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EXCITATION ENERGY TRANSFER AND CHLOROPHYLL ORIENTATION IN THE GREEN ALGA *CHLAMYDOMONAS REINHARDI*

JOHN WHITMARSH* and R. P. LEVINE

The Biological Laboratories, Harvard University, Cambridge, Mass. 02138 (U.S.A.)

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SUMMARY

We have investigated the process of intermolecular excitation energy transfer and the relative orientation of the chlorophyll molecules in the unicellular green alga *Chlamydomonas reinhardi*. The principal experiments involved in vivo measurements of the fluorescence polarization as a function of the exciting-light wavelength in the presence and in the absence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea. We found that as the fluorescence lifetime increases upon the addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea that the degree of fluorescence polarization decreases over the excitation region from 600 to 660 nm. This result, we argue, implies that a Förster mechanism of excitation energy transfer is involved for Photosystem II chlorophyll molecules absorbing primarily below 660 nm. We must add that our results do not exclude the possibility of a delocalized transfer process from being involved as well. Fluorescence polarization measurements using chloroplast fragments are also discussed in terms of a Förster transfer mechanism. As the excitation wavelength approaches 670 nm the fluorescence polarization is nearly constant upon the addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

Experiments performed using either vertically or horizontally polarized exciting light show that the fluorescence polarization increases as the exciting light wavelength increases from 650 to 673 nm. This suggests the possibility that chlorophyll molecules absorbing at longer wavelengths have a higher degree of relative order. Furthermore, these studies imply that chlorophyll molecules exist in discrete groups that are characterized by different absorption maxima and by different degrees of the fluorescence polarization. In view of these results we discuss different models for the Photosystem II antenna system and energy transfer between different groups of optically distinguishable chlorophyll molecules.

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenolindophenol.

* Present address: Centre d'Etudes de Saclay, Service de Biophysique, Department de Biologie, BP no. 2, 91120 Gif sur Yvette, France.

INTRODUCTION

Starting with the work of Arnold and Meek [1], fluorescence polarization studies using photosynthetic organisms have focused on determining how such organisms are able to transfer excitation energy from the light absorbing pigments to reaction center molecules where the energy can be used to do chemically useful work [2–6]. Although the excitation energy transfer process is generally accepted to be due to a resonance interaction between the electric fields of the donor and acceptor molecules, there is insufficient experimental evidence [5, 7–11] to determine whether the excitation energy is transferred by a localized mechanism, by a delocalized mechanism, or by a mixture of the two [12–17]. In the case of transfer by the localized mechanism, hereafter referred to as Förster transfer [14, 16], the excitation energy is considered to be localized on a single molecule, and the transfer process, which occurs after vibrational relaxation ($\approx 10^{-12}$ s), involves jumps from one molecule to another. In the delocalized transfer process the excitation energy is considered as being shared by several molecules and as spreading out over the molecular array as an exciton before vibrational relaxation has occurred.

If the chlorophyll molecules are in the neighborhood of 10–15 Å of one another, an electron exchange mechanism must be taken into account [13, 18, 19]. However, for the exchange mechanism to be involved in the majority of the transfer processes a sizeable fraction of the excited molecules must exist in the triplet state. Since we know of no evidence that the triplet state exists in vivo [20, 21], we do not think it likely that an exchange mechanism is involved in most excitation energy transfer processes in photosynthesis. However, we do not think it should be ignored, for an exchange mechanism may be important in energy transfer processes in the vicinity of a trap where the stringent orientation requirements of the Förster and delocalized processes need not be met.

Although there is disagreement in the literature, it appears that either a delocalized, or a Förster transfer mechanism, or a combination of the two can adequately account for most of the excitation energy transfer in photosynthesis [11–14, 17, 21–25]. In order to investigate this question we have measured the polarization of the steady state fluorescence for cells and isolated chloroplast fragments of *Chlamydomonas reinhardi* as a function of the exciting light wavelength. These measurements were done in the presence and absence of DCMU, an inhibitor of the flow of electrons from Photosystem II. In the presence of DCMU the Photosystem II traps are closed, which causes the fluorescence lifetime to increase by a factor of two or more [26, 27]. Our results show that the addition of DCMU to intact cells causes the polarization to decrease when exciting with light from 600 to 660 nm [5]. We believe that this DCMU-induced decrease in polarization is a consequence of the fact that the excitation energy is not randomized over the molecular array within the chlorophyll fluorescence lifetime. The increase in the fluorescence lifetime, caused by adding DCMU, allows for greater energy transfer so that the excitation energy becomes more randomized causing the fluorescence polarization to decrease. This interpretation implies that a Förster mechanism is active, because if only a delocalized mechanism were involved the excitation energy would be randomized over the molecular array long before fluorescence occurs so that an increase in the fluorescence lifetime would not correspond to a decrease in the polarization [5]. Experiments,

performed using chloroplast fragments, comparing the effect of varying the light intensity or adding an electron acceptor support this interpretation (see Results and Discussion).

