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Pigment organization and energy transfer in green bacteria. 1. Isolation of native chlorosomes free of bound bacteriochlorophyll *a* from *Chloroflexus aurantiacus* by gel-electrophoretic filtration

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A new method, employing a combination of native gel electrophoresis and gel filtration which we call gel-electrophoretic filtration, has been used for the isolation of native chlorosomes from the green bacterium *Chloroflexus aurantiacus*. The chlorosome preparation does not contain a BChl *a* protein complex in contrast to chlorosomes isolated according to previous preparation methods. Due to the lack of the BChl *a* complex these chlorosome preparations lack also absorption and emission bands at 792–795 nm and approx. 805 nm, respectively. The implications of a chlorosome preparation without a BChl *a* protein complex for the understanding of the structural organization of pigments in chlorosomes are discussed.

Introduction

The main light-harvesting antennae of green bacteria consist of bacteriochlorophylls (BChls) organized in so-called chlorosomes which are attached to the inner side of the cytoplasmatic membrane. (see Ref. 1 for a review). The chlorosomes are large compartments which contain several thousand BChl *c* molecules [2]. They are supposedly surrounded by a lipid monolayer [3]. The chlorosomes of the gliding green bacterium *Chloroflexus aurantiacus* contain BChl *c*, which is believed to be attached to rod-shaped polypeptides [3] of ≈ 5 nm diameter. The amino acid sequence of the 5.6 kDa BChl *c* binding protein of *C. aurantiacus* has been determined by Wechsler et al. [4]. A characteristic feature of chlorosomes is their extremely high BChl *c*/protein content of 348 μg BChl/mg protein [5]. It is generally believed that chlorosomes contain a quasi-crystalline baseplate with integrated BChl *a*-protein complexes. According to the current understanding, the BChl *a* in the baseplate should serve two functions: (i) to trap excitons from the BChl *c* in the chlorosome, and (ii) subsequently feeding this energy into the BChl *a* complexes located in the cytoplasmatic membrane [6–8].

Isolation procedures for functional chlorosomes have been described by Schmidt [5] and Sprague et al. [9]. A density-gradient centrifugation method was used in both cases. Feick et al. [10] introduced the use of the detergent miranol as a procedure for the isolation of chlorosomes. A common feature of all the chlorosome preparations published so far consists in a small amount of pigments absorbing at 792–795 nm in intact chlorosomes (corresponding to an absorption a 769 nm in the extract) which is attributed to a BChl *a* complex located in the baseplate. Feick et al. [10] reported a constant molar ratio BChl *c*/BChl *a* of 25 : 1. Measurements of steady-state fluorescence emission and excitation spectra indicate that the energy transfer from BChl *c* to BChl *a* in these isolated chlorosomes has a surprisingly low yield as compared to membranes amounting to between 41% [7] and 55% [6] only. These findings prompt two questions:

(a) is the BChl *a* present in these preparations indeed organized in the baseplate, and

Abbreviations: BChl *a*, bacteriochlorophyll *a*; BChl *c*, bacteriochlorophyll *c*; GEF, gel-electrophoretic filtration; TEMED, *N,N,N′,N′*-tetramethylethylenediamine; LDS, dodecyl sulfate·Li-salt; PMSF, phenylmethylsulfonyl fluoride.

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(b) why is the transfer efficiency from BChl *c* to BChl *a* so low?

In an attempt to address these as well as other questions related to the structure and function of chlorosomes, we present here an isolation procedure for intact chlorosomes free from bound BChl *a*.

