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THE KINETIC RELATIONSHIP BETWEEN THE C-550 ABSORBANCE CHANGE, THE REDUCTION OF Q(ΔA_{320}) AND THE VARIABLE FLUORESCENCE YIELD CHANGE IN CHLOROPLASTS AT ROOM TEMPERATURE

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Summary

The light minus dark difference spectrum and the kinetics of the indicator pigment C-550 have been measured at room temperature in isolated, envelope-free chloroplasts in the presence of 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU). The C-550 spectrum indicates a band shift with peaks at 540 and 550 nm and has an isosbestic point at 545 nm. On the assumption of 400 chlorophyll molecules per electron transfer chain the differential extinction coefficient $\Delta\epsilon(540—550)$ is calculated to be approximately 5 $mM^{-1} \cdot cm^{-1}$. The kinetics of the C-550 absorbance change, occurring upon the onset of continuous illumination, are shown to be biphasic and strictly correlated with the kinetics of the complementary area measured from the fluorescence induction curve under identical conditions and with those of the absorbance increase at 320 nm due to photoreduction of Q. The light-induced change in these three parameters can be described as a function of the variable fluorescence yield change occurring under the same conditions. Such functions are non-linear and reveal a heterogeneous dependence of the variable fluorescence yield on the fraction of closed System II reaction centers.

It is concluded that for every molecule of the primary electron acceptor Q of Photosystem II that is photochemically reduced there corresponds an equivalent change in the absorbance of the indicator pigment C-550 and in the size of the complementary area. Thus, C-550 and area are two valid parameters for monitoring the primary photochemical activity of System II at room temperature.

Abbreviation: DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea.

Introduction

Knaff and Arnon [1] have demonstrated that the primary photochemical activity of System II is reflected by a reversible absorbance change in the green region of the spectrum. The compound responsible for this absorbance change is commonly referred to as C-550 after the wavelength at which it was first detected [1]. Butler and Okayama [2] established that at low temperature the absorbance change of C-550 is a typical band shift. Erixon and Butler [3] concluded from titration experiments that C-550 may be equivalent to the chlorophyll fluorescence quencher and that cytochrome *b*-559 serves as an electron donor for the photoreduction of C-550 at liquid nitrogen temperatures. It has been suggested that the storage of a negative charge on the primary electron acceptor Q of Photosystem II causes, through the influence of a local electric field, the electrochromic band shift of a pheophytin *a* molecule that is closely associated with the reaction center of Photosystem II [4].

The precise correlation of the kinetics of chloroplast fluorescence and of C-550 at room temperature was difficult to establish, mainly because of the small absorbance change of C-550, and therefore, the low signal to noise ratio and also due to the simultaneously occurring and overlapping large absorbance changes of other electron transfer components [5,6]. Thus there are reports in the literature [7,8] expressing doubts concerning the reliability of the C-550 absorbance change as a measure of Q reduction at room temperature.

The kinetics of photochemical energy conversion at System II, occurring upon the continuous illumination of 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU)-poisoned chloroplasts, are biphasic [9,10]. Such biphasic kinetics have been interpreted as underlying the existence of two different types of reaction centers at System II, the α centers and the β centers, the photochemical activity of which can be monitored kinetically [10,11]. Light-induced absorbance difference measurements in the ultraviolet have shown that both types of reaction center complexes reduce, in a primary photochemical step, a special plastoquinone molecule to its plastosemiquinone anion [10].

In this work we present an analysis of the kinetics of the C-550 absorbance change at room temperature occurring upon the continuous illumination of DCMU-poisoned chloroplasts. A comparison of the light-induced kinetics of the C-550 absorbance change with the kinetics of fluorescence induction and of Q photoreduction (ΔA_{320}) is presented. The results indicate that for every molecule of the primary electron acceptor Q that is photoreduced there corresponds an equivalent change in the absorbance of the indicator pigment C-550. It will be concluded that the α and β reaction centers of System II show the C-550 absorbance change upon reduction of their primary electron acceptor and that the area growth above the fluorescence rise curve is a reliable indicator for the reduction at both types of reaction centers.

