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Derivatives of Hydroxyperfluoroisopropyldinitrobenzole Inhibit Electron Transfer in Photosystem II

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We have revealed new highly efficient inhibitors of electron transfer in photosystem II (PSII) of plants – hydroxyperfluoroisopropyldinitrobenzole derivatives, designated as K15-type inhibitors. Their inhibitory effect is based on the redox interactions with PSII reaction centre (RC) components accompanied by formation of a short cyclic electron transfer chain which leads to rapid recombination of separated charge in PSII. This conclusion is supported by the following effects obtained upon treatment of isolated chloroplasts of higher plants or PSII preparations with K15: (1) inhibition of the photoinduced changes in chlorophyll fluorescence yield (ΔF) related to photoreduction of the primary electron acceptor Q_A and photoinduced absorption changes (ΔA) related to photoaccumulation of the primary electron donor P_{680} in its oxidized form P_{680}^+; (2) acceleration of dark relaxation and the decrease of the amplitude of ΔA and ΔF related to photoreduction of the intermediary electron acceptor pheophytin and the decrease in the fluorescence yield when Q_A was prereduced; (3) disappearance of all these effects upon addition of dithionite (which reduces K15 in the dark) accompanied by an accelerated electron donation to RC; (4) coincidence of the concentration dependences of the inhibition by these compounds of both oxygen evolution and the above mentioned reactions related to electron transfer in PSII RC (the value of the inhibition constant K_i was found to be equal to 45 nM for K15).

Keywords: *hydroxyperfluoroisopropyldinitrobenzole, K15 inhibitors, PSII, cyclic electron transfer*

INTRODUCTION

As was shown earlier (Allakhverdiev et al., 1989; Klimov et al., 1989), dinoseb (2,6-dinitro-sec-butyl phenol), a herbicide of the phenolic group, along with blocking the electron transfer between quinones Q_A and Q_B (Trebst and Draber, 1979) is also capable of redox interaction with the reaction centre (RC) components in photosystem II (PSII). This leads to the enhanced oxidation of the reduced pheophytin ($Pheo^-$) and reduction of the oxidized chlorophyll P_{680}^+ (Pheo and P_{680}, intermediary acceptor and primary donor of electron in PSII, respectively) and, as a result, to a more rapid recombination of [$P_{680}^+Pheo^-$] pair. The dinoseb induced cyclic electron transfer which competes with the functional reactions of $Pheo^-$ oxidation and P_{680}^+ reduction and contributes to the total effect of PSII inhibition only at relatively high concentrations of this inhibitor (Allahverdiev et al., 1989; Klimov et al., 1989).

In this article, we summarize recent progress in studies of the inhibitory effect exerted on electron transport in PSII by hydroxyperfluoroisopropyldinitrobenzole (HPFIPDNB) derivatives exhibiting a growth-regulatory and herbicidal activity (Konstantinova et al., 1980). The structural formulas of these inhibitors are illustrated in Figure 1.

RESULTS

Figure 2A shows that compound K15 taken at concentrations of 0.9 μM, 2 μM and 7 μM inhibits the photoinduced evolution of oxygen in pea chloroplasts measured in the presence of ferredoxin and $NADP^+$ by 30%, ~ 60% and ~ 90%, respectively.

Similar dependence was observed for the photoinduced ΔF associated with the photoreduction of Q_A, the primary electron acceptor of PSII (Figure 2B). Compound K15 does not inhibit the $NADP^+$ photoreduction in the presence of the reduced 2,6-dichlorophenolindophenol, i.e., under the conditions of involvement of PSI alone in this reaction (see Table 1).