Materials and Methods

Organism and growth conditions

Chloroflexus aurantiacus strain OK-70-fl was obtained from the Deutsche Sammlung von Mikroorganismen, Braunschweig, F.R.G. Cells were grown at 50 °C in 1200-ml screw-cap bottles under anaerobic conditions. The light intensity was 0.6 W/m^2 of white light from a tungsten light bulb of 40 W. We have used a new growth medium that was modified after Schmidt [5]. This medium contains per 1000 ml H_2O dest: 0.1 g $Na_2HPO_4 \cdot 2\ H_2O$, 0.1 g $MgSO_4 \cdot 7\ H_2O$, 0.008 g NaCl, 0.1 g KNO_3, 0.7 g $NaNO_3$, 0.05 g $CaCl_2 \cdot 2\ H_2O$, 1.0 g glycylglycine (Sigma), 2 g yeast extract (Merck), 5 ml Fe(III)-citrate solution (28% Fe, 100 mg in 100 ml H_2O dest.), and 5 ml trace element solution SL6 (Medium 27 Catalogue of strains 1983, Deutsche Sammlung von Mikroorganismen, Braunschweig, F.R.G.). The latter contains per 1000 ml H_2O dest. the following salts: 0.1 g $ZnSO_4 \cdot 7\ H_2O$, 0.03 g $MnCl_2 \cdot 4\ H_2O$, 0.3 g H_3BO_3, 0.2 g $CoCl_2 \cdot 6\ H_2O$, 0.01 g $CuCl_2 \cdot 2H_2O$, 0.02 g $NiCl_2 \cdot 6\ H_2O$ and 0.03 g $Na_2MoO_4 \cdot 2\ H_2O$. The pH of the solution was adjusted to 8.3 before autoclaving the medium. Each culture vessel containing 1000 ml of this medium was inoculated with 200 ml of a 14-day old culture of *C. aurantiacus*.

Isolation of membranes

Membranes were isolated from cultures which were grown for 3 and 8 days using a procedure modified after Wittmershaus (private communication). Cells from 3000 ml culture were collected by centrifugation in a Beckmann JA 10 rotor (8000 rpm) for 25 min. The pellet was resuspended in a medium of 10 mM Tris-HCl, (pH 8.0) [10], 2 mM ascorbate [8] and 150 mM NaCl in the same volume as the original culture. After centrifugation under the above conditions, the pellet was resuspended in a medium of 10 mM Tris-HCl (pH 8.0), and 2 mM ascorbate, adjusted to an absorbance of 2 per cm at 866 nm. 2 mM PMSF (Serva), 1 mM EDTA, 50 μg/ml DNAase (Sigma) and 50 μg/ml lysozyme (Serva) were added [5]. (Each value refers to the final concentration.) The cells were broken with a Branson sonifier B15 at a sonic power of 40: the cell suspension was divided into 50 ml aliquots and sonicated three times on ice for 3 min with 5 min intervals between successive sonications in order to avoid heating effects. The remaining whole cells and cell debris were removed by centrifugation in the JA 10 rotor (25 min at 10000 rpm).

Gel-electrophoretic filtration

We have modified an electrophoresis system used for native gel electrophoresis into a gel-electrophoretic filtration system. A vertical chamber without a well former in the gel was used. The gel system (3 mm thickness) had the following composition: acrylamide/bisacrylamide (29.2% : 0.8%) 8%, Tris-borate 0.05/0.033 M (pH = 8.7), sucrose 7%, TEMED 0.1%, LDS 0.1%. The electrophoresis buffer contained 0.05/0.033 M Tris-borate (pH 8.7), and 0.1% (w/v) LDS. 1500 μl membranes were incubated with 500 μl sucrose solution (70%) and 500 μl of LDS solution of various concentrations. The incubation was performed for 5 min at 50 °C. Before and after this procedure the suspension was mixed using a whirlmixer. An aliquot of 2.5 ml of the incubated suspension was layered on top of each gel. Electrophoresis was performed at 4 °C at a constant voltage of 300 V. After 1 h the polarity of the power supply was reversed for 1 min and the chlorosomes were collected from the top of the gel with a Pasteur pipette.

Pigment determination

The pigments of whole cells were extracted by adding 10 ml acetone/methanol 7 : 2 (v/v) to a pellet from 5 ml cell suspension. Cells had been harvested at 4000 rpm for 20 min a Beckman table-centrifuge. Cell debris in the extract was removed by the same procedure. The pigments from isolated chlorosomes and from density gradient centrifugation fractions were extracted with methanol alone. The pigment content was calculated using the absorption coefficients published by Feick et al. [10] (BChl *c*: 74 per mM cm at 666 nm, BChl *a*: 68.6 per mM cm at 769 nm). The carotenoid content was calculated using the absorption coefficient given by Schmidt [5]. We verified that the absorption coefficients are not significantly different in methanol and the methanol/acetone mixture.

Absorption spectroscopy

Absorption spectra were measured using either a Cary 17 or a Hitachi 100–80 spectrophotometer. Absorption spectra of whole cells were measured with a suspension of the extracted cells as a reference in order to compensate for scattering artefacts.

