Distribution of intrinsic and extrinsic subunits of the PS II protein complex between appressed and non-appressed regions of the thylakoid membrane: an immunocytochemical study

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The distribution of photosystem (PS) II centers between appressed and non-appressed domains of the thylakoid membranes has been investigated, using immunogold labeling, in *C. reinhardtii* and in spinach. We have used antibodies directed against the two main intrinsic subunits of the PS II center and against two extrinsic subunits of the O_2-evolving site. 90% of the two intrinsic PS II antigens are located in the appressed membranes, in a density 3–3.5-fold higher than in the non-appressed regions. The same distribution is observed for the two extrinsic antigens. This result suggests that the O_2-evolving system is associated with the PS II center, both in appressed and in non-appressed regions of the thylakoid membrane.

Photosystem II subunit *Oxygen evolution site* *Membrane lateral heterogeneity* *Immunogold labeling*

1. INTRODUCTION

The differentiation of the thylakoid membranes between appressed and non-appressed regions is well documented both in higher plants and green algae [1]. The functional significance of this organization in vivo has been discussed in particular with respect to the lateral distribution of the main thylakoid proteins [2].

Recent analysis, using the phase partition technique applied to higher plant thylakoids, supports a model in which most of the PS II centers are localized in the appressed regions of the thylakoid membranes, whereas PS I centers are localized exclusively in non-appressed membranes [3]. An immunocytochemical characterization of these two domains at the ultrastructural level should provide independent evidence for the lateral distribution of the two photosystems in the native membrane. However, techniques using 'en bloc' immunolabeling, before resin impregnation, are not suitable for such an analysis, due to a limited accessibility of the antigens located in the appressed membrane regions. Here, thin sections of Lowicryl embedded material were subjected to immunogold staining, a method by which all structures are equally accessible to antibodies. As a first step towards the identification of the proteins located in either region of the thylakoids, we have checked the distribution of PS II centers by immunogold labeling and have addressed the question as to whether some PS II subunits are localized in the non-appressed regions. To this end, we have compared, both in *Chlamydomonas reinhardtii* and in spinach, the lateral distribution of the two main integral subunits of the PS II reaction center with that of two peripheral subunits involved in electron donation to PS II [4] and presumably parts of the oxygen-evolving site.

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2. MATERIALS AND METHODS

The WT and F34 strains of *C. reinhardtii* were grown in Tris-acetate-phosphate medium under cool fluorescent light (200 lux). Thylakoid membranes were purified as in [5] and broken cells were prepared in the presence of 10 mM $MgCl_2$ as described [6]. Spinach leaves were purchased at the local market and chloroplasts were prepared according to Avron [7], in the presence of 10 mM $MgCl_2$.

The α-5, α-6, α-12 and α-19 antibodies to polypeptides 5, 6, 12 and 19 in *C. reinhardtii* were obtained by courtesy of N.H. Chua. SDS-polyacrylamide gel electrophoresis was performed according to Laemmli [8]. Samples were heated at 100°C for 1 min and run on 7.5–15% polyacrylamide gradient gels, at 4°C to limit proteolysis. Immunoblotting experiments were performed according to Towbin et al. [9].

For immunocytochemical analysis, samples were fixed in 1% glutaraldehyde in 4 mM potassium phosphate buffer, pH 7.0, rinsed in 0.1 M glycine and embedded in Lowicryl K4M, according to Carlemaem et al. [10]. Thin sections were picked up on collodion-coated nickel grids and incubated successively in a Tris buffer containing 10 mM Tris-HCl (pH 7.4), 0.9% NaCl, 0.5% bovine serum albumin, 0.1% Tween 20, and in the same buffer containing specific antibodies (final IgG concentration: 50–500 $\mu g \cdot ml^{-1}$), for 45 min. Then they were rinsed 3 times in the buffer, incubated for 1 h with goat anti-rabbit IgG, and labeled with 10 nm gold granules (GAR G10, Janssen Pharmaceutica, Beerse, Belgium). The grids were rinsed 3 times in the buffer, once in water, then dried.

The thin sections were stained successively with aqueous OsO_4 vapor for 4 h, 5% aqueous uranyl acetate for 1.5 h, and lead citrate for 12–15 s. Micrographs were taken at 60 kV in an EM 400 Philips. Labeling densities were estimated by counting gold granules on appressed and non-appressed membrane regions on several micrographs; membrane lengths of the two regions were measured using a Tektronix coordinate analyser and computer.