Of all known inhibitors of PSII dinoseb is regarded to be the closest to these compounds by its chemical structure. But, as shown in Figure 3, the efficiency of the inhibitory action of compound K15 is much higher (~80-100 times) than that of dinoseb. Furthermore, in contrast to dinoseb, compound K15 suppresses the electron transport and

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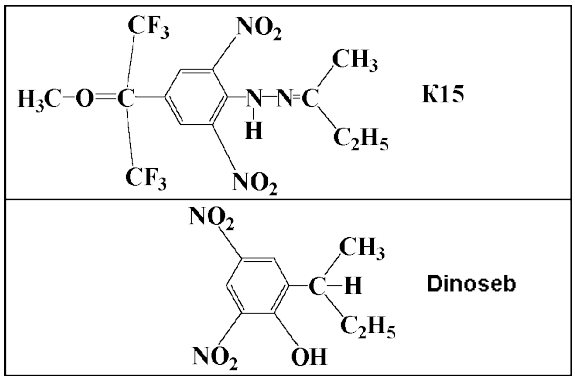

Figure 1. Structural formulas of inhibitor K15 and dinoseb.

photoinduced ΔF in chloroplasts within the same concentration range. Consequently, in contrast to dinoseb (Allakhverdiev et al., 1989; Klimov et al., 1989), in the case of compound K15 the inhibition of electron transport and repression of photoinduced ΔF in PSII appears to be based on the same action mechanism.

Figure 4 shows the results derived from comparison of the effect of compound K15 on F and photoinduced ΔF of PSII subchloroplast particles (DT-20) with that of diuron and dinoseb during measurements with the use of light of saturating intensity. Already at a concentration of 18 nM (curve 3) K15 causes a decrease of the maximum level of fluorescence ($F_M = F_0 + \Delta F$) due to a 15%, decrease of ΔF compared to the control. At concentrations of 80 nM and 360 nM, K15 was found to inhibit the photoinduced ΔF by 57% and 86%, respectively (curves 4 and 5).

It is noteworthy that in contrast to diuron, compound K15 does not virtually affect the magnitude of F_0 and the rate of dark relaxation of ΔF (reflecting reoxidation Q_A^-). The inhibitory effect of K15 on ΔF is totally preserved during prolonged (>5 min) illumination with the actinic light even at a 0.05-0.1 μM concentration of inhibitor (in contrast to the well-known electron acceptor 2,5-dibromo-3-methyl-6-isopropylbenzoquinone (DBMIB) whose addition to DT-20 preserved the fluorescence quenching only during the first 5-10 s of illumination, which then disappeared due to photoreduction of DBMIB). Multiple wash-off of PSII preparations treated with inhibitor K15 (by centrifugation) did not eliminate the inhibitor from PSII, which is evidenced by the preservation of the inhibitory effect.

It is known that the decrease of F_M due to repression of ΔF can occur as a result of inactivation of the PSII electron donor site, e.g., after complete removal of manganese (curve 8). In this case addition of exogenous PSII electron donors restores the magnitude of ΔF to its initial value (curve 8), which was also observed in some earlier studies (Klimov et al., 1982). Figure 3 shows that the efficiency of the inhibition of photoinduced ΔF by K15 does not virtually change upon addition of artificial PSII electron donor - Mn^{2+} (0.1-20 μM, curve 5), sodium ascorbate (2 mM), diphenylcarbazide (1 mM), NH_2OH (1 mM) (data not shown) - irrespective of the sequence of the reagent addition.

Table 1. Decrease of the rate of $NADP^+$ photoreduction by pea chloroplasts upon addition of compounds K15.

Additions	Reduction rate	
	nmole/(mg chlorophyll h)	%
-	117.0	100
K15		
0.6 μM	80.7	69
2 μM	45.6	39
7 μM	11.7	10
+ $DCPIPH_2$ (0.1 mM)	124.0	106