Fluorescence spectra

Corrected fluorescence emission and excitation spectra were recorded on a computer-controlled Spex Fluorolog spectrofluorimeter [11]. An RCA C31034 photomultiplier with a gallium-arsenide photocathode was used as the detector in the photon-counting mode. The wavelength dependence of the sensitivity of the detection system was corrected with reference to a calibrated

tungsten lamp standard tracable to the Physikalische Techn. Bundesanstalt, Braunschweig, F.R.G. In order to avoid self-absorption, the samples were diluted to an absorbance $\leqslant 0.2$ at 740 nm.

Results

The first point to discuss concerns influence of the detergent LDS on the absorption and fluorescence spectra of incubated membranes. At low concentrations (0.1–0.3% LDS) the absorption spectra of incubated membranes show no difference in comparison to the native membranes. At higher detergent concentrations ($> 0.3\%$ LDS) there occurs a denaturation of the antenna complex and the reaction center located in the cytoplasmatic membrane. In contrast the component with the absorption maximum at 790–800 nm shows a different behaviour. In one experiment, e.g., the ratio A_{740}/A_{800} after incubation with 0.1% LDS was 14.2. At higher detergent concentrations (0.5–5%) we find that the ratio A_{740}/A_{800} was stabilized at 31.2 ($\sigma = 1.4$). This component which is due to the BChl *a* 790 antenna was stable under the conditions of the incubation procedure. The A_{740} from the BChl *c* was stable as well. Studies of the fluorescence emission show also a different behaviour of these components. At low concentrations of LDS (0.1–0.3%), where the absorption spectra are not affected, we find an energetic uncoupling of the chlorosome from the cytoplasmic components. Fluorescence excitation spectra with a detection wavelength at 740 nm show a maximum at 460 nm (BChl *c*) in the short wavelength region, spectra with a detection wavelength at 884 nm a maximum at 375 nm (BChl *a*). At high detergent concentrations, where the cytoplasmatic components were totally denaturated, emission spectra with an excitation wavelength of 460 nm show in all cases peaks at 750 nm and a shoulder at 800 nm. The ratio F_{802}/F_{750} decreases from 1.3 (0.1% LDS) to 0.5 (5% LDS). In contrast to the stabilized absorption of the chlorosomal components the energetic coupling decreases for these components continuously with higher detergent concentrations.

The absorption spectrum of a chlorosome preparation obtained by the gel-electrophoretic filtration procedure after incubation of the membranes with a concentration of 0.1% (v/v) LDS is shown in Fig. 1A (full line). The long wavelength absorption band shows a maximum of approx. 740 nm which is characteristic for intact chlorosomes (see Table I). The absorption of the methanol extract is given as a dashed line. Basically identical spectra were obtained in the range of LDS concentration from 0.1 to 5% during incubation. For comparison the absorption of raw membranes is given in Fig. 1B. A characteristic feature of these chlorosome preparations is the absence of a shoulder in the 792–795 nm region which is present in all previous preparations. The absorption ratio A_{740}/A_{795} is very high ranging to ratio of 620 ± 200 from chlorosomes prepared with membranes 1 and 550 ± 230 from chlorosomes prepared with membranes 2 (see Table I). The wide range of this ratio is explained mainly by the fact that the very small absorption of 795 nm is determined only with a fairly large error. A_{740}/A_{795} ratios calculated from published spectra are approx. 19 [10], 12 [12], 13 [5], and 19 [9]. Comparison of these results with our data shows drastic differences. In previous preparations the ratio of BChl *c*/BChl *a* calculated from the pigment extract is higher than the A_{740}/A_{795} ratio calculated from the corresponding chlorosomes [5,10]. As shown in Fig. 2, this is not the case for our preparations. It is clear from these data that BChl *c* and the small BChl *a* content are uncorrelated in our chlorosome preparations. The highest BChl *c*/BChl *a* ratio (determined from the extract) in our preparations is 280, indicating the virtual

