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Functional size of the thylakoid phosphatases determined by radiation inactivation

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Radiation inactivation technique was employed to determine the functional size of phosphatases from thylakoid membrane. The enzymatic activities of phosphatases decayed in a simple function with the increase of radiation dosage. D_{37} values of 18.8 ± 2.4–14.1 ± 1.5 Mrad were obtained, using phosphoserine, phosphothreonine, *p*-nitrophenol phosphate, and phospho-histone V-S, respectively, as substrates. The molecular masses of 48.2 ± 6.3–61 ± 5.7 kDa were yielded by target theory analysis. We thus speculate that the thylakoid alkaline phosphatase is probably a monomer while acid phosphatase is functionally a dimer in situ.

Phosphatase; Thylakoid; Functional size; Radiation inactivation

1. INTRODUCTION

Illumination of spinach thylakoid membrane leads to the phosphorylation of the two main phosphoproteins, the light-harvesting chlorophyll *a/b* protein complex of photosystem II (LHC II)[2] and the 9-kDa polypeptide [1]. The activity of protein kinase was activated by plastoquinone pool reduced by light [2] or reductants [3]. It is believed that the phospho-LHC II is responsible for the regulation of absorbed excitation energy [4–7]. The protein kinase from spinach thylakoid has been purified and well characterized [8,9].

On the other hand, the protein phosphatase was supposed to be responsible for the dephosphorylation of thylakoid phosphoproteins. The existence of thylakoid-associated protein phosphatase was demonstrated [10,11]. Unlike the protein kinase, the protein phosphatase activity was not regulated by light and photosynthetic electron transport [12]. Besides, the protein phosphatase activity is inhibited by NaF and molybdate ions [13]. Recently, Kieleczawa et al. [14] have successfully isolated an alkaline phosphatase of molecular weight 51,500 from pea thylakoid. However, an earlier report by Rengasamy et al. [15] indicated the molecular weight of thylakoid acid phosphatase was 27,000. Nevertheless, exact information on the identity, function and structure of thylakoid phosphatases is still lacking.

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Abbreviations: Chl, chlorophyll; LHC II, light-harvesting chlorophyll protein complex of PS II.

In this communication, we determined the functional size of the thylakoid phosphatases as 48–61 kDa by radiation inactivation technique. We speculate that the thylakoid alkaline phosphatase is probably a monomer while acid phosphatase is a functional dimer in situ.

2. MATERIALS AND METHODS

2.1. *Preparation of thylakoid membrane*

Fresh spinach (*Spinacia oleracea* L.) was obtained from the local market. Spinach leaves of 100 g were deveined, chopped into pieces, and ground for 15–20 s in 250 ml Buffer I containing 0.3 M sucrose, 30 mM Tris-HCl (pH 7.8), 10 mM NaCl, 3 mM $MgCl_2$, and 0.5 mM EDTA and filtered through 4 layers of cheesecloth. The filtrate was centrifuged at 11,000 × *g* for 15 min and resuspended in Buffer I. The resuspended solution was centrifuged at 500 × *g* for 3 min. The pellet was discarded and the supernatant was centrifuged again at 2,300 × *g* for 5 min. The pellet was resuspended in Buffer II consisting of 0.2 M sucrose, 20 mM Tris-HCl (pH 7.6), 10 mM NaCl, and 5 mM $MgCl_2$. After washing twice with Buffer II at 13,300 × *g* for 1 min, the thylakoid membranes were resuspended in a solution containing an equal amount of Buffer II and storage buffer which was composed of 10% glycerol, 10 mM NaCl, and 25 mM Tris-HCl (pH 7.5).

2.2. *Preparation of phospho-histone V-S*

[^{32}P]Phospho-histone V-S was prepared according to the method of Yang et al. [16] with minor modifications. Two mg histone V-S was incubated in a reaction medium containing 20 mM Tris-HCl (pH 7.0), 1.5 mM Mg acetate, 1 mM DTT, 0.15 mM [γ-^{32}P]ATP and 40 μg/ml C subunit of cAMP protein kinase. After 3 h incubation at 30°C, 20% trichloroacetic acid was added to precipitate the histone. The pellet was redissolved in 1 ml 50 mM Tris-HCl (pH 8.0) and subjected to a second trichloroacetic acid precipitation. The pellet was then washed several times with acetone, and resuspended in 0.5 ml 500 mM Tris-HCl (pH 8.0). The solution was dialyzed once against 250 ml 50 mM Tris-HCl (pH 8.0), and twice against 250 ml of 5 mM Tris-HCl (pH 7.0). The denatured histone was finally spun down with an Eppendorf centrifuge and was ready for phosphatase assay or was stored at −25°C for later use. Approximately 20 nmol P_i was incorporated into 1 mg histone.

