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Biochimica et Biophysica Acta 1412 (1999) 94–107

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Spectroscopic and molecular characterization of a long wavelength absorbing antenna of *Ostreobium* sp.

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Received 21 January 1999; received in revised form 3 May 1999; accepted 4 May 1999

Abstract

One of the strains of the marine green alga *Ostreobium* sp. possesses an exceptionally large number of long wavelength absorbing chlorophylls (P. Haldall, Biol. Bull. 134, 1968, 411–424) as evident from a distinct shoulder in the absorption spectrum at around 710 nm while in the other strain this shoulder is absent. Therefore, *Ostreobium* offers a unique possibility to explore the origin of these red-shifted chlorophylls, because strains with and without these spectral forms can be compared. Here, we characterize these red forms spectroscopically by absorption, fluorescence and CD spectroscopy. In the CD spectra at least three spectroscopic red forms are identified which lead to an unusual room temperature fluorescence spectrum that peaks at 715 nm. The gel electrophoretic pattern from thylakoids of *Ostreobium* sp. shows an intense band at 22 kDa which correlates with the presence or absence of long wavelength absorbing pigments. By protein sequencing of the N-terminus of the 22-kDa polypeptide and sequence alignments, this was identified as an Lhca1-type light-harvesting complex. The abundance of this polypeptide – and a possibly co-migrating one – in *Ostreobium* sp. indicates an antenna size of approximately 340 chlorophyll molecules (Chl *a* and Chl *b*) per PS II_α reaction center, which is significantly larger than in higher plants ($\approx$240). The red forms are more abundant in the interior of the thalli where a 'shade-light' light field is expected than in the white-light exposed surface. This demonstrates that algae exist which may be able to up-regulate the synthesis of large amounts of LHCI and associated red forms under appropriate illumination conditions.

Keywords: Absorption spectrum; Amino acid sequence; Far red antenna; Fluorescence spectra; Green algae; Light-harvesting complex, type Lhca1

1. Introduction

The reaction centers (RCs) of photosystem I (PS I) and photosystem II (PS II) of oxygen-evolving organisms are surrounded by chlorophyll (Chl) *a*-containing light-harvesting complexes (LHCs), forming the photosynthetic units (PSU) of PS I and PS II. The LHCs have been classified into LHCI and LHCII according to their physical association with

Abbreviations: CD, circular dichroism; Chl, chlorophyll; DM, dodecyl-maltoside; LHC, light-harvesting complex; PS I, photosystem I; PS II, photosystem II; PSU, photosynthetic unit; RC, reaction center; RT, room temperature; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis

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0005-2728/99/$ – see front matter
PII: S0005-2728(99)00061-4

RC I or RC II, respectively, even though it is known that (phosphorylated) LHCII can also bind to PS I. In many cases, the photosynthetic units have been isolated and spectroscopically investigated. Spectral decomposition of the absorption spectra showed that the PSUs contain a broad distribution of Chl *a* spectral forms, some of which have the absorption maximum of the Q_y transition significantly more to the red than the primary donors P680 and P700 [1–4]. The presence of these red-shifted pigments (with respect to the primary donors) is generally more pronounced in PS I than in PS II. Compared to the total number of light-harvesting Chls, the fraction of the red forms is generally small, making up no more than a few percent.

According to current knowledge, the excited states equilibrate very rapidly (<15 ps) between the red forms and the bulk chlorophylls [5,6]. Although the equilibration is not instantaneous, stationary fluorescence spectra can be described by the assumption of thermal equilibrium as shown by the validity of the Stepanov relation [3,7,8]. The competence of the red forms to elicit the primary charge separation with high quantum efficiency in open PS I upon excitation at wavelengths >715 nm has been demonstrated [2,3,5,9]. Their contribution to the primary charge separation can be explained by the thermal energy of the surroundings that provides the energy to overcome the energy gap between the energetically low-lying red forms and the higher lying primary donors. At 77 K the thermal energy is not sufficient to allow uphill energy transfer from the red forms. Consequently, the red forms represent a poorly quenched energy trap at low temperature. Thus, at low temperature, the red forms manifest themselves by their enhanced red-shifted fluorescence in the 715–730 nm region [10,11].

Long wavelength absorbing Chls in oxygen evolving organisms are not ubiquitous. Different cyanobacteria, for example, possess different amounts and different compositions of the red form composition. On the one hand, *Synechocystis* has few and only slightly red-shifted pigments [12–14], whereas *Spirulina platensis* has in addition to these a further red form that is extremely red-shifted to 738 nm [15–17]. *Pseudoanabaena*, similar to *Spirulina* (F760 at 77 K), also has a very red fluorescence maximum F750 at 77 K [18]. On the other hand, in *Gloeobacter*, red forms are absent [19]. Higher plants PS I has a few red forms with absorption maxima between 700 and 720 nm [20,21], and also in PS II red forms have been demonstrated [22,23]. Algae are known in which the red forms are totally absent, e.g. the Prasinophycea *Mantoniella squamata* [24], whereas other algae possess them, as in higher plants [25–27].

The physiological role of these long wavelength absorbing pigments is presently not very clear and will be taken up in the discussion, and, clearly, the discussion must refer to ambient temperature. There are several reasons for difficulties in clarifying the physiological role of the red forms. First, the red forms represent only a small fraction of the total antenna pigments. Second, some organisms possess them, while others do not. Third, due to their small concentration little is known about their expression and regulation.

It is often difficult to analyze the long wavelength absorbing pigments by absorption techniques due to their relatively weak absorption signal and the presence of scattering artifacts. This problem, however, is greatly reduced in siphonal marine green alga *Ostreobium*, in which some strains possess a very intense absorption at long wavelengths, which give rise to an easily detectable shoulder near 710 nm [28,29].

The genus *Ostreobium* lives in an endozoic habitat under extreme low-light conditions, e.g. on chalky substrates but the living environment is known for only few strains. For instance, the strain *Ostreobium quekettii* lives within shells, whereas *Ostreobium* sp. lives within corals usually under a layer of dinoflagellates. Through spectral filtering of the dinoflagellates, 680 nm light is removed by the Q_y-band of the bulk Chl and predominately light of wavelengths >700 nm reaches the underneath *Ostreobium* cells. The alga *Ostreobium* sp., which we used for our study, was isolated from a coral found in a coral reef in Chile. The other strain studied was *O. quekettii* which is found within shells from the North sea. To our knowledge, no spectral characterization of this latter alga has been published.

