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MEASUREMENT OF DEGREE OF CHLOROPHYLL FLUORESCENCE POLARIZATION IN RELATION TO THE REGULATION OF EXCITATION ENERGY TRANSFER BETWEEN PHOTOSYSTEMS I AND II IN PEA CHLOROPLASTS

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The degree of chlorophyll fluorescence polarization (p) at 740 nm was measured at room temperature for pea chloroplasts subjected to various conditions. (1) In agreement with previous published observations, p decreased when the Photosystem (PS) II traps were closed by illumination in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea. (2) Under these conditions, the magnitude of p was also sensitive to the presence of salts. Under conditions when 'spillover' of the excitation energy from PS II to PS I was low, p was also low, being consistent with increased migration of energy between the PS II light-harvesting chlorophylls. In contrast, when spillover was at a maximum p increased. (3) The change in p in the presence of salts was dependent on the concentration and valency of the cations in such a way as to suggest the changes were mediated through electrostatic forces. The dependency of p on ionic composition of the experimental medium was closely related to the associated changes in fluorescence yield. (4) Membrane stacking, caused by lowering pH of the chloroplast suspension, did not induce a significant change in p, suggesting that this pH-induced process is different from the membrane stacking brought about by manipulating the salt levels. (5) Incubation of thylakoids with ATP induces light-dependent phosphorylation of the light-harvesting chlorophyll-protein complexes, and regulates excitation energy transfer between PS I and PS II (Bennett, J., Steinback, K.R. and Arntzen, C.J. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 5253–5257). Under conditions which bring about this phosphorylation it was found that p increased to a value indicative of spillover.

Introduction

The distribution of excitation energy between PS I and PS II of isolated envelope-free chloroplasts can be regulated by cations [1–4]. This process is accompanied by a significant change in the morphology of thylakoid membranes; namely, membrane stacking and unstacking [5–7] and changes in the distribution of various-sized particles within the plane of the membrane [8–10]. Efficiency of energy transfer from PS II to PS I at the light-harvesting pigment level (spillover) will be determined by the distance between the associated pigment-protein complexes which exist as intrinsic components of the membrane. Indeed, the relationship between the cation-induced changes in the membrane structure and the changes in energy transfer between PS II and PS I have been extensively studied theoretically and experimentally in terms of a model involving lateral diffusion of pigment-protein complexes within the membrane [11–14].

Abbreviations: PS, photosystem; Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; Mes, 2-(*N*-morpholino)ethanesulphonic acid.

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Energy transfer among chlorophyll molecules in the photosynthetic membrane has been investigated by adopting several approaches, but of special importance are the measurements involving the detection of chlorophyll fluorescence, including fluorescence yield, time course of fluorescence changes, excitation and emission spectra, fluorescence life-times and fluorescence polarization [15–19].

In the present work, using isolated pea thylakoid membranes, we have measured the degree of polarization (p) of steady-state room-temperature chlorophyll fluorescence for different degrees of spillover between PS II and PS I as brought about by varying the cationic composition of the suspension medium. We have also studied p under membrane-phosphorylation conditions when spillover of excitation energy from PS II to PS I has been reported to occur [20].

Materials and Methods

Envelope-free chloroplasts were obtained from pea leaves using a method described previously [21].

Chlorophyll fluorescence was measured at room temperature using a Perkin Elmer spectrofluorometer, MPF-44A. For determining the steady-state fluorescence yield, chloroplasts were excited with blue actinic light (peak wavelength, 400 nm; half-bandwidth, 20 nm) and the fluorescence was detected at 685 nm (half-bandwidth, 10 nm).

For measuring p of chlorophyll fluorescence, narrow-band red excitation light (peak wavelength, 670 nm; half-bandwidth, 10 nm) was used corresponding to the Q_y red absorption band of chlorophyll in vivo [22]. Since chlorophyll fluorescence has Q_y polarization, energy transfer from the accessory pigments to Chl *a*, or excitation into higher singlet bands of chlorophyll having a different polarization, did not contribute to the p reported in this paper.

Fluorescence was viewed at 740 nm (half-bandwidth, 10 nm) which is the peak of a satellite band of chlorophyll fluorescence at room temperature, and mostly originates from the light-harvesting chlorophyll-protein complexes associated with PS II [23,24]. Two polaroid filters (a polarizer and an analyser) were put in front of, and behind, a quartz cuvette and manually rotated to obtain vertically and horizontally polarized light. p was estimated according to the following equation:

$$p = \frac{I_{vv} - I_{vh} \times (I_{hv})/(I_{hh})}{I_{vv} + I_{vh} \times (I_{hv})/(I_{hh})}$$

where I is the intensity of fluorescence and the subscripts v (vertical) and h (horizontal) show the geometry of the polarizer and the analyser.

