Light Induced Changes in the Conformation of Spinach Thylakoid Membranes as Monitored by 90° and 180° Scattering Changes: A Comparative Investigation

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Z. Naturforsch. **39 c**, 1120–1127 (1984); received August 6, 1984

Conformational Changes, Thylakoids, Scattering Changes, Comparison

A series of investigations on light induced thylakoid membrane conformational changes has been carried out using scattering changes measured either at 90° or 180° (transmittance changes) as criteria for changes in membrane structure upon illumination. These two phenomena were found to react similarly to a range of photosynthetic inhibitors, to changes in the ionic (Mg^{2+}, H^+) concentration, and to have similar difference spectra. However, it was found possible to distinguish these two phenomena by the differences in the kinetics of the light induced rise times. This could be shown most clearly using an Aminco DW2 spectrophotometer with the measuring beam deflected through 90° by front face mirrors. The dark decay time of the two scattering changes was similar.

These results are discussed in relation to the hypothesis that the light induced narrow angle 90° scattering response represents microconformational membrane changes in response to proton uptake, and the 180° scattering changes represent a mixed response including changes in the stacking of thylakoids.

Introduction

There are at least two different classes of light dependent structural changes observed in thylakoid membranes. Rapid ($t_{1/2}$ 20–100 ms) conformational changes in some externally located peripheral membrane components (ferredoxin NADP reductase, coupling factor complex) which have been linked to the activation of these enzymes [1, 2]. Slower (s-min) conformational changes of membrane structure including a decrease in intergranal distance and stacking changes. These structural changes have been correlated to changes in the lumen microenvironment due to ionic and water fluxes during illumination and have been monitored by changes in light scattering [3–5] and by ultrastructural studies combined with microddensitometry [6, 7].

The light induced scattering changes in thylakoid membranes were first reported by Packer [8] and have been the subject of extensive research, firstly on thylakoids [3, 4], and later on intact chloroplasts [9, 10] and on intact leaves [11–13] and algal thalii [14]. Convincing evidence has been obtained that scattering changes measured at 90° reflect microconformational changes in membrane structure in response to proton influx [3, 5, 15]. From the theoretical work of Latimer [16, 17] and Thorne [18, 19], it is known that there is a strong angular dependency to the scattering response. Thus, in discussing scattering changes the angle of scatter and the pathlength of scattered light should always be defined. Light induced scattering changes measured at 180° (transmittance mode) are not necessarily synonomous with 90° scattering although the two phenomena usually occur in parallel.

We have recently observed that short time glutaraldehyde treatment of spinach thylakoids selectively abolished light induced scattering changes measured at 180° whereas 90° scattering changes were relatively unaffected [20]. This result stimulated us to undertake a detailed series of investigations on the light induced scattering changes both at 90° and 180° in both intact and freshly shocked

Abbreviations: ASC, sodium ascorbate; 9AA, nine amino acridine; DAD, 2,3,5,6-tetramethyl-β-phenylene diamine; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-β-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCCD, N,N′-dicyclohexyl carbodiimide; DNP-INT, 1,3-dinitrophenyl ether of iodonitrothymol; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; Fecy, potassium fericyanide; GM_D, gramicidin$_D$; MV, methylviologen (1,1′-dimethyl-4-4′-bipyridinium dichloride); NH_4Cl, ammonium chloride; nig, nigericin; PAR, photosynthetically active radiation (400–700 nm); TBT, tri-*n*-butyltin chloride; Val, valinomycin.

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spinach chloroplasts, in order to gain additional insight into the physical nature of these two processes. These experiments were carried out under normal osmotic conditions and in the absence of any freely permeable weak acids or amines which lead to nonspecific thylakoid swelling upon illumination [5].

Materials and Methods

Chloroplast isolation

Spinach (*Spinacia oleracea* L. hybrid 524) was grown in a greenhouse with supplementary lighting (total PAR 100–200 W m^{-2}) under a short day regime (10/14 h L/D cycle).