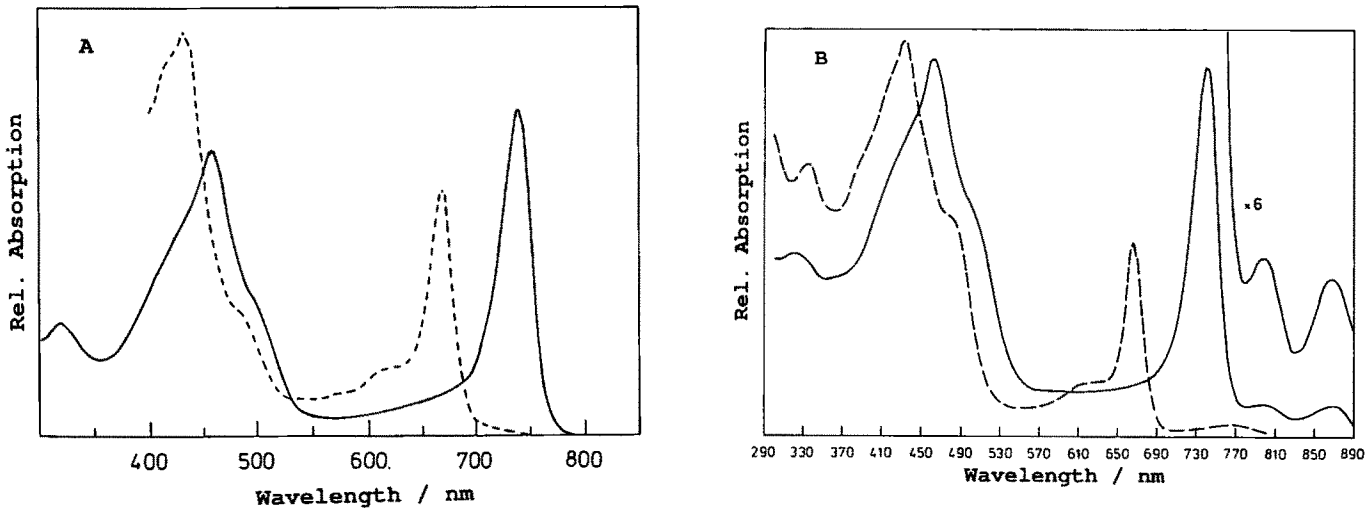

Fig. 1. Room temperature absorption spectra (full line) of intact isolated chlorosomes (A) and isolated membranes (B) from *C. aurantiacus*. The respective absorption spectra of the methanol extracts are shown in dashed line.

TABLE I

Absorption (λ^{abs}_{max}) and fluorescence emission maxima (λ^{em}_{max}) of whole cells, membranes and chlorosomes of C. aurantiacus from two different membrane preparations (membranes 1 and 2)

Also given are the ratios of long-wavelength to 740 nm absorption bands, the ratios $F_{747:806}$ for the fluorescence band intensities and the BChl *c*/carotenoid ratio. Positions of absorption and fluorescence maxima have an error of ±1 nm.

Sample		Whole cells	Membrane 1	Chlorosomes from membrane 1 [a]	Membrane 2	Chlorosomes from membrane 2 [a]
λ^{abs}_{max}	I	740	740	738	740	739
	II	800	799		800	
	III	866	866		866	
λ^{em}_{max}	I	750	754	749	754	747
	II	803	802		802	
	III	884	884		884	
$A_{740:795(800)}$ [b]		5.8	8.2	550 ±230	8.95	620 ±200
$A_{740:790}$				440 ±250		450 ±300
$F_{747:803}$		0.7	0.41	7.6± 1	0.42	5.4± 1
BChl *c*/carotenoid (w/w)		2.5	2.4	4.2± 1.2	3.0	3.4± 1

[a] The error ranges indicated are statistical errors from different preparations.

[b] The ratio was calculated with A_{740} and the maximum near 800 nm for membranes and whole cells.

absence of BChl *a*. In contrast the amount of carotenoids is clearly proportional to the BChl *c* content as seen from Fig. 3. Table I summarizes the results for two different preparations. The BChl *c*/carotenoid ratio of 3.4 ± 1.1 and 4.2 ± 1 (w/w) is somewhat lower than the published ratio of 10.5 [5]. This indicates that carotenoids form an integral part of the chlorosomes.

