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# AN EQUILIBRIUM MODEL FOR ELECTRON TRANSFER IN PHOTOSYSTEM II ACCEPTOR COMPLEX: AN APPLICATION TO *CHLAMYDOMONAS REINHARDTII* CELLS OF D1 MUTANTS AND THOSE TREATED WITH FORMATE\*

BY

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KEY WORDS: Chlorophyll fluorescence decay; Photosystem II; Electron acceptor complex; Herbicide resistance; D1 mutants; (*Chlamydomonas reinhardtii*).

## ABSTRACT

Chlorophyll *a* fluorescence yield decays in photosynthetic systems, after single-turnover flashes, are usually analyzed in terms of three or four exponentials related to several pathways for reoxidation of  $[Q_A]$ . We term this the independent decay model. In this paper we have examined these fluorescence decays in the wild type and five mutants of *Chlamydomonas reinhardtii* in an alternate fashion that we call the equilibrium model since it includes apparent equilibria reactions between  $Q_A^-Q_B^-$  and  $Q_AQ_B^-$  as well as electron flow out of the  $Q_AQ_B^-$  complex. It is, however, based on the assumption that the rate constants of binding and unbinding of plastoquinone cancel out and don't affect the results. This analysis allows us to predict average lifetime for the forward electron transfer ( $\tau_{ab}$ ) from  $Q_A^-$  to  $Q_B^-$  and  $Q_B^-$ , the apparent equilibrium constant ( $k_{ab}/k_{ba}$ ), the average lifetime ( $\tau_{PQ}$ ) of electron flow out of PS II, mainly to the plastoquinone (PQ) pool; and the ratio of slow to fast photosystem II reaction centers. A comparison of these results with those obtained by the independent decay model (GOVINDJEE *et al.*, 1992) will also be presented.

In view of the fact that three independent parameters ( $\tau_{ab}$ ,  $\tau_{ba}$  and  $\tau_{PQ}$ ) are calculated in this paper from a single measurement of Chl *a* fluorescence yield decay, the following results are presented here so that they may be checked by other measurements and methods. Assuming the intersystem energy transfer parameter, *p*, to be 0.5, the D1 mutants Ar-207 (F255Y)<sup>4</sup>, Br-202 (L275F), and Dr-18 (V219I) had an almost unimpaired forward electron transfer ( $Q_A^-Q_B^{(-)} \rightarrow Q_AQ_B^{(-)}$ ) lifetime ( $\tau_{ab}$ , 600 – 950  $\mu$ s), and an almost unchanged ratio (0.1 - 0.2) of the slow to the fast PS II centers compared to that of wild type. However, the mutants Ar-204 (G256D) and DCMU-4 (S264A) had a several fold higher forward electron transfer lifetime (2,000  $\mu$ s), altered apparent equilibrium constant for  $Q_A^-Q_B^- = Q_AQ_B^-$  reaction (lowest in S264A and G256D, intermediate in V219I, and highest in F255Y), and an abnormally high ratio (0.6 - 0.8) of the slow to the fast PS II centers.

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<sup>4</sup> Mutants were named as follows: name of the wild type amino acid in single letter code, followed by the number of the amino acid and then the single letter code of the mutated amino acid.

Addition of 100 or 200 mM formate led to an increase in the lifetime of the forward ( $Q_A^-$  to  $Q_B$ ) electron transfer in the wild type (from 640 to 990  $\mu$ s) and in the five mutants, with the largest change in the S264A (from 1,400  $\mu$ s to  $\sim$  30 ms), and the smallest in the L275F (from 950  $\mu$ s to 1.4 ms) mutant. Formate treatment (bicarbonate depletion) also led to an increase in the lifetime of the electron transfer to the PQ pool by a factor of 1.5 to 5 depending upon the mutant, a decrease in the apparent equilibrium constant for  $Q_A^-Q_B = Q_AQ_B^-$  reaction (by a factor of 2 to 6) and a noticeable increase in the fraction of slow centers. All effects were reversed by 20 mM bicarbonate.

We confirm the earlier conclusion of GOVINDJEE *et al.* (1992) that the amino acid S264, but not F255 and V219, plays an important role in the binding and the function of bicarbonate and plastoquinone in PS II. The roles of L275 and of G256 remain to be further examined since L275 showed much smaller formate/bicarbonate effect than the wild type and since aspartic acid is drastically different from glycine in G256D.

### RÉSUMÉ

La décomposition des émissions de fluorescence de la chlorophylle *a* des systèmes photo-synthétiques après un flash unique saturant est d'habitude analysée par trois ou quatre exponentielles, qui sont en relation avec les différents modes de réoxydation du  $[Q_A^-]$ . Ce modèle est appelé le modèle indépendant de décomposition. Dans la présente publication nous avons testé ces décompositions des émissions de fluorescence chez *Chlamydomonas reinhardtii* (type sauvage et cinq mutants) d'une manière différente que nous appellerons le modèle d'équilibre, car nous tenons compte des réactions d'équilibres apparents entre le  $Q_A^-Q_B$  et  $Q_AQ_B^-$  et, en plus, d'un flux d'électrons quittant le complexe  $Q_AQ_B$ . Ce modèle suppose que les constantes de vitesse de liaison et de dégagement de la plastoquinone s'anéantissent et n'influencent pas les résultats. Cette analyse nous permet la prédiction du temps de vie moyen pour le transport d'électrons en avant ( $\tau_{ab}$ ) du  $Q_A^-$  vers le  $Q_B$  et le  $Q_B^-$ , la constante d'équilibre apparente ( $k_{ab}/k_{ba}$ ), le temps de vie moyen ( $\tau_{PQ}$ ) d'électrons quittant le PS II, essentiellement vers le pool de la plastoquinone (PQ) ainsi que le rapport entre les centres rapides et lents du photosystème II. Nous présenterons en plus une comparaison de ces résultats avec ceux obtenus par le modèle indépendant (GOVINDJEE *et al.* 1992).

Du fait que dans la présente publication les trois paramètres indépendants ( $\tau_{ab}$ ,  $\tau_{ba}$  et  $\tau_{PQ}$ ) sont obtenus à partir d'une seule mesure de décomposition des émissions de fluorescence de la chl *a*, nous présentons nos résultats pour qu'ils puissent être vérifiés par d'autres mesures et méthodes. Si l'on suppose que le paramètre de transfert d'énergie ( $p$ ) entre les systèmes est de 0.5, les mutants D1 Ar-207 (F255Y)<sup>1</sup>, Br-202 (L175F) et Dr-18 (V219I) montrent un transfert d'énergie en avant ( $Q_AQ_B^{(-)} \rightarrow Q_A^-Q_B^{(=)}$ ) pratiquement inchangé (temps de vie moyen  $\tau_{ab} = 600 - 950 \mu$ s) et, en plus, un rapport des centres réactionnels du PS II rapides et lents pratiquement égal à 0.1 à 0.2 en comparaison avec le type sauvage. Par contre, chez les mutants Ar-204 (G256D) et DCMU-4 (S264A), on observe un temps de vie moyen du transport d'électrons en avant augmenté plusieurs fois ( $\tau_{ab} \approx 2'000 \mu$ s), des constantes d'équilibre apparentes du  $Q_A^-Q_B = Q_AQ_B^-$  modifiées (la plus faible chez S264A et G256D, intermédiaire chez V219I et la plus élevée chez F255Y), et des rapports exceptionnellement élevés (0.6 – 0.8) de centres réactionnels rapides et lents du PS II.

