Stimulatory Effects of an Ammonium Salt Biocide on Photosynthetic Electron Transport Reactions

Klaus P. Bader and Susanne Höper
Fakultät für Biologie, Lehrstuhl Zellphysiologie, Universität Bielefeld, Postfach 10 01 31,
D-33501 Bielefeld, Bundesrepublik Deutschland

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Alkylbenzyldimethylammonium chloride (ABDAC, zephirol) has been shown to improve the functioning of the photosynthetic apparatus of the filamentous cyanobacterium *Oscillatoria chalybea* (Bader, K. P. (1989) Biochim. Biophys. Acta *975,* 399–402). This biocide exerts stimulatory effects on various electron transport reactions in *Oscillatoria chalybea* and chloroplasts from higher plants. By means of oxygen evolution measurements and of repetitive flash-induced absorption spectroscopy we were able to demonstrate an impact of the drug on the major complexes of photosynthetic membranes, *i.e.* the water splitting complex, photosystem II and photosystem I. Both, P_{820}- and X_{320}-absorption change signals were enhanced by the addition of ABDAC. Along with the quantitative analysis we investigated the relaxation kinetics of the signals and observed a substantial stabilization of the oxidized states of the respective redox components in the presence of the ammonium salt. Under appropriate conditions the relaxation kinetics of the absorption signals were significantly slowed down. ABDAC also affects photosystem I in *Oscillatoria chalybea,* but only under conditions, where a donor/acceptor system *i.e.* an isolated photosystem I reaction with photosystem II being disconnected was measured. Electron transport through the whole chain *i.e.* with water as the electron donor yielded no effect of the quaternary ammonium salt. It is suggested that this is due to an extremely bad linkage between the two photosystem, each of which, however, shows good reaction rates, when separately measured. The described interactions of the biocide with photosynthetic membranes are not restricted to *Oscillatoria chalybea* but are also observed with higher plant chloroplasts. In these systems, ABDAC enhances X_{320}- and P_{700}-signals to a comparable extent. In this case the P_{700}-signal is stimulated in assays with electrons which are furnished from water which hints at good coupling between the two photosystems in our tobacco chloroplast preparations.

Introduction

Quaternary ammonium salts have been shown to exert a variety of effects on biological membranes and membrane-associated processes. In general, such substances are used as antimicrobials to prevent bacterial growth in solutions or suspensions. Different chemicals of this type have been tested for biological activity on yeast and have been found to inhibit respiration, amino acid transport and cell growth in general (Skala *et al.,* 1988). Depending on the concentration, a variety of almost "unspecific" influences on the integral structure of the membranes and the overall viability of the respective organism was described (Kolodynski *et al.,* 1985). In some cases such chemicals seem to have a fungicidal beside the bactericidal activity. Also, the hemolysis of erythrocytes was described (Kleszczynska *et al.,* 1981). These authors tentatively localize the site of action of the ammonium salts in the lipid phase of the erythrocyte-membrane. There are relatively few reports on the interference of quaternary ammonium salts with photosynthetic membranes and processes. One of the relevant effects in this context consists of the uncoupling of photophosphorylation induced by zwitterionic buffer systems like tetraethylammonium chloride (Gross, 1972). The influence of a specific quaternary ammonium salt, namely alkylbenzyldimethylammonium chloride (ABDAC) on photosynthetic oxygen evolution in the cyanobacterium *Oscillatoria chalybea*

Abbreviations: ABDAC, alkylbenzyldimethylammonium chloride; asc, ascorbic acid; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP, dichlorophenolindophenol; JWB, *Nicotiana tabacum* var. John William's Broadleaf; MV, methylviologen; pBQ, *para*-benzoquinone; PS, photosystem; P_{700}, reaction center of photosystem I; P_{680} (P_{820}), reaction center of photosystem II; X_{320}, absorption change of the first quinone acceptor of photosystem II.

Reprint requests to Priv. Doz. Dr. Klaus P. Bader.

