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

II. The Modification of the Functional Integrity of the Photochemical Active Centers of System II by α-Bromo-α-benzyl-Malodinitril and Accompanying Effects on Chloroplasts

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Photosynthetic Electron Transport, System II, α-Bromo-α-benzyl-malodinitril-Effects

The effect of α-bromo-α-benzylmalodinitril (BBMD) on the oxygen evolution and on the absorption changes at 515 nm and 704 nm has been investigated in spinach chloroplasts. It has been found:

1. Under repetitive flash excitation conditions, where the back reaction around system II is practically excluded for kinetical reasons, BBMD does not restore the 515 nm absorption change in DCMU poisoned chloroplasts.
2. Under single flash excitation conditions, where the back reaction around system II becomes prominent in the presence of DCMU, BBMD moderately inhibits this back reaction. The deleterious effect is pronounced by preillumination with short flashes during the BBMD incubation period of the chloroplasts in the absence of DCMU.
3. Incubation of the chloroplasts with BBMD leads to an activity loss of oxygen evolution which increases with the time t_d between the repetitive short excitation flashes and with the dark incubation time. Preillumination during the incubation period with BBMD significantly enhances the effect.
4. In the absence of artificial electron acceptors BBMD suppresses in DCMU poisoned chloroplasts the 704 nm absorption change reflecting an internal cyclic electron flow around system I. On the other hand the linear electron transport at system I mediated by DCIP plus ascorbate as electron donor couple and benzylviologen as electron acceptor is not disturbed by BBMD.
5. BBMD incubation of chloroplasts accelerates the decay rate of the field indicating 515 nm absorption change.

Based on these experimental findings the conclusion has been drawn, that — in contrast to the assumption of Brandon and Elgersma (Biochim. Biophys. Acta **292**, 753–762 [1973]) — BBMD does not accept electrons from the primary electron acceptor X 320 of system II in DCMU poisoned chloroplasts. BBMD rather acts as a system I electron acceptor.

Furthermore, BBMD exerts deleterious effects on the center of photosystem II, accompanied by a weak ADRY-effect on the water-splitting enzyme system Y. As a tentative explanation of the BBMD-action on system II it is assumed that BBMD transforms the photochemical centers of system II into dissipative energy sinks.

Introduction

Within the photosynthetic electron transport chain of higher photoautotrophic organisms system II plays a central role because it generates by light excitation strong oxidizing equivalents which are able to use water (*via* a complex enzyme system, s. ref. 1) as the natural electron source for the reduction of $NADP^+$ (for review s. ref. 2). The functional integrity of native system II electron transport from water to plastoquinone pool can be selectively modified by different classes of chemical effectors (s. Fig. 1): Inhibitors (DCMU- or Tris-type), donors or acceptors and ADRY agents. However till now no substance has been found which directly and rapidly accepts electrons from the primary

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Abbreviations: ADRY, *A*cceleration of the *D*eactivation *R*eactions of the water-splitting enzyme system *Y*; BBMD, α-bromo-α-benzylmalodinitril; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenolindophenol; DPC, 1,5-diphenylcarbazide; MV, methyl viologen.

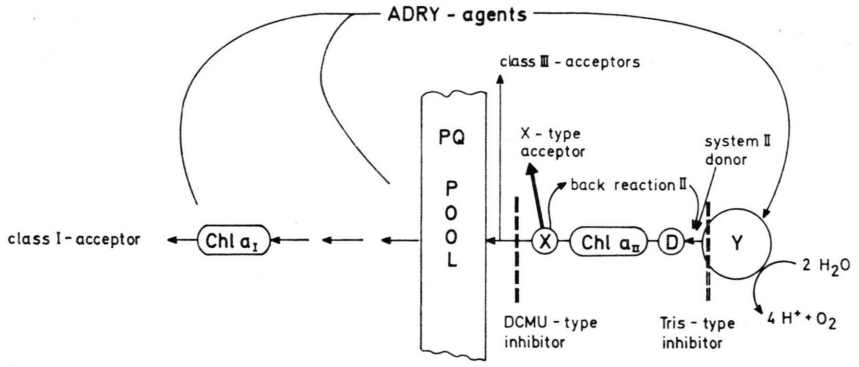

Fig. 1. Simplified scheme of system II electron transport and its modification by exogeneous effectors. Chl a_I, chlorophyll a_I; PQ, plastoquinone pool interconnecting at least 10 electron transport chains [3]; X, electron acceptor X 320 [4, 5]; chl a_{II}, chlorophyll a_{II}; D, electron donor [1, 6]; Y, watersplitting enzyme system [1], class I and class III electron acceptors [7, 8], system II donors [9–11], DCMU-type inhibitors [12–15], Tris-type inhibitors [16,17] and ADRY-agents [18,19] are modifiers of native system II electron transport, back reaction occuring between primary electron acceptor X and donor D [20, 21].

acceptor of system II, identified by Witt and coworkers as to be a special plastoquinone molecule called X 320 [4, 5]. In the following we will designate an agent able to accept electrons from the primary system II acceptor X 320 as an X-type acceptor. The discovery of an X-type acceptor would be desirable for the investigation of system II, because a combination of an X-type acceptor with a DCMU-type provides an unequivocal functional separation of system II from the overall electron transport thereby avoiding the application of harmful isolation procedures [22–24]. An ideal X-type electron acceptor is characterized by two properties:

1. It should accept electrons from X 320 with a transfer rate comparable to that of electron transport from X to PQ which has been found to occur with a half time of 0.6 ms [4, 25].
2. It should not interfer with the electron transport from water to X 320.