We have selected these two strains because the former one has large amounts of long wavelength absorbing pigments and the latter one negligible amounts. These long wavelength absorbing pigments are organized in chloroplasts embedded in a siphon-

uous filament whose organization is absolutely identical in both strains, and therefore optimal conditions for a comparative study are given. The comparison of their spectral properties in absorption and fluorescence spectra (room temperature (RT) and 77 K), as well as their protein composition allowed us to correlate the red forms with the occurrence of the specific polypeptide Lhca1. Analysis of the intense CD signal associated with the red forms suggests that they are spectrally heterogeneous, with transitions near 707, 720 and 735 nm being detected. On the basis of sensitivity of the CD bands to detergent and comparison with higher plant CD spectra of thylakoids, we can find no evidence supporting the idea that the red forms represent transitions of excitonically coupled pigments.

2. Materials and methods

O. quekettii (strain B 14.86 [30]) was obtained from the 'Sammlung von Algenkulturen' at the University of Göttingen, whereas the other strain *Ostreobium* sp. was isolated by Prof. Schnetter from a coral in the pacific ocean near Chile. Both algae were grown photoautotrophically at 15°C in the medium described by Müller [31] under a strict regimen of 16 h of light and 8 h of dark. The light intensity did not exceed 20–30 μW cm^{-2}. For cultivation, a thallus was cut into small pieces and these were transferred to fresh medium until the pieces reached the size of the initial sample (approximately 1–2 cm in diameter) after about 6–8 weeks. The cells were broken by sonication in a homogenization buffer (0.05 M Tricine-KOH pH 8.0, 0.075 M KCl, 0.5 M mannitol and 0.2 mM Pefabloc).

The Chl *a* and Chl *b* concentrations of the broken thalli were measured after extraction with 80% acetone/water (v/v) using the extinction coefficients given by Porra et al. [32].

Absorption spectra were recorded with an Aminco DW-2000 spectrophotometer or an EG and G OMAIII instrument (model 1460). The Aminco measured spectra were corrected for scattering by subtraction of a straight line which was a tangent to the absorption at 750 nm, whereas for the OMA measured spectra, the opal glass technique was used to minimize scattering. Quantum-corrected fluorescence emission spectra were measured with a custom-built high-sensitivity flash-fluorimeter. The excitation light was filtered through a 441 nm narrow band interference filter (Schott), and the fluorescence was detected at 90° with respect to the exciting light. For fluorescence measurements the absorption of the samples was adjusted to <0.15 at the maximum of the Q_y-band. All spectroscopic measurements were carried out on sonicated thalli. For fluorescence measurements at 77 K disrupted thalli were frozen in the dark in homogenization buffer. Addition of the cryoprotective agent glycerol did not change the spectra.

Fluorescence induction measurements were performed according to [24] and analyzed as described before [33]. The excitation wavelength was 675.5 nm with an intensity of 1.4 mW cm^{-2}. Fluorescence light was detected under 90° through a broad-band interference filter (680–720 nm, Dr. H. Anders, Nabburg, Germany). To perform the analysis of the induction kinetics under single turnover conditions, 20 μM DCMU was added to the dark-adapted sample.

Measurements of the fluorescence decay kinetics in the picosecond time range were performed with a setup described before [34] and the data were analyzed as described in [35].

Circular dichroism spectra were measured in a Jasco-600 spectropolarimeter with a bandwidth of 2 nm. Samples were routinely placed at about 2 cm from the photomultiplier and the optical density was about 0.45 at the Q_y absorption maximum.

For gel electrophoresis, the samples were prepared by disrupting the thalli in ice-cold homogenization buffer and filtering the slurry through a nylon mesh (diameter 50 μm). The suspension was centrifuged at $5000\times g$ for 20 min at 4°C. The chloroplasts containing fraction was washed twice with shock buffer (0.01 M Tricine-KOH pH 8,0, 0.2 mM Pefabloc), resuspended in another buffer (0.4 M sucrose, 0.01 M Tricine-KOH pH 8.0, 5 mM $MgCl_2$, 50 mM NaCl, 0.2 mM Pefabloc) and collected at $10\,000\times g$ for 20 min at 4°C. The Chl *a* concentration was adjusted to about 1 mg/ml before samples were frozen at −80°C. Proteins were separated for 18 h at 25 mA on 8–21% SDS-PAGE according to Fling and Gregerson [36] and stained with Coomassie brilliant blue. For blotting, proteins were electrotransferred to a PVDF membrane (Immobilon-P, Millipore) in

blot-buffer (10 mM $NaHCO_3$, 3 mM Na_2CO_3, 10% methanol). Following electrotransfer, the membrane was stained with Coomassie and the band at 22 kDa was cut and sequenced. An Applied Biosystems 473A gas phase sequencer was used for the N-terminal protein sequencing. Finally, the SWISSPROT and EMBL databases were searched for proteins related to the amino acid sequence of the 22-kDa band. For this purpose, the multiple alignment program MACAW [37] was used.

3. Results

An essential feature in this study is the comparative characterization of two strains belonging to the same genus, which live in nearly identical environments, but differ significantly in their absorption spectrum.

To achieve low optical densities for the spectroscopic measurements it was necessary to break the thalli into single filaments, because even small pieces cut from the thallus showed high optical density which may give rise to a disturbing sieve effect. Nevertheless, even single filaments appeared dark-green in the light microscope due to the densely packed chloroplasts in the cell. Breaking the thalli by mild sonication yielded short recalcitrant filaments which still contained the chloroplasts. Only few chloroplasts were released by this treatment. If the sonication was more intense or longer, or if glass beads were used to break the thalli, the filaments were fractionated into smaller particles and more chloroplasts were released, but the harsher treatment resulted in a loss of the red shoulder. Thus, some distortion of our spectra by the sieve effect was unavoidable.

