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Analysis of fluorescence induction curves from pea chloroplasts. Photosystem II reaction centre heterogeneity

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The fluorescence induction kinetics of DCMU-blocked pea chloroplasts were determined under various redox conditions. At ambient redox potentials the usual rapid kinetics were observed, followed by further slow changes. These slow changes had significant effects on a determination of the complementary area over the induction curve, and hence on the proportion of α to β centres determined as in Melis and Homann (Melis, A. and Homann, P.H. (1975) Photochem. Photobiol. 21, 431–437). An increase as small as 2% in the ratio of the maximum variable fluorescence to the maximum total fluorescence ($F_{v\,max}/F_{max}$) resulted in an increase in the area over the induction curve of over 150%. Furthermore, determinations of the proportion of β centres were virtually impossible because of the nonlinear nature of a first-order plot of complementary area growth. At redox potentials less than 0–50 mV, a slow negative fluorescence change was observed after the initial fast rise. This quenching was found both in the presence and absence of redox mediators, and made determinations of F_{max} difficult. Various attempts to eliminate this quenching met with only moderate success. It is concluded therefore that accurate studies of PS II heterogeneity based on area growth determinations suffer from an inability to determine F_{max} precisely.

Introduction

The induction of chlorophyll fluorescence from dark-adapted DCMU-blocked chloroplasts has been used extensively to monitor changes associated with the primary reactions of Photosystem II [1]. Analysis can provide information of two types. First, the fluorescence levels attained on first illuminating dark-adapted chloroplasts (F_0) and the maximum fluorescence level attained (F_{max}) provide an indication of the efficiency with which the trapped light energy is used in the photosystem [2–4]. Secondly, calculations involving the kinetics of the rise in fluorescence give information on the rate at which the primary acceptors become reduced. In recent years, an increasing effort has been made to categorise the electron acceptor (or acceptors) associated with the oxidation of the Photosystem II reaction centre chlorophyll P-680 (for a review, see Ref. 5). Because Photosystem II reaction chlorophyll fluorescence yield is related to the degree of reduction of the acceptor [6], many attempts have been made to relate the kinetics of fluorescence induction with those of the reduction of the acceptor.

Two lines of evidence have indicated that Photosystem II centres are heterogenous, or at least do not consist of a single centre functionally con-

Abbreviations: DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; PS, Photosystem; P-680, primary electron donor chlorophyll of PS II; Tricine, *N*-(2-hydroxy-1,1-bis(hydroxymethyl)ethylglycine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; Mops, 4-morpholinepropanesulphonic acid.

nected to a single electron acceptor [7]. Firstly, the fluorescence yield has been measured at various redox potentials. Upon lowering the redox poise, two components are found: the first (normally designated Q_H) has a midpoint potential of approx. 0 mV at pH 7.0, and the second (Q_L), a midpoint potential of -250 mV [8–11]. Both these values, and the proportion of the fluorescence that each acceptor quenches, are variable. Altering the redox poise affects other indicators of the photocentre as well: the membrane potential-indicating 515 nm carotenoid shift [12], the absorbance changes at 550 nm [13] as well as the fluorescence lifetime [14] are all sensitive to redox potential, as are EPR signals associated with pheophytin [15] and a Q-Fe complex [16].

Secondly, it is found that the kinetics of the variable fluorescence induction in a dark-adapted, DCMU-blocked chloroplasts are in general not first order, suggesting that the idea of an 'isolated' photosynthetic unit containing a single reaction centre and a single electron acceptor is untenable. The sigmoidicity of the induction curves has been interpreted in four ways: (a) each PS II reaction centre is able to reduce more than one acceptor [17,18]; (b) each PS II photosynthetic unit contains more than one reaction centre [19–22]; (c) sigmoidicity is a consequence of the rapid movement of excitations round a pigment bed containing many reaction centres [23]; and (d) the PS II centres are heterogenous [24].

A good deal of interest has been centred on this last model, in which the analyses are based on the deconvolution of the fluorescence induction kinetics (redrawn as the increase in the complementary area over the curve) into two phases, and it is assumed that each phase is attributable to one kind of PS II centre (α and β centres). In this terminology, the α centres give rise to the fast, sigmoidal rise in the fluorescence induction curve, and the β centres contribute to a slower, first-order phase [24].