2.3. *Activity assay and protein determination*

The phosphatase activity of thylakoid membrane was assayed at 30°C for 15 min by measuring P_i release from substrates. The reaction was started by addition of the thylakoid membrane. The reaction mixture contained 50 mM Tris-MES, 0.1 M sorbitol, and 1 mM $MgCl_2$, thylakoid membrane (400 μg Chl/ml), and substrates. The concentrations of the substrates were 8 mM phosphothreonine and phosphoserine, 10 mM *p*-nitrophenol phosphate, and 10 μg/ml histone V-S (approximately 2.5–3 × 10^3 cpm), respectively. The pH of the reaction medium was adjusted to optimal values for individual substrates as indicated in table legend. P_i release was measured using either a method as described by LeBel et al. [17] or scintillation counter. The phosphatase activity using *p*-nitrophenol phosphate as substrate was determined spectrophotometrically as the rate of decrease at 405 nm with extinction coefficient of 18,300 $mM^{-1} \cdot cm^{-1}$.

The Chl concentration of isolated chloroplasts was measured using the equations of Arnon [18]. Protein concentration was determined according to the modified method of Lowry [19].

2.4. *Irradiation procedure and calculation of functional size*

Irradiation was performed with a ^{60}Co irradiator (~1,000 Ci) at our institute. The dose rate was 1.14 ± 0.08 Mrad/h which was determined by the method of Hart and Fricke [20] using an Fe^{2+}/Fe^{3+} or Ce^{4+}/Ce^{3+} couple. Thylakoid membranes (1 mg/ml) were irradiated at −18 to −25°C maintained by cryothermostat. The control samples were run concurrently under the same conditions but without irradiation.

Molecular mass (functional size) was calculated from the equation of Beauregard and Potier [21]:

$$\log m = 5.89 - \log D_{37,t} - 0.0028t$$

where m is the functional size in daltons, $D_{37,t}$ is the dose of radiation in megarads required to reduce the activity to 37% of that found in unexposed control at temperature t (°C).

3. RESULTS AND DISCUSSION

Thylakoid protein kinase could phosphorylate endogenous protein substrates, such as LCHII and 9-kDa polypeptides, at the threonyl residue near the N-terminus [20–24]. They could also phosphorylate exogenous protein substrates, such as casein and histone, at seryl residues. In addition, *p*-nitrophenol phosphate is regarded as a common substrate for general phosphatases. We therefore used phosphoserine, phosphothreonine, *p*-nitrophenol phosphate, and phospho-histone V-S as substrates in this study.

When thylakoid membrane was exposed to high energy γ-ray irradiation, the phosphatase activities were reduced with increasing radiation dosage. Fig. 1 depicts the dose–response of dephosphorylation activity of phospho-histone V by thylakoid membrane. The phosphatase activity decayed as a simple exponential function of dosage, allowing straightforward application of the target theory for determination of the functional size involved. The D_{37} value of 18.8 ± 2.4 Mrad was obtained from the survival curve of thylakoid phosphatase using phospho-histone V as substrate. By the equation of Beauregard and Potier [21], a functional mass of 48.2 ± 6.3 kDa was calculated. Furthermore, the functional size of phospho-histone phosphatase is independent of the pH of the reaction medium (data not shown). To verify whether this technique is suitable under our conditions, an internal standard glucose-6-phosphate dehydrogenase was included and its functional mass determined (Fig. 1, open circles). Similarly, the functional size of 110 kDa was obtained for glucose-6-phosphate dehydrogenase, as compared to 104 kDa measured by conventional biochemical methods. We are therefore convinced that this technique is feasible within the limits of accuracy to determine the functional mass of thylakoid phosphatase. Furthermore, Table I shows the D_{37} values and the calculated functional size of the thylakoid phosphatase using different substrates. The D_{37} values for phosphothreonine, phosphoserine, and *p*-nitrophenol phosphate as substrates are 16.2 ± 1.5, 14.1 ± 1.5, and 14.3 ± 1.5 Mrad, respectively. These values give the functional size of phosphatase(s) as 54.1 ± 5.0, 59.4 ± 6.3, and 61.3 ± 6.4 kDa, respectively.

