Occupation of the QB-binding Pocket by a Photosystem II Inhibitor Triggers Dark Cleavage of the D1 Protein Subjected to Brief Preillumination*

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The D1 protein of the photosystem (PS) II reaction center turns over very rapidly in a light-dependent manner initiated by its selective and specific cleavage. The cleavage of D1 was studied by using a PS II inhibitor, N-octyl-3-nitro-2,4,6-trihydroxybenzamide (PNO8), as a molecular probe. The following results were obtained. (i) D1 was selectively cleaved into 23-kDa N-terminal and 9-kDa C-terminal fragments in complete darkness by PNO8 at a single site in a D-E loop connecting membrane-spanning helices D and E. (ii) The cleavage was much lower than that required for oxygen evolution. (iii) The effect of preillumination was slowly lost during brief period before the addition of PNO8 in darkness. (iv) The light intensity of preillumination required for the cleavage was much lower than that required for O₂ evolution. (v) The light-triggered cleavage of D1 was observed in thylakoids, PS II membranes, and PS II core particles, but not in purified PS II reaction centers. More than 60% of D1 was cleaved into the two fragments with no other by-products. (vi) The cleavage reaction revealed a marked pH dependence that was considerably different from that for inhibition of PS II activity. The results are interpreted as indicating that the binding of PNO8 to the QB-binding pocket triggers proteolytic cleavage of D1 that has been previously modified during illumination.

Photosystem (PS) II is composed of at least 20 proteins, including the reaction center D1 and D2 proteins, embedded in thylakoid membranes (1). D1 is involved in the binding of all redox-active components but QA quinone required for primary charge separation and electron transport from water to QA quinone (2, 3). When photosynthetic oxygen-evolving organisms are exposed to excess light with an intensity that over saturates photosynthesis, electron transport is inhibited at the acceptor side of PS II (4–7). In damaged PS II, D1 is selectively degraded, while other PS II proteins are generally preserved in an intact state (5–8). These processes are termed photoinhibition. Under high light conditions, the translation rate of D1 mRNA is 50–100 times greater than those of other PS II proteins, and defective PS II is repaired by inserting newly synthesized D1 into photo inhibited PS II (9–11). PS II activity can remain uninhibited since the rates of both denovo synthesis of D1 and its reassembly are coordinated with the D1 degradation rate (12). Therefore, the rate of the light-dependent turnover of D1 may be determined by its degradation rate.

D1 is considered to be irreversibly modified during light-induced inactivation of PS II to become a target of the following pathway toward proteolytic breakdown (5–8). Active oxygen species are believed to be involved in damaging D1 both in vivo and in vitro (13–16). However, what damage specifically leads to degradation of D1 and how it is selectively degraded remain unclear. Light-induced degradation of D1 is retarded by photoaccumulation of plastoquinol at the QA site (17) or by the addition of herbicides such as 3-(3,4-dichlorophenyl)-1,1-dimethyleurea and atrazine (9, 11, 18–22). Therefore, it has been proposed that degradation of damaged D1 is regulated by conformation of the acceptor side of PS II, which is affected by QA site occupation by plastoquinone or herbicides (23). Evidence for changes in the conformation of the QA site induced by herbicide binding includes the apparent influence of herbicides on the accessibility of trypsin to the target arginine residue located near the QA site in D1 (18, 24). Therefore, it has been hypothesized that D1 with conformationally modified QA sites is selectively degraded by a further proteolytic process.

Degradation of D1 is initiated by cleavage at specific sites, resulting in several distinct fragments. Extensive in vivo and in vitro studies have shown that the primary cleavage occurs at the loop domain connecting transmembrane helices D and E, yielding N-terminal and C-terminal fragments with apparent molecular masses of 23 kDa (25–29) and 10 kDa (27, 28, 30). However, the precise site of cleavage has not been determined. Since this loop domain serves as the binding site for QA plastoquinone as well as various kinds of herbicides, primary cleavage in this region appears consistent with a conformational change around the QA site, leading to subsequent degradation of photodamaged D1.

