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The modification of atrazine binding by the redox state of the endogenous high-spin iron and by specific proteolytic enzymes in Photosystem II membrane fragments and intact thylakoids

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Atrazine-binding properties reflecting the fine structure of the binding niche within the 32 kDa polypeptide, D1, were analyzed in thylakoids and Photosystem II membrane fragments from spinach as a function of two parameters: (i) the redox state of the endogenous iron located between Q_A and Q_B at the Photosystem II acceptor side, and (ii) proteolytic degradation by the specifically acting enzymes trypsin, glutaminic acid-specific, lysine-specific and arginine-specific proteinases. It was found (a) Fe^{2+}-oxidation by $K_3[Fe(CN)_6]$ significantly reduces atrazine binding in Photosystem II membrane fragments. The $K_3[Fe(CN)_6]$ effect exhibits a marked pH dependence attributable to different percentages of Fe^{3+} formation. Thylakoids hardly show any modification of atrazine binding by $K_3[Fe(CN)_6]$; (b) proteolytic enzymes which are expected to interact with specific sites of the stroma-exposed loop between transmembrane helices IV and V of polypeptide D1 affect the atrazine-binding properties quite differently. The protective action of $CaCl_2$ to proteolytic degradation markedly depends on the nature of the enzyme used; (c) the degradation of the atrazine binding by a lysine-specific proteinase in samples from spinach, containing a lysine-free D1 polypeptide, is largely retarded if the atrazine-binding sites are occupied during the proteolytic treatment. The mechanistic implications of these findings are discussed.

Introduction

During the last couple of years remarkable progress has been achieved in understanding the mechanism of herbicide interaction with Photosystem II (PS II) of cyanobacteria and higher plants. PS II herbicides prevent electron transport between the primary plastoquinone acceptor Q_A of the reaction center and a plastoquinone pool molecule bound to a specific site, referred to as Q_B site within the polypeptide D1 of the reaction center complex. A competitive herbicide binding at the Q_B site is currently assumed to be responsible for this blockage although allosteric effects cannot be totally neglected (for recent review, see Ref. 1). Detailed structural schemes were proposed [2,3] for the binding niche of Q_B and PS II herbicides. The models are mainly based on three different lines of experimental evidence: (a) the striking sequence homologies and hydropathy pattern similarities between the L- and M-subunits of the reaction centers of purple bacteria and the PS

Abbreviations: PS II, Photosystem II; Mes, 4-morpholineethanesulphonic acid; Tricine *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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II polypeptides D1 and D2, respectively, of cyanobacteria and plants [4,5], (b) the X-ray structure of *Rhodopseudomonas viridis* reaction centers [6] and (c) the point mutations of D1 in herbicide resistant mutants [7–9]. Essential structural elements of the binding niche are the membrane spanning helices IV and V of D1 containing histidine ligands of the high-spin Fe^{2+} and of Q_B and the connecting loop between these two helices which contains a helix parallel to the thylakoid membrane [2].

Accordingly, the affinity of herbicide binding can be modulated by structural changes of this polypeptide either by direct modifications or indirectly through the interaction of D1 with other proteins or its lipidic environment. The redox state of the non-heme iron located between Q_A and Q_B is another parameter which can affect herbicide binding [10,11]. As an extension of our previous studies [11–14] we report here on effects of various polypeptide modifications by specifically acting proteolytic enzymes and of Fe^{2+} oxidation on the binding affinity of atrazine. In order to monitor possible indirect effects of the lipidic surrounding comparative studies were performed in normal thylakoids and PS II membrane fragments obtained by Triton X-100 solubilisation.

Materials and Methods

Thylakoids and Triton X-100 PS II membrane fragments were prepared according to Winget et al. [15] and a modified procedure of Berthold et al. [16], respectively, as described in Ref. 17. Proteolytic treatments were performed in suspensions containing the sample material (50 μg chlorophyll/ml, 10 mM NaCl, 5 mM $MgCl_2$, 0.33 M sorbitol, commercially available (Boehringer) proteinases of 0.25 units of proteolytic activity and either 50 mM Mes/NaOH (pH = 6.0) or 50 mM Tricine/NaOH (pH = 7.5)). Herbicide binding was determined by a method similar to that outlined in Ref. 18. In order to prevent light effects [17] the samples (50 μg chlorophyll/ml) were added under very dim green light to the suspension medium containing [^{14}C]atrazine and incubated for 5 min in complete darkness at 25°C. After addition of modifying substances and appropriate incubation times (see figure legends) the samples were centrifuged at 12000 × *g* for 5 min. In order to improve the accuracy, both the supernatant and the pellet were analyzed for the content of [^{14}C]atrazine: 800 μl of the supernatant were transferred to 10 ml of a scintillation mixture (Fa. Packard Emulsifier 299). Likewise the pellet was resuspended in 800 μl buffer and transferred to 10 ml scintillation mixture. After at least 30 min dark incubation at 4°C the counts of each sample were determined (Packard model Tricarb).

