Development of the 33-, 23- and 16-kDa polypeptides of the photosynthetic oxygen-evolving system during greening

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The accumulation of the 33-, 23- and 16-kDa polypeptides of the oxygen-evolving complex has been compared with the development of oxygen evolution activity during greening. Both the 33- and 23-kDa proteins are present in etioplast membranes whereas the 16-kDa species is detectable only in trace amounts. The accumulation of the 3 polypeptides' greening is asynchronous. Only the 16-kDa polypeptide is formed in concert with the appearance of oxygen evolving activity.

Thylakoid development *Photosynthetic oxygen evolution* *33-kDa protein* *23-kDa protein* *16-kDa protein* *Western blotting*

1. INTRODUCTION

The structure of the PS II oxygen-evolving system in thylakoids consists of the reaction center and light-harvesting assemblies, intimately associated with the water-splitting complex which contains the 33-, 23- and 16-kDa polypeptides and probably also a so far unidentified Mn-carrying protein [1]. Studies of the oxygen evolution mechanism have so far been focussed on the 33-, 23- and 16-kDa proteins since these are hydrophilic and are bound to the inner membrane surface, and are thus amenable to resolution-reconstitution experiments. Extraction of either inside-out thylakoids or oxygen-evolving particles with concentrated NaCl releases the 23- and 16-kDa species and inhibits oxygen evolution [2,3]. Activity is reportedly restored either by rebinding the 23-kDa protein [2,3], by high chloride concentrations [4,5] or by 10 mM $CaCl_2$ [6,19], suggesting that one function of the 23-kDa species is to increase the affinity of the catalytic center for essential Ca and chloride. Additional release of the 33-kDa protein occurs upon sonication [7] or treatment with high Tris [8] or $CaCl_2$ [9] concentrations. Provided Mn is retained during extraction, activity can apparently be restored by rebinding the 33-kDa [10] or 23-kDa species [7] or simply by high chloride concentrations [11]. Thus, a somewhat confused picture is emerging which nonetheless suggests that the 33- and 23-kDa proteins are non-catalytic but are in some way involved in the retention or binding of Mn, Ca and chloride at the active center. The role of the 16-kDa protein is completely unknown and only one report has appeared showing any beneficial effect in the reconstitution of oxygen evolution activity [12].

One means of probing the essentialness or otherwise, and possible roles, of the 33-, 23- and 16-kDa proteins is to compare their accumulation during greening with the development of oxygen evolution activity. Here, this correlation has been studied during prothylakoid differentiation under either continuous or intermittent light conditions, using

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Abbreviations: PS, photosystem; IML, intermittent illumination; LiDS–PAGE, lithium dodecyl sulphate-polyacrylamide gel electrophoresis

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the sensitive immunological technique of Western blotting for unambiguous detection of the polypeptides.

2. EXPERIMENTAL

Barley seedlings (*Hordeum vulgare* cv. Clipper) were grown for 6–7 days in darkness then transferred to an IML regime consisting of cycles of 2 h darkness interrupted by 2 min light of intensity 90 $\mu E \cdot m^{-2} \cdot s^{-1}$ [13]. Where indicated, the plants were further exposed to continuous illumination at 170 $\mu E \cdot m^{-2} \cdot s^{-1}$. Intact chloroplasts [13] and etioplasts [14] were isolated as described then membrane fractions were prepared by osmotic lysis in 10 mM NaCl and 5 mM Tricine–NaOH, pH 7.5, for 15 min followed by centrifugation at 10000 × *g* for 10 min. Control thylakoids were