It does not seem possible at present to confirm a direct correspondence between the α and β centres and Q_H and Q_L. Inasmuch as the β component of the fluorescence induction can be eliminated at redox potentials above those for the Q_H component [25] it seems likely that Q_H and Q_L are both associated with the α centres.

Both kinetic analyses and redox titrations have been widely used to study PS II reaction centre heterogeneity. Unfortunately, however, there is evidence to suggest that the reliability of both of these approaches needs further investigation [26–28]. This paper reports on analyses of the fluorescence induction curves, with close attention being given to the determination of F_{max} and the growth of the complementary area. The results, together with those on the effects of redox mediators, are discussed in terms of the analysis of chlorophyll fluorescence inductions in general, and the determination of α and β centres in particular.

Materials and Methods

Chloroplasts were isolated from 12–16 day-old pea seedlings grown in a growth chamber with 12 h per day of illumination at 1.5 mW/cm^2 from high-pressure mercury lamps, and at a temperature of 23°C. Approx. 30 g of pea shoots were ground for 6 s with a Polytron tissue homogeniser in 180 ml of an ice-cold solution containing 0.33 M sorbitol/0.005 M Na_2HPO_4/0.05 M $MgCl_2$/0.1% NaCl/0.2% sodium ascorbate (pH 6.5). After filtering through 12 layers of cheesecloth, the homogenate was centrifuged for 2 min at $1500 \times g$ and the chloroplast-containing pellet resuspended with an artist's brush in approx. 3 ml of a resuspension medium containing 0.2 M sucrose/0.01 M Tricine (pH 7.8)/0.01 M NaCl/0.005 M $MgCl_2$ at an approximate chlorophyll concentration of 1–2 mg/ml. The chloroplast stock was stored on ice in the dark until use.

Intact chloroplasts were prepared as described by Stokes and Walker [29]. With both intact chloroplasts and normal chloroplasts, the chloroplasts were osmotically shocked by dilution into 1.5 ml distilled water or a 100-fold dilution of the resuspension medium before being made up to the appropriate concentration with double strength resuspension medium.

Chloroplasts depleted of cations were prepared by resuspending the pellets in 180 ml of a solution containing 0.1 M sucrose/0.01 M NaCl/0.01 M Mops (pH 7.0), and leaving on ice and in the dark for at least 5 min. The chloroplasts were subsequently centrifuged for 3 min at $1500 \times g$ and the pellets resuspended in the normal resuspension

medium, but lacking $MgCl_2$. Cyanide-treated chloroplasts were prepared as described by Bell et al. [30], and assayed for the inhibition of non-cyclic electron transport (H_2O to methyl viologen, or H_2O to potassium ferricyanide) using a Rank oxygen electrode [31]. Only preparations that exhibited an inhibition of over 95% were used.

Redox mediator stock solutions were made up to 0.006 M in ethanol or water as appropriate. Indigotetrasulfonic acid was synthesised as in Juillard [32].

Fluorescence induction curves were measured in a laboratory-constructed fluorimeter [33]. The illumination was by broad-band blue light provided by a 150 W projector bulb and defined by a Balzers Calflex-C filter, an Oriel 70318-SWP 600 cut-off filter, a Corning 4-96 filter, and two BG-18 filters. The photomultiplier signal was normally stored in a transient recorder (Datalab Models DL901 or DL922) and transferred to a PDP 11/34 minicomputer (Digital Equipment Corp.) for analysis. For traces with acquisition periods exceeding 5 s and using a split-time base, the photomultiplier signal was fed directly into the computer through an analogue-to-digital converter.

For the analysis of the maximum fluorescence and the area accumulation (see Fig. 6 and Table II), the area increment was calculated for each of the 1024 points of data using the minicomputer. Corrections for the relative values due to split-time bases were made in the analysis programmes as appropriate. Values for F_{max} at the chosen time were determined as the mean of 51 points centered at the time selected.