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was described by Bader 1989. Careful analyses of the effect of ABDAC concentrations lower than the ones usually described for such disinfectants revealed a positive impact on the oxygen evolution pattern induced by short (5 μs) saturating Xenon flashes. The oscillation of such a sequence according to the Kok model (Kok *et al.,* 1970) is always strongly damped with *Oscillatoria chalybea* (Bader *et al.,* 1983) and can be significantly improved by addition of the drug. This effect could be shown to correspond to a reduction of the miss parameter to about one half (Bader, 1989). It should be noted again that the described effects were clearly distinct from the ones described for ammonia and substituted amines by Beck and Brudvig (1986 and 1987) or Förster and Junge (1986). There is also no obvious parallel to the rapid binding of NH_3 to the S_2-state described by Velthuys (1975).

In the present paper we characterize the interference of this biocide and fungicide with the electron transport system of photosynthesis. As an approach to localize the phenomenon within the electron transport chain we chose absorption spectroscopic assays beside oxygen evolution measurements. An important point of interest was of course the question, whether the described phenomena are restricted to *Oscillatoria chalybea* or, whether such effects can also be observed with agriculturally and industrially more relevant higher plants.

Materials and Methods

Cyanobacteria: Oscillatoria chalybea was obtained from the algal collection in Göttingen (Germany) and cultivated on different nitrogen sources (nitrate, ammonium or nitrogen-free) in the medium. This has been described in detail (Bader *et al.,* 1983; Bader and Schmid, 1989).

Thylakoid preparations of the filamentous cyanobacterium were obtained according to the method described by Bader, Thibault, and Schmid (1983). Usually, the filaments were harvested after 25 days of growth, gently homogenized and treated with glucuronidase (Merck, Germany) to remove the mucoid layer on the filaments and with cellulase Onozuka R-10 (Kinki Yakult, Japan) and lysozyme (Sigma, Germany) to digest the cell walls.

Tobacco: Photosynthetically active chloroplasts from the Connecticut cigar variety *Nicotiana tabacum* var. John William's Broadleaf were isolated according to the procedure described by Homann and Schmid (1967).

The reaction assays for spectroscopic measurements usually contained *Oscillatoria* protoplasts equivalent to 30 μg chlorophyll *a* or tobacco chloroplasts equivalent to 40 μg chlorophyll $a+b$ in 4 ml reaction buffer (pH 7.5) 0.06 M Tricine and 0.03 M KCl. Upon dilution with the hypotonic reaction buffer the chloroplasts (as well as the protoplasts from *Oscillatoria chalybea*) burst thus liberating the thylakoids. Specific reagents were used at concentrations as indicated in the figure legends.

Oxygen evolution measurements were carried out on the rapid 3-electrode system (flash electrode) described by Schmid and Thibault (1979) to measure single S-state transitions upon flashes (5 μs) and in a normal Clark-type electrode from Rank Bros. (England) to determine oxygen evolution rates under continuous light. Details are included in the figure legends and in the text.

Absorption change reactions were measured in a set-up according to the principles according to Rüppel and Witt (1969) and to Witt (1971) suited for a wide range of absorption changes both in the UV- and in the visible light region. The complete device consisted of two separate axes with background illumination of the appropriate wavelength. For visible light measurements (P_{700} and P_{820}) Schott interference filters DAL 698/HW 20/46% (IL 699.5/HW 11/48.5%, respectively) and MA 7-05 829.8/HW 5.5/45% (IL 834.5/HW 11/39%, respectively), in front of the silicium photodiode (EG & G, SGD 444 F) were used. UV-absorption changes can be examined by light from a mercury lamp (HBO 100 W, Osram) condensed by a lens-system and passing through a monochromator before hitting the reaction assays. The absorption changes were registered *via* a photomultiplier (No. 9658, EMI Electronics Ltd.) using high tension of 1000–1500 V. The signals were recorded and converted *via* a signal averager from Tracor, Northern. Thus, signals with a largely improved signal to noise ratio at the respective wavelength could be obtained as average signals of 100–300 (in the case of P_{700} or P_{820}) or 500–1000 (in the case of X_{320}) flashes. These flashes were provided by a Xenon-lamp (EG & G Multiflash Model 553) with an intensity of 20 joules and a half-width of *ca.* 20 μs.