Intact chloroplasts were isolated by grinding (50 g chopped leaves/150 ml grinding medium, 5 × 1 s full speed) in a Braun blendor using a modified version of the medium "A" of Jensen and Bassham [21] as described by Demmig [22]. The brei was filtered through eight layers of muslin and one layer of 25 µm mesh size nylon cloth, and the homogenate spun for 30 s, 3000 g. The pellet was gently resuspended and washed once in the grinding medium without added ascorbate, and resuspended in a minimal amount of medium to about 2 mg Chl ml^{-1}. Chloroplasts thus isolated were between 70–85% intact as estimated by ferricyanide reduction [23]. Typical rates of CO_2 dependent O_2 evolution were between 100–200 µmol O_2 h^{-1} mg Chl^{-1}.

Spectrophotometric measurements

1) Narrow angle 90° scattering changes

These were measured in an Aminco SPF 5000 spectrofluorometer. The pathlength from the cuvette to the emission photomultiplier tube (protected by a 2 mm slit) was 35 cm. Actinic light was supplied to the top of the cuvette by a Schott KL 1500 fibre optic light source filtered through a Calflex K9 infra red cut off filter and a Schott RG 630 red cut off filter. Light was focused onto the cuvette *via* a fish eye lens. Where necessary, the actinic light was attenuated by Schott neutral density filters. Excitation and emission photomultiplier tubes were protected against actinic light by Corning 4303 blue cut off filters. The fluorometer was operated in the ratio mode with an output of between 0–100 MV. The cuvette contained 1.5 ml total volume, thermostated at 20 °C. Scanning spectra were either obtained point by point or scanned continuously, the excitation and emission monochromators (1 nm and 5 nm slit width) being first set to the same wavelength. The scanning spectra were stored and manipulated on a Hewlett Packard HP85 minicomputer interfaced to the spectrofluorometer.

2) Wide angle 90° and 180° scattering changes

These were measured in an Aminco DW2 spectrophotometer operated in the split beam mode. Actinic light was provided to the side of the cuvette from an incandescent light source (100 W, 250 V) filtered through 3 cm water and a Calflex K9 cut off filter to absorb infra red radiation, and a Schott RG 630 red cut off filter. The photomultiplier was protected from the actinic light by a combination of Schott BG 38 and Corning 4303 blue cut off filters. The measuring beam was deflected through 90° where necessary by front face mirrors. The distance from the cuvette to the photomultiplier was 2 cm. The cuvette contained 2.5 ml of solution and was thermostated at 20 °C. The cuvette geometry was identical for both machines.

3) 9AA fluorescence quenching

Light induced 9AA fluorescence quenching was measured in the spectrofluorometer. 9AA fluorescence (1 µM) was excited at 400 nm (1 nm slit width) and the emitted fluorescence measured at 480 nm (40 nm slit width). Actinic light was supplied as for narrow angle 90° scattering measurements.

Light induced, thylakoid mediated proton uptake was measured as in [24] chlorophyll concentration was determined according to Arnon [25].

Intact chloroplasts were pipetted into half the appropriate cuvette volume (1.25 ml or 0.75 ml) containing shocking medium (0–10 mM $MgCl_2$, 10 mM MES/NaOH pH 6.0–7.0, Hepes/NaOH pH 7.0–8.0, tricine/NaOH 8.0–9.0), ruptured by stirring for 10 s, then the volume made up by the addition of an appropriate amount of double strength medium (660 mM sorbitol, 20 mM buffer).

Inhibitor solutions were added in methanolic solution (final methanol concentration < 1%) and incubated in the dark for 1 min before the onset of illumination.

Results

The first figure shows the spectra obtained at 180° (transmittance mode) between 400–560 nm. One minute of continuous illumination induced a general increase in apparent absorbance (curve 2) compared to the dark control (curve 1) which was only partly reversed by a subsequent three minute dark period (curve 3). Curve 2 shows the attributes typical of a scattering spectrum in that all apparent absorbance values were positive with no isosbests. There were broad apparent peaks of absorption in the region 500–540 nm with a subsidiary peak at 450 nm. The light/dark difference spectra (curves 2-1, 2-3) also show typical properties of a scattering spectra in that again all values were positive. Again there were apparent absorbance maxima between 500–540 nm, and at 450 nm.

