Polarized fluorescence measurements on ordered photosynthetic antenna complexes

Chlorosomes of *Chloroflexus aurantiacus* and B800-B850 antenna complexes of *Rhodobacter sphaeroides*

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ABSTRACT We have used a new and relatively easy approach to study the pigment-organization in chlorosomes from the photosynthetic bacterium *Chloroflexus aurantiacus* and in B800-850 antenna complexes of the photosynthetic purple bacterium *Rhodobacter sphaeroides*. These particles were embedded in compressed and uncompressed gels and the polarized fluorescence was determined in a 90° setup. Assuming both a rotational symmetric distribution of the particles in the gel and of the transition dipole moments in the particles, the order parameters $\langle P_2 \rangle$ and $\langle P_4 \rangle$, describing the orientation of the symmetry axis of the particles with respect to the direction of gel expansion can be determined. Moreover, the direction parameters, describing the orientation of the absorption and emission dipole moments with respect to the symmetry axis of the particles can be obtained.

The value of $\langle P_2 \rangle$ is essential for quantitative interpretation of linear dichroism measurements and usually it is estimated from theoretical approaches, which may lead to incorrect results. For the rod-like chlorosomes the value of $\langle P_2 \rangle$ appears to be the same as predicted by the theoretical approach of Ganago, A. O., M. V. Fok, I. A. Abdourakhmanov, A. A. Solov'ev, and Yu. E. Erokhin (1980. *Mol. Biol. [Mosc.]*. 14:381–389). The agreement with linear dichroism results, analyzed with this theoretical approach shows that the transition dipole moments are indeed in good approximation distributed in a rotationally symmetric way around the long axis of the chlorosomes. Moreover, it appears those BChl c molecules, which fluoresce, are oriented in the same way with respect to the symmetry axis as the rest of these pigments, with the dipole moments close to parallel to the long axis.

The B800-850 complexes appear to orient like discs, whereas the transition dipoles of the BChl *a* 800- and 850-nm bands are oriented almost perpendicular to the symmetry axis. These findings are in agreement with the minimal model for these complexes proposed by Kramer, H. J. M., R. van Grondelle, C. N. Hunter, W. H. J. Westerhuis, and J. Amesz (1984. *Biochim. Biophys. Acta.* 156–165).

The amount of orientation of the particles appears to vary for different gels and it is lower than predicted by the theory of Ganago et al., showing that application of their approach for these particles leads to incorrect interpretations.

The approach that is used in this study allows determination of orientations of those dipole moments, which transfer their excitation energy to the fluorescing species, in contrast to linear dichroism measurements, where the orientations of all absorbing dipole moments are studied. For the polarized fluorescence measurements, the amount of orientation of the particles is determined experimentally, whereas for linear dichroism this amount has to be estimated from theoretical models. The value of $\langle P_2 \rangle$ that can be determined from the fluorescence measurements can, however, also be used for a quantitative interpretation of the linear dichroism results.

INTRODUCTION

Polarized light spectroscopy is often applied to study the structure of biological macromolecules and macromolecular complexes. For instance, in photosynthesis research use is made of linear dichroism (ld) and polarized fluorescence. For ld measurements the particles have to be aligned and a variety of techniques is available (for a review see, e.g., Breton and Vermeglio, 1982). A method which is often applied is alignment in squeezed polyacrylamide gels (Abdourakhmanov et al., 1979). The gels, which are transparent down to 250 nm, are squeezed in two mutual perpendicular directions with a factor $n^{1/2}$ and expand with a factor n (squeezing factor or degree of compression) in the z-direction, usually called the orientation axis of the gel. The orientation of the particles in the gel is in general considered to be rotationally symmetric around this axis. When light is incident along the x-axis the amount of ld (ΔA) is defined as $\Delta A = A_z - A_y$, where $A_{y,z}$ is the absorption for light polarized along the y,z-axis. For such rotationally symmetric distributions, the following relation holds (see also van Gurp et al., 1988)

$$(A_z - A_y)/(A_z + 2A_y) = \Delta A/3A_{iso} = \langle P_2(\cos(z,\mu)) \rangle = S_\mu , \quad (1)$$

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0006-3495/91/05/992/10 $2.00

where A_{iso} is the isotropic absorption before compression, corrected for the changed thickness of the gel. The unit vector μ denotes the direction of the transition dipole moment. The second-rank Legendre polynomial $P_2(x)$ is defined as $P_2(x) = \frac{1}{2}(3x^2 - 1)$ and the brackets denote an ensemble average over all contributing transition dipoles. It is clear that S_μ depends both on the orientation of the macromolecules or macromolecular complexes (henceforward called "particles") and the orientation of the dipoles in these particles. Ganago et al. (1980) presented mathematical expressions to describe the orientational behavior of rod-like and disc-like particles in polymer gels. They assumed that the particles contain an axis of symmetry, such that all orientations of the transition dipoles are equally probable around this axis. This axis is assumed to be the long axis in a rod or the normal to the plane of a disc. In that case one can write the well-known addition theorem as (see, e.g., van Gurp et al., 1988)

$$S_\mu = \langle P_2 \rangle \langle P_2(\cos\beta^\mu) \rangle , \tag{2}$$

where

$$\langle P_2 \rangle = \frac{1}{2}\langle 3\cos^2\beta - 1 \rangle . \tag{3}$$

In Eq. 3 β denotes the angle between the symmetry axis of the particle and the orientation axis of the gel (see Fig. 1). β^μ is the angle between the transition dipole moment and the symmetry axis of the particle (see Fig. 2). In general, one is interested in the value of $\langle P_2(\cos\beta^\mu) \rangle$ and thus $\langle P_2 \rangle$ should be known. Ganago et al.