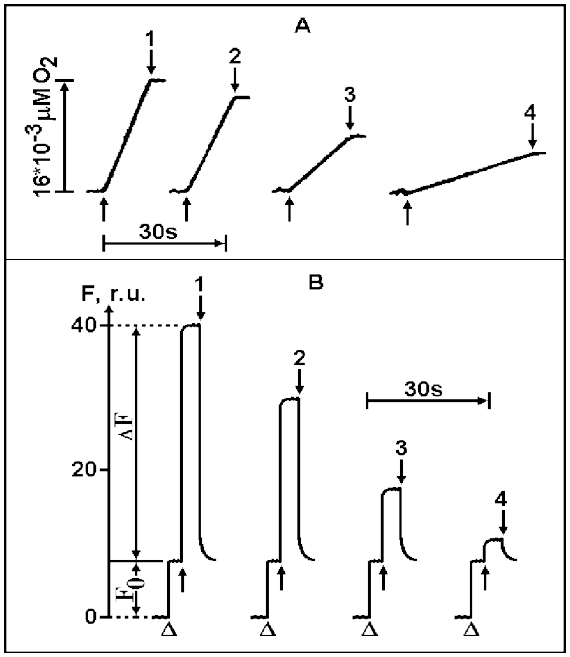

Figure 2. Effect of compound K15 at concentrations of 0.6 μM (2), 2 μM (3) and 7 μM (4) on the photoinduced electron transfer in pea chloroplasts measured by: A, the rate of oxygen evolution in the presence of 20 μM ferredoxin, 20 μM ferredoxin-$NADP^+$ reductase and $NADP^+$ (5 mg·ml^{-1}); B, photoinduced changes of chlorophyll fluorescence yield in PSII due to photoreduction of Q_A. 1, control (measurement in the absence of K15). Here and below the triangles indicate the moments of switching on the measuring light (λ = 490 nm; 0.15 J m^{-2} s^{-1}) which induces the chlorophyll fluorescence (λ > 650 nm); the upward and downward arrows indicate switching the actinic light (λ > 600 nm; 100 J m^{-2} s^{-1}) on and off, respectively. Chlorophyll concentration, 100 μg ml^{-1}; 20°C.

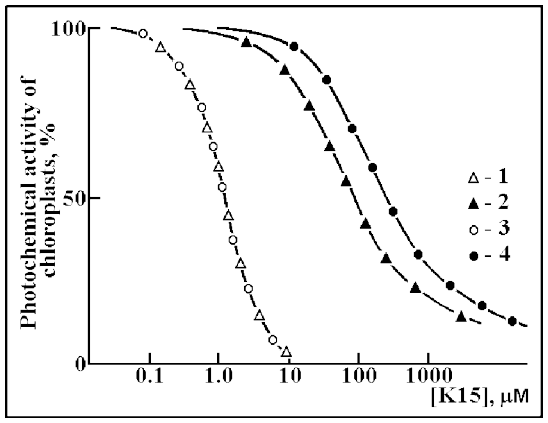

Figure 3. Dependence of photochemical activity of pea chloroplasts estimated from the rate of oxygen evolution in the presence of exogenous electron acceptors (ferredoxin, ferredoxin-$NADP^+$ reductase and $NADP^+$) (3, 2) and from photoinduced ΔF of PSII chlorophyll related to photoreduction of Q_A (3, 4) on concentration of K15 (1, 3) and dinoseb (2, 4). Chlorophyll concentration, 100 μg ml^{-1}; 20°C. For the conditions of measurements see the legend to Figure 1.

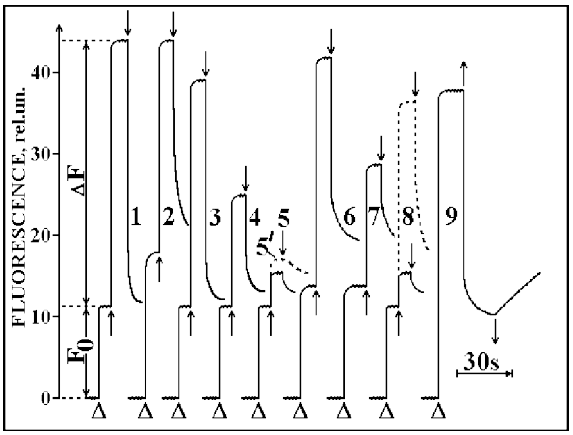

Figure 4. The level of dark fluorescence (F) and the kinetics of photoinduced changes F (ΔF), related to photoreduction of Q_A in PSII, under anaerobic conditions in subchloroplast DT-20 particles (1-7, 9; solid lines) before (1) and after addition (2) of 0.5 μM diuron; compound K15 added at concentrations of 20 nM (3), 80 nM (4) and 360 nM (5) in the absence of other additives and after addition of 0.8 mg ml^{-1} of dithionite to (5) (9); dinoseb at concentration of 1 μM (6) and 5 μM (7) and in DT-20 particles after a complete removal of Mn (8). Curves 5' and 8' (dashed line) are the same as curves 5 and 8 but after addition of 20 mM $MnCl_2$. For designations and measurement conditions see the legend to Figure 1. Chlorophyll concentration, 10 μg ml^{-1}; 20°C.

