication). As depicted in Fig. 55, there is an indirect repeat of chemically similar and identical amino acid residues of the sequence Val 13 to Gln 22, which is inversely repeated from Gln 26 to Thr 35. The symmetry is especially interesting considering possible pigment-binding amino acid residues other than histidine. As proposed by Wechsler et al. (1985), the amide oxygens of glutamine and asparagine
may serve as fifth ligand to the Mg atom in BChl c. Similarly, the carboxyl oxygen of aspartic acid or glutamic acid might act as ligands to the Mg cation. As depicted in Fig 55. Gln 15 and Gln 22 have symmetric counterparts in Gln 33 and Gln 26, respectively. Furthermore, Asp 18 corresponds to Asn 30. The observed symmetry constitutes an argument in favour of an α-helical arrangement of the 5.7 kDa polypeptide. As described by Wechsler et al. (1985), the α-helical arrangement of the polypeptide results in a preferred ordering of the nucleophilic residues on one side of the helix. The symmetric order of the residues in the primary structure results in a repetition of pigment binding sites along the helix axis.

A possible role of aromatic amino acid side chains in modulating spectral properties of pigment molecule has been postulated by Brunisholz et al. (1984), Brunisholz et al. (1985) and Brunisholz and Zuber (1988). Trp 25, which has no counterpart in the observed symmetry of the 5.7 kDa polypeptide, and Phe 21 and Trp 27, which are in symmetric positions, may play a role in modifying the spectral properties of a BChl c molecule. Above that, these residues could serve as an energetic funnel: They may have the function to transfer energy from BChl c molecules to the B796 BChl α-protein complex, which is the next transmitter in the down-hill energy transfer.

Additionally, the symmetric structure of the 5.7 kDa polypeptide, with His 24 approximately in the middle, is also revealed by the hydropathy plot of the 5.7 kDa polypeptide (Fig. 56). In summary, the symmetry centred at His 24 may be an indication for the functional role of this residue and of the other nucleophiles in pigment-binding.

Fig. 54. α-helix and β-sheet potential within a window of 7 amino acids for the 5.7 kDa polypeptide from C. aurantiacus (Chou and Fasman, 1978).
Fig. 55. Amino acid sequence of the 5.7 kDa polypeptide from *C. aurantiacus*. An indirect repeat of chemically similar and identical amino acid residues (no computer-aided interpretation) is indicated by arrows. The symmetry axis located at His 24 is drawn in. Symmetric residues with pigment-binding capacity are connected by lines.

Fig. 56. Hydropathy plot (Kyte and Doolittle, 1982) for the 5.7 kDa polypeptide from *C. aurantiacus*. The average hydropathy was determined within a window of 7 amino acids.
1.2. Comparison of the 5.7 kDa polypeptide with the 6.3 kDa polypeptides from Chlorobiaceae

Amino acid sequence analyses indicated that the 5.7 kDa polypeptide of *C. aurantiacus* chlorosomes and the smallest and most abundant polypeptides in the chlorosomes of Chlorobiaceae (6.3 kDa) were related by a homology of maximally 30% (Wagner-Huber et al., 1988, 1990). The histidine residue and the two adjacent amino acids are conserved in all species of the Chlorobiaceae and *C. aurantiacus*. This supports the BChl-binding function of the histidine residue. The polypeptides of the Chlorobiaceae species also possess a discernible symmetry. The +2 and -2 positions on either side of the histidine are glutamic acid in all BChl c and d containing species of the Chlorobiaceae analysed so far (glutamine in *C. aurantiacus*) and only the BChl e containing species *Chlorobium phaeovibrioides* has alanine in the +2 position. At the +1 position all polypeptides have a tryptophan, which might be of importance for a directed energy transfer (energetic funnel).

Nevertheless, the pigment-binding modes in the chlorosomes of *C. aurantiacus* and the Chlorobiaceae are expected to be different because of the differences in the pigment-protein ratios and in rod diameters of the chlorosome core.

