The significance of the kinetic analysis of fluorescence induction in DCMU-inhibited chloroplasts in terms of photosystem 2 connectivity and heterogeneity

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A study has been made on the slow component (β_{max}) of chlorophyll fluorescence induction curves exhibited by DCMU-inhibited pea thylakoids. In the absence of high levels of screening cations, β_{max} is high and the fluorescence yield low, while the addition of K^+, Mg^{2+} or Tris(ethylenediamine) cobaltic(III) cation (TEC^{3+}) decreases β_{max} and increases fluorescence yield. These changes are inhibited when the thylakoids are fixed with glutaraldehyde. By comparing cation- and light-harvesting chlorophyll *a/b*–protein (LHCP) phosphorylation-induced changes it can be seen that β_{max} correlates with changes in the overall kinetics of photosystem (PS) 2 photoreduction, as indicated from the normalised area over the induction curve (Anorm). When the plastoquinone (PQ) pool is chemically reduced by dithionite, prior to the initiation of the curve, both F_o and F_m are increased and the slow component removed. These observations are used to question the concept of two structurally distinct PS2 centres [Arch. Biochem. Biophys. (1978) 190, 523–530] and to discuss the use of β_{max} in monitoring PS2 organisation.

Photosynthesis *Chlorophyll fluorescence* *Induction curve* *Biphasic kinetics* *Membrane organisation* *Structural heterogeneity*

1. INTRODUCTION

Room temperature chlorophyll fluorescence induction curves measured with DCMU-poisoned chloroplasts reflect the photoreduction of the photosystem two (PS2) primary electron acceptor Q [1]. The curve is not indicative of a single first-order photochemical event. In the presence of sufficient salt to facilitate membrane appression, the major part of the curve is sigmoidal which is followed by an extended slow phase. This biphasic nature can readily be detected by analysing the normalised area growth above the curve [2,3] and has been attributed to two forms of PS2 [2,4] termed α- and β-centres. By using semilog plots and assuming that the slow component is exponential, it has been possible, by extrapolating this phase to zero time, to determine the relative contribution of the β-centres (β_{max}).

The differences between the two PS2 centres have been widely investigated. Authors in [5], using membrane fractionation techniques, have shown that only β-centres are found in stromal membrane fragments, while granal lamellae are enriched in α-centres. Using tobacco mutants, which show different ratios of the two centres, it has been found that β-centres have less light-harvesting antenna pigments [6] which are composed of chlorophylls showing different absorption characteristics to those associated with α-centres [7]. Also from these studies it was shown

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea; LHCP, light-harvesting chlorophyll *a/b*–protein complex; PS, photosystem; Q, primary acceptor of photosystem 2; F_m, maximum fluorescence; F_o, initial fluorescence; F_v, $F_m - F_o$; A_{max}, unnormalised area over induction curves; A_{norm}, normalised A_{max}

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that a relative increase in β-centres could be correlated with decreasing amounts of appressed membranes [8]. Further studies have shown other important differences which include an absence of the herbicide binding protein, B, associated with β-centres [9] and the insensitivity of β-centre chlorophyll fluorescence to changes in Mg^{2+} concentration [10]. Differences in the redox properties on the acceptor side of α- and β-centres (Q_α and Q_β, respectively) have also been reported [11,12]. The E_m for Q_β has been found to be +120 mV [13] but this observation does not clearly fit with previous redox titrations of chlorophyll fluorescence which indicate two quenchers with E_m-values of −250 mV and 0 mV [14].

In contrast to the interpretation of [3,5], in [15] (using PS2 particles from the blue green alga *Phormidium laminosum*) it was suggested that to obtain heterogeneity in the induction curve does not require either the presence of appressed and non-appressed membranes nor differences in antenna size. The explanation in [15] is that the presence and extent of the two phases are a consequence of differing degrees in PS2 connectivity. Moreover, other observations such as changes in α- and β-levels brought about by either phosphorylation of the light-harvesting chlorophyll *a/b*–protein complex (LHCP) or by differences in cation levels [16,17] do not support the idea of two distinct structural forms of PS2 as suggested in [5].