3. RESULTS AND DISCUSSION

The immunoblotting experiments shown in fig.1 give some characteristics of the 4 antibodies to the PS II subunits of *C. reinhardtii* used here.

As shown by Chua and Blomberg [11], we observe that α-5 and α-6 antibodies cross-react with spinach antigens and are monospecific both in *C. reinhardtii* (we have checked the monospecificity of the 4 antibodies with purified thylakoids as well as with broken cell preparations from *C. reinhardtii*) and in spinach. They react, respectively, with 50-kDa and 47-kDa PS II subunits in *C. reinhardtii*, and with 47-kDa and 43-kDa PS II subunits in spinach. The higher molecular mass subunit in the two organisms is the location site of both P680 and the pheophytin acceptor molecule [12,13], while the lower molecular mass subunit was shown to participate in the fixation site of the primary quinone acceptor molecule in *C. reinhardtii* [13]. Both are integral membrane components and build up the 'core' of the PS II reaction center [14].

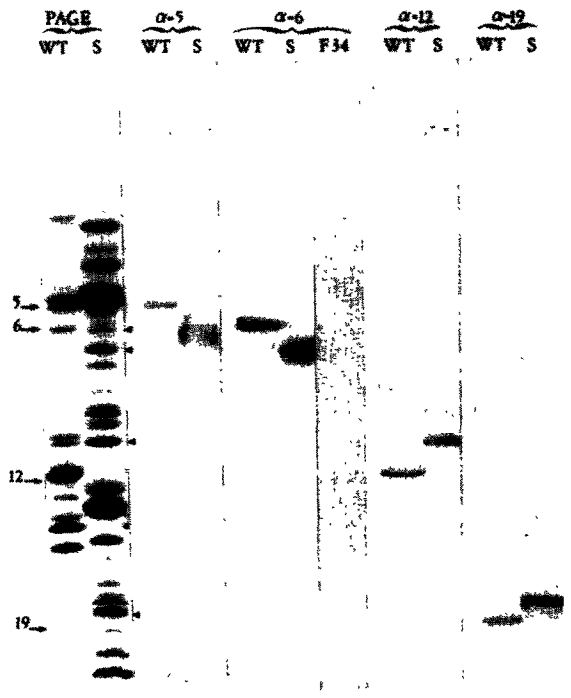

Fig. 1. Left: electrophoretogram of thylakoid membrane polypeptides of *C. reinhardtii* (PAGE WT) and spinach (PAGE S). Right: characterization of the α-5, α-6, α-12 and α-19 antibodies on *C. reinhardtii* (WT) and on spinach (S). The α-6 antibody has also been characterized on the F34 mutant (F34). Reacting polypeptides in *C. reinhardtii* are numbered according to [5] and in spinach, the cross-reacting bands are indicated by arowheads.

