Studies on the Nature of the Primary Reactions of Photosystem II in Photosynthesis

I. The Electrochromic 515 nm Absorption Change as an Appropriate Indicator for the Functional State of the Photochemical Active Centers of System II in DCMU Poisoned Chloroplasts

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Photosynthesis, System II, Primary Reactions, System II-Indicator

The field indicating electrochromic 515 nm absorption change has been measured under different excitation conditions in DCMU poisoned chloroplasts in the presence of benzylviologen as electron acceptor. It has been found:

1. The amplitude of the 515 nm absorption change is nearly completely suppressed under repetitive single turnover flash excitation conditions which kinetically block the back reaction around system II (P. Bennoun, Biochim. Biophys. Acta **216**, 357 [1970]).
2. The amplitude of the 515 nm absorption change measured under repetitive single turnover flash excitation conditions which allow the completion of the back reaction during the dark time between the flashes (measuring light beam switched off) amounts in the presence of 2 μM DCMU nearly 50% of the electrochromic 515 nm amplitude obtained in the absence of DCMU. In DCMU poisoned chloroplasts this amplitude is significantly decreased by hydroxylaminhydrochloride, but nearly doubled in the presence of DCIP+ascorbate.
3. The dependence of the 515 nm amplitude on the time t_d between the flashes kinetically resembles the back reaction around system II. The time course of the back reaction can be fairly described either by a second order reaction or by a two phase exponential kinetics.
4. 1,3-dinitrobenzene (DNB) or α-bromo-α-benzylmalodinitril (BBMD) reduce the 515 nm amplitude in DCMU poisoned chloroplasts, but seem to influence only slightly the kinetics of the back reaction.
5. The dependence of the 515 nm amplitude on the flash light intensity (the amplitude normalized to 1 at 100% flash light intensity) is not changed by DNB.

Based on these experimental data it has been concluded that in DCMU poisoned chloroplasts the amplitude of the 515 nm absorption change reflects the functional state of photosystem II centers (designated as photoelectric dipole generators II) under suitable excitation conditions. Furthermore, it is inferred that in DCMU poisoned chloroplasts the photoelectric dipole generators II either cooperate (probably as twin-pairs) or exist in two functionally different forms. With respect to BBMD and DNB it is assumed that these agents transform the photoelectric dipole generators II into powerful nonphotochemical quenchers, which significantly reduce the variable fluorescence in DCMU-poisoned chloroplasts.

Introduction

In the electron transport pathway of the primary reactions of higher photoautotrophic organisms electrons are transferred from water to NADP$^+$ by the action of two light reactions operating in series. These reactions are coupled with the generation of ATP (for review s. ref. 1). At the centers of photosystem I and II the electronic excitation energy of chlorophyll a_{I}[2-5] and chlorophyll a_{II}[6-8], respectively, is transformed into the electrochemical energy of electrical dipoles anisotropically arranged perpendicular to the plane of the thylakoid membrane[9-12]. According to this functional mechanism the centers of photosystem I and II will be designated in the following as *photoelectric dipole generators* I and II, respectively.

The photoelectric dipole generators I and II are the fundamental operational units of the primary energy transformation step of photosynthesis. For

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Abbreviations: BBMD, α-bromo-α-benzylmalodinitril; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DNB, 1,3-dinitrobenzene.

the sake of efficiency the photoelectrical dipole generators I and II are coupled with two essential operational units (s. Fig. 1):

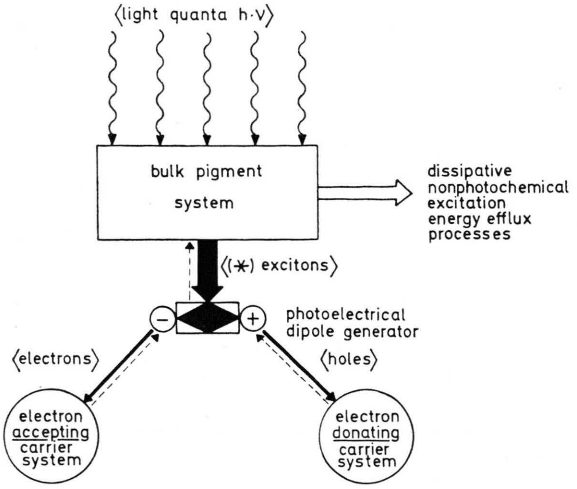

Fig. 1. Simplified scheme of the functional organization of the photosynthetic unit. For the details see text. Thick arrows indicate the main reaction pathway, thin broken lines indicate backfluxes of the electrical charges and excitons.

A. The *bulk pigment system* which regulates the *influx of excitations* generated by light quanta into the photoelectric dipole generators.
B. The *electron carrier systems* which regulate the *electrical charge* (electrons and holes) *efflux* out of the photoelectrical dipole generators.

Hence, the 3 operational units – bulk pigment system, photoelectric dipole generator and electron carrier systems – build up a molecular machinery (called photosynthetic unit) for the transformation of light quantum energy into electrochemical energy. One of the central problems of photosynthesis research is the elucidation of the mechanism of the molecular machineries of system I and II, respectively. Relatively little is known about the functional mechanism of the photosynthetic unit of system II. One way to attack the problem of the complex mechanism of photosynthetic unit II is the isolation of intact operational units and to analyze their properties.