An intriguing similarity between the 5.7 kDa polypeptide and the 6.3 kDa polypeptides is the fact that they are both synthesized as precursors of 79 amino acids that are subsequently processed at their C-termini to mature proteins of either 52 residues (*C. aurantiacus*) or 58 or 59 residues (Chlorobium species) (Chung et al., 1994). As pointed out by the authors, carboxy-terminal processing events are relatively rare in bacteria (C-terminal processing of the D1 protein of PSII in cyanobacteria, α/β polypeptides of the core complex in *Rhodospirillum rubrum*).

Summarizing the similarities between the 5.7 kDa polypeptide from *C. aurantiacus* and the 6.3 kDa polypeptides from Chlorobiaceae it is very likely that these polypeptides serve related functions in the chlorosomes.

1.3. The 11 and 18 kDa polypeptides

The amino acid sequences of the 11 and the 18 kDa polypeptide will be available in the near future as a result of the cloning and sequencing of the respective genes by Niedermeyer and Feick (EMBO Workshop 'Green Bacteria and Heliobacteria', 1993 in Nyborg, Denmark). As reported by Niedermeyer, the two sequences showed no
significant homologies to any known pigment-binding protein. The original function proposed by Feick and Fuller (1984), according to which these polypeptides form the chlorosome envelope, was thus confirmed. Nevertheless, it is surprising that degradation of the 11 and 18 kDa polypeptide with trypsin did not show noticeable changes in the chlorosome shape and size on electron micrographs (Fig. 41b). A possible explanation may be that the remaining fragments were sufficient to maintain the overall chlorosome structure.

1.4. Minor protein components in the chlorosomes

Low light chlorosomes contained several proteins in addition to the most abundant 5.7 kDa polypeptide and the 11 and 18 kDa polypeptides. All of these were present in much smaller amounts compared to the main polypeptides. In three cases N-terminal sequences of proteins with apparent M_r of 70000, 18000 and 8000 were determined. No significant sequence homologies to known protein sequences were found in the data bases. The functions of the low abundance proteins are not known. In any case, a pigment-binding protein has to be a highly abundant component in order to build up an antenna system of repeating units. In that sense, polypeptides of low abundance are not likely to have pigment-binding functions.

1.5. The base-plate protein

The energy collected by the BChl c-antenna is further transferred onto the BChl a-containing B790 complex (base-plate) within the chlorosome. BChl a as part of the chlorosomes is thought to be associated with a 5.8 kDa polypeptide in the pigment-protein complex of the base-plate (Feick and Fuller, 1984). The polypeptide pattern of high light chlorosomes with a strong B790 absorption band, i.e. a low BChl c to BChl a ratio, comprised the three major chlorosome proteins (18, 11 and 5.7 kDa), but there was no valuable candidate for the base-plate protein in the 6-8 kDa range (Fig. 53). The two bands which were located between the 11 and the 5.7 kDa band were identified as the B806-866-β (lower band) and an unknown amino acid sequence (upper band).
The N-terminus of the unknown sequence was determined, namely SVKESGGIFGMFREVFFWEQV-NWAEE, and it remains unclear, whether this represents the base-plate protein or whether the base-plate protein is located among the higher molecular weight polypeptides, which were not resolved in the SDS-PAGE system used.

IV.2. Spectroscopic properties of low light chlorosomes

2.1. Circular dichroism of chlorosomes

The CD spectrum of a single BChl molecule in solution occurs because of the presence of asymmetrically substituted carbon atoms in the molecule. In solution the CD bands are either positive or negative as shown for BChl a in ether (Philipson and Sauer, 1972, Fig. 7). Upon the formation of dimers or oligomers the CD spectrum becomes more complicated. The different bands split into two (in the case of dimers) or more (in the case of oligomers) components with different signs, so that the CD bands appear S-shaped, as shown for the BChl a dimer in CCl4 (Sauer, 1972). The magnitude and the order of the sign (+/-, -/+ ) depend on the geometry of the dimer or oligomer. Higher aggregates of BChl a showed conservative1 S-shaped CD signals (Gottstein and Scheer, 1983).