90° light-scattering changes in chloroplasts were monitored using the same spectrofluorometer by setting the wavelengths of both the excitation and emission monochromators at 540 nm (half-bandwidths, 2 nm).

Trypsin treatment of the thylakoid membrane was carried out as previously described [25].

The aging treatment of chloroplasts was done by incubating the chloroplasts at 25°C in the standard suspension buffer (see below) with the concentration of chlorophyll at 3.5 μg Chl/ml.

The standard reaction mixture for fluorescence measurements contained broken chloroplasts equivalent to 10 μg Chl/ml, 100 mM sorbitol and 1 mM Tris-HCl (pH 7.5). Other specific conditions are described in the figure legends. Salts were added by microsyringe after which the suspension was stirred briefly. Fluorescence was measured after 5 min incubation of chloroplasts with salts at 21°C. The temperature of the cuvette was kept constant during the measurement by flowing water through a cuvette jacket connected to a Grant Instruments thermostatically controlled water bath.

Results and Discussion

Dependency of p on the redox state of the PS II reaction centre

Under the condition when the ionic content of the suspension medium is kept constant, the chlorophyll fluorescence yield is dependent only on the redox condition of the PS II reaction centre. Strong actinic light, or the addition of DCMU at limiting light intensities, causes closure of the PS II traps, resulting in an increase in the fluorescence yield. On the other hand, in the absence of DCMU the addition of an artificial electron acceptor causes oxidation of the primary electron acceptor of PS II so as to bring about a concomitant decrease in the fluorescence yield.

In line with changes in the intensity of the emission, p showed a significant change depending on the open or closed state of the PS II traps. In condition A in Table I, thylakoids were stacked due to the

TABLE I

EFFECTS OF DCMU AND POTASSIUM FERRICYANIDE ON THE RELATIVE YIELD AND p OF CHLOROPHYLL FLUORESCENCE

KCl (10 mM) and $MgCl_2$ (10 mM) were included in the standard reaction mixture to obtain chloroplasts which had their thylakoid membranes stacked and were in a minimum spillover condition. For A, the intensity of the actinic light was reduced to 15% of the saturating level to elicit large effects of DCMU addition on the fluorescence yield and p. Saturating actinic light was used in condition B. Data are the average of five measurements ± S.D.

Conditions	p (%) ± S.D.	Relative yield of variable fluorescence
A Control	5.9 ± 0.8	63
+ DCMU (33 μM)	2.5 ± 1.0	100
B Control	1.7 ± 0.6	97
+ $K_3Fe(CN)_6$ (1 mM)	5.1 ± 0.8	56

presence of 10 mM $MgCl_2$ and spillover of the excitation energy from PS II to PS I was low. In this experiment the chloroplasts were excited by low-intensity actinic illumination so that PS II reaction centres tended to be in a partially open state. Consequently, the fluorescence yield was kept reasonably low due to the efficiency of trapping and the relative degree of fluorescence polarization was high. Under this condition the average number ($\bar{n}$) of the excitation energy transfer (hopping) among chlorophyll molecules should be small. (The relationship between p and $\bar{n}$ has been considered in a previous paper [26]; also, see Ref. 27.) The addition of DCMU under the same illumination conditions induced an increase in the fluorescence yield and a decrease in p (Table I). The smaller p can be explained by the increased migration of the excitation energy among the light-harvesting PS II chlorophylls, where the excitation energy can no longer be trapped by the closed PS II reaction centres. It is worth noting that p under conditions of infinite migration of the excitation energy among the light-harvesting chlorophylls (i.e., in the presence of DCMU) was not 0% as might be expected, but about 2%. This probably reflects either the random orientation of the thylakoid membranes in the suspension (an effect which will always give a positive p; i.e., 'geometric photoselection' [28]) or a residual polarization specific to the instrument we used. In contrast to the effect of DCMU, the addition of potassium ferricyanide under high-intensity illumination induced a decrease in fluorescence and an increase in p (Table I, B). The DCMU and ferricyanide effect on the chlorophyll fluorescence polarization reported above can readily be explained by their ability to alter the redox state of the PS II traps and is consistent with previous arguments [29]. It seems very unlikely that changes in the orientation of chlorophyll molecules in the membrane or changes in the orientation of thylakoid membranes in the suspension (which would also be effective in changing p) can explain the data in Table I. Overall, the results support the previous work of Lavorel [18] and Mar and Govindjee [19] who used intact algae.

