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Binding of Novel Inhibitors of Electron Transfer in Photosystem 2, Derivatives of Perfluoroisopropyldinitrobenzene, with Polypeptide D2 of the Reaction Center

S. K. Zharmukhamedov[1], M. S. Kristin[1], Li Shuqin[2], S. I. Allakhverdiev[1], and V. V. Klimov[1]*

[1]*Institute of Basic Biological Problems, Russian Academy of Sciences, Pushchino, Moscow Region, 142290 Russia; E-mail: klimov@issp.serpukhov.su*
[2]*Institute of Botany, Chinese Academy of Sciences, 100093 Beijing, China; E-mail: lishuqin1965@yahoo.com.cn*

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Abstract—A binding site for novel inhibitors of K15 type (derivatives of perfluoroisopropyldinitrobenzene) with the components of reaction center (RC) of photosystem 2 (PS-2) of higher plants has been investigated. It has been shown that multiple washing the PS-2 submembrane chloroplast fragments (BBY-particles) treated with the K15 inhibitor, including multiple dilution in buffer in the presence of high concentrations of mono- and divalent ions, prolonged (up to 2-5 h) incubation, centrifugation, and subsequent resuspension in buffer deprived of the inhibitor, does not lead to restoration of functional activity of the PS-2. After addition of dithionite, inducing reduction and consequent decomposition of the inhibitor, and subsequent removal of dithionite by washing, the functional activity of PS-2 was completely restored. Incubation in the presence of sodium dodecyl sulfate (SDS), leading to solubilization of the sample to the level of protein components, induced the appearance of a fraction of free K15 retaining the initial inhibitory efficiency. To create a covalent binding of the inhibitor with protein, retained under the conditions of denaturing SDS polyacrylamide gel electrophoresis, the azido-containing analog of K15 (K15-N_3) was used. The need for radioactive label for identification of K15 was avoided by the revealed ability of K15-type inhibitors to emit fluorescence, which retained its features under the experimental conditions. With the technique of photoaffinity binding and denaturing SDS-PAGE in the presence of 6 M urea of submembrane chloroplast fragments enriched in PS-2 the D2-polypeptide, an integral component of the reaction center of PS-2, has been shown to be a binding site for K15-type inhibitors. This conclusion is in agreement with a suggestion (put forward in our earlier publications) that K15-type inhibitors are bound to PS-2 reaction center, replacing Q_A in its binding site. Hence, an agent specifically binding to polypeptide D2 has been found for the first time. The data are compared with information about inhibitory action and binding sites of the known inhibitors of electron transfer in PS-2.

Key words: photosystem 2, electron phototransfer, electron transport inhibitors, binding site

It was shown earlier [1-6] that K15 type inhibitors, derivatives of perfluoroisopropyldinitrobenzene (PFIPDNB), blocked with high efficiency electron transfer at the level of photosystem 2 (PS-2). The inhibitory mechanism of K15 type compounds differed significantly from that of the known inhibitors, diuron and atrazine, blocking reoxidation of the reduced Q_A^-, the primary plastoquinone electron acceptor of PS-2 [7]. Diuron and atrazine type inhibitors are known to be bound with the so-called herbicide-quinone binding, D1 polypeptide (32 kD) of the PS-2 reaction center (RC) [8-10]. They compete with each other [11], with exogenous plastoquinones [12], as well as with Q_B, the secondary plastoquinone electron acceptor of PS-2 [13, 14], for a binding site on this protein. As to inhibitory mechanism of K15-type substances, it was suggested that they are capable of replacing the primary plastoquinone electron acceptor, Q_A, from its binding site on the D2 polypeptide [1, 5, 6]. The following experimental data [1-6] support this assumption: 1) high affinity of K15-type compounds to the reaction center of PS-2; only one molecule of inhibitor per RC is enough to get the inhibitory effect; 2) suppression of photoreduction of Q_A, revealed as selective decreasing of the value of photoinduced changes of PS-2 chlorophyll fluorescence yield (ΔF), related to photoreduction of Q_A; 3) the capability of K15-type agents to redox interaction with reduced pheophytin ($Pheo^-$), the primary electron acceptor of PS-2; 4) the ability of K15 to stimulate photoreduction of cytochrome b_{559} in the isolated complex of D1/D2-cytochrome b_{559} reaction center of

* To whom correspondence should be addressed.

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PS-2 (deprived of Q_A), and thus to serve as an effective electron carrier between $Pheo^-$ and cytochrome b_{559} like Q_A, retained in subchloroplast membrane fragments and core complexes of PS-2; 5) the ability of K15 to maintain inhibitory action during a long period of illumination at the ration K15/RC close to 1 : 1 (or lower), in spite of redox nature of its interaction with the components of RC. The value of the redox potential of the pair $K15/K15^-$ is −0.53 V [3]. Due to its low redox potential K15 (unlike Q_A, which has a redox potential of −0.13 V), taking an electron from $Pheo^-$, cannot hold it for a sufficiently long period, and as a consequence the electron, transferred from $Pheo^-$ to K15, rapidly returns to $P680^+$ or Z^+, thus upsetting basal electron transport in PS-2 [1-6]. Also, it is known that the D2 polypeptide is most likely the binding site for Q_A [15, 16]. Consequently, serving as a "faulty" PS-2 primary electron acceptor, the derivatives of PFIPDNB as analogs of Q_A could be bound to a site different from the diuron-binding site. Furthermore, D2 protein (on which Q_A is located), and not D1 (on which Q_B is located) could be the polypeptide carrying the binding site for PFIPDNB.

The goal of this work was to investigate binding of the novel K15 type inhibitors with the polypeptides of PS-2 reaction center of plants.

