Studies on the Functional Mechanism of System II Herbicides in Isolated Chloroplasts

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The effect of specific proteolytic enzymes on variable fluorescence, \( p \)-benzoquinone-mediated oxygen evolution, PS II herbicide (atrazine and bromoxynil) binding, and protein degradation has been analyzed in isolated class II pea chloroplasts. It was found that:

1. Trypsin and a lysine-specific protease effectively reduce the maximum chlorophyll-\( a \) fluorescence yield, whereas the initial fluorescence remains almost constant. At the same number of enzymatic activity units both proteases have practically the same effect.

2. Trypsin and a lysine-specific protease inhibit the \( p \)-benzoquinone-mediated flash-induced oxygen evolution with trypsin being markedly more effective at the same number of activity units of both enzymes. Unstacked thylakoids exhibit a higher sensitivity to proteolytic degradation by both enzymes.

3. Trypsin and a lysine-specific protease reduce the binding capacity of \( ^{14}C \)atrazine, but enhance that of \( ^{14}C \)bromoxynil (at long incubation times trypsin treatment also impairs bromoxynil binding). At the same specific activity a markedly longer treatment is required for the lysine-specific protease in order to achieve the same degree of modification as with trypsin.

4. Trypsin was found to attack the rapidly-turned-over \( 32 \text{kDa} \) protein severely, whereas the lysine-specific protease does not modify this polypeptide. On the other hand, the lysine-specific protease attacks the light harvesting complex II.

5. Under our experimental conditions an arginine-specific protease did not affect chlorophyll-\( a \) fluorescence yield, \( p \)-benzoquinone-mediated oxygen evolution, herbicide binding and the polypeptide pattern.

Based on these results a mechanism is proposed in which an as yet unidentified polypeptide with exposable lysine residues, as well as the lysine-free "\( Q_b \)-protein" regulate the electron transfer from \( Q_A \) to \( Q_b \) and are involved in herbicide binding.

Introduction

Among the great variety of herbicides interfering with plant metabolism at different stages, products are widely used in modern agriculture which effectively block photosynthetic electron transport between the primary and secondary plastoquinones (referred to as \( Q_A \) and \( Q_b \)) at the photosystem II acceptor side (for review see [1]). Based upon results obtained in mildly trypsinized chloroplasts, \( Q_A \) and \( Q_b \) are inferred to be bound to a proteinaceous complex that functions [2]: (a) as regulator for the electron transport between \( Q_A \) and \( Q_b \), (b) as barrier to exogeneous redox components and protons, and (c) as binding area for PS II herbicides that interrupt electron transfer from \( Q_A \) to \( Q_b \). Later is was postulated [3] that \( Q_b \) is not a tightly bound specific plastoquinone, but a molecule from the plastoquinone pool which statistically occupies, via noncovalent interactions, a specific binding site. After reduction by \( Q_A \) the semiquinone form \( Q_b^- \) becomes tightly bound and stabilized. A second transfer step from \( Q_A \) reduces \( Q_b^- \) to the quinol, which is released via an exchange reaction with another plastoquinone molecule from the pool. PS II herbicides are assumed to "compete" with plastoquinone for binding to the herbicide/quinone binding environment. This idea is in line with recent findings of a seemingly competitive interaction between binding of quinones and herbicides [4]. Photoaffinity labeling experiments with different types of PS II herbicides have shown that the "clas-
sical" inhibitors (DCMU, atrazine) predominantly bind to a 32 kDa polypeptide, whereas phenol-type inhibitors are associated, among others, with 40 to 50 kDa components which may be related to the system II reaction center complex (for recent review see [5]). Therefore, more than one polypeptide seems to participate in creating the environment to which quinones and herbicides bind.