BChl c essentially shows the same behaviour as BChl a (Olson and Pedersen, 1988, 1990). Several studies revealed a concentration dependence of the CD strength of BChl c in water-saturated CCl4 due to the formation of dimers and higher aggregates. (Olson and Pedersen, 1990, Uehara and Olson, 1992). The extent of aggregation was controlled by the solvent, the concentration of BChl c and the type of BChl c (different substituents at positions 4 and 5 on ring II and III) used. According to the present state of knowledge, the CD spectra of the chlorosomes in C. aurantiacus and the Chlorobiaceae are caused by aggregates of (two or more) strongly interacting BChl c molecules.

1 the negative and the positive portion of the CD signal sum to zero
2.1.1. Sign-reversed spectra

Previously published CD spectra of chlorosomes isolated from *C. aurantiacus* showed S-shaped signals centred around 740 nm with negative bands at about 752 nm and positive bands at about 735 nm (Betti et al., 1982, Van Dorssen et al., 1986, Blankenship et al., 1988, Brune et al., 1990). In contrast to that, sign-reversed CD spectra, which correspond to the spectrum of low light chlorosomes (Fig. 21), with the longest wavelength band positive, were obtained by Niedermeyer et al. (1992). Furthermore, there is a single report by Olson et al. (1985) on chlorosomes isolated from *Chlorobium limicola* with a positive maximum at 753 nm and a negative band at 724 nm. According to Brune et al. (1990), however, Chlorobium chlorosomes could possibly undergo a sign reversal due to their apparent instability. The difference between the two types of CD spectra of Chloroflexus chlorosomes mentioned above is not clear. The CD spectrum of isolated low light chlorosomes compares well to the spectrum of the freshly prepared whole-membrane fraction (Fig. 22), which is an indication of the intactness of the preparation. Moreover, proteolytic degradation of Chloroflexus chlorosomes resulted in an increase in rotational strength rather than a sign reversal. Niedermeyer et al. (1992) observed a 10–23-fold increase in CD intensity upon proteolytic treatment of chlorosomes with thermolysin. They suggested that the increase in rotational strength was due to a different aggregation size or geometry of BChl c in the digested chlorosomes.

As revealed by the present investigation, different growth conditions had an effect on the intensity of the near infra-red CD signal, but not on the order of the sign (-/+ or +/-).

2.1.2. Increased rotational strength

As shown in Fig. 28, the CD spectrum of low light chlorosomes remained almost unchanged upon proteolytic treatment with trypsin. This would suggest that the 11 and 18 kDa polypeptides, which were degraded, did not contribute to the pigment organization. Nevertheless, tryptic fragments of the two proteins were still present and could have structural functions. The results obtained with proteinase K support the possibility that the 5.7 kDa polypeptide plays an important role in the organization of the pigments. Its degradation obviously led to an irreversible increase in the rotational strength of the BChl c molecules. This was evident in the digestion of native chlorosomes in 10 mM Tris/HCl, where the 5.7 kDa polypeptide was only partly
degraded, and even more pronounced in the presence of 59 mM 1-hexanol, where the 5.7 kDa polypeptide was totally degraded (Fig. 57). Saturating concentrations of 1-hexanol obviously made the 5.7 kDa polypeptide susceptible to degradation by proteinase K. The electron micrographs (Fig. 41d) revealed that the proteinase K treatment in 59 mM 1-hexanol produced an inhomogeneous distribution of large aggregates.

Fig. 57. Experimental scheme of the combined treatment of low light chlorosomes using both 1-hexanol and proteolytic enzymes.