The effects of various herbicides and PS II inhibitors on the stability of D1 were studied in an attempt to demonstrate that its degradation can be induced in the absence of photoinhibitory conditions through changes in the conformation of the D-E loop domain. Recently, we demonstrated that under dark conditions, D1 is selectively and specifically cleaved into two fragments with apparent molecular masses of 23 and 9 kDa when the QA site is occupied by PNO8, a highly potent PS II inhibitor that specifically inhibits electron transport between QA and QB (31). PNO8 belongs to the category of phenol-type PS II inhibitors (32) because it contains the characteristic phenol nucleus.
and other structural features essential for phenol-type inhibitors (33). Furthermore, PS II of atrazine-resistant plants has increased sensitivity both to phenol-type inhibitors and to PNO8 due to replacement of Ser-264 by Gly in the D1 protein (32). PNO8 has a unique chemical structure due to its phloroglucinol nucleus. In a previous study, PNO8-dependent cleavage of D1 was suppressed at low temperatures and by inhibitors of serine-type proteases, but was not inhibited by the absence of oxygen (31). The 23- and 9-kDa fragments are also generated as degradation products by photoinhibitory illumination in the absence of PNO8 (27, 28). Therefore, binding of PNO8 to the Q₉ site seems to induce conformational changes that are similar to those induced by photodamage in the D-E loop domain, triggering proteolytic cleavage. However, the population of D1 cleaved by PNO8 was limited to a minor fraction of the total PS II, despite PNO8 binding at Q₉ sites in all PS II centers (31). This suggests that PNO8-dependent cleavage of D1 requires certain specific conditions.

In this study, the selective and specific cleavage of D1 induced by PNO8 is further characterized. It is demonstrated that PNO8 binding to the Q₉ site leads to cleavage of the fraction of D1 that has been modified during illumination before the addition of PNO8. Possible mechanisms to explain the effects of preillumination are discussed. In addition, the site of PNO8-induced cleavage in D1 was studied to further characterize the proteolytic mechanism.

**EXPERIMENTAL PROCEDURES**

**Preparations and Plant Materials**—Thylakoid membranes were prepared from mature spinach as described previously (31). They were resuspended in 400 mM sucrose, 15 mM NaCl, 5 mM MgCl₂, and 40 mM MES/NaOH (pH 6.5) and stored in liquid nitrogen. BBY-type O₂-evolving PS II membranes were prepared from spinach and seedlings of wheat by solubilization with Triton X-100 as described previously (34). O₂-evolving PS II core particles were fractionated by centrifugation following solubilization of the PS II membranes with n-heptyl thiogluco side according to the method of Enami et al. (35) with modifications. PS II reaction center complexes were prepared from PS II membranes following Triton X-100 solubilization according to the method of Namba and Satoh (3). Samples were stored in liquid nitrogen until use. After thawing, samples were washed and suspended in assay buffer containing 400 mM sucrose, 20 mM NaCl, and 40 mM MES/NaOH (pH 6.5). All procedures were carried out under a dim green light or in complete darkness, unless otherwise noted. The samples were then incubated in the dark for at least 2 h at 0°C, after which further treatments were applied.