3.1. Stationary spectra

A comparison of the absorption spectra *Ostreobium* sp. (Fig. 1a, solid line) and *O. quekettii* (Fig. 1a, dashed line) reveals striking differences. Although both spectra display the same maximum at 678 nm, the main absorption band of *Ostreobium* sp. is broader than the one of *O. quekettii* and has an intense shoulder at around 710 nm which is lacking in *O. quekettii*. The excess absorption of *Ostreobium*

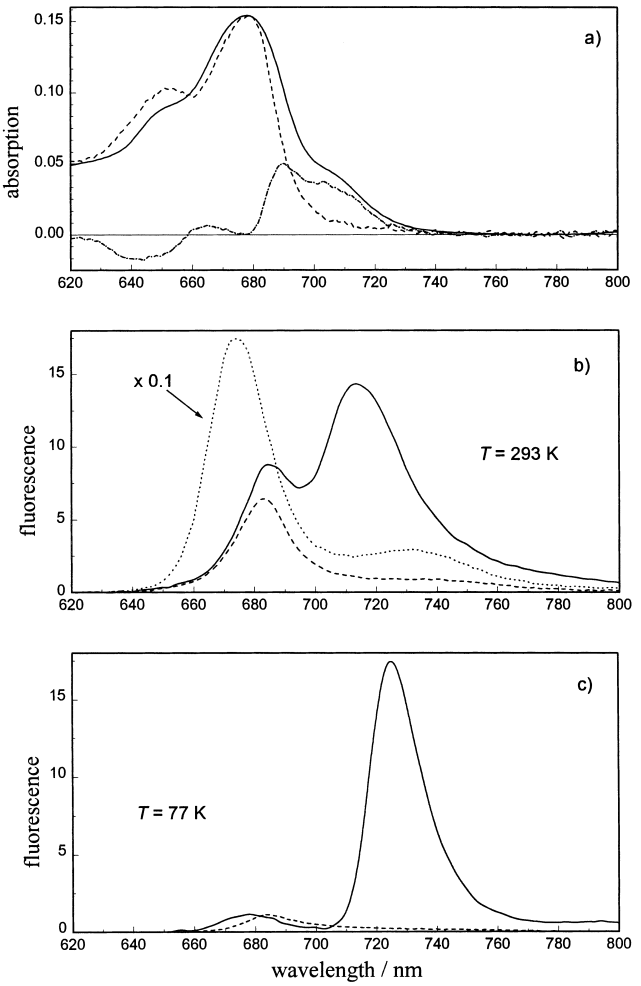

Fig. 1. Comparison of the absorption and fluorescence spectra of *Ostreobium* sp. and *O. quekettii*. (a) RT absorption spectra of *Ostreobium* sp. (solid line) and *O. quekettii* (dashed line) normalized to the peak maximum at 678 nm. The dash-dotted line is the difference spectrum. (b) Quantum-corrected RT fluorescence spectra of *Ostreobium* sp. (solid line), *O. quekettii* (dashed line) and Chl *a* in methanol (dotted line). The spectra were corrected for the absorbed photon flux and excitation wavelength. (c) 77 K fluorescence of *Ostreobium* sp. (solid line) and *O. quekettii* (dashed line). The spectra were scaled to the same height of their maxima around 676–678 nm.

sp. at wavelengths >700 nm is assigned to the presence of long wavelength absorbing Chls. Another marked difference occurs at the typical Q_y-absorption of Chl *b* at around 650 nm. It is obvious that *Ostreobium* sp. possesses much less Chl *b* than *O. quekettii*. All these differences are also apparent in the difference spectrum (Fig. 1a, dash–dot line). To check for scattering artifacts, some absorption spectra were recorded with a photospectrometer

equipped with an Ulbricht sphere. These spectra were slightly narrower, but essentially similar in shape (data not shown).

Fluorescence spectra at RT were recorded from filaments at very low chlorophyll concentration. The quantum corrected fluorescence spectrum of *Ostreobium* sp. (Fig. 1b; solid line) shows a peak at 685 nm and a second one of nearly two-fold amplitude at 714 nm, whereas the spectrum of *O. quekettii* (dashed line) shows only one peak at 684 nm and little emission at 714 nm. In some cases, we have noted emission spectra from *Ostreobium* sp. in which the 685-nm band was reduced to a shoulder with a 714/685 nm ratio of up to 4. This ratio was slightly greater with 475 nm excitation than with 440 nm excitation (data not shown) in agreement with previous results using isolated PS I complexes [20,21]. The amplitude of the fluorescence spectrum was independent of the redox conditions (± dithionite). We did not succeed in finding F_o conditions for recording fluorescence spectra.

Additionally, we also measured the fluorescence spectra of Chl *a* in methanol (Fig. 1b, dotted line). This allowed us to calculate a rough estimate of the fluorescence lifetimes from the integrated areas. Assuming a lifetime for Chl *a* in methanol of 5.6 ns the mean fluorescence yield of *Ostreobium* sp. corresponds to 800 ps and of *O. quekettii* to 225 ps. Time-resolved fluorescence decay measurements of thin pieces of whole thalli showed multiple phases in the 200–1000-ps range at all emission wavelengths.

The 77-K fluorescence spectra of *Ostreobium* sp. (Fig. 1c, solid line) and *O. quekettii* (Fig. 1c, dashed line) are also basically different. The fluorescence spectrum of *Ostreobium* sp. shows a small peak at 678 nm and a large one at 725 nm. In contrast, the 77-K fluorescence of *O. quekettii* shows only one peak at 683 nm thus demonstrating the insignificance of red forms in this strain. Both spectra have been normalized to the peak around 680 nm.