Materials and Methods

Chloroplasts were isolated from leaves of peas (*Pisum sativum*) following a procedure previously described [10]. The reaction mixture contained chloroplasts suspended in the isolation buffer (0.4 M sucrose, 50 mM Tricine, 10 mM

NaCl and 5 mM $MgCl_2$ at pH 7.8), 20 μM gramicidin, 15 μM DCMU and 2.0 mM $K_3Fe(CN)_6$. DCMU and potassium ferricyanide were added simultaneously before the transfer of the mixture into the cuvette. The chlorophyll concentration of the sample was 25 μg/ml. Absorbance difference spectrum and kinetics of the indicator pigment C-550 were measured with a dual-beam dual-wavelength apparatus in which the two beams (measuring and reference) were fixed with respect to each other at a distance of 10 nm resulting from a double-exit slit of the same monochromator. The half-bandwidth of each of the two beams was approximately 2 nm. The two beams were chopped alternatingly at a frequency of 2500 Hz and passed through the same cuvette (1.0 cm optical pathlength). The resulting modulated photomultiplier signal was electronically resolved into the measuring and reference components and the light-induced difference between the two signals was recorded. Actinic illumination was provided in the red region of the spectrum by a combination of filters (a cut-off RG 630 Schott, a K-6 Balzers and a SP 658 short wavelength pass) having a half-bandwidth of 20 nm and a peak at 645 nm. The photomultiplier tube (EMI 9558 QB) was protected from the actinic illumination by CS 4-96 Corning filters. Fluorescence induction measurements were performed under identical experimental conditions with the same apparatus. In this case the photomultiplier tube was protected from the actinic light by a RG 715 Schott cut-off filter. To improve the signal to noise ratio the frequency response of the apparatus was limited to 30 Hz for the C-550 measurements. The signal to noise ratio was further improved in the kinetic measurements by using a signal averager (Nicolet Instrument Co., Model 527). Spectrophotometric measurements of Q reduction were performed with a single beam apparatus in the near ultraviolet (320 nm). The details of this apparatus and the method used have been presented elsewhere [10].

Results

The absorbance difference spectrum and the kinetics of the indicator pigment C-550 occurring upon the onset of continuous illumination, were measured with isolated chloroplasts in the presence of gramicidin, DCMU and potassium ferricyanide. Under these conditions the contribution of the transthylakoid field indicating absorbance changes [12] are eliminated and the cytochromes of the photosynthetic apparatus are in the oxidized state (Refs. 1, 10, 13, see also Fig. 1) along with a large fraction of the *P*-700 pool [10]. A chemically induced complete oxidation of *P*-700 is difficult to obtain. Under our conditions approximately 15% of the *P*-700 remained in the reduced state and, therefore, was photooxidized upon onset of illumination in parallel with the reduction of the primary electron acceptor of Photosystem II. This residual *P*-700 photooxidation did not interfere with our measurements in the green part of the spectrum (although of the order of $5 \cdot 10^{-5}$ ΔA at 545 nm) since the spectrum of *P*-700 is relatively flat in this region [14,15] and thus any significant contribution of *P*-700 and/or of other disturbances were eliminated by the dual-beam dual-wavelength method (see Materials and Methods). Under these conditions we measured the absorbance difference spectrum of Photosystem II activity in the green (510—570 nm) region where C-550 shows