Redox titrations were performed with the mediators indicated using one of two methods. In the first, the sample was made up in 3.0 ml in a 1.0 cm^2 cell that had been modified to take a combination electrode (EIL Analytical Instruments), a stream of oxygen-free N_2 gas, and a 3 mm magnetic stirring bar. Adjustments of the redox poise were made by microlitre additions of either 0.5 M sodium dithionite (in 0.5 M Tris buffer, pH 7.5) or 0.1 M potassium ferricyanide. Drifts in the redox potential were minimised by the use of a laboratory-constructed automatic redox-potential stabiliser which consisted of a motor-driven microlitre syringe controlled by the error signal between the output of the redox electrode amplifier (Electronic Instruments Model 7030) and a preset vlaue.

To minimise variations between samples and to reduce times of incubation and dark adaptation, a second method was sometimes used with similar results. In these cases, 60 ml of the reaction sample were placed in a light-tight glass vessel. The vessel lid incorporated a combinationa redox electrode and ports for the addition and removal of samples. Stirring and anaerobic conditions were effected by bubbling with N_2 gas. After the desired treatment, a 2 ml aliquot (of the sample) was removed from the vessel with a syringe and injected into the cell described above.

In experiments in which extra illumination was provided during the fluorescence induction, the illumination was provided by a He-Ne laser at an intensity sufficient to give an approx. 40% increase in the F_{max} level.

Results

Fig. 1 shows the induction of chloroplast fluorescence from dark-adapted, DCMU-blocked chloroplasts suspended in a 'high salt' medium

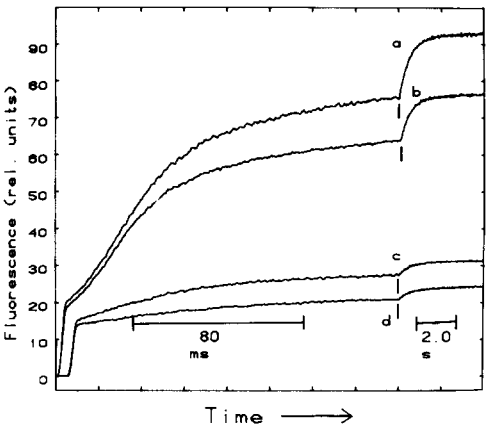

Fig. 1. Fluorescence induction curves in DCMU-blocked chloroplasts at ambient redox potential. Data were collected with a split time-base with the time values before and after the break as indicated on the figure. Light intensity was 820 $\mu W/cm^2$. Reaction mixture (3.0 ml) contained chloroplasts equivalent to 10 μg/ml; 0.1 M sucrose; 0.01 M Hepes (pH 7.0); 0.01 M NaCl; 33 μM DCMU; 5 μM gramicidin; and 0.005 M $MgCl_2$ (except trace d). Trace a, intact chloroplasts shocked immediately before the assay; trace b, normal chloroplasts; trace c, normal chloroplasts plus 10 μM 1,4-naphthoquinone; trace d, chloroplasts depleted of cations.

(trace a). The induction was sigmoid. The time base of the data acquisition was changed towards the end of the first-rise phase to demonstrate that there was a significant slow secondary rise that accounts for about 20% of the total signal. Etienne [34] observed a similar, but much smaller, secondary rise. Sigmoidicity of the induction was seen both when the chloroplasts were subjected to osmotic shock during the preparation (trace a) or immediately before the addition of DCMU (trace b). Shocking essentially intact chloroplasts into a low-cation medium eliminated the sigmoidicity as expected [22], but the secondary rise remained (trace d).

Other investigators have shown that upon a slight lowering of the redox potential of the chloroplasts (to 20–50 mV), the slow fluorescence rise can apparently be eliminated [25]. These potentials are above the midpoint potentials for the reduction of Q_H and Q_L [8,9]. In titrations of this type, lipophilic redox mediators are normally added to facilitate the equilibration of endogenous pools of membrane-bound electron carriers with the external medium. The problems associated with the addition of mediators have only recently been given serious consideration [9,26,28,35]. Trace c of Fig. 1 illustrates the effect of a relatively low concentration (10 μM) of a typical mediator, 1,4-naphthoquinone ($E_{m7.0} = +36$ mV), on the fluorescence induction at ambient redox conditions. Both the F_0 level and the total amount of variable fluorescence ($F_{v\,max}$) were smaller – in the latter instance, by 75–80%. The slow phase was still present, but the sigmoidicity was greatly reduced [22].