Because of these properties, DCMU would not inhibit system II electron transport mediated by an X-type electron acceptor. Recently it has been inferred indirectly – mainly based on fluorescence and oxygen uptake measurements – that α-bromo-α-benzyl-malodinitril (BBMD) acts as an X-type acceptor [26]. However, as has been discussed in part I of this series [27] and as has been already shown by Joliot *et al.* [28], fluorescence data do not allow an unequivocal determination of the functional state of system II electron transport. Hence, we reinvestigated the effect of BBMD in order to clarify the action of BBMD on system II.

By the use of the 515 nm absorption change which indicates under suitable conditions the functional integrity of system II [27] we came to the conclusion that BBMD does not act as an exogeneous X-type acceptor. BBMD rather acts as an inhibitor of system II accompanied by the introduction of an energy wasting leakage mechanism on photosynthetic units II. Furthermore, BBMD enhances the permeability of the thylakoid membrane. Based on measurements of light induced absorption changes at 704 nm the conclusion has been drawn that BBMD acts in addition as an electron acceptor of system I.

Materials and Methods

The spinach chloroplasts were prepared according to the method of Winget *et al.* [29] as is described in part I of this series [27].

Reaction mixture

The standard reaction mixture for the oxygen measurements contained chloroplasts (50 μM chlorophyll), 0.3 mM $K_3[Fe(CN)_6]$ + 0.3 mM $K_4[Fe(CN)_6]$ as electron acceptor, 10 mM KCl, 2 mM $MgCl_2$ and 20 mM N-tris(hydroxymethyl)-methylglycine (Tricine)-NaOH, pH = 7.5. For the measurements of absorption changes at 515 nm and 704 nm, resp., the reaction mixture contained chloroplasts (10 μM chlorophyll) and benzylviologene as electron acceptor (100 μM) instead of ferri- and ferrocyanide, other additions as for the oxygen measurements.

Measurements

The oxygen measurements were performed with a Clark-type electronde (IL 125 B Instrumentation La-

boratory Inc. Watertown) by a repetitive technique as is described in ref. 18.

The absorption changes at 515 nm and at 704 nm were measured with a repetitive flash spectroscopic technique similar to that published in ref. 30. The signals were averaged in a Fabri-Tek Mod. 1062 plus 952. Frequently the electrical bandwidth ranged from 0 – 5 kHz. The details of the measuring device for the analysis of the back reaction in the presence of DCMU are described elsewhere [27]. The optical pathlength was 20 mm, the bandwidth of the monitoring light was 10 nm. The exciting xenon-lamp flashes were passed through a Schott filter RG 1/2 mm for the 515 nm-measurements and through a Schott filter BG 28/4 mm for 704 nm-measurements. The flash duration was approx. 20 μs, saturating intensity.

For the exclusion of fast field indicating absorption changes at 515 nm with decay kinetics in the time range of microseconds (see Results and Discussion) ultra short flashes with a duration of 0.4 μs [31] were used in combination with an electrical bandwidth of 500 kHz.

All measurements were carried out at room temperature.

Results

The effect of BBMD on DCMU inhibited system II electron transport

In order to decide whether BBMD acts as an X-type acceptor it remains to be shown in which way system II electron transport is influenced by DCMU-type inhibition. For this investigation an unequivocal indicator for system II electron transport is required. As has been discussed in part I [27] and in l. c. [28] the variable fluorescence yield which is practically exclusively caused by system II fails as an indicator for system II activity under certain conditions. Similarly measurements of oxygen evolution would not provide conclusive data if in addition BBMD selectively influences the watersplitting enzyme system Y or if a Mehler type reaction [32] would be superimposed by BBMD. It has been shown elsewhere [27] that the amplitude ΔA_0 of the electrochromic 515 nm absorption change indicating the electrical field across the thylakoid membrane [33, 34] can be used as an indicator for the functional integrity of the primary events of system II electron transport. In Fig. 2 a a typical 515 nm signal under standard conditions is shown. DCMU-type inhibition completely abolishes the signal under

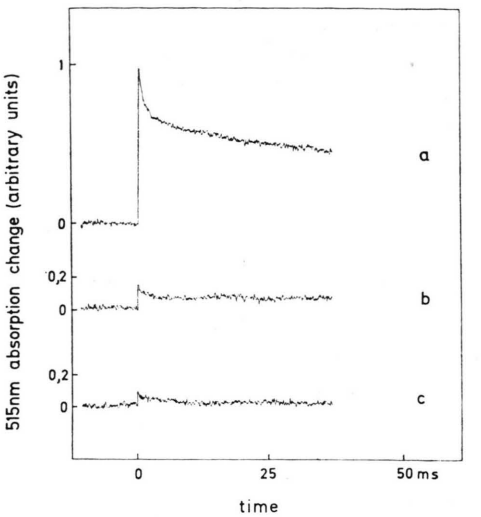

Fig. 2. Absorption change at 515 nm as a function of time in chloroplasts of spinach. a. Without DCMU and BBMD; b. with 2 μM DCMU; c. with 2 μM DCMU+50 μM BBMD. 16 signals were averaged, time t_d between the flashes 2 s. Other experimental conditions as described in Materials and Methods.

