Studies on Herbicide Binding in Photosystem II Membrane Fragments from Spinach

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The mechanism of atrazine binding and its modification by Chelex-100-induced Ca^{2+} depletion and proteolytic degradation by trypsin, was analyzed in PS II membrane fragments from spinach. It was found:

1) Chelex-100 treatment leads in a comparatively slow process ($t_{1/2}$ = 5–10 min) to Ca^{2+} removal from a site that is characterized by a high affinity as reflected by K_D values of the order of 10^{-7} M. The number of these binding sites was found to be almost one per PS II in samples washed twice with Ca^{2+}-free buffer.

2) Chelex-100 treatment does not affect the affinity of atrazine binding but increases the susceptibility to proteolytic attack by trypsin.

3) The electron transport activity is only slightly affected by Chelex-100 treatment.

4) The atrazine binding exhibits a rather small T-dependence within the physiological range of 7 °C to 27 °C.

The implications of these findings for herbicide binding are discussed.

Introduction

Photosynthetic water cleavage into dioxygen and metabolically bound hydrogen takes place in a membrane-bound protein complex referred to as photosystem II (for a short review see ref. [1]).

All functional redox groups that participate in the overall reaction sequence, which comprises a) light-induced charge separation and stabilization, b) water oxidation and c) plastoquinone reduction, are currently assumed to be incorporated into a membrane-spanning heterodimer of polypeptides D1 and D2, each of which contains about 350 amino acids [2, 3]. Of special relevance for herbicide research is the functional and structural organization of plastoquinol formation. This process occurs within a special pocket designated as the Q_B site which is also the target site for many herbicides (for review, see ref. [4, 5]). The Q_B site is formed by the stromal side of transmembrane helices IV and V and the corresponding surface exposed loop of polypeptide D1 [5]. Plastoquinone, which is moderately bound to the Q_B site (*i.e.* Q_B), becomes reduced to plastoquinol *via* a two step univalent redox reaction sequence. A highly stabilized semiquinone ($Q_B^{-\cdot}$) is formed as an intermediate. The semiquinone of another tightly bound plastoquinone ($Q_A^{-\cdot}$) acts as the one-electron reductant in this reaction sequence. $Q_A^{-\cdot}$ is generated in the fast step of stabilization of the primary charge separation [6]. The plastoquinone Q_A is tightly bound to a site mainly located in polypeptide D2 [2]. A high spin, non-heme Fe^{2+} coordinated with four histidines of transmembrane helices IV and V of D1 and D2, respectively, is located between Q_A and Q_B [2, 3]. The physiological role of this Fe^{2+} atom is not yet clear (for review see ref. [7]).

A number of powerful herbicides prevent plastoquinone binding to the Q_B site thereby blocking plastoquinol formation [4]. The interaction of these compounds with photosystem II strongly depends upon the structure of the binding domain as reflected by the existence of different single site mutations that can drastically change the efficiency of inhibition by herbicides [8–11]. Furthermore, the herbicide binding also depends on the redox state of the non-heme iron [12, 13].

Abbreviations: atrazine, 2-ethylamino-4-chloro-6-isopropylamino-1,3,5-triazine; Q_A, Q_B, primary and secondary plastoquinone of photosystem II; PS II, photosystem II; AAS, atomic absorption spectrometry; MES, 4-morpholino-ethane sulfonic acid; DCIP, 2,6-dichlorophenolindophenol; DPC, 1,5-diphenylcarbazide; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea.

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It was recently shown that in suspensions of PS II membrane fragments the proteolytic degradation of the Q_B site by trypsin [14, 15] is not only affected by the occupation state of this site [16] but also by the presence of Ca^{2+} ions [17]. The protective effect of Ca^{2+} was shown to depend on the nature of the proteolytic enzyme [13] and therefore seems to imply that specific conformational changes of the Q_B site could be involved.

In this study we further analyzed the effects of Ca^{2+} on herbicide binding and the modifications by mild trypsin treatment. In addition an attempt was made to determine the thermodynamics of herbicide binding by measurements of the temperature dependence.

Materials and Methods

Triton X-100 PS II membrane fragments were prepared by a modified procedure of Berthold *et al.* [18] as described in ref. [19]. Bound Ca^{2+} was removed by dark incubation of PS II membrane fragments with Chelex-100 at 0 °C under slow stirring for the times indicated in the figures.

At a sample concentration of 400 µg chlorophyll/ml in 50 mM MES/NaOH (pH = 6.5), 100 mg Chelex-100/ml were added. After Chelex treatment samples were spun down at 15,000 × *g* for 5 min and subsequently washed at least twice with buffer solution (50 mM MES/NaOH, pH = 6.0).