Lowering the redox potential to values sufficient to eliminate the slow phase [25] introduced a second effect: a slow quenching of the maximum fluorescence (Fig. 2). It has been proposed [28] that this slow quenching requires the presence of redox mediators. Table I and Fig. 2 show, however, that in the absence of mediators the quenching was much smaller than when mediators were added, but was present nonetheless.

The slow quenching was insensitive to added ATP (data not shown) and was completely insensitive to the ionophore gramidicin (Fig. 2). The slow quenching of fluorescence was also independent of the reactions on the oxidising side of PS II. In the

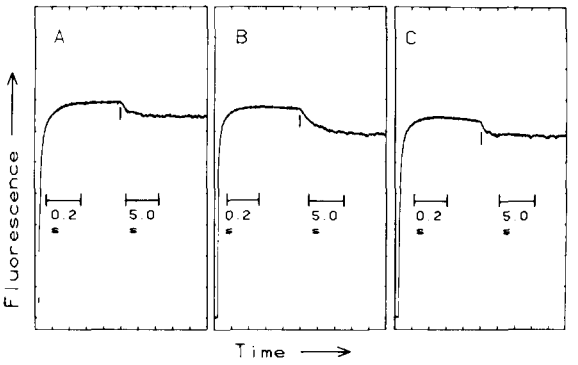

Fig. 2. Fluorescence induction curves in DCMU-blocked chloroplasts at a redox potential of +50 mV. Light intensity was 820 μW/cm^2. Reaction mixture contained chloroplasts equivalent to 10 μg/ml Chl; 0.01 M sucrose; 0.01 M NaCl; 0.01 M Hepes; 0.005 M $MgCl_2$; 33 μM DCMU. Redox mediators absent. Trace A, control; trace B, control plus 3.3 mM NH_2OH; trace C, control plus 5 μM gramicidin.

presence of hydroxylamine, an agent which prevents the oxidation of water [36], the slow decrease observed was just as large. The kinetics of the decrease were slower, however, suggesting that the addition of NH_2OH had some small effect.

The fluorescence decline is best observed at negative redox potentials (Fig. 3). However, the

TABLE I

EFFECT OF PHOTOSYSTEM I MODIFIERS ON THE SLOW FLUORESCENCE QUENCHING

Reaction conditions: chloroplasts equivalent to 10 μg/ml Chl; 0.1 M sucrose; 0.01 M Tricine (pH 7.8); 0.01 M NaCl; 0.005 M $MgCl_2$; 10 μM anthraquinone-2-sulfonic acid; 10 μM 2-hydroxy-1,4-naphthoquinone; 10 μM 2,5-dihydroxynaphthoquinone. Preillumination was for 10 min with light at 718 nm (6.0 W/m^2) provided by a tungsten lamp and an interference filter. Exciting light intensity was 600 μW/cm^2. Redox potential was approx. −170 mV. The fluorescence decrease was determined as the extent of the decrease from F_{max} at 5 s after light on.

Treatment	Percentage of fluorescence decrease
Normal	
plus mediators	10
minus mediators	3
KCN	
plus mediators	10
minus mediators	1
718 nm preillumination	
plus mediators	11
minus mediators	0