Effects of cations on p

In agreement with the work of Wong and Govindjee [24,30], we have found that p is also highly dependent on the ionic condition of the chloroplast suspension (see Table II). In a chloroplast suspension where no salt was added (except for the basic buffer solution, 1 mM Tris-HCl, pH 7.5), thylakoids would be stacked [14,31–33] and have a high fluorescence yield [34,35]. Under these conditions, a low p was observed. The addition of 10 mM KCl induces unstacking of thylakoids [7,32,33,36] and a lowering of the fluorescence yield [31–35] and associated with these changes a high p was observed (Table II). Further addition of 10 mM $MgCl_2$ causes membrane stacking again [7,33,36] and the fluorescence yield and p changes accordingly (Table II).

The dependency of p on salt levels was demon-

TABLE II

EFFECTS OF KCl AND $MgCl_2$ ON p

Data are the average of five measurements ± S.D.

Conditions	p (%) ± S.D.	Relative yield of variable fluorescence
Control	3.8 ± 0.5	95
+ KCl (10 mM)	5.7 ± 1.0	59
+ KCl (10 mM) + $MgCl_2$ (10 mM)	2.3 ± 0.6	100

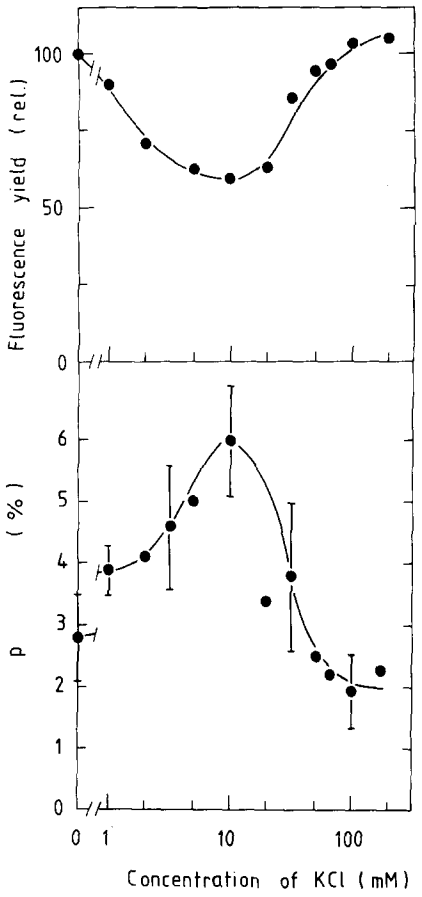

Fig. 1. Effects of various KCl concentrations on the yield and p of chlorophyll fluorescence. The bars show standard deviations with three measurements.

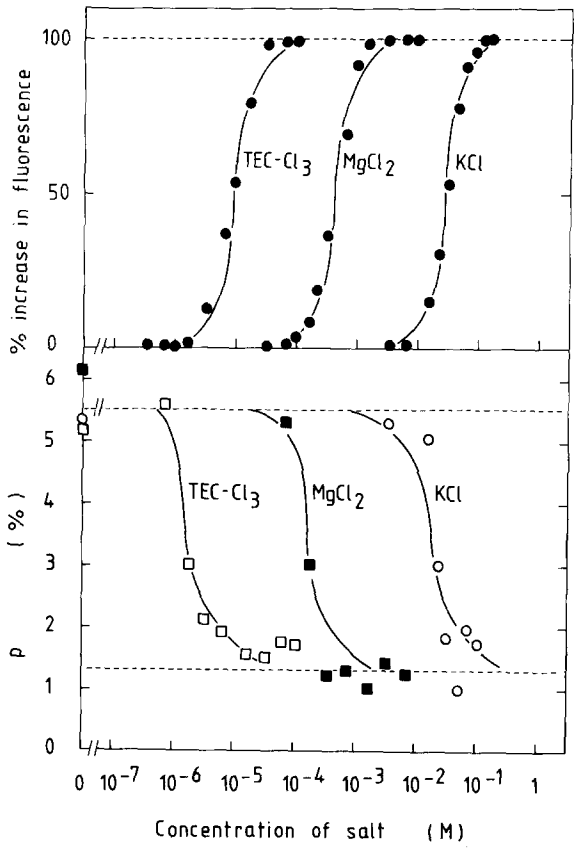

Fig. 2. Dependency of yield and p of chlorophyll fluorescence on the valency and concentration of cations added to the chloroplast suspension. Tris(ethylendiamine)cobalt (III) (TEC) was used as a trivalent cation.

strated further by changing the concentration of KCl in the chloroplast suspension (Fig. 1). The minimum fluorescence yield observed at a KCl level of about 10 mM corresponds to the state when efficient spillover of excitation energy from PS II to PS I occurs and the thylakoid membranes are unstacked [14,36]. Under this condition, p was a maximum as expected if spillover from PS II to PS I reduces the excitation migration among the PS II light-harvesting chlorophylls. Decreasing spillover by manipulating the KCl levels therefore has the effect of increasing the yield of fluorescence and its degree of depolarization.