repetitive flash excitation (time t_d between the flashes short in comparison to the time of the back reaction, s. ref. 27), s. Fig. 2 b. Recently it was clearly shown [35] that in the presence of DCIP + $K_3[Fe(CN)_6]$ only system II is functionally operative under repetitive flash excitation conditions and that under these circumstances the amplitude of 515 nm absorption change amounts nearly 50% of the amplitude measured if both light reactions are active. Hence, if BBMD really would act as an ideal X-type acceptor, in the presence of BBMD about 50% of the normal 515 nm signal should reappear in DCMU inhibited chloroplasts due to the restoration of system II electron transport which is known to contribute to 515 nm absorption change to the same degree as system I electron transport [36]. However, as Fig. 2 c shows, we did not observe any restoration of the 515 nm signal by BBMD in the presence of DCMU. Hence, we conclude that BBMD cannot act as an ideal exogeneous X-type electron acceptor. Because of the limited time resolution of every flash photometer equipment (our fast apparatus has a time resolution of 1 μs) from a theoretical standpoint an X-type acceptor action of BBMD cannot be totally excluded. However, an X-type acceptor function of BBMD would be in correspondence with the experimental findings of Fig. 2 c only, if simultaneously two aditional conditions are satisfied:

a. The electron transfer from X 320 to BBMD occurs in a time shorter than the time resolution of the measuring apparatus.
b. The reduced form of BBMD has to penetrate the thylakoid membrane as a negatively charged anion (radical?) extremely fast in order to decay the electrical field (instantaneously built up by the action of the electric dipole generators, s. ref. 27, 37) at a rate exceeding the time resolution of the measuring device.

These strong conditions are probably not realized (s. Discussion) so that we exclude an X-type acceptor function of BBMD.

If the earlier reported decrease [26] of electron transport to exogeneous electron acceptors like DCIP or ferricyanide cannot be explained by a competitive X-type acceptor function of BBMD one would anticipate that BBMD exerts inhibitory effects.

The effect of BBMD on oxygen evolution and on the system II electron transport

Generally 3 different sites of inhibition of system II can be distinguished (s. Fig. 1):

1. Inhibition of the electron transport on the reducing side of system II, *i. e.* blockage of charge transfer between the primary electron acceptor X 320 and plastoquinone pool (DCMU-type inhibition).
2. Inhibition of the electron transport on the oxidizing side of system II (Tris-type inhibition), *i. e.* blockade of charge transfer between the electron donor D of system II and the water-splitting enzyme system Y.
3. Inhibition of the function of photochemical active reaction center II itself (X-Chl a_{II}), designated as photoelectric dipole generator II because its function is the charge separation leading to the formation of an electric dipole arranged perpenticular to the thylakoid membrane, s. ref. 27.

Furthermore, the loss of biological activity by an exogeneous effector can occur in two different modes of action:

a. True inhibitory-type activity loss.
   The inhibitor I binds to a specific reactive site (or sites). This inhibitory effect arises comparatively rapidly and depends on the fraction p of reactive inhibitory sites occupied by I. Frequently this effect is reversible.
b. Denaturation-type activity loss.
   This effect arises relatively slowly (minutes to hours). It is caused by a structural denaturation (often irreversible) of large parts or of the whole ensemble (*e. g.* the whole water-splitting enzyme system Y is destroyed by chaotropic agents, s. ref. 38).

Hence, in respect to an inhibitory effect of BBMD mainly two questions has to be answered: A. Where is the site of BBMD action? B. Which mode of activity loss is caused by BBMD?

DCMU-type inhibition is characterized by a light induced fast rise of variable fluorescence yield to a high level [39–41]. However in correspondance with Brandon and Elgersma [26] we did not observe such an effect in the presence of BBMD. Hence, the conclusion has been drawn that the mode of action of BBMD does not resemble that of a DCMU-type inhibitor. In order to decide whether BBMD acts as a Tris-type inhibitor or as an inhibitor of photochemical active system II reaction center itself comparative studies of oxygen evolution and 515 nm absorption change has been performed.

In Fig. 4 the effect of BBMD on the average oxygen yield per flash in a sequence of illumination periods of short flashes (s. Fig. 3) is shown. During the course of the experiments in the absence of BBMD a slight denaturation-type activity loss occurs due to aging processes. The slower decrease of the average oxygen yield per flash at $t_d = 100$ ms is explainable by the functional coupling of different electron transport chains *via* a common plastoqui-

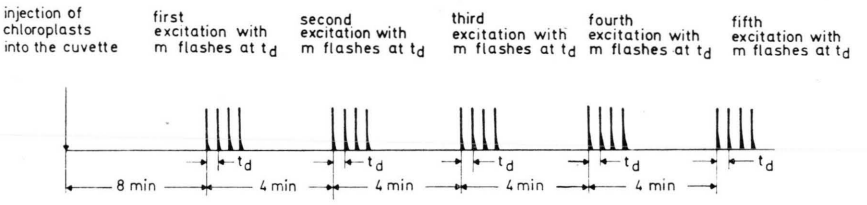

Fig. 3. Flash excitation conditions for the experiments of Figs 4–6.

