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A method for analysis of fluorescence induction curve from DCMU-poisoned chloroplasts

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The kinetic analysis of fluorescence induction curve from DCMU-poisoned chloroplasts involves the calculation of the growth of the complementary area above the curve with time. It has been shown that extreme care must be taken in the estimation of the maximum fluorescence yield (F_m), in order to obtain accurate values of the area and the rate constants. Prolonging illumination time to obtain more accurate F_m value, however, met the problems of finding an adequate time of illumination and the presence of very slow fluorescence rise or quench, which was not related to the redox changes of Photosystem II acceptors. In this report, a mathematical analysis method is introduced. It enabled us to determine the F_m value accurately from a fluorescence induction curve obtained after a relatively short period of illumination (2 s). Using the new method, we found that the rate constant of the slowest phase was much smaller than any previously estimated value. Moreover, we found that the fluorescence curve consisted of three instead of two phases. It appears that the existing hypotheses dealing with two phases may have to be re-examined.

Introduction

The fluorescence induction of chloroplasts in the presence of DCMU is thought to reflect the reduction of the primary electron acceptor of PS II, Q_A [1]. The induction apparently is not generated by a single first-order photochemical event. A method for analysis of the kinetics of the fluorescence induction has been worked out by Melis and Homman [2,3]. It involved the calculation of the growth of the normalized complementary area, defined by the fluorescence induction curve and the line parallel with the maximum level of fluorescence (F_m), with time. The semilogarithmic plot of such an analysis revealed two kinetically different phases: a rapid sigmoidal phase followed by a slow exponential phase. The nature of these two phases has been widely studied and attributed to the two forms of PS II, termed PS II_α and PS II_β, respectively [4–7].

Nevertheless, the determination of the area above the induction has a pitfall, the asymptotic level F_m. Bell and Hipkins [8] showed that an increase as small as 2% in the ratio of the maximum variable fluorescence to the maximum total fluorescence resulted in an increase in the area over 150%. Moreover, Sinclair and Spence [9], using theoretical curve, demonstrated that an error of as little as 0.6% in the estimate of F_m could cause the slope of the slow phase (k_β), which was derived from kinetic analysis of the area growth, to be overestimated by 42%. It is therefore clear that, in order to obtain accurate values of the complementary area and the rate constants of the fluorescence induction, extreme care must be taken in the estimation of the F_m value. Bell and Hipkins [8] and Sinclair and Spence [9] both stressed the importance of using long period of illumination (approx. 20 s) when performing fluorescence induction measurement. Their objective was to obtain a value as close to the 'correct' value of F_m as possible.

However, using long period of illumination may still have flaws. First, it is difficult to determine an adequate time of illumination. Sinclair and Spence [9] tried to extend their measurement until the results did not change any further. This was not entirely successful, since the rate constants of the phases decreased with longer period of illumination. They therefore had to regard their rate constant values as being too large. Furthermore, a very slow but significant increase of fluorescence has sometimes been observed over a time scale of about 1 min [8,10,11]. This increase, because of

Abbreviations: A_{norm}, normalized complementary area above the fluorescence induction curve; A_1, total complementary area; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethyl urea; F_m, maximum fluorescence yield; PS II, Photosystem II.

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its slowness, was believed not related to the redox changes of Q_A. On the other hand, very slow fluorescence quench has also been observed by some investigators [8,12]. These complicated variations in fluorescence make the F_m determination obtained after long-time illumination not dependable.

In this report, a mathematical analysis method is introduced. It enabled us to determine the F_m value accurately from a fluorescence induction curve obtained after a relatively short period of illumination (2 s).

Materials and Methods

Spinach was obtained from local market. Deveined leaves were ground for 20 s with a blender in a medium containing 0.4 M sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EDTA and 20 mM Tris-HCl (pH 7.8). The slurry was filtered through eight layers of cheesecloth followed by centrifugation at $5000 \times g$ for 5 min. The pelletted chloroplasts were resuspended in a medium containing 0.2 M sucrose, 10 mM NaCl, 5 mM $MgCl_2$ and 20 mM Tris-HCl (pH 7.8). Chlorophyll concentration was estimated according to Arnon [13]. All steps were performed at 4°C under dim light.