2.1.3. Giant circular dichroism

According to Keller and Bustamante (1986) there is a correlation between the CD intensity and the particle size of large chiral objects. A tenfold increase in the CD upon aggregation is common. The signal can be positive or negative and the band shape of a large aggregate often bears no resemblance to the CD of the constituent molecule. The authors have developed a theory with the central idea that when the dimensions of a chiral object are similar to the wavelength of the incident light, the large scale handedness of the object will have a much greater effect in enhancing or suppressing the absorption of
circularly polarized light than when the chiral object is small compared to wavelength. Furthermore, Keller and Bustamante (1986) stated that in order to get large CD magnitudes, distant parts of the system must be significantly coupled to each other. In the chlorosomes large CD magnitudes were only observed after the degradation of the 5.7 kDa polypeptide. In the degraded state in 59 mM 1-hexanol the band shape of the giant signal was nonconservative and changed to a conservative signal after dilution to 29.5 mM 1-hexanol. Although the molecular arrangement that gave rise to the giant CD signal in digested chlorosomes is not known, one can assume that in native chlorosomes the array of interacting BChl c molecules is much smaller (compared to the wavelength of the incident light of about 700 nm) than in proteinase K treated chlorosomes. As long as the 5.7 kDa polypeptide is still present in the chlorosome the CD signal of chlorosomes treated with 59 mM 1-hexanol returns to its original shape and original magnitude after dilution to 29.5 mM 1-hexanol. But when the 5.7 kDa polypeptide is degraded the BChl c molecules are allowed to interact strongly and produce irreversibly large CD magnitudes. This result strengthens the possibility of a BChl c-binding function of the 5.7 kDa polypeptide. BChl c aggregates could interact with the polypeptide via a single interaction site (e.g. the histidine residue), or via hydrophobic interactions, or via several nucleophilic residues, such as asparagine and glutamine residues, as proposed by Wechsler et al. (1985). In all cases the degradation of the 5.7 kDa protein would allow the pigments, which were originally separated in smaller clusters, possibly the rod-shaped elements, to form huge clusters by pigment-pigment interaction leading to a very strong increase in rotational strength.

2.2. 'Monomeric' state of BChl c in the chlorosomes

Treatment of chlorosomes with saturating concentrations of 1-hexanol caused a reversible blue shift of the Qy-transition (Matsuura and Olson, 1990). It appeared that there were only two species involved in the conversion (Fig. 33). The two species represented BChl c in two different aggregation states. The absorption band at 740 nm stemmed from a higher aggregate of BChl c (Brune et al., 1987b). The dissociation in 59 mM 1-hexanol gave rise to a form of the pigment absorbing at 670 nm. This species might represent the monomeric state of BChl c (Olson and Pedersen, 1988, 1990). Several pieces of evidence, however, indicated that the monomeric state was somehow organized in a molecular order. For instance, the electron micrographs (Fig. 41c)
revealed that these structures resembled swollen chlorosomes. According to the results obtained by SEC on Sephacryl S500, the chlorosomes in 59 mM 1-hexanol stuck together and were eluted in the void volume. No liberated BChl c was detected in the chromatography. Similarly, Matsuura and Olson (1990) showed by density gradient centrifugation that BChl c was still trapped in a larger molecular structure. Moreover, high energy transfer efficiency was observed from BChl c to the base-plate BChl a in treated chlorosomes (see also section III.3.3.). In addition, Matsuura and Olson (1990) observed only little fluorescence from the monomeric form in the chlorosome compared to BChl c in organic solvents. Yet, this was in contrast to our observation (Fig. 36). When excited in the Soret-band, BChl c in the hexanol treated chlorosomes gave rise to very strong, temperature dependent fluorescence at 676 nm, which can be expected for the dissociated BChl c aggregate.

All the same, a strong argument in favour of a molecular order of BChl c molecules in the hexanol treated chlorosomes was the fact that the hexanol treatment was reversible with respect to the absorption and the CD properties. In addition, LD measurements confirmed that the orientation of the Q_y-transitions was restored (Matsuura et al., 1993). Finally, the 'monomeric' form of BChl c in the chlorosomes still showed a weak CD signal around 740 nm (Fig. 35). The origin of this signal remained unclear. It may be speculated that it represented a minor fraction of BChl c bound to the 5.7 kDa polypeptide, which was hardly seen in the absorption spectrum, but visible in the CD spectrum.

2.2.1. Two spectral forms of BChl c in native chlorosomes

Matsuura et al. (1993) detected two major spectral forms of BChl c in native chlorosomes. One absorbing at 744 nm and the other at 727 nm. The 744 nm form had its Q_y-transition oriented completely parallel to the longest axis of the chlorosome and the 727 nm form was oriented slightly less parallel to the longest axis. The authors also observed that the 744 nm form could partly be converted to the 727 nm form at non-saturating concentrations of 1-hexanol. In the present investigation the 727 nm absorbing form was observed after dilution of hexanol treated chlorosomes to 29.5 mM 1-hexanol, accompanied by incomplete restoration of the 740 nm band.
2.2.2. Fluorescence lifetimes of \( \text{BChl c} \)

