Significance of Photosystem II Core Phosphorylation Heterogeneity for the Herbicide-Binding Domain

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Photosystem II Core, Phosphorylation, Heterogeneity, Phosphatase,  
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In recent papers the heterogeneous nature of photosystem (PS) II core phosphorylation has  
been revealed (Giardi et al., BBRC 176, 1298–1305 (1991); Plant Physiol. 100, 1948–1954  
(1992)). In this paper the action of endogenous and exogenous phosphatases both on the  
distribution of phosphorylated PS II core populations and on herbicide-binding activity in pho­  
tosystem II preparations from Spinacia oleracea L. has been investigated. The results indicate  
that these phosphatases modify the photosystem II core phosphorylation heterogeneity at a  
different level. Dark incubation causes a partial dephosphorylation of D3 and D2 proteins by  
endogenous phosphatase(s) and changes the relative distribution of phosphorylated photosys­  
tem II core populations, while the action of the alkaline phosphatase leads to extensive de­  
phosphorylation and to the detachment of PsbH protein from the photosystem II core. De­  
phosphorylation by the two alternative methods results in a differential modification of herbi­  
cide-binding activity. It is suggested that photosystem II heterogeneity with respect to the  
herbicide action, observed in vivo, could be a consequence of PS II core phosphorylation  
heterogeneity.

Introduction

In recent papers the heterogeneous nature of photosystem II (PS II) core phosphorylation has  
been shown. Four PS II core populations characterized by the differing extent of phosphorylation  
on CP43, D2, D1, and PsbH proteins have been isolated from grana regions of spinach thylakoids [1,  
2]. So far the properties of these isolated PS II core populations can be summarized as follows: i)  
their relative distribution responds to conditions that regulate the activity of light-induced kinase [2,  
3]; ii) at least two populations are detected in thylakoids adapted to complete darkness [3]; iii) these  
complexes differ in their sensitivity to photoinhibitory conditions [4]; iv) they show varying ability to  
transfer electrons from diphenylcarbazide (DPC) to dichlorophenol indophenol (DCPIP), the most  
phosphorylated population being inactive [2, 3]; v) moreover, their affinity constants for the bind­  
ing of PS II-directed herbicides are quite different [1, 3]. It is generally accepted that PS II herbicides  
such as phenylurea, triazines, and phenolic compounds compete with plastoquinone (PQ) at its  
Qb-binding site preventing oxidation of reduced

Materials and Methods

Phosphorylation and isolation of membranes

Phosphorylation of spinach (Spinacia oleracea L.) leaves was performed in vivo by incubation  
in the presence on [3P]orthophosphate (5000 Ci/mmol) as previously described [9]. Isolated  
spinach thylakoids were resuspended in the buffer containing 50 mM Tricine (pH 7.5), 15 mM NaCl,  
5 mM MgC12 and 0.1 M sucrose and immediately solubilized for isolation of PS II membranes [1, 3].  
Dephosphorylation experiments were performed by the two alternative methods, dark incubation of  
thylakoids for 3 h or treatment of PS II particles (0.5 mg/ml chlorophyll) for 10 min with a highly  
purified alkaline phosphatase (Sigma) resuspended in the above buffer (pH 7.9) at 15 units/ml. The
PS II particles, solubilized in 1% n-dodecyl β-D-maltoside (0.5 mg Chl/ml), were applied to the cathode region of a flat-bed of granulated gel as described [1]. Under these conditions four distinct, differently phosphorylated PS II core populations were separated [2].

**Herbicide-binding measurements**

The binding experiments were performed as reported by Tischer and Strotmann [10] for PS II-enriched membranes. The herbicide binding to isolated PS II core was evaluated by determining the distribution of radiolabelled herbicide (initial concentration 10 μM) associated to the PS II core populations on the isoelectrofocusing bed according to Giardi et al. [1].

**Chlorophyll (Chl) and electron transfer**

These measurements were carried out as reported by Hipkins and Baker [11]. Electron transfer from DPC (150 μM) to DCPIP (100 μM) was measured spectrophotometrically at 600 nm in samples of 15 μg Chl/ml under 1200 μE/m² s illumination in 1 cm optical path at 4 °C.

**SDS-PAGE and immunoblot**

SDS-PAGE in the presence of 6 M urea was performed using a 12–17% linear acrylamide gradient. Densitometric analyses of Coomassie-stained gels were carried out using a Shimadzu CS 9000. For immunoblot the resolved proteins were transferred to a nitrocellulose filter and probed with antibodies. Quantification of 9 kDa protein (psbH gene product) was performed by immunoblot of a serial dilution.