Alkylbenzyldimethylammonium chloride (ABDAC; zephirol) was obtained from Merck-

Schuchardt, Germany. "Alkyl" means a mixture of side chains from C_8 to C_{18}. For this reason an average molecular weight of 365 g/mol was used for preparation of the applied solutions.

Results and Discussion

The filamentous cyanobacterium *Oscillatoria chalybea* exhibits a flash-induced oxygen evolution pattern which due to a high contribution of missed transitions appears to have a periodicity higher than 4 and a specifically strong damping of the oscillation (Fig. 1; *Control*). Mathematical fit of the sequences clearly showed that this is due to a miss parameter of around 35% in such preparations (Bader, 1989) whereas for *Chlorella* a miss parameter of 15–17% is calculated under our conditions. All S-states appear relatively stable in *Oscillatoria chalybea* in comparison to green algae or higher plant chloroplasts, as the deactivation kinetics of the S-states (S_2 and S_3 in particular) are significantly slower than in other organisms. This slow deactivation can be further slowed down by the addition of ABDAC with the result that dark adaptation times of 15 min are not sufficient to reach dark equilibrium of the S-states (Bader, 1989). Moreover, the functioning of the oxygen evolving apparatus of *Oscillatoria chalybea* is substantially improved by addition of ABDAC (Fig. 1 and Bader, 1989). It is generally observed in such cases that the maximum of the sequence is increased and shifted from the 4th to the 3rd flash, whereas the minimum is decreased and shifted from the 7th to the 6th flash. (This is equivalent to a nearly 50% reduction of the miss parameter.) Under optimal conditions, the ratio of the maximum to the minimum flash yield (Y_{max}/Y_{min}) increases from around 1.5 to 5–6. Used in appropriate concentrations, ABDAC strongly improves the efficiency of the oxygen evolving system in *Oscillatoria chalybea.* An important feature for this was the observation that ABDAC substantially stabilizes all S-states and particulary the states S_2 and S_3 (Bader, 1989). A stimulatory effect is not restricted to flash light but can as well be observed under continuous light illumination as substantiated by an increase of the Hill reaction rates. All pBQ-Hill reactions show the expected sensitivity towards DCMU and DBMIB, respectively (Table I).

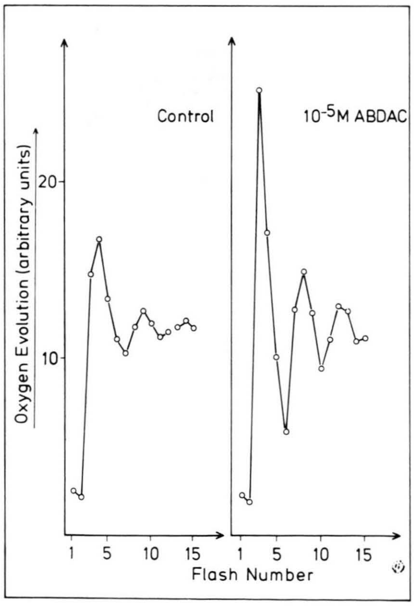

Fig. 1. Oxygen evolution pattern observed with thylakoids from *Oscillatoria chalybea* upon illumination with a train of short saturating light flashes spaced 300 ms apart (Control) and pattern in the presence of 10^{-5} M ABDAC. It should be noted that under these conditions the oscillation of the sequence is largely improved, whereas the steady state amplitudes as such remain unaffected.