Fluorescence induction was performed at room temperature using a homemade fluorometer. Chloroplast suspension was dark-adapted for at least 30 min. After dilution of chloroplasts to 2 μg Chl per ml in a 1-cm cuvette, 10 μM DCMU and 2 mM NH_2OH were added. There was another 1-min dark period before the onset of illumination. Chloroplast sample was excited by a broadband blue light ($\lambda_{max} = 440$ nm, 2.5 W/m^2). In order to obtain fairly homogeneous illumination, a plane mirror was put on the opposite side wall of the cuvette to reflect the passed exciting light back to the cuvette. The fluorescence induction, started by the opening of a shutter (opening time, 2 ms), was collected at 90° by a photomultiplier (Model 7206, Oriel) shielded by an interference filter (685 nm, Schott). The signals were amplified and digitized by an A/D converter (12 bit resolution, 30 μs conversion time). The data were then stored and processed by a microcomputer (IBM PC/AT compatible). 6000 data points were obtained in 2 s period of measurement.

Results

Fig. 1 shows a fluorescence induction curve from DCMU-poisoned chloroplasts. The illumination time was 2 s.

To proceed with the kinetic analysis of Melis and Homann [3], the maximum fluorescence level (F_m) must be determined first. If F_m was obtained by taking the mean value of 100 data points around 0.8 s, we found that the fluorescence induction curve could be resolved into two kinetically different phases. The slower β-phase

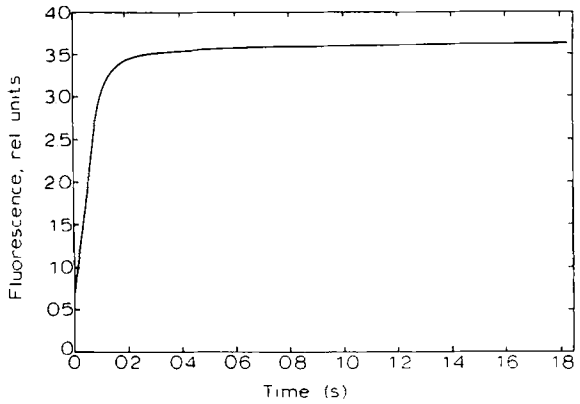

Fig. 1. The fluorescence induction curve of DCMU-poisoned chloroplasts. The measurement was carried out as described in Materials and Methods.

was an exponential phase with a rate constant (k_β) of 5.95 s^{-1}. The relative concentration (the relative contribution to the complementary area, β%) of PS II_β was about 29.3% of total PS II reaction centers. The faster α-phase was sigmoidal in shape, which revealed the cooperative nature of PS II_α, with a rate constant (k_α) of 17.6 s^{-1}. The rate constants and the relative concentrations of the two kinds of PS II obtained here were very similar to those of McCauley and Melis [14], in which the F_m value was also taken from the fluorescence yield at about 0.8 s (see Fig. 1) of Ref. 14).

However, if F_m was obtained by taking the mean value of 100 data points around 1.8 s, we obtained a different set of data. The rate constants were 2.16 s^{-1} and 16.8 s^{-1} and the relative concentrations were 28.8% and 71.2%, respectively. Only k_β was significantly lowered. Obviously, the value of k_β could become even lower, if F_m was taken from the data of longer illumination time, which presumably yielded a value closer to the 'correct' F_m value. Nevertheless, for the reasons given in the introduction section, long-time illumination should be avoided. Therefore, the question is: can we accurately determine the F_m value and the rate constants using data obtained from a relatively short time of measurement? Furthermore, it has been shown that a small error in the F_m determination would cause a very large error in the estimation of k_β. Hence, even though we try to determine F_m with care, it is still apt to obtain a 'wrong' k_β. It would be better then to reverse the analysis procedure, i.e., to determine accurately k_β first, then F_m.