**Radioactivity measurements**

Radioactivity was determined by scintillation counting using Optiphase Safe (LKB) as the cocktail and a Packard tri-carb 2200 CA liquid scintillation analyzer. Counting efficiencies were determined on similar samples containing radioactive standards. Radiolabelled polypeptides were visualized by autoradiography of Coomassie-stained gels using hyperfilm TM MP (Amersham). The autoradiograms were scanned in a Shimadzu 9000 densitometer.

**Herbicides and acceptors**

Bromoxynil, 9.0 Ci/mol, terbutryn, 9.8 Ci/mol, and chlorbromuron, 8.6 Ci/mol, were a kind gift from Ciba-Geigy; ioxynil, 9.5 Ci/mol, was a gift from May and Baker Ltd. (2',3'-3H)imidazole, 490 Ci/mol, was a kind gift from Dr. W. Oettmeier of the Ruhr-Universität (Bochum, Germany). DCPIP, 2,6-dichlorophenol indophenol, and DPQ, decyplastoquinone, were purchased from Sigma.

**Results and Discussion**

Fig. 1 shows the typical pattern of phosphorylated PS II core preparations isolated from spinach PS II particles using the isoelectrofocusing (IEF) and sucrose gradient ultracentrifugation method previously reported [2, 3]. These PS II core populations have been referred to as cores a, b, c and d in increasing order of the extent of their phosphorylation. The complexes were composed of CP47, CP43, D2, D1, Cyt b559 proteins present in the same stoichiometry but with a different degree of phosphorylation. Moreover, the presence of a protein at about 9 kDa, attributed to PsbH phosphoprotein, was revealed and its content in each core was inversely related to the phosphorylation of D1 and D2 proteins. The complex with the more acidic isoelectric point (pI), i.e. complex d, showed the highest content of radioactivity localized mainly on D1. This activity was 40 times that found in complex a (see ref. [2–4]). To elucidate

![Fig. 1. SDS-PAGE (A) and autoradiography (B) analyses of heterogeneous PS II core populations obtained by IEF of PS II particles.](image-url)
the effect of the phosphorylation process in modifying the herbicide-binding activity, we proceeded to examine the consequences of PS II dephosphorylation by using endogenous phosphatase(s) and an alkaline exogenous phosphatase. For this purpose spinach leaves were phosphorylated in vivo by incubation in $[^{32}P]$orthophosphate. To inactivate the kinase(s) and to obtain some degree of dephosphorylation of PS II core proteins by endogenous phosphatase(s), prior to PS II particle extraction, phosphorylated thylakoids were incubated in complete darkness for some hours (Fig. 2, lanes 1 and 2, method A). Under our conditions, dark incubation of thylakoids never completely reverted phosphorylation of the PS II cores. Among the phosphorylated PS II core proteins, dephosphorylation was more evident in D$_1$ and D$_2$ proteins; a decreased content of the most phosphorylated core complex d was also observed (Scheme 1). In order to obtain complete dephosphorylation, PS II particles isolated from phosphorylated thylakoids were treated with a highly purified alkaline phosphatase. After 10 min of incubation, complete dephosphorylation was observed (Fig. 2, lane 3, method B). Since the alkaline phosphatase is not selective, it caused extensive dephosphorylation but, surprisingly, increased the number of PS II core populations obtained by isoelectric focusing (IEF) (Scheme 1). The pattern of PS II core populations changed both in number and in the isoelectrofocusing point of the populations. Eight PS II core fractions were isolated and analyzed by SDS-PAGE and immunoblot with antibodies against the main PS II polypeptides. From Fig. 3, showing the results of a serial dilution with antibodies against 9 kDa, it is clear that these PS II core populations differ in the relative content of PsbH protein. We conclude that also the separation of differently phosphorylated PS II core populations obtained by isoelectrofocusing was mainly a consequence of the different content of PsbH protein bound to each PS II core population. The electron transfer activity of differently phosphorylated membranes and their herbicide-binding activity have also been studied. Table I reports the electron transfer activity of phosphorylated PS II core populations and the inhibition of this activity by the herbicide ioxynil. In order to observe the herbicide inhibition in isolated PS II cores, low concentrations of reagents DPC and DCPIP had to be used.

Fig. 2. Autoradiography of phosphorylated and dephosphorylated membranes. Lane 1, PS II particles from phosphorylated thylakoids; lane 2, PS II particles dephosphorylated by method A; lane 3, PS II particles dephosphorylated by method B.