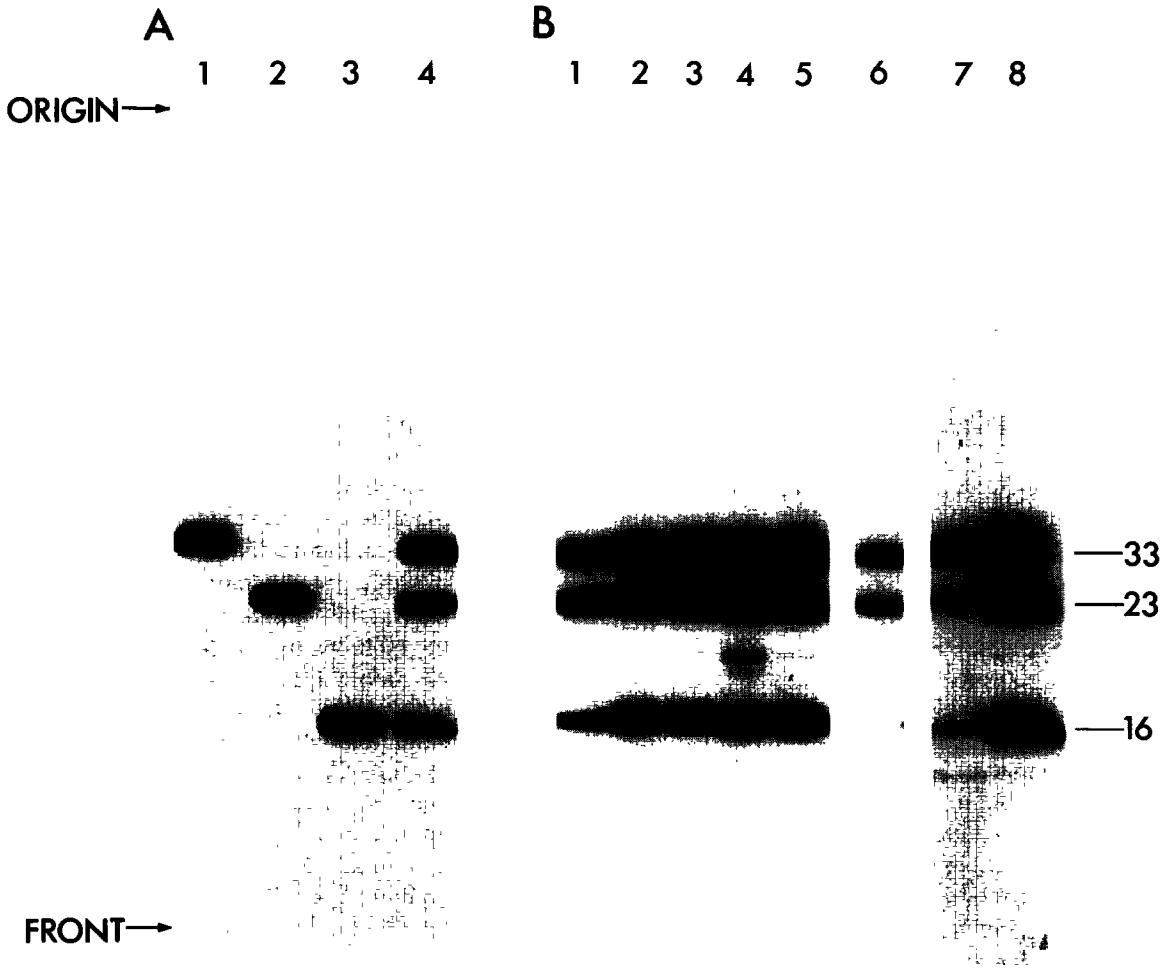

Fig.1. Development of the 33-, 23- and 16-kDa polypeptides of PS II during intermittent illumination of dark-grown barley seedlings. (A) Specificity of the antisera. Samples (60 μg protein) of continuous-light thylakoids were electrophoresed by LiDS–PAGE then the lanes were processed individually by Western blotting using 1:800 dilutions of the antisera against the 33 (lane 1), 23 (lane 2) or 16 (lane 3) kDa proteins. Lane 4 was processed with a combination of the antisera. (B) Prothylakoids were prepared from IML plants and the 3 polypeptides were detected by LiDS–PAGE and Western blotting. Lanes 1–4, 12, 24, 36 and 48 IML cycle prothylakoids, respectively; lane 5, control (continuous-light) thylakoids; lane 6, etioplast membranes; lanes 7–8, 120-μg samples of etioplasts and control thylakoid membranes, respectively. Both lanes were processed at 1:400 dilutions of the antisera.

isolated from plants grown in the greenhouse using a 16 h per day photoperiod.

Antisera against the 33-, 23- and 16-kDa proteins were raised in rabbits following injection of the purified proteins [4]. PS II oxygen evolution was assayed in 50 mM sorbitol, 10 mM NaCl, 0.5 mM NH_4Cl, 10 mM Tricine–NaOH, pH 7.4, using dimethylbenzoquinone as acceptor [15]. Procedures for LiDS–PAGE and Western blotting [13] are described elsewhere.

3. RESULTS

The specificities of the antisera raised against the 33-, 23- and 16-kDa polypeptides were first examined by Western blotting using thylakoids from control (continuous-light) plants as the antigen for electrophoresis. Each antiserum was highly specific and showed reactivity with only the single band amongst the thylakoid polypeptide profile (fig.1A, lanes 1–3). Where the antisera were combined only the 3 sharp bands were seen on blots (lane 4); thus, the mixture of antisera enabled a simultaneous examination of the 3 polypeptides and was therefore employed routinely.

Fig.2. Development of the 33-, 23- and 16-kDa polypeptides during continuous illumination of 12 IML cycle barley seedlings. Samples (60 μg protein) of prothylakoid membranes were electrophoresed by LiDS–PAGE then subjected to Western blot analysis. Lanes 1–5, 30 min, 3, 6, 10 and 16 h light, respectively; lane 6, control (continuous-illumination) thylakoids.