For tryptic degradation sample material (50 µg chlorophyll/ml, 10 mM NaCl, 0.33 M sorbitol, 50 mM MES (pH = 6.0) was incubated with trypsin (Boehringer) at a ratio of 0.25 U trypsin/50 µg chlorophyll for 30 min and afterwards spun down at 15,000 × *g* and washed with suspension buffer.

Ca^{2+}-binding experiments were performed by incubation with [^{45}Ca]$CaCl_2$ of the sample in the dark at 0 °C. Atrazine binding was determined by a method similar to that outlined in ref. [20]. The details are described in ref. [13]. For some experiments, the samples were incubated at different temperatures in complete darkness.

The Ca^{2+} content of the samples were measured by atomic absorption spectrometry (Perkin Elmer model AAS 300) at 1500 °C with a graphite cuvette technique. DCIP-reduction rates were measured spectrophotometrically as outlined in ref. [21].

Results

For the determination of the Ca^{2+}-binding properties PS II membranes were washed twice with Ca^{2+}-free buffer solution (50 mM MES/NaOH, pH = 6.0) in order to remove loosely bound Ca^{2+}. After this treatment these control samples were found to contain 4.2 ± 0.3 Ca^{2+} per 400 chlorophyll molecules. Different treatments with EDTA, EGTA or Chelex-100 were tested for their chelator ability to remove Ca^{2+} from washed PS II membrane fragments. The best data were obtained with Chelex-100. Incubation with Chelex-100 and subsequent washing with Ca^{2+}-free buffer solution led to a time-dependent Ca^{2+} release. This is shown in Fig. 1 where after 20–30 min of incubation with Chelex-100 the Ca^{2+} content drops to a level of about 3 Ca^{2+}/400 Chl (2.7 ± 0.3).

Taking into account measurements of the average oxygen yield per flash the present data indicate that in our spinach PS II membrane fragments after Chelex-100 treatment 2–3 Ca^{2+} are tightly bound per PS II reaction center. This value is slightly higher than latest results of 2 Ca^{2+}/PS II [22–24]. Regardless of this difference, the data of Fig. 1 suggest that after washing twice with Ca^{2+}-free buffer PS II membrane fragments contain about 1 bound Ca^{2+}/PS II that can be removed by Chelex-100. The question arises whether this Ca^{2+} has any functional relevance.

At first, we determined the binding properties of the Chelex-100-extractable Ca^{2+}. Binding studies

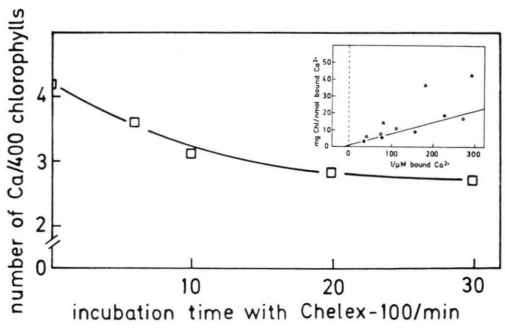

Fig. 1. Number of bound Ca/400 chlorophylls as a function of incubation time with Chelex-100 in washed PS II membrane fragments from spinach. Insert: Double reciprocal plot for binding of [^{45}Ca]$CaCl_2$ to washed PS II membrane fragments control (△), 10 min (●) and 30 min (○) incubation with Chelex-100. Experimental details as described in Materials and Methods.

were performed with [^{45}Ca]$CaCl_2$. The results are depicted in the insert of Fig. 1. In Chelex-100-treated samples the number of binding sites were of the order of one Ca^{2+} per (600 ± 300) chlorophylls and the affinity can be described by a K_D value of (7 ± 3) × 10^{-8} M. In contrast, the [^{45}Ca]$CaCl_2$ binding in control samples was markedly smaller and the data scattering did not permit to calculate reliable values for the number of binding sites and K_D.

Based on these data of Fig. 1 we conclude that Chelex-100 treatment leads in a comparatively slow process to the extraction of about 1 Ca^{2+}/PS II from binding sites that are characterized by rather high affinity as reflected by the K_D values of the order of 10^{-7} M. This value resembles that of strong PS II herbicides [13, 20] and is markedly lower than K_D values in the range of 50–100 μM and 1–3 mM, reported in the literature for Ca^{2+} effects on O_2-evolution rates and $P680^+$-reduction kinetics, respectively in variously treated samples [22, 25–27].