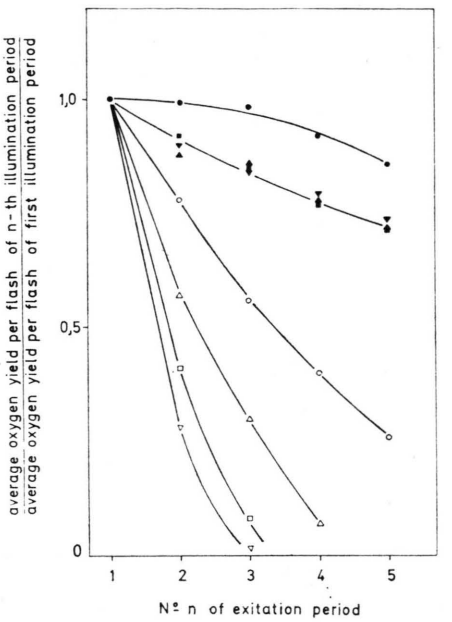

Fig. 4. Relative average oxygen yield per flash as a function of the number of the flash light excitation period and of the time t_d between the flashes in the absence and in the presence of 250 μM BBMD (○ ●, t_d = 100 ms; △, ▲, t_d = 250 ms; □ ■, t_d = 500 ms; ▽ ▼, t_d = 1000 ms) full symbols indicate experiments without BBMD, open symbols those with 250 μM BBMD. 120 flashes per illumination period (s. Fig. 3). Other experimental conditions as described in Materials and Methods.

none pool [3] and by the fact that at $t_d = 100$ ms the effect of the rate limiting step of whole electron transport is not fully eliminated. Hence, a small decrease of the number of functional intact water-splitting enzyme systems Y does not change the average oxygen yield per flash at shorter times t_d. In the presence of BBMD two effects are observed: a. The decrease of the average oxygen yield per flash with increasing illumination periods is significantly pronounced indicating a denaturation-type activity loss due to the action of BBMD. b. The activity loss is strongly dependent on the time t_d between the flashes. These results favor the assumption of the contribution of a light dependent reaction to the activity loss. However, the decrease of the average oxygen yield per flash with increasing time t_d between the flashes and with increasing illumination period could be explained also by an ADRY effect (s. ref. 18) increasing in its efficiency with duration of the BBMD incubation. If the observed decrease of oxygen yield would be exclusively caused by an activity loss phenomenon then the amplitude of 515 nm absorption change should be congruent with the average oxygen yield per flash in its dependence on the time t_d between the flashes and on the number n of illumination period.

As is shown in Fig. 5, in the absence of BBMD the amplitude ΔA_0 of 515 nm absorption change remains constant, in contrast to the observed decrease of oxygen yield per flash (s. Fig. 4). The

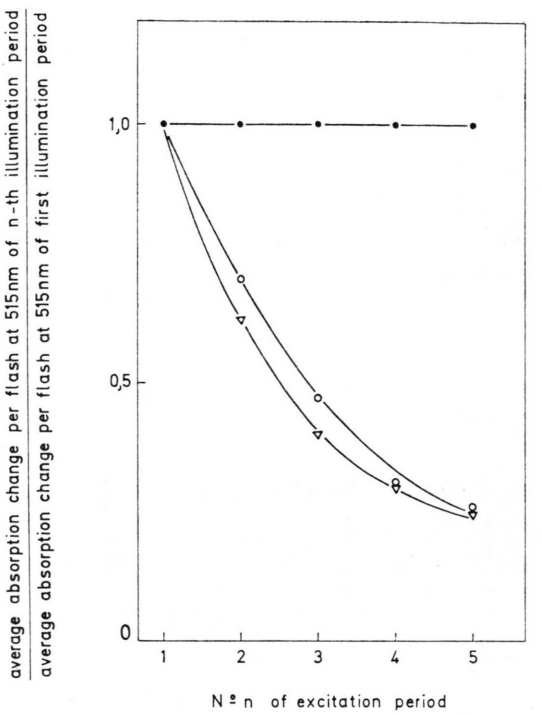

Fig. 5. Relative average amplitude ΔA_0 of the field indicating 515 nm absorption change as a function of the number of the flash light excitation period and on the time t_d between the flashes in the absence and in the presence of 250 μM BBMD. (○ ●, t_d = 100 ms; ▽, t_d = 1000 ms; open and full symbols as in Fig. 4, without BBMD the relative average amplitude remains constant for t_d = 100 ms and 1000 ms, respectively.) 128 flashes per illumination period (s. Fig. 3). Other experimental conditions as described in Materials and Methods.

different behaviour can be explained by the existence of internal donors which are able to feed electrons into system II if the watersplitting enzyme system Y is destroyed (similar effects arise in Tris-washed chloroplasts, s. ref. 42). On the other hand, in the presence of BBMD the amplitude ΔA_0 of 515 nm absorption change drastically decreases with increasing illumination period n, but the dependence on the time t_d between the flashes becomes very small. Hence, one can conclude, that BBMD exerts as well a denaturation-type activity loss as an

ADRY-type effect. The ADRY like effect is shown in Fig. 6 for different incubation conditions. In all cases the average oxygen yield per flash decreases with increasing t_d (curves a – c), whereas the amplitude ΔA_0 of 515 nm absorption change is independent of t_d (curve d). The decrease rate of the oxygen yield (curve a) increases with dark incubation time (curve b) and is more pronounced by preillumination (curve c).