The functional separation of the photoelectric dipole generator II connected with its bulk pigment system from the electron carrier systems is possible by the use of suitable chemical effectors. DCMU-type inhibitors[13–17] are known to block the electron efflux out of the photoelectric dipole generator II, whereas the functional integrity of the photoelectric dipole generator II per se remains practically uneffected[18, 19]. Because of the pecularities of the watersplitting enzyme system Y[20] – which requires the accumulation of 4 holes to be able to perform the water oxidation – the hole produced simultaneously with the electron at the photoelectric dipole generator II cannot be transformed into the terminal product oxygen in DCMU poisoned algae and chloroplasts. Furthermore, DCMU has been found to influence also the reactions in the watersplitting enzyme system Y[21–23]. Hence, DCMU-type inhibition provides a tool for the isolation of photoelectric dipole generators II connected with its quantum influx operational unit (bulk pigment system). In this way DCMU blocked chloroplasts should be very useful for the investigation of the mechanism of the photoelectric dipole generators II and the functional correlations between them and the bulk pigment system. For these studies an analytical method is required which allows the unequivocal determination of the functional state of photoelectric dipole generator II. In principle two different methods should be applicable:

a. The indirect methods using the degree of the dissipative nonphotochemical excitation energy efflux processes as an indicator of the functional state of photoelectric dipole generator II. The indirect methods are applicable only if there exists an exact stoichiometrical relation between the degree of a special dissipative nonphotochemical excitation energy efflux process and the level of functional activity of photoelectric dipole generator II.
b. The direct methods using the state of the functional elements of photoelectric dipole generator II itself as an indicator.

In the present paper we will show, that the amplitude of the field indicating electrochromic 515 nm absorption change can be used as a direct measure of the functional state of photoelectric dipole generator II. Furthermore, the action of substances which influence the functional connection between the bulk pigment system and the photoelectric dipole generator II is investigated and discussed.

Materials and Methods

Preparation of the chloroplasts

The chloroplasts were prepared from market spinach according to the method of Winget *et al.*[24], except that 10 mM ascorbate was present during the grinding of the spinach. For the storage in liquid nitrogen 5% dimethylsulfoxide was added. After thawing of the frozen chloroplasts, the activity (Hill reaction rate and average oxygen yield per flash) was nearly the same as for freshly prepared chloroplasts.

Reaction mixture

The standard reaction mixture for the measurements of absorption changes at 515 nm contained: Chloroplasts (10 μM chlorophyll), 20 mM N-tris(hydroxymethyl)-methylglycine (Tricine)-NaOH, pH 7.5, 100 μM benzylviologen, 10 mM KCl and 2 mM $MgCl_2$. For the oxygen measurements the reaction mixture contained chloroplasts (50 μM chlorophyll) and 0.3 mM $K_3[Fe(CN)_6]$ plus 0.3 mM $K_4[Fe(CN)_6]$ as electron acceptor instead of benzylviologen, other additions as for measurements of absorption changes.

Measurements

The absorption changes at 515 nm were measured with a repetitive flash spectroscopic technique similar to that published in ref. 6. The optical pathlength of the cuvette was 20 mm, the bandwidth of the measuring light 10 nm. The exciting xenon-lamp flashes (duration 20 μs) were passed through a Schott filter RG 1/2 mm, saturating light intensity was used. For the light saturation curve in Fig. 8 the flash intensity was reduced by grey filters Schott NG 5. The signals were averaged in a Fabri-Tek Mod. 1062. The electrical bandwidth of the apparatus ranged 0 – 5 kHz.

For the observation of the 515 nm-absorption changes under excitation conditions which allow the internal back reaction in system II completely or to some degree (in the presence of DCMU) the measurements were performed as indicated at the top of Fig. 4. The chloroplasts dark adapted for 30 s were preilluminated by a saturating flash f_p. At a time t_d after f_p the analyzing flash f_a of saturating intensity was fired. The 515 nm absorption change initiated by this analyzing flash f_a was detected. It was found that the extent of the measured absorption change does not alter for times $t_d \geqq 30$ s between f_p and f_a.

In order not to disturb the preillumination conditions the measuring light beam (intensity approx. 50 ergs/cm^2·s) was switched on only 0.2 s before the measuring (*i. e.* before f_a) by an electrical triggered shutter. The intensity was low enough so that practically it did not excite system II during these 0.2 s.

One second after the analyzing flash f_a the measuring light beam was switched off. In order to keep the photomultiplier in its working state at an anode current of 100 μA during the dark time when the measuring light beam is switched off, an auxiliary light beam (with equal intensity) was given onto the photomultiplier. At the time when the measuring light beam was switched on the auxiliary beam was switched off with an own shutter. Some uncontrolled differences in the light intensities of the two beams and the jitter between opening and closing of the two shutters caused some disturbances of the steady state current level of the photomultiplier. These disturbances were balanced during the 0.2 s until the analyzing flash f_a was fired.

The oxygen measurements were performed with a Clark-type electrode (IL 125 B Instrumentation Laboratory Inc., Watertown) by a repetitive technique as was described in ref. 25. All measurements were carried out at room temperature.