MATERIALS AND METHODS

Chloroplast membrane fragments enriched in PS-2 (BBY-particles) and containing 180-220 chlorophyll molecules per reaction center were prepared from pea (*Pisum sativum* L.) chloroplasts as described previously [17]. Complete removal of Mn from BBY-particles was achieved using 1 M Tris-HCl buffer (pH 8.0), and 0.5 M $MgCl_2$ as described earlier [18]. All measurements were made using reaction medium A containing 20 mM Tris-HCl buffer (pH 7.8), 35 mM NaCl, and 2 mM $MgCl_2$. The photoinduced changes in fluorescence yield (ΔF) of chlorophyll ($\lambda > 660$ nm) related to photoreduction of PS-2 primary electron acceptor, Q_A, were measured in a 1-cm cuvette using a phosphoroscopic set-up [19]. The PFIPDNB derivatives were prepared and purified as described in [20]. The inhibitors were dissolved in dimethyl sulfoxide, the final concentration of which in samples did not exceed 1%. Triton X-100, digitonin, Tris, and diuron were from Serva (Germany) and other reagents were of domestic manufacture and of extra pure or chemically pure grade.

Photoaffinity labeling experiments using K15-N_3, the azide-containing analog of K15 inhibitor, were carried out with slight modifications as described earlier [10]. Suspension of subchloroplast membrane fragments of PS-2 in medium A (chlorophyll concentration was 50 μg/ml) was adapted in the dark for 10 min. Then K15-N_3 was added at the ratio one molecule of the inhibitor per a reaction center of the PS-2. The resulting mixture was incubated in the dark at room temperature for 5 min, transferred into the thermostat, and incubated there for no less than 15 min while their temperature reached 4°C. For activation of the azido-group the mixture was illuminated for 18 min with UV-light obtained from an OI-18 luminescent lamp (LOMO) (the source of light was a SVD-120A lamp). The band of UV-light with maximum transmission at near 370 nm (maximum of azido-group absorbance) was isolated with a UVS-6 light filter (thickness 3 mm; maximum transmission at 365 nm). The samples were washed three times with buffer lacking the inhibitor by means of 20-times dilution and subsequent centrifugation.

The pellet obtained after final centrifugation was cleared from the light-harvesting pigment–protein chlorophyll *a/b* binding complex (LHC-2), as described previously [21] and used for SDS-PAGE. Electrophoresis was carried out in 12-15% polyacrylamide gel in the presence of 6 M urea, 2% SDS, and 3% mercaptoethanol following the method of Laemmli [22].

For obtaining densitograms that revealed the contribution of polypeptide bands in polyacrylamide gel after electrophoresis, the gels were scanned at 280 nm using a scanning device on a Specord M-40 (Germany). To determine allocation of fluorescence at 450 nm, emitted by K15 inhibitor covalently bound to polypeptide, polyacrylamide gel was carefully cut into 2 mm pieces, the pieces dissolved in 1 ml 17% hydrogen peroxide at 55°C as described earlier [23], and characteristic fluorescence with maximum at 450 nm (with wavelength of excitation 315 nm) was registered on a Hitachi-850 spectrofluorimeter (Japan).

RESULTS

1. Reversibility and character of binding of PFIPDNB derivatives with components of the reaction center of PS-2. Binding a majority of the presently known inhibitors of electron transfer in PS-2 with chloroplasts, subchloroplast membrane fragments (heavy particles, enriched in PS-2) or core complexes of PS-2 occurs due to formation of a noncovalent bond [24]. The most widely know photosynthesis inhibitors, derivatives of urea (diuron) and triazine (atrazine) families, are characterized in particular by this type of binding with the components of PS-2. This means that these inhibitors can be easily removed from PS-2 by a washing procedure or by addition of competing alternative agents (other inhibitors) for the binding site. The washing procedure is dilution of the membrane with bound inhibitor by solution devoid of the inhibitor. Thus, binding the substance with membrane preparation can be considered as reversible if it is easily and completely forced out or if its concentration is decreased proportionally to the dilution of the membrane preparation with the

bound substance in the larger volume of solution deprived of the inhibitor [25].

Using the approaches described above it was shown that traditional inhibitors of PS-2, the derivatives of urea (diuron) and triazine (atrazine) families, were bond with PS-2 reversibly. An interesting situation occurs with phenolic family inhibitors. Binding of dinoseb with PS-2 membrane is noncovalent, and therefore it can be considered as reversible. However, dinoseb could not be removed easily from PS-2 membrane by washing as easily as with diuron or atrazine, although dinoseb was replaced from its binding site by competing with these agents. In this connection it was decided to designate the binding of phenolic family inhibitors of electron transfer in PS-2 as conditionally reversible [24].

Figure 1 shows data of experiments carried out to clarify the extent of reversibility of binding the K15-type inhibitors with subchloroplast membrane fragments enriched in PS-2. The value of photoinduced changes of PS-2 chlorophyll fluorescence yield (ΔF) related to photoreduction of the primary electron acceptor, plastoquinone Q_A, was selected for measurement of functional activity. The inhibitor K15 at the concentration of 3 μM completely suppresses the photoinduced ΔF (curve *2*) corresponding fully with data published by us previously [1, 2]. We tried to remove the inhibitor with multiple (up to five times) subsequent washings by means of 10-20-fold dilution by buffer A, prolonged (up to 30 min) incubation, and subsequent sedimentation of PS-2 membrane fragments by centrifugation. All washing procedures were carried out at the temperature of about 4°C. The efficiency of inhibition of functional activity of PS-2 by K15 at the temperature 20-25°C was comparable with that at 4°C. The percent inhibition of photoinduced ΔF of the treated by K15 subchloroplast membrane fragments of PS-2 after all these washing procedures remained unchanged (compare curves *2* and *4*). The washing procedure itself did not induce any suppression of the functional activity of PS-2 (curve *3*). The supernatant obtained after each centrifugation was tested for the presence of K15. For this purpose "fresh" noninhibited subchloroplast membrane fragments of PS-2 were resuspended into the obtained supernatant and their functional activity was compared to that of PS-2 particles resuspended in a buffer free from the inhibitor. Functional activity in both cases was practically the same (not shown). Furthermore, there was no fluorescence at 450 nm, characteristic to K15 in each supernatant (data about spectral properties of K15 are presented below).