3.2. Fluorescence induction

In order to get an estimation of the size of the

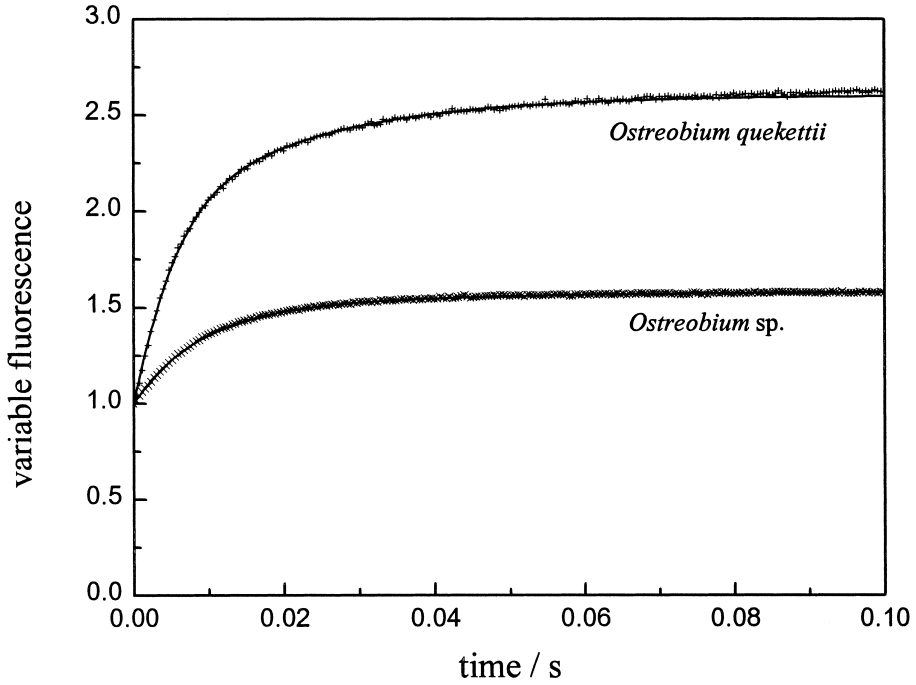

Fig. 2. Fluorescence induction curves (dots) from *Ostreobium* sp. and *O. quekettii* suspended in homogenization buffer and 20 μM DCMU. The experimental curves were fitted by a theoretical curve (solid line) composed of α- and β-centers according to the connected units theory described in [38]. The excitation wavelength was at 675.5 nm and the total energy flash was 1.4 mW cm^{-2}. For *O. quekettii* (upper curves) the best fit was obtained with 25% β-centers having an 80% quantum yield and $F_m/F_o = 2.6$. For *Ostreobium* sp. (lower curves) the best fit was obtained with 25% β-centers having a 45% quantum yield and $F_m/F_o = 1.6$.

PSU, we performed fluorescence induction measurements, using a laser line at 675.5 nm for excitation. At this wavelength, mainly Chl *a* is excited with Chl *b* excitation being negligible ($\approx 7\%$). The dilution of the samples was such that in the projection through the measuring cuvette, the filaments were well separated from each other. This minimized fluorescence reabsorption artifacts as much as possible.

The fluorescence induction curves of *Ostreobium* sp. and *O. quekettii* are shown in Fig. 2. The F_m/F_o ratio in *Ostreobium* sp. was approximately 1.6. The highest value we ever achieved was 2.0. In contrast, the F_m/F_o ratio in *O. quekettii* was >2.6. Both fluorescence induction curves were fitted with theoretical curves (solid lines) composed of α- and β-centers according to the connected units theory [38]. To determine the antenna size, we used the following equation to convert the time scale into the dimensionless quantity hits per PSU, z:

$$z = N_{Chla} \cdot \sigma_{Chla} \cdot I \quad (1)$$

in which $N_{\mathrm{Chl}\ a}$ is the number of pigments in the PSU, σ the absorption cross section of a single pigment at the excitation wavelength, and I the photon flux per unit area. The term $\sigma_{\mathrm{Chl}\ a}$ was calculated from

$$\sigma_{Chla} = \frac{10^3 \cdot \varepsilon \cdot \ln 10}{N_A} \quad (2)$$

in which N_A is Avogadro's number and ε is the apparent molar absorption coefficient of Chl *a* in the chloroplasts as determined from the comparison from acetone and in vivo absorption spectra.

Assuming that only Chl *a* is excited at 675.5 nm, we obtain for the α-centers Chl *a* antenna sizes of $N_{\mathrm{Chl}\ a} = 220$ in *Ostreobium* sp. and *O. quekettii*. Together with the Chl *a*/*b* ratios – 1.9 for *Ostreobium* sp. and 1.1 for *O. quekettii* as determined by acetone extraction (see Section 2) – the following Chl *a*+*b* antenna sizes are calculated: $N_{\mathrm{Chl}\ a+b} = 340$ in *Ostreobium* sp. and $N_{\mathrm{Chl}\ a+b} = 420$ in *O. quekettii*. Both numbers are significantly greater than for higher plants [39].

3.3. CD measurements

In the hope of gaining more information on the red absorption wing of *Ostreobium* sp., we have measured the circular dichroism (CD) spectrum. It is sometimes suggested that the very red-shifted spectral forms may be due to excitonically interacting dimers [12,17,40] or else they could represent Chls in a strongly polarized or charged environment (for review see [41]). Either of these physical situations could conceivably give rise to red-shifted, dichroic, absorption transitions. The CD spectrum for the Q_y region of *Ostreobium* sp. is presented in Fig. 3a, together with the absorption spectrum and the so-called anisotropy spectrum (CD/A). As is well known for higher plant thylakoids the CD spectrum of *Ostreobium* sp. is markedly non-conservative [42,43] and displays clear peaks near 652 nm (−), 676 nm (−), 690 nm (+) and 707 nm (+). In addition, a

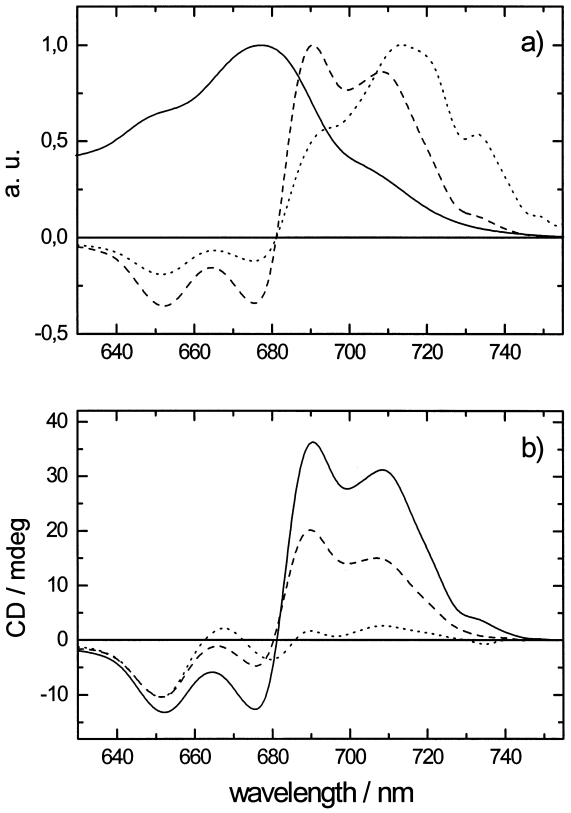

Fig. 3. (a) Absorption (solid line), CD (dashed line) and CD anistropy (CD/A; dotted line) spectra of *Ostreobium* sp. thylakoids in the Q_y region. Spectra are normalized at their maxima which for the absorption spectrum was 0.45 OD and for the CD spectrum was 36 mdeg. (b) The effect on the CD spectrum of incubating *Ostreobium* sp. thylakoids with the detergent dodecyl-maltoside. Untreated membranes (solid line); membranes incubated with dodecyl-maltoside 0.015% (dashed line); membranes incubated with dodecyl-maltoside 0.025% (dotted line).