Recently fluorescence polarization measurements have been used not only to study excitation energy transfer but also to determine the relative orientation of the light absorbing molecules [4, 28–33]. In our experiments we find that the fluorescence polarization observed while exciting either with vertically or with horizontally polarized light increases by a factor of two as the excitation wavelength increases from 650 to 673 nm. These results provide strong evidence that there is a substantial degree of relative order among the Photosystem II chlorophyll molecules and are consistent with an antenna model in which chlorophyll molecules absorbing at longer wavelengths exhibit a higher degree of relative order than those chlorophyll molecules absorbing principally at shorter wavelengths in the 650–673 nm region. Furthermore, comparison of our experimental results with simple models of antenna and trapping molecules for Photosystem II lead us to conclude that the chlorophyll molecules exist in physically discrete groups characterized by different absorption maxima and by different degrees of minimum fluorescence polarization. In some cases optically different groups of chlorophyll molecules are unable to share excitation energy.

THEORY

Since an understanding of the relationship between the fluorescence polarization and energy transfer is essential to our arguments, we include a discussion of the dependence of the polarization on the energy transfer process. Explanations of the different transfer mechanisms have been presented in detail in the literature and will not be repeated here [13–17].

Fluorescence polarization

For non-rotating molecules the polarization of the fluorescence reflects the degree to which the excitation energy has become distributed over the molecular array before fluorescence occurs [34]. Vertically polarized light incident upon a group of pigments creates an anisotropy among the excited molecules because transition dipoles aligned nearly parallel to the polarization of the incoming light absorb much more strongly than those transition dipoles aligned nearly perpendicular to the polarization of the incoming light. If no excitation energy transfer occurs only those molecules that absorb a photon can emit light; therefore, due to the anisotropy created by the vertically polarized exciting beam, the fluorescence is also partially vertically polarized.

If, however, excitation energy transfer does occur, then the initial anisotropy created by the exciting light will decrease and the fluorescence polarization, P (see Material and Methods), will decrease as well. If enough transfer processes occur, the polarization will decrease to a minimum value, P_M, defined to be the minimum value that the polarization exhibits as the number of energy transfer processes increase. For homogeneous, randomly oriented molecules between which excitation energy transfer occurs, the minimum polarization is zero. A non-zero minimum polarization indicates that the sample exhibits some net and/or relative molecular order. As an example, consider the molecular arrangement shown in Fig. 1. In Case A, the transi-

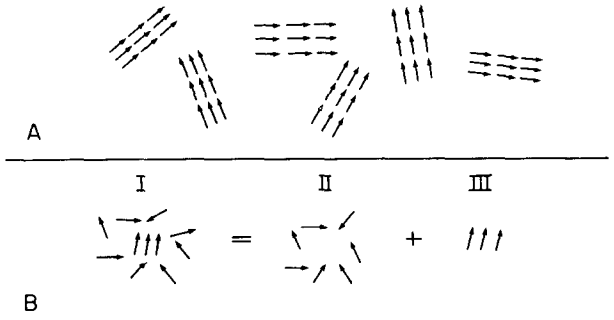

Fig. 1. Non-zero fluorescence polarization due to the orientation of the molecules. Consider the exciting light to be vertically polarized and coming out of the plane of the paper and the fluorescence to be observed in the same direction. The arrows represent the absorption and emission transition dipoles of the molecules. (A) Groups of molecules oriented nearly parallel to the exciting light will absorb more strongly than those groups oriented close to perpendicular to the exciting light. Since no energy transfer is allowed between different groups, and energy transfer within a group does not decrease the initial anisotropy, the polarization observed should be 0.5. The orientation of the groups is intended to be random. (B) The molecules in Group I are equivalent to the randomly oriented molecules in Group II plus the vertically oriented molecules in Group III. Even though extensive energy transfer may randomize the excitation energy over the array the polarization would still be greater than zero due to fluorescence from molecules in Group III.

tion dipoles are all parallel within a group of molecules so that energy transfer between molecules in the same group produces no change in the polarization. Since the groups themselves are randomly oriented and no energy transfer occurs between different groups, the minimum polarization is 0.5. Note that the dichroic ratio of this sample is 1, even though a large degree of relative order exists. In Case B, extensive energy transfer would cause the excitation energy to be randomly distributed over the molecules. Since the transition dipoles are preferentially oriented toward the vertical axis, the fluorescence would be partially polarized vertically. Hence, the polarization of the fluorescence from a group of molecules which share excitation energy depends not only upon the extent of the energy migration but also upon the orientation of the molecules.