Trypsin treatment was found to interrupt Q\textsubscript{A} reoxidation in the same manner as DCMU in normal chloroplasts [6], to decrease the binding of DCMU-type inhibitors [7] and, concomitantly, to increase the accessibility of Q\textsubscript{A} to K\textsubscript{3}[Fe(CN)\textsubscript{6}] [8]. Recently, a lysine-specific protease was shown to induce a partial recovery of oxygen evolution with K\textsubscript{3}[Fe(CN)\textsubscript{6}] as acceptor in chloroplasts inhibited by SN 58132 [9] despite the fact that the 32 kDa polypeptide does not contain any lysine [10]. The effect of the inhibitor ioxynil was not changed. On the other hand, an arginine-specific protease appears to be inefficient [9]. These findings might suggest that the effect of herbicides is modified by a polypeptide that contains surface-exposed lysine residues.

In order to confirm this idea, the effect of specific proteolytic enzymes on chlorophyll-\textit{a} fluorescence, electron transport, herbicide binding, and polypeptide-degradation patterns have been analyzed. The data presented favor the involvement of, among others, a protein with surface-exposable lysine residues in creating the quinone/herbicide binding environment.

Materials and Methods

Class II pea chloroplasts were prepared as described in [4]. Mild proteolysis was performed in the dark at room temperature by incubation of chloroplasts (50 \(\mu\)g \cdot ml\(^{-1}\)) with trypsin or the lysine- or arginine-specific protease of 0.25 activity units per ml (unless indicated otherwise) in a reaction medium containing 10 mM NaCl, 5 mM MgCl\(_2\), 0.3 M sorbitol and 50 mM Tricine-NaOH, pH = 7.6, and, when applicable, the labeled inhibitor ([\(^{14}\)C]atrazine or [\(^{14}\)C]bromoxynil). Where indicated, MgCl\(_2\) was omitted from the reaction medium. The time of incubation with a protease is indicated in the Figure legends.

The chlorophyll \textit{a} fluorescence measurements were performed as in [9]. For measurements of flash-induced \(\text{O}_2\)-evolution, a Clark-type electrode was used which has been described in [12]. The analysis of the polypeptide degradation pattern by SDS/PAGE was performed as in [11].

The arginine- and lysine-specific proteases were obtained from Boehringer, Mannheim, West-Germany.

Results

In order to study the functional role of different polypeptides for the electron transfer between Q\textsubscript{A} and Q\textsubscript{B} and the action of PS II herbicides, modifications were analyzed that are caused by specific enzymes attacking proteins selectively at the level of lysine or arginine or both (in the case of trypsin).

I. Effects on variable fluorescence

The variable fluorescence yield, \(F_v\), and its modification by cations (as well as grana stacking) was found to be highly sensitive to tryptic digestion [13]. The effects were inferred to be caused by a specific attack on lysine residues near the C-terminal end of the light harvesting complex of system II (LHC II) (the structure of the C-terminus is (Lys or Arg)-Ser-Ala-Thr-Thr-Lys-Lys [14]). Accordingly, trypsin and a lysine-specific protease should exert almost the same effect on the variable fluorescence, while an arginine-specific protease could be inactive. The data obtained in pea chloroplasts suspended in the reaction medium containing 5 mM MgCl\(_2\) are depicted in Fig. 1. At the same specific activity, trypsin and lysine-spe-
cific protease induce a rapid, almost parallel degra-
dation of the variable fluorescence due to the
decrease of the maximum level, \( F_{\text{max}} \), whereas the
initial level, \( F_0 \), remains nearly unaffected. In con-
trast to that, at an even tenfold higher activity, the
arginine-specific protease does not modify fluo-
rescence emission. As the LHC-II is present mainly in
the appressed membrane region, the arginine-spe-
cific protease might be prevented from being active
simply by steric hindrance. In order to eliminate
this effect, chloroplasts were prepared in an Mg\(^{2+}\)-
free medium and resuspended for the fluorescence
measurements in the reaction buffer described in
Materials and Methods, but lacking MgCl\(_2\). In this
case, the variable fluorescence yield is significantly
smaller than in the control but with respect to the
attack of proteolytic enzymes, the arginine-specific
protease was found to be completely inactive (data
not shown). Also, this enzyme did not prevent the
Mg\(^{2+}\)-induced increase of \( F_{\text{max}} \) and \( F_v \), while trypsin
and the lysine-specific protease completely elimi-
nated this effect. These results show that LHC II
does not contain surface-exposed arginine residues
which can be attacked by proteolytic enzymes.