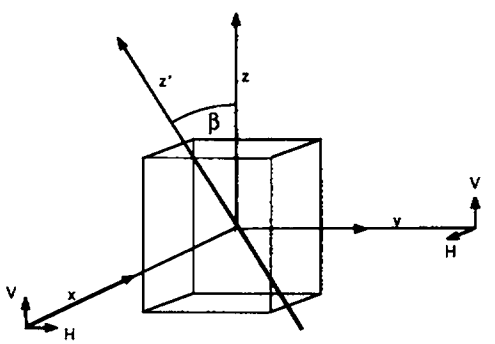

FIGURE 1 Definition of directions for the experimental setup. Light is incident along the x-axis and detected along the y-axis. Polarization directions are either vertical (V) or horizontal (H). The gel is compressed along both the x- and y-axis and expands along the z-axis. The angle between the orientation axis of the gel and the symmetry axis z' of the particle is called β. Particles are assumed to be distributed around the z-axis in a rotationally symmetric way and their orientational distribution is given by the distribution function $f(\beta)$.

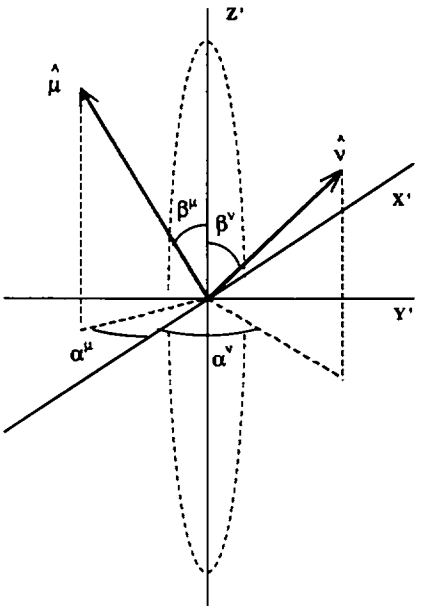

FIGURE 2 Definitions of angles, describing the orientations of the absorption dipole moment μ and the emission dipole moment ν. Only one example for both μ and ν is given. The particle symmetry axis is called the z'-axis. For rotationally symmetric disc-like particles this axis is the normal to the plane through the disc. The x'- and y'-axis can be chosen arbitrarily for rotationally symmetric particles.

(1980) give expressions for $\langle P_2 \rangle$, being equal to $\frac{1}{2}(3T[n] - 1)$ for rods with a value between 0 and 1 and equal to $\frac{1}{2}(3L[n] - 1)$ for discs with a value between -0.5 and 0. Analytical expressions for both $T(n)$ and $L(n)$ are given by these authors. The expressions for $\langle P_2 \rangle$ can be calculated according to:

$$\langle P_2 \rangle = \frac{1}{2}\int_0^\pi \sin\beta d\beta P_2(\cos\beta') . \tag{4}$$

For instance, for rods β' is related to β according to the following relation:

$$\cos^2\beta' = (n^3\cos^2\beta)/[(n^3 - 1)\cos^2\beta + 1]. \tag{5}$$

The angle β between the symmetry axis of the particle and the orientation axis of the gel before compression changes to β' after compression. Likewise $\langle P_4 \rangle$, which is the second term in the expansion of the orientational distribution and which will be used below, can be calculated by replacing $P_2(\cos\beta')$ by $P_4(\cos\beta')$ in Eq. 4.

For the derivation of their results, Ganago et al. (1980) assume that the orientation of the particles follows exactly the deformation of the gel. This can be visualized by drawing an imaginary line or plane in the gel and by looking how the orientation of this line or

plane changes upon compression of the gel. This is illustrated by van Amerongen et al. (1988) for a gel, which is compressed in one direction and expands in one perpendicular direction. It is assumed that a rod-like (disc-like) particle with the same orientation as the line (plane) also aligns in the same way as the line (plane). Moreover, it is assumed that the particles are not deformed upon gel compression and that they are rotationally symmetric around either the long axis of the rod or the normal to the disc. Using the same assumptions, an orientational model was obtained for gels that are compressed in one direction and expand in one perpendicular direction (van Amerongen et al., 1988). However, these assumptions are not always justified, especially when the particles are not rod- or disc-like. Also, if an axis exists around which the transition dipoles are distributed in a rotationally symmetric way, this need not necessarily be the long axis or the normal. Therefore, in general the obtained absolute value for $\langle P_2 \rangle$ should be considered as an upper limit and the corresponding value for $\langle P_2(\cos \beta^u) \rangle$ as a lower limit (see Eq. 2). However, for many applications a more accurate determination of $\langle P_2 \rangle$ is desirable. Under certain conditions this can be achieved, making use of polarized fluorescence measurements in a 90° fluorescence setup, using both an unsqueezed and a squeezed gel. The basic theory that it is needed is given by van Gurp et al. (1988). Here we present how it can be applied to squeezed gels and which assumptions have to be made. The method is applied to interpret the ld and polarized fluorescence measurements on the rod-like chlorosomes from *Chloroflexus aurantiacus*. These light-harvesting chlorosomes are flat elongated sacs with rounded ends and measure about 100 × 30 × 10 nm^3 as determined by electron microscopy (Staehelin et al., 1978). They contain thousands of BChl *c* molecules as the most important pigments. As will be shown, excellent agreement exists between the experimentally determined values of $\langle P_2 \rangle$ and the calculated ones. The determined values for $\langle P_2 \rangle$ can be used for interpretation of linear dichroism results. Small differences, however, exist for the values of $\langle P_4 \rangle$. The transition moments corresponding to the 460 and 740 nm BChl *c* absorption bands are oriented along the long axis of the chlorosomes in agreement with previous results (van Amerongen et al., 1988).

In addition, we studied B800-850 antenna complexes of the photosynthetic purple bacterium *Rhodobacter sphaeroides*. These particles appeared to behave like discs in their ordering, but not to the same extent as predicted by the theory of Ganago et al. (1980). The obtained orientations of the dipole moments are in agreement with a model proposed by Kramer et al. (1984).