In order to establish the existence of the Förster transfer process in photosynthesis, we may compare the dependence of the fluorescence polarization on the fluorescence lifetime for the delocalized and Förster transfer processes. If energy migration were due solely to a delocalized mechanism, then, since the excitation energy is randomized over the molecular array long before fluorescence occurs, an increase in the fluorescence lifetime would not cause the polarization to decrease. The polarization would remain constant, upon the addition of DCMU, at its minimum value. If, however, a Förster transfer mechanism is involved (though not necessarily exclusively), then the transfer process may be slow enough so that the excitation energy is not completely randomized over the molecular array within the fluorescence lifetime. In this case, an increase in the amount of energy transfer, caused by adding DCMU for example, would result in a decrease in the polarization. Therefore, if the fluorescence polarization for a photosynthetic organism is a function of the fluorescence lifetime, and it can be shown that no other depolarization effects are active (e.g. conformational changes, artifacts, see Results and Discussion), then

we argue that a Förster mechanism is involved in the transfer process. Note that this argument does not exclude the possibility of a delocalized process from being involved as well.

Information about the relative orientation of the absorbing molecules can be obtained by observing the polarization while exciting with horizontally polarized light. The fluorescence polarization excited by horizontally polarized light depends upon the anisotropy of the molecules [33] and to a lesser extent upon the energy transfer processes [35]. A randomly oriented sample exhibits zero polarization upon excitation with horizontally polarized light. A non-zero polarization indicates that the transition dipole moments of the absorbing molecules exhibit some net order.

MATERIALS AND METHODS

Cells and chloroplast fragments

The wild-type strain (137c) of *C. reinhardi* was used in the experiments described here. Cells were cultured in 300 ml of Tris–acetate–phosphate medium under conditions previously described [36] and harvested during the logarithmic phase of growth. The intact cells were washed and suspended in fresh Tris–acetate–phosphate for the fluorescence polarization measurements. The resuspended cells were stirred and kept in the light prior to the measurements. Chloroplast fragments were prepared by sonic disruption [37] and kept at 0 °C in the dark. For the polarization measurements, the fragments were suspended in a phosphate buffer, pH 7.0, consisting of 0.33 M KH_2PO_4, 0.67 M KCl and 8.0 mM $MgCl_2$. For all measurements of fluorescence polarization the chlorophyll concentration never exceeded 3 μg/ml. Chloroplast fragments exhibiting an impaired rate of oxygen evolution were obtained by letting isolated chloroplasts stand for several hours at 0 °C.

Chlorophyll concentration was measured by a modification [38] of the procedure of Mackinney [39].

Apparatus

The instrument used to measure the fluorescence polarization is based upon a design suggested by Dr Gregorio Weber (Fig. 2). The fluorescence polarization is defined by the following relationship:

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} = \frac{I_p}{I_c}, \qquad \begin{aligned} I_p &= I_{\parallel} - I_{\perp} \\ I_c &= I_{\parallel} + I_{\perp} \end{aligned}$$

where $I_{\parallel}$ = intensity of the emission parallel to the direction of partial polarization and $I_{\perp}$ = intensity of the emission perpendicular to the direction of partial polarization. This instrument measures the polarized component of the fluorescence, I_p, directly rather than the more common method of measuring the components $I_{\parallel}$ and $I_{\perp}$ separately and taking their difference.