minor, but fully reproducible band, is evident near 735 nm (+). The 652-nm band, clearly associated with Chl *b*, is also present in higher plant thylakoids and PSI 200 isolated from higher plants (R. Croce, doctoral thesis, University of Milan, 1997), as are the bands near 676 and 690 nm [42,43]. On the other hand, the 707-nm and minor 735-nm bands are completely absent in higher plant thylakoids and are clearly associated with the red absorbing Chl forms in the *Ostreobium* sp. membranes. The anisotropy spectrum increases in intensity across the positive CD bands to attain a maximum value near 712 nm, i.e. somewhat red-shifted with respect to the 707-nm band. In addition, the very long wavelength band near 735 nm is clearly evident and, interestingly, a distinct shoulder in the 720 nm region also emerges. We therefore tentatively conclude that the red absorption wing of these membranes is spectrally heterogeneous and contains a minimum of three red-shifted chlorophyll forms with absorption maxima near 707, 720 and 735 nm. This conclusion is supported by a gaussian decomposition analysis of the all positive CD spectral interval (685–750 nm). Such an analysis requires the presence of sub-bands at exactly these wavelengths (data not presented). We would also point out that an excellent description of the red absorption tail is also provided by sub-bands at 707, 720 and 735 nm (data not presented). We emphasize that it is impossible to obtain an acceptable gaussian description without the 720-nm band.

Circular dichroic measurements of particulate samples, such as thylakoid membranes, may be distorted by the phenomenon of circular differential scattering [44]. To check this, we compared the spectra measured under the normal measuring conditions (sample about 2 cm from the phototube) with measurements performed on the same sample placed at about 16 cm from the phototube (data not shown). When the sample was placed far from the phototube, the spectrum was slightly red-shifted. This was greatest at the zero crossover wavelength where a 3.5-nm shift was observed while the 707-nm band was shifted by about 2 nm to the red. Apart from this, only minor changes in the relative band intensities were observed. Thus we conclude that the red CD bands are not substantially distorted by circular differential scattering. In addition, when samples were incubated with 0.02% dodecyl-maltoside (DM), which greatly reduces scattering, the same three CD anisotropy structures as described in Fig. 3a and b, in the red region, were clearly present.

We have also studied the effect of the detergent DM on the CD signal of *Ostreobium* sp. membranes (Fig. 3b). Data are presented for two DM concentrations near the critical micellar concentration. It is evident that with the exception of the 652-nm band, the intensity of all other CD structures is markedly sensitive to detergent treatment, with the 735-nm band displaying the greatest sensitivity. The decrease in intensity of the long wavelength CD bands upon detergent treatment is paralleled by a decrease in the absorption intensity in the red wing (data not presented). These data show that the 652-nm band (Chl *b*) is independent of the CD bands associated with the red forms.

3.4. SDS-PAGE

To investigate whether the long wavelength forms found in *Ostreobium* sp. are related to a specific protein, we solubilized broken filaments with SDS, which had been subjected to hypertonic shock me-

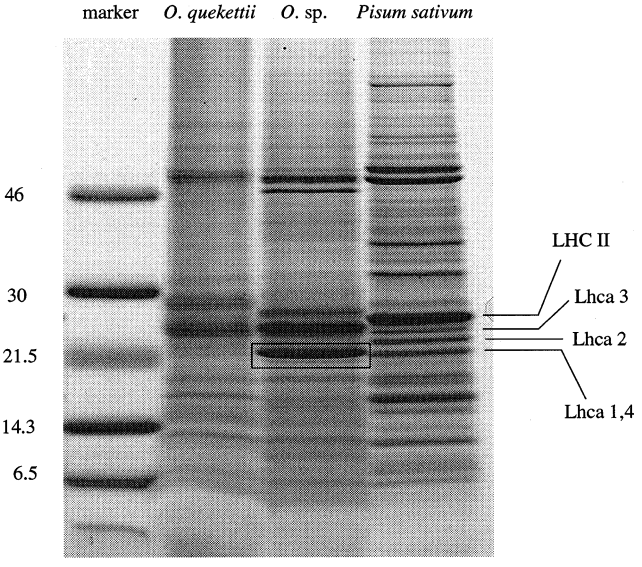

Fig. 4. SDS-PAGE of thylakoids from *O. quekettii*, *Ostreobium* sp. and *Pisum sativum*. The molecular weight of the molecular markers is indicated on the left side of the gel. Lanes were loaded with 210, 255 and 80 μg protein of *O. quekettii*, *Ostreobium* sp. and *Pisum sativum*, respectively. For the assignment, see text. The framed 22-kDa band was sequenced.

a) Alignment with the N-terminal sequence of Lhca 1

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A. Ostreobium     1 FAREGTWIPGQEKPEWLPEGIPGNFGYDPLGLAREP 36                          Id    Si
                    :  | |:|| : | |||: :|||:|:||| |::||        Helix B             18/34; 9/34
B. Chlamydomonas  30 KAGNWLPGSDAPAWLPDDLPGNYGFDPLSLGKEPASLKRFTESEVIHGRWAMLGVA   88
                    : |:||| :| :|    ||:||:|||||:  |                              16/34; 7/34
C. Nicotiana     42 FSMSAEWMPGQPRPPYLDGSAPGDFGFDPLGLGEVPSNLERYKESELIHCRWAMLAVP  100
                    : | ||: :| :|    ||:||:|||||:  |                              15/34; 7/34
D. Arabidopsis    46 AHWMPGEPRPAYLDGSAPGDFGFDPLGLGEVPANLERYKESELIHCRWAMLAVP  100
                    : |:||| :|  |    ||:||:||| |:  |                              15/34; 6/34
E. Pinus          46 SAEWMPGQPRPPHLDGSAPGDFGFDPLRLGVIPENLERYKESELIHCRWAMLAVP  101
                    : |:||| :|  |    ||:||:|||||  |                              17/34; 5/34
F. Hordeum         1 SAEWFPGQPRPAHLDGSSPGDFGFDPLGLATVPENFERFKESEIYHCRWAMLCVP   57
                    : |:||| :| :|    ||:||:|||||:  |                              16/34; 7/34
G. Lycopersicon   45 MSADWMPGQPRPSYLDGSAPGDFGFDPLGLGEVPANLERYKESELIHCRWAMLAVP  100
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b) Alignment with the N-terminal sequence of Lhca 4