Results and Discussion

The amplitude of the field indicating 515 nm absorption change as an indicator for the functional state of photoelectric dipole generator II in DCMU blocked chloroplasts

Generally the photoelectric dipole generator II is the molecular arrangement of 3 functional elements:

a. A part which is able to build up – either *via* direct absorption of light quanta or *via* singlet-singlet-energy transfer – a localized excited electronic state. This part very probably consists of a special form of chlorophyll a, called Chl a_{II} [7, 8].

b. The negative pole (primary electron acceptor) designated by the symbol X 320 because it has been found, that the primary electron acceptor of system II is a special plastoquinone molecule identified in its reduced form as a semiquinone anion with an absorption band around 320 nm [26–29].

c. The positive pole (primary electron donor). The nature of this part is not unequivocally known. According to recently obtained results [8, 60] it is probable that elements a. and c. are realized by the same chlorophyll molecule (Chl a_{II}), as is already known for photoelectric dipole generator I, s. ref. 1. Hence, in the following we will designate the primary donor of system II as Chl a_{II}.

Therefore, irrespective of the spin multiplicity of the excited state (indicated by *) which ultimately leads to charge separation, the function of photoelectric dipole generator II can be described by:

$$\mathrm{X\,320\,Chl\,a_{II}} \xrightarrow{h\nu_{II}} \mathrm{X\,320\,Chl^*\,a_{II}} \xrightarrow{k} \mathrm{X\,320^-\,Chla_{II}^+}.$$

The transformation of an exciton into the geometrically separated electrical charges (electrical dipole $\mathrm{X\,320^-\,Chl\,a_{II}^+}$) is possible only, if both poles, X 320 and $\mathrm{Chl\,a_{II}}$, are discharged. Hence, the photoelectric dipole generator II is functionally active in the state $\mathrm{X\,320\,Chl\,a_{II}}$, whereas $\mathrm{X\,320^-\,Chl\,a_{II}^+}$, $\mathrm{X\,320^-\,Chl\,a_{II}}$ and $\mathrm{X\,320\,Chl\,a_{II}^+}$ represent functionally inactive states.

Excitation with a single turnover flash leads to transformation of all functionally active photoelectric dipole generators II into the inactive dipole state $\mathrm{X\,320^-\,Chl\,a_{II}^+}$. The dipoles $\mathrm{X\,320^-\,Chla_{II}}$; are anisotropically arranged perpendicular to the plane of the thylakoid membrane. Thus an electrical field is built up across the thylakoid membrane [9–12]. Therefore the fraction of functionally active photoelectric dipole generators II can be directly determined by measuring of one term of the following 3 terms: 1. The amount of reduced X 320 generated by a single turnover flash or 2. the amount of oxidized $\mathrm{Chl\,a_{II}}$ generated by a single turnover flash or 3. the size of the electrical field across the thylakoid membrane built up by a single turnover flash under conditions where all of the photoelectric dipole generators I are in a functionally inactive state before the flash.

Under these 3 terms the electrical field is most easily detectable. Hence, we have investigated the possibility to use the electrical field as an indicator of the functional state of photoelectric dipole generator II. It has been shown, that an electrical field across the thylakoid membrane can be measured on the basis of electrochromic absorption changes which extent is proportional to the electrical field strength [1, 30, 31]. These field indicating absorption changes are frequently detected at 515 nm. The rise time of the changes has been found to be $\leqq 20$ ns [10]. This fact support the conclusion that the generation of the electrical field is directly coupled with the function of the photoelectric dipole generators I and II, resp. [9]. In normal chloroplasts the electrical field decays in a time range >1 ms [30]. Therefore, because the rise time is shorter than the decay time by at least 4 orders of magnitude [10, 30], the initial amplitude of the field indicating 515 nm absorption change (in the folowing symbolized by ΔA_0) is not influenced by the decay kinetics. The observed initial amplitude is identical with the true initial amplitude ΔA_0 if the time resolution of the apparatus is high enough in order to observe the real decay kinetics. This condition is fulfilled by our flash photometer equipment.

Hence, the measured initial amplitude ΔA_0 caused by the analyzing flash is a direct measure of the number of electrical dipoles generated at the photochemically active centers I and II, resp. In this way ΔA_0 represents the number of photoelectric dipole generators which were functionally active just before the analyzing flash f_a. In order to avoid any misinterpretation, it should be emphasized that only the initial amplitude ΔA_0 is related to the functionally active state of the photoelectric dipole generators, because the decay of the electrical field across the thylakoid membrane is caused by ion transport processes which are determined by the membrane permeability only.

According to the current hypothesis about the action of inhibitors like DCMU [13–17] it is possible to block the photoelectric dipole generator II at its negative pole, that means to prevent the reoxidation of the reduced form of the primary electron acceptor $\mathrm{X\,320^-}$. Therefore, in the presence of DCMU only *one* single turnover in the photoelectric dipole generator II should be possible. However, it has been found, that in DCMU poisoned algae and chloroplasts $\mathrm{X\,320^-}$ can be reoxidized by the oxidizing side of system II *via* a slow internal back reaction [18]. Hence, in order to prove, that the amplitude ΔA_0 of the 515 nm absorption change can be used indeed as a suitable indicator of the functional state of photoelectric dipole generator II in DCMU blocked chloroplasts (the situation in algae is even more complicated), it remains to be shown that under conditions allowing the completion of the back reaction in repetitive single turnover flashes the full photosystem II induced 515 nm absorption change occurs, whereas under conditions avoiding the influence of the above mentioned back reaction the absorption change should disappear. According to earlier findings of Schliephake *et al.* [9] indicating that systems I and II, respectively, contribute to nearly the same degree to electrical field generation, it is anticipated, that the amplitude of 515 nm ab-