We analyzed and tried to characterize the phenomenon by measuring distinct parts of the electron transport chain with absorption spectroscopic techniques. Thus, we investigated the influence of ABDAC on the 820 nm absorption change representative for the oxidation of the PS II reaction center chlorophyll$_{aII}$. This signal can be attributed to the forward reaction of photosystem II from P_{680} to Q_A as well as to the corresponding back reaction. Analyses of the time constants and relaxation kinetics allow the discrimination between the involved reactions. Also, PS I contributes to the signal detected at 820 nm (Mathis and Conjeaud, 1978; van Best and Mathis, 1978). Fig. 2 shows an increase in the P_{820} absorption change by more than 50% in the presence of the drug indicating that the ABDAC action might be on the PS II reaction center itself. This could be substantiated by the stimulation of the respective X_{320}-signal (see next paragraph). The signals are strongly DCMU-sensitive, what has to be expected for a photosystem II reaction. The effect of ABDAC as being a stabilizer of the oxidized S-states has been suggested in consequence of the flash experiments upon oxygen evolution in a preceding paper (Bader, 1989). This interpretation can

Table I. Effect of Alkylbenzyldimethylammonium chloride on the oxygen evolution in a pBQ-Hill reaction and on the oxygen uptake in a MV-Mehler reaction with thylakoids from *Oscillatoria chalybea.* The reactions were carried out in a Clark-type electrode.

ABDAC [M]	pBQ-Hill reaction	pBQ-Hill reaction +DBMIB	pBQ-Hill reaction +DCMU	$H_2O \rightarrow MV$	$DCPIP \rightarrow MV$
0	127	43	19	87.4	212.8
1.4×10^{-6}	147	–	–	–	–
2.7×10^{-6}	136	42	22	80.4	297.9
6.8×10^{-6}	190	–	–	–	–
1.4×10^{-5}	213	55	20	70.8	419.2
2.0×10^{-5}	184	–	–	–	–
2.7×10^{-5}	76	37	6	57.7	432.0
1.4×10^{-4}	–	–	–	23.6	632.0
2.7×10^{-3}	–	–	–	4.4	795.9

Inhibitor concentrations were 5×10^{-5} M for DBMIB and 10^{-5} M for DCMU, respectively. Donor/acceptor concentrations were 1 mM for pBQ, 0.1 mM for MV, 0.1 mM for DCPiP and 5 mM for ascorbate, respectively. Oxygen evolution/uptake rates in μmoles $O_2 \cdot$ mg $Chl^{-1} \cdot h^{-1}$.

be further substantiated by the effect on the relaxation kinetics of the absorption change signals. Addition of ABDAC at the optimal concentration of 10^{-5} M significantly slows the relaxation kinetics of the signals down. This observation refers to a general response of the involved membrane complexes towards the drug and might not be restricted to the PS II forward reaction from P_{680} *via* pheophytin to Q_A, as we can not resolve the ns-range of the P_{820} signal.

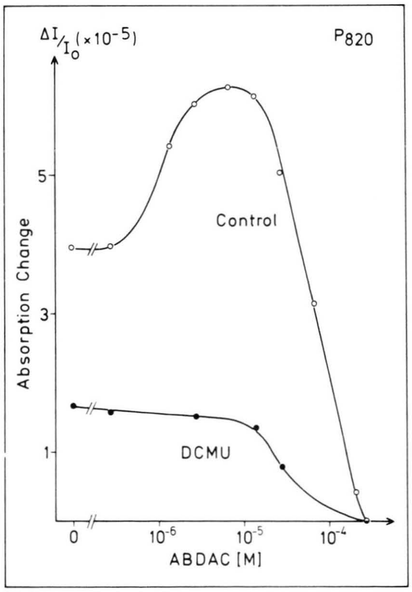

Fig. 2. Effect of ABDAC on the P_{820}-absorption change in thylakoids from nitrate-grown *Oscillatoria chalybea* and the influence of 10^{-4} M DCMU on the signals.