Treatment with PNO8 and with Lysylendopeptidase—The sample suspension was incubated with PNO8 in complete darkness at 25°C. PNO8 was dissolved in dimethyl sulfoxide and this was added to the sample such that the final concentration of dimethyl sulfoxide in the suspension mixture was <1% (v/v). For preillumination, the sample suspension at 25°C was illuminated with white light (50 mW/cm²) unless otherwise noted) filtered through heat-absorbing and neutral density filters prior to the addition of PNO8 in the dark. The sample concentrations during PNO8 treatment were 400, 200, 80, and 4 μg of Chl/ml for thylakoid membranes, PS II membranes, O₂-evolving core particles, and reaction center complexes, respectively. The following buffers were used to treat the samples with PNO8 at various pH values: 40 mM MES/NaOH for pH 4.5–6.8, HEPES/NaOH for pH 7.1–7.5, and Tricine/NaOH for pH 8.0–8.5, supplemented with 400 mM sucrose and 20 mM NaCl. After incubation, the treated membranes were quickly frozen and stored in liquid nitrogen until protein analysis. Digestion of D1 cleaved by PNO8 was limited to a minor fraction that are similar to those induced by photodamage in the D-E centers (31). This suggests that PNO8-dependent cleavage of D1 is induced by PNO8 binding to the QB site, leading to cleavage of the D1 protein. The 23- and 9-kDa fragments are also generated as degradation products by photoinhibitory illumination with nitro blue tetrazolium chloride and bromochloroindolyl phosphate (Sigma). Densitometric determination of immunoreacted bands was carried out using an image scanner (ATTO densitograph system).

Measurement of PS II Activity—O₂ evolution was measured at 25°C using a Clark-type oxygen electrode placed in assay buffer supplemented with 0.5 mM phenyl-p-benzoquinone as an electron acceptor.

Preparation of PNO8—PNO8 was synthesized and separated using a silica gel column (38). PNO8 was then highly purified by high performance liquid chromatography with a semicarbazide silica gel column (PEGsil ODS, Senshu Pack) using acetonitrile/H₂O/formic acid (87.5:12.4:0.1, v/v) as the solvent system. The structure of synthesized PNO8 was confirmed by H NMR, 1R, and mass spectroscopy. No derivatives were detected during storage in dimethyl sulfoxide.

**RESULTS**

Fig. 1 shows the effects of preillumination on PNO8-induced cleavage of D1. PS II membranes were illuminated with white light at 50 mW/cm² for 0.5 s in the absence of PNO8. PNO8 was added, and the membranes were further incubated for 60 min in the dark. The treated membranes were then subjected to SDS-PAGE, and D1 and its cleavage products were detected immunologically using D1 antiserum. As reported previously, incubation in the dark of membranes with PNO8 but without preillumination induced cleavage of D1, yielding two fragments of 23 and 9 kDa (Fig. 1, lane c). The band of the 23-kDa fragment was detected as a doublet due to interference of the psbS gene product (39) superimposed on the fragment. When the PS II membranes were illuminated prior to the addition of PNO8, the quantity of the two bands of the fragments increased markedly to at least five times greater than the quantity induced without preillumination (lane d). Since the positions of the fragments in the preilluminated membranes were exactly the same...
It is unlikely that loss of D1 cleavage is due to the absence of O$_2$ but does not induce the cleavage of D1. Perhaps this is due to large perturbations on the acceptor side of PS II that occur during preparation, but we cannot rule out the possibility that PNO8 is incapable of binding to the Q$_b$ site in the reaction center complexes. Alternatively, some proteinaceous factor responsible for D1 degradation may be absent in these preparations.

Fig. 2. Effects of preillumination on the cleavage of spinach D1 induced by PNO8 in various preparations. Thylakoid membranes (lanes a–c), PS II membranes (lanes d–f), O$_2$-evolving PS II core particles (lanes g–i), and PS II reaction center complexes (lanes j–l) are shown. Samples were illuminated with white light (50 mW/cm$^2$) for 0.5 s (lanes b, e, h, and k) or 0.5 s (lanes a, c, d, f, g, i, j, and l) at 25 °C and then incubated for 60 min at 25 °C with (lanes b, c, e, f, h, i, k, and l) and without (lanes a, d, g, and j) the addition of PNO8. D1 and its degradation products were detected with D1 antiserum. Sample and PNO8 concentrations during treatment were 400 µg, 200, 80, and 4 µg of Chl/ml and 10, 10, 50, and 100 µM for the thylakoid membranes, PS I membranes, O$_2$-evolving PS II core particles, and PS II reaction center complexes, respectively. The respective sample quantities loaded in each SDS-PAGE well were 2.5, 1, 0.3, and 0.04 µg of Chl for the thylakoid membranes, PS II membranes, PS II core particles, and PS II reaction center complexes, bands of approximately 42, 34, 22, and 15 kDa seen in lanes g–l were due to nonspecific immunoreaction with unidentified substances.