The addition of various salts changed p depending on the valency of the cations used, and again, its value was closely associated with changes in the fluorescence yield (Fig. 2).

Effects of trypsin and aging treatments on chlorophyll fluorescence polarization

Cation-induced membrane stacking and fluorescence changes in chloroplasts can be inhibited in several ways. Mild trypsin treatment of thylakoids has been shown to digest partially the exposed segment of the light-harvesting Chl *a*/Chl *b*-protein complex [37] and increase the amount of negative electrical charge exposed on the membrane surface [25]. It has been argued that the trypsin-treated thylakoids lose their ability to stack in the presence of salts because of the resulting increase in electrostatic repulsion between adjacent membranes [39].

Aging of chloroplasts seems to decrease the fluidity of the lipid phase of the thylakoid membrane, judging from measurements of the degree of fluores-

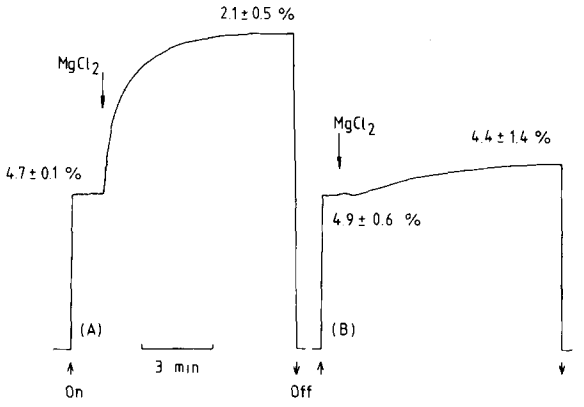

Fig. 3. Time course of Mg^{2+}-induced fluorescence change in broken chloroplasts treated without (A) or with (B) trypsin. Both chloroplast samples were incubated with 10 mM KCl for 5 min at room temperature before subjecting them to illumination. p was measured before and after adding $MgCl_2$. The data are the average of three measurements ± S.D.

cence polarization of 1,6-diphenylhexatriene [40,41]. It has been argued that a decrease in the membrane fluidity might retard the reorganization of the chlorophyll-protein complexes in the thylakoid membrane on adding cations, resulting in the observed inhibition of membrane stacking and fluorescence changes [40].

Both trypsin and aging treatments were found to reduce significantly the change in p on addition of Mg^{2+} (Figs. 3 and 4). These results confirm the idea

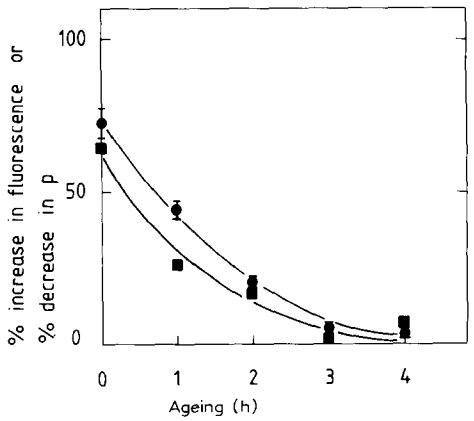

Fig. 4. Effects of aging on the fluorescence yield and p values monitored by the sensitivity of the two parameters to the addition of 10 mM Mg^{2+}. Data are the average of three experiments. (•) % increase in fluorescence, (▪) % decrease in p.

presented above that the change in the degree of polarization in PS II chlorophyll fluorescence is dependent on the efficiency of excitation energy spillover from PS II to PS I.