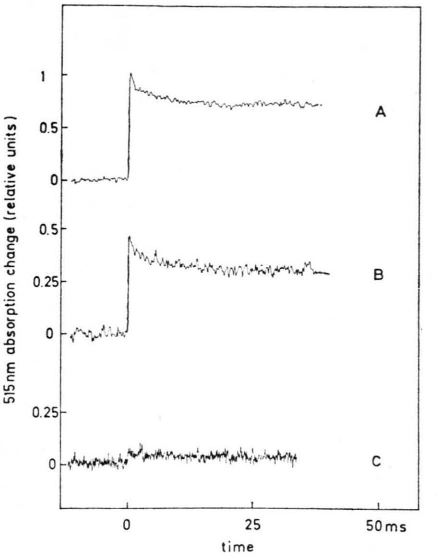

Fig. 2. Absorption change at 515 nm as a function of the time in spinach chloroplasts under different excitation conditions (s. Materials and Methods). A. without DCMU, time t_d between the flashes 30 s, measuring light beam switched on only for a short time for measuring of the contribution of each flash to the 515 nm absorption change without DCMU, 16 flashes averaged; B. with 2 μM DCMU, excitation as in A; C. with 2 μM DCMU, $t_d = 2$ s, measuring light beam on during the course of the experiment. Other experimental conditions as described in Materials and Methods.

sorption change in DCMU poisoned chloroplasts unter conditions of fully active back reaction amounts nearly 50% of the amplitude found in DCMU free chloroplasts. In Fig. 2 the 515 nm absorption changes measured under different conditions are depicted. The amplitude of the 515 nm absorption change obtained under repetitive single turnover flash excitation conditions is independent of the time t_d between the flashes in the range of $2 \leqq t_d \leqq 30$ s (irrespective of the duration of the weak detecting light beam) in the absence of DCMU, where practically all photoelectric dipole generators I and II are functionally active. This signal is shown in Fig. 2 A. The 515 nm absorption change is practically completely suppressed by 2 μM DCMU at $t_d = 2$ s and weak detecting light beam switched on during the course of the experiment (Fig. 2 C). On the contrary, if the detecting light beam is switched on only during the measurement of the signal of each flash (s. Materials and Methods) and at $t_d = 30$ s the 515 nm absorption change in the presence of 2 μM DCMU reappears to nearly 50% (Fig. 2 B) as has been expected.

However, these results are not sufficient to provide an unequivocal proof, that the amplitude ΔA_0 of the 515 nm absorption change measured in DCMU poisoned chloroplasts under suitable excitation conditions really reflects the functionally active state of the photoelectric dipole generator II. First it was to be shown that this signal indicates an electrical field across the thylakoid membrane. In Figs 3 A and 3 B it is seen, that the ionophor valinomycin accelerates drastically the decay of the 515 nm absorption change measured under back reaction supporting conditions in DCMU poisoned chloroplasts (a small remaining constant signal uneffected by valinomycin which is also found in DCMU free chloroplasts does not belong to the electrochromic absorption change, s. ref. 31). Hence, the measured 515 nm absorption change mainly reflects the electrical field across the thylakoid membrane.

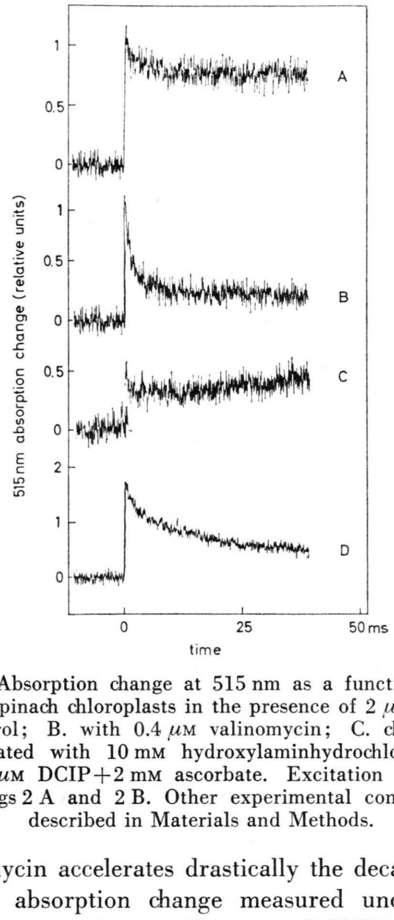

Fig. 3. Absorption change at 515 nm as a function of the time in spinach chloroplasts in the presence of 2 μM DCMU. A. Control; B. with 0.4 μM valinomycin; C. chloroplasts preincubated with 10 mM hydroxylaminhydrochloride; D. with 10 μM DCIP+2 mM ascorbate. Excitation conditions as in Figs 2 A and 2 B. Other experimental conditions as described in Materials and Methods.

Because of the contribution of photoelectric dipole generators I and II to the electrical field, the result of Fig. 2 B per se does not allow an unequivocal interpretation. This result can be explained by 3 different mechanisms:

A. During the dark time $t_d = 30$ s between the flashes the functional integrity of photoelectric dipole generator II could be fully restored by the above mentioned back reaction around system II, whereas photoelectric dipole generator I is nonfunctional because chlorophyll a_I (P 700) remains to be oxidized (the presence of benzylviologen as autooxidizable electron acceptor should prevent an electron cycling around system I, s. ref. 32).
   Hence, in this case the 515 nm signal of Fig. 2 B would be caused practically exclusively by the operation of photoelectric dipole generator II.
B. Photoelectric dipole generator II is nonfunctional, whereas oxidized P 700 could become reduced during the dark time of $t_d = 30$ s. In this case the observed 515 nm signal of Fig. 2 B would be an indicator of the action of photoelectric dipole generator I only.
C. In a hybrid mechanism including the partial function of mechanism A and B, respectively, both photosystems could contribute to the signal of Fig. 2 B.