L'adjonction de formiate à 100 ou 200 mM provoque une augmentation du temps de vie du transport d'électrons en avant ( $Q_A^-$  vers  $Q_B$ ) chez le type sauvage de 640 à 990  $\mu$ s ainsi qu'aux cinq mutants testés. Le plus grand changement est observé chez S264A (de 1.4 à  $\sim$  30 ms), le plus petit chez L275F (de 950  $\mu$ s à 1.4 ms). Le traitement au formiate (appauvrissement en bicarbonate) conduit à une augmentation du temps de vie pour le transport d'électrons vers le pool de la PQ de 1.5 à 5 fois selon le mutant, une diminution des constantes d'équilibre apparentes de la réaction  $Q_A^-Q_B = Q_AQ_B^-$  d'un facteur de 2 à 6 ainsi qu'à une augmentation remarquable en centres lents. Tous ces effets sont réversibles par le bicarbonate.

<sup>1</sup> Les mutants sont désignés comme suit: en tête le nom de l'acide aminé (en code de lettre unique) du type sauvage suivi par le nombre de l'acide aminé, ensuite le code de l'acide aminé muté.

## INTRODUCTION

Chlorophyll *a* (Chl *a*) fluorescence yield decay, after a saturating single-turnover flash, is often used to monitor electron transfer from  $Q_A^-$  to  $Q_B$  or  $Q_B^-$ , where,  $Q_A$  and  $Q_B$  are primary and secondary plastoquinones of Photosystem II (PS II) [LAVOREL and ETIENNE 1977, VAN GORKOM 1986, EATON-RYE and GOVINDJEE 1988]. When reaction center Chl *a* of PS II is in the reduced state,  $P_{680}$ , Chl *a* fluorescence yield is low when  $[Q_A]$  is high and high when  $[Q_A]$  is low [DUYSENS and SWEERS 1963, VAN GORKOM 1986]. Analyses of such measurements provide information on the functioning of the electron acceptor side of the PS II [CAO and GOVINDJEE 1990, ETIENNE *et al.* 1990]. Such analyses are usually in terms of three or four exponential decays. We have dubbed this analysis to be independent decay analysis or the model used for the analysis to be independent decay model. In this paper, we have examined a specific analysis that we call an equilibrium model. We also present an equivalence between the two models that we suggest should be tested with further analysis and experimentation. A comparison between the results obtained by the independent model (GOVINDJEE *et al.* 1992) and those by the equilibrium model (this paper) are also presented. The test material was *Chlamydomonas reinhardtii* wild type cells, five D1 herbicide-resistant mutants of the same, and formate-treated cells.

## MATERIAL AND METHODS

Wild type *Chlamydomonas reinhardtii* cells and five D1 mutants (V2191, F255Y, G256D, S264A and L275F) were grown autotrophically in a tris-phosphate medium [GORMAN and LEVINE 1965, GOVINDJEE *et al.* 1991] for 2 days at 25°C (day) and 22°C (night). Cultures were illuminated for 16 h with fluorescent white light and kept in darkness for 8 h. When formate was used it was at a concentration of 100 or 200 mM, and was added in dark. A minimum of 15 min dark incubation preceded measurements.

The decay of Chl *a* fluorescence yield was measured exactly as described earlier [GOVINDJEE *et al.* 1992] (Fig. 1). The dark time was 30 s between 16 sets of measurements averaged, but the dark time between each flash was 1 s. The curve fitting was made using the commercial non-linear data analysis program ENZFITTER [LEATHER-BARROW 1987]. For curve fitting the data sets were reduced to 250 data points using a smoothing program with a logarithmic time scale with more points at short and less points at long time intervals in order to precisely determine the parameters for the fast components. Datapoints up to 170  $\mu$ s were discarded in order to avoid any gating or other artifacts.

## THEORY AND ANALYSIS

Chl *a* fluorescence yield decays with, at least, triphasic kinetics (see *e.g.* [CAO and GOVINDJEE 1990, ETIENNE *et al.* 1990]). The slow kinetics with a lifetime in the 1-2 s range has been suggested to be due mainly to a back reaction of  $Q_A^-$  with the oxygen

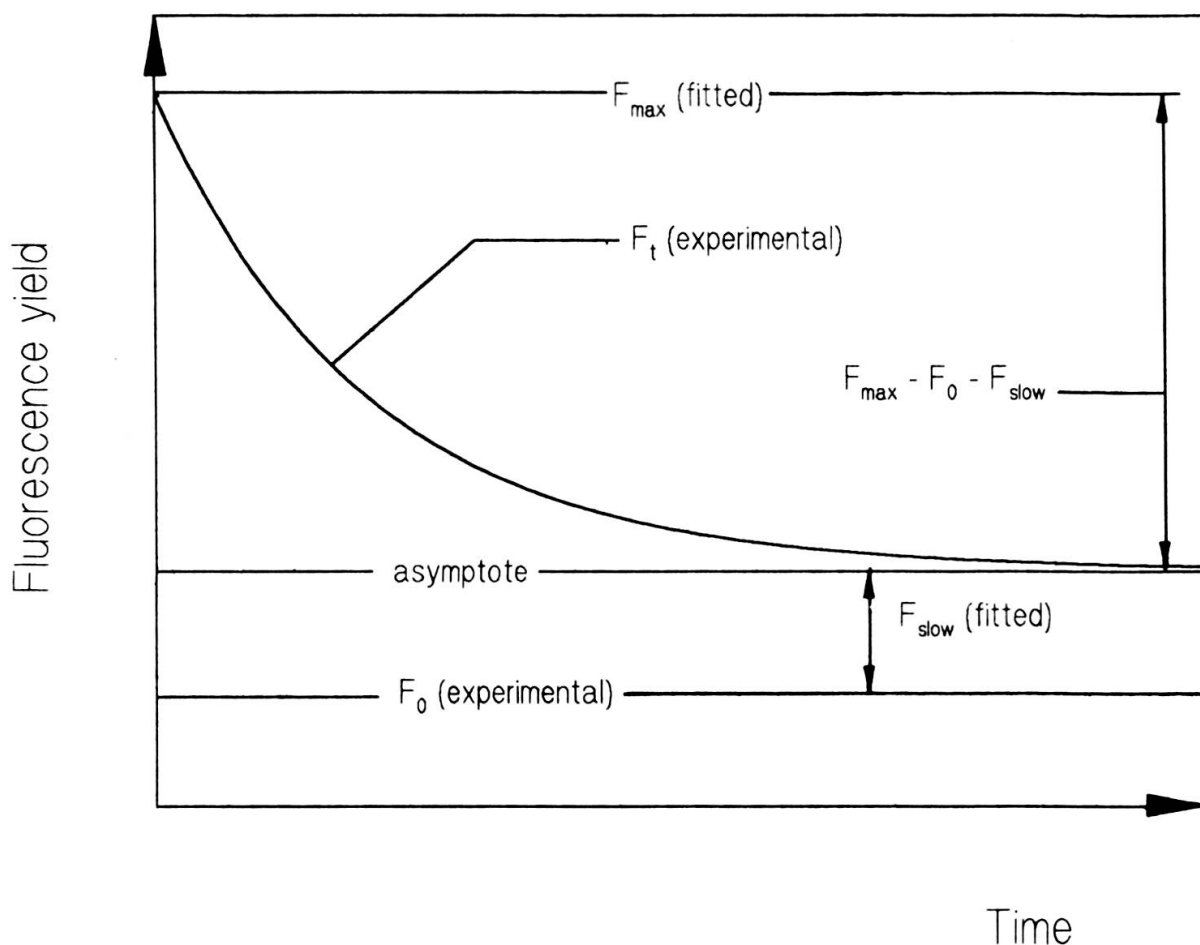
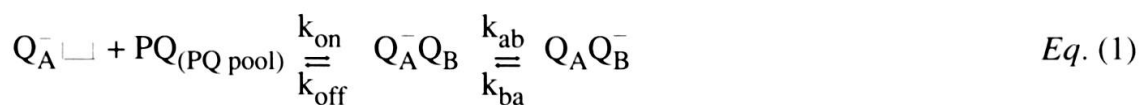