The narrow angle 90° scattering spectra obtained between 400–560 nm agred in general with that obtained in the 180° mode, both the continuously scanned spectra (Fig. 2a) and a point by point spectrum (Fig. 2b) again showed all positive values with a general scattering maximum in the region between 510–520 nm with a secondary maximum at 450 nm. The light-dark difference spectrum showed a maximum around 520 nm, again with a secondary maximum at 450 nm.

The amplitude of the light induced scattering changes was relatively independent of chlorophyll concentration between 1.5–50 μg Chl ml^{-1} (Fig. 3). A concentration of 15 μg Chl ml^{-1} was thus chosen for all experiments. There was a striking requirement for divalent cations, particularly Mg^{2+}, to elicit the light induced scattering responses both at 90° and 180° (Fig. 4). The dark scattering level at both 90° and 180° representing the degree of thylakoid stacking, and the light induced scattering changes shared a parallel requirement for Mg^{2+}.

The 90° scattering component saturated at very low light intensities (5 W m^{-2}) which, in these thylakoids, corresponded to the saturation of light dependent proton uptake into the thylakoids. The 180° scattering component was saturated at slightly higher light intensities (20–30 W m^{-2}, Fig. 5). The pH dependency of the two scattering components is shown in Fig. 6. The 90° component showed a scattering maximum between pH 6.0–6.5 (twice the level at pH 8.0), as does light dependent proton uptake. The 180° component also showed a scattering maximum around pH 6.0, but this was less pronounced than for the 90° component (40% in-

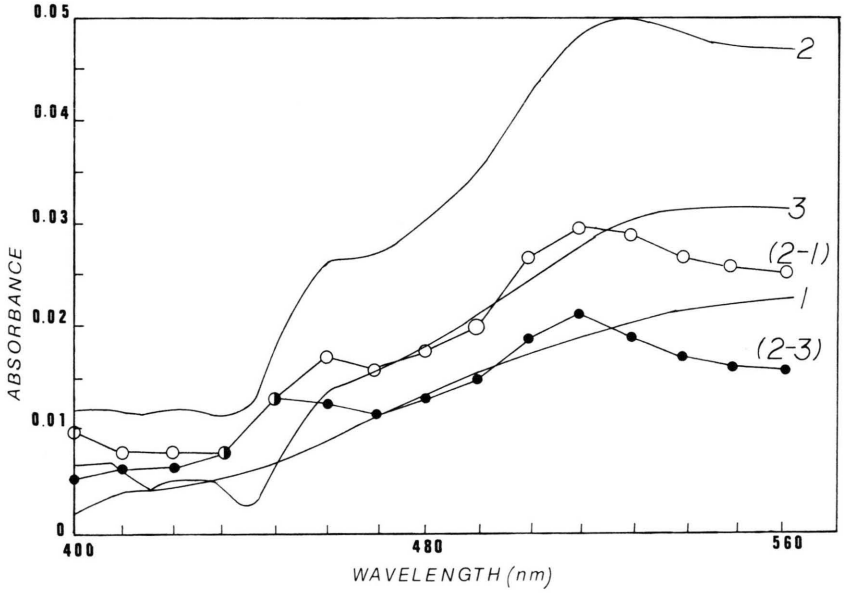

Fig. 1. Spectrum of transmittance (180°) changes between 400–560 nm. The cuvette (2.5 ml volume) contained 0.33 M sorbitol, 10 mM $MgCl_2$, 10 mM HEPES/NaOH pM 7.6, 0.1 mM MV, 15 μg Chl ml^{-1} broken chloroplasts. Scanning speed was 100 nm min^{-1}. Curve 1, dark level (3 min dark adapted); curve 2, after illumination (50 Wm^{-2}, 630–800 nm); curve 3, after 3 min dark period; curve 2-1, difference spectrum 2-1 (point by point); curve 2-3, difference spectrum 2-3 (point by point).