Results

In the present study two basically different modifications of the PS II acceptor side were analyzed for their effects on atrazine binding: (a) $K_3[Fe(CN)_6]$ induced changes of the redox state of the high-spin iron located between Q_A and Q_B, and (b) mild degradation of polypeptides by different specifically acting proteolytic enzymes. Fig. 1 shows atrazine binding in thylakoids and PS II membrane fragments at pH = 7.5 in the absence or presence of 0.5 mM $K_3[Fe(CN)_6]$. A significant decrease of the affinity due to $K_3[Fe(CN)_6]$ addition is observed in PS II membrane fragments while only marginal effects arise under the same conditions in thylakoids. Different lines of indirect [20,21] or direct [22,23] evidence clearly indicate that $K_3[Fe(CN)_6]$ is able to oxidize the endogenous Fe^{2+} located between Q_A and Q_B into Fe^{3+} in thylakoids and PS II membrane fragments. Based on these findings the data of Fig. 1 suggest that the redox state of the endogenous iron markedly affects the atrazine-binding affinity in PS II membrane fragments as reflected by an increase of the dissociation constant K_i whereas in thylakoids the atrazine binding appears to be almost invariant to Fe^{2+} oxidation (for details see Discussion). These differences between thylakoids and PS II membrane fragments are indicative for small but detectable changes of atrazine interaction with its binding site. It appears reasonable to assume that this phenomenon is due to a modification of the natural lipidic environment caused by Triton X-100 solubilization. This idea is supported by slightly higher K_i values in PS II membrane fragments compared with thylakoids even if the iron stays in its reduced normal state, Fe^{2+}.

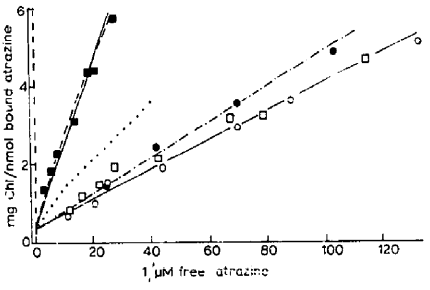

Fig. 1. Double reciprocal plot for binding of [^{14}C]atrazine to thylakoids (open symbols) and PS II membrane fragments (closed symbols) in the absence (circles) or presence (squares) of 0.5 mM $K_3[Fe(CN)_6]$ at pH = 7.5. Experimental conditions as described in Materials and Methods. The fitting curves (for details, see Discussion) for the data obtained with PS II membranes were calculated according to Eqns. 1 and 3 with $p = 0$ and $K_I(Fe^{2+}) = 1.4 \cdot 10^{-7}$ (·-·-·), $p(Fe^{3+}) = 1$ and $K_I(Fe^{3+}) = 7 \cdot 10^{-7}$ M (———), $p(Fe^{3+}) = 0.85$ and $K_I(Fe^{3+}) = 1.4 \cdot 10^{-6}$ M (- - - - -) or $p(Fe^{3+}) = 0.5$ and $K_I(Fe^{3+}) = 3.5 \cdot 10^{-6}$ M (·····). $[X_0]$ was in all cases 3.2 nmol atrazine/mg Chl.

The details of the above mentioned 'lipidic' effect will not be analyzed in this study. Fig. 2 summarizes the effect of $K_3[Fe(CN)_6]$ on atrazine binding in PS II membrane fragments at two different pH values and in the absence or presence of 10 mM $CaCl_2$. The data obtained permit the following conclusions: (a) if Fe^{2+} remains reduced Ca^{2+} does not affect the binding affinity of atrazine at pH = 6.0 and 7.5, (b) the effect of $K_3[Fe(CN)_6]$ addition is more pronounced at pH = 7.5 than at pH = 6.0, and (c) Ca^{2+} does not affect or only slightly modifies the affinity for atrazine binding in samples with oxidized Fe^{3+}. A numerical analysis reveals that these effects can be consistently explained by a dependence of the percentage of Fe^{3+} formation on pH and to a much smaller extent on $CaCl_2$ (see Discussion).

A different way to modify the microenvironment of the binding site is a mild proteolytic degradation. Basically, two different types of modification can be distinguished: (a) cleavage of the D1 polypeptide itself or (b) degradation of closely associated subunits which could interfere through indirect structural interactions with the Q_B site. Phenomenon b will be referred to as allosteric type effect. Thylakoids or PS II membrane fragments from spinach provide ideal samples in order to separate proteolytic effects on atrazine binding due to modification types 'a' and 'b' because spinach D1 does not contain any lysine [4,5]. Therefore, in this study a lysine specific proteinase was used to characterize allosteric type modifications. Before describing results related to 'type b' modifications by a lysine specific protease it appears worthwhile to analyze some 'type a' phenomena.