The results obtained by steady state fluorescence anisotropy measurements were ambiguous. The fluorescence lifetime of \( \text{BChl c} \) in methanol is \( \tau = 2.6 \) ns, or \( \tau = 6.5 \) ns in methylene chloride/5% methanol (Brune et al., 1988). The lifetime of \( \text{BChl c} \) is considerably shorter in the chlorosomes: \( \tau = 30-70 \) ps (Brune and Blankenship, 1987a). It follows that the anisotropy is high in the native chlorosome, because the fluorophore does not significantly change its orientation within 30-70 ps. On the other hand the fluorophore in solution may not remain stationary during the much longer lifetime of fluorescence. It is not possible to decide, whether the observed differences of anisotropy (Table 10) were due to different fluorescence lifetimes or to different freedom of movement of the fluorophores.

IV.3. Response to different growth conditions

3.1. Variations in the ratio of \( \text{BChl c} \) to \( \text{BChl a} \)

\( \text{C. aurantiacus} \) is capable of adapting to different anaerobic growth conditions with remarkable variations in the pigment content. Especially the \( \text{BChl c} \) to \( \text{BChl a} \) ratio can vary over a wide range. In contrast to the Chlorobiaceae, where \( \text{BChl a} \) always represents a minor fraction (5-10%) of the total \( \text{BChl} \) present (Sybesma, 1970), \( \text{BChl a} \) in \( \text{C. aurantiacus} \) may represent only a minor fraction, or it may be present in equal or even greater amounts than \( \text{BChl c} \) (Pierson and Castenholz, 1974). The synthesis of \( \text{BChl c} \) and \( \text{BChl a} \) seemed to be controlled independently. Pierson and Castenholz (1974) observed that specific \( \text{BChl} \) contents increased with decreasing light intensity and decreasing growth rate and that the \( \text{BChl c} \) to \( \text{a} \) ratio increased considerably at the same time.

When the light intensity was increased or the temperature of growth decreased, the cells responded with a decrease in \( \text{BChl c} \) content (Schmidt et al., 1980). Ultrathin section electron micrographs showed that cells with very low \( \text{BChl c} \) contents contained chlorosome attachment sites or base-plates but almost no chlorosomes. Upon reactivation of the \( \text{BChl} \) synthesis the specific \( \text{BChl c} \) content increased and chlorosomes became
visible again in thin-sectioned cells, while the BChl a content of the CM remained almost constant. Schmidt et al. (1980) suggested that BChl c was incorporated into preformed chlorosomes when BChl c synthesis was stimulated by transfer of cells grown at low temperature and 1,500 lx to high temperature and 400 lx. It remained unclear whether the observed changes in pigmentation were directly induced by light intensity or originated from different growth rates as a consequence of changes in illumination or temperature of growth.

Low light intensity in batch cultures of *C. aurantiacus* results from self-shading at high cell density. Photosynthesis is then limited by the amount of light received and one would expect that the bacteria adapt to low light intensity by increasing the amount of light-harvesting pigments. However, Oelze et al. (1991) reported that the BChl c to BChl a ratio of Chloroflexus cells remained relatively constant over 4 days of growth in batch cultures in complex medium containing casamino acids (equal to 'low light conditions' in the present investigation). This means that low light intensity as a consequence of self-shading did not affect this ratio.

### 3.2. Variable size of the chlorosome

Oelze and Fuller (1987) demonstrated that BChl c increased exponentially in continuous culture in response to a decreased growth rate. The same was found in batch cultures, where lowered growth rates due to limited availability of nutrients resulted in higher specific BChl c contents. Based on morphometric measurements of chlorosomes in cells grown in continuous cultures at various growth rates Golecki and Oelze (1987) found out that chlorosomes increased in volume as well as in numbers when the cells produced more BChl c. Above that, the authors put forward a variable degree of packing of BChl c in chlorosomes, which could account for the incorporation of higher amounts of BChl c into the chlorosomes.