We assume that the slow β-phase is a simple exponential phase. It can be written as

$$F(t) = F_\beta(1 - e^{-kt}) \quad (1)$$

where F_β is the maximum fluorescence of β-phase, k is the rate constant and t is time. The total complementary area (A_t) of this exponential curve is F_β/k. How-

ever, if we stop the measurement at a certain time T, then the total complementary area becomes

$$A_t = \frac{F_\beta}{k} - \frac{F_\beta \cdot e^{-kT}}{k} - F_\beta \cdot T \cdot e^{-kT} \quad (2)$$

and the complementary area as a function of time is

$$A(t) = \frac{F_\beta}{k} - \frac{F_\beta \cdot e^{-kt}}{k} - F_\beta \cdot t \cdot e^{-kT} \quad (3)$$

then

$$\ln(1 - A_{norm}) = \ln\left(1 - \frac{A(t)}{A_t}\right)$$

$$= \ln \frac{1 + k(T-t) - e^{k(T-t)}}{1 + kT - e^{kT}} \quad (4)$$

The derivative of Eqn. 4 with respect to t, which defines the slope, is

$$\text{slope}(t, T) = \frac{-k(1 - e^{k(T-t)})}{1 - e^{k(T-t)} + k(T-t)} \quad (5)$$

We thus obtain a slope function which depends on time t as well as the measuring time T. In principle, for a given T and a slope at t, we can calculate, from Eqn. 5, the rate constant k.

Since the fluorescence induction is not composed of a single phase, Eqn. 5 is valid only for the last slow exponential phase and when the inverse of the rate constant of the preceding fast phase is much shorter than the measuring time T, so that the fast phase has negligible influence on the last part of the fluorescence curve.

To demonstrate how Eqn. 5 could be used in the kinetic analysis of a fluorescence curve, we generated a theoretical curve, which was the sum of two exponential phases (α and β, see Fig. 2).

$$F(t) = F_\alpha(1 - e^{-k_\alpha t}) + F_\beta(1 - e^{-k_\beta t}) \quad (6)$$

The rate constants ($k_\alpha = 17\ s^{-1}$ and $k_\beta = 2\ s^{-1}$) and the relative contributions to the complementary area

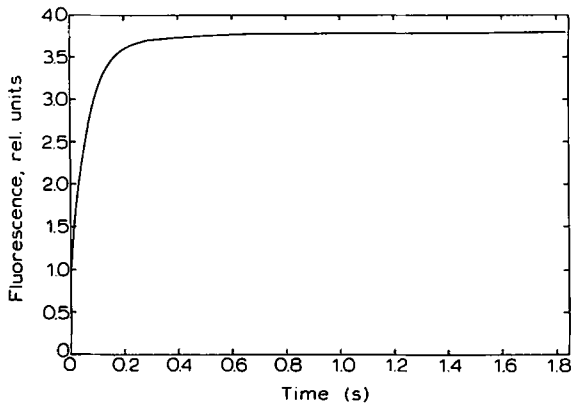

Fig. 2. The theoretical curve, which is the sum of two exponential phases. See text for detail.

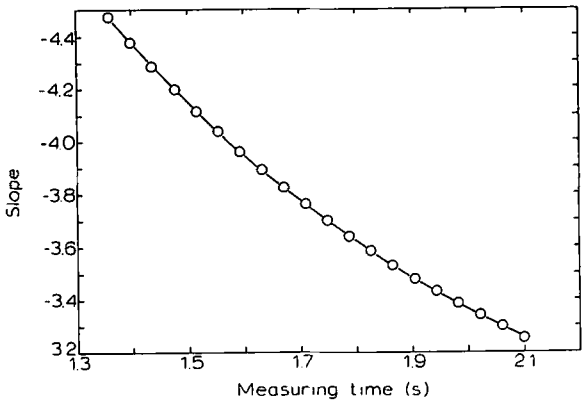

Fig. 3. The data points were derived from the theoretical curve of Fig. 2 using our analysis method. The curves of $\ln(1 - A_{norm})$ vs. time t were plotted for different measuring time T. The slope at $t = 0.6\ T$ of the curves vs. T are shown as data points. The curve was derived for Eqn. 5 with the rate constant $k = 0.2\ s^{-1}$ and $t = 0.6\ T$. See text for detail.

above the curve (70% of α-phase and 30% of β-phase) were approximations of the second set of data obtained above.