MATERIALS AND METHODS

BChl *a* containing chlorosomes from *Chloroflexus aurantiacus* were prepared as described by Griebenow and Holzwarth (1989). The buffer contained 10 mM Tris.HCl and the value of the pH was 8.7. The detergent *N*-lauryl-β-Iminodipropionic Acid was used with a detergent/BChl *c* molar ratio of 13:1. B800-850 antenna complexes from *Rhodobacter sphaeroides* were essentially prepared as described by Frank et al. (1987) and were further purified by FPLC (R.W. Visschers, manuscript to be published). The buffer used was 20 mM Tris.HCl with pH = 8.0 and 0.1% (wt/vol) LDAO (*N,N*-Dimethyldodecylamine-*N*-Oxide).

Uncompressed gels were prepared in a glass, quartz, or perspex cuvette of 1.0 cm × 1.0 cm. Other gels were prepared in a press with maximum dimensions in both the *x*- and *y*-direction of 1.25 cm. Compressed gels were put in a perspex cuvette of dimensions 1.0 cm × 1.0 cm, corresponding to a maximum squeezing factor of $n = 1.56$. The polyacrylamide/water gels contained 14.5% (wt/vol) acrylamide from Serva (Heidelberg, Germany), 0.5% (wt/vol) *N,N*-methylenebisacrylamide, 0.1% *N,N,N',N'*-tetramethyl-paraphenylene-diamide, both from Merck-Schuchardt (Hohenbrunn, Germany) and 0.02–0.1% (wt/vol) ammoniumpersulphate from J. T. Baker Chemicals B. V. (Deventer, the Netherlands). The buffers were the same as mentioned above.

Linear dichroism spectra were recorded on a homebuilt spectropolarimeter and absorption spectra on a Cary 219 spectrophotometer. Fluorescence data were measured on a home-built fluorimeter with a 90° setup, using photoncounting. Sheet-polarizers in the excitation and detection branch were placed either vertically or horizontally. The gel was placed in the sample holder with its *z*-axis (direction of gel expansion) vertically. Absorption values were typically 0.05.

THEORY

In the following we shall confine ourselves to the cases, where particles are rotationally symmetric and also their distribution is rotationally symmetric around the orientation axis of the gel. In that case the orientational distribution of the particles can be described by (van Gurp et al., 1988):

$$f(\beta) = \sum_{\substack{L=0 \\ (L \text{ even})}}^{\infty} 1/2\,(2L+1)\,\langle P_L \rangle\, P_L(\cos\beta) \tag{6}$$

$$\langle P_L \rangle = \int_0^{\pi} P_L(\cos\beta)\, f(\beta) \sin\beta\, d\beta. \tag{7}$$

For $\langle P_2 \rangle$ the expression was given in Eq. 3 and $\langle P_4 \rangle$ is defined as:

$$\langle P_4 \rangle = 1/8\,\langle 35\cos^4\beta - 30\cos^2\beta + 3 \rangle. \tag{8}$$

The experimental setup that is used in this study is given in Fig. 2. We shall make use of the fact that the measured light intensity I'_{ij} with excitation light polarized along the vector $\hat{\mathbf{e}}_i$ and the emission light detected with a polarizer along the vector $\hat{\mathbf{e}}_j$ is given by

$$I'_{ij} = k_{ij}\,\langle (\hat{e}_i \cdot \hat{\mu})^2\, (\hat{e}_j \cdot \hat{\nu})^2 \rangle \tag{9}$$

where $\hat{\mu}$ and $\hat{\nu}$ are unit vectors in the direction of the absorption and emission transition dipole, respectively. The proportionality constants k_{ij} depend amongst others on the lamp intensity, detector sensitivity, extinction coefficients, concentrations, and fluorescence quantum yields. We shall now define the corrected intensities as $I_{ij} = I'_{ij}/k_{ij}$, which only depend on the orientation of absorption and emission dipoles. For the fluorescence intensities I_{ij} $(i,j = v,h)$ the following relations hold (van Gurp et al., 1988):

$$I_{vv} = C\,(1 + 2S_{\mu} + 2S_{\nu} + 4G_0) \tag{10a}$$

$$I_{vh} = C\,(1 + 2S_{\mu} - S_{\nu} - 2G_0) \tag{10b}$$

$$I_{hv} = C\,(1 - S_{\mu} + 2S_{\nu} - 2G_0) \tag{10c}$$

$$I_{hh} = C\,(1 - S_{\mu} - S_{\nu} + G_0 - 3G_2). \tag{10d}$$

By definition the parameter C is equal to 1, but it is included here for later use. S_{μ} is the same as S_{μ} in Eq. 1 except that there it is determined by all dipole moments contributing to the absorption at a particular wavelength and here only by those which absorb light which eventually leads to fluorescence. S_{ν} is defined as $\langle P_2\rangle\langle P_2(\cos\beta^{\nu})\rangle$, where β^{ν} is the angle between ν and the symmetry axis of the particle. When all dipole moments are oriented randomly in the sample, both S_{μ} and S_{ν} are 0. When the dipole moments are perfectly parallel to the z-axis, S_{μ} and S_{ν} are 1. G_0 and G_2 are the so-called correlation functions, correlating the orientation of $\hat{\mu}$ and $\hat{\nu}$ in a given particle. For definitions of G_0 and G_2 see van Gurp et al. (1988).