II. Effects on PS II electron transport

The oxygen evolution induced by repetitive
flashes was shown to be rather resistant to mild
trypsin treatment provided that \( K_{3}[\text{Fe(CN)}_6] \) is
added as exogeneous electron acceptor, whereas a
rapid decline is observed in the presence of \( p \)-
benzoquinone (\( p \)-BQ) [2]. This effect can be reason-
ably explained assuming that \( p \)-BQ functions as
electron acceptor only if the binding site for endoge-
neous plastoquinone is intact. Accordingly, the
effect of different proteolytic enzymes on the
oxygen evolution mediated by \( p \)-BQ has been mea-
sured in stacked and unstacked pea chloroplasts.
The data depicted in Fig. 2 reveal that trypsin as
well as the lysine-specific protease markedly inhibit
the reaction, whereas the arginine-specific protease
had no effect, even at a tenfold higher enzymatic
activity and regardless of the stacking state of the
thylakoid membranes. This shows that the polypep-
tides required for the acceptor function of \( p \)-BQ do
not contain surface-exposed arginine residues that
can be attacked with the specific proteolytic enzyme
used in this study.

Compared with the effects on the variable fluo-
rescence, two remarkable differences are observed
for the action of trypsin and the lysine-specific
protease on oxygen evolution with \( p \)-BQ as electron
acceptor: (a) trypsin exhibits a significantly higher
activity and (b) the modification by trypsin as well
as the lysine-specific protease occur at an increased
rate in unstacked compared to stacked thylakoids.
Therefore, the results depicted in Fig. 2 are not
simply caused by effects of LHC II on the electron
transfer to \( p \)-BQ. Furthermore, a proteolytic cleav-
age at the 32 kDa-herbicide binding polypeptide
cannot be responsible for the blockage of electron
transport because this protein was reported to be
free of lysine [10].

III. Effects on herbicide binding

The herbicide binding studies were performed as
in [15]. Two compounds were used: \(^{14} \text{C}-\text{labeled}
 atrazine and bromoxynil. The data are depicted in
Fig. 3. In the case of atrazine binding, the well-known
pattern is observed when using trypsin as proteolytic
enzyme. With increasing incubation time the num-
ber of herbicide binding sites becomes diminished
concomitant with an increase of the dissociation
constant, \( K_d \). Qualitatively the same effects are
observed for the lysine-specific protease. In contrast
to that, the arginine-specific protease does not exert
any influence, even at a tenfold higher enzymatic activity.

A more complex situation arises for bromoxynil binding. The data shown in Fig. 4 indicate that after a short trypsin-treatment the binding capacity increases due to a tighter attachment (K_d-decrease) rather than a change in the number of binding sites. The same effect can be achieved with lysine-specific protease. After extensive trypsin treatment the binding capacity of bromoxynil becomes diminished too, but this effect will not be discussed here. As in the case of atrazine binding, the arginine-specific protease was totally ineffective in modifying bromoxynil-binding properties.

IV. Effects on the polypeptide pattern

The effects of the proteolytic enzymes on p-BQ-mediated oxygen evolution and on the herbicide binding capacity suggest that surface-exposed lysine residues play an essential role in the structural integrity of polypeptide(s) that affect electron transport and interact with herbicides at the acceptor side of system II. On the other hand, atrazine is claimed to be a prototype for herbicides which specifically bind to a 32 kDa-polypeptide [17, 18] that was reported to be free of lysine [10]. In order to be able to draw a reasonable conclusion, it remains to be proven that the lysine-specific protease actually does not affect the 32 kDa-herbicide binding protein. Therefore, comparative measurements were performed for analysis of the proteolytic cleavage pattern of trypsin and the lysine-specific protease (for the sake of completeness the effect of the arginine-specific protease was also measured).