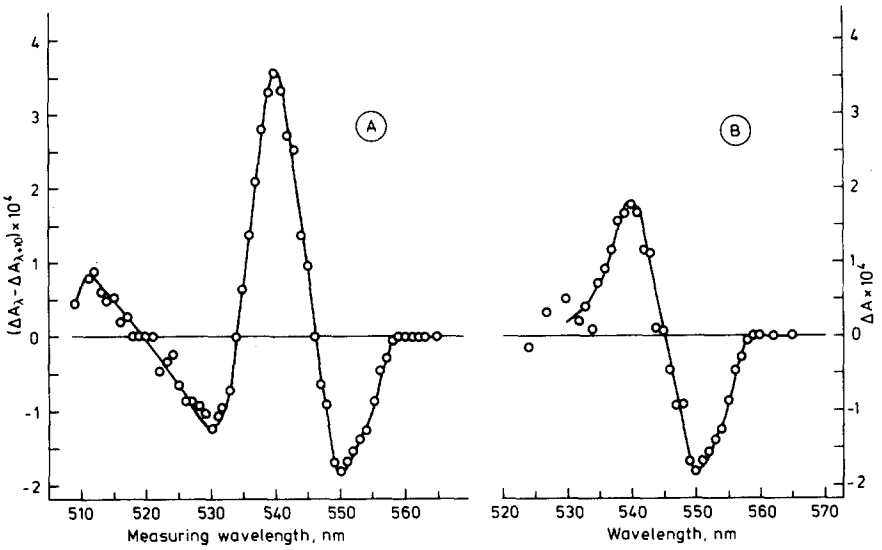

Fig. 1. (A) Light minus dark dual-beam difference spectrum of chloroplasts measured in the presence of 20 μM gramicidin, 15 μM DCMU and 2 mM $K_3Fe(CN)_6$. The abscissa indicates the wavelength position of the measuring beam while the reference beam was positioned at 10 nm distance towards longer wavelengths of the measuring beam. (B) Absorbance difference spectrum calculated from the data of (A). For other experimental conditions see Materials and Methods.

characteristic light minus dark absorbance difference changes.

The experimentally obtained double-beam difference spectrum of C-550 is shown in Fig. 1A. In such a presentation the abscissa represents the wavelength of the measuring beam and, therefore, the reading at a given wavelength, for example at 540 nm, indicates the amplitude of the value $\Delta A_{540} - \Delta A_{550}$. The spectrum of Fig. 1A was obtained by scanning both measuring and reference beams ($\Delta\lambda = 10$ nm) along the wavelength axis. Readings were taken at 1 nm intervals. Important features of the double-beam difference spectrum of Fig. 1A are the zero relative change for measuring wavelengths longer than 560 nm, the negative peaks at 530 and 550 nm ($\Delta A_{530} - \Delta A_{540}$ and $\Delta A_{550} - \Delta A_{560}$, respectively) and the positive peak of large amplitude at 540 nm ($\Delta A_{540} - \Delta A_{550}$). Secondary features include a positive peak at 510 nm ($\Delta A_{510} - \Delta A_{520}$) which is not of interest in this study. The light-induced difference spectrum can be calculated point by point from the double-beam difference spectrum of Fig. 1A. Such a calculation is based on the assumption that the absolute change in the wavelength region between 560 and 570 nm is zero. Although this assumption may not be strictly correct, it appears justified by the fact that the double-beam difference spectrum values ($\Delta A_\lambda - \Delta A_{\lambda+10}$) are zero for 558 nm $< \lambda <$ 580 nm. The absolute absorbance change ΔA_λ can then be calculated from the measured changes ($\Delta A_\lambda - \Delta A_{\lambda+10}$) by proceeding in steps of 1 nm from the long wavelength side of the experimental spectrum (i.e. 560 nm) to shorter wavelengths while adding the previously determined values at 10 nm longer wavelengths. With the above assumption of $\Delta A_\lambda = 0$ for 560 nm $< \lambda <$ 570 nm, it automatically follows that for 550 nm $< \lambda <$ 560 nm the calculated absolute absorbance changes plotted in Fig. 1B are identical to the experimental relative

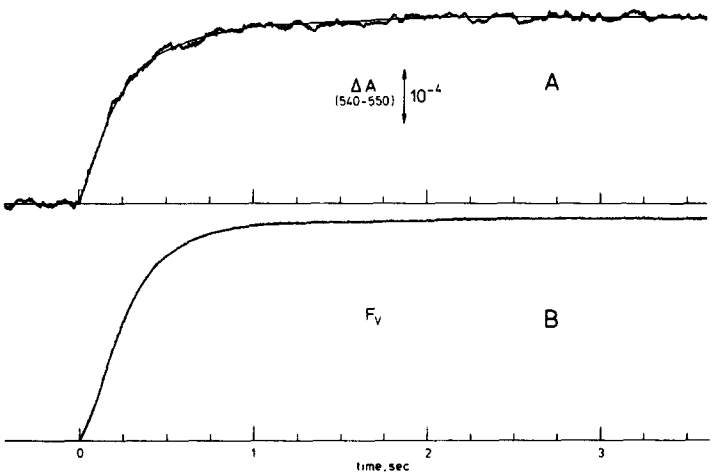

Fig. 2. (A) Kinetics of the C-550 absorbance change ($\Delta A_{540-550}$) induced by continuous illumination and (B) of the variable fluorescence yield of chloroplasts measured under identical conditions (onset of actinic illumination at zero time). The noisy trace represents the experimentally obtained data. The kinetic data of C-550 shown in Fig. 3 were obtained from the thin smooth superimposed line. The absorbance change measurements were obtained by averaging the kinetics of 36 freshly prepared samples. The fluorescence induction curve is the average of four measurements.