The addition of reducing agent, dithionite, led to increased chlorophyll fluorescence yield to the maximal level (F_{max}) (curves *5* and *6*), independently of the presence in the medium of K15. The increasing in fluorescence to F_{max} in the presence of dithionite (in the absence of K15) has been repeatedly described earlier for different types of PS-2 preparations [26]. This occurs because

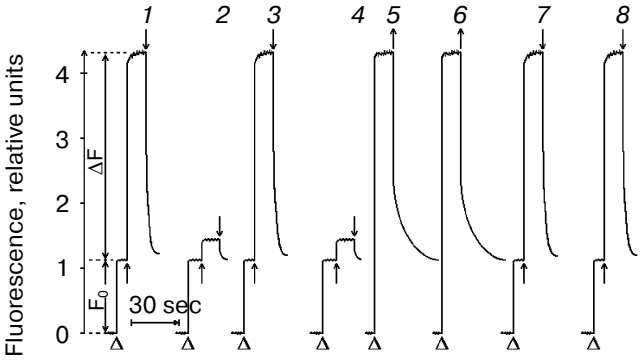

Fig. 1. Value of the dark level of fluorescence (F) and kinetics of photoinduced changes of yield of F (ΔF) related to photoreduction of the primary electron acceptor of PS-2, plastoquinone Q_A of subchloroplast membrane fragments (BBY-particles) in the absence of K15 (*1*, *3*, *5*, *7*) and in the presence of 3 μM inhibitor K15 (*2*, *4*, *6*, *8*) in the absence of other additions (*1-4*, *7*, *8*) and in the presence of 1 mg/ml dithionite (*5*, *6*). Kinetics *3-8* were measured after incubation for 30 min in the dark at temperature 4°C and subsequent procedure of washing free from the inhibitor as described in the text. Open triangles show the time of switching on measuring light (λ = 490 nm, 0.15 $J \cdot m^{-2} \cdot sec^{-1}$) which excites PS-2 chlorophyll fluorescence (λ ≥ 650 nm), arrows directed up and down indicate the time of switching on and off the actinic light (λ > 600 nm, 10^2 $J \cdot m^{-2} \cdot sec^{-1}$). Chlorophyll concentration was 10 μg/ml.

dithionite completely reduces Q_A in the dark. If dithionite is removed for example by the washing procedure described earlier, the functional activity of PS-2 membranes treated with K15 (curve *8*) will be recovered to the level of the control PS-2 preparations (curve *7*). Assuming that dithionite induced chemical destruction of K15 by a redox mechanism, spectral "model" experiments were performed. The experiments showed that absorbance of K15 solution totally disappeared after a short-term (about 1-2 min) incubation of the solution of this inhibitor in the presence of dithionite (not shown).

It is known that specific binding can to considerable extent depend upon the value of pH, type of buffer, the presence of di- or/and monovalent cations (Ca^{2+}, Mg^{2+}, Na^+, K^+), and sulfhydryl compounds (mercaptoethanol, dithiothreitol) [25]. We carried out experiments similar to those described above but with different compositions, at different pH values, and in the presence of the mentioned cations and reagents. Many of the conditions (alkaline pH, high concentration of salts) induce suppression of functioning of oxygen-evolving complex (OEC) of PS-2 due to the greatest vulnerability of the PS-2 donor side to the affect of the damaging factors [27]. To exclude the influence of these conditions on OEC, we have avoided using the "native" capable of photosynthetic oxygen evolution PS-2 preparation and performed the experiments on PS-2 membranes lacking Mn (after complete removal of endogenic Mn from OEC), in the presence of an artificial electron donor, sodium ascorbate. We have shown

earlier that efficiency and the mechanism of inhibitory action of K15-type substances remained unchanged on this type of PS-2 preparations; a similar situation was observed in the presence of artificial electron donors of PS-2 [1, 2, 5]. We found that efficiency of inhibition of PS-2 functional activity by K15 did not decrease over a wide range of applied conditions, namely: at pH values from 5.0 to 9.0; in the presence of high concentrations (up to 0.3 M) of Na^+, 50 mM Ca^{2+}, or Mg^{2+}.

In addition to the mentioned, similar experiments towards the removal of K15 by washing procedure were also made in the presence of anionic detergent, sodium dodecyl sulfate (SDS), which was applied for separation of polypeptides for SDS-PAGE, inducing denaturation of the functional complex of PS-2. In this case after the first washing the fluorescence at 450 nm characteristic for K15 was revealed in the supernatant. In addition, its intensity (in the supernatant) could be compared to that of the initial concentration of the inhibitor (in a buffer, before subchloroplast membrane fragments of PS-2 were resuspended in it). This indicates that K15 leaves its binding site with the component(s) of PS-2 reaction center and is found in the supernatant. Therefore, for formation of a covalent bond of the inhibitor with its binding polypeptide(s), which may be resistant under denaturing conditions it is necessary: 1) to use technique of photoaffinity binding base on K15-N_3, the azide containing analog of K15, and 2) to attach a radioactive or fluorescent label to the inhibitory molecule to facilitate identification of the inhibitor.