For a direct proteolytic attack on D1 the loop between transmembrane helices IV and V is of special relevance because this part is assumed to play a key role for the structural integrity of the

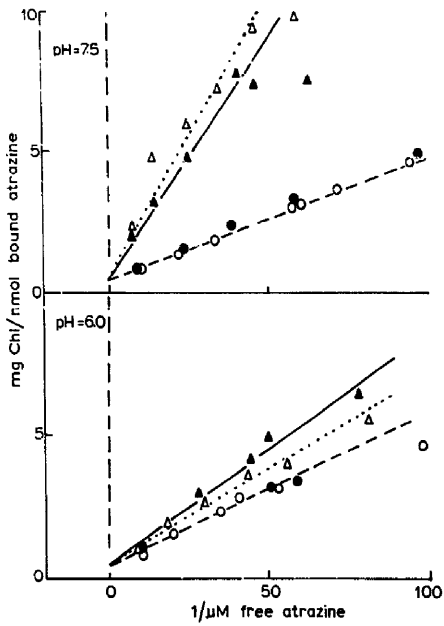

Fig. 2. Double reciprocal plot for binding of [^{14}C]atrazine to PS II membrane fragments at pH = 6.0 and pH = 7.5 in the absence (circles) and presence (triangles) of 0.5 mM $K_3[Fe(CN)_6]$ and in the absence (open symbols) and presence (closed symbols) of 10 mM $CaCl_2$. Experimental conditions as described in Materials and Methods. The curves were calculated with the following values at pH = 6.0: $K_I(Fe^{2+}) = 1.55 \cdot 10^{-7}$ and $K_I(Fe^{3+}) = 1.4 \cdot 10^{-6}$ M; $p = 0$ (- - - - -), $p(Fe^{3+}) = 0.4$ (·····) and $p(Fe^{3+}) = 0.5$ (———). At pH = 7.5: $K_I(Fe^{2+}) = 1.4 \cdot 10^{-7}$ M and $K_I(Fe^{3+}) = 1.4 \cdot 10^{-6}$ M; $p = 0$ (- - - - -), $p(Fe^{3+}) = 0.85$ (·····) and $p(Fe^{3+}) = 0.80$ (———).

herbicide binding niche [2]. Accordingly this segment which is accessible from the outer surface of the thylakoid membrane appears to be the primary target to direct proteolytic attack. This idea has been confirmed for trypsin [24] and other proteinase(s) responsible for endogenous degradation of D1 [25]. The loop segment comprises two interesting structural elements: (a) an amphipathic helix probably oriented parallel to the membrane plane; and (b) a region rich in serine, threonine and glutamic acid which is postulated to form a PEST-type sequence that is especially sensitive to endogenous proteolytic degradation of D1 [25]. The loop contains inter alia arginine and glutamic acid residues and is therefore expected to be susceptible to selective modifications by trypsin and other commercially available enzymes specifically cleaving proteins at the level at arginines and glutamic acids.

Beyond the primary structure of D1 other parameters are of relevance for the proteolytic cleavage pattern: (i) the tertiary structure of both, the target region segment and of the proteolytic enzyme; and (ii) the diffusion kinetics of the proteolytic enzymes into the partition. Both parameters could be affected by bivalent cations. Accordingly experiments were performed in the absence and presence of $CaCl_2$. Typical results obtained with trypsin in PS II membrane fragments are summarized in Fig. 3. The data obtained clearly show that the extent of a trypsin-induced modification of atrazine binding markedly depends on the addition of $CaCl_2$. In the presence of $CaCl_2$ the deleterious effect of trypsin on the affinity of atrazine becomes significantly impaired compared to that in control samples without $CaCl_2$. On the other hand, if $CaCl_2$ is added after starting the tryptic treatment, a stimulation of the degradation process is observed as the proteolytic activity of trypsin itself becomes stimulated by $CaCl_2$. This proteolytic pattern is practically independent of the absence or presence of 5 mM $MgCl_2$ in the assay (data not shown). The protective function is inferred to be caused by Ca^{2+}-induced structural changes. Measurements of fluorescence induction curves and their modifications by trypsin, Ca^{2+} and other bivalent cations led to the conclusion that already a very short proteolytic treatment is sufficient to eliminate the Ca^{2+} protection and