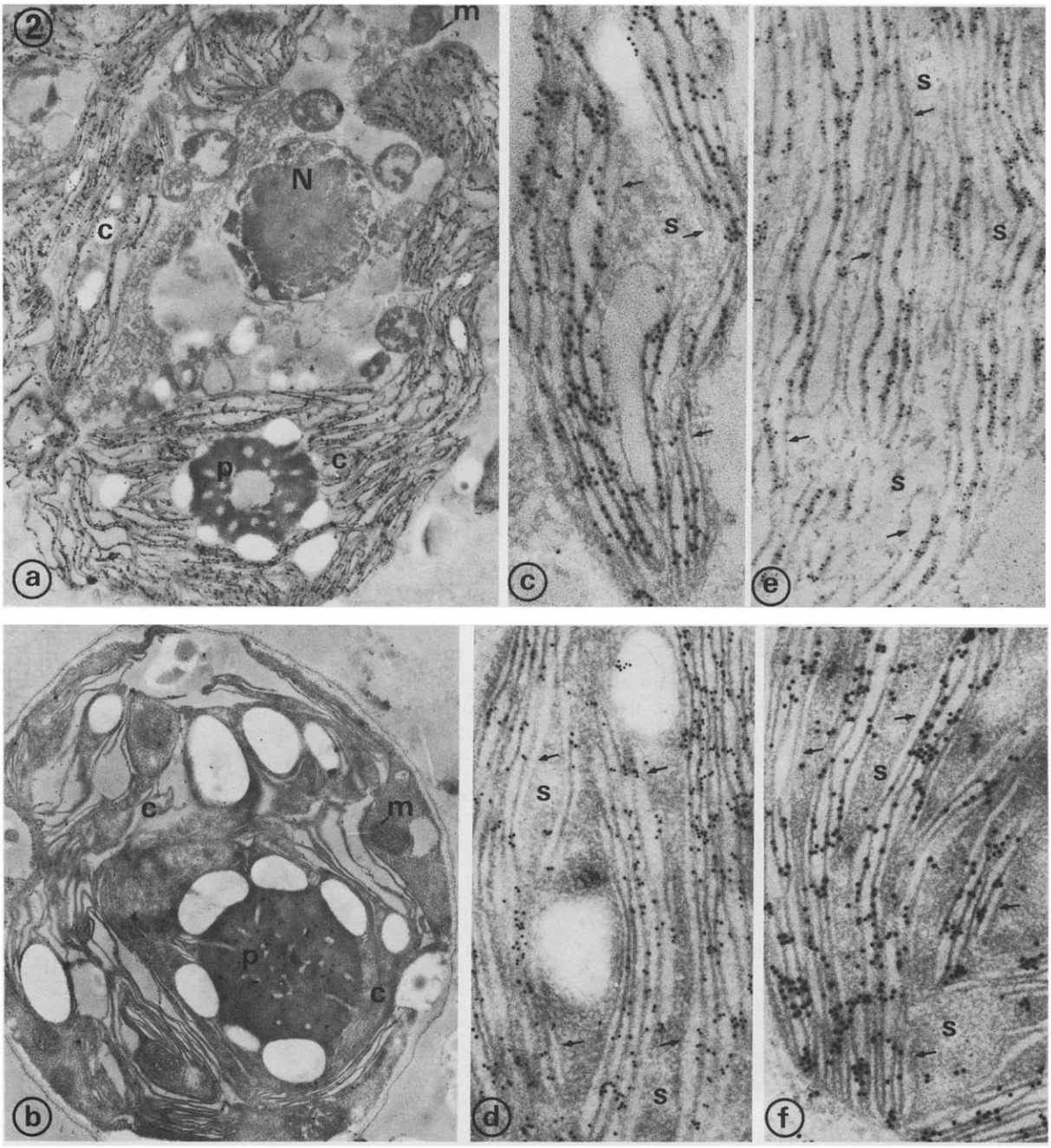

Fig.2. Immunocytochemical localization of the PSII subunits in *C. reinhardtii.* (a, c). WT labeled with α-12 antibody. In (a), note the high density of gold particles on the thylakoid membranes and the low background on other cell structures. (b) F34 mutant labeled with α-6 antibody. In contrast with (a), very few gold particles are visible both on the thylakoid membranes and on other cell structures. (d,e,f). WT thylakoid membranes labeled with the α-6, α-19 and α-5 antibodies, respectively. Most of the labeling occurs in the appressed regions whereas the stroma-exposed membranes are slightly labeled (arrows). c, chloroplast; m, mitochondria; N, nucleus; p. pyrenoid; s, stroma. Magnification: a,b, × 12125; c–f; × 43650.

The α-12 and α-19 antibodies are both monospecific in *C. reinhardtii* and are directed against the 30.5-kDa and 20-kDa polypeptides, respectively. In spinach thylakoids, the former antibody cross-reacts with a 33-kDa band, whereas the latter cross-reacts with a 23-kDa band. Polypeptides 12 and 19 in *C. reinhardtii* are, together with polypeptide 24, peripheral membrane subunits associated with PS II centers and are in reduced amounts in mutants unable to evolve oxygen [4]. They correspond to the 33-, 23- and 16-kDa polypeptides which were recently shown to be involved in the organization of the O_2-evolving site in higher plants thylakoids [15].