The excitation light is produced by an air cooled tungsten–halogen lamp. The polarized monochromatic light incident on the sample is obtained by a polarizer, P_1, a water filter, interference filters and a monochrometer. Light emitted by the sample is observed perpendicular to the exciting light. Before being collected at a photomultiplier, the emitted light passes through a $\frac{1}{2}$-waveplate rotating at frequency

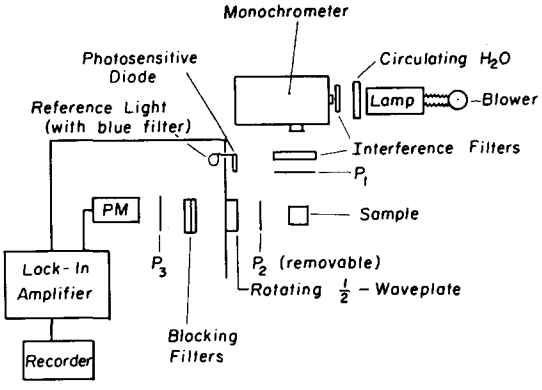

Fig. 2. Block diagram of the instrument used to measure the fluorescence polarization. Lamp, tungsten–halogen (Sylvania); grating monochrometer, Bausch and Lomb 500 nm; P_1, P_2 and P_3, polaroid sheet polarizer HN32; interference filters, Baird Atomic, Thin Films Industry and Ealing; blocking filters, Corning colored glass filters 4-77 and 2-64; PM, EMI 9558 photomultiplier; lock-in amplifier, Princeton Applied Research HR 8; recorder, Esterline Angus Speed Servo; 1/2-waveplate, Industrial Optical Laboratories, 694 nm.

f (76 Hz), a set of blocking filters, and a polarizer P_3. The blocking filters only allow light above 680 nm to pass through so that the exciting light is removed from the signal. The rotating $\frac{1}{2}$-waveplate causes the polarized component of the fluorescence, I_p, to rotate at frequency $2f$. The polarizer P_3 causes the rotating linearly polarized light, I_p, to become modulated. Hence, the light leaving the photomultiplier consists of a steady component proportional to the unpolarized fluorescence and a modulated component proportional to the I_p. The signal from the photomultiplier together with a reference signal of frequency $4f$ is sent to a lock-in amplifier. The reference signal is produced by a small light, a photosensitive diode, and a chopper connected to the rotating $\frac{1}{2}$-waveplate. The lock-in amplifier separates the modulated signal, proportional to I_p, from the steady signal and sends a signal, V_p, to the recorder. The signal V_p is directly proportional to I_p so that we can write

$$I_p = \gamma V_p, \text{ where } \gamma = \text{constant.}$$

In order to determine the combined fluorescence, I_c, a polarizer, P_2, is inserted between the sample and the $\frac{1}{2}$-waveplate. The polarizer allows only vertically polarized light to pass. The signal, V_c, recorded in this situation is related to the total fluorescence in the following manner

$$I_c = \gamma(\alpha V_c - V_p)$$
$$\alpha = 2.66 \pm 0.02$$

The factor α is included because P_2 attenuates the fluorescence. Since P_2 is parallel to I_p the term $(-V_p)$ must be included before multiplying through by the factor γ [35]. Finally, the polarization is given by

$$P = \frac{I_p}{I_c} = \frac{V_p}{\alpha V_c - V_p}$$

The data analysis was performed on a Nova computer made by General Data. All absorption measurements were carried out on a Cary 14 recording spectrophotometer.

Measurements

The intact cells were placed in a cuvette and agitated by stirring or bubbling prior to making the measurements. All measurements were made after the fluorescence had reached a constant level. We found that the cells must be stirred continuously while measuring the polarization using horizontally polarized exciting light. The signal V_p was measured over a period of 30–120 s, followed, within 15 s, by a determination of the signal V_c over a 30 s period. DCMU was then added to a final concentration of $1 \cdot 10^{-5}$ M and the procedure was repeated. The fluorescence polarization for chloroplast fragments was measured in the same way except that the fragments were neither stirred nor bubbled.

A major problem in determining the polarization of the fluorescence is to insure that scattered exciting light, which is highly polarized, is not detected. In order to check for this possibility, we doubled the number of interference filters and blocking filters. The additional filters cause any scattered light to be reduced by a factor of at least $1 \cdot 10^{-3}$ while reducing the fluorescence by a factor of 0.2. If part of the measured signal were due to scattered exciting light then the additional filters would cause the measured polarization to change. We performed this check upon our measurements and found that the polarization remained constant upon doubling the number of filters.

As a check upon any systematic errors due to our instrument we measured the fluorescence polarization for chlorophyll in acetone using horizontally polarized exciting light. Since in this case the fluorescence is unpolarized any polarization caused by our instrument would be revealed. We found the polarization to be $0.00 \pm 0.05\,\%$.

Another possible artifact is depolarization by scattering of the fluorescence within the chloroplast. However, since we always measure the polarization in the same region of the fluorescence spectrum (a peak of 702 nm with a 20 nm $\frac{1}{2}$-bandwidth) and we are concerned with differences between different polarizations, we do not think this artifact, if it exists, has any effect upon the interpretation of our data.