Fig. 3. Immunoblot of PS II core populations obtained by IEF of PS II particles dephosphorylated by method B, using antibodies against PsbH protein.

RELATIVE % DISTRIBUTION OF PSII POPULATIONS

Scheme 1
Table I. Electron transfer and its inhibition by herbicides in differently phosphorylated PS II core populations. Electron transfer rates were measured from DPC (150 μM) to DCPIP (100 μM). The activity is represented as μmol DCPIP reduced/mg Chl h. % Inhibition represents inhibition observed in the presence of herbicide ioxynil (10 μM). n.d.: not determined. The values of electron transfer are an average of five independent experiments; SE approx. 13%.

<table>
<thead>
<tr>
<th>Core populations</th>
<th>Electron transfer</th>
<th>% Inhibition</th>
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<tbody>
<tr>
<td>a</td>
<td>215</td>
<td>100</td>
</tr>
<tr>
<td>b</td>
<td>143</td>
<td>21</td>
</tr>
<tr>
<td>c</td>
<td>167</td>
<td>26</td>
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<tr>
<td>d</td>
<td>10</td>
<td>n.d.</td>
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Phosphorylation resulted in a reduced ability, about 18%, to transfer electrons from DPC to DCPIP, compared to control thylakoids obtained from leaves dark-adapted for 12 h (Table II). A similar electron transfer reduction, about 15%, was observed in PS II particles treated with the alkaline phosphatase (Table II). Fig. 4 shows the effect of dephosphorylation obtained by the two methods on the binding of herbicide bromoxynil to PS II particles. While dephosphorylation obtained by dark adaptation of thylakoids decreased the binding affinity with no effect on the number of herbicide-binding sites, the dephosphorylation promoted by the alkaline phosphatase caused a great decrease in the number of binding sites, about 50%, with modest effect on the binding affinity. Due to the alkaline pH used for activation, all the controls were performed at the same alkaline pH; moreover, similar results were obtained using an acidic phosphatase (data not shown). We also determined the herbicide-binding capacity of the PS II core populations after dephosphorylation using a recent method based on the observation that PS II-directed herbicides, applied on the isoelectrofocusing plate together with solubilized PS II particles, migrate in close association with the PS II core populations [1]. This herbicide binding was thought to be specific because it was absent in membranes isolated from the atrazine-resistant mutant Senecio vulgaris. Fig. 5 shows that the distribution of radiolabelled herbicide terbutryn among the focused PS II core populations seems to be correlated to the content of PsbH protein. This correlation has been confirmed with other classes of PS II herbicides such as phenylurea and phenolic herbicides as well as using an acidic phosphatase (data not shown). This observation is in accordance with the current idea that an overlapping binding domain participates in the binding of herbicides [5, 12]. Thus, one of the possible explanations for the differential effects on herbicide-binding domain observed after dark incubation and dephosphorylation by exogenous phosphatase is that the former method acts on D₁ and D₂ and the latter on all PS II core polypeptides. In our opinion these different mechanisms could explain the contradictory conclusions reported in the literature concerning the effect of phosphorylation on herbicide-binding activity [3, 6–8]. Our results indicate that herbicide-binding activity of PS II
cores directly responds to the modification of phosphorylation heterogeneity.

The PsbH protein has been detected in thylakoids as a 9 kDa phosphoprotein and has subsequently been shown to be a protein of photosystem II [13]. Although the precise function of this protein is unclear, it has been suggested that it plays a role in regulating and stabilizing secondary electron transfer at the level of the two plastoquinone acceptors QA and QB [14]. This suggestion is in accordance with our experimental observations. As we have demonstrated, both complete dephosphorylation and high level of phosphorylation of PS II core proteins lead to the detachment of PsbH protein from the core and, perhaps as a consequence, to a decreased electron transfer activity (Tables I and II), the binding of PsbH protein being favoured by intermediate levels of phosphorylation. This is also supported by the observed involvement of PsbH protein in the susceptibility of PS II to photoinhibition [4, 15].

In conclusion our results suggest that PS II core phosphorylation-dephosphorylation process could be a mechanism of electron transfer regulation through a modification of the QB herbicide-plastoquinone-binding domain, the binding affinity of this site being regulated by phosphorylation on D1/D2 and the consequent association of PsbH protein to the PS II core. Furthermore these results confirm our previous suggestion [3] that phosphorylation of PS II core polypeptides explains in part the heterogeneity of PS II in vivo, observed when these inhibitors are used [16].

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