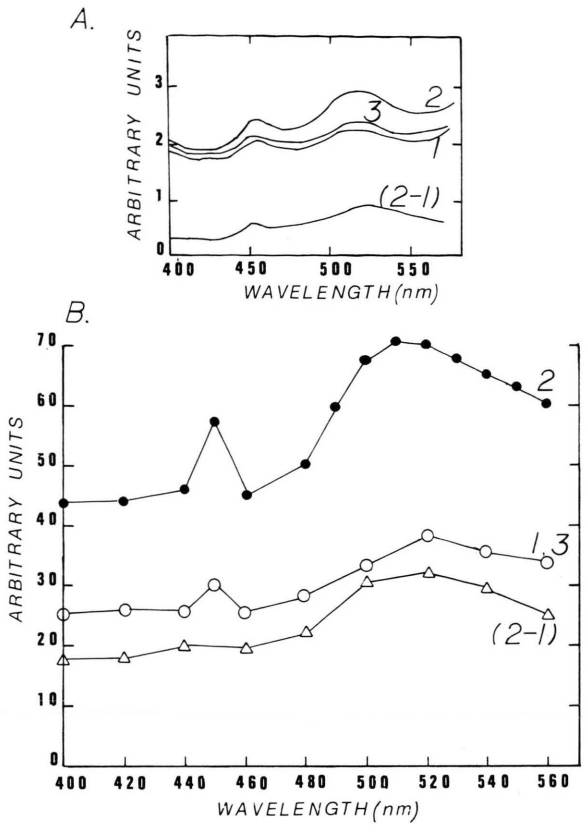

Fig. 2. 90° scattering spectrum between 400–560 nm. The cuvette (1.5 ml volume) contained 0.33 M sorbitol, 10 nM $MgCl_2$, 10 mM HEPES/NaOH pH 7.6, 0.1 mM MV, 15 µg Chl ml^{-1} broken chloroplasts. Scanning speed was 100 nm min^{-1}. A. Continuous scanning spectra: Curve 1, dark level (3 min dark adapted); curve 2, after 1 min continuous illumination (50 Wm^{-2}, 630–800 nm); curve 3, after a 3 min dark period; curve 2-1, difference spectrum between curves 2 and 1. B. Point by point scanning spectra: Curve 1, dark level (3 min dark adapted); curve 2, after 1 min continuous illumination (50 Wm^{-2}, 630–800 nm); curve 3, after a 3 min dark period; curve 2-1, difference spectrum between curves 2 and 1.

crease compared to the response at pH 8.0). Light dependent proton uptake and nine amino acridine fluorescence quenching representing proton gradient formation showed an antiparallel relationship as would be expected.

Thus far, the two scattering components showed similar characteristics. However, it proved possible to more clearly separate the two components kinetically (Fig. 7). The typical narrow angle 90° scattering signal showed a rapid rise ($t_{1/2}$ 1–5 s) to a steady state after between 10–20 s. The $t_{1/2}$ decay time was between 15–30 s. There was apparently no component of the electrochromic shift present in this response as valinomycin present in sufficient quantities to collapse the field (5×10^{-7} M–5×10^{-6} M) had no affect on the scattering response. Furthermore the scattering response measured at 500 nm and 480 nm, near the isosbest and negative absorption peak for the electrochromic shift showed no changes in the kinetics of the rise time. Addition of 5×10^{-7} M nigericin alone or in combination with 5×10^{-7} M valinomycin abolished the 90° scattering response.