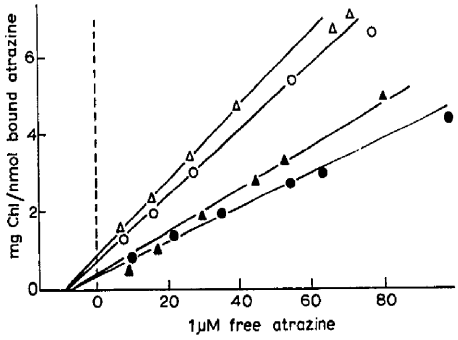

Fig. 5. Double reciprocal plot for binding of [^{14}C]atrazine to PS II membrane fragments (top) and thylakoids (bottom) at pH = 7.5. Control (○), treatment with arginine specific proteinase (●), treatment with lysine specific proteinase (■). Experimental conditions are as described in Materials and Methods.

that this effect is not highly Ca^{2+} specific. The most simple explanation for these findings is the assumption that bivalent cations induce tight membrane appression which drastically reduces the accessibility of the target site(s) to trypsin [26]. This interpretation would imply a general protecting effect of bivalent cations against an attack by all proteolytic enzymes interacting with the PS II acceptor side. In order to analyze the proteolytic degradation of D1 in more detail, the modification of atrazine binding was measured in the presence of other specifically acting proteinases which are expected to attack the loop between helices IV and V.

The results obtained with a glutamic acid specific proteinase from *Staphylococcus aureus* are summarized in Fig. 4. The curves on top reveal that this enzyme properly reacts with thylakoids leading to a remarkable decrease of the atrazine-binding affinity. This phenomenon is only marginally affected by the presence of atrazine during proteolysis. In thylakoids where the proteolytic attack is assumed to be restricted to the stroma side of the membrane, the PEST-type region appears to be the most likely target because of its high content of glutamic acid residues. Further glutamic acid residues of D1 are located at the lumenal side [2]. Therefore, these residues could act as an additional target in PS II membrane fragments provided that they become accessible to

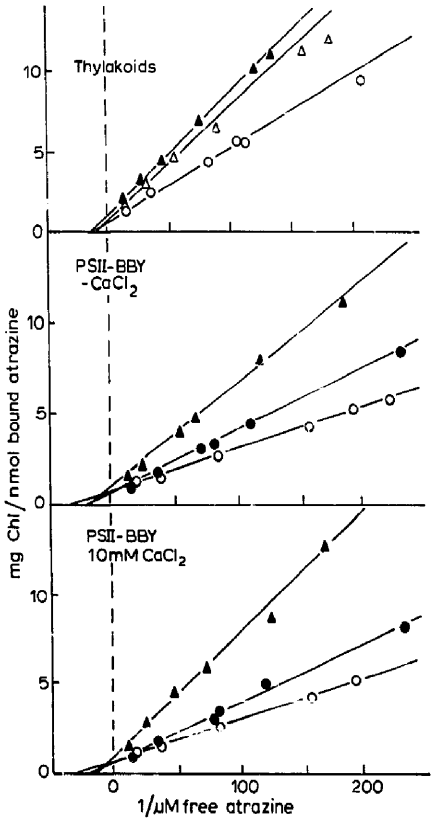

Fig. 4. Double reciprocal plot for binding of [^{14}C]atrazine to thylakoids and PS II membrane fragments in the absence or presence of 10 mM $CaCl_2$ at pH = 7.5. Control (○), treatment with glutamine specific proteinase of 1 unit (●) and 3 units (▲) of proteolytic activity; proteolysis with 3 units in the presence of atrazine in thylakoids (△). Experimental conditions are as described in Materials and Methods. BBY, called after the authors of Ref. 16.

the proteolytic enzyme. The experiments performed with PS II membranes are depicted in Fig. 4, middle and bottom. The data show almost the same pattern as in thylakoids. This finding indicates that the exposure of the lumenal side to the glutamic acid-specific proteinase does not markedly affect the modification of the atrazine-binding affinity. D1/D2 shielding by the extrinsic polypeptides of 17, 23 and 33 kDa is the most likely explanation for this phenomenon, because an analogous effect was recently reported for the reactivity of antibodies with D1 segments located at the lumenal side of the membrane [24]. A more interesting effect, however, is the lack of any protection by $CaCl_2$ to a modification of the atrazine-binding site by the glutamic acid-specific proteinase. This finding is not easily reconcilable with the simple model of tight membrane appression which prevents any contact between D1 and extrinsic proteolytic enzymes. One might as-