On the one hand, from Eqn. 5, if we let $k = 2\ s^{-1}$ and $t = 0.6$ T, we could generate a theoretical curve of slope as a function of the measuring time T as shown in Fig. 3. On the other hand, for any given T, we could, from the theoretical curve of Fig. 2, obtain a semilogarithmic plot of the $(1 - A_{norm})$ vs. time and calculate the slope at $t = 0.6\ T$. These are shown as data points in Fig. 3. Here, we chose to take the slope at $t = 0.6\ T$. In principle, t should be chosen as close to T as possible, in order to have the least overlap with the preceding fast phase. However, as t approaches T, $A(t)$ also approaches to the total complementary area A_t. In this case, $\ln(1 - A(t)/A_t)$ becomes very large and sensitive to the signal noise in real measurement. It usually makes the slope determination impossible. We found that $t = 0.6\ T$ was a proper position under our experimental conditions.

From Fig. 3, we can find that the data points fit the theoretical curve of Eqn. 5 well. Thus, from a fluorescence induction measurement, we can always obtain a set of data points similar to those shown in Fig. 3 simply by varying the measuring time T, then the rate constant of the slow phase can be determined from the curve of Eqn. 5 giving the best fit of these data points using computer.

Fig. 4 shows the result of such a process with the fluorescence induction curve taken from Fig. 1. The curve, which was derived from Eqn. 5 with the rate constant k_β equaled $1.1\ s^{-1}$, gave the best fit of the data points obtained with the measuring time T longer than 1.3 s. The k_β value was 5.4 times smaller than that of the first set of data obtained above.

The next step is to determine the 'correct' F_m value. There are several ways to do it. Only one method is discussed here. We have assumed that the last part of

fluorescence induction can be described by a simple exponential function. Since the measurement stopped at 2 s, and the fluorescence yield at 2 s ($F(2\text{ s})$) was lower than the 'correct' F_m value, then the last part of fluorescence curve could be written as

$$F(t) = F(2\text{ s}) + C(1 + e^{-k_\beta(2s-t)}) \quad (7)$$

where C is the difference between F_m and $F(2\text{ s})$. Thus, for any given t ($2\text{ s} > t > 1.3$ s under our conditions), we could obtain C by solving Eqn. 7. After averaging for many different t, the value of C was determined precisely.

After knowing the 'correct' F_m ($= F(2\text{ s}) + C$) value, the growth of the complementary area with time ($A(t)$) was calculated and normalized by the total complementary area, which is

$$A_t = \int_0^{2s} (F_m - F(t))\,dt + C/k_\beta \quad (8)$$

The semilogarithmic plot of $(1 - A_{norm})$ vs. time is shown in Fig. 5a. We found that the slow phase was linear all the way to 1.9 s. k_β was found to be 1.08 s^{-1}, which was very close to the value obtained by curve fitting as shown in Fig. 4. It thus confirmed our mathematical analysis method. The β% was 29%.

After subtracting the influence of the slow β-phase from the induction curve, we generated a new semilogarithmic plot of $(1 - A_{norm})$ vs. time (see Fig. 5b). The plot revealed that the remaining fluorescence curve was biphasic. We designated the slower phase as α–s-phase, with the rate constant ($k_{\alpha-s}$) to be 8.45 s^{-1} and the relative concentration (α–s%) to be 19.3%. Eliminating the influence of the α–s-phase, in addition to the first, from the induction curve, led to a new semilogarithmic plot of $(1 - A_{norm})$ vs. time (see Fig. 5c). The α-phase appeared to have the rate constant 17.3 s^{-1} and α% was 51%. Note that the downwardly concave curve, which corresponds to the sigmoidal shape of the initial part of fluorescence rise, suggests the cooperative nature of PS II_α.

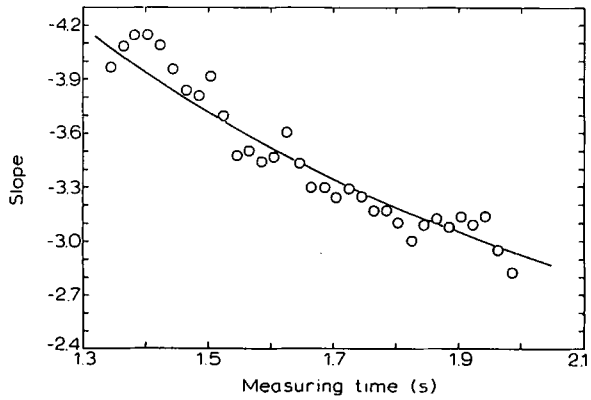

Fig. 4. The data points were derived from the fluorescence induction curve of Fig. 1 using our analysis method. The curve was derived from Eqn. 5, which, with $k = 1.1$ s^{-1}, gave the best fit of the data points.

