Modification of the chloride requirement for photosynthetic O_2 evolution

The role of the 23 kDa polypeptide

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In thylakoid preparations from spinach and the halophyte *Avicennia marina* a correlation is observed between functional O_2 evolution at low chloride concentrations and the presence of the 23 kDa protein. Addition of spinach 23 kDa protein to polypeptide-depleted halophyte inside-out thylakoid vesicles lowers their chloride requirement for optimal O_2 evolution activity from 250 to 5 mM. It is suggested that the specific role for the 23 kDa protein is to increase the affinity of the water oxidation site for chloride.

Photosynthetic oxygen evolution *Chloride requirement* *23 kDa protein* *Western blotting* *Inverted thylakoid vesicle* *Phase partitioning*

1. INTRODUCTION

The mechanism of photosynthetic O_2 evolution, arguably one of the most important biological processes, has remained elusive despite considerable research effort. Manganese [1] is known to be essential, and there is now evidence that 2–4 manganese atoms per O_2-evolving site are required [2]. Chloride is also essential [3] but the amounts necessary appear to be variable, and optimal concentrations in vitro range from 10 to 20 mM [4] in salt-sensitive species up to 100–500 mM [5–7] in salt-tolerant species. There is no general agreement concerning the amount of chloride associated with each O_2-evolving complex, but recent binding studies have suggested 5 or 37 Cl^- per centre [8,9].

Recently, 3 polypeptides of 33, 23 and 16 kDa, located at the inner thylakoid surface, have been shown to be associated with the O_2-evolving complex [10,11]. When isolated under oxidising conditions, the 33 kDa protein contains manganese and is therefore a possible candidate for the assumed mangano-protein of O_2 evolution [12]. No prosthetic groups have been found for the 23 or 16 kDa protein [10] and their precise role in the water oxidation process is so far unclear.

We present here evidence that the 23 kDa protein modulates the chloride requirement for maximal O_2 evolution. By using sensitive immunological techniques for polypeptide detection we show that the presence of this protein in normal and inverted thylakoids of mangrove and spinach was essential for O_2 evolution at low chloride concentration.

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Abbreviations: AMPD, 2-amino-2-methyl-1,3-propanediol; LiDS–PAGE, lithium dodecyl sulphate-polyacrylamide gel electrophoresis; FeCN, ferricyanide; PBQ, phenyl-*p*-benzoquinone; Chl, chlorophyll

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2. MATERIALS AND METHODS

Spinach (*Spinacia oleracea*) was grown hydroponically. Mangrove leaves (*Avicennia marina*) were collected from a mangrove swamp in Cullendulla Creek, Bateman's Bay, Australia.

Spinach and mangrove thylakoids were prepared as in [6,13]. Intact mangrove chloroplasts were isolated in essentially the same way as thylakoids except that 1 M sorbitol was present throughout the preparation procedure (M.C. Ball, personal communication). Additionally, mangrove thylakoids were obtained from these intact chloroplast preparations by brief osmotic shock in 50 mM Hepes–AMPD buffer (pH 7.3). Spinach and mangrove inside-out thylakoid vesicles were prepared by phase partitioning using a recently developed high-yield method [13].

The 33, 23 and 16 kDa proteins were purified by ion-exchange chromatography of a crude spinach chloroplast protein extract [14,15]. The proteins were desalted by ultrafiltration and dissolved in distilled water. Antisera monospecific for each of these proteins were obtained by standard immunological techniques after injection into rabbits. LiDS–PAGE was carried out using 10–22% polyacrylamide gradient slab gels [16]. Western blotting was performed as in [17] using a mixture of the 3 antisera at 1/800 dilutions.

O_2-evolution activity was measured in an O_2 electrode, illuminated with saturating red light, using 25 mM Hepes–AMPD buffer (pH 6.8) and 1 mM FeCN plus 0.2 mM PBQ as electron acceptors. Chlorophyll and NaCl concentrations and pH are given in the figure legends. Chlorophyll was determined as in [18].

3. RESULTS

Thylakoid membranes of *A. marina* have been shown to be salt-tolerant so that maximal rates of O_2 evolution are obtained at 250–500 mM NaCl (table 1), but this activity is inhibited at low chloride concentrations [5–7]. By contrast, spinach thylakoids show high rates of O_2 evolution at low, and marked inhibition at high, chloride concentrations (table 1) [6]. In case these differences derived from an altered polypeptide structure of the O_2 evolving complex, we compared the polypeptide composition of mangrove and spinach

Table 1

Oxygen-evolution activity of mangrove and spinach thylakoids (μmol O_2 evolved·mg $Chl^{-1}\cdot h^{-1}$)

Assay medium (25 mM Hepes–AMPD, pH 6.8)	Spinach	Mangrove	Mangrove[a]
No added NaCl	230	0–41	225
Plus optimal NaCl	275	174	211
Optimal NaCl (mM)	10–25	300–500	25–500