To check for possible functional changes due to high affinity Ca^{2+}-binding sites by Chelex-100, the electron transport activity (DCIP reduction with DPC as donor) was measured as a function of incubation time with the chelator. The normalized DCIP-reduction rate is compared in Fig. 2 with the depletion of Ca^{2+} induced by Chelex-100 (Fig. 1). Fig. 2 shows that a small activity loss of about 20% is caused by Chelex-100 treatment but this effect is kinetically not related to the removal of

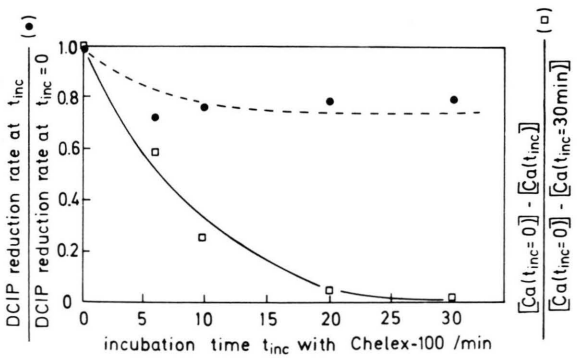

Fig. 2. Normalized DCIP-reduction rate (●) and content of extractable Ca^{2+} as a function of incubation time with trypsin in washed PS II membrane fragments (□). Experimental details in Materials and Methods.

Ca^{2+} from its high affinity binding site(s). The activity loss is comparable with findings reported previously for the effect of EDTA on O_2 evolution [28]. Readdition of 10 mM $CaCl_2$ did not restore the activity, but further suppressed the DCIP-reduction rate. This inhibitory effect of Ca^{2+} is also observed in non-treated PS II membrane fragments. It seems to be specific for the action of DCIP because in the presence of other electron acceptors a slight stimulation is often observed, especially in the case of $K_3[Fe(CN)_6]$ (data not shown).

Based on the above results the Chelex-100 extractable Ca^{2+} is inferred to be not directly correlated with the electron transport activity. Although this Ca^{2+} does not seem to play a key functional role it may still be important in order to maintain the structure of a polypeptide domain that indirectly affects the reaction pattern of PS II. The Chelex-100-extractable Ca^{2+} site may be located at the acceptor side of PS II because the loop between transmembrane helices IV and V of polypeptide D 1 which in part forms the Q_B site(s) contains a number of amino acid residues (Glu, Gln, Asn) that could act as ligands for Ca^{2+} coordination. A structural change by Ca^{2+} binding may affect the herbicide affinity of the Q_B site. The properties of atrazine binding and its modification by mild trypsin treatment were used to test possible effects due to Ca^{2+} extraction by Chelex-100. The results are depicted in Fig. 3. After extensive Chelex-100 treatment leading to complete removal of the extractable Ca^{2+}, there is no effect on the binding properties of atrazine. This finding indicates that the occupation state of this Ca^{2+}-binding site does not significantly modify the structure of the atrazine-binding site. On the other hand, the data of Fig. 3 show that compared with the non-treated control, the degradation of the atrazine-binding domain by mild proteolytic treatment is stimulated after Chelex-100-induced removal of Ca^{2+}. Therefore, the occupation of the Chelex-100-extractable high affinity Ca^{2+}-binding site could be of structural significance for the susceptibility to trypsin. However, if one takes into account that Ca^{2+} addition to non-treated PS II membrane fragments highly retards the susceptibility to tryptic attack on the atrazine-binding site, a more complex pattern emerges for the Ca^{2+}-induced effects on the PS II acceptor side (see Discussion).

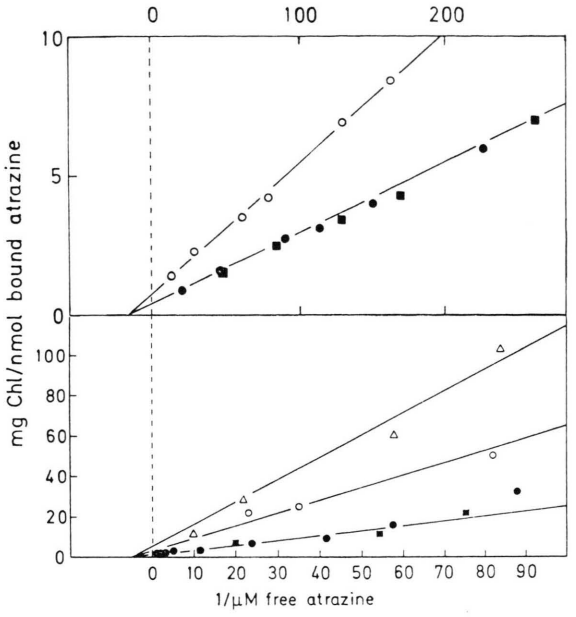

Fig. 3. Double reciprocal plot of [^{14}C]atrazine binding to normal (top) and washed (bottom) PS II membrane fragments. Top: Non-treated PS II membrane fragments without (●) and after trypsination in the absence (○) or presence (■) of 10 mM $CaCl_2$. Bottom: Control washed samples (●) and Chelex-100-pretreated samples (■). The binding properties of trypsinized samples are represented by open symbols: washed (○) and Chelex-100 (△) pretreated samples. Experimental details as outlined in Materials and Methods.