absorbance changes shown in Fig. 1A. The results of such an analysis of the experimental data of Fig. 1A is shown in Fig. 1B, which shows a typical C-550 band shift with maxima at 540 and 550 nm, isosbestic point at 545 nm and zero relative change at 560 nm [4,13]. The reliable deconvolution of the C-550 spectrum for wavelengths lower than 530 nm was difficult to obtain mainly due to the scattering of the experimental points in Fig. 1A. The differential extinction coefficient of the C-550 band shift $\Delta\epsilon(540-550)$, is calculated to be approximately 5 $mM^{-1} \cdot cm^{-1}$ assuming 400 chlorophyll molecules/reaction center of System II. This value agrees with the results of Knaff and Arnon [1] in chloroplasts but not with those of van Gorkom [4] in System II particles.

Using the above experimental conditions we measured the kinetics of C-550 ($\Delta A_{540} - \Delta A_{550}$) induced by a continuous actinic illumination (Fig. 2A). Fig. 3A shows the kinetics of C-550 plotted on a logarithmic scale. The logarithmic plot shows that the kinetics of C-550 display a biphasic pattern. The kinetics of the C-550 band shift measured at other wavelengths ($\Delta A_{530} - \Delta A_{540}$ and $\Delta A_{550} - \Delta A_{560}$) were also found to be biphasic and identical to those shown in Fig. 3A.

Under identical experimental conditions we also measured the induction curve of the chloroplast fluorescence. The variable part of the fluorescence curve (see Fig. 2B) is a measure of the rate of photoreduction of the primary electron acceptor of System II [16]. Information about the kinetics of photochemical energy conversion at System II can be obtained from the complementary area growth over the fluorescence induction curve [9]. In Fig. 3B a plot of the area growth is presented. A comparison of the data in Fig. 3A and B shows that the area growth and the C-550 absorbance change are kinetically identical processes. It was found that the rate of both phases in the kinetics followed the $I \times t$ law, indicating that the phenomena studied were of photo-

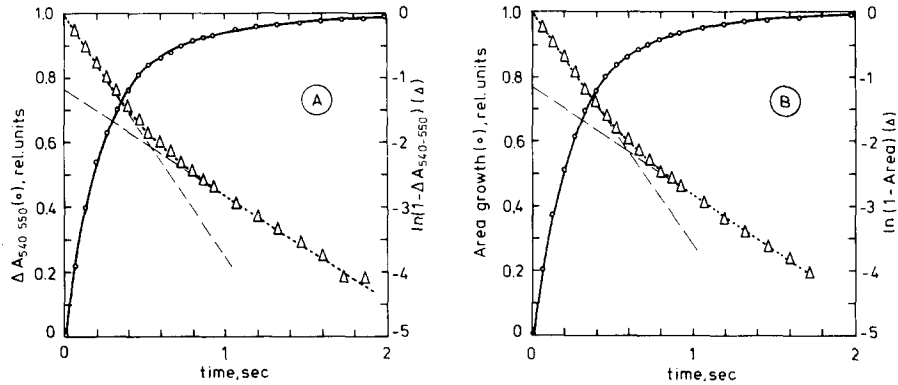

Fig. 3. (A) The kinetics of the C-550 absorbance change ($\Delta A_{540-550}$, circles) and a logarithmic plot (triangles) showing the biphasic pattern of its kinetics. (B) The time course of the complementary area growth (circles) and a logarithmic plot of the area growth data (triangles).

chemical nature. From the kinetic data of Fig. 3 we have calculated that the slow phase monitors the photochemical activity of approximately 35% of the System II reaction centers (see also Ref. 10). Considerations similar to those of Fig. 3 have shown that these 35% of the System II reaction centers controlled only about 15% of the total variable fluorescence emission (results not shown). An explanation of such results is given in the discussion.