Wide angle 90° scattering could be readily resolved into two components (Fig. 7). A rapid ($t_{1/2}$ rise 1–5 s) and a slow component ($t_{1/2}$ 15–20 s). Again, the fast rise component appeared not to represent the electrochromic shift as measurements at 500 nm and 480 nm showed identical kinetics and 5×10^{-7} M valinomycin had no affect on the response. Again, addition of 5×10^{-7} M nigericin alone or in combination with valinomycin abolished the scattering responses. The slow rise component of the wide angle 90° scattering component appeared to be identical to the slow rise component measured at 180° in the same instrument. Again the dark decay time of this slow component ($t_{1/2}$ 15–30 ×) was the same as for the narrow angle 90° scattering. The electrochromic shift could be observed in measurements at 180°, as witnessed by the characteristic spike on illumination. This could be abolished by 5×10^{-7} M valinomycin, but a rapid rise component still remained which was probably the overlapping rapid rise 90° component. Interpretation of the 180° component was complicated by the fact that valinomycin promotes a degree of light induced thylakoid swelling which varies between different thylakoid preparations. The slow 180° rise component was thus unaffected or increased by 5×10^{-7} M valinomycin. The addition of 5×10^{-7} M nigericin, alone or in combination with valinomycin abolished the slow scattering changes, but alone had no affect on field formation.

When the Aminco DW2 was used in the dual wavelength mode, the slow 180° rise component was eliminated (figure inset). The residual slow rise component at 518 nm, which had an amplitude of about 70% of the electrochromic shift, had a $t_{1/2}$ of about 5 s. Again the dark decay time ($t_{1/2}$) was between 15–30 s.

Finally, Table I shows the effect of various inhibitors of photosynthetic electron transport, proton

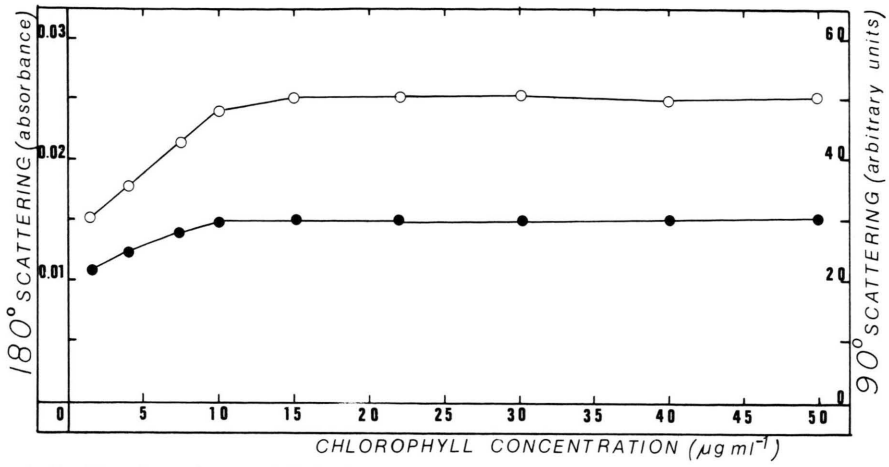

Fig. 3. The dependence of light induced scattering changes (90°, 180°) at 540 nm on chlorophyll concentration. Experimental conditions were as for Figures 1 and 2. Light induced scattering changes were measured as the difference between the dark level and after 40 s (90°) and 60 s (180°) illumination with red light (50 Wm^{-2}, 630–800 nm). ○—○ 90° Scattering; ●—● 180° scattering.

