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

Maria Teresa Giardi

Institute of Biochemistry and Ecophysiology of Plants, CNR Area della ricerca di Roma, Via Salaria km 29.3, 00016 Monterotondo Scalo, Italy

Z. Naturforsch. **48c**, 241–245 (1993); received November 9, 1992

Photosystem II Core, Phosphorylation, Heterogeneity, Phosphatase, Herbicide-Binding Domain

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 photosystem 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 D_1 and D_2 proteins by endogenous phosphatase(s) and changes the relative distribution of phosphorylated photosystem II core populations, while the action of the alkaline phosphatase leads to extensive dephosphorylation and to the detachment of PsbH protein from the photosystem II core. Dephosphorylation by the two alternative methods results in a differential modification of herbicide-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, D_2, D_1 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 binding 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 Q_B-binding site preventing oxidation of reduced Q_A [5]. In the present work, by comparative studies with isolated PS II particles and PS II core, the modification induced by dephosphorylation treatments both on PS II core phosphorylation heterogeneity and on herbicide-binding domain is investigated. The experimental results suggest that the action of exogenous and of endogenous phosphatases is different. This observation could explain the contradictory conclusions reported in literature concerning the effect of PS II phosphorylation on the herbicide-binding activity [6–8].

Verlag der Zeitschrift für Naturforschung, D-W-7400 Tübingen
0939–5075/93/0300–0241 $ 01.30/0

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 [^{32}P]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 $MgCl_2$ 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 $\mu E/m^2$ 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 (*psb*H 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)i-dinoseb, 490 Ci/mol, was a kind gift from Dr. W. Oettmeier of the Ruhr-Universität (Bochum, Germany). DCPIP, 2,6-dichlorophenol indophenol, and DPQ, decylplastoquinone, 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, D_2, D_1, Cyt b_{559} 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 D_1 and D_2 proteins. The complex with the more acidic isoelectric point (p*I*), *i.e.* complex **d,** showed the highest content of radioactivity localized mainly on D_1. This activity was 40 times that found in complex **a** (see ref. [2–4]). To elucidate

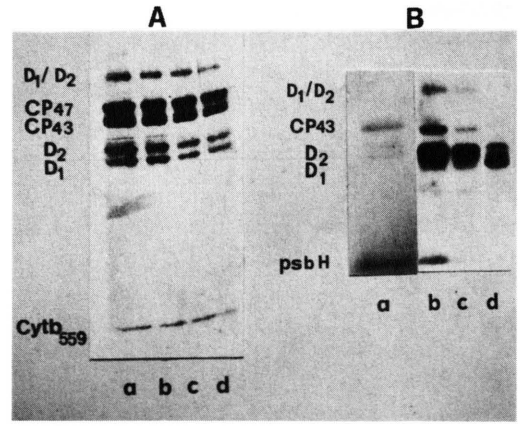
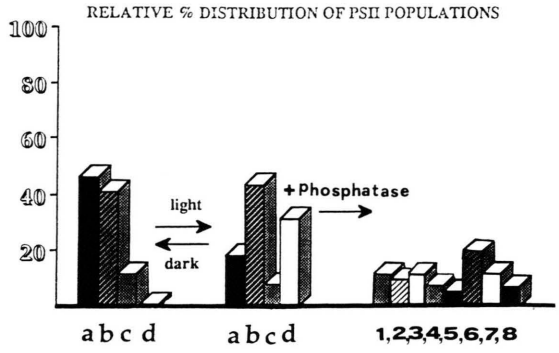


Fig. 1. SDS-PAGE (A) and autoradiography (B) analyses of heterogeneous PS II core populations obtained by IEF of PS II particles.

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



Scheme 1

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.

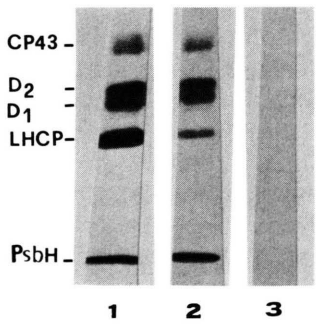


Fig. 2. Autoradiography of phosphorylated and dephosphorylated membranes. Lane 1, PS II particles from phosphorylated thylakoids; lane 2, PS II particles dephosphorylated by method A; lane 3, PS II particles dephosphorylated by method B.

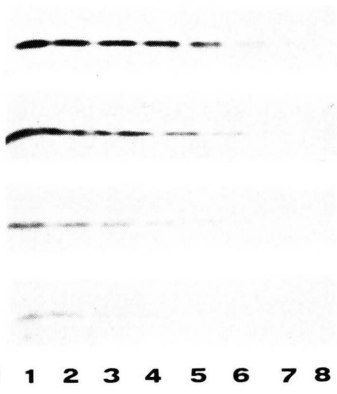


Fig. 3. Immunoblot of PS II core populations obtained by IEF of PS II particles dephosphorylated by method B, using antibodies against PsbH protein.

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%.

Core populations	Electron transfer	
	Control	% Inhibition
a	215	100
b	143	21
c	167	26
d	10	n.d.

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

Table II. Electron transfer activity of PS II particles measured as reported in Table I. The values are an average of 10 experiments. SE approx. 8%.