Fig. 3. Effect of light intensity during preillumination on the cleavage of spinach D1 by PNO8. The accumulation of the 23-kDa fragment (C) and O$_2$ evolution activity (△) was measured at various light intensities. The 23-kDa fragment was detected with D1 antiserum and quantified densitometrically. The sample quantity loaded in each SDS-PAGE well was 1 µg of Chl. For PNO8 treatment, the PS II membranes were illuminated at 25 °C for 10 s, followed by incubation in the dark at 25 °C for 60 min after the addition of 10 µM PNO8. The sample concentration was 200 µg of Chl/ml for both the PNO8 treatment and the O$_2$ evolution measurement. Maximum O$_2$ evolution was 750 µmol of O$_2$/mg of Chl/h when measured under saturating light conditions with samples of 10 µg of Chl/ml.

Fig. 4. The stimulation effect of preillumination persisted for a surprisingly long time even at room temperature and decayed exponentially, with a decay half-time of ~1 h at 25 °C (Fig. 4, inset). No D1 fragments were detected following preillumination or during incubation in the dark in the absence of PNO8.

Fig. 5A shows the effect of repetitive illumination on the PNO8-dependent cleavage of D1. PS II membranes were preincubated for 0.5 s and then incubated in the dark after the addition of PNO8. During incubation in the dark, the sample suspension was illuminated intermittently every 30 min for...
0.5 s. The quantity of the 23-kDa fragment that accumulated during each 30-min incubation period in the dark was nearly the same after the first and second illuminations, decreased progressively after each successive illumination, and showed virtually no change after the fifth illumination. The quantity of D1 decreased to 40% of the original after the fifth illumination, but no fragments other than the 23- and 9-kDa fragments were detected. It should be noted that no fragments were detected even after incubation for 6 h in the absence of PNO8 (data not shown). Fig. 5A also shows the accumulation of the 23-kDa fragment following a single preillumination. The 23-kDa fragment appeared with no lag time and reached a constant level after 150 min, in good agreement with the kinetics for its formation in the absence of preillumination (31). This indicates that preillumination does not stimulate the rate of the cleavage reaction, but instead increases the number of PS II susceptible to PNO8 treatment. As shown in Fig. 5B, the quantity of the 23-kDa fragment that accumulated during repetitive illumination showed an inversely proportional relationship to the quantity of D1. It therefore can be concluded that the PNO8-dependent cleavage of D1 at a single site is solely the direct cause of the decrease in the quantity of D1.

The results also show that the effect of repetitive illumination is cumulative with respect to stimulating the cleavage of D1. Since the light intensity of each illumination was near the saturation level for cleavage, the cumulative nature of the preillumination effect may imply that modification of D1 can be reversibly and repeatedly effected by illumination, thus resulting in a state that is cleaved upon binding of PNO8. The first illumination was in the absence of PNO8, but successive illuminations were in the presence of PNO8, a potent PS II inhibitor. This indicates that preillumination stimulates the PNO8-dependent cleavage of D1 even when electron transfer from 

\[ Q_0^{-} \] to 

\[ Q_0 \] is interrupted. This is consistent with the finding that preillumination stimulated the cleavage of D1 in core particles that had been depleted of a plastoquinone molecule at the 

\[ Q_0 \] site, as shown in Fig. 2.