For an isotropic sample $S_{\mu} = S_{\nu} = 0$ and $G_0 = G_2 = r/2$, where r is the fluorescence anisotropy defined as $(I_{vv} - I_{vh})/(I_{vv} + 2\,I_{vh})$. The anisotropy r is determined experimentally by $(I'_{vv} - kI'_{vh})/(I'_{vv} - 2kI'_{vh})$, where k is the correction factor for the polarization sensitivity of the setup. Substitution in Eqs. 10*a–d* with $C = 1$ gives the correction factors k_{ij} in the uncompressed gel, according to:

$$I'_{vv}/k_{vv} = 1 + 2r \tag{10e}$$

$$I'_{vh}/k_{vh} = 1 - r \tag{10f}$$

$$I'_{hv}/k_{hv} = 1 - r \tag{10g}$$

$$I'_{hh}/k_{hh} = 1 - r. \tag{10h}$$

Assuming that these remain unchanged they can also be applied to the case of the compressed gels. However, variations in the lamp intensity and detection sensitivity must be rigorously excluded during the experiment. Moreover, the concentration must be made exactly equal in both gels and the fluorescence quantum yields of the particles should be perfectly identical. It is very difficult, if not impossible, in practice to verify if all these parameters indeed are unchanged. Therefore, by using the constants k_{ij} obtained for the uncompressed gel for the correction of the intensities for the compressed gel, we find the parameters I_{ij}, which are related to S_{μ}, S_{ν}, G_0, and G_2 according to Eqs. 10*a–d* but now C is not necessarily equal to 1. The inclusion of the same parameter C in all Eqs. 10*a–d* is strictly spoken only correct if the ratio of vertically and horizontally incident light is the same throughout the sample for the compressed and uncompressed gel. However, for dichroic samples this ratio changes throughout the sample and the effect is larger when higher concentrations are used. This is the major reason why samples with low absorptions were used. If the absorption is lower than 0.05, the ratios of incident horizontally and vertically light intensities are almost equal everywhere in the compressed and uncompressed gel. The calculated parameters can be used iteratively to correct for intensity ratio differences, but even for the chlorosomes, which give rise to very dichroic compressed gels, the changes after correction are very small.

There are now four equations with five unknowns and these equations cannot be solved without further assumptions, unless one of the five parameters can be determined independently. For the parameters S_{μ}, S_{ν}, G_0, and G_2 the following relations hold (van Gurp et al., 1988):

$$\begin{aligned}
S_{\mu} &= \langle P_2\rangle\langle P_2(\cos\beta^{\mu})\rangle \\
S_{\nu} &= \langle P_2\rangle\langle P_2(\cos\beta^{\nu})\rangle \\
G_k &= G(k0)\langle P_2(\cos\beta^{\mu})P_2(\cos\beta^{\nu})\rangle \\
&\quad + 3\,G(k1)\langle\sin\beta^{\mu}\cos\beta^{\mu}\sin\beta^{\nu}\cos\beta^{\nu}\cos(\alpha^{\mu} - \alpha^{\nu})\rangle \\
&\quad + 3/4\,G(k2)\langle\sin^2\beta^{\mu}\sin^2\beta^{\nu}\cos 2(\alpha^{\mu} - \alpha^{\nu})\rangle,
\end{aligned} \tag{11}$$

where k is either 0 or 2. Expressions for $G(ij)$ can be found in van Gurp et al. (1988) and depend on $\langle P_2\rangle$ and $\langle P_4\rangle$ α^{μ} and α^{ν} are the azimuthal angles for $\hat{\mu}$ and $\hat{\nu}$. So the four parameters S_{μ}, S_{ν}, G_0, and G_2 are determined by seven parameters, namely $\langle P_2\rangle$, $\langle P_4\rangle$, and the five expressions between brackets, depending on the angles β^{μ}, β^{ν}, α^{μ}, and α^{ν}, which cannot all be extracted from the measured intensities. It is, however, possible to simplify Eqs. 11 considerably by assuming that rapid energy transfer takes place (i.e., transfer rate is much faster than the rate of fluorescence) among all of the pigments in the particles, constituting at least a minimal unit of rotational symmetry, provided that such a rotationally symmetric unit exists. In that case the orientations of absorption and emission dipoles within the particle are no longer correlated and the correlation functions G_k in Eq. 11 reduce to separate ensemble averages over the orientations of absorption and emission dipoles. Using the rotational symmetry, integration over the angles α now eliminates the latter two elements of G_k in Eqs. 11,

so that one obtains:

$$S_\mu = \langle P_2 \rangle \langle P_2(\cos \beta^\mu) \rangle$$
$$S_\nu = \langle P_2 \rangle \langle P_2(\cos \beta^\nu) \rangle$$
$$G_k = G(k0) \langle P_2(\cos \beta^\mu) \rangle \langle P_2(\cos \beta^\nu) \rangle. \quad (12)$$

Using the explicit expressions for $G(k0)$ one finds for the correlation functions G_0 and G_2:

$$G_0 = [1/5 + 2/7\langle P_2 \rangle + 18/35\langle P_4 \rangle] \langle P_2(\cos \beta^\mu) \rangle \langle P_2(\cos \beta^\nu) \rangle \quad (13)$$

$$G_2 = [1/5 - 2/7\langle P_2 \rangle + 3/35\langle P_4 \rangle] \langle P_2(\cos \beta^\mu) \rangle \langle P_2(\cos \beta^\nu) \rangle. \quad (14)$$

Note that rotationally symmetry for either the absorption or the emission dipole moments alone is sufficient for Eqs. 13 and 14 to hold.

For an uncompressed gel both $\langle P_2 \rangle$ and $\langle P_4 \rangle$ are equal to 0 so that G_0 and G_2 reduce to:

$$G_0 = G_2 = r/2 = 1/5 \langle P_2(\cos \beta^\mu) \rangle \langle P_2(\cos \beta^\nu) \rangle. \quad (15)$$

