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ANALYSIS OF CHLOROPHYLL FLUORESCENCE QUENCHING BY DBMIB AS A MEANS OF INVESTIGATING THE CONSEQUENCES OF THYLAKOID MEMBRANE PHOSPHORYLATION

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The effect of Mg^{2+} concentration and phosphorylation of the light harvesting chlorophyll *a*/*b* protein on the ability of DBMIB to quench chlorophyll fluorescence of isolated pea thylakoids has been studied. Over a wide range of Mg^{2+} concentrations (5–0.33 mM), the observed changes in fluorescence yield are mirrored by similar changes in the quenching ability of DBMIB, indicating that the cation-induced phenomenon involves alterations in radiative lifetimes. In contrast, phosphorylation at 10 mM Mg^{2+} brings about a lowering of the chlorophyll fluorescence yield, while having no effect on the quenching capacity of DBMIB. This result can be interpreted as a phosphorylation-induced decrease in PS II absorption cross-section. At Mg^{2+} levels between 5 and 1 mM, phosphorylation leads to a change in the quenching of fluorescence by DBMIB, when compared with non-phosphorylated thylakoids. At these cation levels, the degree of DBMIB-induced quenching cannot wholly account for the observed changes in chlorophyll fluorescence due to phosphorylation. It is concluded that the phosphorylation- and Mg^{2+}-induced changes in fluorescence yield are independent but inter-related processes which involve surface charge screening as emphasised by the change in cation sensitivity of the DBMIB quenching before and after phosphorylation.

Introduction

The ability of oxygen-evolving photosynthetic organisms to regulate excitation energy distribution between the two photosystems to maintain maximal photosynthetic efficiency under varying light qualities was first demonstrated by Bonaventura and Myers [1]. Initially, it was believed that this regulatory process was controlled in some way by light-induced ionic movements within the chloroplast [2]. During the past few years, however, it has been argued that it is the reversible phosphorylation of LHC [3–6] which brings about the in vivo redistribution of excitation energy. The change in the surface electrical charge characteristics of the phosphorylated species is thought to cause the lateral movement of a mobile pool of LHC [7,8] from the appressed to the non-appressed membrane regions where it then acts as a Photosystem I (PS I) antenna pigment. Recent work has utilised the analysis of room temperature chlorophyll fluorescence induction curves in DCMU-inhibited thylakoids [9,10] to investigate the above concept. From these studies, a cation

Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PS, Photosystem; LHC, light harvesting chlorophyll *a*/*b* protein; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl-glycine.

dependence on the extent of the fluorescence change due to LHC phosphorylation shows characteristics which are expected for a process under electrostatic control [11]. It was concluded that at high salt levels, phosphorylation mainly affects the absorption cross-section of Photosystem II (PS II), while at lower cation concentrations, the observed additional fluorescence quenching was probably a consequence of increased spillover from PS II to PS I [10]. The data reported below provide further evidence supporting these previous conclusions. In this study, we have compared the ability of the quinone, DBMIB, to quench chlorophyll fluorescence from phosphorylated and non-phosphorylated thylakoids suspended in a media containing different levels of Mg^{2+}. This approach, recently used by Jennings and co-workers [13], allows a distinction between chlorophyll fluorescence quenching due to changes which bring about decreases in radiative lifetime (e.g., spillover) or due to alterations in absorption cross-section.

Materials and Methods

Chloroplasts were prepared from pea leaves as described previously [12]. To investigate the effect of protein phosphorylation, thylakoid membranes were prepared as follows. Intact chloroplasts were osmotically shocked and suspended in buffer to give the following final concentrations: 30 μg chlorophyll/ml, 10 mM Tricine (pH 8.2, KOH), 0.33 M sorbitol, 0.5 mM $MgCl_2$, 0.5 mM NADPH, 4.2 μM *Spirulina maxima* ferredoxin, 10 mM NaF and 150 μM ATP were added to produce the phosphorylated samples while non-phosphorylated samples had no ATP added. The thylakoids were incubated in the dark for 30 min to allow phosphorylation to occur before being diluted as described previously [10] for measurement. To investigate the effect of Mg^{2+} levels on the fluorescence yield, samples were prepared as described previously [10]. Chlorophyll fluorescence was measured at room temperature by illuminating the samples with blue-green light transmitted by a 4 mm Schott BG18 glass filter (intensity 1.3 $W \cdot m^{-2}$) and the resulting signal was detected via a photomultiplier protected with a Balzer 686 nm interference filter. DBMIB titrations were accomplished by the sequential addition of 0.67 μM DBMIB to continually stirred samples in the presence of 20 μM DCMU.