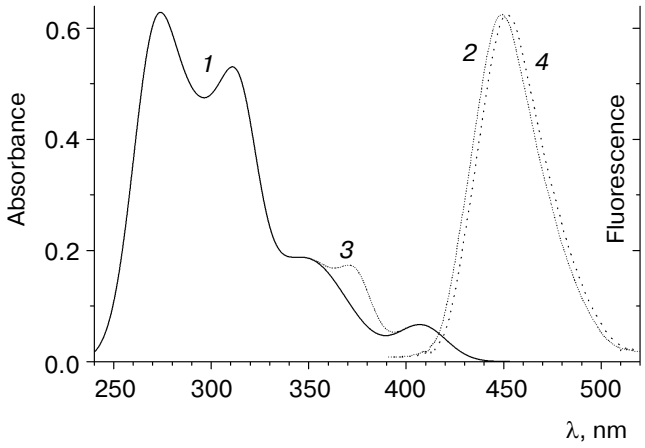

Fig. 2. Spectra of absorbance (*1*, *3*) and fluorescence (*2*, *4*) of the inhibitor K15 at the concentration of 10 μM (*1*, *2*) and the compound K15-N_3, the azide-containing analog of the inhibitor K15, at the concentration of 10 μM (*3*, *4*). Fluorescence was excited by light at 315 nm. The spectrum shown by line *3* differs from that shown by line *1* by the presence of an additional peak with absorption maximum at approximately 370 nm. The measuring medium contained 20 mM Tris-HCl buffer (pH 7.8), 35 mM NaCl, and 2 mM $MgCl_2$. All measurements were carried out in 1-cm quartz cuvettes. Fluorescence was measured from the front wall of the cuvette at temperature 20°C. Fluorescence spectra were normalized at the maximum near 450 nm.

2. Spectral properties of K15 type inhibitors. We investigated the spectral properties of K15 type inhibitors, including a rather large group of their azide-containing derivatives. The class of inhibitors revealed by us contains 26 compounds. They differ from each other in the substituents located on the right or on the left from the basic part of a molecule of perfluoroisopropyldinitrobenzene [2]. All K15 type inhibitors have analogous characteristic absorbance spectra, which is similar to that of K15, presented in Fig. 2 (continuous line *1*). The absorption spectrum was measured in medium A containing 20 mM Tris-HCl buffer, pH 7.8, 35 mM NaCl, 2 mM $MgC1_2$, and 10 μM K15 in a 1-cm quartz cuvette. It has four maxima at ~275, ~315, ~350, and ~410 nm. This is in good agreement with the data published on absorbance spectra for the range of aromatic compounds having molecular structure similar to K15 type inhibitors. For example, it was shown that the absorption maximum in the range of 340-350 nm and the shoulder in the range of 410 nm were characteristic for derivatives of 2,4-dinitro-5-fluoro-aniline [28]. Attaching an azido group to the molecule of K15 type inhibitor led to the appearance of an additional peak with maximum at approximately 365-370 nm in the absorption spectrum (line *3*). It was shown earlier that attaching an azido group to the molecule in the case of aromatic compounds was accompanied by the appearance of an additional band in the absorption spectra in the range from 300 to 400 nm [29].

The studied inhibitors are fluorescent. We investigated carefully the ability of K15 type inhibitors to emit fluorescence in different media. The fluorescence spectrum of K15 is presented by line *2* having a maximum at approximately 450 nm (excitation wavelength, 315 nm) and line half-width of 40 nm. The fluorescence spectrum of K15 presented in Fig. 2 was measured in medium containing 20 mM Tris-HCl buffer, pH 7.8, 35 mM NaCl, 2 mM $MgC1_2$, and 10 μM K15, from the front wall of a 1-cm quartz cuvette at 20°C. The fluorescence spectrum of K15-N_3, the azide-containing analog of K15, practically coincides with that of the initial inhibitor, K15 (Fig. 2, line *4*).

Conversion of azide into a highly reactive nitrene (–N:) was monitored by decreasing absorbance at 365-370 nm during illumination by UV-light with the wavelength 365 ± 5 nm, that corresponded to absorbance maximum of the azide group in the azide-containing analog of K15. In similar experiments we determined previously the duration of illumination by UV-light required for total conversion of azide group to nitrene under conditions used for photoaffinity binding. We established that after 18 min of illumination the band at approximately 370 nm had disappeared completely, while the absorbance spectra changed insignificantly as a whole.

Using the technique of photoaffinity binding, the azide containing analog of K15 and bovine serum albumin as a model of polypeptide of PS-2 reaction center, we ensured that fluorescence at 450 nm, characteristic for K15 type inhibitors, retained the formed covalent inhibitor–protein complex. In this model experiment we monitored the properties of fluorescence at 450 nm characteristic for the K15 inhibitor–polypeptide complex, at each stage both during electrophoresis and during elution of this polypeptide complex from the gel. Fluorescence at 450 nm of the inhibitor–polypeptide complex in a buffer in which the extraction of the complex from polyacrylamide gel was performed (see "Materials and Methods") retained the maximum at 450 nm (Fig. 3, line *1*, excitation wavelength 315 nm), and was reproducibly registered by the spectrofluorimeter. Thus, the revealed capability of K15 type inhibitors to emit characteristic fluorescence avoided the necessity of attaching a radioactive label to the inhibitor molecule for its identification in the polypeptide band on a polyacrylamide gel.