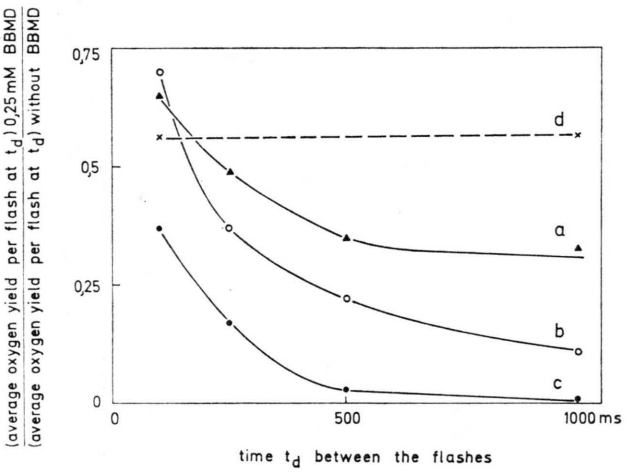

Fig. 6. Average oxygen yield per flash in the presence of 250 μM BBMD related to the average oxygen yield per flash in the absence of BBMD as a function of the time t_d between the flashes. (▲, First illumination period after 8 min dark incubation with 250 μM BBMD; ○, first illumination period after 16 min dark incubation with 250 μM BBMD; ●, third illumination period, 16 min after addition of chloroplasts to the 250 μM BBMD containing suspension,s. Fig. 3). 120 flashes per illumination period, other experimental conditions as described in Materials and Methods. Dotted line and crosses represent the corresponding values for the relative average amplitude of 515 nm absorption change in the presence of 50 μM BBMD (the molar ratio of BBMD : Chl was the same as is described for the oxygen measurements (see Materials and Methods). First illumination period (16 flashes) after 8 min dark incubation.

The interpretation of oxygen measurements in the presence of BBMD could be complicated if BBMD would act as an electron acceptor competing with $K_3[Fe(CN)_6]$ and if in addition the reduced form of BBMD would be reoxidized by O_2 more efficiently than by $K_3[Fe(CN)_6]$. Under these conditions an oxygen uptake would be superimposed on the oxygen evolution, so that the measured net oxygen production would not represent the real amount of oxygen evolved by system II. However, a BBMD induced oxygen uptake is probably very small in the presence of relatively high concentrations of $K_3[Fe(CN)_6]$ (under our conditions for oxygen measurements the concentration of O_2 is lower than the $K_3[Fe(CN)_6]$ concentration by at least two orders of magnitude). Hence, we assume, that the oxygen measurements are not significantly influenced by a possible BBMD induced oxygen uptake.

The ADRY-type effect indicates that BBMD modifies the watersplitting enzyme system Y. Hence, it could be possible that the inhibitory effect also restricts to system Y (Tris-type inhibition). However, in agreement with the results of Brandon and Elgersma [26] we observed, that the electron transport of system II cannot be restored by donors like hydroxylamine or 1,5-diphenylcarbazide (results not shown here). This result supports the conclusion that the inhibitory effect induced by BBMD is located close to the photochemical active center of system II. In order to show, that really the reaction center itself is modified by BBMD, we investigated the influence of BBMD on the system II activity which is restored after illumination of DCMU inhibited chloroplasts due to the comparitively slow back reaction [20] including only the primary electron acceptor X 320 and the primary electron donor D of system II, respectively (s. Fig. 1).

In order to eliminate practically a kinetical limitation of the restoration of system II caused by back reaction, the time t_d between the flashes was 30 s and the measuring beam of weak light intensity has been switched on only during the measurement of the contribution of each flash to the 515 nm absorption change (single flash excitation condition, for details and apparative realization s. ref. 27).

In Fig. 7 the amplitudes ΔA_0 of the 515 nm absorption change, representing the number of active system II generators under single turnover flash excitation conditions and in the presence of DCMU and of a suitable system I electron acceptor (s. ref. 27), have been presented for different BBMD incubation conditions. Dark incubation of chloroplasts in the presence of BBMD (with or without DCMU) significantly reduces the number of functional intact system II electric dipole generators as is shown by comparison of Fig. 7 A and 7 B and C. This activity loss is drastically enhanced by preillumination with single turnover flashes (Fig. 7 D). If however DCMU is present during the preillumination period, so that electron transport is prevented, the effect of preillumination on the activity loss practically disappears (Fig. 7 E). This indicates, that intact elec-