A decision about the type of mechanism being responsible for the 515 nm absorption change of Fig. 2 B should be possible by the use of artificial electron donors. It is known that the back reaction around system II is partly suppressed by system II electron donors like hydroxylamine [18, 33]. Hence, a decrease of the amplitude ΔA_0 of the 515 nm absorption change – under the experimental conditions of Fig. 2 B – would be expected if mechanism A is realized. By contrast, the amplitude should remain constant for mechanism B because hydroxylamine cannot oxidize P 700 in the dark.

A completely reverse situation would arise in the presence of a system I electron donor like the couple DCIP + ascorbate. Since system I electron donors are not expected to influence the back raction around system II, an increase of the amplitude ΔA_0 of the 515 nm absorption change should be observed for mechanism A, but a constant amplitude for mechanism B.

In Fig. 3 the obtained results are depicted. It is seen that hydroxylamine significantly reduces the amplitude ΔA_0 of the 515 nm absorption change (Fig. 3 C), whereas DCIP + ascorbate nearly doubles the signal (Fig. 3 D). The DCIP + ascorbate effect strongly supports the assumption that mechanism A is practically exclusively responsible for the signal of Fig. 2 B. If this interpretation is correct one would expect that the dependence of the amplitude ΔA_0 of the 515 nm absorption change on the time t_d between the flashes should reflect the rate of the back reaction around system II which is known to occur in a few seconds [18, 19]. Furthermore, because the donor couple DCIP + ascorbate reacts very fast with oxidized P 700 [34] in comparison to the back reaction, the 515 nm amplitude ΔA_0 as a function of t_d should comprise two components of approximately the same amplitude: A time independent part (for $t_d \geqq 100$ ms) representing the function of photoelectric dipole generator I and a component with the same dependence of t_d as mentioned above indicating the kinetics of photoelectric dipole generator II restoration by the back reaction.

Fig. 4 (bottom curve, open circles) demonstrates that in the presence of 2 μM DCMU, but in the absence of system I electron donor, the amplitude ΔA_0

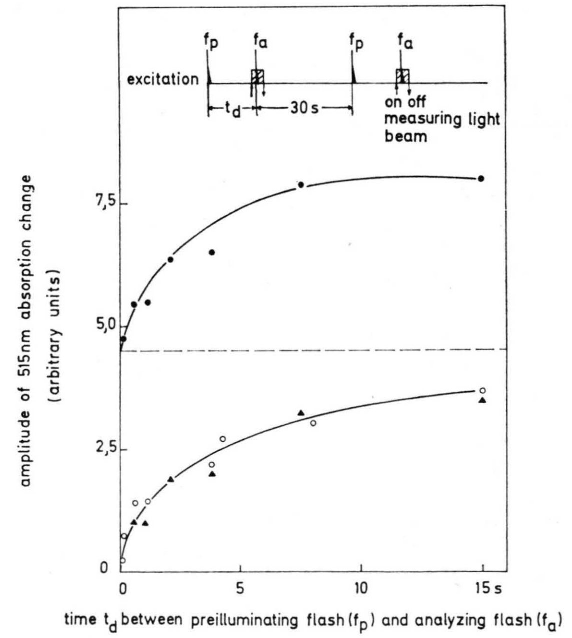

Fig. 4. Amplitude of the 515 nm absorption change as a function of the time t_d between the preilluminating flash f_p and the analyzing flash f_a in spinach chloroplasts in presence of 2 μM DCMU. ○, control; ●, with 10 μM DCIP +2 mM ascorbate; ▲, with 10 μM DCIP+2 mM ascorbate, separated time dependent phase. Excitation conditions as described in the top of the figure, 16 analyzing flashes were averaged. The amplitudes have been corrected by subtraction of the contribution of the valinomycin insensitive nonelectrochromic absorption change at 515 nm (s. text). Other experimental conditions as described in Materials and Methods.

of 515 nm absorption change has a restoration time of $\tau_{1/2} \approx 2$ s in correspondence with the kinetics of the back reaction around system II (the 515 nm amplitude ΔA_0 has been corrected for components which are not field indicating by the addition of valinomycin in the same way as is shown in Fig. 3 B.

In the presence of a system I electron donor (10 μM DCIP + 2 mM ascorbate) a two component kinetic pattern arises. The results of Fig. 4 (top curve, full circles) show that the amplitude of the time independent phase (independent for $t_d \geqq 100$ ms) indicated by the dotted line nearly equals that of the time dependent phase. Furthermore, it is seen that the kinetics of the separated time dependent component (closed triangles) and of the time dependent component found in the absence of system I electron donors are practically identical. Hence, it is proved, that in the presence of benzylviologen and DCMU only photoelectric dipole generator II is operative and that under these conditions its functional integrity is unequivocally determined by the amplitude ΔA_5 of the field indicating 515 nm absorption change.