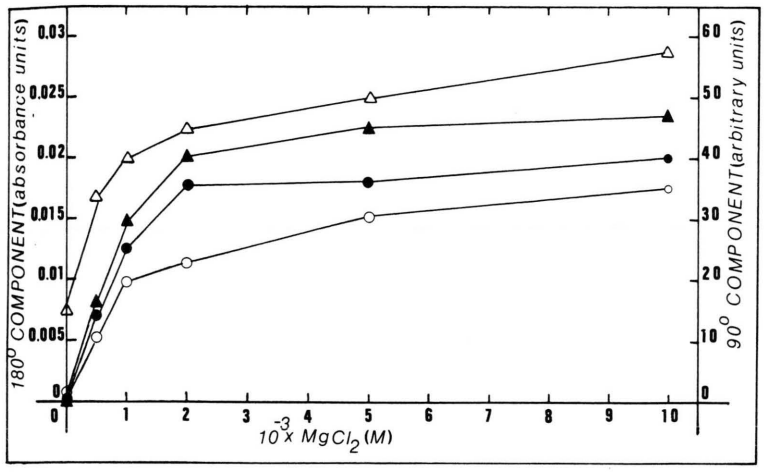

Fig. 4. The dependence of light scattering changes (90°, 180°) at 540 nm on magnesium concentration. Dark scattering levels (90°, 180°) were normalized against 0 mM $MgCl_2$. Known aliquots of $MgCl_2$ were added, and the new dark scattering level monitored over a 5 min incubation period. Light induced scattering changes (90°, 180°) were measured as in Fig. 3. Experimental conditions were as in Figs. 1 and 2. ○—○ 180° Dark scattering level; ●—● 180° light induced scattering change; △—△ 90° dark scattering level; ▲—▲ 90° light induced scattering change.

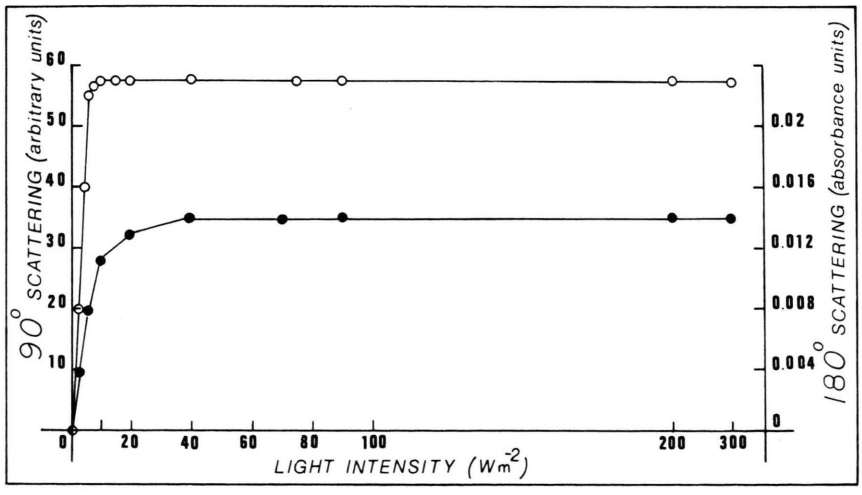

Fig. 5. The dependence of light induced scattering changes (90°, 180°) at 540 nm on light intensity. Experimental conditions as in Figs. 1 and 2. ○—○ 90° Scattering; ●—● 180° scattering.