Here, we further investigate the interpretation of the α- and β-centre concept and conclude that the heterogeneity is a consequence of an incomplete DCMU-blocking of PS2 centres.

2. MATERIALS AND METHODS

Intact chloroplasts were prepared from pea leaves as in [18] and resuspended as a concentrated stock in 0.33 M sorbitol (pH 7.5, Tris) and 3 mM $MgCl_2$. The chlorophyll concentration was determined as in [19].

To investigate the effect of cations, thylakoids were prepared as follows. Intact chloroplasts were subjected to an osmotic shock at twice the final cation concentration for 15 s and finally resuspended to give the following final concentrations: 5 μg chl/ml, 10 mM tricine (pH 8.2, KOH), 0.33 M sorbitol and either 5 mM Mg^{2+}, 0.1 mM Tris(ethylenediamine) cobaltic(III) cation (TEC^{3+}), 100 mM K^+ or 5 mM K^+. All samples were dark-adapted for 4.5 min, 20 μM DCMU was then added and fluorescence measured after a total dark time of 5 min.

Gluteraldehyde fixation was used to investigate the requirement for membrane reorganisation in bringing about changes in β_{max}, as well as to investigate the reported Mg^{2+} dependence on α-centre fluorescence. Shocked chloroplasts were resuspended as above in either 1 mM $MgCl_2$ or 5 mM KCl and allowed to undergo membrane reorganisation for 5 min before the addition of 1% (v/v) gluteraldehyde. After a further 4 min incubation to fix the membranes, 5 mM $MgCl_2$ was added to some of the low cation samples and after 5 min 20 μM DCMU was added and fluorescence was measured. Control samples were those incubated in the absence of gluteraldehyde for a total dark time of 14 min.

The magnesium and phoshorylation induced changes in the slow phase, as seen in fig.1, were performed as in [16].

To investigate the effect of the redox state of the plastoquinone (PQ) pool on the induction curve, chloroplasts were shocked as in the cation experiments and resuspended in the same medium containing 5 mM Mg^{2+}. 20 μM DCMU was introduced after 4 min dark incubation followed by

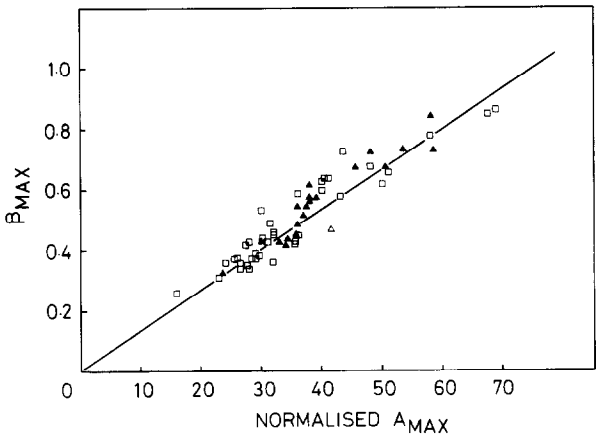

Fig.1. The correlation of the slow phase (β_{max}) with the normalised A_{max}. The data were obtained by either cation-induced (—□—) or LHCP phosphorylation-induced (—▲—) fluorescence changes. The line is the least squares fit to the experimental points with a correlation coefficient (r) of 0.94.

2.4 mM sodium dithionite (freshly prepared for each sample at a concentration seen to reduce PQ). Illumination proceeded after a further 2 min dark time.

All induction curves were initiated, monitored and analysed as in [16]. The analysis was by the method in [3].

3. RESULTS

Table 1 shows that the level of screening cations influences the β_{max}-value and the fluorescence yield. It can be seen that low β_{max}-values are obtained when thylakoids are suspended in the presence of any cation at sufficient concentrations which allow membrane appression. However, when in a low cation medium, which facilitates thylakoid unstacking and the lateral reorganisation of protein complexes [20] the β_{max}-value increases and the fluorescence yield diminishes. Table 2 shows that when unstacked thylakoids are fixed by gluteraldehyde, the subsequent addition of Mg^{2+} does not lead to a change in β_{max} nor to an increase in fluorescence yield. These results support the concept that the changes in β_{max} reflect an alteration within thylakoid arrangement and that this reorganisation also leads to the change in fluorescence yield.