Fig. 6 shows the pH dependence of the cleavage of D1 and the inhibition of O2 evolution activity by PNO8. In this experiment, PS II membranes were incubated with PNO8 for 60 min with no preillumination to minimize any possible effect of pH on the preillumination process. D1 cleavage, as measured by the formation of the 23-kDa fragment band, was relatively low below pH 7 and increased steeply below pH 7, reaching a plateau at around pH 4.5. This is not ascribed to a pH-dependent change in the capability of PNO8 to bind to the 

\[ Q_0 \] site since the
potential of PNO8 as a PS II inhibitor showed a constantly high level between pH 4.5 and 7.0 and decreased above pH 7.0. Preillumination was found to stimulate the PNO8-dependent D1 cleavage at pH 5.0 as well as at pH 6.5 (data not shown). Throughout the pH range examined in this study, only the 23- and 9-kDa fragments were detected as cleavage products of D1, indicating that the cleavage site remains the same and that the pH dependence of the formation of the 23-kDa fragment reflects a cleavage reaction at a single site.

The origin of the 23- and 9-kDa fragments induced by PNO8 treatment was determined using antibodies specific to the D1 residues Thr-227–Glu-235 (anti–(227–235)), Phe-239–Asn-247 (anti–(239–247)), and Glu-333–Ala-344 (anti–(333–344)). As shown in Fig. 7, both anti–(227–235) and anti–(239–247) reacted with the 23-kDa band, but not with the 9-kDa band (lanes d and f), while anti–(333–344) reacted with the 9-kDa band, but not with the 23-kDa band (lane h). Therefore, the 23- and 9-kDa bands correspond to the N-terminal and C-terminal fragments of D1, respectively. The results also indicate that the cleavage by PNO8 takes place at the C-terminal side of residue 248.

The cleavage site acted on by PNO8 in D1 was further analyzed by limited proteolysis using lysylendopeptidase, as shown in Fig. 8. D1 from spinach does not contain a lysine residue, while D1 from wheat contains one at position 238 in the D-E loop connecting helices D and E (42). When D1 from wheat is cleaved at Lys-238 by digestion with lysylendopeptidase, two polypeptides with molecular masses of 26.0 and 12.0 kDa as determined by amino acid sequences should be produced (42). After digestion of control membranes by the peptidase, the band corresponding to native D1 completely disappeared, and two new bands are observed with apparent molecular masses of 20.5 and 12.5 kDa (Fig. 8A, lane c). Since anti–(227–235) reacted with the 20.5-kDa band, but not with the 12.5-kDa band (Fig. 8B, lane c), the origins of the 20.5- and 12.5-kDa bands can be assigned to the 26.0-kDa N-terminal and 12.0-kDa C-terminal polypeptides, respectively. Incubation of wheat membranes with PNO8 resulted in the formation of the two bands with apparent molecular masses identical to those of the PNO8-induced fragments of spinach D1 (Fig. 8A, lane b). Since anti–(227–235) reacted with the 23-kDa band, but not with the 9-kDa band (Fig. 8B, lane b), it can be concluded that PNO8 induced cleavage of wheat D1 at the same site as it did for spinach D1.

After digestion with the peptidase, the 23-kDa fragment disappeared (Fig. 8A, lane d; B, lane d), but the 9-kDa fragment was not affected (Fig. 8A, lane d). The disappearance of the 23-kDa fragment implies that this fragment contained Arg-238 and was digested to the 26.0-kDa N-terminal polypeptide and a residual small polypeptide, which was not resolved.
Dark Degradation of D1 Protein Tagged by Light

by the SDS-PAGE system used in this study. With the SDS-PAGE system containing high concentrations of urea, all the hydrophobic proteins of PS II show that a linear relationship exists between the logarithm of relative mobility and molecular mass deduced from the amino acid sequences (43). We found in our gel system that the two peptides induced by digestion with lysylendopeptidase also participated in the linear relationship. As determined from the difference of the migration distance in the gel between the bands for the 23-kDa fragment and the 26.0-kDa N-terminal peptide, the molecular mass of the 23-kDa fragment is larger by 2.3 kDa than that of the 26.0-kDa N-terminal polypeptide. Taking into account the amino acid sequence of D1, it can be concluded that cleavage occurs around Leu-258, although the exact site is not yet known due to the relatively large ambiguity in determining the molecular mass from a protein band on SDS-PAGE.