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A  Ostreobium   1 FAREGTWIPGQEKPEWLPEGIPGNFGYDPLGLAREP 36
                  | |:||   | :|   :||: |:|||||| :|          Helix B                16/34; 6/34
B´ Pinus       49 VEAKGEWLPGLSSPSYLNGSLPGDNGFDPLGLAEDPESLKWYVQAELVNGRWAMLGVAGMLIP 111
                  | |:||   |::|   :|   |:||||  :|                                   14/34; 6/34
C´ tomato 1     1 KKGQWLPGLASPDYLDGSLPDGNGFDPLGLVEDPENLKWFIQAELVNGRWAMLGVAGMLLP 61
                  | |:||   | :|   : |: |:|||||| :|                                   14/34; 6/34
D´ tomato 2     1 KKGEWLPGLTSPTYLNGSLAGDNGFDPLGLAEDPENLRWFVQAELVNGRWAMLGVAGMLLP 61
                  | |:|| : | :|   : |: |:|||:|| :|                                   14/34; 8/34
E´ Hordeum      1 AKGSWLPGLQSPAYLDGSLAGDNGFDPLALAEDPEDLRWFVQAELVNGRWAMLGVAGMLIP 61
                  | |:||   |::|   : |: |:|||||| :|                                   15/34; 7/34
F´ Arabidopsis 50 EAKKGEWLPGLASPDYLTGSLAGDNGFDPLGLAEDPENLKWFVQAELVNGRWAMLGVAGMLLP 113
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Fig. 5. Alignment of the N-terminal sequence of the 22-kDa band of *Ostreobium* sp. (A) with various LHCI polypeptides. (a) Alignment with Lhca1 proteins: B, *Chlamydomonas rheinhardtii* EMBL X65119, SWISSPROT Q05093 [61]; C, *Nicotiana tabacum* EMBL X64198, SWISSPROT Q40512 [75]; D, *Arabidopsis thaliana* EMBL X56062, SWISSPROT Q01667 [76]; E, *Pinus sylvestris* EMBL X58515, SWISSPROT Q02069 [77]; F, *Hordeum vulgare*, personal communication with Dr. J. Knoetzel; G, *Lycopersicon esculentum*: EMBL J03558, SWISSPROT P12360 [78]. (b) Alignment with Lhca4 proteins: B′, *Pinus sylvestris* EMBL Z17226, SWISSPROT Q07489 [45]; C′ and D′, *Lycopersicon esculentum* Lhca4*1 and Lhca4*2 [79]; E′, *Hordeum vulgare* personal communication with Dr. J. Knoetzel; F′, *Arabidopsis thaliana* EMBL M63931 [80]. The gray shaded area shows the beginning part of the helix B. Thirty-four of a total of 36 letters of the N-terminus were used to produce significant alignments. In the two last rows, identical and similar amino acids are given: the first ratio (Id) gives the number of identical amino acids in the aligned sequence, the second ratio (Si) the number of similar amino acids.

dium before the detergent treatment. The protein pattern from *Ostreobium* sp., as well as from *O. quekettii* and *Pisum sativum* as references are shown in Fig. 4. Marked differences can be seen throughout the entire length of the gel. We shall focus attention on that part of the gel in which the light-harvesting polypeptides are known to be located. In the 20–28-kDa region of the lane loaded with thylakoids from

P. sativum up to four distinct bands are resolved which can be assigned to LHCII of PS II (28 kDa), Lhca3 (26 kDa) and Lhca2 (25 kDa) of PS I [45]. The band at 22 kDa can be assigned to Lhca1,4 due to their similar molecular weight [46]. In the polypeptide pattern of *Ostreobium* sp., three bands are discernible in the region in question. The intense band at 25 kDa is most likely LHCII. Another band at 22 kDa (framed) is present at a comparable high intensity. This band is hardly recognizable in the lane loaded with *O. quekettii*. According to the molecular mass of the framed band in *Ostreobium* sp., it seems to be an LHCI protein.

The occurrence of the strong 22-kDa band in the *Ostreobium* strain that possesses the long wavelength absorbing pigments and the lack of this band in *O. quekettii* with no or only few red forms suggests that this band is the light-harvesting complex carrying the red forms. From other studies with barley and tomato chloroplasts it is known that red forms originate from the association of Lhca1 with Lhca4 [46,47]. We therefore attempted to sequence this particular band.

3.5. Sequencing and alignment

The sequencing of the 22-kDa band of *Ostreobium* sp. yielded only one protein, without evidence for heterogeneity. The N-terminus could be determined up to a length of 36 amino acids. To identify the protein its sequence was aligned with the N-terminal regions of various other LHCI polypeptides for which the sequences are known (Fig. 5). An inspection of the frequency of the identities and similarities with Lhca1 (last two lanes) shows highest similarity with Lhca1 from *Chlamydomonas rheinhardtii*, whereas the number of identical or similar amino acids with Lhca4 is generally lower. It is worth noting that the xanthophyll binding motif GFDPL is present, which is known to be highly conserved in both Lhca1 and Lhca4 proteins [48]. Thus, we identify the strong 22-kDa band as a Lhca1 protein.

3.6. Other experimental observations

For our experiments, we used exclusively 1–2-month-old *Ostreobium* sp. that had a dark-green appearance. The filaments used for the experiments were cut preferentially from the interior of the thallus. Filaments collected from the surface of the algae either showed no red shoulder or only a very weak one. This seems to indicate that the occurrence of the red forms in this strain may depend on the light regime.

The reference strain, *O. quekettii*, was cultured under the same light conditions and harvested after the same growth period. However, we never observed a red shoulder, even if filaments from the dark interior were collected.

4. Discussion

4.1. Circular dichroism and the origin of the red shift

The present CD analysis indicates that the red-shifted spectral forms of *Ostreobium* sp. are strongly dichroic with an intense maximum near 707 nm (Fig. 3a). Other CD bands are also evident near 720 and 735 nm, even though these longer wavelength transitions are not resolvable in the absorption spectra. As there is no significant contribution of circular differential scattering to the CD signal in this spectral interval, we conclude, as has already been demonstrated for PS I red forms from higher plants and cyanobacteria [3,9,12,49,50], that the unusually intense red absorption tail of *Ostreobium* sp. is markedly heterogeneous. It should be mentioned that CD band structures at wavelengths which are very close to 707, 720 and 735 nm have also recently been observed in a PS I-200 preparation from maize (Tagliabue and Jennings, unpublished observation) thus suggesting that the red form heterogeneity of *Ostreobium* sp. may be very similar to that of higher plants. The present results also indicate the potential of CD to identify absorption transitions in strongly dichroic spectral regions, which is due to strong spectral overlap, may be otherwise difficult to identify.