One point of interest involves the fact that the polarization observed for isolated chloroplast fragments is generally twice as great as that observed for intact cells. It may be that the higher polarization value results from inactive chlorophyll (i.e. from chlorophyll that is unable to share its excitation energy with a reaction center) and that this inactive chlorophyll is produced by the isolation process. Two other experimental observations support this suggestion. First, the relative fluorescence yield for chloroplast fragments is 1.5–2.5-times as great as for intact cells to which DCMU has been added for the same chlorophyll concentration. Second, the polarization for chloroplast fragments measured using 650 nm exciting light increases as the time the chloroplasts spend in the sonicator increases. However, this observation may well be explained by the fact that the chloroplast fragments would presumably cause less scattering of the fluorescence. Nevertheless, we suggest that measurements of the fluorescence polarization using isolated chloroplasts may be misleading because of the presence of "inactive chlorophyll", hence making it difficult to make reliable

statements concerning the transfer of excitation energy or the orientation of the fluorescing molecules.

RESULTS AND DISCUSSION

The results of measurements of the fluorescence polarization for intact cells and isolated chloroplast fragments of *C. reinhardi* are given in Tables I–V.

Excitation wavelength 600–660 nm

Of particular interest to us is an earlier observation [5] that the addition of $1 \cdot 10^{-5}$ M DCMU to algae causes the fluorescence polarization to decrease when exciting with 632.8 nm light. We have shown this to be the case, using intact cells of *C. reinhardi*, in the excitation region from 600 to 660 nm (Table I). In the presence of DCMU the Photosystem II traps are closed and are therefore no longer able to trap the excitation energy. As a consequence there is a greater amount of energy transfer, which we believe, in turn, causes the excitation energy to become more randomized over the molecular array causing the fluorescence polarization to decrease.

A decrease in the fluorescence polarization can also be induced in isolated chloroplast fragments by increasing the intensity of the exciting light at 650 nm (Table II). As the intensity of the exciting light increases, dark reactions become rate limiting and the Photosystem II traps are no longer as effective in trapping the excitation energy. As a consequence the fluorescence lifetime increases and the amount

TABLE I

The in vivo polarization of the steady state fluorescence is shown as a function of the excitation wavelength for intact cells of *C. reinhardi* in the presence and in the absence of $1 \cdot 10^{-5}$ M DCMU. All values are the average of at least four measurements. The decrease in the polarization induced by DCMU is shown as the average of the difference for individual measurements. The ratio of the relative fluorescence yield with DCMU compared to the relative fluorescence yield without DCMU is also shown. The 1/2-band-width of the exciting light was 10 nm for wavelengths below 650 nm and 3.3 for wavelengths of 650 nm and above. The intensity of the exciting light varied between $1 \cdot 10^{14}$ and $1 \cdot 10^{15}$ photons/cm^2 per s.

Excitation wavelength (nm)	P (%) normal	P (%) with DCMU	Decrease (%)	$\frac{I_T \text{ with DCMU}}{I_T \text{ normal}}$
600	1.3±0.05	0.7±0.05	46±5	2.9
615	1.7±0.05	1.2±0.05	30±4	3.0
625	1.9±0.05	1.5±0.05	22±4	2.2
640	1.8±0.05	1.3±0.05	26±4	2.8
645	1.8±0.05	1.4±0.05	25±4	2.7
650	1.8±0.05	1.4±0.05	23±4	2.1
653	2.0±0.05	1.6±0.05	20±3	2.0
657	2.3±0.05	2.0±0.05	15±3	1.9
660	2.5±0.05	2.3±0.05	9±3	2.0
664	3.3±0.06	3.0±0.06	9±2	1.9
667	3.8±0.07	3.6±0.06	5±2	1.9
670	4.2±0.07	4.2±0.07	1±2	2.0
673	4.6±0.1	4.5±0.07	2±3	1.8

TABLE II

The fluorescence polarization as a function of exciting light intensity is shown for isolated chloroplast fragments of *C. reinhardi*. The chloroplast fragments were suspended in a phosphate reaction buffer. The sample was changed for each measurement. The values shown are the average of three measurements. I_0, the maximum intensity of the exciting light, equals $1 \cdot 10^{15}$ photons/cm^2 per s.