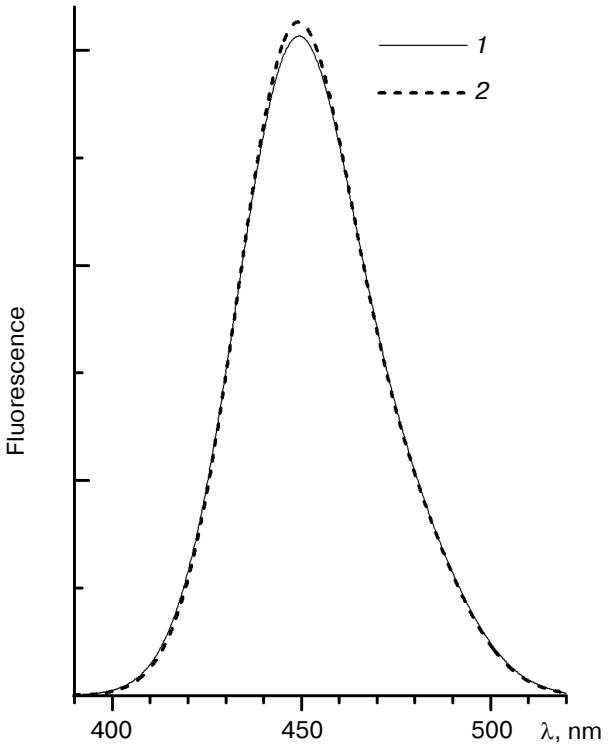

Fig. 3. Fluorescence spectra of covalent complex of the inhibitor K15–bovine serum albumin (*1*) and covalent complex of the inhibitor K15–polypeptide with apparent molecular weight 32 kD (*2*). Both complexes were eluted from the corresponding band of polyacrylamide gel after photoaffinity binding of K15-N_3 with bovine serum albumin or with subchloroplast membrane fragments (BBY-particles) and SDS-PAGE. Fluorescence was excited by light at 315 nm. Measuring medium contained 20 mM Tris-HCl buffer (pH 7.8), 35 mM NaCl, and 2 mM $MgCl_2$. Fluorescence was measured from the front wall of a 1-cm quartz cuvette at temperature 20°C. Fluorescence spectra were normalized at the maximum near 450 nm.

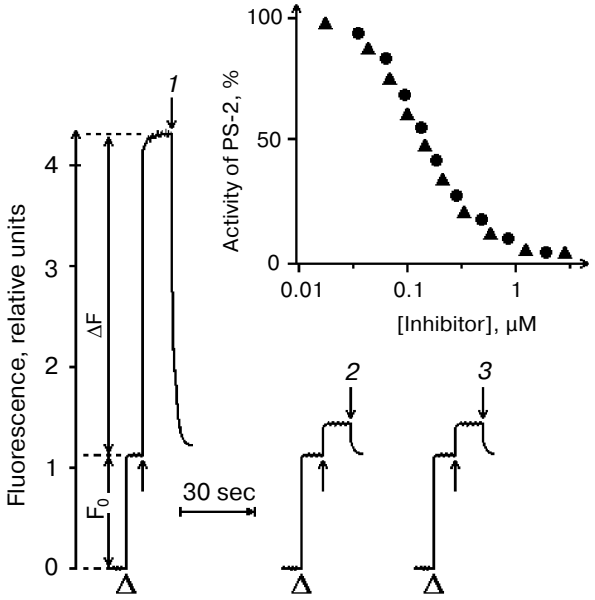

Fig. 4. Value of the dark level of fluorescence (F) and kinetics of photoinduced changes of yield of F (ΔF) related to photoreduction of the primary electron acceptor of PS-2, plastoquinone Q_A of subchloroplast membrane fragments (BBY-particles) without any additions (*1*) and in the presence of 3 μM inhibitor K15 (*2*) or 3 μM of its azide-containing analog, inhibitor K15-N_3 (*3*). Open triangles show the time of switching on measuring light (λ = 490 nm, 0.15 $J \cdot m^{-2} \cdot sec^{-1}$) which excites PS-2 chlorophyll fluorescence (λ ≥ 650 nm), arrows directed up and down indicate the time of switching on and off the actinic light (λ > 600 nm, 10^2 $J \cdot m^{-2} \cdot sec^{-1}$). Chlorophyll concentration was 10 μg/ml. The insert shows the dependence of functional activity of PS-2 on the concentration of K15 (black triangles) and K15-N_3 (black circles).

3. Comparison of the efficiency and the inhibitory mechanism of the initial compound K15 and its azide-containing analog on subchloroplast membrane fragments of PS-2. To use azide-containing analog of K15 in photoaffinity binding experiments it is necessary to make sure that it acts on PS-2 according to the same inhibitory mechanism as the initial inhibitor K15. Figure 4 shows the results of comparison of the inhibitory action of K15 and its azide-containing analog. Both inhibitors induce a decrease of positive ΔF, while the level of "constant" fluorescence (F_0) does not change (curves *2* and *3*). The rate of dark decay of ΔF reflecting reoxidation of the reduced Q_A also does not change. Such influence on the PS-2 chlorophyll photoinduced ΔF is one of the characteristic manifestations of the inhibitory action of K15 type compounds on PS-2 [1, 2]. This indicates that inhibition in both cases occurs according to the same mechanism. Dependencies of efficiency of inhibition of PS-2 functional activity measured by the PS-2 characteristic pho-

toreaction as described earlier [1] for the two inhibitors practically coincide (insert).

4. Photoaffinity binding of PFIPDNB derivatives with PS-2 preparations. The experimental data described above allowed us begin study of photoaffinity binding of K15-N_3, the azide-containing analog, with the PS-2 subchloroplast membrane fragments.