PS II particle types	Electron transfer
Phosphorylated	254
Dephosphor., method A	310
Dephosphor., method B	262

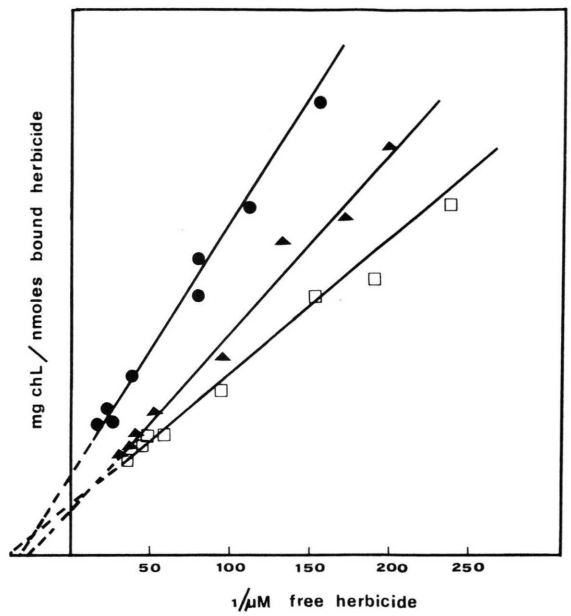


Fig. 4. Double reciprocal plots of bound *vs.* free herbicide bromoxynil to PS II particles: triangles, phosphorylated; squares, dephosphorylated by method A; circles, dephosphorylated by method B.

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 D_1 and D_2 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

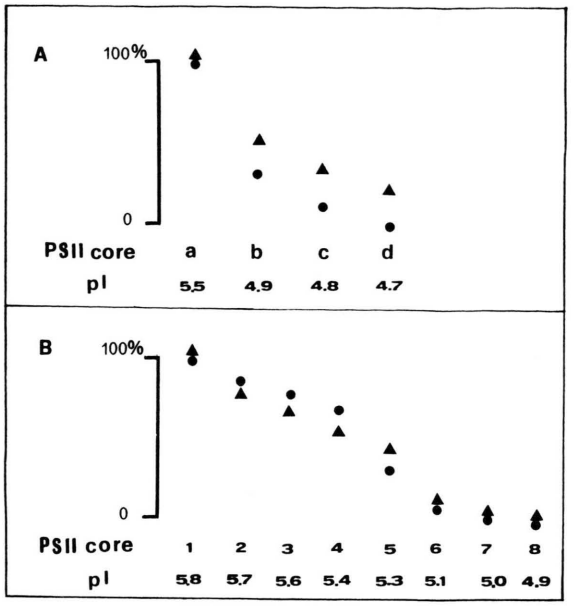


Fig. 5. % Recovery of radioactive herbicide associated to PS II core populations (triangles) and % recovery of PsbH protein (circles) per unit chlorophyll relative to the PS II core population with higher p*I*. A, PS II core populations from phosphorylated PS II particles; B, PS II core populations from PS II particles dephosphorylated with method B (see Scheme 1).

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 Q_A and Q_B [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 Q_B herbicide-plastoquinone-binding domain, the binding affinity of this site being regulated by phosphorylation on D_1/D_2 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].

Acknowledgements

The author thanks Dr. G. Di Marco for a critical reading this manuscript; Prof. P. Böger (Universität Konstanz, Germany) for a gift of PsbH protein antibodies; Prof. W. Oettmeier (Ruhr-Universität, Bochum, Germany) for a gift of dinoseb herbicide, and E. Brizzi for technical assistance.

[1] M. T. Giardi, J. Barber, M. C. Giardina, and R. Bassi, Z. Naturforsch. **45c,** 366–372 (1990).
[2] M. T. Giardi, F. Rigoni, R. Barbato, and G. M. Giacometti, Biochem. Biophys. Res. Commun. **176,** 1298–1305 (1991).
[3] M. T. Giardi, F. Rigoni, and R. Barbato, Plant Physiol. **100,** 1948–1954 (1992).
[4] M. T. Giardi, Proceeding of the IXth International Congress on Photosynthesis, Tokyo, Japan, in press (1992).
[5] G. Renger, Physiol. Vég. **24,** 509–520 (1986).
[6] W. F. Vermaas, K. E. Steinback, and J. Arntzen, Arch. Biochim. Biophys. **23,** 226–237 (1984).
[7] M. Hodges, A. Boussac, and J. M. Briantais, Biochim. Biophys. Acta **894,** 138–145 (1987).
[8] M. Harrison and J. F. Allen, Biochim. Biophys. Acta **1058,** 289–296 (1991).
[9] G. Owens and I. Ohad, J. Cell Biol. **93,** 712–718 (1982).
[10] W. Tischer and H. Strotmann, Biochim. Biophys. Acta **460,** 113–123 (1977).
[11] M. F. Hipkins and N. R. Baker, in: Photosynthesis Energy Transduction: A Practical Approach, IRL Press, Oxford 1986.
[12] W. Draber, K. Tietjen, J. F. Kluth, and A. Trebst, Angew. Chem. **30,** 1621–1633 (1991).
[13] J. Bennett, Annu Rev. Plant Physiol. **42,** 281–311 (1991).
[14] M. Hodges, N. K. Packam, and J. Barber, FEBS Lett. **181,** 83–87 (1985).
[15] M. Kuhn, A. Thiel, and P. Böger, Z. Naturforsch. **43c,** 413–417 (1988).
[16] M. T. Black, T. H. Brearly, and P. Horton, Photosynth. Res. **8,** 193–207 (1986).