FIG. 1.

Schematic diagram defining the fluorescence signals used in the text.  $F_{\max}$  = maximum fluorescence;  $F_0$  = minimum fluorescence when all  $Q_A$  is in the oxidized state;  $F_t$  = experimental fluorescence decay;  $F_{\text{slow}} \geq 1$  s component, reflecting the slow PS II centers.

evolving state  $S_2$  in the centers in which  $Q_A$  is not connected to  $Q_B$  and the PQ pool and, thus, includes the kinetics of the so-called inactive (non  $Q_B$ ) PS II centers [MELIS 1991]. On the other hand, the decay of Chl *a* fluorescence yield in subms times after a single turnover flash reflects the kinetics of reoxidation of  $Q_A^-$  by  $Q_B$  (after 1st flash) or by  $Q_B^-$  (after 2nd flash) [see *e.g.* ROBINSON and CROFTS 1987]. At longer times (ms range) the variable fluorescence reflects the variable  $[Q_A^-]$  which is in equilibrium with  $Q_B^-$  [see *e.g.* CROFTS and WRAIGHT 1982]. Furthermore fractions of centers that contain empty  $Q_B$  sites bind plastoquinone from the PQ pool forming  $Q_A^-Q_B$ . Thus:



Here,  $k_{on}$  and  $k_{off}$  are rate constants for the binding and unbinding of the PQ molecule to the  $Q_B$  site and  $k_{ab}$  and  $k_{ba}$  are the forward and backward rate constants for electron flow between  $Q_A^-Q_B$  and  $Q_AQ_B^-$ . The net conversion rate of  $Q_A^-Q_B$  to  $Q_AQ_B^-$  is much faster than that for the binding of PQ. As noted above, the fastest decay component is associated with  $Q_A^-Q_B$  conversion to  $Q_AQ_B^-$ , the ms and the s components, without further precision, with the equilibrium  $[Q_A^-]$  that includes the component of  $[Q_A^-]$  recombining with  $S_2$  state in slow and/or inactive PS II centers.

### 1. INDEPENDENT DECAY MODEL

Even though the reaction scheme of Eq. (1) corresponds to our current understanding of the acceptor side (without the protonation events) of PS II, its application to the measured Chl *a* fluorescence decay is not obvious. However, the fluorescence decay curves can be deconvoluted in terms of three empirical and independent first order exponentials (see *e.g.* CAO and GOVINDJEE 1990). We have termed this the independent decay model, and it reveals, in our view, three empirical amplitudes of three components ( $A_{fast}$ ,  $A_{intermediate}$  and  $A_{slow}$ ) and their empirical lifetimes ( $\tau_{fast}$ ,  $\tau_{intermediate}$  and  $\tau_{slow}$ ):

$$\frac{F(t) - F_0}{F_{max} - F_0} = \left[ A_{fast} \cdot e^{-t/\tau_{fast}} + A_{int} \cdot e^{-t/\tau_{int}} + A_{slow} \cdot e^{-t/\tau_{slow}} \right] \quad Eq. (2)$$

Here  $F(t)$  = fluorescence yield at time  $t$ ,  $F_{max}$  = maximum fluorescence yield;  $F_0$  = minimum fluorescence yield;  $A$ 's represent the relative amplitudes and  $\tau$ 's the lifetimes of fast, intermediate (int) and slow components; and  $A_{fast} + A_{int} + A_{slow} = 1$ . Equation (2) gave excellent fits with  $\tau_{slow} = 1 - 2$  s, but within the measuring interval (maximum 20 ms) and the observed noise level, it cannot be distinguished from  $\tau_{slow}$  of infinity. Thus,  $A_{slow} \cdot e^{-t/\tau_{slow}}$  could be replaced simply by the asymptote:

$$A_{slow} = F_{slow} / (F_{max} - F_0).$$

With this model one is unable to directly relate the above defined parameters to specific reactions of PS II, although the  $\tau_{fast}$  may mainly refer to average lifetime of reoxidation of  $Q_A^-$  by  $Q_B$  and  $Q_B^-$  and the ratio of the  $A_{slow}/A_{int}/A_{fast}$  to the ratio  $Q_{Ainactive}/Q_{A^-} / Q_AQ_B$  at time zero. Results, obtained by the independent decay model, on wild type and five D1 mutants of *Chlamydomonas reinhardtii* have been published elsewhere (GOVINDJEE *et al.* 1992).

### 2. THE "ONE WAY MODEL"

According to the biochemical reaction scheme in equation 1, it is possible to obtain a direct link to the empirical independent model: assuming that plastoquinone is in excess and that  $k_{on} \gg k_{off}$ ;  $k_{ab} \gg k_{ba}$  and  $k_{ba} \gg k_{on}$ , then the biphasic fluorescence decay becomes a sum of two exponentials on the top of a slow ( $\tau_{slow} > 2s$ ) decay as in the

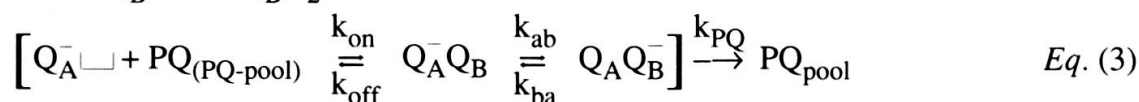
independent model. The  $Q_A^-$  with a vacant site for  $Q_B$  ( $Q_A^-\square$ ) reacts with plastoquinone in a pseudo first order reaction to  $Q_A^-Q_B$  which is transformed to  $Q_AQ_B^-$ . This two step reaction is governed by the rate limiting step, therefore, by  $k_{on}$  (experimentally by  $\tau_{int}$ ).  $Q_A^-Q_B$  decays immediately as a real first order reaction to yield  $Q_AQ_B^-$ . This reaction is governed by  $k_{ab}$  (experimentally by  $\tau_{fast}$ ).

This model allows an estimate of the relative concentrations of  $Q_A^-\square$  and  $Q_A^-Q_B$  at time zero as well as an estimate of the forward reactions defined by  $k_{on}$  and  $k_{ab}$ . This model does not explicitly consider any equilibria as do the following models.