A stimulated PS II oxidation with the increased electron transport rates caused by the addition of ABDAC should also be recognizable as Q_A reduction. Photoreduction of Q_A by PS II *via* pheophytin yielding the semiquinone anion leads under repetitive flash illumination to an increase of the UV-absorption around 320 nm (Stiehl and Witt, 1968; Stiehl and Witt, 1969; van Gorkom, 1974). *Oscillatoria chalybea* exhibits this absorption change signal in the presence of an electron acceptor like *e.g.* *p*-benzoquinone (Fig. 3). ABDAC-induced stimulation, however, seems to require an open *i.e.* unoccupied manganese binding site within the mem-

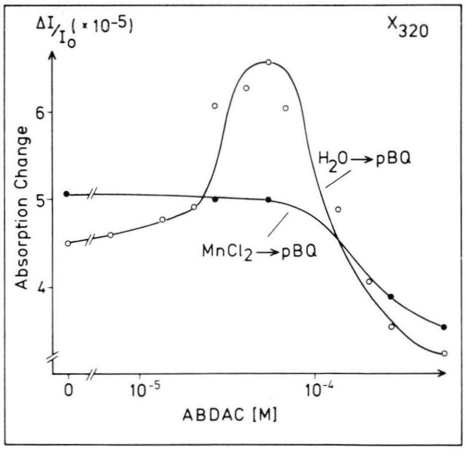

Fig. 3. Influence of ABDAC on the X_{320} absorption change in thylakoids from *Oscillatoria chalybea* in the presence of an electron acceptor (10^{-4} M pBQ) without and with addition of manganese (10^{-3} M).

brane system, as addition of ABDAC to the reaction assay $MnCl_2 \rightarrow X_{320} \rightarrow$ pBQ does not show any stimulation, whereas the reaction $H_2O \rightarrow X_{320} \rightarrow$ pBQ appears strongly enhanced upon addition of the chemical (Fig. 3). In the latter case, the control signals (in the absence of ABDAC) are substantially smaller due to the lack of stimulation by manganese addition.

We failed to observe a stimulating effect of ABDAC on the P_{700}-absorption signal of *Oscillatoria chalybea* at any of the tested concentrations. Also, pre-incubation of the assay with the biocide in the dark and in the light (which had been shown to facilitate the appearance of the effect *i.e.* to decrease the optimal concentration of the drug by one order of magnitude for P_{820} (results not shown) and for O_2-evolution measurement (Bader, 1989)) did not yield any effect in the relevant concentration range. The absence of any stimulating effect in this context might be related to an extremely bad linkage of the electron transport between the two photosystems. If one measures the P_{700}-absorption signal in the absence of any donor/acceptor system, one determines the oxidation of PS I which is re-reduced by electrons coming from water. Under the assumption that a system contains a substantially leaky electron transport between the two photosystems one should expect a relatively low absorption signal that should be insensitive to any stimulation within PS II. After the addition of DCPIP/asc, MV and DCMU, however, an increase in the signal should be observed due to the better supply with electrons. Fig. 4 supports this interpretation. The same reaction assay that exhibits a relatively low P_{700}-signal can be stimulated nearly 3-fold by the addition of a donor/acceptor system. Furthermore, this condition shows that a stimulating effect of ABDAC can also be observed for PS I (Fig. 4). Apparently, similar structures in the membrane system of both photosystems undergo conformational changes upon addition of ABDAC leading in both cases to an improved electron transport. We suggest that the interference takes place *via* adsorption of the molecule to the membrane where the polar components of the biocide play a role. In a second phase the alkyl chain of the biocide specifically penetrates into the hydrophobic parts of the membrane system.