Exciting light intensity at 650 nm	Polarization (%)
$0.22I_0$	3.6
$0.50I_0$	3.5
$0.75I_0$	3.4
$1.0I_0$	3.3

of energy transfer increases. Again we argue that the increased number of transfer processes causes the excitation energy to become more randomly distributed over the array and results in lowering the fluorescence polarization. Using intact cells we found that the polarization measured as a function of the exciting light intensity at 650 nm remains approximately constant over the range from $2 \cdot 10^{14}$ to $1 \cdot 10^{15}$ photons/cm^2 per s (unpublished). Since we could not provide monochromatic light bright enough to saturate photosynthesis we could not observe a polarization decrease.

Using isolated chloroplast fragments which had an impaired rate of oxygen evolution, we were able to show that the addition of DCIP (which acts as an electron acceptor for Photosystem II) causes the polarization to increase (Table III). The fluorescence polarization for aged chloroplast fragments in phosphate buffer was 3.0 % and the rate of electron transport was very low. Upon the addition of DCIP, which acts as an electron acceptor for Photosystem II and increases the flow of electrons from Photosystem II, the polarization increased to 3.5 %. Since DCIP increases the trapping efficiency of the Photosystem II traps the excitation energy is trapped more rapidly and therefore is less randomized over the molecular array. Since the excitation energy is less randomized the fluorescence polarization is greater. The addition of DCMU to the sample consisting of chloroplast fragments and DCIP causes the fluorescence polarization to decrease to 3.0 % as expected.

TABLE III

The fluorescence polarization is shown for isolated chloroplast fragments under various conditions. (A) Chloroplast fragments in phosphate buffer exhibiting impaired rates of oxygen evolution. (B) Chloroplast fragments in phosphate buffer with 0.2 M DCIP added so that Photosystem II is active. (C) Same as B, except $1 \cdot 10^{-5}$ M DCMU has been added so that Photosystem II is blocked. Excitation wavelength 650 nm.

	Chloroplast fragments in		
	A phosphate buffer	B phosphate buffer + 0.2 M DCIP	C phosphate buffer + 0.2 M DCIP + $1 \cdot 10^{-5}$ M DCMU
Polarization (%)	3.0	3.5	3.0

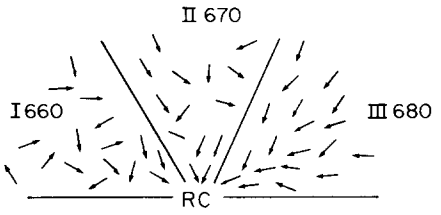

Fig. 3. One of several models of the Photosystem II chlorophyll molecules which is consistent with the data presented here. Regions I, II and III consist of chlorophyll molecules absorbing primarily around 660, 670 and 680 nm, respectively. The arrows represent the red transition dipoles of the chlorophyll molecules. We have tried to indicate that as the absorbing wavelength increases (Region III) the relative order increases. This would cause longer wavelength absorbing pigments to have a greater interaction energy and could account for their redshift relative to pigments in Region I.

Although the explanation of the changes in the polarization and the fluorescence lifetime in terms of the state of the Photosystem II traps is adequate to account for our experimental results, two alternative explanations must be considered.

The first explanation attempts to account for the DCMU-induced polarization decrease by assuming that the polarized component of the fluorescence, I_p, is from inactive chlorophyll [5] or is from Photosystem I. In both cases the addition of DCMU would cause the fluorescence, I_c, to increase by a factor of two to three but would have no effect upon the polarized component of the fluorescence since any fluorescence from Photosystem I or inactive chlorophyll is not sensitive to the state of the Photosystem II reaction centers. Since the polarization is equal to the ratio of I_p to I_c, the addition of DCMU would cause it to decrease by one-half to one-third. However, Table IV shows that the polarized component of the fluorescence doubles upon the addition of DCMU when exciting at 640 nm. Therefore, assuming that DCMU acts only on Photosystem II fluorescence and has a negligible effect on any fluorescence from inactive chlorophyll or Photosystem I, we can conclude that the major contribution to the polarized component of the fluorescence is from Photosystem II. However, it is still possible to account for the DCMU-induced decrease

TABLE IV

Typical values of individual measurements of the fluorescence polarization for intact cells of *C. reinhardi* in the presence and absence of DCMU are shown. Observe that the polarized component of the fluorescence, I_p, doubles upon the addition of $1 \cdot 10^{-5}$ M DCMU while exciting at 640 nm.