### 3. THE EQUILIBRIUM MODEL

We present below what we believe is a more realistic approach: to derive a mathematical relationship based on equation (1) considering, in particular, the equilibrium reaction  $Q_A^-Q_B = Q_AQ_B^-$ , and, then, to fit the experimental fluorescence yield decay data with that predicted by the calculated equations. We have termed this the equilibrium model.

Equation (1) can be rewritten to include a rate constant  $k_{PQ}$  for electron transfer to the PQ pool and/or a leak (Eq. 3). The latter is justified even for our first flash data because in intact cells a significant fraction of centers remain in  $Q_B^-$  state in the dark [see *e.g.* XU *et al.* 1989], and thus, electron transfer to the PQ pool does occur. Electron transfer from the  $Q_AQ_B^-$  to the PQ pool occurs only after two electrons have accumulated on  $Q_B^{2-}$  and  $Q_BH_2$  has been formed.



After a flash,  $Q_A^-$  is formed in three types of centers: (a) slow or inactive ( $Q_A^-$ —non  $Q_B$ ) PS II centers; ; (b) active PS II centers in which  $Q_B$  is not bound (labeled as  $Q_A^-\square$ ); and (c) active PS II centers in which  $Q_B$  is bound ( $Q_A^-Q_B$ ). In (a) decay of  $Q_A^-$  is slow ( $\tau_{slow} > 1$  s) and during our measurement, it is essentially constant. In (b), no fluorescence yield change occurs; and in (c) decay of  $[Q_A^-]$  occurs as affected by reactions shown in equation (3). The rate constants  $k_{on}$  and  $k_{off}$  cancel out when an equilibrium situation related to PQ binding and unbinding and those related to  $Q_A^-$  equilibrium with  $Q_B^-$  are considered. If this assumption turns out to be incorrect, further modifications will have to be made.

Analysis of changes in total  $[Q_A^-]$  as  $[Q_A^- + Q_A^-Q_B]$  in Eq. (3), thus, leads to the following solution for the fraction of closed centers at time t:

$$q(t) = \frac{[Q_A^-]_t}{[Q_A^-]_{t=0}^{max}} = \frac{(1 - A_{slow})}{k_{ab} + k_{ba}} \left[ k_{ba} + k_{ab} e^{-t(k_{ab} + k_{ba})} \right] e^{-t k_{PQ}} + A_{slow} \quad Eq. (4)$$

$$\text{Here, } A_{slow} = \frac{[Q_A^-]_{t>2s}}{[Q_A^-]_{t=0}^{max}}$$

Assuming that the probability of energy transfer, among PS II units,  $p = 0$  (see *appendix*),

$$\frac{F_{(t)} - F_0}{F_{\max} - F_0} = \frac{(1 - A_{\text{slow}}) k_{ab}}{k_{ab} + k_{ba}} \left[ e^{-t(k_{ab} + k_{ba} + k_{PQ})} \right] + \frac{(1 - A_{\text{slow}}) k_{ba}}{k_{ab} + k_{ba}} e^{-t k_{PQ}} + A_{\text{slow}} \quad \text{Eq. (5)}$$

$F_{(t)}$  and  $F_0$  are available from the data, and these experimental data are fitted with  $F_{\max}$ , and the three rate constants  $k_{ab}$ ,  $k_{ba}$  and  $k_{PQ}$ , directly or after conversion into  $[Q_A^-]$  assuming  $p = 0.5$  (JOLIOT and JOLIOT 1964); also see *appendix*.

Since the first term will give higher preexponential and smaller lifetime than the second term, an equivalence between the independent decay (Eq. 2) and the equilibrium model (Eq. 5) may be suggested. The equivalence is:

$$A_{\text{fast}} \equiv \frac{(1 - A_{\text{slow}}) k_{ab}}{k_{ab} + k_{ba}} \quad \text{Eq. (6A)}$$

$$\tau_{\text{fast}} \equiv \frac{1}{k_{ab} + k_{ba} + k_{PQ}} \quad \text{Eq. (6B)} \quad \text{Eq. (6)}$$

$$A_{\text{int}} \equiv \frac{(1 - A_{\text{slow}}) k_{ba}}{k_{ab} + k_{ba}} \quad \text{Eq. (6C)}$$

$$\tau_{\text{int}} \equiv \frac{1}{k_{PQ}} \quad \text{Eq. (6D)}$$

Thus,

$$\frac{A_{\text{fast}}}{A_{\text{int}}} \equiv \frac{k_{ab}}{k_{ba}} = K_{\text{eq}} \quad \text{Eq. (7)}$$

$$k_{ab} \equiv \left( \frac{1}{\tau_{\text{fast}}} - \frac{1}{\tau_{\text{int}}} \right) \frac{K_{\text{eq}}}{1 + K_{\text{eq}}} \quad \text{Eq. (8A)} \quad \text{Eq. (8)}$$

$$k_{ba} \equiv \left( \frac{1}{\tau_{\text{fast}}} - \frac{1}{\tau_{\text{int}}} \right) \frac{1}{1 + K_{\text{eq}}} \quad \text{Eq. (8B)}$$

With the above equivalence, it is possible to understand the differences in results by the independent decay model (GOVINDJEE *et al.* 1992) and the equilibrium model (this paper). With the assumptions made in this paper, only equation (5) can provide

information on  $k_{ab}$  and  $k_{ba}$  separately. Whether the values, predicted in this paper, are true rate constants remains to be established by direct measurements of the quinone complexes. Furthermore, to obtain a complete picture, new analyses are needed that will include separate data for  $Q_A^-$  to  $Q_B$  or  $Q_B^-$  electron flow as well as the effects of protonation and of the binding and unbinding of plastoquinone at the  $Q_B$ -site.

#### 4. THE TWO EQUILIBRIA MODEL

This model is the full mathematical description of the biochemical reaction scheme of equation 1. It has been elaborated by BAROLI (1992) and by CROFTS *et al.* (1992). A single fluorescence decay curve does not allow one to solve the equations. Additional information is needed as reported by these authors. As the reaction scheme of equation 1 ends with an equilibrium, the concentration of  $Q_A^-$  (with or without  $Q_B$ ) can never reach true zero after any illumination. This means that the fluorescence decay curve should not reach the  $F_0$  level of dark adapted cells with all reaction center being open! Since the original  $F_0$  level is restored after a flash, we introduced an additional 'one way' reaction with the rate constant  $k_{PQ}$  in our equilibrium model.

### RESULTS AND DISCUSSION

Of the four models mentioned earlier, we discuss our results here only according to the two extreme versions *i.e.*, the *independent* model and the *equilibrium* model.