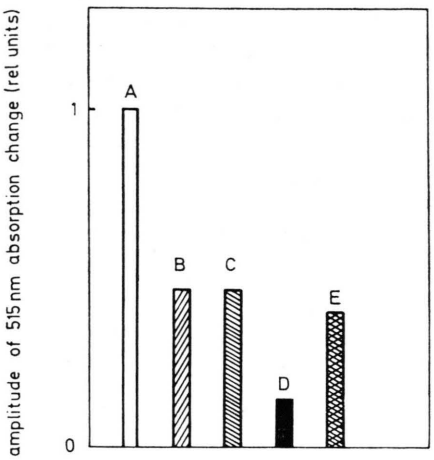

Fig. 7. Average amplitude ΔA_0 of 515 nm absorption change in presence of 2 μM DCMU under excitation conditions where only the back reaction around system II is activated (s. ref. 27). 16 signals were averaged in each experiment, time t_d between the excitation flashes 30 s, weak intensity measuring light has been switched on 200 ms before each flash and has been switched off 1 s after each flash, for the measuring device s. ref. 27, chloroplast suspension as described in Materials and Methods. A. Without BBMD; B. with 50 μM BBMD, 8 min dark incubation in the presence of DCMU and BBMD; C. with 50 μM BBMD, 8 min dark incubation in the presence of BBMD. Addition of 2 μM DCMU after the incubation period, just before the measurement; D. with 50 μM BBMD, during the incubation period of 8 min in the presence of BBMD preillumination with 512 flashes, time t_d between the flashes 300 ms. Addition of 2 μM DCMU after the incubation period, just before the measurement; E. with 50 μM BBMD, during the incubation period of 8 min in the presence of BBMD and 2 μM DCMU preillumination with 512 flashes, time t_d between the flashes 300 ms.

tron transport is required for the light induced activity loss of system II.

The results reported above clearly show, that BBMD acts as an inhibitor of the photoelectric dipole generators of system II accompanied by a slight ADRY-type effect on the watersplitting enzyme system Y, but we did not observe an X-type acceptor action of BBMD. However it could be possible, that BBMD exerts an electron acceptor effect on system I which is known to generate a strong reducing power [43–47].

Effect of BBMD on system I electron transport

In order to clarify a possible effect on system I we measured the influence of BBMD on the light induced 704 nm absorption change in the presence of DCMU (which prevents electron flow from system II) under different conditions for the mediation of system I electron transport.

In the absence of an artificial electron acceptor (natural system I acceptors including the enzyme system mediating the reduction of $NADP^+$ and $NADP^+$ itself have been lost during the isolation procedure of the chloroplasts) a short cyclic electron flow around system I arises [43]. The absorption change caused by this cyclic reaction is shown in Fig. 9 a. The addition of BBMD to these DCMU blocked chloroplasts practically abolishes the 704 nm absorption change in the absence of system I electron donors (Fig. 9 b). Exactly the same result is obtained by the addition of benzylviologen [43] instead of BBMD (Fig. 9 c). Because we found in the absence of system I electron acceptor an increase of the amplitude of 704 nm absorption change by the donor couple DCIP + ascorbate, the effect of BBMD on the light induced 704 nm absorption change (Fig. 9 b) can be explained either by a system I electron acceptor function or by an inhibitory effect. However, the addition of DCIP + ascorbate to DCMU inhibited chloroplasts in the presence of BBMD fully restores the 704 nm absortion change. This result proves that BBMD acts as an electron acceptor of system I and not as an inhibitor. Brandon and Elgersma [26] also have shown, that BBMD does not inhibit system I.

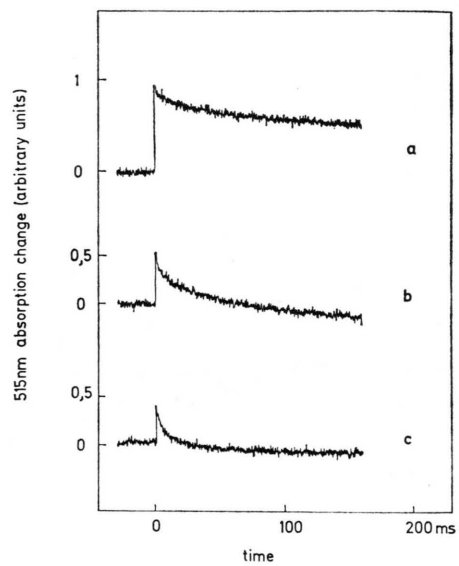

Fig. 8. Absorption change at 515 nm as a function of time in spinach chloroplasts. a. Without BBMD; b. with 50 μM BBMD; c. with 250 μM BBMD. 16 signals were averaged, time t_d between the flashes 2 s. Dark incubation time 8 min Other experimental conditions as described in Materials and Methods.

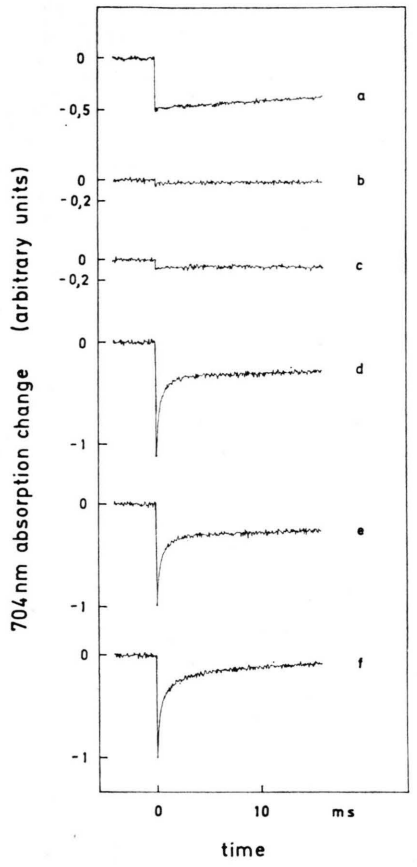

Fig. 9. Absorption changes at 704 nm as a function of time in spinach chloroplasts in the presence of 2 μM DCMU. a. Without external electron acceptor; b. with 50 μM BBMD; c. with 100 μM benzylviologen; d. with 50 μM DCIP+2 mM ascorbate; e. with 50 μM BBMD and 50 μM DCIP+2 mM ascorbate; f. with 100 μM benzylviologen and 50 μM DCIP +2 mM ascorbate. 128 signals were averaged, time t_d between the flashes 1 s. Other experimental conditions as described in Materials and Methods. The authors would like to thank Dr. W. Haehnel for supporting the measurements of Fig. 9 d—9 f. An analysis of the kinetics of these data will be given by Dr. W. Haehnel elsewhere.