[a] Thylakoids 'gently' prepared from intact chloroplasts in 1 M sorbitol media and assayed in the presence of 1 M sorbitol

thylakoid preparations. To determine unequivocally the presence of the 33, 23 and 16 kDa polypeptides we employed the sensitive technique of Western blotting immunoelectrophoresis. As can be seen from fig.1, lane 1, spinach thylakoids contain the 33, 23 and 16 kDa polypeptides. Mangrove thylakoid preparations, however, although containing the 33 kDa protein, are completely deficient or very depleted of the equivalent 23 and 16 kDa polypeptides compared with spinach thylakoids as judged by Western blotting (fig.1, lane 3) and stained LiDS–PAGE (not shown). On the other hand, *A. marina* thylakoids prepared from intact chloroplasts and kept at 1 M sorbitol possess all 3 polypeptides (fig.1, lane 4). (Note that the apparent molecular mass values of the 33 and 23 kDa species are altered to 34 and 22 kDa, respectively, in mangroves.) These gently prepared thylakoids remain tolerant to high NaCl concentrations (100–500 mM), but notably, also show comparatively high rates at low chloride (table 1, M.C. Ball, personal communication). Thus, in the halophytes there is a correlation between the presence of the 23 and/or 16 kDa proteins and a low chloride requirement for O_2-evolution activity.

To study this correlation further we compared the properties of inverted vesicles prepared from both mangrove and spinach thylakoids. In such inverted thylakoid membrane vesicles the O_2-evolving complex is exposed to the external medium and becomes accessible to manipulation so that individual polypeptides can be removed or added [10]. In spinach the 33, 23 and 16 kDa

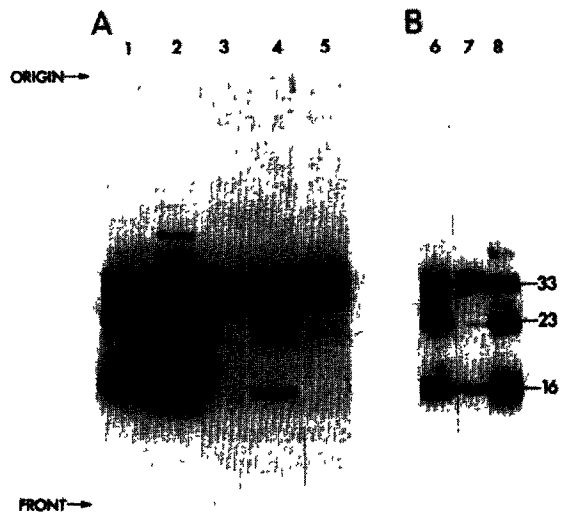

Fig.1. Detection by Western blotting immunoelectrophoresis of the 33, 23 and 16 kDa polypeptides of Photosystem II in thylakoid membranes from spinach and mangroves. Each lane contained sample equivalent to 10 μg Chl. A and B are autoradiographs from separate experiments. Lane 1, spinach thylakoids; lane 2, inverted spinach thylakoid vesicles; lane 3, mangrove thylakoids; lane 4, mangrove thylakoids prepared in the presence of 1 M sorbitol; lane 5, inverted mangrove thylakoid vesicles; lane 6, spinach thylakoids; lane 7, salt-washed inverted spinach thylakoid vesicles; lane 8, inverted mangrove thylakoid vesicles isolated after reconstitution with the 23 and 16 kDa proteins from spinach.

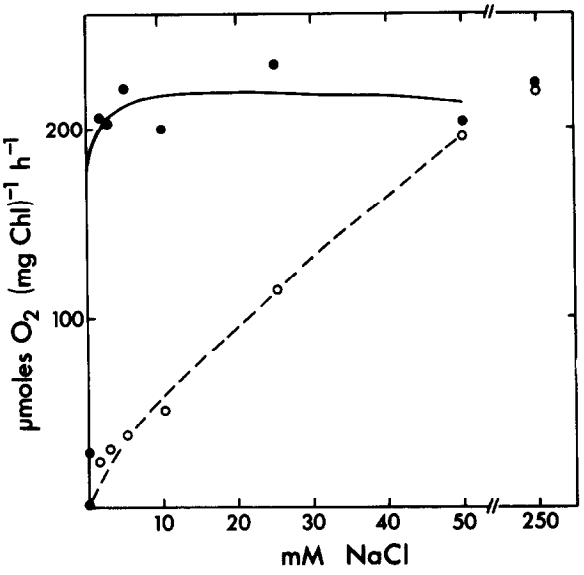

Fig.2. O_2-evolution activity as a function of NaCl concentration in control (o---o) and reconstituted (●——●) inside-out vesicle preparations from mangroves; 7.5 μg Chl/ml; added 23 kDa polypeptide fraction was 0.25 mg protein/mg Chl. Assay buffer (2 ml final volume) was 25 mM Hepes–AMPD (pH 6.8) with FeCN plus PBQ as electron acceptors.