It was shown earlier [1] on thylakoids that K15 type inhibitors suppressed electron transfer at the level of PS-2 and did not influence the components of the photosynthetic electron-transport chain at the level of plastoquinone-plastocyanin oxidoreductase (cytochromes b_6/f complex) and photosystem 1. It is reasonable to suppose that the binding site for these inhibitors is also located on PS-2 polypeptide(s). Recently, a wide range of different kinds of preparations enriched in PS-2 ("heavy" subchloroplast membrane fragments, BBY-particles, "core" complexes of PS-2, isolated D1/D2-cytochrome b_{559} complexes of the reaction center of PS-2) was obtained. The polypeptide composition and functional properties of these preparations have been much studied. BBY-particles are preparations in which the photosystem retains "native" characteristics (containing active reaction centers and being connected with its secondary electron donors and acceptors, as well as light-harvesting pigment–protein chlorophyll *a/b*-binding complex (LHC-2)). On the other hand, carrying out photoaffinity binding experiments on BBY-particles (not thylakoids) allows us to exclude possible nonspecific binding. Therefore, experiments designed to reveal the binding site of K15 type inhibitors in the BBY-particles were selected. LHC-2 is composed of several polypeptides with molecular weight in the range 29-24 kD and binding more than half of the PS-2 chlorophyll molecules. The presence of LHC-2 polypeptides on SDS-PAGE hampered the well-defined revealing of D1 and D2 polypeptide bands. Therefore, upon photoaffinity binding we purified BBY-particles from LHC-2 as described earlier [21], i.e., actually we isolated the so-called "core" complexes of PS-2 (PS-2 complexes deprived of LHC-2) from these BBY-particles. The resulting preparations were subjected to denaturing SDS-PAGE to reveal the polypeptide which carries the binding site for K15 type inhibitors.

Figure 5 shows densitograms after SDS-PAGE of PS-2 preparations (obtained by the procedures described above for LHC-2 removal) which were not subjected to photoaffinity binding (*1*) and after photoaffinity binding with K15-N_3 (*2*). The polypeptide composition of the PS2 preparations, represented by densitogram *1*, correlates well with that described previously for a similar type of PS-2 preparation [30, 31]. It consists of polypeptides D1, D2, CP43, CP47, water-soluble polypeptides of OEC (33, 24, and 18 kD) as well as apo-protein of cytochrome b_{559}. The polypeptide band in the region 60 kD corresponds to heterodimer of D1- and D2-polypeptides [31, 32].

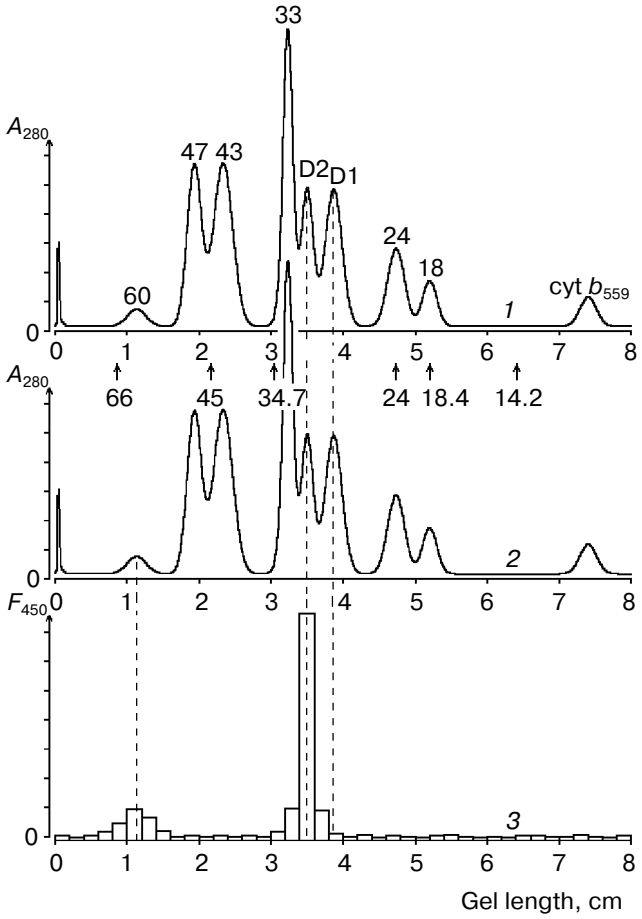

Fig. 5. Comparison of densitograms of gels (scanned at wavelength 280 nm) after denaturing SDS-PAGE in the presence of 6 M urea of PS-2 preparations (BBY-particles after removal of LHC-2) (lines *1*, *2*) and distribution of fluorescence with maximum at 450 nm (λ of excitation is 315 nm) emitted by the inhibitor–polypeptide complex in different sites of the gel (2) (line *3*). Gel (1) was obtained in the absence of other additions (line *1*); gels (2) and (3), after photoaffinity binding with K15-N_3, the azide-containing analog of the K15-type inhibitors (lines *2*, *3*).

Comparison of the positions of respective bands with similar relative electrophoretic mobility in these two gels shows that they practically coincide. This indicates that the photoaffinity binding of the azide-containing analog does not change the relative electrophoretic mobility of the PS-2 polypeptides.

Figure 3 (line *2*) shows fluorescence emission spectra of the inhibitor–PS-2 polypeptide complex, which was isolated with digestion of one 2-mm piece of gel containing polypeptides of PS-2 after photoaffinity binding with inhibitor. The spectrum coincides with the corresponding spectra, characteristic for the inhibitor–polypeptide complex described above. The intensity of fluorescence at 450 nm (excitation wavelength 315 nm) characteristic of

the inhibitor–PS-2-polypeptide complex was measured for each 2-mm piece of gel. The result is presented as a fluorogram (Fig. 5, line *3*).