The measurement of the fluorescence induction is the easiest of all parameters reflecting the primary System II activity in the presence of DCMU. Therefore, it is important that a direct comparison of the kinetics of the C-550 band shift with those of the variable fluorescence yield change provides an empirical expression of the function C-550 = f(F_v). Fig. 4A (circles) shows the kinetic relationship between C-550 and variable fluorescence yield. Fig. 4A (triangles) shows the kinetic relationship between the complementary area and the

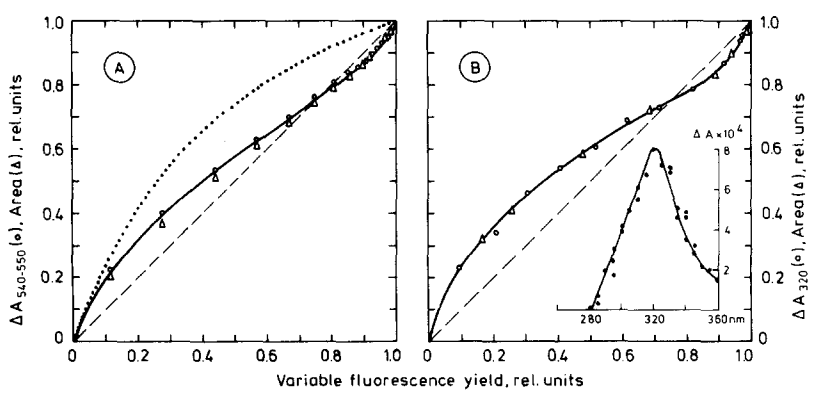

Fig. 4. (A) The relative C-550 absorbance change (circles) and growth of the complementary area (triangles) as a function of the corresponding variable fluorescence yield change. The dotted line represents the fraction of closed System II centers as a function of the relative fluorescence yield change calculated according to the statistical pigment bed model [19,22] for a value of the excitation energy trapping probability K_p = 0.65. (B) The fraction of reduced Q (ΔA_{320}, circles) and of the complementary area growth (triangles) as a function of the corresponding variable fluorescence yield change. The inset shows part of the light minus dark difference spectrum of the primary electron acceptor Q of Photosystem II.

variable fluorescence yield change. As already expected from the results of Fig. 3, the two functions (C-550 and AREA) display the same dependency on the variable fluorescence yield. Fig. 4A (circles) suggests that the relationship between C-550 and F_v is not a mathematically simple one. An initially large positive deviation from linearity is followed by a smaller negative one occurring at the end of the induction phenomenon. The positive and negative deviations from linearity of the function C-550 = f(F_v) can be directly attributed to the two kinetic phases shown in Fig. 3A and B and therefore, to the photochemical activity of the α and β reaction centers of System II.

The reduction of the primary electron acceptor Q of Photosystem II is accompanied by characteristic absorbance difference changes in the near ultraviolet (see insert of Fig. 4B and also Ref. 4). In a previous study [10] the kinetics of Q reduction have been measured in spinach chloroplasts and found to agree well with the kinetics of the complementary area growth. In this work, we show the experimentally obtained relationship between Q reduction (ΔA_{320}) and the variable fluorescence yield change (Fig. 4B, circles) and the relationship between the complementary area growth and the variable fluorescence yield change measured under identical conditions (Fig. 4B, triangles). Within the experimental error, the two phenomena are identical.

A direct comparison of the results of Fig. 4A and B shows that the kinetic patterns of Q reduction, C-550 absorbance change and AREA growth are very similar. It is indicated that all three phenomena can be validly used as a measure of the progress of the System II primary photochemistry. Furthermore, the results show that for every molecule of Q that is photoreduced there corresponds an equivalent change in the absorbance of the indicator pigment C-550 and an equivalent growth of the complementary area.

The empirical relationship of C-550, Q(ΔA_{320}) and AREA versus fluorescence yield obtained in Fig. 4 shows the fraction of System II centers closed as a function of the fluorescence yield change during the light-induced reduction of the primary electron acceptor of Photosystem II. It is apparent that there is a heterogeneous dependence of the fraction of closed System II reaction centers on the variable fluorescence yield change.