polypeptides are still present in highly purified inverted vesicles (fig.1, lane 2). These inverted vesicles show considerable O_2-evolution activities even in the absence of added NaCl (see also fig.3) and become inactivated at NaCl concentrations above 100 mM (not shown). By contrast, inverted vesicles from the salt-tolerant mangrove lack or are very depleted of the 16 and 23 kDa polypeptides, but retain the 33 kDa protein (fig.1, lane 5). These vesicles had very low O_2-evolution activities at low NaCl concentrations and maximal rates were reached only above 50 mM NaCl (fig.2). Addition of the purified 23 kDa polypeptide from spinach to the polypeptide-deficient inside-out membrane vesicles from the halophyte caused a dramatic decrease in the chloride requirement (fig.2), such that maximal activity was obtained at 5 mM NaCl and substantial rates were measurable at only 1 mM NaCl. It should be emphasized that addition of the 23 kDa protein alone was sufficient to lower the chloride requirement to this extent, whereas neither the 16 kDa polypeptide by itself nor a heated aliquot of the 23 kDa protein had any effect. Addition of both polypeptides together gave the same results as addition of the 23 kDa protein alone. Chloride specificity was demonstrated by assay in the presence of equivalent concentrations of Na_2SO_4, which failed to cause the increase of activity observed with the chloride salt (not shown). Western blots of the reconstituted vesicles showed clearly that the spinach polypeptides had indeed bound to the mangrove membranes (fig.1, lane 8 *vs* 5).

Salt washing of the spinach vesicles inhibited O_2-evolution activity considerably as shown in [10] and largely removed the 23 and 16 kDa polypeptides (fig.1, lane 7). This inhibition was most evident when O_2-evolution activity was assayed at low chloride concentrations (fig.3, inset). This means that the chloride requirement for maximal O_2-evolution activity after removal of the polypeptides was increased significantly from 10 to 50 mM (fig.3).

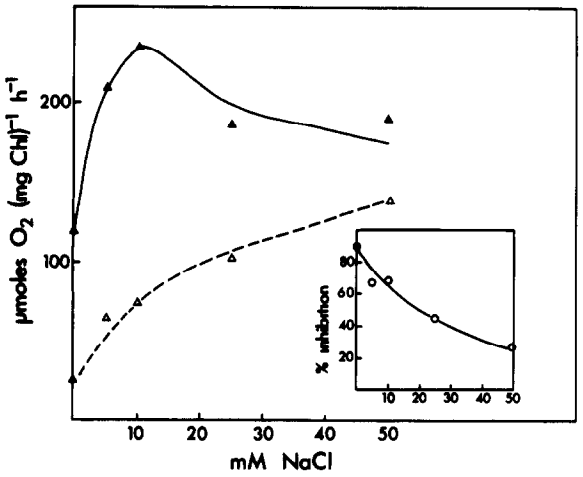

Fig.3. Dependence of O_2-evolution activity of inverted spinach thylakoid membranes on NaCl concentration in the assay medium. (▲——▲) Controls, (△---△) salt-washed (23 and 16 kDa polypeptide-depleted). Inset: % inhibition of O_2-evolving activity in salt-washed as compared to control vesicles as a function of NaCl concentration during the assay. Assay conditions as in fig.2.

4. DISCUSSION

Inhibition and reconstitution experiments on inside-out vesicles and O_2-evolving particles have previously shown that the 23 kDa protein has a function in photosynthetic O_2 evolution [10,11,19,20]. Our experiments show that:

(i) Both high chloride-requiring thylakoids and inverted thylakoid membrane vesicles from a salt-tolerant species lose the 23 and 16 kDa polypeptides during preparation;

(ii) Reconstitution of these vesicles with purified 23 kDa protein derived from spinach membranes lowers their chloride requirement for maximal O_2-evolution activity drastically, i.e., from 250 to 5 mM;

(iii) Gently prepared mangrove thylakoids show high O_2-evolving activity at low chloride concentrations and contain both (23 and 16 kDa) polypeptides;

(iv) Inverted spinach thylakoid vesicles depleted of these two polypeptides require high chloride concentrations for maximal O_2 evolution activity.

These results clearly establish a role for the 23 kDa protein of Photosystem II in modifying the chloride requirement of O_2 evolution. They also explain why this protein can participate in the water oxidation reaction despite its apparent lack of electron transporting prosthetic groups. We found no evidence for the involvement of the 16 kDa protein in chloride binding and its precise role in O_2 evolution remains to be elucidated.

The specific mechanism for this modification of chloride requirement by the 23 kDa protein is not yet clear. This protein may itself be a high-affinity chloride-binding polypeptide since it contains 12 mol% of the cationic amino acids, lysine and arginine [14] and thus could assist in chloride binding through Coulombic interactions. Alternatively, the presence of the 23 kDa protein in the oxygen-evolving complex may increase the affinity for chloride of the water oxidation site itself. In both cases this protein would have to be in close proximity to the manganese centre and, by inference, the water oxidation site. A close structural association between the 23 kDa and the 33 kDa protein (currently the most likely candidate to carry manganese [11,12]) has been shown [21].

Our results show clearly that the 23 kDa protein is obligatory for oxygen evolution at low but not high chloride concentrations. This may explain conflicting evidence concerning the importance of this protein, which has variously been suggested to have an obligatory [21,22] or supplementary role [11] in O_2 evolution. When the 23 kDa polypeptide is absent, very high chloride concentrations are required for optimal activity showing that the actual chloride binding at the water oxidation site is of low affinity.

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