A possibility for explaining the large CD signal of thylakoids has been suggested by Garab and coworkers [42,43]. This is based on collective absorption phenomena due to long distance radiative coupling which may occur in large particles, the dimensions of which are greater than 50 nm [51,52]. Barzda et al. [42] have demonstrated that Mg-induced grana stacks show a large increase in the CD bands near

650 nm (−), 675 nm (−) and 688 nm (+). These data are interpreted in terms of 'psi type' CD signals due to collective absorption phenomena within the grana. We would point out, however, that grana formation due to Mg addition has no significant influence on the absorption spectrum of higher plant thylakoids apart from a small particle flattening effect [53]. In the case of *Ostreobium* sp. Mg addition only slightly modified the CD spectrum and had very little effect in the major red-shifted band structure (data not presented). We therefore conclude that collective absorption phenomena within the thylakoid membranes are probably not involved in generating the intense red absorption tail.

Circular dichroism signals in chlorophyll–protein complexes are usually discussed in terms of excitonic interactions between pigments [54–57]. As it has been suggested that the PS I red forms could in fact be due to chlorophyll dimers which interact excitonically [12,17,40], we will now discuss the CD data in this context. In this hypothesis two possibilities exist: (1) the excitonic coupling is between Chl *a*-monomers which absorb at similar energies and gives rise to two energetically split excitonic bands, with the red forms being the low energy bands and either of the two negative bands at 652 or 676 nm being associated with the high energy transition; and (2) the excitonic interaction is between monomers with markedly different site energies, e.g. a Chl *b* molecule absorbing near 650 nm and a red absorbing Chl *a* molecule. In this case, the excitonic interaction would give rise to a CD spectrum with positive and negative lobes energetically close to the monomer energies [58]. This latter possibility is interesting in the context of the suggestion from both chlorophyll/protein reconstitution experiments [47] and fluorescence excitation spectroscopy [20,21] that a special interaction might exist between Chl *b* and the red absorbing Chl *a* forms. Our experiments on detergent sensitivity demonstrate that while the red CD bands are destroyed by incubation with DM, the 652-nm band is not. Thus, we conclude that this latter band is probably not due to an excitonic interaction involving the red transitions. On the other hand, the intense negative band at 676 nm displays a similar sensitivity to DM as the red bands. However, we would point out that a pronounced CD signal at this wavelength is present also in higher plant thylakoids in which the positive bands at 707 nm and above are not present [42]. It would therefore seem unlikely that the 676-nm band is due to an excitonic interaction involving or generating the red forms. Thus, while the CD of the red forms in *Ostreobium* sp. is intense, we are unable to find evidence that this is due to excitonic interactions between pigments. We tend to conclude that the red-shifted Chl *a* molecules in *Ostreobium* sp. probably arise from specific pigment protein interactions.

4.2. Spectral decomposition

Spectral decomposition of the measured absorption spectra was made with Chl *a* and Chl *b* solvent spectra. We obtained three pigment pools with absorption maxima at 652 nm for Chl *b*, 678 nm for bulk Chl *a* and 706 nm for the red pigment Chl *a* pool. Then, $N^{652}_{\text{Chl } b}$, $N^{678}_{\text{Chl } a}$ and $N^{706}_{\text{Chl } a}$ are the number of pigments in a pool (= pool size) having identical spectra. In the case of *Ostreobium* sp., the pools have the following sizes: 156, 339 and 72, respectively. The same treatment with absorption spectra from *O. quekettii* yielded the sizes of 256 and 375 for the 652 nm (Chl *b*) and the 678 nm (bulk Chl *a*) pools. From the number of pigments bound to three pigment pools we estimated, by comparing the areas, that up to 21% of all Chl *a* molecules can be red-shifted ones. If the core complexes of PS I (100 Chl *a*) and PS II (45 Chl *a*) are assumed not to contain red-shifted forms and the red forms are ascribed only to Lhca1,4, we arrive at the conclusion that approximately 8 Chl *a* (out of probably 18 Chl *a* [47]) in the Lhca1,4 heterodimer are red forms. This number is much higher than 2 which are expected, if red forms would just originate from a Chl *a* dimer formation at a contact site of the heterodimer.

4.3. Location of the red forms

While red forms are known to exist in both the core and outer antenna of higher plant PS I (for review see [40]) it has been recently suggested by Croce et al. [59] that at least 80% are in fact in the LHCI complexes. The potential carriers of the red forms in the peripheral complexes of PS I are the four known subunits Lhca1–4 [45]. Specifically, an isolated LHCI-680, that is composed of the subunits

Lhca2 and Lhca3, shows a 77-K fluorescence peaking at 680–690 nm [60] and, hence, contains no red forms. This shows that association of LHCs does not necessarily produces red forms. Another complex, termed LHCI-730, shows a 77-K fluorescence peaking at 730 nm and, hence, carries red forms. This complex is composed of the subunits Lhca1 and Lhca4 [45,46]. It is reasonable to assume that the red forms in algae are also due to these latter two subunits, since the polypeptide composition of algal chloroplasts is very similar to that of higher plants [61,62]. Thus, according to our present understanding the subunits responsible for the red forms in the peripheral antenna complexes of PS I are heterodimers of Lhca1 and Lhca4 (but see [81]).

The N-terminal sequencing of the 22 kDa protein band from *Ostreobium* sp. yielded an amino acid sequence of a single polypeptide. However, it is possible that the band contains two co-migrating similar proteins, since Lhca1 and Lhca4 are known to co-migrate in SDS-PAGE [46]. Attempts to sequence the upper and lower half of the band separately, with the idea to identify a second protein with only slightly different mobility, were not successful. The fact that we found only one sequence in the band could be due to the presence of only one protein or, if two proteins were present, the second co-migrating polypeptide is not detectable due to a blocked N-terminus. For the time being, the question whether we have to deal with only Lhca1 or with Lhca1 plus Lhca4 remains open.

