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Rapid Report

Determination of the molecular size of the binding site for the manganese-stabilizing 33 kDa protein in Photosystem II membranes

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A radiation-inactivation study revealed that the manganese-stabilizing 33 kDa protein (MSP) binds to a site of only 13 kDa in $CaCl_2$-treated Photosystem (PS) II complexes. From the coincidence of this size with molecular weight it appears that a cytochrome *b*-559 heterodimer is responsible for the functional binding of MSP to the PS II reaction center.

Biochemical research on PS II has accelerated significantly since the successful isolation of PS II complexes (see Ref. 1 for review). Isolated PS II core complexes capable of evolving oxygen are composed of about 10 protein subunits [2]. The manganese-stabilizing protein (MSP) is one of the best-characterized proteins of the core complex with respect to molecular properties and functions in the evolution of oxygen [1,3]. In the oxygen-evolving PS II core complex, three subunit species have been shown to be localized near MSP: CP47, from studies of chemical cross-linking [4] and immunological reactivity [5]; CP43, from results of differential digestion with a specific proteinase [6]; and cytochrome *b*-559, from an assay of direct binding to PS II reaction-center complexes [7] and from chemical cross-linking data (Enami et al., unpublished data). MSP can be detached from its binding site among these neighboring proteins in such a way that its reintegration with PS II membranes depleted of MSP restores oxygen-evolving activity [3]. However, the functional binding site has still not been unequivocally identified. MSP is not a redox-active component itself [8,9] but forms a complex with other component(s) to shield the manganese catalytic center, providing the environment that is essential for the overall water-oxidation function [1,3]. Thus, identification of the binding protein participating in the functional association of PS II with MSP should help in understanding the biochemical mechanism of oxygen evolution. Using a radiation-inactivation method we have made an estimate of the size in situ of the protein in PS II that binds MSP. This method allows determination of the molecular mass of the binding site exclusively, within a structure that includes proteins that are loosely or nonfunctionally associated with the protein of interest [10,11]. Molecular masses of membrane-integral receptor proteins for asialoglycoprotein [12], insulin [13], and high-density lipoprotein [14] have been determined by similar radiation-inactivation studies. Decay of the binding that survives after a specific dose of radiation allows calculation of the functional size of the component of the membrane that is required for the reconstitution of the intact complex, and we show here that the binding site for MSP is comparable in size to the cytochrome *b*-559 heterodimer.

PS II membranes from spinach were prepared by the method of Kuwabara and Murata [15] and treated with 1 M $CaCl_2$ by the method of Ono and Inoue [8]. MSP was isolated from the resultant extract by column chromatography on DEAE-Toyopearl 650M (Tosoh Co., Tokyo, Japan) in 10 mM Tris-HCl (pH 8.0). After washing of the column with 10 mM Tris-HCl (pH 8.0)/0.1 M NaCl, MSP was eluted by increasing the concentration of NaCl to 0.5 M. $CaCl_2$-treated PS II membranes (0.24 mg of chlorophyll (Chl)) in 0.25 ml of incubation medium which contained 0.4 M sucrose, 40 mM Mes-NaOH (pH 6.0) and 10 mM NaCl, were frozen in liquid N_2 and irradiated for various times