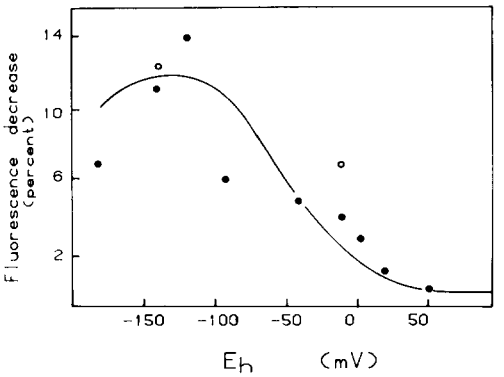

Fig. 3. Effect of redox potential on the slow fluorescence decline. Redox titration was performed reductively (●) or oxidatively (○). Reaction mixture contained chloroplasts equivalent to 10 μg/ml Chl, 0.1 M sucrose, 0.01 M NaCl, 0.01 M Tricine (pH 7.8), 0.005 M $MgCl_2$, 10 μM indigotetrasulfonic acid, 10 μM anthraquinone-2-sulfonic acid, 10 μM 1,4-naphthoquinone, 10 μM 2-hydroxy-1,4-naphthoquinone, 10 μM 2,5-naphthoquinone and 10 μM 1,2-naphthoquinone. The ordinate is the percentage of decrease in fluorescence intensity from F_{max} at 16 s after light on.

actual midpoint potential of the change varies greatly from preparation to preparation.

The proportions of cyclic and non-cyclic electron transport can have an effect on the spillover of energy from PS II to PS I and a concomitant effect on the fluorescence [37]. Blocking electron

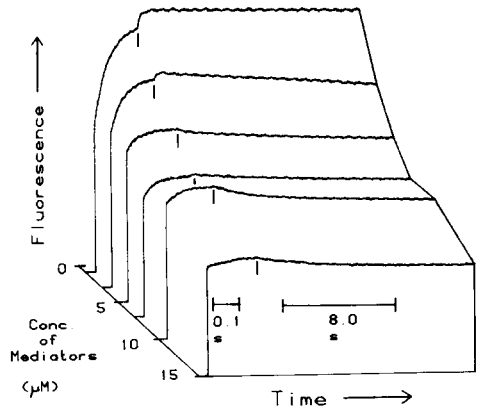

Fig. 4. Effect of mediator concentration on the fluorescence-induction curves. Reaction mixture as in Fig. 3 with the addition of mediators at the concentrations as indicated on the figure. Time ranges before and after the break are indicated on the figure. Light intensity was 800 μW/cm²; redox potential was approx. −140 mV.

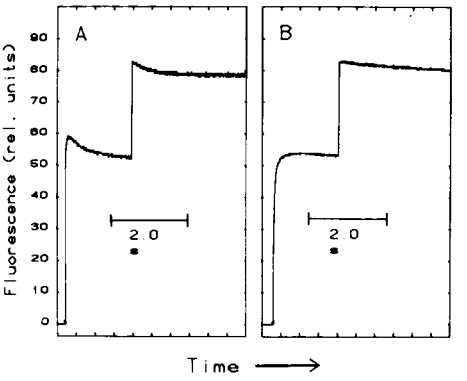

Fig. 5. Effect of additional illumination on the slow fluorescence decline. Assay medium as in Fig. 2. Extra illumination was provided by a HeNe laser. The maximum intensity of Part A was 66% of that of part B due to the quenching of the mediators. Trace a, plus mediators (10 μM) and no DCMU; trace b, no mediators and 6.6 μM DCMU.

transport at plastocyanin using KCN [30] and oxidising the pools of electron carriers between the photosystems by preilluminating with light specific

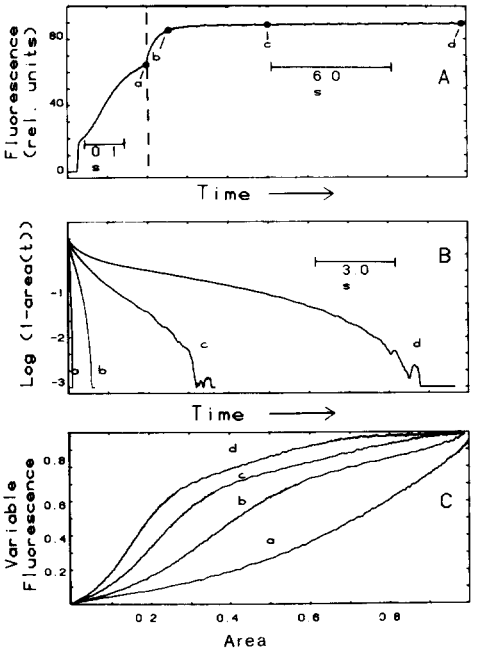

Fig. 6. Analysis of a fluorescence induction curve. Assay conditions as in Fig. 2. (A) Induction curve in split-time base mode. Vertical dashed line indicates point of the time break. The points labelled a, b, c, and d indicate the values used in Parts B and C and Table II. (B) First-order plot of the area growth determined as in Melis and Homann [24]. (C) Variable fluorescence versus Area.