Despite the fact that the region of 30-34 kD did not show complete resolution of polypeptides D1 and D2, the comparison of fluorescence at 450 nm in gel (fluorogram) with polypeptide bands (densitograms) showed that gel zone with fluorescence at 450 nm emitted by the inhibitor–PS-2-polypeptide complex corresponded to polypeptide band in the region of 30-34 kD with less relative electrophoretic mobility (32 kD polypeptide D2). It should be noted the absence of a shoulder on the fluorogram corresponds to the polypeptide in the region of 32-30 kD with higher relative electrophoretic mobility (30 kD polypeptide D1). Furthermore, the emission of fluorescence by polypeptide band in the region of 65-55 kD, corresponding to heterodimer of the D1 and D2 polypeptides [31, 32], was observed.

DISCUSSION

1. Binding of a substance with membrane preparations is considered as reversible if the substance is easily and completely forced out (for example, [^{14}C]naphthalene-1-acetyl acid, NAA, is easily forced out from membrane preparations by excess of unlabeled NAA, irrespective of the order in which the reagents were added) or if the concentration of the substance bound with the membrane preparation decreases proportionally to the dilution in a larger volume of solution lacking the inhibitor [25].

The fact that multifold dilution of PS-2 subchloroplast membrane fragments does not result in release of K15 connected to them without photoaffinity binding shows that the binding of investigated PFIPDNB derivatives with PS-2 is irreversible. The rate and reversibility of binding are used as indicators that formation of a covalent bond does not occurred, while retaining of a bond in the presence of high concentrations of mono- and divalent cations indicates its nonionic nature [25]. The impossibility to remove K15 from PS-2 subchloroplast membrane fragments with multiple washing (as described above) shows the irreversibility of binding of PFIPDNB derivatives with PS-2 and the formation of a covalent binding. At the same time, the covalent bond is retained under denaturing conditions (for example, during solubilization of PS-2 into the composing polypeptide components). This did not occur in our case. During incubation of PS-2 in the presence of SDS K15 passes easily into solution. The fact that high concentrations of mono- and divalent cations do not disturb binding of K15 with PS-2 indicates its nonionic nature. On one hand, since the bond of K15 type inhibitors with PS-2 components is not covalent, it may be regarded as reversible. Because these inhibitors are not removed from PS-2 by washing, their binding may be considered as irreversible.

Thus, based on the experiments performed it can be concluded that binding of K15 type inhibitors with PS-2 components, though very firm, is not covalent, and has nonionic nature and may be considered as conditionally reversible.

The results coincide well with the literature data. Binding of the known inhibitors of PS-2 is known to occur due to the formation of hydrogen bridges [24]. The inhibitors of diuron and atrazine types are considered to bind reversibly, whereas there is evidence for binding of phenol type inhibitors (dinoseb) as being irreversible [33].

2. The K15 type inhibitors are relatively complex organic molecules, derivatives of perfluoroisopropyldinitrobenzene containing a wide range of substitutes like alkyl-, hydroxyl-, amino-, fluorocarbon-, keto-, and other groups [2]. It was shown earlier that the nitro group has a tendency to suppress fluorescence. For example, nitrobenzene, nitronaphthalene, nitrophenol, and picric acid fluoresce have quite low quantum efficiency, therefore these compounds are considered as weak or even non fluorescing (see, for example [34, 35]). However, the nitro group does not necessarily totally quench fluorescence of compounds, especially if a molecule contains a substituent promoting increased fluorescence intensity. Hence, for example, 1-nitro-2-naphthylamine fluoresces, like a whole range of dyes containing a nitro group [36]. Alkyl substitution induces an increase of quantum efficiency of fluorescence of benzene derivatives. The intensity of fluorescence of fluoro-substituted compounds is similar to that of the corresponding hydrocarbons. Amino- and dimethylamino-groups induce a considerable increase in fluorescence yield of benzene derivatives and a shift in fluorescence spectra closer to the visible range. Hydroxyl- and methyl-groups available in a molecule induce an increase of fluorescence intensity of aromatic compounds and a shift of their luminescence spectra into the region of longer wavelengths [36]. It has been established that on increasing size of an aromatic or heterocyclic system the influence of groups preventing fluorescence emission is considerably attenuated. Intense fluorescence of these compounds has been noted [37]. For example, several derivatives of nitrostilbene (4-dimethylamino-4′-nitrostilbene), and the derivatives of nitroaniline (*m*-nitrodimethylaniline) had rather intensive fluorescence [38]. The presence of "fluorescence activating" substitutes shifted fluorescence spectra into the region of the longer wavelength [37]. The literature describes examples where nitro- and dinitro-derivatives of benzene are used as effective fluorescing labels for analysis of a whole range of biological components. The derivatives of nitro- and/or dinitrobenzene have been shown to be able to emit characteristic fluorescence with such high quantum yield that it allows researchers to reveal labeled components in micro- and even picomolar concentrations. Thus, a whole range of nitrobenzene derivatives are used as effectively fluorescing labels for

fluorometric determination of amphetamine and its analogs [39], primary and secondary amines in blood and urine [40], carbamates, ureas, organophosphorus compounds, aliphatic amines, aldehydes, ketones, diphenyls, some compounds having pharmacological significance [41], and amino acids [42, 43]. Sodium dantrolene (1-{[5-*p*-nitrophenyl)furfurylidene]amino}hydantoin sodium hydrate), a nitrobenzene derivative used as medicinal preparation, fluoresces (λ_{ex} = 395 nm; λ_{em} = 530 nm) with efficiency high enough for analytic identification of it in blood and urine by spectrofluorimetry [44, 45].