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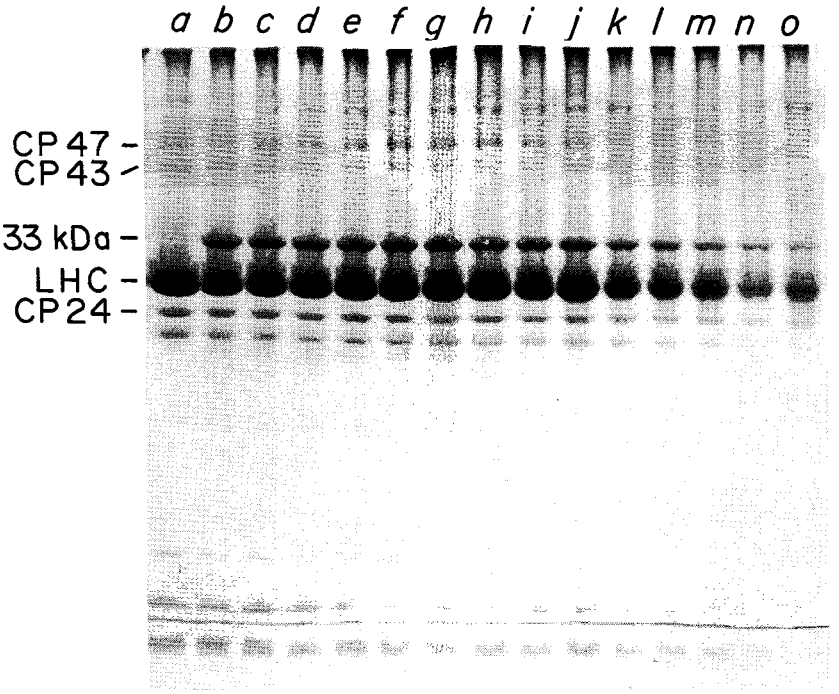

Fig. 1. Results of SDS-polyacrylamide gel electrophoresis of $CaCl_2$-treated PS II membranes (14.5 μg Chl) that were exposed to various doses of radiation and subsequently reconstituted with MSP, as described in the text. Lane *a*, $CaCl_2$-treated PS II membranes without irradiation and reconstitution; lanes *b*–*o*, reconstituted $CaCl_2$-treated PS II membranes that had been irradiated at −150°C with radiation doses of 0, 2.8, 5.6, 8.4, 11.2, 16.8, 22.4, 28.1, 42.1, 56.1, 70.1, 84.2, 98.2 and 112.2 Mrad, respectively.

from 0.5 to 20 min by 1.5 MeV electrons at a dose rate of 5.61 Mrad (56.1 kGy) min^{-1}, as described previously [16]. Irradiated samples were annealed overnight at −85°C prior to the assay, of the capacity of PS II membranes for reconstitution with isolated MSP. Control samples were treated identically but without irradiation.

Survival of the MSP-binding protein was evaluated by ability of the protein in PS II membranes to rebind MSP. $CaCl_2$-treated PS II membranes (20 μg of Chl) were incubated with 3 μg of MSP for 2 h at 5°C in 0.5 ml of the incubation medium and then centrifuged at $10\,000 \times g$ for 20 min. Pellets were resuspended in the incubation medium (1 ml) and centrifuged again as above. The resultant pellets were subjected to SDS-polyacrylamide gel electrophoresis and relative amounts of MSP were quantified by integration of the absorbance at 570 nm in densitograms of bands stained with Coomassie brilliant blue R-250, as described elsewhere [16].

$CaCl_2$-treated PS II membranes were reconstituted with MSP (compare lanes *a* and *b* in Fig. 1). The amount of rebound MSP was almost equal to the natural level of MSP in untreated PS II membranes despite the incubation of $CaCl_2$-treated PS II membranes with MSP at a 5-fold molar excess with respect to its binding site (data not shown; Ref. 17). With increases in the dose of radiation lesser amounts of MSP became associated with the $CaCl_2$-treated membranes, indicating damage to the MSP-binding protein in PS II membranes, as shown in Fig. 2. The rate of disappearance of the band of MSP is inversely proportional to the molecular weight of the receptor protein, as described by an empirically derived equation: molecular mass $= 6.4 \cdot 10^{11}/D_{37}/S_t$, where S_t is a temperature-sensitivity factor of 3.15 for ionizing radiation at