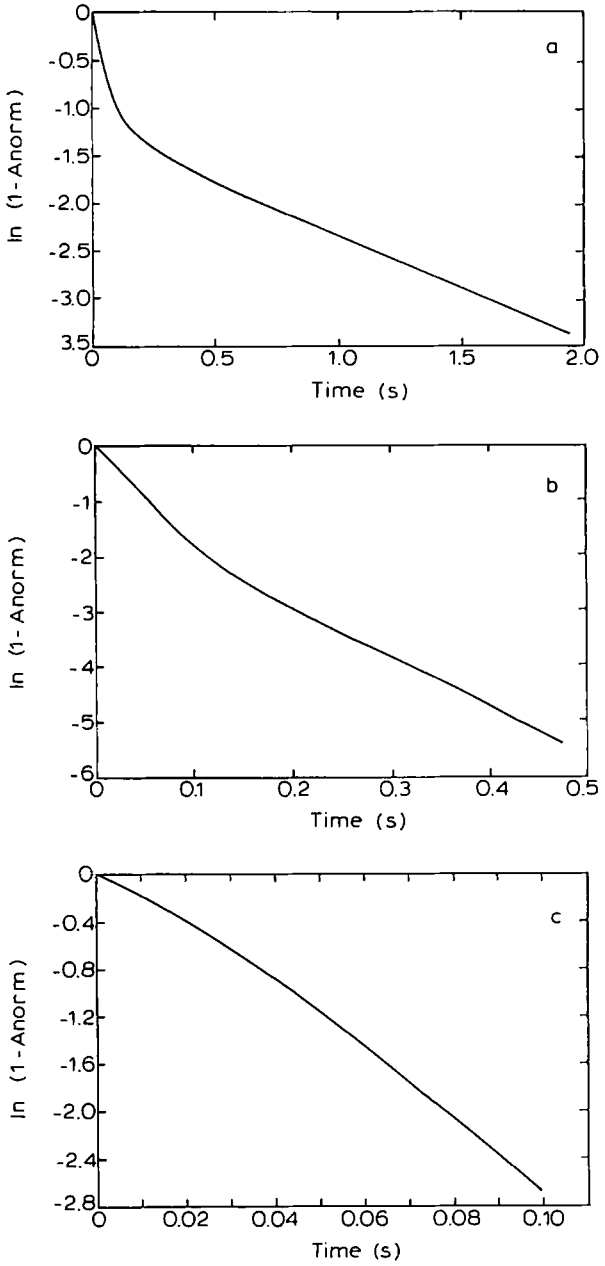

Fig. 5. (a) First-order analysis of the kinetics of the growth of the complementary area over the fluorescence induction curve of Fig. 1. The F_m value was determined using our analysis method. (b) First-order analysis of the kinetics of the area growth after eliminating the influence of the β-phase. (c) First-order analysis of the kinetics of the area growth of the α-phase after eliminating the influence of the α–s and β-phases.

Discussion

It was found that, in the analysis of the fluorescence induction curve from DCMU-poisoned chloroplasts, accurate determination of the F_m value is essential for obtaining correct value of the complementary area above

the fluorescence curve and the rate constants of the phases [8,9]. A long period of illumination when performing fluorescence experiment was proposed to solve this problem. However, the F_m determination after a long illumination time can become troublesome. This is due to the difficulty in finding an adequate illumination time, and sometimes the presence of very slow fluorescence rise or quench, of which the causes are still not clearly understood.