Addition of dithionite (≅ 0.8 mg ml^{-1}) totally eliminates the inhibitory effect of K15 on ΔF of PSII. Fluorescence rises to its maximal level, whereas the application of the actinic light causes a decrease of fluorescence (curve 9) related, as shown earlier in (Allakhverdiev et al., 1989; Klimov et al., 1989), to the photoreduction of Pheo. Dithionite induces the dark reduction of K15, which is evidenced by the disappearance of characteristic absorption bands in the range of 200-500 nm with the maximum at 410 nm (not shown).

It is known that the decrease of F_M due to repression of ΔF can occur as a result of inactivation of the PSII electron donor site, e.g., after complete removal of manganese (curve 8). In this case addition of exogenous PSII electron donors restores the magnitude of ΔF to its initial value (curve 8), which was also observed in some earlier studies (Klimov et al., 1982). Figure 3 shows that the efficiency of the inhibition of photoinduced ΔF by K15 does not virtually change upon addition of artificial PSII electron donor - Mn^{2+} (0.1-20 μM, curve 5), sodium ascorbate (2 mM), diphenylcarbazide (1 mM), NH_2OH (1 mM) (data not shown) - irrespective of the sequence of the reagent addition.

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Figure 5 shows the variation of I_{50} (the inhibitor concentration inducing a 50% inhibition) for compound K15 as a function of the concentration of DT-20 particles expressed as the level of chlorophyll they contain. The magnitude of I_{50} approximated to the zero chlorophyll concentration, as it was done earlier for other inhibitors (Tischer and Strotmann, 1977; van Rensen et al., 1978; Fedtke, 1985), has values of 45 nM for K15, whereas the values of pI_{50} defined as $-\log I_{50}$ (Fedtke, 1985) are 7.4 for K15. The data presented in Figure 5 also indicate that one inhibitor molecule is bound to one RC of PSII.

It is known (Klimov et al., 1985, 1986) that photoreduction of Pheo to $Pheo^-$ in PSII may also occur in the absence of dithionite - when anaerobic conditions are created. In this case the value of fluorescence also reaches the level F_M as a result of Q_A photoreduction to Q_A^- by a weak measuring light, whereas during the illumination with the acting light one can observes a decrease of fluorescence (-ΔF) and changes in absorption (ΔA) related to re versible photoreduction of Pheo (Figure 6).

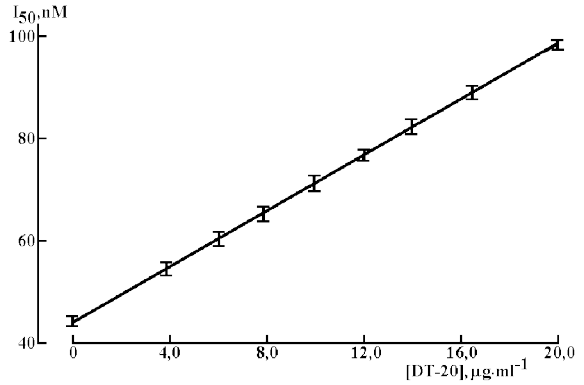

Figure 5. Dependence of the K15-induced 50% - inhibition of photoinduced ΔF (I_{50}) in DT-20 particles on their concentration expressed as concentration of chlorophyll they contain (μg ml^{-1}).