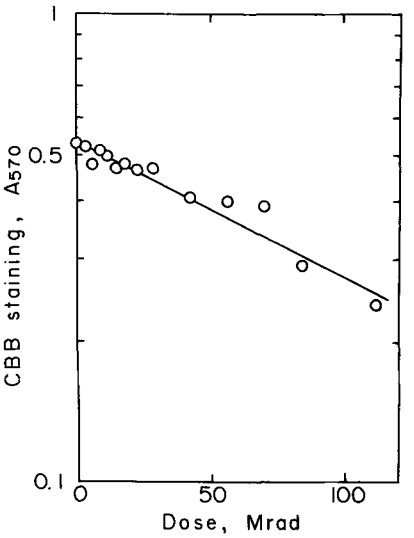

Fig. 2. Radiation-inactivation of the reconstitution of $CaCl_2$-treated PS II membranes with MSP. The extent of inactivation of the binding protein was deduced from the rate of disappearance of MSP in the densitogram of the stained gel shown in Fig. 1.

−150°C and D_{37} is the dose of radiation that is required for the decomposition of a protein to 37% of its original amount [10,18]. D_{37} for this function is estimated to be 153 Mrad at −150°C, so that it appears that a protein with a molecular mass of 13 (±1.4) kDa (average of three determinations) is responsible for the binding of MSP to the PS II membranes. Such a small size indicates that the MSP-binding unit may be composed of only a single subunit protein rather than a protein complex of PS II. As shown in Fig. 1, discrete bands of proteins from PS II membranes became diffuse and heterogeneous as a result of the random cleavage of peptide backbones by bombardment with high-energy electrons [16]. From a similar calculation, the rate of disappearance of the bands of CP47 (56.2 kDa [19]) and CP24 (21 kDa [20]) yields molecular masses of 55 kDa and 23 kDa, respectively, a validation of this method for the analysis of the molecular masses of intrinsic membrane proteins (data not shown).

Among the protein subunits adjacent to MSP that were mentioned above [4–7], the cytochrome *b*-559 heterodimer has a molecular mass of 13.5 kDa [21], which is similar to the functional size of 13 kDa deduced for the MSP-binding protein in PS II membranes. The possibility that the 13 kDa functional mass is that of a peptide segment of some molecule with a larger subunit can be ruled out. Given that the binding energy of MSP to PS II membranes is 10 kcal M^{-1} [17], the contact surface area is assumed to be about 500 $Å^2$ [22]. The complementary surfaces between associated protein molecules are consisted of multiple strands of aligned peptides [23]. Thus, the protein–protein interaction requires the integrity of the entire structure of the binding proteins. In the above-mentioned determinations of functional size of receptor molecules by radiation-inactivation of the ability to bind ligand molecules [12–14], the size obtained in each case corresponded closely to the molecular mass of each respective receptor or to that of its oligomeric form, with none of the functional sizes corresponding to segments of the receptor molecules.

MSP that had been unfolded by the oxidation of a single disulfide bridge failed to bind to PS II membranes in reconstitution experiments [24]. Moreover, MSP that had lost a small NH_2-terminal segment, with the rest of the structure intact, was no longer able to bind with MSP-depleted PS II membranes [25]. These findings indicate that the entire structure of the MSP molecule may stabilize a binding niche that is composed of peptide strands and includes the NH_2-terminal segment of MSP. A topological study of cytochrome *b*-559 in the thylakoid membranes showed that the COOH-terminal half of cytochrome *b*-559 is exposed to the luminal surface [26]. This hydrophilic region is enriched in acidic amino-acid residues, whereas the NH_2-terminal segment of MSP is positively charged at neutral pH. It is likely that MSP and cytochrome *b*-559 associate initially as a result of an electrostatic attraction between local opposite charges.

Cytochrome *b*-559 has been reported to play only an indirect role in PS II: in protection from photodamage [27] or in the cyclic flow of electrons around the photochemical reaction center [28]. However, MSP appears to associate primarily with cytochrome *b*-559 in $CaCl_2$-treated PS II membranes. This interaction suggests the participation of cytochrome *b*-559 in some other important function in the oxygen-evolving reaction.

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