The regeneration kinetics of the functionally active state of photoelectric dipole generator II in DCMU poisoned chloroplasts

After having shown that the amplitude ΔA_0 of the electrochromic 515 nm absorption change in DCMU poisoned chloroplasts directly reflects the functionally active state of photoelectric dipole generator II, we will now use this tool for the investigation of the kinetics of the back reaction leading to regeneration of this functionally active state. The kinetic pattern of the back reaction should provide information about the functional organization of photoelectric dipole generators II, because in DCMU poisoned chloroplasts the electrical charge efflux systems (plastoquinone pool and indirectly the water splitting enzyme system) are functionally separated from photoelectric dipole generator II. In principle, two different problems of functional organization have to be considered:

A. The *uniformity* problem;
B. the *cooperativity* problem.

To resolve the uniformity problem it remains to be clarified whether only one type of photoelectric dipole generators II does exist in DCMU poisoned chloroplasts. For the cooperativity problem one has to ask whether there exists any form of cooperativity between the photoelectric dipole generators II.

If there would exist only one type of photoelectric dipole generators II and if in addition they would act as noncooperative entities, the kinetics of the back reaction have to be of first order. In Fig. 5 the dependence of the valinomycin corrected 515 nm amplitude ΔA_0 (see Figs 3 A and B) on the time t_d between preilluminating and analyzing flash is given in a semilogarithmic plot. It is seen that a simple exponential kinetics do not arise. The experimental data can be described by a two phase exponential kinetics with half times of $\tau_{1/2} = 170$ ms and $\tau_{1/2} = 5$ s. A two phase first order kinetics favors the assumption of the existence of two different types of noncooperative photoelectric dipole generators II, each characterized by its own back reaction kinetics. The existence of two different types of configurations of the photoelectric dipole generator II has been already discussed by Vermeglio and Mathis [35] in order to explain their experiments of cytochrome b 559-oxidation at 218 °K. In their model the efflux rate of holes out of the photoelectric dipole generator II is determined by its configurational state. Etienne postulates the existence of two forms of photoelectric dipole generators II in DCMU blocked chloroplasts in the presence of hydroxylamine [36].

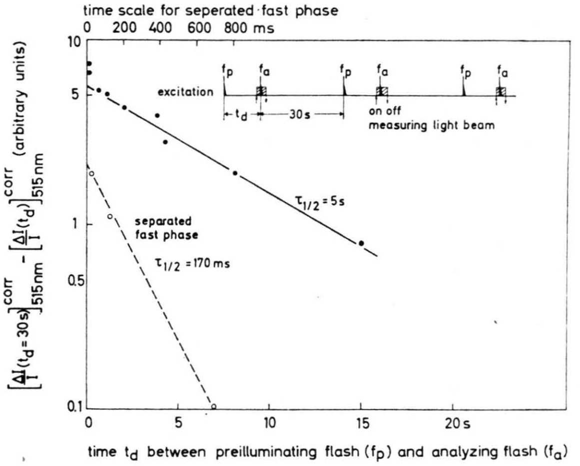

Fig. 5. Difference of the amplitudes of 515 nm absorption change at $t_d = 30$ s and at variable t_d, respectively, as a function of the time t_d between the preilluminating flash f_p and the analyzing flash f_a in spinach chloroplasts in presence of 2 μM DCMU. Experimental conditions and amplitude correction as in control experiment of Fig. 4 (open circles). Ordinate: logarithmic.

If one takes into account that DCMU not only blocks the electron efflux out of the photoelectric dipole generator II, but also influences the reactions of the holes[21–23], two functional states O and O′ can be postulated, which are characterized by back reaction half times of 170 ms and 5 s. Since the recombination rate between X 320⁻ and Chl a_{II}^+ at 77 °K has been determined to be 30 times faster[37] than the fast phase of the back reaction at room temperature, we can conclude, that the state O does not represent an ideal isolated photoelectric dipole generator II in the form

$$_{\mathrm{DCMU}}\,|\,\mathrm{X}\,320^-\,\mathrm{Chl}\,a_{II}^+\,|\,_{\mathrm{DCMU}}\,.$$

Therefore, if our assumption about the existence of two forms of photoelectric dipole generator II states O and O′ is correct, these forms are distinguished by the mode of reaction of the holes stored in the secondary donor systems (watersplitting enzyme system Y?) with the electron blocked at the negative pole X 320 of photoelectric dipole generator II.

However, as is shown in Fig. 6 (bottom curve) the results also fairly agree with a second order kinetics. A second order type back reaction is only possible, if there occurs a cooperativity between different photoelectric dipole generators II. The extensive studies of Joliot *et al.*[38] and Kok *et al.*[39] exclude any form of statistical cooperation of the holes generated by different photoelectric dipole generators II. Furthermore, the cooperativity of the electrons of photoelectric dipole generators II which occurs *via* the common plastoquinone pool[40] is interrupted in the presence of DCMU. Hence, we concluded that a cooperativity can occur only between a small number of photoelectric dipole generators II, In this respect it is interesting to know that DCMU titration experiments supported evidence for a possible connection of two photoelectric dipole generators II to a functional unit[41]. However, the present data do not provide sufficient evidence for the existence of a binary cooperativity.