This expression for r for an uncompressed gel together with the four corrected intensities in Eqs. 9*a–d* are sufficient to determine the remaining unknown parameters C, $\langle P_2 \rangle$, $\langle P_4 \rangle$, $\langle P_2(\cos \beta^\mu) \rangle$, and $\langle P_2(\cos \beta^\nu) \rangle$. In solving the equations we make use of the fact that

$$[G_0(c) - 6G_2(c)]/G_0(u) = -5 + 10\langle P_2 \rangle \quad (16)$$

and

$$S_\mu(c) S_\nu(c)/G_0(u) = 5 \langle P_2 \rangle^2, \quad (17)$$

where (c) and (u) denote compressed and uncompressed gels. $G_0(u)$ can be determined experimentally. Together with this parameter, the parameters k_{ij} can be determined, which can be used to calculate the corrected intensities for the compressed gel. $G_0(c)$, $G_2(c)$, $S_\mu(c)$, and $S_\nu(c)$ can now be expressed in terms of the corrected intensities and the unknown parameter C. Substitution of these parameters into Eqs. 16 and 17 leads to two equations with the unknowns C and $\langle P_2 \rangle$. Because of the quadratic form of the second equation there are two sets of solutions which can now be determined. Below it will be demonstrated how for two specific cases a choice can be made between these two sets. Eventually one finds: $\langle P_2 \rangle$, $\langle P_4 \rangle$, $\langle P_2(\cos \beta^\mu) \rangle$, $\langle P_2(\cos \beta^\nu) \rangle$, and C. Note that repeating the experiment at different absorption wavelengths should lead to identical values for $\langle P_2 \rangle$, $\langle P_4 \rangle$, and $\langle P_2(\cos \beta^\nu) \rangle$ if the same emission dipoles are involved. This leads to an internal check of the consistency of the interpretation of the results.

Particles in which the transition moments have a rotationally symmetric distribution will lead to different polarized fluorescence intensities as compared with particles which lack this symmetry. If the transition moments are not distributed in a rotationally symmetric way around a symmetry axis and if the energy transfer is not fast enough, then the determined parameters $G_0(c)$, $G_2(c)$, $S_\mu(c)$, and $S_\nu(c)$ are not correct. A consequence is that S_μ and S_ν will not agree with linear dichroism results. However, if agreement does exist, then this indicates that there is rotational symmetry around the orientation axis and that fast energy transfer occurs.

RESULTS

Chlorosomes from *Chloroflexus aurantiacus*

Measurements were performed on chlorosomes from *Chloroflexus aurantiacus*. The absorption and ld spectrum are similar to those given by van Amerongen et al. (1988). Our intention was to obtain accurate intensities to test the applicability of the method and not to find detailed spectral information for the particles. The chlorosomes were excited at 466 nm (bandwith = 13 nm) (absorption was ~0.05) and the fluorescence was observed at 751 nm (bandwidth = 13 nm). After determining one set of the intensities I'_{vv}, I'_{vh}, I'_{hv}, and I'_{hh} for both the compressed and uncompressed gel, together with the dark intensities (measured with excitation light blocked) the measurements were repeated until a sufficient amount of data had been accumulated. Note that differences in the lamp intensity in the case of compressed and uncompressed gels are corrected for by the constant C. Per set of measurements $\langle P_2 \rangle$, $\langle P_4 \rangle$, $\langle P_2(\cos \beta^\mu) \rangle$, $\langle P_2(\cos \beta^\nu) \rangle$, and C were determined. The procedure was repeated for all the other sets of measured intensities. As an example the results for the first and last (20th) set of results of a series of measurements, after correction for the dark signal, are given in Table 1. Note that there are several hours in between these two series of measurements. Per set of data there are two possible solutions for the desired parameters. One of these can be discarded since the second Legendre polynomial for the absorption and emission dipole moments can never be less than −0.5. For the measurements, corresponding to the data in Table 1, there is a decrease in detected light intensity for the compressed gel during the experiment, but the only factor that changes systematically is C. Therefore, the value of C has to be determined experimentally and taking it to be unity is not justified. The fact that C changes during the experiment may be due to changes in the absorption and/or the fluorescence quantum yield. However, the amount of orientation of the chlorosomes does not change and the orientation of the probed absorption and fluorescence dipole moments remain the same within the particles. In Table 2 the average results of the

TABLE 1 **Measured intensities I'_{ij} (number of counted photons in 30 s, corrected for dark signals, which were always < 10% of the total intensities) for chlorosomes in compressed and uncompressed gels at the beginning (1) and at the end (20) of a series of 20 measurements**

	Uncompressed				Compressed			
Experiment	I'_{vv}	I'_{vh}	I'_{hv}	I'_{hh}	I'_{vv}	I'_{vh}	I'_{hv}	I'_{hh}
1	55037	6282	27333	6586	94030	6187	30225	4075
20	54912	6492	26231	6524	81955	5805	26095	3599
Experiment	r	$\langle P_2 \rangle$	$\langle P_4 \rangle$	$\langle P_2(\cos\beta^{\mu}) \rangle$	$\langle P_2(\cos\beta^{\nu}) \rangle$		C	
1	0.270	−0.155	0.326	−0.741	−0.912		1.103	
	0.270	0.293	0.060	0.770	0.877		0.934	
20	0.269	−0.154	0.304	−0.742	−0.906		0.978	
	0.269	0.289	0.038	0.770	0.873		0.831	

Excitation is at 466 nm and detection at 751 nm. The degree of compression n is 1.56. T = 20°C. The parameters calculated from the first two lines of intensities are given in the last four lines. Mathematically, there are two possible solutions per series of measurements for these parameters. Only solutions shown in the bottom row for each experiment are physically significant.

analysis for two different degrees of compression, n = 1.36 and n = 1.56 are presented. The amount of orientation of the chlorosomes increases significantly, upon increasing n, whereas the values of the direction parameters $\langle P_2(\cos\beta^{\mu}) \rangle$ and $\langle P_2(\cos\beta^{\nu}) \rangle$ hardly show a change. This shows that the orientation of the dipole moments in the chlorosome remain the same upon compression of the gel, and only the amount of orientation changes. Different experiments with other gels also led to very similar results for these two direction parameters. The only factor that varied significantly for the various gels was C. There is rather good agreement between the values of $\langle P_2 \rangle$, calculated according to the theory of Ganago et al. (1980) and the determined ones. The calculated and determined values of $\langle P_4 \rangle$, however, deviate for n = 1.56, showing that the chlorosomes do not exactly follow the deformation of the gel in the way described in the introduction. Ld measurements gave $\Delta A/(3A_{iso}) = \langle P_2 \rangle \langle P_2(\cos\beta^{\mu}) \rangle$ = 0.22 at 466 nm and 0.25 at 741 nm for n = 1.56. These values are the same as S_{μ} (466 nm) and S_{ν} (751 nm), which can be calculated from Table 2, indicating the consistency of this approach.