Exciting wavelength	P (%)	P (%) with DCMU	Decrease (%)	$\frac{I_p \text{ with DCMU}}{I_p \text{ normal}}$	$\frac{I_c \text{ with DCMU}}{I_c \text{ normal}}$
640 nm	1.79	1.27	29	1.9	2.7
	1.71	1.32	23	2.2	2.8
	1.81	1.28	29	2.1	3.0
	1.78	1.38	23	2.1	2.7
670 nm	4.09	4.08	0	2.0	2.0
	4.29	4.21	2	1.8	1.9
	4.18	4.10	2	1.9	1.9
	4.18	4.21	−1	2.1	2.1

TABLE V

The in vivo fluorescence polarization for *C. reinhardi* using horizontally polarized exciting light is shown. Note that the polarization increases slightly upon the addition of DCMU. The values shown are the average of four measurements. The ratio of the relative fluorescence yield with DCMU compared to the relative fluorescence yield without DCMU is also shown.

Excitation wavelength (nm)	P (%) normal	P (%) with DCMU	$\frac{I_c \text{ with DCMU}}{I_c \text{ normal}}$
650	0.42±0.05	0.47±0.05	2.1
660	0.56±0.05	0.58±0.05	2.1
670	0.85±0.05	0.87±0.05	2.1

in the polarization by assuming that the polarization from active chlorophyll is constant upon the addition of DCMU and that the decrease in the polarization is a consequence of polarized fluorescence from inactive chlorophyll [5]. However, the amount of fluorescence from inactive chlorophyll which must be assumed is unreasonably high (at least 30 % of the total fluorescence while exciting at 441 nm must be from inactive chlorophyll) [35] and the variation in the amount of fluorescence due to inactive chlorophyll is so great (it varies from 12 % to 1 % over the excitation range from 600–673 nm) [35] that we do not think this interpretation is tenable.

The second explanation of the DCMU-induced decrease in the fluorescence polarization depends on changes in the orientation of the chlorophyll molecules in the chloroplast membrane. We think that this explanation is somewhat difficult to uphold in view of the fact that the decrease in the polarization can be induced by high light intensity as well, but nevertheless, in order to investigate this possibility, we measured the polarization using horizontally polarized exciting light as a function of wavelength in the presence and absence of DCMU (Table V). If DCMU-induced conformational changes were causing a decrease in the polarization while using vertically polarized exciting light, then we would expect a similar result using horizontally polarized exciting light. The results in Table V show that DCMU causes the polarization to increase slightly*. This leads us to believe that the DCMU-induced decrease in the polarization observed while exciting with vertically polarized light is not a consequence of changes in the chlorophyll orientation.

As a further check on changes in orientation produced by DCMU, we observed the light scattering from intact cells at 545 nm. The addition of DCMU caused no observable change in the intensity of the scattered light.

We have attempted to demonstrate that the changes in the fluorescence polarization caused by DCMU, DCIP and by increasing the excitation light are a consequence of changes in the state of the Photosystem II traps. This interpretation implies that a Förster transfer mechanism must be active, since if the only mechanism involved were a delocalized process the excitation energy would be randomized over the molecular array before the fluorescence occurs. Accordingly, we proposed that a Förster transfer mechanism is involved in excitation energy transfer between Photosystem II chlorophyll molecules absorbing in the region from 600 to 660 nm. We

* We propose to discuss the small DCMU-induced increase in the polarization in another paper.

want to emphasize that our results cannot exclude the possibility that a delocalized transfer process may be involved in this region as well. For example, a few molecules grouped together could be interacting via strong coupling and transfer excitation energy to a neighboring group by Förster transfer.

Exciting light wavelength 670–673 nm

For pigments absorbing light around 670 nm the polarization is essentially unchanged upon the addition of DCMU (Table I). In order for the polarization to remain virtually constant as the amount of energy transfer approximately doubles, the excitation energy must be completely distributed over the molecular array before fluorescence occurs, which means that the observed polarization is at its minimum value (P_M). This result is consistent with either a delocalized transfer mechanism or a Förster transfer mechanism. In order for Förster transfer to be involved it must be fast enough to cause the polarization to reach its minimum value before fluorescence occurs.

The effect of DCMU at 670 nm on intact cells (Table I) raises a point concerning the existence of fluorescence from either inactive chlorophyll or Photosystem I. Unless the polarization of the fluorescence from inactive chlorophyll or from Photosystem I exhibits the same polarization as the active Photosystem II fluorescence or is much less intense than the Photosystem II active fluorescence, the addition of DCMU would increase the ratio of Photosystem II active fluorescence to the fluorescence from inactive chlorophyll or Photosystem I fluorescence. This would cause the polarization to decrease. Since DCMU causes essentially no change, we believe that any fluorescence from inactive chlorophyll or from Photosystem I when exciting at 670 nm either has a polarization very close to 4.2 % or its intensity must be small compared to the Photosystem II fluorescence intensity [35].