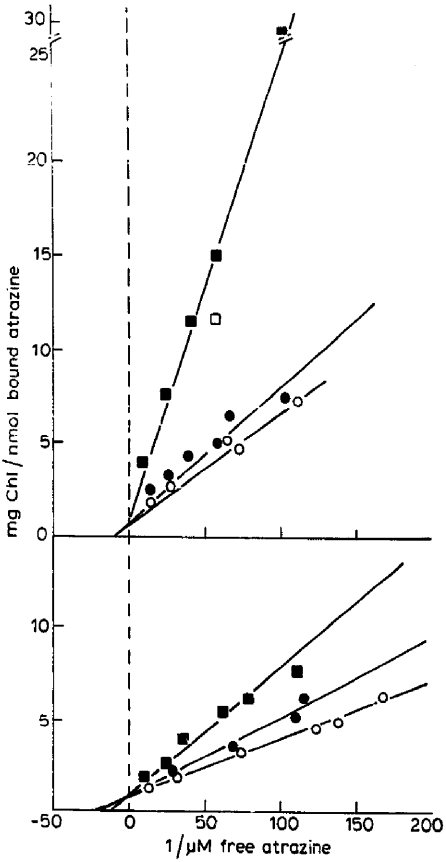

Fig. 5. Double reciprocal plot for binding of [^{14}C]atrazine to PS II membrane fragments (top) and thylakoids (bottom) at pH = 7.5. Control (○), treatment with arginine specific proteinase (●), treatment with lysine specific proteinase (■). Experimental conditions are as described in Materials and Methods.

sume that Ca^{2+} induces additionally more specific structural changes also in the surrounding of the target polypeptide itself which lead to different responses of glutamic acid-specific proteinase and trypsin, respectively. If the latter effect really dominates, then $CaCl_2$ has to cause rather specific structural changes because the target sites of D1 for both proteolytic enzymes are located at the loop between helices IV and V. As an alternative explanation, which is in line with the membrane appression hypothesis, one might suggest that trypsin and the glutamic acid-specific proteinase are characterized by different structural parameters (size and/or shape) giving rise to largely different diffusion coefficients in the partition. To address this problem, experiments were performed with an arginine-specific proteinase from glandula submaxillaris of mice. This enzyme should directly attack the arginine-containing loop between helices IV and V of D1 provided that the target sites can be reached. The results obtained are depicted in Figs. 5 and 6. Surprisingly, the arginine-specific proteinase was found to exhibit only weak effects on atrazine binding in thylakoids and in PS II membrane fragments. Ca^{2+} addition is practically without influence. The absence of a significant modification was previously reported also for pea thylakoids [14]. The most trivial explanation of this phenomenon would be the assumption that the arginine-specific proteinase is not very active in attacking native proteins despite its high reactivity with the model substance. In order to check this point the proteolytic activity of the enzyme was analyzed in thylakoid membranes and isolated CF_0F_1. The Coomassie blue-labeled gel electrophoresis pattern depicted in Fig. 7 indicates some modifications in thylakoids in the range of 47 kDa and 32–34 kDa (see arrows) induced by the arginine-specific proteinase. However, the changes are small, compared with the action of other proteolytic enzymes like trypsin which has the same arginines as proteolytic target sites in the lysine free D1 of spinach. The CF_0F_1 did not reveal changes due to the presence of the proteolytic enzyme. Based on these data the arginine-specific proteinase from glandula submaxillaris is inferred to require for its proteolytic activity a specific structural array of the arginines in the polypeptide. Probably, D1 does only marginally satisfy these structural requirements, so that its atrazine affinity remains almost unaffected by the arginine-specific proteinase while trypsin exerts a

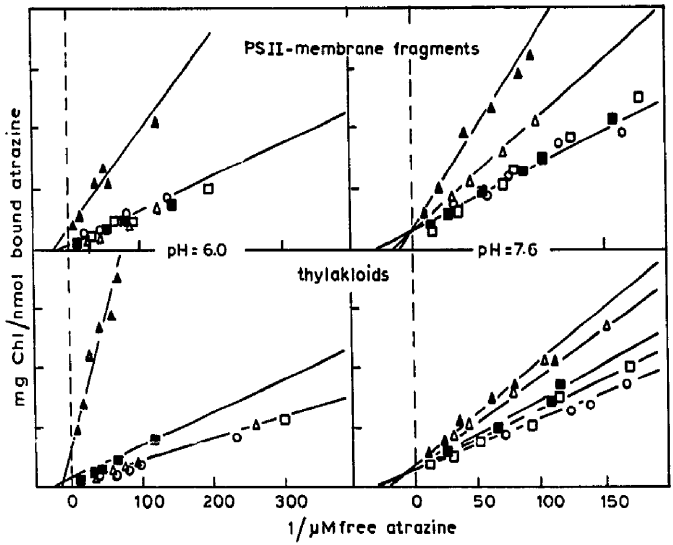

Fig. 6. Double reciprocal plot for binding of [^{14}C]atrazine in PS II membrane fragments and thylakoids at pH = 6.0 and 7.5. Control (○); lysine-specific proteinase before (▲) and after (△) atrazine addition; arginine-specific proteinase before (■) and after (□) atrazine addition.