It would be interesting to know, whether an increase in BChl c content is related to the synthesis of a specific chlorosome protein, namely the possible BChl c-binding protein. Oelze (1992) has developed a continuous culture of *C. aurantiacus*, where it is possible to induce BChl c synthesis by the addition of 5-aminolevulinate (ALA), without changing the growth rate. Quantitative data on chlorosome size and numbers on electron micrographs showed that the addition of ALA mainly caused the volume of the chlorosomes to increase. Interestingly, this was not accompanied by a proportional
increase of the chlorosome polypeptides, indicating variable pigment-protein stoichiometries (Oelze et al., 1992).

It appears that the photosynthetic apparatus in C. aurantiacus can respond to different growth conditions by varying the BChl c to a ratio. This can be accomplished on the one hand by varying the numbers of chlorosomes per RCs or the chlorosome size (Golecki and Oelze, 1987). On the other hand it is possible that the BChl c to a ratio within the chlorosome itself varies. In the present investigation a drastic change in the proportion of the B740 complex to the B790 complex could be observed in the high light chlorosomes. A variation of the amount of B740 complex relative to the amount of B790 complex may also be interpreted as a change of chlorosome size, which affects the two antenna complexes differently. This is in agreement with the findings of Schmidt et al. (1980), who observed base-plate structures or attachment sites with little or no chlorosomes in cells with a low BChl c to a ratio.

Furthermore, it has now become evident in the work of Oelze et al. (1992) and in this investigation (see below), that the stoichiometry between BChl c and the chlorosome proteins can vary considerably.

### 3.3. Influence of the growth medium

The growth conditions affected the BChl c to a ratio within the chlorosome of C. aurantiacus. In contrast to my observations, a constant ratio of BChl c to BChl a of 25:1, which was independent of growth conditions, was reported earlier by Feick et al. (1982). It is not clear, how the growth conditions of high light grown cells affected the BChl c to a ratio in the present study. Equally, one can only speculate about the reason for the lower pigment-protein ratio in high light chlorosomes compared to low light chlorosomes: The growth medium was nutrient-limited in several respects. It contained only 2 mM L-glutamate as the major carbon and nitrogen source. Glutamate was chosen, because it proved to be less efficient in stimulating BChl formation (Oelze and Söntgerath, 1992). It is the precursor for BChl c synthesis in the C5 pathway of tetrapyrrol formation and at the same time the major carbon source in the high light medium. Hence, it is possible that BChl c synthesis was impaired because protein and BChl c synthesis competed for the same substrate (Oelze et al., 1991, Oelze, 1992). Glycyl-glycine as the buffer substance, can only be consumed by C. aurantiacus after hydrolysis to glycine. Oelze et al. (1991) showed that glycyl-glycine was only used in the
latest stage of growth in batch cultures. Apart from glutamate and glycine, acetate also functioned as a carbon source. Its presence stimulated cell protein formation and was shown to avoid adaptation of the cells to an amino acid, when this amino acid was offered as the only amino acid in continuous culture (Oelze and Söntgerath, 1992). Finally, the high light medium had no inorganic nitrogen source and only restricted amounts of vitamins provided by yeast extract and biotin.

IV.4. Structural differences between low light and high light chlorosomes

4.1. Pigment-protein stoichiometry

The observed variations in the stoichiometry between pigments and proteins of low light and high light chlorosomes, especially between BChl c and the 5.7 kDa polypeptide reflected structural differences in the B740 complex of C. aurantiacus grown under different conditions. Low light chlorosomes had a molar ratio of BChl c molecules to the 5.7 kDa polypeptide in the range of 15:1 (Eckhardt et al., 1991) to 21:1 (this work). The exact value for high light chlorosomes could not be determined, but judging from the staining intensities on SDS-PAGE the ratio was about five times lower for the 22 h high light chlorosomes compared to the low light sample. The absorbance ratio of A740 nm to A268 nm (there was no absorbance maximum at 280 nm) also differed by a factor of five between the low and the 22 h high light chlorosomes. If the 5.7 kDa polypeptide is supposed to bind BChl c, variable pigment-protein stoichiometries can be explained by an oligomeric arrangement of BChl c molecules that interact with the 5.7 kDa polypeptide. The number of BChl c molecules forming the oligomer may well be variable in a range of approximately 4 or 5 to 20 or more molecules.