Discussion

The results of this investigation show that the absorbance change of the indicator pigment C-550 is a good monitor of the System II primary photochemical activity, at least under the conditions employed in this work. Investigations on the same subject have been performed in the past by other authors [7] who reached a conclusion different from ours. Ben-Hayyim and Malkin [7], using a camera shutter technique, reported that the kinetics of the C-550 absorbance change occurred in two distinct phases, a first photochemical one (following the $I \times t$ law) and a second thermal one. However, the most likely explanation of their results is that the thermal phase reflected the oxidation of cytochrome *f* which apparently was not fully oxidized by the preillumination treatment. This interpretation of their data is supported by their own difference spectra which show a peak at about 555 nm, an indication of cytochrome *f* oxidation and from the elimination of their thermal phase by ferri-

cyanide. Therefore, we believe that our two phases are contained in Ben-Hayyim and Malkin's first phase and were not resolved by their approach. The above criticism concerning overlapping cytochrome *f* changes does not apply to the work of Katoh and Kimimura [8] since their chloroplast samples had been pretreated with an adequate amount of potassium ferricyanide which effectively eliminated the oxidation-reduction reactions of cytochromes. They concluded that C-550 is a better indicator for the primary photoact of System II than the fluorescence yield since the latter, but not the former, was easily depressed by ultraviolet irradiation of the chloroplasts. It must be concluded, therefore, that the fluorescence yield is a more complex and a less direct monitor of the System II energy conversion and as such it should be used only with freshly isolated chloroplasts with unimpaired Photosystem II activity.

The present study also shows that the absorbance change kinetics of C-550 are biphasic in agreement with similar observations on the kinetics of $Q(\Delta A_{320})$ and the complementary area growth [9,10]. The analysis of the biphasic kinetic data has been discussed in detail in previous publications [9—11,17]. The findings of this work give new information about the structural organization of the α and β reaction center complexes. It may be concluded that the α and β centers of Photosystem II possess in addition to a special plastoquinone molecule that acts as a primary electron acceptor [10], a pigment molecule, possibly pheophytin [4], that is located near or at the reaction center, which shows a blue shift at 545 nm under the influence of Q^- and which could possibly play an important role in the System II primary photochemical event such as participation in the transitory transfer of an electron from the primary electron donor *P*-680 to Q [18]. Such a possibility has been suggested also from the light minus dark difference spectrum of Photosystem II which showed a previously unreported band at 430 nm [10].

The kinetic relationship between the fraction of the closed System II reaction centers and the variable fluorescence yield change depends on the organization and interaction of the different photosynthetic units, i.e. the bulk chlorophyll molecules that harvest and transfer the excitation energy to their respective reactions centers. In the past, several authors have suggested for the organization of the units of Photosystem II a common pigment bed matrix in which several reaction center complexes compete for the available excitation [19—22]. The predictions of such a statistical pigment bed model required a non-linear relationship between the fraction of closed System II centers and the variable fluorescence yield change such as the one shown by the dotted line in Fig. 4A. The experimental results of Fig. 4 show that there is only partial agreement with the expectations of the statistical pigment bed model. During the initial phase of the System II photochemistry the fraction of centers closed is considerably larger than the corresponding relative change of the fluorescence yield thus resulting in a 'positive deviation' from linearity in the Q^- vs. F_v plot which is in accordance with the statistical pigment bed model. However, in the later part of the curve a 'negative deviation' is observed, which does not agree with the predictions of the statistical pigment bed model.

The findings of this work and earlier results [10,17] suggest a basic difference in the photosynthetic unit structure and organization of the α and β centers. It appears that the α centers are imbedded in a statistical pigment bed