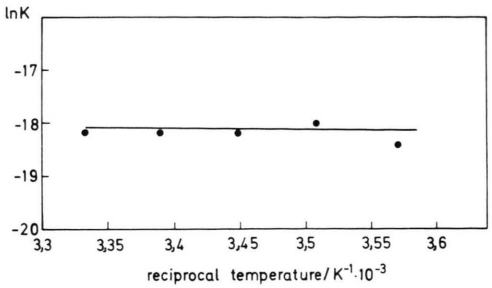

Fig. 4. Semilogarithmic plot of K_D values as a function of reciprocal temperature. The K_D values were determined from a Scatchard analog plot for [^{14}C]atrazine binding to PS II membrane fragments. Experimental details as described in Materials and Methods.

The atrazine binding to PS II is characterized by a K_D value of about 5×10^{-8} M which corresponds to a ΔG° value of the order of -40 kJ/mol for the reaction $I_{free} + X \rightleftharpoons X\,I$, where I_{free} reflects the free atrazine and X the binding site. To analyze the driving force of this process an attempt was made to determine ΔH° and ΔS° by measuring the T-dependence of atrazine binding. Unfortunately, only a rather small T-range can be covered because a recent study led to the conclusion that a structural change of functional relevance takes place at the Q_B site at temperatures around 21 °C [29]. Our measurements of atrazine binding did not reveal a significant T-dependence of the K_D values in the range of 7 to 27 °C as shown in Fig. 4.

Within the limits of experimental uncertainties the ΔH° values are comparatively small (<10 kJ/mol). This finding indicates that entropic effects essentially contribute to atrazine binding. At higher temperature enthalpy contributions to ΔG° seem to dominate but in this case significant unphysiological structural changes are expected to take place (data not shown).

Discussion

In the present study the binding of Ca^{2+} and extraction by Chelex-100 treatment were analyzed in relation to effects on the atrazine-binding site and its degradation by trypsin. The existence of one high affinity Ca^{2+}-binding site per PS II was observed after Chelex-100 treatment of PS II membrane fragments washed twice with Ca^{2+} free buffer. This site is formed in a comparatively slow process with $t_{1/2}$ of 5–10 min. The most simple interpretation of this finding is the assumption that this Ca^{2+}-binding site is occupied, under normal conditions. The removal of Ca^{2+} from this site does not affect atrazine binding but markedly increases the susceptibility to tryptic degradation. Therefore, the conclusion can be drawn that Ca^{2+} is not a structural determinant for the functional integrity of the Q_B site itself. On the other hand, the significant increase of the proteolytic attack on the atrazine-binding site in Chelex-100-treated samples indicates that the binding of Ca^{2+} to its high affinity site affects the structure of the PS II acceptor side.

Other Ca^{2+}-induced effects on the acceptor side were reported in this (Fig. 3 top) and previous studies [13, 17, 26]. Recently, further evidence was obtained that Ca^{2+} modifies the acceptor side reactions of different exogenous redox substances [30].

The nature of these different Ca^{2+} effects have to be clarified especially their specificity for Ca^{2+} remains to be checked. However, regardless of the underlying molecular mechanism, the effect(s) of Ca^{2+} on the PS II acceptor side should be taken into consideration if, based on measurements of oxygen evolution rates, conclusion are drawn about the role of Ca^{2+} as cofactor in the process of water cleavage. In this respect a very important fact has to be emphasized. Latest studies reveal that different treatments leaving the Ca^{2+} content unaffected cause a strong inhibition of O_2 evolution that can be reversed by Ca^{2+} addition [31, 32].

These findings clearly show that artificial structural changes of functional significance can be induced without Ca^{2+} loss but subsequent regeneration by Ca^{2+} addition. Similar effects were shown to exist in the case of Cl^- [33].

Another interesting structural effect should be briefly mentioned. It was shown that the occupation of the Q_B site either by plastoquinone or by a herbicide leads to structural changes that retard the proteolytic attack by trypsin [16]. This raises the question about the possibility of an allosteric effect of the Q_B site on donor side reactions of PS II because all functional redox groups participating in water cleavage are currently assumed to be located in a heterodimer of polypeptides D1 and D2 [2, 3]. Our latest data indicate that at least in the case of DCMU the electron transfer rate from Tyr-161 of polypeptide D1 to $P680^+$ remains invariant to herbicide binding in the Q_B site [34].

The observation of a rather weak *T*-dependence of atrazine binding in the physiological range of 7 °C to 27 °C suggests that the process is dominated by an entropy increase. Different parameters (solvent effects, structural changes of the Q_B site, hydrogen-bond formation, etc.) can contribute to this effect. For a more detailed analysis experiments are desirable with different mutants modified at specific sites of the loops between transmembrane helices IV and V.

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