Chlorophyll orientation and grouping

In cases where extensive energy transfer has completely randomized the excitation energy over the molecular array, the fluorescence polarization can be used as an indication of molecular order. In this situation the fluorescence polarization is at a minimum (P_M) and any non-zero value indicates an anisotropy among the pigments. Table I shows that the polarization for intact cells is 4.6 %. Upon the addition of DCMU, which allows for approximately twice as much energy transfer, the polarization decreases to 4.5 %. Since increasing the amount of energy transfer by a factor of two leaves the polarization essentially unchanged*, we believe that the polarization observed is near its minimum value and is a consequence of order among the red transition dipoles of the chlorophyll molecules which are emitting the light absorbed at 673 nm.

At shorter wavelengths DCMU causes the polarization to decrease, which indicates that the excitation energy is not completely randomized over the array. All we can conclude is that the minimum polarization is less than the polarization

* We believe that the small decrease in the polarization (0.1 %) induced by DCMU is due to absorption of 673 nm light by chlorophyll molecules absorbing principally around 660 nm. Pigments absorbing principally at 660 nm exhibit a DCMU-induced decrease in the polarization. Therefore, a small amount of 673 nm light absorbed by these pigments could account for the small polarization decrease that we observed.

measured in the presence of DCMU. Table I shows that the minimum polarization decreases from 4.5 % at 673 nm to below 1.4 % at 650 nm. This observation leads us to conclude that the red transition dipoles of the Photosystem II chlorophyll exhibit a non-random orientation. Furthermore, it appears that molecules absorbing at longer wavelengths may exhibit a higher degree of orientation than those molecules absorbing at shorter wavelength in the absorption region from 650 to 673 nm. This last suggestion is speculative, since a highly ordered sample could equally well exhibit a zero minimum polarization. It also assumes that the absorption and emission transition dipole moments of a single chlorophyll molecule between 650 and 673 nm are nearly parallel. However, in view of fluorescence polarization data for chlorophyll in vitro [41] and recent experimental work [33], we feel that it is a valid suggestion.

Measurements using horizontally polarized exciting light can also reveal an anisotropy that may exist among the absorbing pigments. Table V shows that the polarization increases from 0.42 % at 650 nm to 0.85 % at 670. Any non-zero value of the fluorescence polarization using horizontally polarized exciting light is a consequence of order among the pigments. Therefore, the data in Table V provide further evidence that the red transition dipoles of chlorophyll in Photosystem II are oriented and also support the suggestion that the order increases as the absorption maximum of the molecules increases.

Our results can be used to test the validity of different chlorophyll antenna models. For example, a homogeneous random array of chlorophyll molecules is clearly too simple a system to explain our wavelength-dependent results. An antenna system in which longer wavelength absorbing pigments are more closely related to the trapping centers [42] is also inconsistent with our results. In this model we would expect that as more energy transfer occurs the excitation energy absorbed at shorter wavelengths (650 nm) would be transferred to longer wavelength chlorophyll. Therefore, adding DCMU while exciting at 650 nm should cause the polarization to become more like the polarization exhibited while exciting at longer wavelengths. Table I shows that the opposite effect occurs.

This last observation leads us to believe that chlorophyll molecules are arranged in different groups. Let us compare the effect of DCMU on fluorescence polarization of molecules absorbing light at 657 nm and 670 nm. Upon the addition of DCMU the polarization decreases from 2.3 % to 2.0 % for molecules absorbing at 657 nm, whereas the polarization remains essentially constant (4.2 %) for molecules absorbing at 670 nm. Consider now the effect of energy transfer between the two different groups of pigments. If excitation energy were being transferred to any great extent from molecules absorbing at 657 nm to molecules absorbing at 670 nm, then we would expect the addition of DCMU to cause the polarization to increase, since transferring excitation energy to 670 nm pigments should have the same effect as exciting the 670 pigments directly. If energy transfer were taking place in the opposite direction, from 670 nm pigments to 657 nm pigments, then we would expect the addition of DCMU to cause the polarization to decrease.

These considerations lead us to the tentative conclusion that the chlorophyll molecules in Photosystem II exist in discrete groups characterized by different absorption maxima and by different degrees of fluorescence polarization and that excitation energy is not shared between some groups absorbing at different wavelengths.

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