B800-850 complexes from *Rhodobacter sphaeroides*

For the measurements on B800-850 antenna complexes the fluorescence was detected at 860 nm. The samples were excited at 512, 580, 590, 596, 605, and 800 nm. Both for excitation and detection a bandwidth of 11 nm was used. The absorption and ld spectrum are, for instance, given by Kramer et al. (1984). At some excitation wavelengths two sets of possible solutions for $\langle P_2 \rangle$, $\langle P_4 \rangle$, $\langle P_2(\cos\beta^{\mu}) \rangle$, $\langle P_2(\cos\beta^{\nu}) \rangle$, and C were obtained, whereas for other wavelengths only one possible set remained because the other set contained a value for $\langle P_2(\cos\beta^{\mu,\nu}) \rangle$ lower than −0.5, which is not physically significant. Because $\langle P_2 \rangle$, $\langle P_4 \rangle$, $\langle P_2(\cos\beta^{\nu}) \rangle$, and C should be independent of the excitation wavelength it is possible to choose between two sets of solutions.

An independent way to make a choice is based on the assumption that the real particle distribution will be such that it resembles a distribution for rods or discs as predicted by the orientation model of Ganago et al. (1980). For instance, results obtained upon excitation at 800 nm for n = 1.56 gave rise to two solutions, namely either $\langle P_2 \rangle$ = −0.146 (disc-like) and $\langle P_4 \rangle$ = −0.033 or $\langle P_2 \rangle$ = 0.188 (rod-like) and $\langle P_4 \rangle$ = −0.290. According to the "Ganago distribution function" one expects that $\langle P_4 \rangle$ = 0.027 when $\langle P_2 \rangle$ = −0.146 (disc), whereas for $\langle P_2 \rangle$ = 0.188 one expects $\langle P_4 \rangle$ = 0.040 (rod). Therefore, we consider the first case to be more likely. When the particles are excited at 590 nm, the same order parameters are found but the positive solution for $\langle P_2 \rangle$ corresponds to a solution for $\langle P_2(\cos\beta^{\mu}) \rangle$ which is less than −0.5, showing that indeed the negative value for $\langle P_2 \rangle$ should be chosen. The results for the different experiments are given in Table 3. Note that in all experiments independent of excitation wavelength or the degree of particle orientation, the value for $\langle P_2(\cos\beta^{\nu}) \rangle$ is the same within experimental error with an average value of −0.42, corresponding to an angle of ~77° with the optical symmetry axis, assuming that all dipole moments have the same orientation with respect to this axis. In the following, the same assumption is made every time an explicit value for an angle is given. Also, the determined values for $\langle P_2 \rangle$ and $\langle P_4 \rangle$ are the same in one gel when

TABLE 2 **Determined parameters for chlorosomes at different degrees of compression n**

n	$\langle P_2^{calc} \rangle$	$\langle P_4^{calc} \rangle$	$\langle P_2 \rangle$	$\langle P_4 \rangle$	$\langle P_2(\cos\beta^{\mu}) \rangle$	$\langle P_2(\cos\beta^{\nu}) \rangle$	C
1.36 ± 0.01	0.193 ± 0.005	0.042 ± 0.002	0.213 ± 0.006	0.036 ± 0.004	0.770 ± 0.008	0.905 ± 0.010	0.98 ± 0.03
1.56 ± 0.01	0.280 ± 0.004	0.088 ± 0.003	0.281 ± 0.002	0.047 ± 0.004	0.784 ± 0.005	0.878 ± 0.003	0.87 ± 0.03

Excitation wavelength is 466 nm. Detection wavelength is 751 nm. The indicated errors are standard deviations of means. $\langle P_2^{calc} \rangle$ and $\langle P_4^{calc} \rangle$ are calculated according to the orientation model of Ganago et al. (1980) as indicated in Theory, assuming the particles are rod-like. For both measurements the value of r was 0.279 ± 0.004 and 0.275 ± 0.002, respectively.

TABLE 3 **Obtained parameters for B800-850 complexes**

Excitation wavelength	r	$\langle P_2 \rangle$	$\langle P_4 \rangle$	$\langle P_2(\cos\beta^a) \rangle$	$\langle P_2(\cos\beta^y) \rangle$	C	x	
nm								
512	−0.06 ± 0.01	−0.16 ± 0.02	−0.04 ± 0.03	0.39 ± 0.03	−0.37 ± 0.04	1.24 ± 0.01	9	b
580	−0.04 ± 0.01	−0.17 ± 0.02	−0.01 ± 0.05	0.27 ± 0.05	−0.38 ± 0.05	1.20 ± 0.01	5	b
580	−0.05 ± 0.01	−0.04 ± 0.01	−0.03 ± 0.03	0.35 ± 0.15	−0.43 ± 0.21	0.85 ± 0.01	3	c
590	−0.07 ± 0.01	−0.15 ± 0.01	−0.02 ± 0.03	0.47 ± 0.06	−0.40 ± 0.03	1.66 ± 0.03	6	a
596	−0.09 ± 0.01	−0.05 ± 0.01	−0.04 ± 0.02	0.61 ± 0.10	−0.40 ± 0.10	0.85 ± 0.01	8	c
605	−0.11 ± 0.01	−0.17 ± 0.01	−0.03 ± 0.01	0.68 ± 0.04	−0.42 ± 0.01	1.27 ± 0.03	4	b
800	0.08 ± 0.01	−0.14 ± 0.01	−0.02 ± 0.03	−0.47 ± 0.03	−0.43 ± 0.01	1.68 ± 0.04	9	a
800	0.08 ± 0.01	−0.04 ± 0.01	−0.04 ± 0.05	−0.46 ± 0.04	−0.44 ± 0.05	0.84 ± 0.01	8	c