The fluorescence emission spectra of isolated chlorosomes, isolated membranes, and intact cells are shown in Fig. 4 for an excitation wavelength of 460 nm, i.e., preferential BChl *c* excitation. Both the membranes and the intact cells show three main emission bands in the recorded wavelength region at 750, 803 and 884 nm. The ratio of the amplitude of the 803 nm band in comparison to the others is slightly higher in the membrane preparation (Fig. 4) as compared to intact cells.

The emission spectrum of our chlorosome preparation in the long wavelength region shows only the predominant 747 nm peak and a shoulder near 760 nm. No indication for the two long-wavelength bands is observed. In particular, the absence of a peak in the 800–810 nm region, present in all previous chlorosome preparations [6,8,13] is revealing. Fluorescence emission spectra at 77 K (not shown) do not show either an emission maximum near 800 nm. In addition, a small peak with the maximum at 677 nm is present in the chlorosome preparation. This is generally attributed to free BChl *c*. Repeated freeze/thaw cycles were observed to increase the amplitude of this band as compared to the main 747 nm emission band. Corrected fluorescence excitation spectra were recorded for chlorosomes and

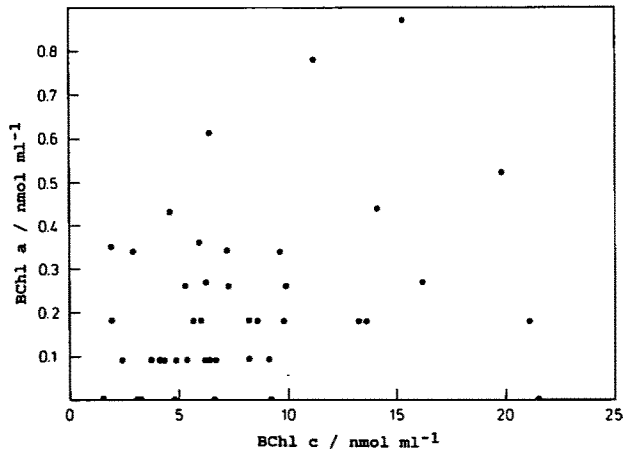

Fig. 2. Plot of BChl *a* vs. BChl *c* content of different (determined from extract) preparations of isolated chlorosomes from *C. aurantiacus*, (see Materials and Methods for details). No correlation seems to be present between these BChls.

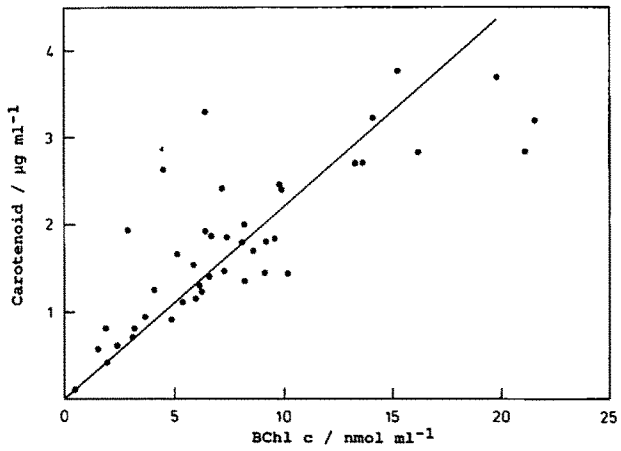

Fig. 3. Carotenoid content vs. BChl *c* content of different preparations of isolated chlorosomes from *C. aurantiacus*, (see Materials and Methods for details). The correlation coefficient for all data is 0.81. These data have been obtained from the same preparations from which the BChl *c*/BChl *a* ratio has been determined (cf. Fig. 2).

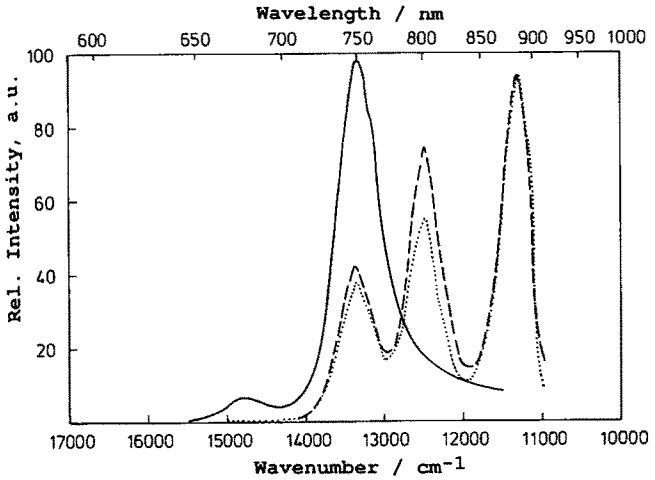

Fig. 4. Corrected fluorescence emission spectra at room temperature with excitation of 460 nm. Isolated chlorosomes (———), isolated membranes (— — —) and whole cells (· · · · · ·) from *C. aurantiacus*.