#### WILD TYPE CELLS

Table 1 shows the calculated results from the independent (GOVINDJEE *et al.* 1992) as well as equilibrium models (this paper) for the various kinetic parameters for the wild type cells of *C. reinhardtii*. The major observations are: (1) No significantly large differences occur in the parameters when  $[Q_A^-]$  ( $p = 0.5$ ) is used or direct fluorescence ( $p = 0$ ) is used. The ratio of  $A_{fast} : A_{int} : A_{slow}$  is about 0.65 : 0.20 : 0.15 and the ratio of slow to fast centers is 0.20-0.24. (2) The predicted average  $\tau_{ab}$  (as  $1/k_{ab}$ ), for electron flow from  $Q_A^-$  to  $Q_B$  and to  $Q_B^-$ , is about two times higher than  $\tau_{fast}$  (640  $\mu s$  vs 320  $\mu s$ ,  $p = 0.5$  and 410  $\mu s$  vs 260  $\mu s$ ,  $p = 0$ ). This is fully understandable in terms of Equation 6B. The predicted  $\tau_{ba}$  (as  $1/k_{ba}$ ) is about 1,200  $\mu s$ ,  $\tau_{PQ}$  (as  $1/k_{PQ}$ ) is 1600 – 1800  $\mu s$  and apparent  $K_{eq}$  is 2-3. This agrees with the  $A_{fast}/A_{int}$  being about 3 (Eq. 7). Further direct measurements of the quinone complexes are needed to confirm the validity of these predictions.

#### $Q_A^-$ DECAY CURVES AND CALCULATED PARAMETERS

Fig. 2 shows fluorescence decay curves for the wild type and four mutants (L275F, F255Y, S264A and G256D) after the third flash (after GOVINDJEE *et al.* 1992). The data points, and the best fits of both the models to the data, are shown in the figure.

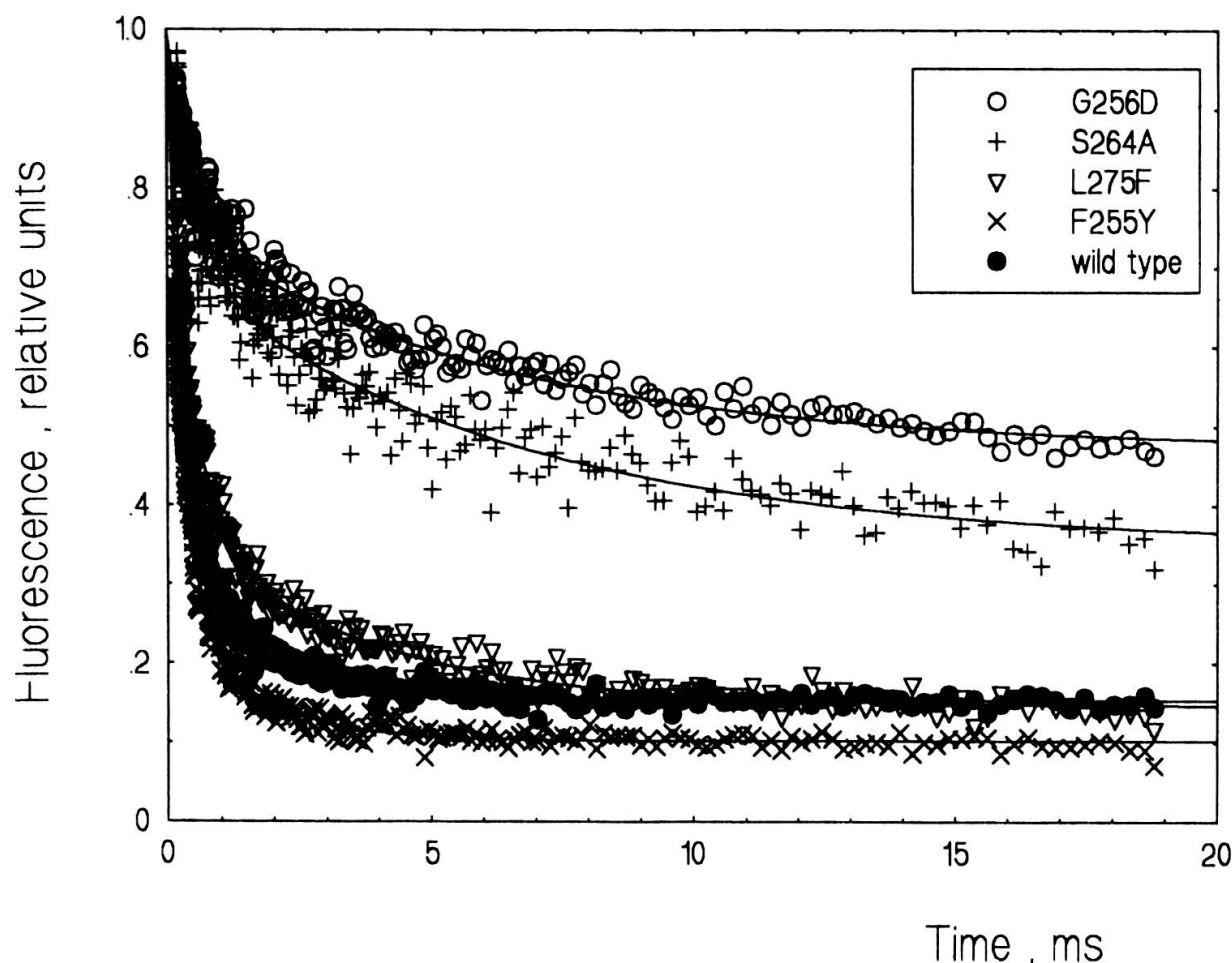


FIG. 2.

Chlorophyll fluorescence decay after the last of the three flashes in the wild type and four D1 mutants (F255Y: Ar-207; L275F: Br-202; S264A: DCMU-4; G256D: Ar-204) of *C. reinhardtii*. Drawn lines are fits for the data (see Tables 1 and 2 for values of constants and other parameters). Dark adapted cells were exposed to 3 flashes (dark time between flashes, 1s); after 30 s dark time, another set of 3 flashes was given, and then, 16 sets were averaged for the last of the three flashes. (After GOVINDJEE *et al.* 1992).

Calculated parameters for  $p = 0$  and  $p = 0.5$  for both the models are shown in Table 2. The major effects, or the absence of the effects, of the mutations are obvious: V219I, F255Y and L275F are like the wild type (cf. with Table 1). Thus, these amino acids may be of marginal importance for the electron transfer from  $Q_A^-$  to  $Q_B^-$  and for the  $Q_B$  binding. However, in the G256D and S264A mutants the fast fluorescence component is slower and the amplitude of this component is larger than that in the wild type, as already shown earlier (GOVINDJEE *et al.* 1992).

TABLE 1.

Kinetic parameters for the wild type *C. reinhardtii* fluorescence decay after the third flash, from independent (columns 2–6, 11) and equilibrium model (columns 7–10).  $\tau_{\text{slow}} \geq 1$  s in all cases.

1	2	3	4	5	6	7	8	9	10	11
p	$A_{\text{fast}}$	$A_{\text{int}}$	$A_{\text{slow}}$	$\tau_{\text{fast}}$ ( $\mu\text{s}$ ) $\pm 50 \mu\text{s}$	$\tau_{\text{int}}$ ( $\mu\text{s}$ ) $\pm 200 \mu\text{s}$	$\tau_{\text{ab}}$ ( $\mu\text{s}$ ) $\pm 50 \mu\text{s}$	$\tau_{\text{ba}}$ ( $\mu\text{s}$ ) $\pm 200 \mu\text{s}$	$\tau_{\text{PQ}}$ ( $\mu\text{s}$ ) $\pm 200 \mu\text{s}$	$K_{\text{eq}}$	$\frac{A_{\text{slow}}}{1 - A_{\text{slow}}}$
0	0.63	0.22	0.15	260	1,520	410	1,200	1,520	2.9	0.18
0.5	0.66	0.21	0.13	320	2,070	640	1,190	1,790	1.9	0.16

TABLE 2.