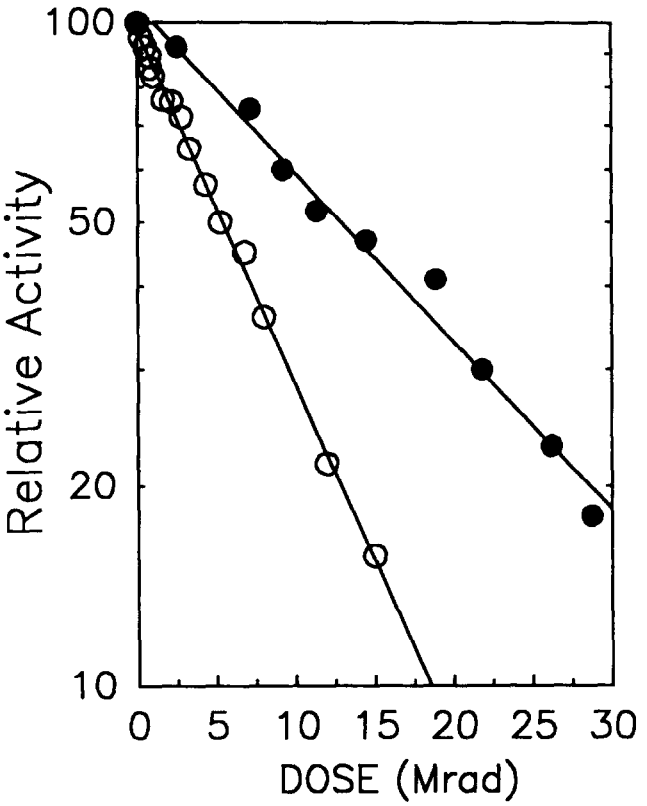

Fig. 1. Radiation inactivation of the thylakoid phosphatase. Reaction conditions were as described in section 2. All data points are means of 4 assays with lines fitted by regression analysis ($r > 0.98$). The intersections of regression lines at 37% control activity give the D_{37} dose values. The functional size was calculated using the equation of Beauregard and Potier [21]. (○) Glucose-6-phosphate dehydrogenase; (●) phospho-histone V-S.

Thylakoid phosphatase has a broad spectrum of substrate specificity [15]. In addition to hydrolyzing phosphoesters, they also dephosphorylate phosphoproteins. The pH optima for each kind of substrate are quite different. Some researchers have suggested that

Table I

The functional size of thylakoid phosphatases

Substrate	pH	D_{37} (Mrad)	Functional size (kDa)
Phosphothreonine	6.5	14.1 ± 1.5	59.4 ± 6.3
Phosphoserine	6.5	16.2 ± 1.5	54.1 ± 5.0
p-Nitrophenol phosphate	8.0	14.3 ± 1.5	61.3 ± 6.4
Phosphohistone V-S	7.0	18.8 ± 2.4	48.2 ± 6.3

The reaction conditions for irradiation of thylakoid membrane and measurements of enzymatic activities using different substrates were as described in section 2. The D_{37} values were obtained from the best-fitted linear regression of the mean of at least 4 assays ($r > 0.98$) for each phosphatase. The functional size was calculated according to the equation of Beauregard and Potier [21].

they might be a group of distinct proteins. Recently, Kieleczawa et al. [15] successfully isolated an alkaline phosphatase of molecular weight 51,500 from pea thylakoid. However, earlier work by Rengasamy et al. [15] indicated that the molecular weight of acid phosphatase was 27,000. Nevertheless, our data reveal that the functional magnitude of phosphatase activities using these substrates at different pH 'values' are very similar, in the range of 48–61 kDa. We speculate that the thylakoid alkaline phosphatase is probably a monomer, while acid phosphatase is functionally a dimer in situ.

It is well known that the phosphorylation of the thylakoid protein is regulated by the electron transport reaction and the redox state of the plastoquinone pool [2,3]. However, the dephosphorylation of the thylakoid proteins is independent of the electron transport and the energization state of the thylakoid membrane [12]. Whether the dephosphorylation of the thylakoid proteins is regulated by another system is still unknown. However, from the radiation inactivation analysis, we observed a linear dose–response relationship and obtained only a small functional size of either monomer or dimer for phosphatase reactions. We therefore believe that the regulation system, if present, is not directly involved in the enzymatic activities of phosphatase under the conditions investigated.

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