Addition of K15 at a concentration of 38 nM leads to a dramatic (15-20-fold) increase of the rate of dark relaxation ΔA and -ΔF which is indicative of the increased rate of dark oxidation of reduced Pheo (Figure 6A, B). The increase of K15 concentration from 65 nM to 360 nM (Figure 6) results in a considerable decrease of -ΔF and ΔA as well as in a lower level of F_M. It should be noted that in this case the illumination with the actinic light does not lead to the appearance of photoinduced rise of F associated with the photoreduction of Q_A. On the other hand, when K15 is replaced by a PSII electron acceptor inducing the dark oxidation of Q_A^-, for example DCPIP, such a decrease in F_M under anaerobic conditions is accompanied by the appearance of photoinduced increase of F (Figure 6A, curve 6). Application of 50-100 μM diuron did affect neither the time course, nor the magnitudes of ΔA and -ΔF related to the photoreduction of Pheo or F_M under anaerobic conditions.

Other experiments showed that compound K15 at concentrations up to 100 μM did not inhibit the fluorescence of chlorophyll in the light-harvesting pigment-protein complexes isolated from pea chloroplasts, as described in (Klimov et al., 1982, 1989; Allahverdiev et al., 1989) as well as the fluorescence of chlorophyll solution in 1% Triton X-100.

It was shown earlier that the photoinduced -ΔF associated with the reversible photoreduction of Pheo may be observed in the presence of a strong reductant, dithionite (Klimov et al., 1979, 1989). Investigation of this photoreaction in DT-20 preparations freed of Mn (Klimov et al., 1982) showed that addition of 0.5 μM K15 (as well as addition of Mn^{2+} (Klimov et al., 1982)) leads to a 1.5-2-fold increase of the Pheo photoreduction rate (Figure 7), whereas the rate of dark

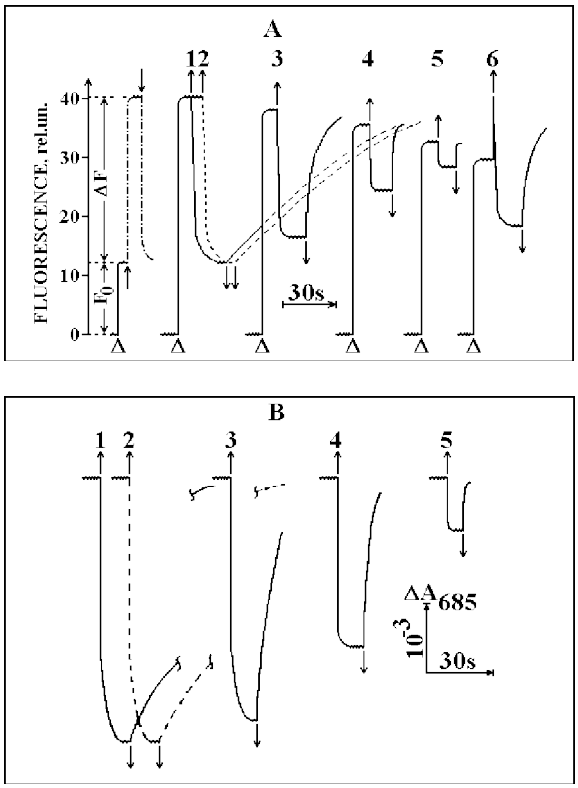

Figure 6. The level of F and the kinetics of photoinduced -ΔF (A) and absorbance changes at 685 nm (B), related to pheophytin photoreduction in PSII, in DT-20 particles under anaerobic conditions (upon addition of 10 mM glucose, ~50 U ml^{-1} glucose oxidase, ~1000 U ml^{-1} catalase, 5 mM sodium ascorbate and 1 μM CCCP) without other additives (1) and after addition of compound K15 at concentrations of 38 nM (3), 65 nM (4) and 360 nM (5); 10 μM DCPIP (6) or 50 nM diuron (2). A, the dash-dot line shows the time course of ΔF related to photoreduction of Q_A in an identical sample placed under aerobic conditions. Chlorophyll concentration, 10 μg ml^{-1}; 20°C.