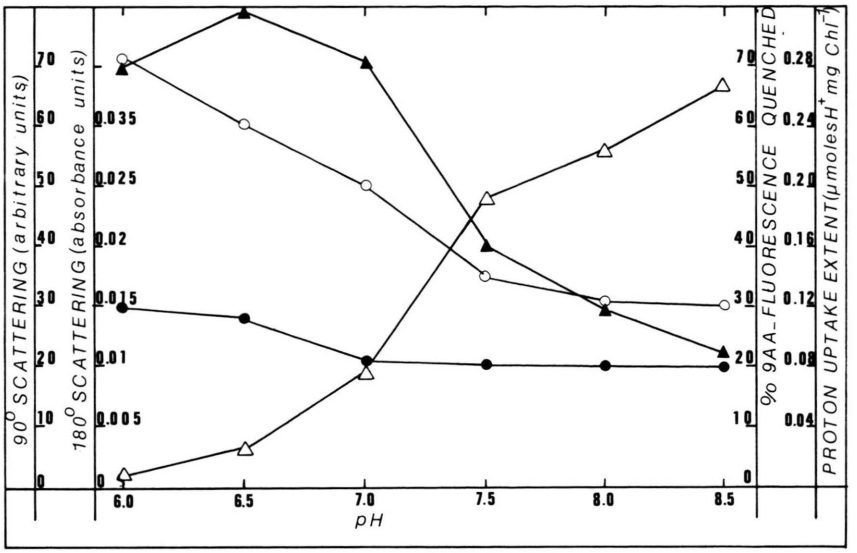

Fig. 6. The dependence of light induced scattering changes (90°, 180°), proton pumping and 9AA fluorescence quenching on pH. Experimental conditions were as in materials and methods and the previous figures. For 9AA fluorescence measurements 1 μM 9AA was added to the medium. For proton uptake measurements the buffer concentrations was reduced to 1 mM. ○—○ Light induced 90° scattering; ●—● light induced 180° scattering; △—△ light induced 9AA fluorescence quenching; ▲—▲ light induced proton uptake.

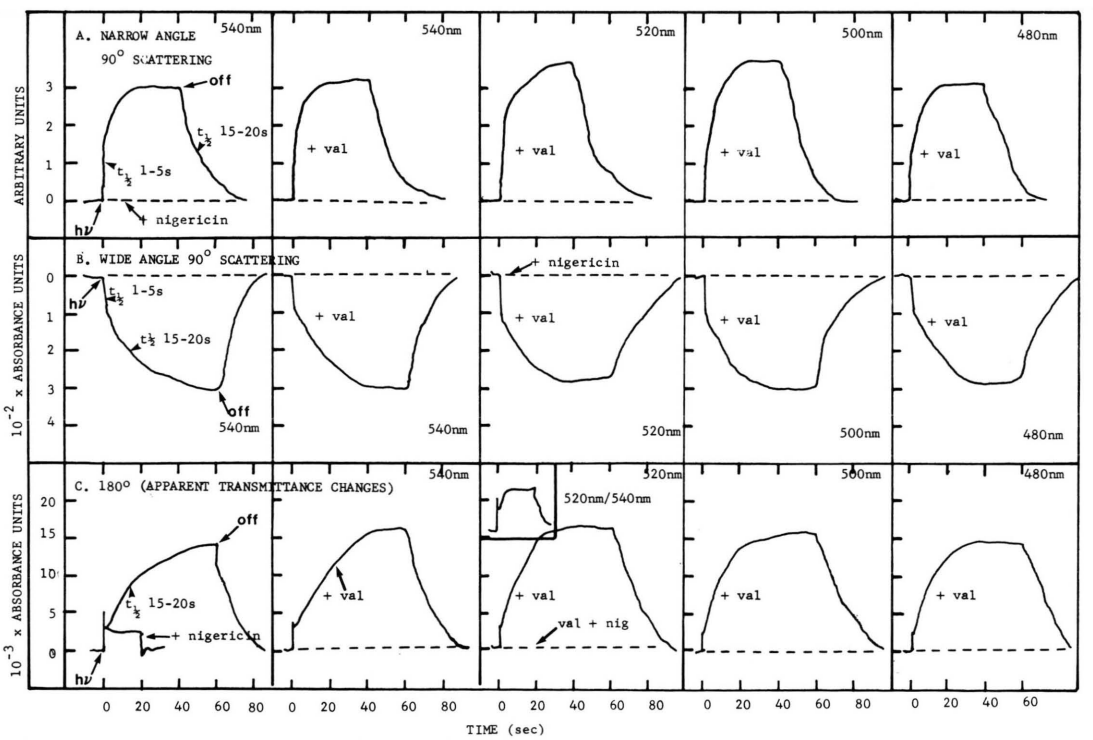

Fig. 7. Time course and wavelength dependency of light induced scattering changes (90°, 180°). Where indicated 5×10^{-7} M nigericin and/or 5×10^{-7} M valinomycin when added in the dark 1 min before the onset of illumination. The inset shows the apparent transmittance changes in the dual wavelength mode (520 nm/540 nm) in the absence of inhibitors. A. Light induced narrow angle 90° scattering changes; B. light induced wide angle 90° scattering changes; C. light induced transmittance (180°) changes.