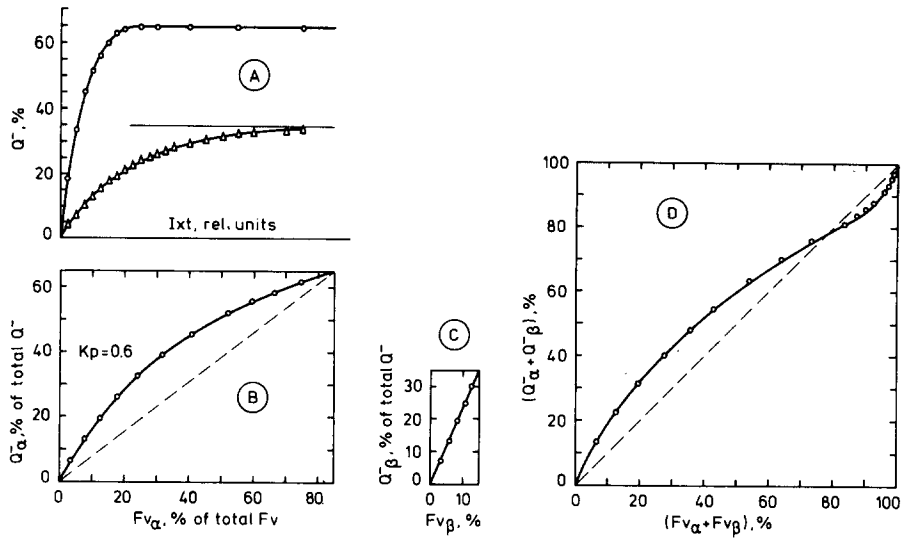

Fig. 5. (A) The solution of Eqn. 1 (circles) and Eqn. 2 (triangles) given in text. The value of cI_β (see Eqn. 2) was arbitrarily taken equal to the slope of the slow phase in the logarithmic plots of Fig. 3. (B) The relationship between Q^- and F_v according to the statistical pigment bed model and (C) according to the separate unit model. (D) Combination of the plots shown in (B) and (C) taking into account the time relationship (A).

while the β centers are located in separate units. Fig. 5 shows that with such an explanation the shape of the Q^- vs. F_v curve and particularly the 'negative deviation' in the later part of the plot can be satisfactorily explained. In Fig. 5 the construction of a theoretical Q^- vs. F_v relationship is based on the following assumptions (see also [10]): (a) the closing of the α and β centers occurs in parallel upon illumination, (b) the α and β centers exist at a relative concentration of 65 and 35%, respectively, (c) the effective optical cross-section of the α centers is three times that of the β centers, (d) α centers are imbedded in a statistical pigment bed and β centers in separate units, (e) the α centers control 85% of the variable fluorescence yield emission (F_v) while the β centers control the remaining 15% and (f) the interunit excitation energy transfer probability (or probability of trapping) K_p is 0.6 for the α centers. The photoreduction of the α centers will be described by Eqn. 1:

$$\frac{dQ_\alpha^-}{dt} = cI_\alpha \frac{K_p(1 - Q_\alpha^-)}{1 - K_p Q_\alpha^-} \tag{1}$$

where Q_α^- is the fraction of reduced α centers, c is a constant, I_α is the fraction of the absorbed light intensity available at the α centers and K_p the probability of excitation trapping ($0 < K_p < 1$). The exponential photoreduction kinetics of the β centers will occur according to the first-order rate law of Eqn. 2:

$$\frac{dQ_\beta^-}{dt} = cI_\beta(1 - Q_\beta^-) \tag{2}$$

where Q_β^- is the fraction of reduced β centers and I_β is the fraction of the absorbed light intensity available at the β centers ($I_\alpha = 3I_\beta$). Fig. 5A shows graphically the solution of Eqns. 1 and 2 for the final values $Q_\alpha^-(t: \infty) = 0.65$ and $Q_\beta^-(t: \infty) = 0.35$, respectively. Note that the complete reduction of Q_α occurs at a much shorter time than the reduction of Q_β. Fig. 5B shows the relative amount of reduced α centers as a function of the relative fluorescence yield change expected from the statistical pigment bed model. Fig. 5C shows the linear dependence of the relative amount of reduced β centers on the variable fluorescence yield as required by the separate unit model. Fig. 5D gives the relationship between the total fraction of reduced Q ($Q^- = Q_\alpha^- + Q_\beta^-$) as a function of the total variable fluorescence yield change ($F_v = F_{v(\alpha)} + F_{v(\beta)}$). For such a plot values of Q_α^- and Q_β^- that correspond in time were obtained from Fig. 5A while the respective fluorescence yield values $F_{v(\alpha)}$ and $F_{v(\beta)}$ were obtained from the diagrams of Fig. 5B and C. The outcome of such a synthetic presentation (Fig. 5D) agrees well with the experimental data of Fig. 4, showing initially a large positive deviation from linearity followed by a smaller negative one.

We conclude that the heterogeneity of Q photoreduction can be satisfactorily explained by a heterogeneity in organization of the pigment units serving two types of System II reaction centers. The experimental data support a model in which α centers are embedded in a statistical pigment bed and β centers are located in separate pigment units [17].

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