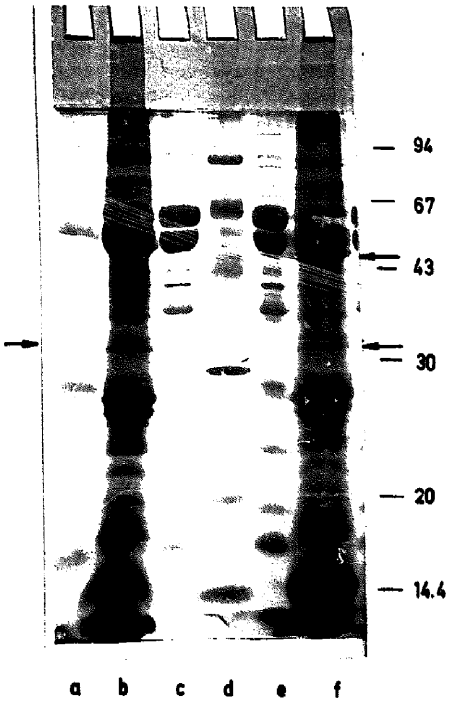

Fig. 7. Effect of protein treatment with arginine-specific proteinase. Coomassie brilliant blue stained SDS-PAGE: (a) 10 μg Arg-C proteinase; (b) control, untreated thylakoid membranes; (c) control, untreated CF_0F_1 ATPase; (d) marker proteins; (e) CF_0F_1 ATPase treated with Arg-C protease; (f) thylakoid membranes treated with Arg-C protease. Incubation conditions were: 2 h at 25° C at pH = 8.0. Values on the right are kDa.

drastic effect. In addition to this specific structural effect of the target polypeptide also differences of the transport rate to D1 might contribute to the remarkably different effects of trypsin and the arginine-specific proteinase. If one considers the effect of trypsin, glutamic acid-specific and arginine-specific proteinases and their response to Ca^{2+} addition, a combination of both transport limitations, and structural parameters of D1 seem to provide a likely answer. It has to be emphasized that in addition allosteric type effects might contribute to the different levels of D1 modification by trypsin, glutamic acid-specific and arginine-specific proteinases caused by different degradation patterns of closely related polypeptides. Further experiments are required to clarify this point.

Beyond changing the atrazine binding by a direct attack on D1, allosteric-type effects have to be taken into account, i.e., polypeptides other than D1 become modified and influence the atrazine-binding site without affecting the primary structure of D1. As spinach D1 is totally free from lysine, this indirect effect can be separately analyzed by using a lysine-specific proteinase provided that the enzyme changes atrazine binding. This was found to be the case, because recent results show that a lysine-specific proteinase markedly reduces the inhibition of PS II electron transport by isopropyl-*N*-(3-ethylcarbamoyloxyphenyl)carbamate (SN 58132) and the affinity of atrazine binding [12,14]. Within the framework of recent findings about the structure of the PS II reaction center [27] polypeptide D2 appears to be the likely target of the lysine-specific proteinase. This is in line with recent findings [28] on proteolytic degradation of D2. For the sake of comparison the data obtained with the lysine-specific proteinase are depicted in the same figures as the results with the arginine-specific proteinase. Fig. 5 shows that at pH = 7.5 the lysine-specific proteinase appears to be rather active in thylakoids and PS II membrane fragments. The most simple explanation of this phenomenon is a degradation of D2 which is expected to affect the structure of the binding niche in D1 because of the strong interaction between these two polypeptides [2,6]. The lysine-specific proteinase is therefore an invaluable tool to analyze the structural interdependencies between D1 and D2 in sample prepared from thylakoids because D1 does not contain lysine. Accordingly, this enzyme was used to study another mechanistically important question: do there occur structural changes due to an occupation of the binding niche with atrazine which alter the susceptibility of D2 (or another closely related polypeptide other than D1) towards proteolytic degradation? To check this point and simultaneously to detect pH induced structural changes experiments were performed under conditions where the order of addition of atrazine and the proteolytic enzyme in PS II membrane fragments and thylakoids is varied at pH = 6.0 and 7.5. The results obtained and summarized in Fig. 6

exhibit a number of interesting features: (a) the extent of impairment of the atrazine-binding affinity by the lysine-specific proteinase markedly depends on the presence or absence of the inhibitor during the digestion period (low concentrations of methanol/ethanol do not affect the activity of the lysine specific proteinse); (b) the effect of inhibitor binding is much more pronounced at pH = 6.0 in PS II membrane fragments and in thylakoids; (c) the general pattern of the proteolytic modification of atrazine binding affinity as a function of pH and inhibitor binding is the same in PS II fragments and thylakoids but quantitative differences still exist.