4.2. Aggregate size of BChl c

There must be a considerable difference in the aggregation state of BChl c between the low light and the high light chlorosomes, which resulted in different relative intensities of either CD signals. The CD intensity of low light chlorosomes only doubled upon
treatment with proteinase K compared to a fivefold increase in the high light chlorosomes. There is some evidence that BChl c in the chlorosome is organized as oligomers (Brune et al., 1987b, Fetisova and Mauring, 1992) which may be bound to protein in order to maintain parallel transition moments between clusters of BChl c (Van Amerongen et al., 1988, Fetisova et al., 1988). There are several reports that aggregates of BChl c in hexane (Blankenship et al., 1988, Griebenow et al., 1991) exhibited higher rotational strength than BChl c in chlorosomes of *C. aurantiacus*. This was also observed for BChl c in aqueous suspension, or after LDS treatment by Niedermeyer et al. (1992). In addition, these authors noted a drastic increase in rotational strength after proteolytic treatment using thermolysin. This is consistent with the increase in CD strength measured after degradation of the 5.7 kDa polypeptide by proteinase K (see section III.3.2.3.). Similarly, GEF-chlorosomes\(^1\) (Griebenow and Holzwarth, 1989) showed stronger CD bands than chlorosomes isolated with detergents and density gradient centrifugation, according to a comparative study by Griebenow et al. (1991).

It is generally accepted that formation of BChl oligomers results not only in a bathochromic shift of the Q\(_y\)-transition of the monomeric pigment but also in hyperchromicity and increased rotational strength (Gottstein and Scheer, 1983, Scherz and Parson, 1984). In an aqueous aggregate of a chloroform/methanol extract from *C. aurantiacus* chlorosomes, however, the rotational strength was lower than in the chlorosomes (Miller et al., 1993). Moreover, chlorosomes from *Chlorobium limicola* were reported by Olson et al. (1985) to have a stronger CD signal than oligomers in methylene chloride/hexane (1:200).

It can be concluded that, if the intensity of the CD signal around 740 nm in the chlorosomes of *C. aurantiacus* is depending on the aggregate size of BChl c (as demonstrated in section IV.2.1.3. and also by Niedermeyer et al., 1992), the BChl c oligomers in high light chlorosomes are smaller than in low light chlorosomes. In that sense, the degradation of the 5.7 kDa polypeptide in high light chlorosomes would produce a stronger increase in rotational strength, because only then the liberated pigments form larger oligomers. Therefore, it seems reasonable to assume that the 5.7 kDa polypeptide is associated with BChl c in the chlorosomes.

\(^{1}\)GEF chlorosomes: Chlorosomes incubated in 0.1% LDS and subjected to gel electrophoresis as described by Griebenow and Holzwarth (1989). The resulting chlorosomes lacked the 11 and the 18 kDa polypeptide, but still contained the 5.7 kDa polypeptide (Eckhardt et al., 1990).
4.3. Structural basis of the chlorosome antenna (B740)

Based on the amino acid sequence of the 5.7 kDa polypeptide and the spectroscopic data discussed above it can be proposed that a certain number of BChl c molecules may be bound to the 5.7 kDa polypeptide. They may be bound, via their Mg atom, to the imidazole nitrogen of His 24, which is also a conserved residue in the sequences of the 6.3 kDa polypeptides of the chlorosomes from Chlorobiaceae (Wagner-Huber et al., 1990). It is also conceivable that the Mg atoms coordinate to the carbonyl oxygens of the amide groups of glutamine and asparagine residues as proposed by Wechsler et al. (1985) or even to the carboxyl oxygens of aspartate and glutamate.

Additional BChl c molecules could be bound as oligomers to the polypeptide-bound BChl c molecules which would result in higher pigment-protein ratios. The additional BChl c molecules could be bound in different ways as depicted in Fig. 57: (1) The hydroxyethyl group of ring I of one BChl c molecule could be involved in a hydrogen bond to the carbonyl oxygen on ring V (C-9 keto group) of a second molecule (Wechsler et al., 1985). (2) The hydroxyethyl group could also interact with the Mg cation of the next BChl c molecule as proposed by Smith et al. (1983). (3) Alternatively, both the hydroxyethyl group and the carbonyl function could interact with the Mg atom as in the model of Bystrova et al. (1979) (not shown). Thus, the chlorosomes could respond to different growth conditions by increasing the number of pigments per polypeptide.