DISCUSSION

This study demonstrates that the cleavage of D1 by PNO8, an inhibitor of electron transport between Qa and Qb, is greatly stimulated by illumination of the sample material before the addition of PNO8, although light is not necessary for the cleavage process. The effect of preillumination decayed exponentially in the dark, and very small quantities of degradation products were induced by PNO8 treatment after prolonged incubation in the dark, as shown in Fig. 4. This implies that PS II is altered as a result of preillumination and that binding by PNO8 can induce the cleavage of D1 only in PS II subjected to preillumination. A straightforward interpretation of the effect of preillumination is that some redox-active component is either photo-reduced or photo-oxidized during preillumination, and this plays a direct role in the cleavage of D1 by PNO8. To assess this possibility, we attempted to identify redox-active components that show a relaxation course after illumination coinciding with the dark decay for the PNO8-dependent cleavage of D1 shown in Fig. 4. The effect of preillumination was still pronounced in the PS II core preparation (Fig. 2), in which the electron transfer from Qa to Qb was largely retarded presumably due to the lack of a plastocyanine molecule in the Qb pocket and the PS II membranes depleted of the manganese cluster by NH2OH treatment (data not shown). It can therefore be concluded that the redox reactions of Qa and the manganese cluster do not affect the effect of preillumination. Y2v, QA, Pheo+, and P680+ are also excluded as candidates since their lifetime is much shorter than that of the preillumination effect (2). Y2v+ is a radical species present on the donor side of PS II and could be formed during preillumination (2). Although Y2v+ cannot be considered as a candidate with respect to its stability, quantitative analysis by EPR showed that 70–80% Y2v is still in an oxidized Y2v+ form in the dark-adapted PS II membranes (data not shown). Y2v+ cannot, therefore, account for the 5-fold increase in D1 cleavage following preillumination (Fig. 1). Cytochrome b6f is also not a likely candidate since it is not reduced or oxidized by illumination at ambient temperatures in preparations with a functional manganese cluster (44). Chlorophyll and carotenoid molecules can be oxidized by PS II under certain conditions (44), but not under the present experimental conditions. Furthermore, no new EPR signal was observed in the PS II membranes following preillumination, indicating that no new organic radical(s) had been formed (data not shown). Thus, these two pigments can both be excluded.

The non-heme iron present on the acceptor side of PS II is usually present in reduced form under darkness, but it has been reported to be partially oxidized gradually by oxygen in PS II membranes and then re-reduced upon illumination (45). It has been proposed that non-heme iron in a reduced form plays a role in the cleavage of D1 via an active oxygen species in vitro system (15). In the present study, however, >80% of the non-heme iron was in a reduced state even after dark adaptation as detected by means of low temperature EPR and Fourier transform IR spectroscopy (data not shown). It is therefore unlikely that the preillumination effect is related to the photoreduction of the non-heme iron.