If cyclic electron flow around system I is induced by DCIP alone [48] BBMD causes a decrease of the amplitude of 704 nm absorption change accompanied by a deceleration of the reduction kinetics of P 700 with increasing number of excitation flashes. Similar effects are observed in the presence of benzylviologen instead of BBMD. This effect can be explained by the assumption that BBMD as well as benzylviologen is a more efficient system I electron acceptor than DCIP. Thus the concentration of the reduced form of DCIP which acts as electron donor for system I during the illumination progressively decreases due to the competitive inhibition of the reduction of oxidized DCIP in the presence of the above mentioned system I electron acceptors.

All the experiments on the 704 nm absorption change are consistent with the conclusion that BBMD acts as a system I electron acceptor.

The effect of BBMD on the electrical field decay

Till now we have considered only local effects of BBMD on specific sites of the electron transport chain. However it could be possible, that BBMD influences also the properties of the whole thylakoid membrane by a change of its permeability. In Fig. 8 a the decay of the field indicating 515 nm absorption change in the absence is compared with the corresponding decay in the presence of 50 μM and 250 μM BBMD, respectively (Figs 8 b and 8 c). It is seen that at moderate levels of system II activity loss the decay rate of the electrical field is significantly enhanced by BBMD. The increase of membrane permeability by a chemical can be achieved a. by a structural modification of the membrane itself leading to an unspecific enhancement of permeability of different ions or b. by an ionophor-type mechanism [47, 48] increasing the permeability of specific ions or c. if the chemical itself can exist in a permeable charged form. BBMD per se seems not to build up anionic or cationic forms in solution which would migrate through the thylakoid membrane. However, it could be possible, that BBMD reduced by system I forms a negatively charged anion with a sufficient high permeability. Furthermore, by the interaction of BBMD with system II either permeable charged forms of BBMD could be generated or this interaction could cause a structural modification of the thylakoid membrane. If a specific interaction between BBMD and system II would be required for the BBMD induced conductance increase of the thylakoid membrane, the effect should not be observed under conditions where only system I is operative. Experiments on DCMU inhibited chloroplasts with a linear system I electron transport mediated by DCIP plus ascorbate as donor and benzylviologen as acceptor do not indicate any influence of BBMD on the decay of the 515 nm absorption change. On the contrary, if a DCIP induced cycle around system I takes place (in the absence of ascorbate and benzylviologen) BBMD accelerates the decay of the 515 nm absorption change, but the amplitude decreases in agreement with the results

obtained for the 704 nm absorption change indicating a competitive inhibition of the DCIP cycle by BBMD. If ascorbate is added, both the amplitude and the kinetics of the control experiment without BBMD reappears. If one supposes that ascorbate does not specifically inhibit the BBMD effect, the results favor the assumption, that the permeability increase is related to the interaction of BBMD with system II. However, the present results do not provide unequivocal informations about the mechanism of the BBMD induced permeability increase of the thylakoid membrane. Further experiments would be necessary to clarify this effect. In the present paper we will not further discuss the action of BBMD on the thylakoid membrane.

Discussion

The results presented in this paper indicate, that BBMD exerts simultaneous effects in chloroplasts. However, we did not find any evidence for the action of BBMD as an X-type acceptor (s. Fig. 1). In the presence of DCMU the 515 nm absorption change is practically completely suppressed under conditions where the influence of the back reaction around system II [20] becomes negligibly small. By the addition of an X-type acceptor the 515 nm absorption change should be restored to nearly 50% [35, 36]. However, we did not observe any restoration of 515 nm absorption change by BBMD. This result can be interpreted either by the fact, that BBMD does not act as an X-type acceptor or by the introduction of additional sereous assumptions including a very fast electron transfer from the natural primary electron acceptor X 320 to BBMD as well as an extremely rapid field decay in the presence of BBMD and DCMU. The transfer time should be shorter than the time resolution of the measuring device, *i. e.* shorter than 1 μs.

The possibility of an X-type acceptor function of BBMD can be excluded for the following reasons:

1. Because the kinetics of the back reaction in the absence of BBMD are slower by at least six orders of magnitude in comparison to the hypothetical transfer time from X 320 to BBMD, BBMD should completely suppress this back reaction. However, as is shown in Fig. 7, in the presence of BBMD and DCMU a significant amplitude ΔA_0 of the 515 nm absorption change indicating the restoration of the functional active state of system II by the back reaction (s. ref. 27) can be observed. Though the extent of the restored system II activity is decreased by BBMD, the occurrence of a significant degree of restoration caused by the back reaction points against the assumption of a very fast electron transfer from X 320 to BBMD in the presence of DCMU. However, it could be argued that in the presence of BBMD only a fraction p of systems II is functionally connected with BBMD acting as an X-type acceptor, whereas 1-p remains in the native state in respect to the electron transport from X 320 to the PQ pool. The fraction 1-p of systems II would show the normal back reaction characteristics, the fraction p of BBMD labeled systems II would be kinetically masked under both, normal and back reaction excitation conditions, respectively. Because the average ratio of BBMD molecules to system II reaction centers is about 2500, it is – irrespective of the distribution function of BBMD over the systems II – extremely improbable that nearly 50% of the systems II are connected with BBMD as X-type electron acceptors, whereas the other 50% remain uneffected. Hence, the conclusion has been drawn that BBMD does not act as an X-type electron acceptor.