Membrane stacking at low pH and p

Stacking of thylakoids can be induced also by decreasing pH of the chloroplast suspension [40,42]. Membrane stacking was monitored by 90° light-scattering changes of chloroplasts at 540 nm. A peak in light scattering occurred at pH 4.3 corresponding to the isoelectric point of the thylakoid membrane (Fig. 5). The fluorescence yield, however, did not change in parallel with the light-scattering change (the

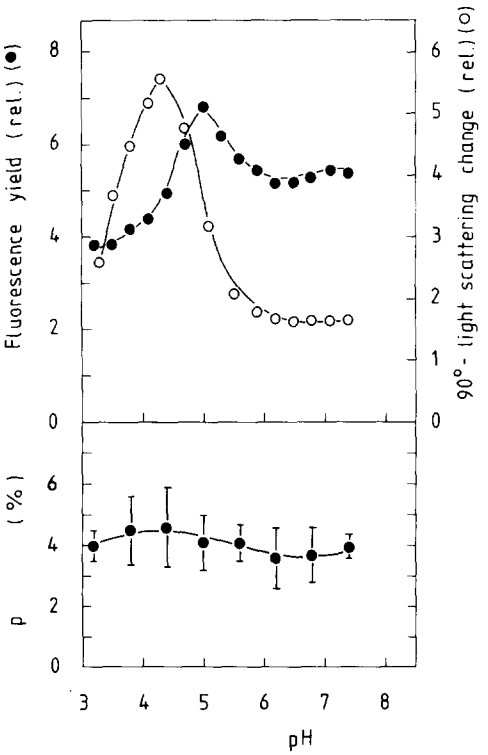

Fig. 5. Dependency of chlorophyll fluorescence yield, 90° light-scattering and p on pH of the chloroplast suspension. The reaction mixture contained broken chloroplasts equivalent to 10 μg Chl/ml, 100 mM sorbitol, 1 mM Hepes, 1 mM Mes, 0.5 mM succinate, 3 mM KOH, 10 mM KCl and 33 μM DCMU. At the beginning the pH of the reaction mixture was 7.5 and thylakoids were initially subjected to unstacking conditions. The pH of the suspension was then transferred to a given value by adding HCl. Data of p are the average of three measurements ± S.D.

peak of the fluorescence appeared at pH 5.0). Thus a different mechanism has been suggested [14] for the pH-induced membrane stacking (charge neutralization) as compared with salt-induced stacking (charge screening). The fluorescence yield over the pH range tested was low, compared with that observed in the presence of cations at pH 7.0, possibly indicating that maximum spillover occurs even at low pH. Consistent with this was the finding that p remained high over the pH range examined with no obvious appearance of a minimum when the pH-induced stacking was a maximum.

p under membrane-phosphorylation conditions

Recently, Bennett et al. [20] have shown an important role of membrane protein phosphorylation in the regulation of excitation energy distribution between PS I and PS II. In the presence of ATP, Mg^{2+} and protein kinase, the light-harvesting Chl *a*/Chl *b*-protein complex becomes phosphorylated under illumination with light below 700 nm. The phosphorylation has been shown to be inhibited by DCMU or by illuminating with PS I light [43]. Measurements of ATP-induced chlorophyll fluorescence quenching at room temperature and chlorophyll fluorescence emission spectra at liquid N_2 temperature indicate the existence of spillover of excitation energy from PS II to PS I when the Chl *a*/Chl *b* complex is phosphorylated. In our experiments, the addition of 400 μM ATP to chloroplasts induced significant quenching of chlorophyll fluorescence and a concomitant increase in p (Table III). The effect of ATP on p was inhibited by DCMU as was the fluorescence quenching.

TABLE III

EFFECTS OF ATP ON p

The reaction mixture contained broken chloroplasts (10 μg Chl/ml), 100 mM sorbitol, 10 mM $MgCl_2$ and 50 mM Tricine-NaOH (pH 7.8). Data are the average of three measurements ± S.D.

Condition	p (%) ± S.D.
Control	2.7 ± 0.7
+ATP (400 μM)	4.4 ± 1.0
+DCMU (33 μM)	2.9 ± 0.2
+DCMU (33 μM) +ATP (400 μM)	2.6 ± 0.7

From the above experiments, it can be concluded that energy-transfer processes which occur within the pigment-protein complexes of the thylakoid membrane can be studied by measuring p. Although p could be affected by a number of factors in addition to energy transfer [41,45], it seems that fixing the excitation and viewing wavelengths at 670 and 740 nm, respectively, simplifies the situation. Using these wavelengths we have been able to monitor fluorescence polarization changes mainly due to energy transfer between the chlorophyll molecules serving as antennae to PS II. Our results are consistent with the concept that varying the ionic nature of the medium in which the thylakoids are suspended leads to changes in energy transfer between the PS II and PS I light-harvesting systems and that these changes in spillover are under the control of electrostatic forces [12,14].

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