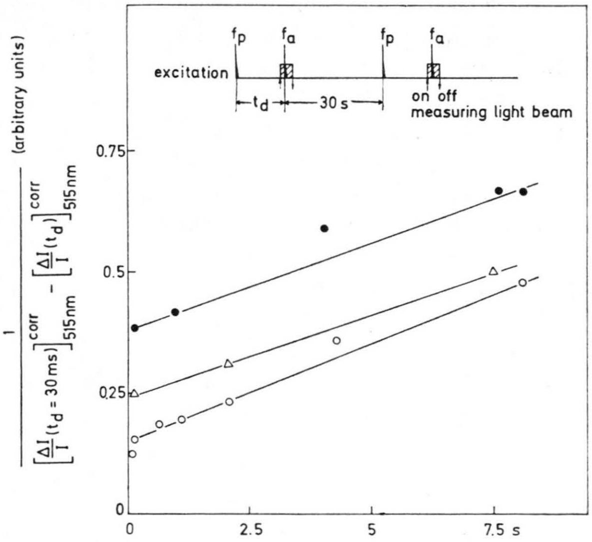

Fig. 6. Reciprocal value of the differences of 515 nm amplitudes at $t_d = 30$ s and at variable t_d, respectively, as a function of the time t_d between the preilluminating flash f_p and the analyzing flash f_a in spinach chloroplasts in presence of 2 μM DCMU. ○, control; △, with 0.4 mM DNB; ●, with 0.13 mM BBMD. Experimental conditions and amplitude correction as in control experiment of Fig. 4 (open circles).

In order to clarify the uniformity and the cooperativity problem further experiments are required. The present investigation clearly shows that in DCMU poisoned chloroplasts either two functional different forms of photoelectric dipole generators II exists or that a small number (probably 2) of them build up a cooperative functional unit.

The effects of DNB and BBMD on the functional interrelationship between the bulk pigment system and the photoelectric dipole generator II in DCMU poisoned chloroplasts

The functional connection between the bulk pigment system and photoelectric dipole generator II gives rise to a strong correlation between the exciton influx into the photoelectric dipole generator II depending on its functional activity state and the dissipative exciton decay processes in the bulk pigment system (s. Fig. 1). If there exists a strong stoichiometric relation between the different dissipative reactions, determined by the properties and the geometrical arrangement of the molecular elements of the bulk pigment system, the following relation should be valid (s. ref. 42):

$$\Phi^{\mathrm{II}}_{\mathrm{PDG}} + \frac{\Phi^{\mathrm{II}}_{\mathrm{k}}}{g^{\mathrm{II}}_{\mathrm{k}}} = 1 \tag{2}$$

where $\Phi^{\mathrm{II}}_{\mathrm{PDG}}$ = quantum yield of the photochemical reaction of the photoelectric dipole generator II, $\Phi^{\mathrm{II}}_{\mathrm{k}}$ = quantum yield of a specific dissipative exciton reaction of type k of the bulk pigment of system II and $g^{\mathrm{II}}_{\mathrm{k}}$ = stoichiometric coefficient. Quantum efficiency measurements[43–45] have shown that for functionally active photoelectric dipole generators II $\Phi^{\mathrm{II}}_{\mathrm{PDG}} \approx 1$, whereas for the functional inactive states $\Phi^{\mathrm{II}}_{\mathrm{PDG}}$ must be zero. It has been found that in normal algae or chloroplasts the variable part of fluorescence emission (k = fluorescence in Epn (2)) arising practically exclusively from system II sufficiently obeys Eqn (2). Therefore, irrespective of the details of fluorescence emission from system II, fluorescence measurements should provide information about the functional state of photoelectric dipole generators II. This has been confirmed by numerous experiments[46–48]. However, the situation becomes complicated if by chemical or physical procedures (*e. g.* by modification of fluorescence quenching properties) the stoichiometric coefficient g_k^{II} will be significantly changed. Among a number of aromatic compounds 1,3-dinitrobenzene (DNB) and *α*-bromo-*α*-benzylmalodinitril (BBMD) have been found to reduce remarkably the variable fluorescence in DCMU-poisoned chloroplasts[49–51]. This effect could be caused either by a modification of the fluorescence quenching properties of the photoelectric dipole generators II or by the creation of artificial energy traps in the bulk pigment system. With respect to quenching of variable fluorescence in DCMU blocked chloroplasts by a modification (chemically or physically induced) of photoelectric dipole generators II in principle two types of mechanism should be considered:

a. Photochemical quenching by a significant enhancement of the turnover rate of photoelectric dipole generator II. This could be achieved either by a drastical acceleration of the back reaction rate or by the action of BBMD or DNB as a powerful acceptor thereby regenerating the high electron efflux rate out of the photoelectric dipole generator II taking place in the absence of DCMU[27, 53].

b. Nonphotochemical quenching by a modified functionally inactive form of photoelectric dipole generatur II.

Hence, it would be useful to ask whether these agents influence directly the functional state of photoelectric dipole generator II. In Fig. 7 the effect of DNB on oxygen evolution and on the amplitude ΔA_0 of the electrochromic 515 nm absorption change is given, indicating that at concentrations $\leqq$ 0.5 mM DNB exerts no effects on the photochemical activities, at higher concentrations inhibitory effects arise. These results are in agreement with earlier findings[54, 55]. By contrast, in DCMU poisoned chloroplasts DNB seems to inhibit about 40% of the photoelectrical dipole generators II at rather low concentrations, whereas the other fraction remains uneffected up to 1 mM DNB. This could be a further hint for the existence of two types of functionally isolated photoelectric dipole generators II in DCMU poisoned chloroplasts. The kinetics of the back reaction presented in Fig. 6 in a second order plot (open triangles) is not seriously changed by DNB except for a small decrease of the rate. For a detailed analysis of the kinetic aspects further experiments are required.