Results

Titrations of the chlorophyll fluorescence of thylakoids suspended in different Mg^{2+} levels with sequential additions of DBMIB are shown in Fig. 1. The graphical representation presented has been discussed by Jennings et al. [13], except we have used modified symbols to designate the maximum fluorescence level before (F_m) and after (F_n) n additions of DBMIB. As explained in detail by Jennings et al. [13], the intercept of the y-axis, at $(F_m - F_n)/F_m = 0$, gives a value which is directly proportional to the extent of the interaction of DBMIB with PS II and for convenience is called the interaction index. It can be seen that at high cation levels where spillover is at a minimum [2,7], the ability of DBMIB to quench fluorescence is greatest, while at minus Mg^{2+} where spillover is at a maximum [2,7], the quenching due to DBMIB is at its lowest. This observation confirms the data previously reported by Jennings et al. [13,14]. From Fig. 1 it can be seen that between 5 and 0.33 mM Mg^{2+}, the decrease in the interaction index is large, while further lowering of the cation level below 0.33 mM produces only small changes. It is

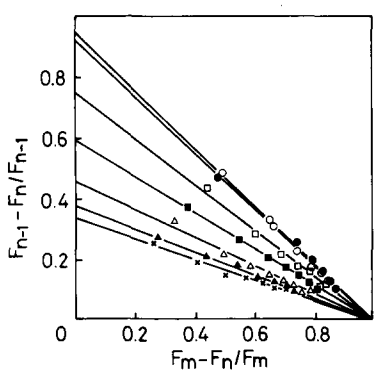

Fig. 1. Titration of chlorophyll fluorescence from thylakoids incubated at different Mg^{2+} concentrations with successive additions of 0.67 μM DBMIB. The $MgCl_2$ concentrations (in mM) are as follows; 5 (○), 2 (●), 0.67 (□), 0.5 (■), 0.33 (△), 0.17 (▲) and 0 (×). F_m, fluorescence level before DBMIB addition; F_n, fluorescence after n additions of quencher. The lines are the least-squares fit to all the experimental datum points.

TABLE I

THE EFFECT OF $MgCl_2$ LEVEL ON MAXIMAL CHLOROPHYLL FLUORESCENCE (F_m) and PS II-DBMIB INTERACTION INDEX

[$MgCl_2$] (mM)	F_m	Interaction index	I_{max}/I_c [a]	F_{max}/F_{mc} [b]
5	64.0	0.95	1.00	1.00
2	64.0	0.92	1.03	1.00
0.67	49.5	0.75	1.27	1.29
0.5	41.1	0.60	1.58	1.56
0.33	32.8	0.46	2.06	1.99
0.17	31.8	0.38	2.50	2.01
0	28.4	0.34	2.79	2.25

[a] I_{max}/I_c represents the ratio of interaction index at 5 mM $MgCl_2$ (I_{max}) with the interaction index at the other cation levels (I_c).
[b] F_{max}/F_{mc} represents the ratio of maximal fluorescence before DBMIB addition at 5 mM $MgCl_2$ (F_{max}) with the maximal fluorescence at the other cation levels (F_{mc}).

at these low Mg^{2+} levels where a decrease in the initial fluorescence level (F_0) of induction curves was observed previously [10]. Using the simple bipartite model [13], it is predicted that the ratio of interaction indices should equal the ratio of the fluorescence yields for any particular pair of conditions. This relationship is respected between 5 and 0.33 mM Mg^{2+}; however, below this concentration, a greater change is seen in the interaction index when compared with fluorescence yield changes (see Table I). This deviation from the simple relationship is probably brought about as a result of decreased coupling between the PS II core complex and LHC which is believed to occur at very low Mg^{2+} concentrations [10,14,15] and hence lead to a deviation from the assumed bipartite model.