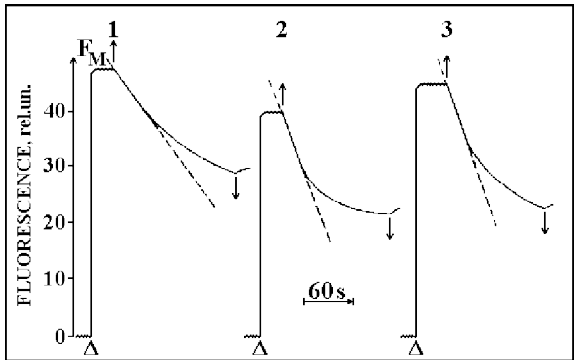

Figure 7. The kinetics of photoinduced fluorescence (F) decrease related to photoreduction of pheophytin in RC of PSII in Mn-depleted DT-20 particles upon addition of dithionite (0.8 mg ml^{-1}) in the absence of other additives (1) and after addition of 0.5 μM K15 (2) or 0.1 mM $MnCl_2$ (3); pH 8.5. For measurement conditions see the legend to Figure 1.

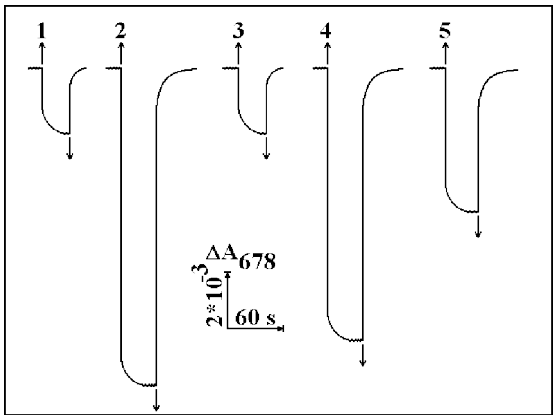

Figure 8. The kinetics of photoinduced absorbance changes at 678 nm related to photooxidation of P_{680} in DT-20 preparations in the presence of 1 mM ferricyanide and 0.1 mM silicomolybdate prior to (2) and after (2-5) complete removal of Mn; the measurement in the absence of other additives (2) and upon addition of 3 μM $MnCl_2$ (3) or compound K15 at concentrations of 22 nM (4) and 80 nM (5).

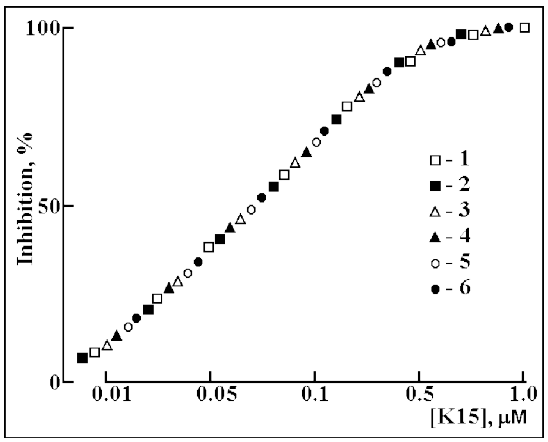

Figure 9. The [K15] dependence of the inhibition (in %) of photoinduced ΔF related to photoreduction of the primary electron acceptor in PSII, plastoquinone Q_A (1), magnitude of F_M under aerobic conditions (2), magnitude F_M under anaerobic conditions (3), photoinduced -ΔF and ΔA_{685} related to photoreduction of the intermediary electron acceptor, pheophytin, under anaerobic conditions (4 and 5, respectively), photoinduced ΔA_{678} related to photooxidation of the primary electron donor in PSII, chlorophyll P_{680} (6). Chlorophyll concentration, 10 μg ml^{-1}.

oxidation of Pheo remains unchanged.

Furthermore, the use of such preparations to study another characteristic photoreaction in PSII - photooxidation of the primary electron donor P_{680} (registered as photoinduced ΔA at 678 nm in the presence of potassium ferricyanide and silicium molybdate) - showed that addition of 22 nM and 80 nM K15 results in the decrease of ΔA_{678} values by 17% and 57%, respectively (Figure 8, curves 4 and 5). It was shown earlier (Allakhverdiev et al., 1985) that a similar decrease of ΔA is observed upon addition of Mn^{2+} (0.1-3 μM) to such preparations, which is conducive to the reactivation of electron donation in RC of PSII.