Discussion

Commonly, the experimental data are analyzed within the framework of a Lineweaver–Burk-type double reciprocal plot. However, depending on the underlying model a strict linear relationship can be expected under special conditions only. In the most simple case one type of binding site does exist. If one considers that a modification of the sample could lead to different states of the binding sites characterized by the dissociation constant $K_1(m)$, depending on the modification degree m, the most generalized form obtained (see Appendix) can be described by Eqn. 1:

$$\frac{1}{[I]_{bound}} = \frac{1}{M([I]_{free})}\left(\frac{K_1(0)}{[X_0]}\frac{1}{[I]_{free}} + \frac{1}{[X_0]}\right) \quad (1)$$

Here $M([I]_{free})$ is a phenomenological integral modification parameter (see Appendix):

$$M([I]_{free}) = \frac{1-\int p(m)\,dm}{K_1(0)+[I]_{free}} + \int \frac{p(m)}{K_1(m)+[I]_{free}}\,dm \quad (2)$$

with $p(m)$ = probability of the binding sites attaining the modification degree m, where m represents the extent of modification of the binding niche of a certain polypeptide D1 (see Appendix), $[X_0]$ is the normalized total number of binding sites and $[I]_{bound}$ and $[I]_{free}$ are the concentrations of bound and free herbicide, respectively. Because of the complexity of a multiparameter system a fitting of the experimental data with the generalized form described in Eqns. 1 and 2 does not permit unequivocal conclusions about the mode of changes of the total ensemble. In some special cases, however, a more detailed analysis can be achieved. A proper example is the effect of the redox state of the endogenous iron located between Q_A and Q_B on herbicide binding (see Figs. 1 and 2). The ensemble can be described by a two-state system with binding constants of $K_1(Fe^{2+})$ and $K_1(Fe^{3+})$, respectively. If $p(Fe^{3+})$ is the percentage of centers with oxidized Fe^{3+}, the integral modification parameters $M([I]_{free})$ simplifies to:

$$\frac{1}{M([I]_{free})} = \frac{K_1(Fe^{3+})+[I]_{free}}{\{1-p(Fe^{3+})\}K_1(Fe^{3+})+p(Fe^{3+})K_1(Fe^{2+})+[I]_{free}} \quad (3)$$

The numerical fitting by Eqns. 1 and 3 of the experimental data in Fig. 1 reveals that a satisfactory description can be achieved by two models: (a) the Fe^{2+} of all centers is oxidized at pH = 7.5 by 0.5 mM $K_3[Fe(CN)_6]$, i.e., $p(Fe^{3+}) = 1$, and the ratio $K_1(Fe^{3+})/K_1(Fe^{2+})$ is about 5; or (b) only 85% of the centers contain Fe^{3+}, and $K_1(Fe^{3+})/K_1(Fe^{2+})$ is about 10. The latter fit leads basically to the same conclusion as that drawn by Diner and Petrouleas [29] on the basis of a totally different approach. A comparison of the experimental data with curves calculated according to Eqns. 1 and 3 also reveals a marked restriction of possible $p(Fe^{3+})$ values. The analysis shows that the results cannot be satisfactorily described by the assumption that only about 50% of the centers are oxidized by $K_3[Fe(CN)_6]$ [23] (see dotted curve in Fig. 1). Taking into account recent results reported by Petrouleas and Diner [23] the remarkable pH dependence of the $K_3[Fe(CN)_6]$ effect on atrazine binding in PS II membrane fragments (see Fig. 2) could be dominated by a decrease of the extent of iron oxidation at low pH. The numerical fit of the data in Fig. 2 confirms this to be the case. Small differences in the extent of iron oxidation can also explain the effect of Ca^{2+}. Therefore, a two-state model properly describes the effects of $K_3[Fe(CN)_6]$-induced iron oxidation in PS II membrane fragments.

In respect to the pH effect another interesting phenomenon arises. It has been suggested that pH-dependent changes of the Fe^{2+} microenvironment give rise to two different $Q_A Fe^{2+}$-EPR signals referred to as '$g = 1.9$' and '$g = 1.8$' signals, respectively [30]. It is assumed that $K_3[Fe(CN)_6]$ cannot oxidize the Fe^{2+} of centers in the '$g = 1.8$' state [23]. The different states of the ligand sphere of Fe^{2+} might also affect the structure of the atrazine binding site. A comparison of the atrazine-binding curves of control samples at pH = 6.0 ('$g = 1.8$' state) and at pH = 7.5 (mainly '$g = 1.9$' state) indicate that slight differences exist [30]. Further studies are required in order to correlate quantitatively the structure of the Fe^{2+} microenvironment with the herbicide-binding capacity.