When Lowicryl sections of broken cells of *C. reinhardtii* are treated for immunogold localization of the 4 PS II subunits, a dense and specific labeling of the thylakoid membranes is observed (fig.2a,c–f). Background labeling on non-thylakoid structures is very low (fig.2a). A quantitative evaluation of this background can be drawn from experiments performed on WT broken cells treated with IgG from a non-immune rabbit (table 1) or on F34 broken cells, lacking in the 47-kDa antigen [5], treated with α-6 antibody (fig.2b and table 1). In both cases, we observe less than 0.3 particles per μm thylakoid membrane.

The main observation drawn from our experiments with *C. reinhardtii* WT strain is that, regardless of the anti-PS II antibody used, appressed thylakoid membranes are much more heavily labeling than non-appressed ones (fig.2). Whereas thylakoids are about 73% stacked in our preparation, we find 90% of the PS II antigens in the appressed membranes (table 1). An identical distribution was observed for the 4 polypeptides under study.

Similar results were obtained for spinach chloroplasts treated with α-5, α-6 and α-12 antibodies (fig.3a–c). A marked labeling of grana stacks is observed as compared to the weak labeling of stroma lamellae. Although cross-reacting on immunoblots, α-19 did not label thin sections of spinach chloroplasts significantly, which might suggest a deleterious effect of the embedding procedure on some antigenic properties of the 23-kDa polypeptide.

This uneven distribution of PS II subunits between appressed and non-appressed thylakoid membranes confirms previous reports drawn from biochemical, ultrastructural (review [16]) or cytochemical [17] data, showing that most of the PS II centers are in the appressed regions of the thylakoid membranes. We find (table 1) that the density of the PS II antigens in the appressed regions is 3–3.5-fold higher than in the non-appressed regions as is the case for EF particles which contain the PS II centers in freeze-fractured thylakoids [18,19]. Although there is conflicting evidence as to whether there is some PS II activity in the non-appressed regions of the thylakoid membrane [20,21], the present study supports the view that a fraction of PS II complexes is localized in these regions.

Table 1

Labeling of appressed and non-appressed membranes with antibodies to PS II subunits in *C. reinhardtii*

Antibody	Membrane length (μm)	% appressed membranes	Labeling density			% particles on appressed membranes
			Appressed membranes (particles/μm)	Non-appressed membranes (particles/μm)	Ratio appressed/ non-appressed	
α-5	561	73.9	3.3	1.2	2.9	89.0
α-6	348	72.5	5.8	1.6	3.6	90.4
α-12	851	73.7	11.9	4.0	3.0	89.3
α-19	339	73.8	8.4	2.5	3.4	90.6
Control: IgG	375	ND	0.2		ND	ND
Control: α-6 on F34	235	ND	0.3		ND	ND