for PS I (Table I) essentially failed to eliminate the slow decrease in the presence of mediators. Only with 718 nm preillumination was the slow decrease eliminated in the absence of mediators.

Fig. 4 shows that the slow quenching was most marked when the concentration of added redox mediators was high. It was also observed that the quenching was essentially independent of the time of incubation with the mediators before the sample was illuminated (data not shown).

The chloroplasts were subjected to an additional actinic light during the quenching (Fig. 5). Under these conditions, the fluorescence quickly attained a new higher level, with no evidence of a gradual increase to a new level that might be expected if the PS II traps had opened during the quenching [38].

Fig. 6 shows a kinetic analysis of a typical trace. To demonstrate the effect of the F_{max} level on the analysis we have arbitrarily selected several points (a–d) as the endpoint of the fluorescence rise.

Discussion

The sigmoidal kinetics in the fluorescence rise of DCMU-blocked PS II centres is well documented [20,39]. Nearly all of the analyses of these kinetics require a good estimate of the complementary area over the curve during the induction. In principle this area determination is not difficult, but there appears to be, in the literature, a lack of attention given to an accurate determination of the F_{max} level. Normally investigators adjust the experimental parameters (e.g., light intensity, chloroplast concentration, time base) until the fluorescence rise appears to be finished during the observation period. This value is then sometimes checked by superimposing a second trace after a given interval with the actinic light still present. For the reasons outline below, we have determined that neither approach is adequate to establish properly the true maximum level of the fluorescence.

Fig. 6 depicts the effect that changes in the level chosen for F_{max} has on the first-order plot of area growth and the graphs of variable fluorescence yield against area for a typical trace. It should be remembered that the choice of a higher F_{max} results in a greater total area, both because of its greater amplitude and because this level is reached in a longer time. A summary of the relative kinetic parameters is presented in Table II. It should be emphasised that a higher $F_{v\,max}$-to-F_{max} ratio cannot be used by itself to establish that the appropriate F_{max} level has been selected. This important point is clearly illustrated in the area calculations shown in Table II where an increase in the $F_{v\,max}$-to-F_{max} ratio of less than 2% (from a value of 0.793 to 0.806) gives an increase in the area determined of 150%. In addition, we have found it difficult subsequently to determine the α-to-β centre ratio using the method of Melis and Homann [24], since an extrapolation to the ordinate on the first-order plot is made virtually impossible due to the non-linear nature of the curve at longer times. One must therefore question the supposition that the heterogeneity can be accounted for by just two distinct types of centre.

Moreover, the plot of variable fluorescence against area has a drastically different shape with different levels of F_{max}. While at the shortest times (points a and b) the curves assume shapes which bracket those often reported for these analyses [22,39], at longer times, the curves are above the line of equality between area and F_v. The interpretation of such curves is not yet clear.

Unfortunately, attempts to eliminate these slow changes introduce additional complications as will be discussed below. Thus the most reasonable approach to analyse the kinetics of fluorescence inductions at ambient redox potentials is to record the fluorescence rise over time ranges well in excess of those normally reported.

TABLE II

A KINETIC ANALYSIS OF THE FLUORESCENCE INDUCTION CURVE OF FIGURE 7

The ratio $F_{v\,max}/F_{max}$ is calculated based on an average for F_{max} of 51 points centered at the channel corresponding to the time indicated. The relative area with respect to the area is determined at point B.