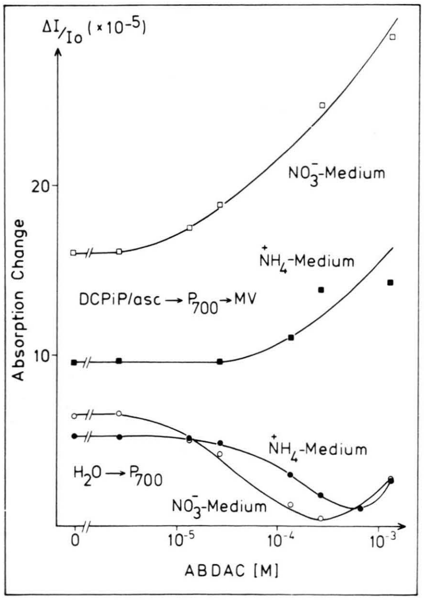

Fig. 4. Effect of ABDAC on the P_{700}-absorption change reactions in thylakoids from nitrate- and ammonium sulfate-grown *Oscillatoria chalybea.* Electron transport through both photosystems was measured by the reaction $H_2O \rightarrow P_{700}$; assays with an artificial donor/acceptor/DCMU-system reflect the electron transport running exclusively through photosystem I. Concentrations were: DCPIP 10^{-4} M; ascorbate 10^{-3} M; MV 10^{-4} M; DCMU 2×10^{-5} M.

The interpretation of a leaky electron transport system between the two photosystems perfectly fits earlier observations by Bednarz *et al.* (1989) who discussed an efficiency of $< 50\%$ for the passage of electrons from water to P_{700}. For *Oscillatoria* as a prokaryote with the photosynthetic, the respiratory and the nitrate reducing electron transport system being located inside the same membranes one can easily imagine that an important share of electrons might be deviated from the photosynthetic to the normal respiratory or even at the plastoquinone site to the alternate respiratory pathway (Bader and Schmid, 1989). Under such conditions, ABDAC even at otherwise optimal concentrations does not exert much of a stimulating effect on the P_{700}-signal due to the limitation of electrons arriving at PS I. These results were substantiated by measurement of $H_2O \rightarrow$ MV and DCPIP/asc → MV Mehler reactions measured as oxygen uptake in a Clark-type electrode that yielded equivalent results (Table I). The electron transport reaction which is dependent on the photoreduction of an electron acceptor located on the acceptor side of PS I but which oxidizes water does not show any ABDAC-induced stimulation. If, however, electrons are directly fed into PS I by DCPIP/asc, oxygen uptake rates are strongly enhanced by addition of the drug.

In order to check the interpretations, given above, on a possible applicability to other photosynthetic systems, we made identical experiments with higher plant chloroplasts. Fig. 5 shows that under conditions where isolated chloroplasts with relatively intact electron transport between PS II and PS I (as evidenced by the stimulation of DCPIP/asc/MV-addition to P_{700} assays) were used, a stimulating effect can be observed for X_{320} and also for P_{700}. The latter must be related to the PS II reaction (as a source of electrons) and so necessarily to a good connection between the two photosystems, as for tobacco chloroplasts no stimulation of an isolated PS I reaction was obtained. Moreover, this result could mean that the PS I complex in tobacco is structured in a way that ABDAC cannot interact with it in a comparable way.

It appears that ABDAC largely improves the activity of the photosystems and the structural organization of membrane complexes of both *Oscillatoria* and tobacco in their functioning by conformational changes. The question that remains open for the moment is, whether this comparable behaviour towards ABDAC is based on identical determinants inside the membrane system or if structurally different but functionally equivalent regions are responsible for the interaction with the biocide molecule. A first explanation for the ABDAC action might be that of an uncoupling effect of photophosphorylation. This might be deduced from observations depicted in Fig. 3, namely that the addition of manganese is required for an optimal X_{320}-signal under our conditions, but which on the other hand prevents any stimulation by ABDAC. The same observations are made for the P_{820}-signals (results not shown). Only in the manganese containing assay absolutely no effect of ABDAC on the UV-absorption change is observed (apart from inhibitions at very high concentrations). This result fits into an observation by Gross who described the reversal of uncoupling effects of zwitterionic buffers by manganese (Gross, 1972). The author described the non-competitive binding and displacement of Mn^{2+}-ions and tetramethylammonium (Gross, 1972). In case of ABDAC stimulation, however, this is clearly not the case, as we investigated and compared the effects of a classical uncoupler (gramicidin) in the absence and in the presence of ABDAC (Fig. 6). Identical results have been obtained fol-