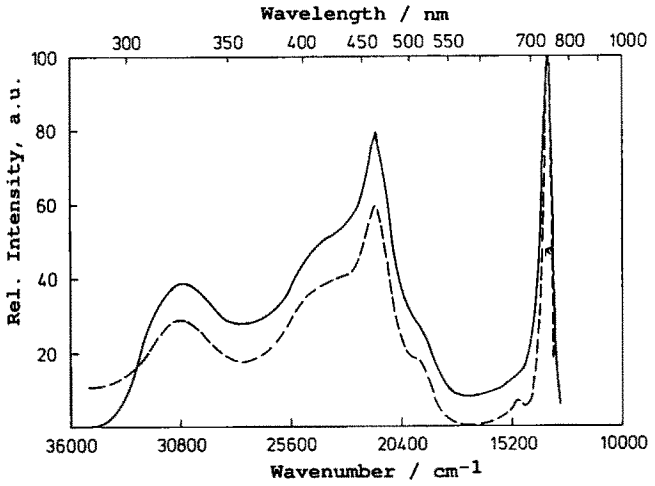

Fig. 5. Corrected fluorescence excitation spectra with $\lambda_{em} = 780$ nm at room temperature. Isolated chlorosomes (— — —) and isolated membranes (———) from *C. aurantiacus*.

membranes (Fig. 5) with emission wavelength of 780 nm at room temperature. The excitation peaks are observed at 325, 420 (free BChl *c*), 460, 670 (free BChl *c*) and 742 nm. A shoulder is observed at approx. 520 nm, which originates from carotenoid absorption and indicates carotenoid → BChl *c* energy transfer.

Discussion

The chlorosomes isolated according to our procedure are intact as judged from the positions of their main absorption band at 740 nm and their main emission peak at 747 nm. The absorption band at 792–795 nm and the corresponding emission peak at ≈ 801–806 nm, which are characteristic of all previous preparations [6,13], are lacking. The fluorescence spectra given in the literature for isolated chlorosomes [6,13] generally show an intensity ratio close to 1 : 1 between the 747 nm and the 802–806 nm emission peaks. Wittmershaus et al. [8] have isolated chlorosomes using an anaerobic isolation procedure. Here the ratio $F_{747:802}$ is significantly lower, thus indicating better energy transfer. These long-wavelength absorption and emission bands are generally attributed to a BChl *a* protein complex located in the base plate of the chlorosomes [10]. From the absorption and fluorescence emission spectra it is clear that such a BChl *a* protein complex is absent in our preparations. This is further supported by the lack of correlation in the BChl *c*/BChl *a* ratio in contrast to reports which found a constant 25 : 1 ratio of BChl *c*/BChl *a* in isolated chlorosomes [10]. All these observations lead us to conclude that our chlorosome preparations are free from bound and/or functional BChl *a*. The extremely small amount of BChl *a* present in our preparations probably represents detached free BChl *a*.

The following hypothesis could explain our results:

(1) We have prepared chlorosomes which lack the whole baseplate.
(2) We have denaturated the BChl *a* protein complex of the baseplate.
(3) We have destroyed the chlorosomes and have instead isolated rod elements [3].
(4) We have prepared chlorosomes which lack the BChl *a* protein complex of the baseplate or there is no BChl *a* protein complex in the baseplate present in vivo.