Our data show that the derivatives of perfluoroisopropyldinitrobenzene (including their azide analogs) also fluoresce, extending experimental possibilities for investigation of the interaction of K15 type inhibitors with the reaction center of PS-2.

3. Generally, attaching an azide group in an inhibitor molecule, as required for photoaffinity binding, does not change the inhibitory mechanism and decreases the efficiency of inhibition only insignificantly (as in the case of traditional inhibitors of PS-2, the derivatives of urea and atrazine [8-10]). In the case of K15 type compounds, investigated by us in this work, attaching an azide group did not influence the mechanism or the efficiency of inhibitory action (Fig. 4). The fact that both azide-free and azide-containing inhibitors induced similar effects on characteristic reactions related to photochemical activity of PS-2 demonstrated this. The above said and the practically total coincidence of the curves of dependence of inhibition of PS-2 functional activity for both inhibitors allowed us to use the azide derivative of K15, K15-N_3, in experiments for revealing the binding site of K15 type inhibitors. The results obtained enable us to use it as a characteristic for the initial inhibitor as well.

4. Earlier [46, 47] during SDS-PAGE an anomalous change in relative electrophoretic mobility of herbicide-quinone-binding polypeptide D1 depending on the concentration of urea in the medium was noted. In the presence of low concentrations (2 M) of urea this polypeptide has lower electrophoretic mobility (32-34 kD) relative to the polypeptide D2 (30 kD). Increased urea concentration in the medium (to 4-6 M), on one hand, promoted a well-defined resolution of polypeptide bands in the region of 30-34 kD. Nevertheless, simultaneously it resulted in changing electrophoretic mobilities of D1 and D2 polypeptides as opposed to 30 kD for D1-polypeptide and 32-34 kD for D2-polypeptide, respectively [47, 48]. These results were repeatedly confirmed by identification of these polypeptides with highly specific monoclonal antibodies [49], by preferential labeling of D1-polypeptide with ^{35}S [50]. Now the presence of high (4-6 M) concentrations of urea during SDS-PAGE is considered to be sufficient condition for identification of polypeptide D1 and D2 in polyacrylamide gel.

The possible application of these data in our experiments is confirmed by the absence of changes in relative electrophoretic mobility of polypeptides after photoaffinity binding of K15 with subchloroplast membrane fragments enriched by PS-2 (Fig. 5).

The fact that in our experiments SDS-PAGE was performed in the presence of high concentration of urea and maximum of fluorescence of compound K15 corresponded to polypeptide band 32 kD (and not to 30 kD) might serve as an indicator that a binding site of PFIPDNB derivatives is apparently located on polypeptide D2. The presence of fluorescence of K15-N_3 in the band 55-65 kD did not contradict the conclusion, since the band belonged to heterodimer of polypeptides D1 and D2 [32].

The absence of fluorescence of K15 at the beginning and end of gel showing the absence of nonspecific binding can be explained by the ratio of inhibitor/RC, which was selected equaled to 1, and by the high specificity of binding of K15 with the polypeptide D2 of the PS-2 reaction center.

At present the D1 polypeptide is considered to be a binding site for the majority of the inhibitors of electron transfer in PS-2 [8-10], containing, probably, different partly overlapping herbicide-binding niches [7, 15, 24, 51].

The existence of several binding sites even for the known inhibitors of diuron and triazine types has been suggested [52-55].

The possible existence of a binding site on the D2 polypeptide for the K15 type inhibitors revealed in our work was also shown for some other traditional inhibitors of the electron transport in PS-2. In addition, it was proved that binding the inhibitor with the D2 polypeptide was no less important for suppression of PS-2 activity than binding with the known site on the D1 polypeptide resulting in no less dramatic consequences for PS-2 (but as a result of inhibition of other reactions of the photosystem). For example, using pea thylakoids and polyclonal antibodies for D1 polypeptide and monoclonal antibodies for D2 polypeptide showed that [^{14}C]atrazine along with the known binding site on D1 polypeptide had a second binding site on the D2 polypeptide. Binding the inhibitor with high affinity site (K_d 80 nM), located on polypeptide D1, blocked the oxidation of reduced primary electron acceptor, plastoquinone Q_A. Binding the inhibitor with low affinity site (K_d 420 nM), located on polypeptide D2, blocked recombination of charges between Q_A^- and S-states of the oxygen-evolving complex of PS-2 [56].

Our conclusion that a binding site for novel K15 type inhibitors is located on the D2 polypeptide, and not on the D1 polypeptide, is indirectly confirmed by the data published earlier about the absence of competition for a binding site between these inhibitors, the derivatives of perfluoroisopropyldinitrobenzene, and traditional herbicide of PS-2 (diuron) [5]. On the other hand, these data serve as confirmation of validity of the idea expressed by

us earlier [1, 5], namely, the inhibitors of K15-type, binding with the polypeptide D2, replace primary electron acceptor, plastoquinone Q_A, from its binding site, likewise diuron or atrazine, binding with the D1 polypeptide, replace secondary electron acceptor, plastoquinone Q_B.

Thus, our data indicate that polypeptide D2 is the binding site for novel inhibitors of K15-type, the derivatives of perfluoroisopropyldinitrobenzene, where the binding is irreversible noncovalent, without ionic nature, and being, apparently, a result of the formation of hydrogen bonds. These results are contrary to some extent to the accepted scheme of herbicide binding in PS-2, but simultaneously are in good agreement with the results on functional measurements, based on the proposed mechanism of inhibitory effect of the compounds on PS-2 [1]. The analysis of publications concerning this matter and the results in this paper allow us to conclude that a chemical agent, which binds selectively with polypeptide D2, an integral component of the reaction center of PS-2, has been revealed for the first time.

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