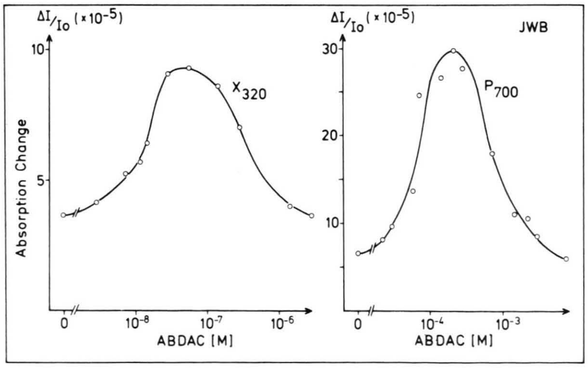

Fig. 5. Effect of the biocide ABDAC on the absorption change reactions X_{320} and P_{700} in chloroplasts from higher plants (*Nicotiana tabacum* var. John William's Broadleaf).

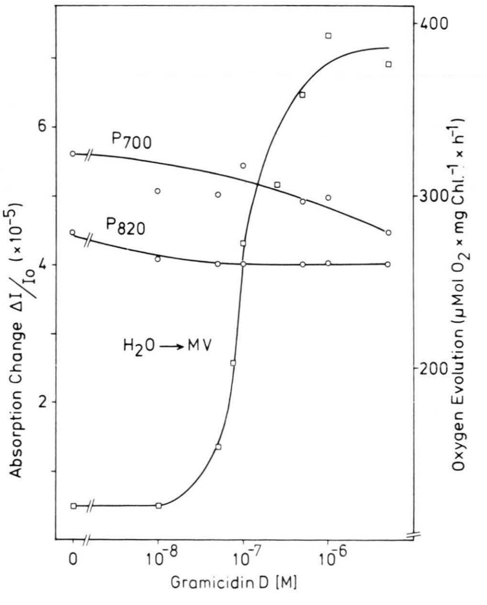

Fig. 6. Comparison of the effects of the uncoupler gramicidin on the MV Mehler reaction measured in a Clark-type oxygen electrode and on the repetitive absorption change signals stimulated by ABDAC. Methylviologen concentration was 10^{-4} M. Note that with the identical thylakoid preparation from *Oscillatoria chalybea* a strong uncoupling effect can be observed for the oxygen uptake, whereas absolutely no effect occurs on the absorption change signals neither for the controls nor for the ABDAC-stimulated signals. Identical results were obtained with chloroplasts from higher plants.

lowing the addition of ammonium-chloride or CCCP as uncoupling agents (results not shown). All of the described effects are clearly distinct from those obtained for higher plant chloroplasts with well-known uncoupling agents like ammonium-ions or gramicidin. Absolutely no increase in any of the recorded P_{700}-signals can be achieved by the addition of the uncoupler, neither in the absence nor in the presence of ABDAC (*i.e.* with the stimulated P_{700}-signals). The uncoupling effect of the applied gramicidin concentrations on oxygen evolution reactions was taken as a control and is therefore also depicted in Fig. 6.

Quaternary ammonium salts interact with photosynthetically active membranes causing stimulation of various electron transport reactions in both cyanobacteria (*Oscillatoria chalybea*) and higher plant chloroplasts (tobacco). If one looks into the problem, it is observed that specifically different concentrations for completely different phenomena (*e.g.* the described stimulatory effects and the usually "intented" damage of cells by ABDAC as being a biocide) have to be taken into consideration. The ranges of these concentrations seem to be directly connected to different phases in the interaction of such molecules within lipophilic and hydrophilic regions of photosynthetic membranes. Here, the specific structure of the biocide molecule with its four ligands appears to play an essential role.

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