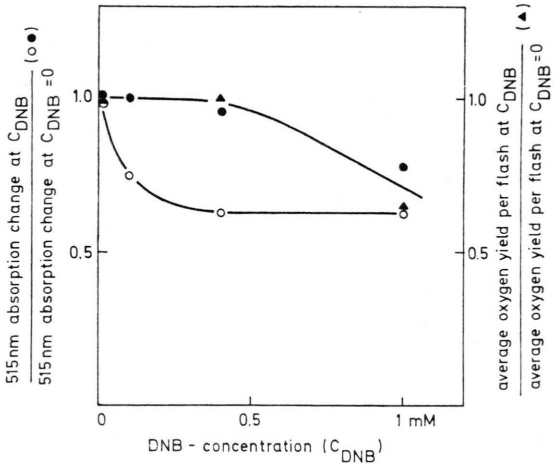

Fig. 7. Relative average oxygen yield per flash and relative amplitude of the 515 nm absorption change as a function of DNB-concentration. ▲, Relative average oxygen yield per flash; ●, relative amplitude of 515 nm absorption change in absence of DCMU; ○, relative amplitude of 515 nm absorption change in presence of 2 μM DCMU. Excitation for the oxygen measurements: 120 repetitive flashes, t_d = 500 ms. Excitation for the 515 nm absorption changes: Same conditions as in Figs 2 A and 2 B. Other experimental conditions as described in Materials and Methods.

A much more dramatic effect on the functional integrity of photoelectric dipole generator II is exerted by BBMD, as has been extensively discussed in part II of this series[56]. BBMD leads to a light dependent destruction of the photoelectric dipole generators II. However, the kinetics and the rate of the back reaction of the photoelectric dipole generators II remaining functionally intact seems not to be remarkably modified (s. Fig. 6, closed circles).

Because BBMD and DNB in DCMU blocked chloroplasts could not be confirmed to accept electrons directly from the photoelectric dipole generator II and because the back reaction rate is sufficiently accelerated neither by BBMD nor by DNB (for the exclusion of an extremely rapid back reaction s. discussion in ref. 56), we have to infer that a photochemical quenching *via* a modified photo-

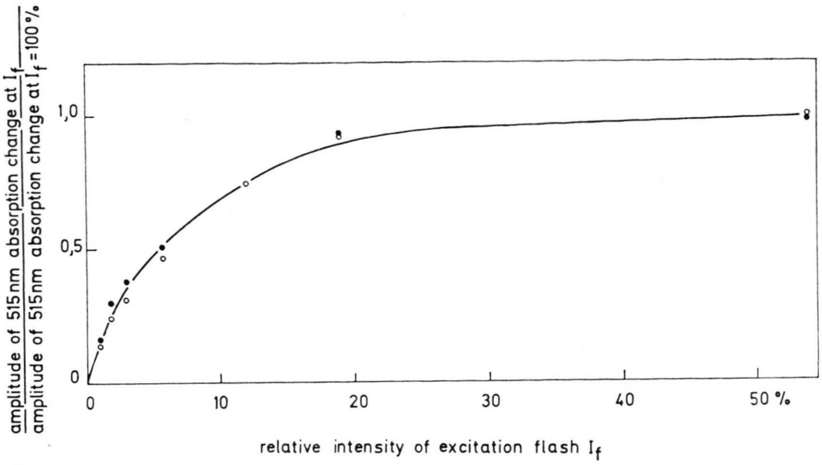

Fig. 8. Normalized amplitude ΔA_0 of 515 nm absorption change as a function of the flash light intensity. ●, Without DNB; ○, with 0.4 mM DNB. The amplitudes obtained at 100% flash light intensity are normalized to 1 (in the presence of 0.4 mM DNB the amplitude amonuts 65% of the control), flash intensity has been reduced by grey filters Schott NG 5. Excitation conditions as in Figs 2 A and 2 B. Other experimental conditions as described in Materials and Methods.

electric dipole generator II can be excluded. Hence, the DNB or BBMD effect could be explained either *via* a nonphotochemical quenching by photoelectric dipole generator II or *via* a bulk pigment phenomenon. If there would be imposed onto the bulk pigment system an energy leak by BBMD or DNB then the ratio of the exciton influx rate into photoelectric dipole generator II related to the rate of dissipative exciton decay in the bulk pigment system should decrease. This effect should cause a decrease of the quantum yield of the photoelectric dipole generator II-reaction which could be indicated by a diminished initial slope in the dependence on the flash light intensity of the 515 nm absorption change. However, Fig. 8 shows, that the normalized curves are practically identical in the absence and the presence of DNB. Therefore, we conclude that the fluorescence quenching induced by lower DNB concentrations in DCMU blocked chloroplasts does not occur mainly by insertion of "DNB-traps" into the bulk pigment system, but rather by transforming the photochemical electric dipole generators II into a form which exerts a powerful nonphotochemical quenching effect. This is in agreement with conclusions drawn by other [54, 55].

Further information about the interrelationship between bulk pigment system and photoelectric dipole generators II should be obtained by the investigation of the regulatory exciton efflux carotinoid reaction, indicated by the fast 515 nm absorption change of type I [57, 58]. This aspect will be discussed in a forthcoming paper [59].

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