Low temperature resonance Raman data supported a coordination of the carbonyl oxygen on ring V to Mg (Lutz and Van Brakel, 1988). At room temperature Hildebrandt et al. (1991) detected two conformational states of the C-9 keto group. On the one hand a conformation free of any intermolecular interaction and on the other hand also a coordination to Mg, but no hydrogen-bonding interaction. In their view the hydroxyethyl group of ring I would exist as a permanent fifth ligand to Mg. Above that, a fraction of BChl c molecules would have a second axial ligand, the C-9 keto group. The findings of Hildebrandt et al. (1991) would be consistent with the binding mode (2). There the Mg atom is coordinated to the hydroxyethyl group and a fraction of BChl c could also have a sixfold coordination.
In the lipid environment the polypeptides with their bound pigments could further interact with one another via polar residues, e.g. glutamine and asparagine residues, which are not involved in BChl c-binding, or serine and threonine. In this way, the BChl c-antenna would be composed of repeating units, which consist of BChl c oligomers of variable length bound to the 5.7 kDa polypeptides.

A model involving polypeptide-bound BChl c oligomers of variable length was also supported by the pronounced differences in the susceptibility of the 740 nm absorption band to proteases. The differences were apparently due to the fact that in low light chlorosomes the 5.7 kDa polypeptide was much less accessible to proteases. It is
probable that the 5.7 kDa polypeptide in low light chlorosomes was protected by the 
bulk of antenna pigments in the form of large oligomers, while in high light chlorosomes 
the protein was still accessible to the enzyme (proteinase K). In high light chlorosomes 
the BChl c oligomers were smaller or even replaced by single BChl c molecules. 
Supporting these findings, proteolytic treatment using thermolysin showed that low light 
chlorosomes were not affected by the enzyme below 40°C and equally, thermolysin 
treatment produced a stronger decrease of the 740 nm band in medium light chlorosomes 
(Niedermeyer et al., 1992).

4.4. Conclusions

The present study has provided indirect evidence for a major role of the 5.7 kDa 
polypeptide in the organization of BChl c in the chlorosomes of C. aurantiacus. This 
was unambiguously revealed by limited proteolysis of the chlorosomes. Furthermore, it 
has become evident that the ratio between the 5.7 kDa polypeptide and BChl c varies 
over a considerable range. In addition, the aggregate size of BChl c in the chlorosomes is 
variable in response to different growth conditions. These observations can be reconciled 
with a pigment-binding mode, in which the 5.7 kDa polypeptide functions as a scaffold 
for BChl c oligomers of variable length. This implies that the spectral properties of 
BChl c in the chlorosomes of C. aurantiacus are determined on the one hand by 
chromophore-protein interactions and above that by chromophore-chromophore 
interactions.

The 11 and the 18 kDa polypeptides are obviously not associated with the antenna 
pigment. Their ratio to the 5.7 kDa polypeptide seems to be constant and more 
experiments are needed to unveil the structural-functional relationship between the 11 and 
the 18 kDa polypeptides and the pigment-protein complex involving the 5.7 kDa 
polypeptide.
References


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Publications


Curriculum vitae

Rainer Patrick Lehmann

Date of Birth: 25 May 1965 in Essen, West Germany.

Education
1971-1977 Primary school
1977-1984 Gymnasium for natural sciences and modern languages, 
in Bersenbrück, West Germany.
1984, June Abitur
1984-1985 Military service

Academic training
1985-1990 Studies of biology at the University of Konstanz, West Germany.
1987 First general degree of biology.
1987-1989 Course contents: Biochemistry, microbiology, plant physiology.
1989-1990 Diploma: Isolation and physicochemical characterization of the 
membrane-bound [Ni Fe]hydrogenase from the sulphur-reducing 
bacterium Sulfurospirillum deleyianum.

1990, November - 1994, April:

Doctorate at the Swiss Federal Institute of Technology (ETH Zürich), 
Institute of Molecular Biology and Biophysics, Prof. H. Zuber.