Kinetic parameters for D1 mutants of *C. reinhardtii* fluorescence yield decay after the third flash, using Equations 2 (GOVINDJEE *et al.* 1992) and 5 (this paper).  $\tau_{\text{slow}} \geq 1$  s in all cases.

SAMPLES	Using independent decay model (after GOVINDJEE <i>et al.</i> 1992)					Using Equilibrium Model (this paper)				
	$A_{\text{fast}}$	$A_{\text{int}}$	$A_{\text{slow}}$	$\tau_{\text{fast}}$ ( $\mu\text{s}$ ) $\pm 50 \mu\text{s}$	$\tau_{\text{int}}$ ( $\mu\text{s}$ ) $\pm 200 \mu\text{s}$	$A_{\text{slow}}$	$\tau_{\text{ab}}$ ( $\mu\text{s}$ ) $\pm 50 \mu\text{s}$	$\tau_{\text{ba}}$ ( $\mu\text{s}$ ) $\pm 200 \mu\text{s}$	$\tau_{\text{PQ}}$ ( $\mu\text{s}$ ) $\pm 200 \mu\text{s}$	$K_{\text{eq}}$ $\frac{A_{\text{slow}}}{1 - A_{\text{slow}}}$
$p = 0^1$										
L275F	0.62	0.24	0.15	390	3,480	0.15	600	1,560	3,450	2.6
V219I	0.51	0.32	0.16	270	2,060	0.16	430	670	1,890	1.6
F255Y	0.72	0.18	0.10	260	1,450	0.10	390	1,610	1,450	4.1
$^{\dagger}$ S264A	0.29	0.35	0.36	560	4,770	0.39	1,000	670	3,000	0.7
G255D	0.24	0.28	0.47	430	5,960	0.47	960	820	5,880	0.9
$p = 0.5^2$										
L275F	0.66	0.22	0.12	530	5,930	0.13	950	1,470	4,350	1.5
V219I	0.55	0.32	0.13	240	2,580	0.15	810	670	2,380	0.8
F255Y	0.77	0.14	0.09	360	2,330	0.09	590	1,920	1,890	3.3
S264A	0.32	0.29	0.39	800	10,390	0.36	2,160	710	4,940	0.3
G255D	0.27	0.30	0.43	560	11,090	0.45	1,920	790	9,090	0.4

<sup>1</sup> Direct Fluorescence data ( $p = 0$ ); <sup>2</sup>  $[Q_A]$  data, with  $p = 0.5$ ; <sup>†</sup> In S264D, a fast component ( $\tau_{\text{fast}} < 100 \mu\text{s}$ ) was observed after the first flash, but it was not further discussed here. The values Fv/Fo in our S264D samples was  $\sim 2.5$ .

RATE CONSTANT FOR FORWARD ELECTRON TRANSFER FROM  $Q_A^-$  TO  $Q_B$ 

In intact cells, the ratio of  $Q_B$  to  $Q_B^-$ , prior to the flash, is about 1, and thus an average of  $\tau_{ab}$  for  $Q_A^-Q_B$  to  $Q_AQ_B^-$  and  $Q_A^-Q_B^-$  to  $Q_AQ_B^{2-}$  is monitored after almost all the flashes (see *e.g.* XU *et al.* 1989). The calculated lifetime of the fast decay component ( $\tau_{fast}$ ) from Equation 2 is not the lifetime of the  $Q_A^-Q_B$  to  $Q_AQ_B^-$  reaction, but, instead, the inverse of the sum of the rate constants  $k_{ab} + k_{ba} + k_{PQ}$  (see Equation 6B). The value of  $\tau_{fast}$  [or  $1/(k_{ab} + k_{ba} + k_{PQ})$ ] and  $\tau_{int}$  (using Equation 2) for  $p = 0$ , after the third flash, are summarized in Table 2. Interestingly, after the third flash, there is no large difference in  $\tau_{fast}$  (or  $1/k_{ab} + k_{ba} + k_{PQ}$ ), but there is a hierarchy (values  $\pm 0.2$  ms) in  $\tau_{int}$  (or  $1/k_{PQ}$ ) among the mutants: F255Y < V219I < L275F < S264A < G256D. The  $\tau_{fast}$  and the  $\tau_{int}$  of the S264A mutant were larger by 2 and 3 times than those of the wild type (cf. Tables 1 and 2). If the fluorescence yield is converted into  $[Q_A^-]$  ( $p = 0.5$ ), qualitatively a similar picture is obtained (see Table 2). The  $\tau_{fast}$  and the  $\tau_{int}$  of the S264A mutant were larger by 2.5 to 5 times than those of the wild type. The lifetime of the forward reaction ( $\tau_{ab}$ ) (*i.e.*,  $1/k_{ab}$ ), an average for  $Q_A^-Q_B$  to  $Q_AQ_B^-$  and  $Q_A^-Q_B^-$  to  $Q_AQ_B^{2-}$ , can be estimated from Equation 5 (see Table 2). Its hierarchy among the mutants, after the third flash, for  $p = 0$ , is: wild type  $\approx$  F255Y  $\approx$  V219I < L275F < G256D  $\approx$  S264A.

The  $\tau_{ab}$  of S264A was 2.5 times that of wild type. This is in agreement with the conclusion of ETIENNE *et al.* (1990). Results in Table 2 show that G256D also had longer  $\tau_{ab}$ . Although the exact values ( $\pm 50$   $\mu$ s) of  $\tau_{ab}$  change slightly when fluorescence yields are converted into  $[Q_A^-]$  ( $p = 0.5$ ), the hierarchy of the mutants does not change significantly (the  $\tau_{ab}$  of the S264A and G256D mutants was about 3.5 times that of the wild type).

## THE EQUILIBRIUM CONSTANT

ETIENNE *et al.* (1990) had suggested that in several D1 mutants (S264A/F255L; S264A; A255V/F211S) of *Synechocystis* 6714, the high fluorescence yield in the ms range, after the flash, was due to a decrease in the equilibrium constant ( $K_{eq} = k_{ab}/k_{ba}$ ). In S264A of *C. reinhardtii*, however, high fluorescence yield in the ms range after the flash was suggested to be due mainly to a decreased  $k_{ab}$ , not due to a change in  $K_{eq}$ . Results, discussed above, suggest that  $K_{eq}$  may also be altered in S264A. The ratio of  $k_{ab}$  to  $k_{ba}$  is  $K_{eq}$  and is equivalent to the ratio of  $A_{fast}/A_{int}$  from equation 7. This equivalence is borne out by our results (Table 2). For example, the hierarchy in  $K_{eq}$  (identical to  $A_{fast}/A_{int}$ ,  $\pm 0.2$ ) among the mutants, after the third flash, is: F255Y  $\approx$  wild type  $\approx$  L275F > V219I > S264A  $\approx$  G256D. The calculated ratio of  $K_{eq}$  in the wild type to that in the S264A mutant was 6 ( $p = 0.5$ ) or 4 ( $p = 0$ ).