Detection wavelength is 850 nm. Standard errors are given. Three different gels have been used for these measurements, which are indicated with *a*, *b*, and *c*. The degree of compression is 1.56. The number of measurements is indicated under *x*.

different excitation wavelengths are used, which is required if the assumptions are correct. C should approximately be equal for various excitation wavelengths when the same gel is studied. Although C varies appreciably for different gels, it is the same for measurements on individual gels. This variation in C is probably due to variation in the amount of fluorescence. For gel *c* the amount of orientation of the particles ($\langle P_2 \rangle = -0.04$) is rather different than for the other two gels ($\langle P_2 \rangle = -0.16$). Therefore, in gel *c*, the particles are oriented differently than in gels *a* and *b*, but the orientation of the dipole moments is the same. Application of the orientation formula of Ganago et al. (1980) leads to $\langle P_2 \rangle = -0.23$ and therefore to wrong interpretation of linear dichroism results for these gels. The value of $\langle P_2 \rangle$ is less negative than the value of −0.23 calculated by the theory of Ganago et al. (1980), whereas $\langle P_4 \rangle$ is lower than the calculated value of +0.07.

It appeared to be difficult to compare the absolute values of S_μ and $\Delta A/(3A_{iso})$, as determined with fluorescence and linear dichroism. Similar to variations in the amount of fluorescence, there is also variation in the height of the absorption and ld from gel to gel, due to partial bleaching of the absorption of all bands in the gels. It is not possible to measure S_μ (fluorescence), ΔA (ld), and A_{iso} on one sample with sufficient accuracy. However, there is qualitative agreement, as the ratio of S_μ and $\Delta A/(3A_{iso})$ at different wavelengths is similar in all samples, despite the variability of the partial bleaching. It seems that S_μ is slightly larger for the 800 than for the 850-nm band, whereas $\Delta A/(3A_{iso})$ is slightly smaller.

DISCUSSION

Chlorosomes

Much attention has been paid in the literature to the ordering of pigments, especially BChl *c*, in chlorosomes both from *Chloroflexus aurantiacus* (Betti et al., 1982; van Dorssen et al., 1986; Fetisova et al., 1988; van Amerongen et al., 1988; Griebenow, K., A. R. Holzwarth, F. van Mourik, and R. van Grondelle, manuscript submitted for publication) and *Chlorobium limicola* (Fetisova et al., 1986; Fetisova et al., 1988). For chlorosomes an extremely high degree of orientation was observed with all the Q_y transition dipole moments oriented essentially along the long axis of the chlorosomes (Fetisova et al., 1986), leading to a very high degree of polarization of the fluorescence (Fetisova et al., 1988). Also, for chlorosomes from *Chloroflexus aurantiacus* in intact cells, a high degree of polarization was observed by the same authors, again indicating a high degree of ordering of BChl *c*. Initially, rather large angles between the BChl *c* Q_y dipole moments (741 nm) and the long axis of the chlorosomes were obtained from ld measurements on chlorosomes in stretched films (Betti et al., 1982) and compressed gels (van Dorssen et al., 1986). However, as we argued before, these estimations are probably much too high and an angle of 17° was found ($\langle P_2(\cos\beta^a) \rangle = 0.87$ [741 nm]) upon orientation in gels which were compressed in one direction and expanded in a perpendicular direction (van Amerongen et al., 1988). Rotational symmetry was assumed and an orientation model, similar to the one of Ganago et al. (1980), was used. The latter model was recently used by Griebenow et al. (manuscript submitted for publication) for compression in two directions and an angle of 15° was found ($\langle P2[\cos\beta^a] \rangle = 0.90$ [741 nm]), in good agreement with previous results (van Amerongen et al., 1988). Again the assumption of rotational symmetry was used.

In the present study the polarized fluorescence of chlorosomes was determined in both compressed and uncompressed gels. Assuming effective rotational symmetry of the chlorosomes and efficient energy transfer between the pigments, we find that ($P_2[\cos\beta^y] = 0.88$) for the Q_y emission dipole moments with this indepen-

dent and completely different method. Therefore, assuming rotational symmetry of the chlorosomes leads to the same average orientations of the absorption and emission dipoles, using three different approaches. As argued in Theory, the agreement between the polarized fluorescence and ld results for chlorosomes in compressed gels strongly indicates that the dipole moments are organized around the long axis in a rotationally symmetric way. Moreover, the energy transfer between these rotationally distributed BChl *c* should be fast. Therefore, one would expect a fast partial depolarization, both in time-resolved fluorescence and pump-probe experiments, probing the BChl *c* excited state, whereas the residual anisotropy should be high. This is in agreement with experimental results (Fetisova et al., 1988; Miller et al., 1990; Lin et al., manuscript to be published). The determined value for $\langle P_2(\cos \beta^{\mu}) \rangle$ at 466 nm is somewhat higher than the one determined from the ld measurements. However, additional bands (e.g., carotenoids) contribute to the absorption at 466 nm. In determining S_{μ} from ld, these bands lower $\langle P_2(\cos \beta^{\mu}) \rangle$, whereas for the fluorescence these might contribute less than the BChl *c* B_y bands, due to $< 100\%$ energy transfer efficiency to the emitting BChl *c*. Indeed, by comparing absorption and excitation spectra of the chlorosomes at 4 K, one can conclude that this efficiency is slightly $< 100\%$ (van Dorssen et al., 1986).

The fact that the orientations for the Q_y transitions obtained from ld and fluorescence measurements agree implies that also the measured values for $\langle P_2 \rangle$ should agree with the calculated ones. As can be seen in Table 2, this is indeed the case, which indicates that the chlorosomes line up in very good approximation as predicted by the theory of Ganago et al. (1980), as least as far as $\langle P_2 \rangle$ is concerned. There is some deviation for $\langle P_4 \rangle$ at $n = 1.56$. The fact that $\langle P_4 \rangle$ is apparently somewhat lower than predicted suggests that the distribution function for the chlorosomes is slightly narrower than the one proposed by Ganago et al. (1980). In other words, particles that are close to parallel to the direction of gel expansion before compression seem to align less well than predicted, whereas particles that are initially oriented more perpendicular align somewhat better than predicted. The narrowest distribution possible, where all particles have the same orientation with respect to the orientation axis and the value of $\langle P_2 \rangle$ is equal to 0.28, would correspond to a value of $\langle P_4 \rangle = -0.39$. This indicates that the deviation for $\langle P_4 \rangle$ is not very large. This deviation does not influence the interpretation of ld results because the amount of dichroism does not depend on $\langle P_4 \rangle$.