Table I. The effect of photosynthetic electron transport inhibitors, uncouplers and energy transfer inhibitors on 90° and 180° scattering changes.

		90° change [%]	180° change [%]
Control		100	100
$H_2O \rightarrow MV$ (basal) ($+P_i$, ADP coupled)		100	100
Uncouplers			
GM_D	5×10^{-6} M	20	20
NM_4Cl	5 mM	0	0
GM_D+	NH_4Cl	0	0
FCCP	5×10^{-6} M	25	25
(val	10^{-6} M)	(100)	(100–150)
nig	10^{-6} M	0	0
Electron transport inhibitors			
DCMU	5×10^{-6} M	0	0
DBMIB	5×10^{-6} M	0	0
DNP-INT	5×10^{-6} M	10	10
Energy transfer inhibitors			
DCCD	5×10^{-6} M	100	100
Dio9	1 μg ml^{-1}	100	100
TBT	5×10^{-6} M	100	100
Cibacron blue	5×10^{-6} M	100	100
Partial reactions			
$H_2O \rightarrow$ DAD/FeCy (5×10^{-6} M DBMIB)		80	80
DCPIP/Asc → MV (5×10^{-5} M DCMU)		50	50

Rates of electron transport $H_2O \rightarrow MV$ basal 20, coupled 80, uncoupled 200 μmol O_2 h^{-1} mg Ch^{-1} $H_2O \rightarrow$ DAD/FeCy 100. All values mean ($n = 3$). S.D. $< \pm 10\%$ DCPIP → MV 300.

gradient formation and ATP synthesis on the 90° and 180° scattering components. In general, anything affecting proton pumping (uncouplers and electron transport inhibitors) inhibited the scattering responses. Energy transfer inhibitors had no affect, or slightly increased the scattering responses. Phosphorylating conditions also had no affect on the scattering responses. Partial electron transport reactions that included a protolytic step also included scattering changes.

Discussion

The study of the light scattering properties of biological particles has been pioneered by Latimer [16, 17], and has been extensively applied to higher plant thylakoids by Thorne and coworkers [15, 18, 19]. Provided the chlorophyll concentration is kept low enough (below about 20 μg Chl ml^{-1}) to approximate to single selective scattering a quantitative analysis is possible [16, 17]. Thorne has explained the scattering spectra obtained by the phenomena of selective dispersion which is caused primarily by the carotenoid pigments in the region 500–550 nm [18]. The dark level of light scattering of a thylakoid suspension is now known to be correlated to the degree of granal stacking and, together with fluorescence yield measurements, can be used to monitor the degree of restacking of unstacked thylakoids on the addition of an appropriate ion concentration [25, 26]. The changes in scattering intensity of thylakoids produced by illumination have also been interpreted by Thorne and coworkers as caused by changes in selective dispersion induced by conformational changes of membrane components caused by protonation of the intrathylakoid lumenal space.

In the presented report we have further characterized the light induced scattering responses of thylakoids at 90° and 180°. The two components show many features in common including similar difference spectra, ionic (Mg^{2+}) requirements and sensitivity to inhibitors. They can, however, be separated kinetically by wide angle 90° scattering, and also by 180° measurements in the dual wavelength mode where the slow scattering changes are subtracted out leaving a fast rise component equivalent to the 90° scattering change [15]. In full agreement with previous workers [3, 5, 15] we conclude that the narrow angle 90° scattering component is a direct reflection of the light induced proton pumping across the membrane. The origin of the 180° component is still not clear but probably involves an element of change of the degree of granal stacking as reported from the ultrastructural work of Murakami and Packer and Miller and Nobel [13, 14]. Support for this hypothesis has come from recent experiments on the correlation between the reconstitution of proton pumping and light induced scattering changes by DCCD treatment of thylakoids physically uncoupled by removal of the CF_1 part of the coupling factor complex [26].

Acknowledgements

We would like to thank Professor Dr. Ulrich Heber for helpful discussions. This research was supported by the award of an Alexander von Humboldt Stiftung to SJC, and by the DFG.

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