The above considerations suggest that none of the redox states and/or redox reactions of any PS II components are directly related to the effect of preillumination. A remaining explanation for the effect of preillumination is that the redox reaction somehow changes D1 so that cleavage can take place upon PNO8 binding. If this is the case, then the putative change in D1 must persist after complete relaxation of the redox reaction itself. Since Qo evolution activity was unaffected by preillumination, the quantity of D1 irreversibly photodamaged during preillumination is too small to account for the amount of D1 cleavage by PNO8. It has been proposed that photodamaged D1 is either protected from or tagged for degradation (46, 47) when it is phosphorylated in vivo, although D1 phosphorylation does not seem to influence PS II activity. Our results are consistent with regulation of D1 degradation by its modification. Preillumination may therefore induce an alteration that does not affect PS II functions, but that makes D1 susceptible to the cleavage. However, an irreversible covalent modification of D1 should not be ascribed to the preillumination effect since there is a dark decay of susceptibility to degradation. The possibility cannot be ruled out, however, that preillumination influences a PS II component other than D1 and that this indirectly affects D1 protein cleavage via PNO8. In previous report, we proposed that the conformation of the Qb site occupied by the PNO8 molecule would be similar to that induced by photoinhibitory treatment, and both allow cleavage of D1 presumably through a similar proteolytic process (31). Taking the above into consideration, it is reasonable to assume that some kind of conformational change in D1 in response to preillumination is required for the degradation of D1 with a modified Qb site induced either by photodamage or PNO8 binding. Further studies are required to assess whether light-dependent D1 phosphorylation or other modifications are involved in both its PNO8-induced degradation and light-dependent turnover.

The site of D1 cleavage promoted by PNO8 has been studied by immunodetection using antibodies to specific amino acid sequences of D1 (Fig. 7) and by the lysine-specific proteolysis of D1 from wheat with lysylendopeptidase (Fig. 8). The results indicate that D1 is cleaved at a single site located in the loop between helices D and E exposed on the stromal side of the thylakoid membranes, yielding a 23-kDa N-terminal fragment and a 9-kDa C-terminal fragment. Based on the change in apparent molecular mass of the N-terminal fragments when digested with lysylendopeptidase, it can be concluded that D1 is cleaved in the vicinity of Leu-258. This region contains the membrane parallel helix, de, and a loop connection between the de loop and helix E (48). Many amino acid residues in this region are implicated in the binding of the Qb quinone and various types of herbicides (49). These residues can also potentially participate in the binding of PNO8 and are thus candidates for the cleavage site. The site for D1 cleavage upon PNO8 treatment is in good agreement with that proposed for its degradation during photoinduced turnover and under strong light photoinhibition (25, 26).

As shown in Fig. 5, >60% of the total D1 protein was cleaved by PNO8, but no other fragments except for the 23- and 9-kDa fragments were detected, indicating that no second-side cleavage takes place. This high specificity suggests that an enzymatic process is involved in the cleavage reaction induced by
PNO8, consistent with our previous observation that the PNO8-dependent cleavage of D1 is suppressed to 40–60% by several inhibitors of serine-type proteases (31). It should be noted, however, that suppression by the inhibitors does not necessarily prove the direct involvement of a protease in the cleavage process by PNO8 since only partial inhibition is achieved. Nevertheless, there is no D1 degradation in reaction center complexes (Fig. 2), from which putative protease (or modification enzyme) may have been removed.

As shown in Fig. 6, the pH dependence of the PNO8-induced cleavage of D1 was considerably different from that of inhibition of PS II activity. Inhibition of PS II began to decrease above pH 7, whereas D1 cleavage was inhibited at lower pH values. Based upon the chemical properties of the PNO8 molecule with a nitro group in a phloroglucinol nucleus, the pKa value of hydroxyl groups in a phloroglucinol nucleus should be lowered to pH 7–8 compared with that in a phloroglucinol nucleus with no strong electron-withdrawing group. Thus, the decrease in the capability of PNO8 to act as a PSII inhibitor in alkaline conditions can be attributed to deprotonation of the particular hydroxyl group, which might influence the binding of D1. In fact, several of the residues of the triad exist in the D-E loop, although there is no amino acid sequence homologous to that of serine-type proteases in D1. Perhaps a structural change induced in the Qb site by photodamage, attack of active oxygen, and PNO8 binding conformationally activates the putative triad, and cleavage of D1 at the D-E loop is autocatalytic.

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