2. Even the assumption of this extremely improbable distribution function of BBMD would not be sufficient to explain all the results. In order to be consistent with the results of Fig. 7 it is necessary to postulate that in addition the factor p depends in a specific manner on the preillumination conditions and on the time of incubation with BBMD, an assumption which is hardly to rationalize.

Hence, the conclusion has been drawn that BBMD does not act as an X-type acceptor. Similarly, in contrast to results recently published by Miles *et al.* [51] we did not find any evidence for an X-type acceptor function of Hg^{2+}-salts.

These results lead to the conclusion that (in contrasts to system I) the primary electron acceptor of system II (X 320) is not freely accessible by external electron acceptors. Because the primary electron acceptor of system II is assumed to be localized near the outer phase of the thylakoid membrane [2, 36, 58] we tentatively infer that X 320 is covered up in chloroplasts by a transport barrier which hinders the electron transfer to external electron acceptors.

On the contrary, in the present paper evidence has been supported for BBMD to be acting as a

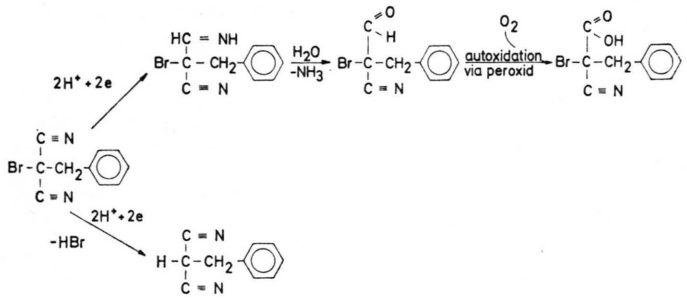

Fig. 10. Proposed reaction scheme for the reduction pathways of BBMD.

system I electron acceptor. The mechanism of the reduction of BBMD is not yet resolved. Two possibilities should be taken into account. There could occur either a reduction of a nitrilo-group or a reductive bromo-elimination. The possible reaction pathways are shown in Fig. 10. If the reduction of BBMD occurs at the nitrilo-group (we prefer this reaction pathway), the reaction sequence probably includes an oxygen consuming step at the level of the aldehyde autoxidation.

The oxygen uptake experiments of Brandon and Elgersma [26] are not easily explainable in the light of our results. However, recently it has been shown by several groups [53–54] that the stoichiometry of oxygen uptake is strongly dependent on the mechanism of oxygen reduction. In this respect the possibility of the generation of superoxide anion radical plays a central role, because different reaction pathways for the radical decay are known, giving rise to different O_2/e ratios. The situation is even more complicated by the dependence on chloroplast preparation of the internal superoxide dismutase activity [54]. A second point which has to be taken into acount, is the function of 1,5-diphenylcarbazide (DPC). The donor function of DPC is not completely system II specific, a partial electron donation occurs also to system I. Furthermore, the oxidation product of DPC reacts in a system I dependent way [55, 56]. We found in flash light experiments, that in DCMU poisoned chloroplasts DPC stimulates an oxygen uptake not only in the presence of BBMD, but also in the presence of methyl viologen (MV). Unfortunately, Brandon and Elgersma [26] did not compare in DCMU blocked chloroplasts (normal or Tris- or BBMD-treated) the action of BBMD and MV, respectively, on the DPC mediated oxygen uptake. Hence, the experiments of Brandon and Elgersma do not provide a proof for their proposed mechanism of BBMD action.

Further experiments are required for the elucidation of the mechanism which is responsible for the BBMD stimulated oxygen uptake. Beyound its acceptor function BBMD does not influence the activity of system I, especially the photoelectric dipole generator of system I (reaction center I) remains uneffected by BBMD.

A completely different pattern arises for system II, where the function of the photoelectric dipole generators II itself is destroyed by BBMD. The activity loss develops relatively slowly and it is significantly pronounced by illumination, indicating that either BBMD is transformed into an active form or that BBMD preferentially reacts with a reactive state of the photoelectric dipole generator of system II. The transformation of these generators into a nonfunctional state leads to photosynthetic units of system II which are characterized by a low yield of variable fluorescence. A deletereous effect on system II with a similar low yield fluorescence characteristics has been reported earlier for lipase treatment of chloroplasts [57]. As it will be discussed in more detail in ref. 27 and in part III (in preparation) of this series BBMD seems to modify the photoelectric dipole generators of system II in such a way that not only their native function (charge separation leading to the formation of a dipole arranged perpendicular to the thylakoid membrane) has been lost, but in addition it also influences the energy distribution within the photosynthetic units of system II.

Probably the BBMD linked nonfunctional photoelectric dipole generators of system II act as dissipative energy sinks (ref. 27 and part III of the series).

In this way BBMD can be used as a tool for the change of energy flow at the centers of photosystem II itself. However, it should be emphasized, that because of its different mode of actions BBMD is not a specific modifier. This restricts the applicability of BBMD in photosynthesis research.

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