In this report, fluorescence measurement was made with a relatively short period of illumination. Nevertheless, the F_m value was determined accurately through mathematical analysis and curve fitting. The reliability of the analysis method was confirmed by using a theoretical curve with known F_m and rate constants. The only requirement is that the rate constant of the last slow exponential phase is much smaller than that of the preceding fast phase, so that the last segment of the curve, on which the fast phase has negligible influence, can be used for analysis. This requirement was satisfied when analyzing the fluorescence induction curve, because the rate constant of the preceding fast phase ($k_{\alpha-s} = 8.45\ s^{-1}$) is much larger than k_β ($1.1\ s^{-1}$). Under this condition, we found that the fluorescence curve obtained after 1.3 s (about 11 times of $1/k_{\alpha-s}$), when used for analysis, yielded satisfactory result.

It has been shown that the k_β is especially prone to error in the analysis of the fluorescence induction curve. If F_m is determined by the fluorescence yield at the end of a fluorescence measurement, then its value is always smaller than the 'correct' F_m value, and the k_β derived from it would always be overestimated. The earlier the fluorescence measurement is terminated, the smaller the F_m value, and the larger the k_β is resulted. It has been shown that an underestimation in F_m as small as 0.6% would cause overestimation in k_β by 42% [9]. On our hand, k_β was $5.95\ s^{-1}$ if F_m was obtained from the fluorescence at 0.8 s. It became $2.16\ s^{-1}$ if F_m was taken at 1.8 s. A continuous decrease of the slope with increasing measuring time is also demonstrated in Fig. 3 and 4 derived from the analysis of the theoretical curve (Fig. 2) and the fluorescence induction curve (Fig. 1), respectively. k_β equaled $1.1\ s^{-1}$ when our analysis method was employed. In contrast, β% was almost invaried. Thus, it was likely that the value of k_β was overestimated by many investigators.

Using our analysis method, we found that the fluorescence curve actually consisted of three phases: a fast sigmoidal α-phase followed by two exponential phases, termed α–s and β-phases, respectively. It was possible that the middle phase (α–s) was missed in the previous detection. This could be attributed to the overestimation of k_β, so that the value of $k_{\alpha-s}$ ($8.45\ s^{-1}$) was not very different from that of k_β ($5.8\ s^{-1}$).

An alternative interpretation of our data would be that the α-phase ($k_\alpha = 17.3\ s^{-1}$) and α–s-phase ($k_{\alpha-s} = 8.45\ s^{-1}$) actually corresponded to the α- and β-phases defined by Melis and Homann [2,3]. The ratio of the rate constants of their α- and β-phases, similar to the ratio of our k_α to $k_{\alpha-s}$, has been shown to be about 2. This has been independently confirmed by determining their k_β value from a measurement of the initial fluorescence yield increase from F_0 to F_{pl} in the absence of DCMU, a method that does not require the estimation of the F_m value [7,16]. Therefore, it was also likely that the very slow β-phase we found ($k_\beta = 1.1\ s^{-1}$), due to the overestimation of k_β, was not resolved from the α–s-phase in the previous analysis. This β-phase probably reflected the photochemical activity of a third as yet unreported group of PS II centers, which had very small light-harvesting antenna. It is worthy to note that there are reports on the existence of stable PS II units with antenna size as little as 50 and 37 chlorophyll molecules [16,17], compared to the antenna sizes of Melis' PS II_α (approx. 230) and PS II_β (approx. 100) [15]. In any case, it appears that the existing hypotheses dealing with only two phases (α and β) may have to be re-examined.

Sinclair and Spence [9], using long period of illumination to determine the F_m value, also found three phases in their fluorescence curves. However, the three phases they found were all simple exponentials. It is well known that the initial part of fluorescence rise is sigmoidal in shape. An exponential α-phase is hard to account for the shape of the curve. This raises a question about their analysis method. Moreover, the rate constants of the three phases they obtained were 9.0, 1.3 and $0.25\ s^{-1}$, respectively. These values, especially of the last two slower phases, were much smaller than our results, even though they regarded them as being too large. The discrepancy could not simply attribute to the different intensity of exciting light used, because we found that k_α was much more sensitive to light intensity than the other two rate constants (data not shown). The discrepancy probably was due to the different methods of analysis. They determined the F_m value after 18 s of illumination, which was 9 times longer than ours. The presence of very slow fluorescence rise in a 10th of a second may be account for the small rate constants they obtained.

Acknowledgements

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