The putative relationship between these polypeptides and the development of oxygen evolution activity was initially examined in plants greened under IML. Since the assembly of the PS II complex under these conditions is quite abnormal [16,17] the IML regime represents an interesting contrast with the continuous-light system (see below). After only 12 IML cycles both the 33- and 23-kDa polypeptides were very prominent on blots (fig.1B, lane 1). Both proteins continued to accumulate during greening (lanes 1–4) and after 48 IML cycles were as abundant as in control thylakoids (lane 4 *vs* 5). In contrast, the 16-kDa protein, though detectable after 12 IML cycles, was low in concentration (lane 1) but increased quite markedly by 24 IML cycles (lane 2) to an amount approximating that in control membranes (lane 5). The accumulation of the 16-kDa protein is therefore asynchronous with the 33- and 23-kDa species but notably, quite closely correlates with the onset of oxygen evolution which also commences between 12 and 24 IML cycles (table 1).

Given the prominence of the 33- and 23-kDa species after only 12 IML cycles, we questioned whether they might also be present in etioplasts and thus synthesized before exposure of the seedlings to light. Indeed, both proteins were clearly present although the 16-kDa band could not be seen under these conditions of detection (lane 6). However, by increasing the sensitivity of detection (see fig.1 legend) the 16-kDa protein was also detectable but was clearly present only in trace amounts compared with control thylakoids (lane 7 *vs* 8). The fact that identical blots were obtained whether intact or osmotically lysed etioplasts were used as antigen (unpublished results) strongly suggests that all 3 proteins are already associated with the prothylakoid membranes.

The relationship between the accumulation of the 3 polypeptides and the onset of oxygen evolu-

Table 1

The development of photosynthetic oxygen evolution in prothylakoids from plants greened under intermittent or continuous illumination

Illumination conditions	PS II activity	
	μmol $O_2 \cdot min^{-2}$ $\cdot$mg protein^{-1}	μmol $O_2 \cdot min^{-1}$ $\cdot$mg chl^{-1}
IML cycles 12	0.01	0.09
IML cycles 24	0.13	0.55
IML cycles 36	0.26	0.65
IML cycles 48	0.34	0.82
12 IML cycles		
+ 1/2 h light	0	0
+ 3 h light	0	0
+ 6 h light	0.23	0.25
+ 10 h light	0.49	0.43
+ 16 h light	0.39	0.31
Control:		
continuous light	0.64	0.50

Intact plastids were prepared from plants after various stages of illumination then prothylakoids were isolated from osmotic lysates and assayed for oxygen evolution activity

tion during greening has been further examined where early stage (12 cycle) IML plants were exposed to continuous illumination. As shown in fig.2 the 33- and 23-kDa proteins were again prominent in the early stages of greening and accumulated further under continuous light. By contrast, the amount of the 16-kDa protein was initially very low but increased most dramatically between 3 and 6 h of illumination (lane 2 *vs* 3). Again, this increase coincided with the onset of oxygen evolution activity, which also begins to develop between 3 and 6 h of illumination (table 1).

4. DISCUSSION

Our findings demonstrate a close correlation between the accumulation of the 16-kDa protein during greening and the onset of PS II oxygen evolution. In contrast with the 33- and 23-kDa polypeptides, which are present at high levels in etioplasts, the 16-kDa species is initially present only in trace amounts, either under continuous or intermittent light conditions, but accumulates quite strongly during development of the photosynthetic activity. These findings are thus strong evidence for an essential role of this protein in the oxygen-evolving complex. The nature of this role still remains unclear, however, and further progress will probably depend on clarification of the so far conflicting results from in vitro reconstitution experiments (see section 1). What is presently certain is that from the developmental standpoint, all 3 polypeptides are synthesized and inserted in barley prothylakoids before oxygen evolution activity becomes measurable.

The detection of the 33-, 23- and 16-kDa polypeptides, and previous reports of the presence of cytochromes *f*, *b*-559$_{LP}$ and *b*-563, and the CF_1-ATPase complex [18], show clearly that some components of the thylakoid photochemical apparatus are present in etioplasts and thus do not require light for their biogenesis. Alternatively, many polypeptides are completely missing at this stage and may eventually be assembled into the multi-subunit, supramolecular membrane complexes in an asynchronous fashion, as is the case for PS I [15]. From the present results, and other developmental studies [16,17], this also seems to be true of the PS II oxygen-evolving complex. As a means of studying the overall assembly of the PS II complex, it will clearly be interesting to correlate the present findings with similar studies on the formation of the reaction center core, and light-harvesting, polypeptides during greening.

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