Point	Time (s)	$F_{v\,max}/F_{max}$	Relative area
A	0.2	0.740	32
B	1.0	0.793	100
C	6.0	0.804	179
D	16	0.806	250

Up to this point, our discussion has been limited to experiments performed at ambient redox potential. In an effort to eliminate the very slow fluorescence rise, we lowered the redox potential in line with the results of Horton [25]. Whereas the slow fluorescence rise did disappear, as can be clearly seen in Figs. 2, 4 and 5, a new complication arises in the form of a slow quenching of the fluorescence. This quenching is seen to its greatest extent in the presence of redox mediators, and to a small, but very reproducible, extent in their absence. Thus once again, it is difficult to determine a true F_{max} for subsequent analysis. While the fluorescence does reach a maximum value, the superposition of a trailing decrease means that the true value (in the absence of the decrease) may have been larger. Thus it appears that previous work which relates to the proportion of α and β centres at various redox potentials [25] may not be applicable to all cases.

An additional problem associated with the use of various redox potentials is a requirement for redox mediators to establish equilibrium. Although the effects of mediators have been reported, the best combination of mediators has yet to be determined [35]. Moreover, previous work has documented complications arising from the use of certain specific mediators like neutral red [10,25] and 2-hydroxy-1,4-naphthoquinone [14]. Since the mediators have a noticeable effect on the kinetics of the fluorescence rise at all redox potentials, one must seriously question the inferences made from experiments which analyse fluorescence kinetics in the presence of mediators.

The precise mechanism by which the slow fluorescence quenching occurs is not clear. As has been reported by others, a role for pheophytin in these effects is unlikely [28,40]. Furthermore, Schultze and Renger [28] have proposed that there is an electron transfer step involved because of a dependence on oxidised redox mediators. But our data show clearly that any such mechanism must taken into account the observation that the decrease is seen (albeit to a much lesser extent) in the absence of mediators (Fig. 2). We show (Fig. 4) that the slow quench depends on the concentrations of mediator present but not on the time of incubation with the mediators before illumination. At this point, many models could be presented to account for this quenching. It could be argued that the mediators are acting by a process which requires the slow diffusion of endogenous or external quenchers to the site of action. This diffusion could be restricted by the proteins surrounding the reaction centre, and since the slow quench does not depend on the incubation time, actinic illumination might be important in permitting access of the quenchers to the correct location. The lack of any observable relationship between the slow quench and light intensity over the range tested (data not shown) suggests that the threshold for this effect may be quite low.

The notion that the slow fluorescence decrease results from a reopening of closed traps [38] is probably not supported by the experiment of giving additional illumination after the slow quench is already underway (Fig. 5). We observe an immediate rise in the fluorescence with no second induction phase. This observation does not, however, preclude the possibility that there is an undetected induction phase during the time of the shutter opening (0.1 s). We also observe that the slow quench is insensitive to ATP and gramicidin and occurs in the presence of DCMU, results which probably rule out explanations based on pH-dependent quenching [38,41], protein phosphorylation [37] and noncyclic electron transport. Finally, an effect on the energy distribution to PS I seems unlikely, because the slow phase is still quite evident after treatments which dramatically alter the rates of electron transport at PS I (Table I).

While we do not propose to abandon the concept of PS II reaction centre heterogeneity, we wish to issue a word of caution to those who would use fluorescence measurements to study it. From the results we present here (especially Fig. 6 and Table II), it is clear that fluorescence induction curves do not offer an ideal technique for investigating the heterogeneity and making quantitative interpretations. Determinations of the absolute area over the induction curves must be done with more attention to the F_{max} level and slow fluorescence decreases.

It is perhaps worth stressing that on a uniform time-scale which includes the entire rise to F_{max}, the sigmoidal rise is normally undetectable. Also the area increase during this first phase is a small

proportion (8–15%) of the total area. Thus kinetic studies of the fluorescence rise which are restricted to the first 90–97% of the total rise in fluorescence level are arbitrarily excluding the information about the majority of the reaction centres of Photosystem II. It is probably only when all of the reaction centres are involved in the analysis that valid inferences can be made about heterogeneity in Photosystem II.

It is important to note that our criticisms do not apply exclusively to models that invoke α and β centres [24]. The need to determine the complementary area is common to most of the major theories outlined to analyse the sigmoidal rise in fluorescence.

Acknowledgements

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