The ability of DBMIB to quench chlorophyll

TABLE II

THE EFFECT OF LHC PHOSPHORYLATION OF THYLAKOID MEMBRANES ON MAXIMAL CHLOROPHYLL FLUORESCENCE (F_m) AND PS II-DBMIB INTERACTION INDEX

[$MgCl_2$] (mM)	Phosphorylation treatment	F_m	Interaction index	I_{nphos}/I_{phos} [a]	F_{nphos}/F_{phos} [b]
10	–	72.0	0.92	1.00	1.16
	+	62.2	0.92		
5	–	72.5	0.90	1.07	1.25
	+	57.8	0.84		
2	–	73.0	0.92	1.30	1.54
	+	47.2	0.71		
1	–	55.0	0.70	1.67	1.67
	+	32.9	0.42		
0	–	30.5	0.33	1.00	1.18
	+	25.8	0.33		

[a] I_{nphos}/I_{phos} represents the ratio of interaction index of non-phosphorylated (nphos) and phosphorylated (phos) samples at the same $MgCl_2$ concentration.
[b] F_{nphos}/F_{phos} represents the ratio of maximum fluorescence yield of non-phosphorylated and phosphorylated samples at the same $MgCl_2$ concentration.

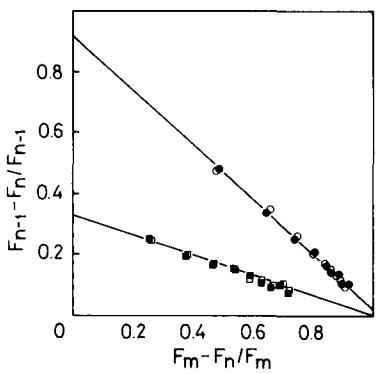

Fig. 2. Titration of fluorescence from thylakoids incubated at 10 mM $MgCl_2$ under phosphorylating (●) and non-phosphorylating (○) conditions and thylakoids resuspended at 0 mM Mg^{2+} under phosphorylating (■) and non-phosphorylating (□) conditions. For 0 mM, the thylakoids were phosphorylated at 10 mM $MgCl_2$ and resuspended in the presence of 1 mM EDTA to remove all $MgCl_2$. The lines are the least-squares fit to all the experimental datum points.

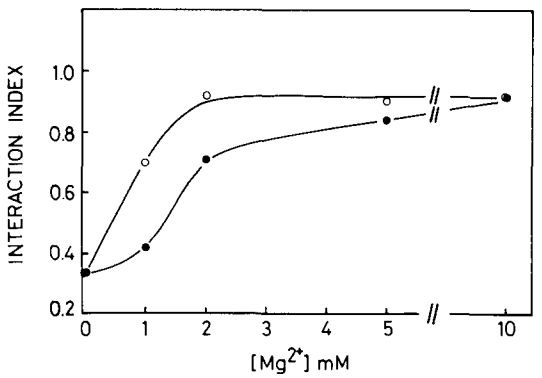

Fig. 4. The interaction index of phosphorylated (●) and non-phosphorylated (○) thylakoids as a function of background $MgCl_2$ level.

fluorescence in thylakoids phosphorylated at 10 mM $MgCl_2$ which have then been resuspended at either 10 mM Mg^{2+} or allowed to unstack in the absence of Mg^{2+} is shown in Fig. 2. It can be seen that in both cases, there is no difference in the quenching capacity of DBMIB between the phosphorylated and non-phosphorylated samples, although the maximal fluorescence yield (F_m) has been attenuated by approximately the same degree in both cases (see Table II). This suggests that the decrease in F_m brought about by phosphorylation is not due to changes in radiative lifetime but is a result of a decrease in PS II absorption cross-section; a process which will not affect the quenching ability of DBMIB. Using similar arguments, Gerola et al. [16] came to the same conclusion about the effect of phosphorylation at a high Mg^{2+} level (10 mM). However, from Fig. 3 it can be seen that LHC phosphorylation can lead to changes in the ability of DBMIB to quench fluorescence and that the extent is dependent on the background cation levels present. Fig. 3a shows that at 5 mM Mg^{2+}, there is now a small decrease in the interaction index. This suggests that at this relatively high Mg^{2+} concentration, protein phosphorylation can lead to small increases in spillover or perhaps other non-radiative decay processes. At lower Mg^{2+} levels (2 and 1 mM), the large decreases in fluorescence due to phosphorylation are mirrored by large decreases in the interaction index when comparing phosphorylated with non-phosphorylated samples (see Fig. 3b, c). From Table II it can be seen that the change in maximal fluorescence before DBMIB addition (F_m) brought about by phosphorylation is not equal to the changes in interaction index. This indicates that a proportion of the fluorescence decrease obtained by phosphorylation is via a process which is not in competition with DBMIB quenching and therefore seems to reflect changes in absorption cross-section of PS II.