Fig.1 shows that the β_{max}-values obtained from thylakoids which have undergone thylakoid reorganisation by either changes in cation levels or by LHCP phosphorylation can be correlated with the normalized area above the induction curve (A_{norm}). This area reflects the time required to reduce all of Q and therefore gives an overall picture of the kinetics of photoreduction [21]. As it is postulated that these two processes probably involve different changes within PS2 organisation [16,22] it is difficult to explain β_{max} being due to a specific form of PS2 centre as in [3,10].

Table 1

Effect of cations on β_{max} and relative fluorescence yield

Condition	β_{max}	Relative fluorescence yield
0.1 mM TEC^{3+}	0.45	120
5 mM Mg^{2+}	0.45	126
100 mM K^+	0.51	100
5 mM K^+	0.83	67

Table 2

The effect of gluteraldehyde fixation on β_{max} and relative fluorescence yield

Treatment			β_{max}	Relative fluorescence yield
1 mM Mg^{2+}	1% Gluteraldehyde	4 mM Mg^{2+}		
+	+	−	0.52	89.0
−	+	−	0.74	75.0
−	+	+	0.74	76.0
+	−	−	0.46	118.0
−	−	−	0.73	82.0
−	−	+	0.46	107.0

Table 3 indicates that various induction curve parameters can be altered if the PQ pool is reduced prior to illumination. Under these reducing conditions the induction curves show an increase in both initial and maximal fluorescence levels so that the ratio F_v/F_m remains unchanged. Also with dithionite present, the unnormalised area (A_{max}) becomes lower indicating that less quanta are now required to photoreduce Q, and the slow phase which is used to detect β_{max} is removed. If the curves are analysed as before, it can be seen from fig.2A that the biphasic nature of the log plot is drastically reduced and that the kinetics of Q photoreduction are significantly increased as shown by the area growth plots in fig.2B.

Table 3

The effect of PQ reduction on induction curve fluorescence parameters

Condition	F_o	F_m	A_{max}	F_v/F_m	β Phase
+ Dithionite	33	98.4	1795	0.67	Absent
− Dithionite	27	84.4	2750	0.68	Present

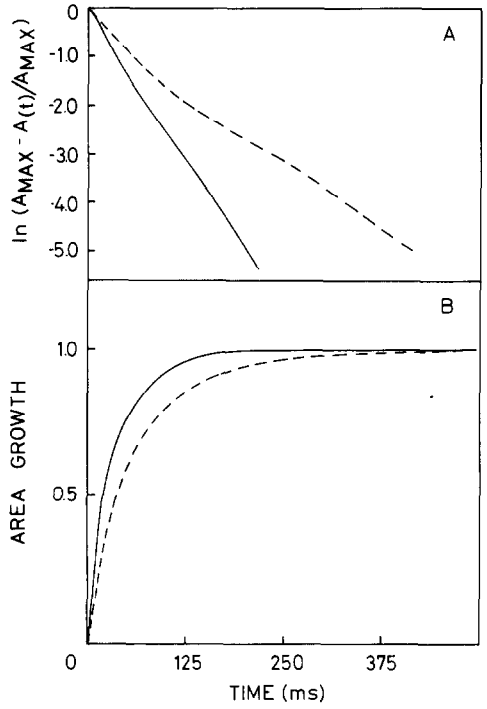

Fig.2. The effect of the presence (——) and absence (– – –) of sodium dithionite on: (a) the $\ln(A_{max} - A(t)/A_{max})$, and (b) the growth of the normalised area over the induction curve (this being representative of Q reduction). The concentration of dithionite used was such that the PQ pool was reduced before and during illumination (not shown).

4. DISCUSSION

These data suggest that the biphasic nature of room temperature fluorescence induction curves from DCMU-poisoned chloroplasts is not due to two distinct structural PS2 centres as proposed in [3,10].