In summary, our predictions, based on the equilibrium model, are that in both S264A and G256D mutants of *C. reinhardtii*, in contrast to other mutants (L275F, V219I and F255Y) rate constants  $k_{ab}$  and  $k_{PQ}$  as well as the apparent equilibrium

constant ( $K_{eq} = k_{ab}/k_{ba}$ ) are 2 to 6 times smaller. As already discussed by GOVINDJEE *et al.* (1992) several authors (see *e.g.* GLEITER *et al.* 1989, OHAD *et al.* 1989, TOAKA and CROFTS 1990) have observed that other herbicide resistant D1 mutants of cyanobacteria, green algae and higher plants, also have reduced equilibrium constants of  $Q_A^-Q_B = Q_AQ_B^-$  reaction.

#### THE RATIO OF SLOW TO FAST PS II CENTERS

It is generally accepted that the slow (second range) component of Chl *a* fluorescence yield decay reflects  $S_2Q_A$  to  $S_1Q_A$  reaction as it is also observed in DCMU-treated samples (see *e.g.* EATON-RYE and GOVINDJEE 1988), and that a portion of this component is suggested to belong to inactive PS II centers (see review by MELIS 1991). VAN RENSEN and SPÄTJENS (1986) had indeed shown that a herbicide-resistant mutant of *Chenopodium album* had a higher proportion of  $\beta$  centers ('inactive'). However, it is not possible to know, without additional measurements, what proportion of the slow component is due to the inactive centers. When the total number of centers was normalized to 1, the ratio of slow to fast components was quite high ( $0.6$  to  $0.9 \pm 0.1$ ) in the S264A and G256D mutants as compared to the other three mutants and the wild type ( $0.11$  to  $0.19$ ). This pattern is independent of the equations used to fit the data. Thus, G256D and S264A have the largest fraction of slow PS II centers among the five mutants examined, and, this is one of the reasons for high  $[Q_A^-]$  at longer times after the flash. Our results on G256D and S264A mutants may suggest a possibility that even a single amino acid mutation may cause structural changes leading to an increase in the fraction of 'slow' centers (GOVINDJEE *et al.* 1992). Such phenomena are not surprising in view of the results on mutants of photosynthetic bacteria (see a review by DEISENHOFER and MICHEL 1991).

#### BICARBONATE-REVERSIBLE FORMATE EFFECT

A bicarbonate-reversible formate effect exists on the electron acceptor side of PS II (see BLUBAUGH and GOVINDJEE 1988). This effect is clearly on the  $Q_AQ_B$  complex (see *e.g.* EATON-RYE and GOVINDJEE 1988). Thus, formate-treated wild type and the herbicide-resistant D1 mutants of *C. reinhardtii* were used for the further application of our equilibrium model.

A bicarbonate (20 mM)-reversible formate (100-200 mM) effect had already been observed in the wild type and the five mutants tested (see GOVINDJEE *et al.* 1992); cf. with Table 4. Analysis of the  $[Q_A^-]$  data of GOVINDJEE *et al.* (1992), using Equation 5 (Table 3), showed that the lifetime of the forward reaction ( $\tau_{ab}$ ), as compared to that of the wild type, was increased 30 times in S264A followed by 3 times in V219I and 1.5 times in L275F. In agreement with the conclusion of Table 1,  $\tau_{ab}$ 's (calculated by the equilibrium model) are much larger than the  $\tau_{fast}$  (calculated by the independent decay model).

TABLE 3.

Kinetic parameters for *C. reinhardtii* fluorescence decay after the third flash, using Equations 2 (GOVINDJEE *et al.* 1992) and 5 (this paper) for  $[Q_A^-]$  data ( $p = 0.5$ ) in formate treated samples.  $\tau_{\text{slow}} \geq 1$  s in all cases.

SAMPLES	Using independent decay model (after GOVINDJEE <i>et al.</i> 1992)					Using Equilibrium Model (this paper)				
	$A_{\text{fast}}$	$A_{\text{int}}$	$A_{\text{slow}}$	$\tau_{\text{fast}}$ ( $\mu\text{s}$ )	$\tau_{\text{int}}$ ( $\mu\text{s}$ )	$A_{\text{slow}}$	$\tau_{\text{ab}}$ ( $\mu\text{s}$ )	$\tau_{\text{ba}}$ ( $\mu\text{s}$ )	$\tau_{\text{PQ}}$ ( $\mu\text{s}$ )	$K_{\text{eq}}$
1. wild type control + 100 mM formate	0.66 0.55	0.21 0.23	0.13 0.22	320 540	2,070 11,040	0.14 0.25	640 990	1,190 1,220	1,790 7,140	1.9 1.2
2. L275Y + 100 mM formate	0.66 0.45	0.22 0.28	0.12 0.27	530 640	5,930 8,370	0.13 0.29	950 1,390	1,470 1,090	4,350 5,880	1.5 0.8
3. V219I control + 200 mM formate	0.55 0.32	0.32 0.40	0.13 0.28	240 770	2,580 13,270	0.15 0.31	810 2,440	670 890	2,380 10,000	0.8 0.4
4. F255Y control + 100 mM formate	0.77 0.40	0.14 0.40	0.09 0.20	360 1,070	2,330 17,410	0.09 0.24	590 2,780	1,920 1,330	1,890 11,110	3.3 0.5
5. S264A control + 200 mM formate	0.32 0.22	0.29 0.41	0.39 0.37	800 710	10,390 68,060	0.36 0.52	2,160 3,330	710 4,170	4,940 25,000	0.3 0.1

TABLE 4.

Kinetic parameters for *C. reinhardtii* fluorescence decay after the third flash, using Equations 2 (GOVINDEE *et al.* 1992) and 5 (this paper) for fluorescence data ( $p = 0$ ) in formate treated samples.  $\tau_{\text{slow}} \geq 1$  s in all cases.

SAMPLES	Using independent decay model (after GOVINDEE <i>et al.</i> 1992)					Using Equilibrium Model (this paper)				
	$A_{\text{fast}}$	$A_{\text{int}}$	$A_{\text{slow}}$	$\tau_{\text{fast}}$ ( $\mu\text{s}$ )	$\tau_{\text{int}}$ ( $\mu\text{s}$ )	$A_{\text{slow}}$	$\tau_{\text{ab}}$ ( $\mu\text{s}$ )	$\tau_{\text{ba}}$ ( $\mu\text{s}$ )	$\tau_{\text{PQ}}$ ( $\mu\text{s}$ )	$K_{\text{eq}}$
1. wild type control + 100 mM formate	0.63 0.51	0.22 0.23	0.15 0.26	260 410	1,520 5,700	0.15 0.27	410 630	1,200 1,430	1,520 5,560	2.9 2.3
2. L275Y + 100 mM formate	0.62 0.42	0.24 0.28	0.15 0.30	390 440	3,480 4,530	0.15 0.31	600 790	1,560 1,200	3,450 4,550	2.6 1.5
3. V219I control + 200 mM formate	0.51 0.29	0.32 0.38	0.16 0.33	270 600	2,060 7,380	0.16 0.33	430 1,410	670 1,110	1,890 7,140	1.6 0.8
4. F255Y control + 100 mM formate	0.72 0.37	0.18 0.37	0.10 0.26	260 680	1,450 7,650	0.10 0.26	390 1,490	1,610 1,470	1,450 7,690	4.1 1.0
5. S264A control + 200 mM formate	0.29 0.18	0.35 0.49	0.36 0.33	560 3,170	4,770 36,800	0.39 0.38	1,000 12,500	670 5,000	3,000 33,330	0.7 0.4