(*ad 1.*) If one accepts the hypothesis that BChl *a* is located in the baseplate, one possible conclusion from our findings would be that we have prepared chlorosomes which lack the baseplate and therefore the BChl *a* complex with absorption and emission maxima at approx. 795 and 805 nm, respectively. Such a conclusion might, however, be preliminary. According to current understanding the baseplate forms a more or less integral part of a chlorosome [3,12]. If these ideas are correct, we find it difficult to imagine how we could remove the baseplate and still maintain functionally intact chlorosomes. This is supported by results from electron microscopy which show that the negatively stained chlorosomes are intact (the results of a comprehensive electron microscopy study will be presented in a forthcoming contribution, unpublished results in collaboration with Dr. J.R. Golecki, Freiburg). The shape of the chlorosomes prepared with gel-electrophoretic filtration shows now differences as compared to the chlorosomes in the isolated membranes.

(*ad 2.*) Denaturation of the BChl *a* protein complex can be excluded on the basis of the studies of the detergent effect on membranes. At low concentration the BChl *a* protein complexes are not affected. At high detergent concentrations we can detect the BChl *a* component with a fluorescence emission at 802 nm in all cases. Isolated chlorosomes prepared with low (0.1% LDS) detergent concentrations during incubation show

no differences to the ones prepared with high concentrations. The specific effect of the new gel-electrophoretic filtration isolation procedure can not be a denaturation effect therefore.

(*ad 3.*) The isolation of rod elements was excluded by negatively stained electron microscopy (see above). The dimensions of our chlorosomes are in the range of 96 nm length and 31 nm width.

(*ad 4.*) The last and most likely possibilities are that either the BChl *a* protein complex is removed from the chlorosome by gel-electrophoretic filtration and is held in the gel matrix or there exists no BChl *a* protein complex in the baseplate at all. We cannot find this complex in the gel matrix after the gel-electrophoretic filtration procedure, but this could be due to the low concentration of the complex and the ineffectiveness of the gel system for this problem. We are not able at present to unequivocally distinguish between these two situations. However, either of these possibilities would allow more easily to understand a variety of experimental observations reported in the literature, including the data from lifetime measurement [7].

Preliminary estimates of the fluorescence yield of our chlorosome preparation gives a value of $\phi_F \leqslant 1\%$ (an exact determination will be reported later). Chlorosomes prepared according to Ref. 10 show similarly low yields. For an isolated antenna complex one would expect a fluorescence yield well above 10% in order to ensure efficient energy transfer to the reaction centers. The formation of a highly efficient quencher of unknown origin during the preparation has been held responsible for the extremely low yield and consequently low fluorescence lifetime of previous chlorosome preparations [7,8]. This remains a puzzling feature of all isolated chlorosomes, including ours.

The BChl *a* protein complex with emission at ≈ 803 nm was found to be not very efficiently coupled to the BChl *c* in the chlorosomes in previous isolations [6,10]. This is surprising if the BChl *a* complex was indeed located in the baseplate. Another possible interpretation would therefore be that previously reported preparations were inhomogeneous, thus containing a majority of chlorosomes of the same type obtained with our method in pure from and a smaller amount of chlorosomes containing also a BChl *a* complex. This BChl *a* complex could originate from the cytoplasmic membrane. This second interpretation would also be supported by the fact that published chlorosome preparations show quite a varying absorption ratio $A_{740:795}$. It would explain recent findings of, e.g., time-resolved fluorescence measurements which found only a small amplitude for long-lived emission components in the 805 nm emission band [7]. If the 805 nm band arose from BChl *a* complexes attached to the majority of the chlorosomes and acting as a terminal energy acceptor in chlorosomes, a predominant amplitude should have been observed for this long lifetime component.

It is not possible from our present data to decide conclusively whether our new chlorosome preparation contains baseplates or not, and consequently we can also not decide whether baseplates do contain the proposed BChl *a* complex emitting around 805 nm or not. Such a decision should be easily possible, however, on the basis of an electron micrographic study. Whatever the result of such experiments will be, our new chlorosome preparation provides a new basis for studies further clarifying questions related to the topography, structure and function of chlorosomes.

The new method has some advantages in comparison to others:

(i) The method is a simple one step preparation. The risk of artificial modifications is therefore minimized.

(ii) Enzymes cannot destroy the chlorosomes because proteins are held in the gel matrix.

(iii) Free pigments, complicating the interpretation of spectroscopic studies, also move into the gel.

(iv) Membrane bound pigment-protein complexes cannot interfere with the chlorosomes.

It may be possible, that the combination of the two principles gel-filtration and gel-electrophoresis in the way described here may provide a useful procedure for the isolation and purification of other large complexes.

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