ND, not determined

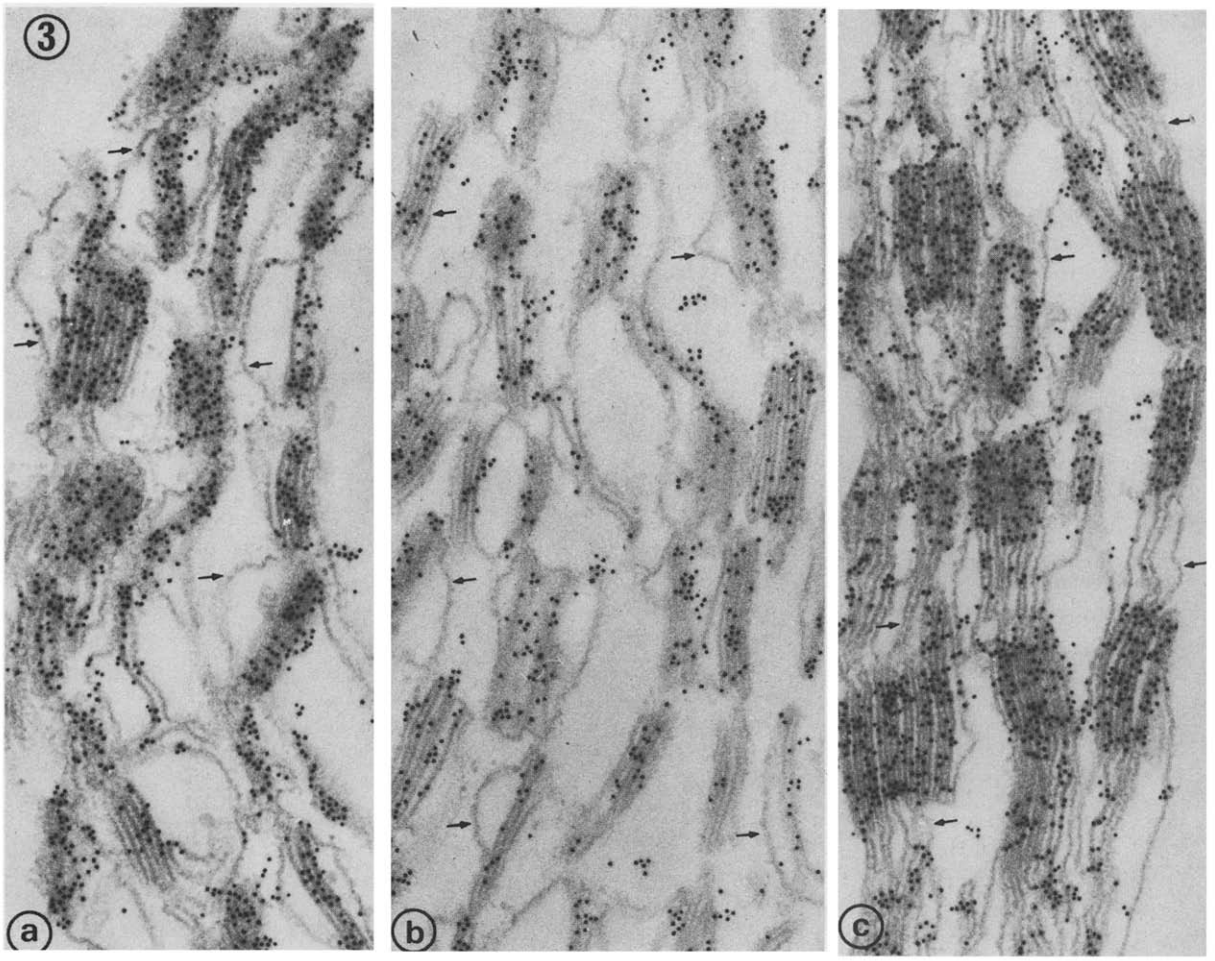

Fig.3. Immunocytochemical localization of the PS II subunits in spinach chloroplasts. The thylakoid membranes are labeled with α-5 (a), α-6 (b) and α-12 (c) antibodies. The appressed membranes are densely labeled whereas the non-appressed ones (arrows) show very few gold granules. Magnification: × 43650.

This presence of PS II complexes in the non-appressed regions can be explained either by a dynamic equilibrium between two sites of PS II, i.e., appressed and non-appressed regions, or by a structural heterogeneity among the population of PS II proteic complexes. The latter view is supported by the difference in size of the EFs and EFu particles associated with the appressed and non-appressed membranes, respectively [18,19]. However, this difference has been understood as a consequence of the uneven distribution of the antenna complex associated with the PS II centers between the two regions of the thylakoids [22].

Functional heterogeneity such as a difference in the organization of the acceptor or donor sides of the PS II centers [20] could provide a basis for a structural heterogeneity among PS II proteic complexes. There are some discrepancies in the estimation of the number of PS II centers in the thylakoid membranes on a chlorophyll basis [23,24]. The lower estimation obtained with measurements of protons or oxygen release could arise from the inability of some PS II complexes, depleted in their peripheral subunits on the donor side, to evolve oxygen. These, if located in the non-appressed membrane regions, would provide the link between a functional and a structural heterogeneity in PS II. This study shows that, in

the native membrane, this is not the case, as far as the 30.5-kDa and 20-kDa peripheral subunits in *C. reinhardtii* (and the 33-kDa subunit in spinach) are concerned: we observed the same distribution of PS II whether we labeled the integral core subunits or the peripheral donor-side subunits. That the 3 peripheral subunits are associated with PS II centers in the non-appressed membranes is also supported by a recent immunological analysis of right-side-out thylakoid fractions from spinach [25].

Here, immunogold localization of 4 distinct PS II subunits on Lowicryl sections gives reproducible and consistent results, supporting a model in which most, but not all, PS II centers are located in appressed regions of the thylakoid membrane. This technique is expected to provide reliable information on the distribution of the other major proteic complexes of the photosynthetic apparatus in the native membrane, as well as on the regulation of membrane stacking and energy distribution.

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