Beyond these effects, Fig. 1 also confirms our previous findings about significant differences between thylakoids and PS II membrane fragments [11]; i.e., the fine structure of the binding niche is affected by the Triton X-100 solubilization step. The most puzzling result is the rather small effect of $K_3[Fe(CN)_6]$ in thylakoids on binding of atrazine, whereas that of DCMU becomes drastically changed as was described recently [10]. The mechanistic implications of this very interesting phenomenon as well as the differences of the fine structure of the binding niche in thylakoids and in PS II membrane fragments remain to be analyzed in more detail in future studies. In contrast to the situation for iron oxidation as a well-defined two-state problem (iron in state Fe^{2+} or Fe^{3+}) the interpretation of data obtained in samples modified by proteolytic enzymes could be much more complex because a multistatic population of the binding site ensemble is expected to occur.

For practical use, however, the numerical analysis can be simplified by the assumption that almost all binding sites are modified, i.e., $p \approx 1$, and one modification degree $\bar{m}$ prevails. This assumption leads to the conventional form (see Ref. 18):

$$\frac{1}{[I]_{bound}} = \frac{K_1(\bar{m})}{[X_0]} \frac{1}{[I]_{free}} + \frac{1}{[X_0]} \qquad (4)$$

A formal analogous relation is obtained for $K_1(m \neq 0)/K_1(0) \gg 1$.

An inspection of the data of Figs. 4–6 indicates that in many cases Eqn. 4 properly describes the experimental data. However, for a more detailed mechanistic analysis much more refined measurements and numerical fittings are required. Regardless of these subtleties two interesting mechanistic conclusions can be drawn from the data presented in this study: (a) Ca^{2+} does not provide a general protection to proteolytic degradation of the herbicide-binding capacity. This Ca^{2+}-action depends on the nature of the proteolytic enzyme; (b) herbicide binding markedly protects the binding site from a modification by a lysine-specific proteinase. This effect is of special interest because the lysine-specific proteinase cannot directly attack the 32 kDa atrazine binding protein D1 and because the effect already arises at very low atrazine concentrations in the assay medium.

'Classical' urea/triazine type inhibitors like atrazine predominantly or exclusively bind to the D1 polypeptide. Accordingly, the data of Fig. 6 suggest that atrazine in the binding niche of D1 structurally affects the target polypeptide of the lysine-specific proteinase. This raises questions about the mutual structural interactions between the binding niche and the target site(s) which is (are) assumed to be located in D2. The experiments described in Fig. 6 were performed at rather small atrazine concentrations corresponding to 0.1–1 molecules per binding site. Urea/triazine type herbicides are characterized by comparatively slow exchange kinetics at the binding site [31]. In the case of 'permanent' atrazine binding during the proteolytic treatment and short range structural interactions the protection to an attack of the lysine-specific protease is expected to depend on atrazine concentration in the above-mentioned range. The data of Fig. 6 do not support this idea. Therefore, more complex structural interactions are required. One might speculate about long-range structural interactions between different systems II or about 'frozen' conformational states after release of atrazine from the binding niche. The present data do not permit any further mechanistic conclusions. Experiments are in progress to clarify these very interesting aspects.

Appendix

For the sake of simplicity only one type of herbicide binding site is assumed to exist in con-

trol samples. It is characterized by a dissociation constant.

$$K_I(0) = \frac{[X][I]}{[X \cdot I]} \tag{A-1}$$

The dissociation constant K_I is determined by the noncovalent interaction potential between the herbicide molecule and the binding niche. In the general case a modification procedure leads to different states of the overall ensemble of binding sites. It is assumed that m describes the extent of modification of an individual binding niche characterized by a 'microscopic' dissociation constant $K_I(m)$, and $p(m)$ represents the fraction of sites in state m. Then one obtains:

$$K_I(m) = \frac{[X(m)][I]}{[X(m) \cdot I]} \tag{A-2}$$

$$p(m) = \frac{[X(m)] + [X(m)I]}{[X_0]} \tag{A-3}$$

where $[X(m)]$ and $[X(m) \cdot I]$ are the number of free and occupied binding sites in state m, respectively, and $[X_0]$ the total number of the ensemble.

Taking into account the relation $[I]_{bound} = \sum_{m=0}^{\infty} [X(m) \cdot I]$ rearrangement of Eqns. A-1–A-3 leads to:

$$[I]_{bound} = [X_0][I]_{free} \left\{ \frac{1 - \sum_{m=1}^{\infty} p(m)}{K_I(0) + [I]_{free}} + \sum_{m=1}^{\infty} \frac{p(m)}{K_I(m) + [I]_{free}} \right\} \tag{A-4}$$

in the general case of a continuous modification one obtains

$$[I]_{bound} = [X_0][I]_{free} \left\{ \frac{1 - \int p(m)\,dm}{K_I(0) + [I]_{free}} + \int \frac{p(m)}{K_I(m) + [I]_{free}} dm \right\} \tag{A-5}$$

Eqn. A-5 directly leads to Eqns. 1 and 2 and Eqn. A-4 to Eqns. 1 and 3 of the text.

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