Pea chloroplasts isolated from plants grown for 3 hours in the presence of [35S]methionine were used, because the 32 kDa-atrazine binding polypeptide is characterized by a very rapid turnover so that a rather specific labeling can be achieved during the comparatively short [35S]methionine supply. Under these conditions, LHC II becomes significantly labeled too, due to the relatively large amount of this protein in the thylakoids. After thylakoid isolation, proteolytic treatment was carried out and the [35S]-labeling of proteins was monitored autoradiographically. The results obtained are depicted in Fig. 5. They reveal a remarkable difference in effects caused by trypsin and lysine-specific protease. In the former case, the 32 kDa peak, reflecting the lysine-free protein, becomes drastically reduced compared to other polypeptides of the control pattern, whereas in the latter
case almost no effect is observed. These data furnish convincing evidence that the rapidly-turned-over 32 kDa-protein is resistant to proteolytic attack by the lysine-specific enzyme.

Discussion

Most of the current models on the functional mechanism of PS II-herbicide action are based on the assumption that only one herbicide or quinone molecule can be bound to the herbicide/quinone binding environment at a time ("competitive" herbicide/quinone interaction) [3]. The binding interaction between different types of herbicides and quinones may be allosteric (binding of one ligand strongly decreases the affinity of another ligand binding to a non-overlapping site) rather than really competitive [16]. Whatever the precise mechanism of herbicide binding and inhibition of QA oxidation may be, the data shown here imply that not only the 32 000 M_r lysine-free protein ("QB-protein") is involved in binding herbicides and QA, but that also (a) protein(s) with (probably surface-exposed) lysine residues participate(s) in creating the herbicide/quinone binding environment: the lysine-specific protease is able to inhibit p-BQ reduction as well as to modify herbicide binding (Figs. 2–4).

One might speculate that a modification of LHC-II, which is present in relatively high amounts in the thylakoid membrane, could induce structural changes in the PS II environment leading to a modification of electron transfer to QB and of the herbicide binding. However, the LHC-II, which is attacked readily by the lysine-specific protease, appears not to be involved in the protease-induced changes in QA and herbicide binding because there is no good kinetic parallelism between the decline in variable fluorescence yield, reflecting LHC-II modification (Fig. 1), and inhibition of p-BQ reduction (Fig. 2). Furthermore, bromoxynil affinity decreases upon unstacking, whereas atrazine binding is not affected [16]; the effects induced by the lysine-specific protease are very different. Therefore, it is more plausible that the lysine-specific protease modifies a proteinaceous component at the PS II acceptor side different from the lysine-free 32 kDa protein. Since the QA oxidation by K_3[Fe(CN)_6] is enhanced by the lysine-specific protease [9], it could be attractive to speculate that the protein to which QA is bound (probably the PS II reaction center protein (H. Y. Nakatani, personal communication)) might be the target for the lysine-specific protease inducing the effects on binding of QA and herbicides. However, as the blockage by ioxynil is not prevented by lysine-specific in contrast to trypsin proteolysis, a simple "unshielding" effect of QA does not explain the data [9], unless ioxynil binding prevents, by conformational changes in the binding environment, an attack by the lysine-specific protease.

One might argue at this stage that the lysine-containing rather than the 32 000 M_r lysine-free protein
is the major regulatory protein of herbicide and quinone binding. However, this is grossly oversimplified. One amino acid in the chloroplast-encoded 32 000 Mr lysine-free “Q_B-protein” appears to be changed in (maternally inherited) triazine-resistant biotypes (L. McIntosh, personal communication), which results in a dramatically changed affinity for most PS II herbicides [19] and a shifted semiquinone equilibrium between Q_A and Q_B [15]. Therefore, we conclude that the herbicide/quinone binding environment is created by more than one polypeptide: the lysine-free 32 000 Mr “Q_B-protein” and a lysine-containing polypeptide are major components. Changes in one of the polypeptides induce a modification of the properties of the herbicide/quinone binding environment (directly or indirectly (allosterically)) resulting in a change in the binding characteristics of many ligands to the environment.

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