Heterodimerization of Lhca1 and Lhca4 has been shown to lead to spectral forms with absorption maxima at 676 nm and a red shoulder around 705 nm [63]. We found in the 77-K fluorescence spectrum of *Ostreobium* sp. one main red peak at 725 nm (Fig. 1c), which is typical for peripheral LHCI. This makes it likely that all long wavelength absorbing pigments present in *Ostreobium* sp. originate from the association of Lhca1 with Lhca4. Further support for this suggestion is given by our reference alga *O. quekettii* in which the lacking 22-kDa protein (Fig. 4) correlates with the missing 725-nm peak in the 77-K fluorescence spectrum (Fig. 1c).

4.4. Association of Lhca1,4 with the photosystems

As indicated above, the red chlorophyll forms absorbing above 700 nm are generally thought to be associated with the antenna system of PS I. In plants, as distinct from cyanobacteria, most of these red forms seem to be associated with the outer antenna complexes collectively known as LHCI [64]. However, in *Ostreobium* sp. several lines of evidence suggest that a significant fraction of these red absorbing forms may in fact also transfer energy to PS II reaction centers. Of particular interest in this respect is the observation of Halldal [28] and Koehne and Trissl (unpublished observation) that the quantum efficiency of oxygen evolution in *Ostreobium* sp. for wavelengths above 700 nm is high. We therefore tentatively suggest, in the case of *Ostreobium* sp., where a massive production of both red spectral forms and Lhca1,4 occurs, that a part of these antenna complexes are energetically coupled to PS II.

Basically long wavelength absorbing pigments have the effect of prolonging the trapping time, decreasing the photochemical quantum yield and increasing the fluorescence yield [65]. However, the drop in photochemical yield may well be overcompensated by the increased absorption [65]. The rather high mean fluorescence quantum yield associated with the red emitting forms is in keeping with antenna coupling to PS II.

4.5. Physiological role of the red forms

The physiological role of red forms in Chl *a*-containing organisms is still a matter of debate. It has been suggested that long wavelength absorbing pigments serve for focusing excitons close to the RC [20,39,66] or may have a protective function [67]. These suggestions are unlikely to meet the point since the photosynthetic unit of PS I has been shown to form a thermally equilibrated system at RT [3]. If so, this means that also the red forms take part in the equilibration and are therefore competent in eliciting the primary charge separation, as was experimentally demonstrated [2].

Alternatively, it has been suggested that the red forms may have an important function in light harvesting in the so-called 'shade-light environments' [65,68–71]. These low-intensity light environments which occur either within or underneath a dense vegetation system are strongly enriched in wavelengths above 690 nm due to wavelength-dependent filtering

and reflectance effects [72]. While measurements on the light environment inside *Ostreobium* sp. mats is lacking, it is expected that it will closely resemble 'shade-light' due to the high filament density and the fact that these mats normally grow under a layer of dinoflagellates [28]. Thus, as demonstrated for leaves growing in 'shade-light' [71], we expect that the red forms in *Ostreobium* sp. will play an important role in light harvesting. In the case of higher plants, as the red forms are thought to be almost exclusively associated with PS I, a potential imbalance in photon absorption between the two photosystems is possible [71]. However, if our suggestion that a significant fraction of the red forms is also energetically coupled to PS II in *Ostreobium* sp., this optical cross section problem may well be circumvented.

Our comparative study shows that in thalli of *O. quekettii*, no red pigments are formed in significant amounts, although this organism lives under similar low-light conditions as *Ostreobium* sp. This shows that red forms are not absolutely necessary for survival in dim light. The question arises why some strains do and others do not develop red forms. A discernible difference in the environments is that *Ostreobium* sp. lives under a layer of dinoflagellates, which act as a spectral filter, whereas *O. quekettii* lives in shells in which this filtering effect is unimportant. Thus, it seems obvious that *Ostreobium* sp. adapted to the red-light climate by up-regulation of a light-harvesting complex that possesses plenty of red forms. In addition to the up-regulation of the Lhca1,4 heterodimer, the alga is able to supply the complex with more than two red pigments. The induction of red forms by exposure to >700 nm light in an other green alga, *Chlorella pyrenoidosa*, has been reported earlier [73]. Recently, Schiller et al. [74] proposed that Chl *a* availability determines the presence or absence of long wavelength absorbing chlorophylls. However, our data indicate that this hypothesis may be true for chlorophyll-limited greening chloroplasts, but cannot be generalized.

The red forms in *Ostreobium* sp. which grew in culture are not equally distributed over the thallus. Filaments inside the thallus developed more red forms than the outer filaments did. They therefore adapt to a light climate in which >700 nm light prevails by increasing the absorption on the bathochromic side. It is conceivable that *Ostreobium* sp. which lives naturally beneath dinoflagellates [28] can develop red forms in all parts of the thallus. In contrast, the interior filaments of *O. quekettii* in culture were not possessing any red forms and seemed to be less well adapted to dim light. However, this strain adapted to the light climate, in which 680 nm light is essentially missing, by increasing the absorption on the hypsochromic side by additional Chl *b*. The high fluorescence yield and low F_m/F_o ratio in fluorescence induction measurements as well as the fact that oxygen is produced when *Ostreobium* sp. is excited with light >700 nm [28] indicate that the Lhca1 complex is physically also connected to PS II.

4.6. Conclusions

In this study, we have shown that a strain of the green alga *Ostreobium* is capable of adapting to low-light conditions ('shade-light') in which wavelengths >700 nm prevail by up-regulating a red form of Chl *a*-containing light-harvesting complex, namely Lhca1 and possibly also Lhca4. In the putative heterodimer consisting of Lhca1 and Lhca4, about 8 out of 18 Chl *a* are red-shifted. The capacity for up-regulation is specific for this strain and is not present in *O. quekettii*. The majority of the red forms is centered at around 706 nm and appears to be photosynthetically competent in both PS I and PS II.

Future research will elucidate in more detail the function of red-shifted chlorophylls in *Ostreobium* sp. The high fluorescence yield and low F_m/F_o ratio in the fluorescence induction measurements together with the observation that oxygen is produced when *Ostreobium* sp. is excited with light >700 nm indicate that the function of these chlorophylls may be different from that which is known from higher plant PS I associated red-shifted pigments.

Acknowledgements

The authors thank Dr. R. Schmid for the performing of protein sequencing, Dr. J. Knoetzel and Prof. F. Koenig for experimental help, and Prof. W. Junge for general support. The financial support of the Deutsche Forschungsgesellschaft (SFB 171, TP-1, and GRK 5/73) is acknowledged.

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