The value of *C* in different experiments appears to vary around unity, which is expected under identical circumstances for compressed and uncompressed gels. The determination of *C* is absolutely necessary and assuming it is unity, can lead to completely erroneous results. In conclusion, the chlorosomes appear to align as predicted by the theory of Ganago et al. (1980), as far as $\langle P_2 \rangle$ is concerned, but there is a small deviation for $\langle P_4 \rangle$ at $n = 1.56$. The very good agreement between the fluorescence and two types of ld measurements indicates that the chlorosomes are effectively rotational symmetric along the long axis. Assuming that the dipoles all have the same angle with respect to the long axis, this angle should be $\sim 16°$. It has been proposed by Mimuro et al. (1989) that a large part of the BChl *c* fluorescence stems from damaged or poorly connected pigments within the chlorosomes. Our results do not support this possibility as the orientations of the fluorescing BChl *c* molecules is very similar to the nonfluorescing BChl *c* molecules, in view of the agreement between fluorescence and ld measurements.

B800-850 antenna complexes

Energy transfer within and pigment organization of B800-850 L(ight)H(arvesting)2 complexes from photosynthetic purple bacteria have been investigated using polarized light spectroscopy (Kramer et al., 1984) and more recently using picosecond laser spectroscopy (Bergström et al., 1986, 1988; Trautman et al., 1990). A minimal model for these complexes was proposed by Kramer et al. (1984), which can explain the energy transfer, the fluorescence depolarization, and ld-measurements. In this model the Q_y dipole moments of the 850 band are distributed with effective rotational symmetry around one axis and fast energy transfer between these pigments is known to occur (Bergström et al., 1986, 1988; Trautman et al., 1990). The complexes are supposed to have a more or less disc-like shape and the normal to this disc is essentially parallel to the normal of the membranes in intact systems. The Q_y transition dipole moments of both the pigments absorbing at 800 and 850 nm are thought to be oriented perpendicular to the normal, although this cannot be concluded directly from the ld measurements when the theory of Ganago et al. (1980) is applied, because in that case the true value of $\langle P_2 \rangle$ should be lower than the one predicted, to explain the observed amount of ld (Kramer et al., 1984).

In this study, the LH2 complexes were excited at a number of different wavelengths (Table 3). For each individual gel the obtained values for $\langle P_2 \rangle$, $\langle P_4 \rangle$, $\langle P_2(\cos \beta^{y}) \rangle$, and *C* are essentially the same for all excitation wavelengths, showing the consistency of the interpretation of the polarized fluorescence results. We find that the

emission dipoles of BChl 850 are oriented nearly perpendicular to the symmetry axis, which is thought to lie along the normal. The average value of $\langle P_2(\cos \beta^{\nu}) \rangle = -0.42$ corresponds to an angle of 77°. The Q_y dipole moments, corresponding to the 800-nm absorption band are even more tilted away from the symmetry axis ($\beta^{\mu} = 81°$).

Again it is demonstrated that C is not necessarily equal to unity and that it can vary for different measurements, but apparently this variation does not significantly influence the results, because the determined value of $\langle P_2(\cos \beta^{\nu}) \rangle$ is the same in all cases. The value of $\langle P_2 \rangle$ is negative as expected for disc-like particles, but less negative than can be calculated for a perfect disc, using the formalism of Ganago et al. (1980). This is not unexpected as recent electron microscopy photographs of LH2 from *Rhodobacter sphaeroides* show (Boonstra, A. F., R. W. Visschers, R. van Grondelle, E. F. J. van Bruggen, and E. J. Boekema, manuscript in preparation) that the complexes have a disc-like shape with a diameter of ~12 nm (including the detergent layer) and a height of 6 nm. The difference between the determined and predicted values of $\langle P_2 \rangle$ and the variation of $\langle P_2 \rangle$ for different gels at the same degree of compression, clearly show that application of the formula of Ganago et al. (1980) may lead to erroneous results for such particles.

In conclusion, the polarized fluorescence measurements on particles oriented in compressed gels in a 90° setup allow a separate determination of $\langle P_2 \rangle$, $\langle P_4 \rangle$, $\langle P_2(\cos \beta^{\mu}) \rangle$, and $\langle P_2(\cos \beta^{\nu}) \rangle$ if one assumes rotational symmetry and sufficient energy transfer. The agreement between fluorescence and ld results for chlorosomes and the fact that the determined parameters C, $\langle P_2 \rangle$, $\langle P_4 \rangle$, and $\langle P_2(\cos \beta^{\nu}) \rangle$ are the same upon excitation at different wavelengths for B800-850 complexes indicate that the assumptions are justified. No orientation model for the particles in the gel is needed as in linear dichroism measurements and the pigment organization can be determined independently. In the future it might be possible to make some approximate estimations of the dimension of particles, based on the determined values of $\langle P_2 \rangle$ and $\langle P_4 \rangle$, but to do so, more particles with known shapes have to be investigated.

The authors thank Mr. K. Griebenow (Max-Planck-Institut für Strahlenchemie, Mülheim a.d. Ruhr) for kindly providing the chlorosomes, Mr. R. W. Visschers for providing the B800-850 antenna complexes, and Mr. F. van Mourik for help with some of the measurements.

This work was supported by the Dutch Organization of Pure Research (NWO) via the Foundation of Biophysics.

Received for publication 17 September 1990 and in final form 2 January 1991.

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