Furthermore, the relationship between  $K_{eq}$  and  $A_{fast}/A_{int}$  is also borne out. However, results from both analyses are qualitatively comparable to the differential sensitivity of bicarbonate-reversible formate effect on Chl *a* fluorescence transient in the same D1 mutants (GOVINDJEE *et al.* 1991). The effect on the amplitude of the fast component was however much less (only 2 fold) and less discriminatory among mutants. In addition,  $K_{eq}$  and  $\tau_{PQ}$  reversibly decreased and increased, also to varying degree upon formate treatment without much discrimination among mutants. Qualitatively, however, these results confirm the importance of L275 and S264 in the bicarbonate-reversible formate effect (GOVINDJEE *et al.* 1991, 1992). Furthermore, the effect of formate seems to mimic the effect of S264A mutation: a decreased  $A_{fast}$ , an increased  $A_{slow}$ , an increased  $\tau_{int}$ , an increased  $\tau_{ab}$ , an increased  $\tau_{PQ}$  and a decreased  $K_{eq}$ . Since formate is expected to remove bicarbonate, it seems logical to suggest that S264 functions in conjunction with bicarbonate to aid in the production of plastoquinol.

#### CONCLUDING REMARKS

Using wild type and five herbicide-resistant D1 mutants of *Chlamydomonas reinhardtii*, we have compared the results of analyses of chlorophyll *a* fluorescence decay by an independent decay model (see GOVINDJEE *et al.* 1992) and that by an equilibrium model (discussed in this paper). The latter, not the former, model is capable of providing information on the rate constants of electron flow from  $Q_A^-$  to  $Q_B^{(-)}$  and for the rate constant for PQ reduction provided the assumption that the plastoquinone binding/unbinding at the  $Q_B$  site does not affect the results is correct. Both models provide information on the apparent equilibrium constant for  $Q_A^-Q_B = Q_AQ_B^-$  reaction. We are fully aware that the current equilibrium model needs to be further refined in order to include (a) separately electron flow from  $Q_A^-$  to  $Q_B$  and from  $Q_A^-$  to  $Q_B^-$ ; and (b) the protonation events involved in PQ reduction. Furthermore, methods must be found to separate the electron 'leak' (*i.e.*, electrons going into dissipative processes) from the reduction of plastoquinone in the PQ pool and to assess the impact of plastoquinone binding and unbinding at the  $Q_B$  site. More importantly, it is obvious to us that predictions from an analysis of a single fluorescence decay curve of the major rate constant needs testing and confirmation by other independent means. Furthermore, quantitative, and even qualitative, differences between those reported here (also see GOVINDJEE *et al.* 1992) and those by BAROLI (1992) and by ETIENNE *et al.* (1990) must be understood before final conclusions on the D1 mutants of *C. reinhardtii* can be made.

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## APPENDIX

On the question of conversion of Chl *a* fluorescence yield to  $[Q_A^-]$

It has been shown earlier that the fraction of the closed reaction centers ( $q_{(t)}$ ) is empirically related, in a hyperbolic manner, to the relative variable Chl *a* fluorescence yield, normalized to the maximum variable fluorescence yield,  $V_{(t)}$ , as follows:

$$V_{(t)} = \frac{F_{(t)} - F_0}{F_{\max} - F_0} = \frac{q_{(t)}}{1 + \text{constant} (1 - q_{(t)})} \quad \text{Eq. (A.1)}$$

where,  $F_{(t)}$  = fluorescence yield at time  $t$ ,  $F_{\max}$  = maximum fluorescence yield when all  $[Q_A]$  is reduced  $Q_A$ ,  $Q_A^-$ ,  $F_0$  = minimum fluorescence yield when all  $[Q_A]$  is oxidized  $Q_A$ . The empirical constant  $c$  in Eq. (A.1) is equal to:

$$c_J = p / (1-p) \quad (\text{Joliot and Joliot 1964}),$$

$$c_P = \frac{p \cdot F_V / F_{\max}}{1 - p \cdot F_V / F_{\max}} \equiv \frac{p \cdot F_V / F_0}{1 + (1-p) \cdot F_V / F_0} \quad (\text{Paillotin 1978}),$$

$$\text{or } c_S = p \cdot F_V / F_0 \quad (\text{Strasser 1978, 1981}),$$

The correlation of the three different concepts is as follows:

$$\frac{1}{c_P} = \frac{1}{c_J} + \frac{1}{c_S} \quad \text{Eq. (A.2)}$$

where  $p$  = probability of excitation energy transfer among PS II units, and  $F_V$  = variable fluorescence yield ( $F_{(t)} - F_0$ ). In this paper, we have used the simplest of the three equations, *i.e.* that of JOLIOT and JOLIOT (1964), as has been done elsewhere (XU *et al.* 1989, GOVINDJEE *et al.* 1992) using the definition  $q_{(t)} = [Q_A^-]_{(t)} / [Q_A^-]_{t=0}^{\max}$ :

$$V_{(t)} = \frac{(1-p) q_{(t)}}{1 - p q_{(t)}} \quad \text{Eq. (A.3)}$$

It is obvious from Eq. A.3 that the relative variable fluorescence of the system under consideration is equal to the fraction of closed reaction centers if *no* energy transfer occurs from one PS II to another, *i.e.*,  $p = 0$ . However, if  $p$  has a positive value,  $V_{(t)}$  is a hyperbolic function of  $q$ . We have used both  $p = 0$  and  $p = 0.5$  in our analyses in this paper.

The transformation of the experimental available fluorescence  $V_{(t)}$  into  $q_{(t)}$  relative concentrations of  $Q_A^-$  can be done in different ways when different species of  $Q_A^-$  are considered in the model:

1. Transformation of the total experimental relative fluorescence  $V_{(t)}$  into an average total relative concentration of all species of  $Q_A^-$

$$q_{(t)} = \frac{[Q_{A_{\text{inactive}}}^- + Q_{A_{\text{open}}}^- + Q_A^- Q_B]}{[Q_{A_{\text{inactive}}}^- + Q_{A_{\text{open}}}^- + Q_A^- Q_B]_{t=0}} (t)$$

This type of conversion, however, should only be used if there is a guarantee that the relative variable fluorescence  $V_{(t)}$  of the sample versus the fraction of closed reaction centers  $q_{(t)}$  is a hyperbolic function according to Eq. (A.3). Using DCMU treated samples, the relative area growth of the kinetics ( $F_{\text{max}} - F_{(t)}$ ) can be taken as a measure for  $q_{(t)}$ . The plot  $V_{(t)}$  versus  $q_{(t)}$  should now show a hyperbolic shape as predicted by Eq. (A.3). This condition is only fulfilled in very rare and special cases. A deconvolution into different unit types already at the level of the fluorescence signal is required.

2. The total experimental variable fluorescence  $F_{(t)} - F_0$  is first considered as the sum of individual components of variable fluorescences which, in a second step, is individually converted into a relative concentration of a  $Q_A^-$  species by the fitting program. This type of transformation takes care of the heterogeneity of photosynthetic units already at the level of the fluorescence emission behaviour of each type of unit as reported earlier for small, big and grouped units (STRASSER 1981).

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