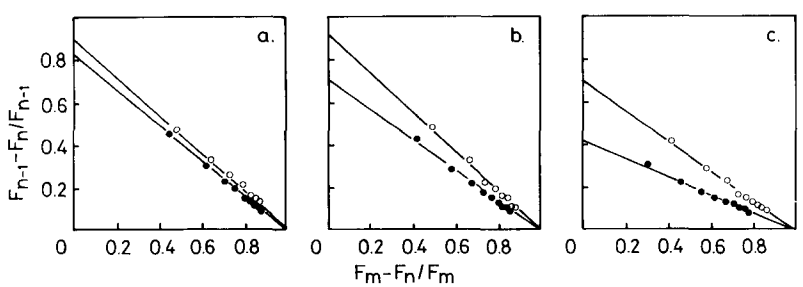

Fig. 3. Titration of fluorescence of thylakoids incubated under phosphorylating (●) and non-phosphorylating (○) conditions with successive additions of 0.67 μM DBMIB at (a) 5 mM $MgCl_2$ (b) 2 mM $MgCl_2$ and (c) 1 mM $MgCl_2$. The lines are the least-squares fit to all the experimental datum points.

Fig. 4 shows the relationship of interaction index with cation level in phosphorylated and non-phosphorylated samples. It can be seen that the Mg^{2+} requirement to bring about the same level of PS II-DBMIB interaction is greater after phosphorylation. The $C_{1/2}$ for non-phosphorylated membranes being 0.8 mM $MgCl_2$ while the $C_{1/2}$ after phosphorylation is 1.7 mM $MgCl_2$.

Discussion

The results presented in this report emphasise that the cation-induced changes in excitation energy distribution are different from the effect of LHC phosphorylation. On lowering the cation-level from 5 mM Mg^{2+} to 0.33 mM Mg^{2+}, the decrease in fluorescence can be explained by increases in spillover from PS II to PS I and other non-radiative decay processes brought about by thylakoid membrane unstacking and lateral protein reorganisation caused by a decreased screening of negative membrane surface charge [11]. Below 0.33 mM Mg^{2+}, there are small changes in chlorophyll fluorescence which do not follow the decrease in interaction index, the interpretation of which is not as yet clear but may involve partial decoupling of LHC from the PS II core complex.

The effect of LHC phosphorylation on chlorophyll fluorescence can be partly explained in terms of changes in the absorption cross-section of PS II. According to the analyses of DBMIB-induced quenching, the absorption cross-section change occurs at all the cation levels investigated and is probably a consequence of the movement of a loosely coupled pool of phosphorylated LHC from the appressed to the non-appressed regions of the thylakoid [8]. It has been suggested that this movement occurs as a consequence of changes in surface charge density [11]. Therefore, it is expected that the fluorescence changes due to phosphorylation will be sensitive to the level of cations present. At 10 mM $MgCl_2$, the decrease in chlorophyll fluorescence can be accounted for by a change in PS II absorption cross-section alone. However, at 5 mM $MgCl_2$, only 65% of the total fluorescence decrease can be attributed to such a change, while an additional quenching is also observed which competes with the DBMIB-induced quenching, and probably reflects an increase in spillover from PS II to PS I. At low Mg^{2+} levels, this additional component becomes more pronounced, as indicated by the significant lowering of the interaction indices. Under these lower cation levels, the phosphorylation process brings about a substantial decrease in membrane stacking comparable with those observed in low-salt media [17]. Therefore, it is likely that this additional component represents a phosphorylation-induced increase in spillover resulting from a randomisation of pigment-protein complexes. However, when phosphorylated thylakoids are resuspended in the absence of $MgCl_2$ (see Fig. 2), a 15% lowering in F_m is still observed when compared to the non-phosphorylated samples. This decrease is attributed to the same affect which is seen when phosphorylated samples are retained at 10 mM $MgCl_2$. This confirms the previous conclusions of Telfer et al. [10] and Horton and Black [18] that the cation and phosphorylation effects are two independent processes.

From the fluorescence and interaction index ratios shown in Tables I and II, it can be calculated that there has been approx. 13% quenching of chlorophyll fluorescence as a result of LHC phosphorylation, bringing about a decrease in the PS II absorption cross-section. This can clearly be seen at all Mg^{2+} levels, except 1 mM, at which a correction has been made to take into account the deviation from the simple relationship between interaction index and fluorescence yield.

As seen in Fig. 4, the $C_{1/2}$ for the change in interaction index is approximately doubled by phosphorylation. This suggests that phosphorylated thylakoids require the presence of higher Mg^{2+} levels to maintain a similar state of membrane organisation compared with the non-phosphorylated membranes, as would be expected for a mechanism involving alterations in electrical surface charge.

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