Tables 1 and 2 emphasize that the relative extent of β_{max} only changed when the membranes were allowed to unstack [18]. The involvement of membrane organisation in the size of the slow phase as measured by β_{max} is shown in table 2. It can be seen that changes in β_{max} are inhibited when the membranes have been fixed with gluteraldehyde. Therefore, by measuring the β_{max} -value an indication of PS2 organisation within the thylakoid membrane can be obtained. However, do these changes reflect the conversion of α-centres into β-centres? Using absorption difference spectroscopy at 320 nm [10], it was suggested that the absolute numbers of α- and β-centres did not change on adding Mg^{2+}. This result, therefore, does not correlate with the chlorophyll fluorescence analysis and they have suggested that the discrepancy is due to an Mg^{2+}-induced change in the emission yield of α-centres. Tables 1 and 2, however, indicate that there is no specific Mg^{2+} effect and that the changes in the fluorescence induction profile probably reflect alterations in membrane organisation (including stacking) which are thought to control the transfer of energy between PS2 and PS2 [20]. Does the biphasic nature of the induction curve represent two distinct forms of PS2 centre? From fig.1 it seems hard to accept such an idea if the concepts of the cation and LHCP phosphorylation-induced membrane changes are valid (see [16,20,22]). Both effects produce changes in β_{max} which lead to a similar effect on the kinetics of Q photoreduction. As these two processes are thought to alter PS2 organisation in different ways it is hard to associate the slow phase with such a specific structural heterogeneity within PS2 centres.

Is there an alternative explanation? Table 3 shows the changes in the induction curves measured when the PQ pool is reduced. It is already well-known that chemical reduction leads to a higher fluorescence yield than that seen when the system is photoreduced. Table 3 shows that the initial fluorescence (F_o) is also altered so that the F_v/F_m ratio remains constant. The A_{max}-value also decreases indicating that less quanta are required to photoreduce Q. The result also seems to indicate that oxidised PQ quenches not only F_m but F_o as well. Fig.2A shows that when PQ is fully reduced the biphasic nature of the log area growth plot is suppressed, the rate of Q reduction is accelerated and the slow phase is removed (fig.2B, table 3). This suggests that the slow phase might be due to a leakage through the DCMU block so that when PQ is reduced by the presence of dithionite it is unable to reoxidise any Q, and therefore Q reduction is faster. Such a conclusion is supported by work carried out on the primary acceptors of PS2. Redox titrations of fluorescence have shown two components which have been called Q_L and Q_H with E_m-values of about −250 mV and 0 mV, respectively [11,16]. However, the redox titration

of the slow phase has been shown to have an E_m of +120 mV [13]. This value is consistent with the involvement of PQ in the manifestation of the slow phase. Authors in [23], while investigating PS2 electron acceptors, found that Q_β did not show a C550 signal, nor did it participate in a transmembrane electron transfer. An explanation put forward was that Q_β did not exist as a primary acceptor but arose from another source like a secondary acceptor or a modification in DCMU-binding. It has been reported [24] that chloroplast intactness changed the extent of the slow component and the slow rise in variable fluorescence concluded to reflect PS2 centres with a low affinity to DCMU.

Therefore, the two components detected by chlorophyll fluorescence induction may only be a consequence of the existence of some PS2 centres which are not easily blocked by DCMU rather than due to the presence of the two specifically different forms of PS2 suggested in [10]. If the number of α- and β-centres remain unchanged as argued in [10], then the various α- and β-centre ratios brought about by various conditions (such as changes in cation level, LHCP phosphorylation, tobacco mutants and membrane fragments) could all be explained by an alteration in the fast component's kinetics due to differences in PS2 connectivity. These differences could be brought about by a number of factors including disconnection of the LHCP from the PS2 core, or by differing degrees of PS2–PS1 energy transfer. By the nature of the analysis such a change in the fast component would mean that the β_{max}-value obtained would be altered. Therefore, based on this conclusion, the analysis as it stands is still a useful tool when investigating PS2 organisation, but we suggest that the concept of two different structural entities of the type proposed in [3,5,10] is probably unlikely.

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