Figure 9 features comparison of the curves showing the dependences of the inhibition of various photoreactions occurring in PSII on the concentration of K15: (1) photoinduced ΔF associated with the photoreduction of Q_A; (2) magnitudes of F_M under aerobic and anaerobic conditions; (3) photoinduced -ΔF and ΔA_{685} associated with the photoreduction of Pheo and (4) photoinduced ΔA_{678} associated with the photooxidation of chlorophyll P_{680}. It should be noted that all these dependences are similar.

DISCUSSION

The inhibition of oxygen evolution and photoreduction of $NADP^+$ in chloroplasts upon addition of K15 is indicative of the ability of these compounds to perturb the electron transport from water to $NADP^+$. On the other hand, the preservation of $NADP^+$ photoreduction by photosystem I from reduced DCPIP in the presence of K15 (see Table 1) shows that this compound acts at the level of PSII.

This suggestion is also supported by the K15-stimulated inhibition of photoinduced ΔF of PSII in chloroplasts (Figure 2) and in subchloroplast preparations of PSII (Figure 4).

Furthermore, both the inhibition of electron transport measured from the oxygen evolution and the repression of ΔF in PSII are apparently based on the common inhibition process which is supported by the similar dependence of these effects on concentration of K15 (Figure 3, curves 1, 2).

On the other hand, in the case of dinoseb the inhibition of electron transport (estimated from the measurements of O_2-evolution) occurs earlier than the repression of ΔF (curves 3, 4). Therefore, the authors of an earlier study (Klimov et al., 1989) concluded that the inhibition of photochemical activity of PSII manifested in the repression of ΔF makes a substantial contribution to the total inhibitory effect only at relatively high levels of dinoseb.

It should be noted that the efficiency of the inhibitory effect of K15 on the activity of PSII is ~80-100 times higher than of dinoseb (Figure 3). The total inhibition occurs upon binding of approximately one inhibitor molecule (K15) per one RC of PSII. Of high interest are the results of comparison of the inhibitory effects exerted on ΔF of PSII by compounds K15, diuron and dinoseb (Figure 4). In contrast to diuron and low levels of dinoseb (Klimov et al., 1989), compound K15 does not increase

the level of F (curves 3-5) which indicates to the absence of blockade of the electron transfer on the acceptor side of PSII. This assumption is also confirmed by the data indicating that, in contrast to diuron (curve 2), K15 does not slow down the dark decrease of ΔF which is known to reflect reoxidation of Q_A^-. This is also supported by the fact that compound K15 (in contrast to diuron and dinoseb) does not disturb, via the electron transport chain, the interaction between PSII and PSI estimated from the characteristic decrease of PSII fluorescence as a result of Q_A oxidation during additional excitation of PSI in chloroplasts (data not shown).

The effect of K15 does not appear to be related to the blockade of the electron transfer on the donor side of PSII, which is supported by the inhibition (and not the increase or at least preservation expected for such a case) of ΔA related to photooxidation of P_{680}. Moreover, after the inhibition of ΔF of PSII by K15 it is not reactivated upon subsequent addition of Mn^{2+} or other electron donors.

On the other hand, the inhibition of ΔF and the decrease of the total F level (as it is the case during the increase of electron transfer from PSII as a result of addition of DCPIP or ferricyanide oxidizing Q_A^- (Izava, 1980)) do not appear to be explainable by the acception of electrons from Q_A^-. This is confirmed by: (1) the absence of the effects of increased rate of dark relaxation ΔF related to photoreduction of Q_A; (2) the absence of positive ΔF, reflecting photoreduction of Q_A, upon addition of K15 under anaerobic conditions, which leads to a decreased F level.

A dramatic increase of the rate of dark relaxation of spectral effects associated with the photoreduction of Pheo as a result of reaction $[P_{680}Pheo]Q_A^- \rightarrow [P_{680}Pheo^-]Q_A^-$ and as a consequence of that - the decrease of these effects (Figure 6) - indicates that in the presence of K15 the life-time of the $Pheo^-$ decreases due to its reoxidation.

Then the decreased values of ΔF under aerobic conditions and lower levels of F under anaerobic conditions may be interpreted as a result of a more rapid decay of $[P_{680}^+Pheo^-]$ pair induced by compounds K15 due to $Pheo^-$ oxidation (which is also confirmed by the K15-induced quenching of the long-lived components of PSII chlorophyll fluorescence (Allakhverdiev et al., 1989)).

It may be suggested that the effect of K15 on the redox state of Pheo is not determined by its direct involvement into oxidation of $Pheo^-$ but this is, e.g., the result of increased O_2 access to $Pheo^-$. However, the preservation of characteristic efficiency of the inhibitory effect of K15 under anaerobic conditions (Figure 6) is an argument against such an assumption.

At the same time the inhibitory effect of K15 cannot be explained solely by the oxidation of $Pheo^-$ with this compound. Then one should observe a decrease of the inhibitory effect of K15 upon prolonged illumination due to accumulation of reduced molecules of the inhibitor which is not the case.

On the other hand, the increase in the rate of Pheo photoreduction upon addition of K15 in Mn-depleted preparations in the presence of dithionite (Figure 7) is a direct indication of the ability of the reduced (by dithionite) K15 form to donate electron(s) to PSII reaction center. The ability of K15 to donate electrons to PSII is supported by the inhibition of ΔA related to photooxidation of P_{680} upon addition of this inhibitor (Figure 8). Furthermore, compound K15 represses both the photoinduced and dark EPR signals II associated with the photooxidation of secondary electron donor Z (data not shown).

The similar concentration dependences of the inhibitory effect of these compounds for all photoreactions considered above are indicative of a common nature of the inhibition (Figure 9).

Thus, electron transfer from $Pheo^-$ to K15 seems to take place with subsequent donation of the electron to Z^+ (or P_{680}^+) which leads to the repression of the basal electron flow through PSII. In other words, compound K15 closes the chain of electron transfer in PSII by accepting an electron from $Pheo^-$ and donating it to Z^+ (or directly to P_{680}^+).

We showed earlier (Allakhverdiev et al., 1989; Klimov et al., 1989) that dinoseb is also capable of redox interaction with PSII reaction center components leading to a more rapid oxidation of $Pheo^-$ and reduction of P_{680}^+ and, as a result, to a faster decay of $[P_{680}^+Pheo^-]$ pair. However, the cyclic electron transfer from $Pheo^-$ to P_{680}^+ with the involvement of dinoseb may compete with the functional reactions of $Pheo^-$ oxidation and P_{680}^+ reduction and make a substantial contribution to the total effect of inhibition of electron transfer in PSII by dinoseb only at relatively high concentrations of this inhibitor. Moreover, in the case of the hydroxyperfluoroisopropyldinitrobenzole derivatives studied the redox interaction with PSII RC components, leading to the cyclic electron transfer seems to underlie their inhibitory action.

It is not ruled out that a K15 molecule reaching into RC of PSII performs the role similar to that of Q_A or even it replaces Q_A. This suggestion is supported by the following data: (1) the ability of these compounds to interact with the primary electron acceptor $Pheo^-$; (2) high affinity of these compounds to RC of PSII - one inhibitor molecule per RC is sufficient for producing the effect; (3) high constant of inhibitor binding to PSII (which is expressed in its retention by RC even despite the multiple attempts to wash the inhibitor out); (4) the ab-

sence of a decrease of the inhibitory effect upon a prolonged illumination (despite the redox nature of its interaction with RC components); (5) the ability of these compounds to inhibit the electron transport in isolated RC of PSII (D1/D2/Cytb559 complexes) free of Q_A (Nanba and Satoh, 1979). Apparently, the redox potential of K15 is around -450 mV which is supported by the data indicating that dithionite (but not sodium ascorbate) eliminates its inhibitory effect on PSII. Consequently, after oxidation of $Pheo^-$, K15 (in contrast to Q_A having a potential of about -130 mV) is unable to keep electron for a sufficiently long time, and the electron goes back to P_{680}^+ or Z^+ which leads to a disturbance of the basal electron transfer in PSII.

Thus, we revealed new highly efficient inhibitors of electron transfer in plant photosystem II - derivatives of hydroxyperfluoroisopropyldinitrobenzole - K15 whose action appears to be based on their redox interaction with the RC components of PSII and activation of a cyclic electron transfer. The inhibition constant for K15 is 45 nM.

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