The release of a 10-kDa polypeptide from everted photosystem II thylakoid membranes by alkaline Tris

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The release of polypeptides from inside-out thylakoid vesicles and photosystem II by alkaline Tris treatment was reinvestigated, using SDS–polyacrylamide gel electrophoresis (PAGE) in the presence of urea, with highly increased resolution in the low molecular mass region. In addition to the 33-, 23-, and 16-kDa proteins of the oxygen-evolving complex, a 10-kDa polypeptide was released. This 10-kDa polypeptide is an entrinsic polypeptide located at the inner grana thylakoid surface and with a likely role in photosynthetic oxygen evolution.

Thylakoid polypeptide *Water oxidation* *Photosystem II* *10-kDa polypeptide* *Tris washing* *Oxygen-evolving complex*

1. INTRODUCTION

Studies concerning the composition of the photosynthetic oxygen-evolving complex have lately been focused on the 33-, 23-, and 16-kDa polypeptides (cf. [1]). Removal of these polypeptides from photosystem II preparations, exposing the inner thylakoid membrane surface, leads to inactivation of oxygen-evolving activity (cf. [1]). Partial restoration of the lost activity in the treated photosystem II preparations has been obtained with each of the 3 proteins [2–5]. At present, most data suggest that these proteins are involved in mediating the calcium and/or chloride necessary for optimal oxygen evolution [6–10]. The 33-kDa protein has also been proposed to be associated with manganese [11]. Thus, the 33-, 23-, and 16-kDa proteins appear as extrinsic membrane proteins located at the inner thylakoid surface with a likely function in photosynthetic oxygen evolution.

Abbreviations: MES, 2-(*N*-morpholino)ethanesulphonic acid; PMSF, phenylmethylsulfonylfluoride

Here, we present evidence for a fourth extrinsic photosystem II polypeptide located at the inner thylakoid surface, and with a possible involvement in oxygen evolution. This polypeptide, with an apparent molecular mass of 10 kDa, is enriched in the appressed thylakoid region and released from everted photosystem II membranes by alkaline Tris washing, but not with high concentrations of NaCl or $CaCl_2$.

2. MATERIALS AND METHODS

Thylakoid membranes were prepared from spinach. Inside-out vesicles were prepared by phase partition, and stroma lamellae vesicles by differential centrifugation [12]. Photosystem II particles were prepared according to the detergent method of [13], modified as in [14]. To remove excess detergent the photosystem II particles were washed in 40 mM MES (pH 6.5), 5 mM $MgCl_2$, 600 mM sucrose after the detergent extraction, prior to the salt and Tris washings.

Washings of the various thylakoid preparations were performed at 500 μg Chl/ml for 30 min, at 4°C in room light. The media used were (a) 1 M

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NaCl–10 mM MES, pH 6.5; (b) 1 M $CaCl_2$–10 mM MES, pH 6.5; or (c) 0.8 M Tris–HCl, pH 8.4.

SDS–urea PAGE was run in the buffer system of [15], with a 12–22.5% polyacrylamide gradient and 4 M urea. The gels were stained in Coomassie brilliant blue R-250. The polypeptides were quantified using an LKB laser gel scanner. Western blotting was performed as in [16] using peroxidase-linked secondary antibodies and 4-chloro-1-naphthol as substrate.

Mn was determined by atomic absorption using graphite oven technique.

3. RESULTS AND DISCUSSION

By alkaline Tris washing of inside-out vesicles and photosystem II particles the 33-, 23- and 16-kDa proteins are released from the inner thylakoid surface (cf. [1]). This is also shown by SDS–urea PAGE in fig.1, lanes 4 and 5, whereas the polypeptide pattern of Tris-washed inside-out vesicles is compared with untreated vesicles. By inclusion of urea in the gel the resolution of polypeptides in the low molecular mass region is greatly enhanced. This increased resolution revealed that a 10-kDa polypeptide was released by Tris washing from the inside-out vesicles in addition to the other 3 polypeptides (fig.1, lanes 4 and 5). Quantification of the 10-kDa polypeptide revealed that approximately 80% of this polypeptide was released. The same treatment of intact thylakoids (fig.1, lanes 1 and 2) and right-side out vesicles (not shown) caused no release of the 10-kDa polypeptide, indicating that it is located at the inner thylakoid surface. The 10-kDa polypeptide was enriched in the inside-out thylakoid vesicles, which are derived from the photosystem II-rich appressed regions, but practically absent in the photosystem I-rich stroma lamellae vesicles (fig.1, lanes 3 and 4). This localizes the 10-kDa polypeptide to the appressed thylakoid region, which in turn suggests a function in photosystem II. It should be noted that the stroma lamellae vesicles contain a polypeptide at 10.5 kDa with a migration clearly distinct from the 10-kDa polypeptide and with a marked depletion in the inside-out vesicles.

The release of the extrinsic 33-, 23- and 16-kDa proteins has not only been reported from inside-out thylakoid vesicles but also from photosystem II preparations isolated by detergent fractionations

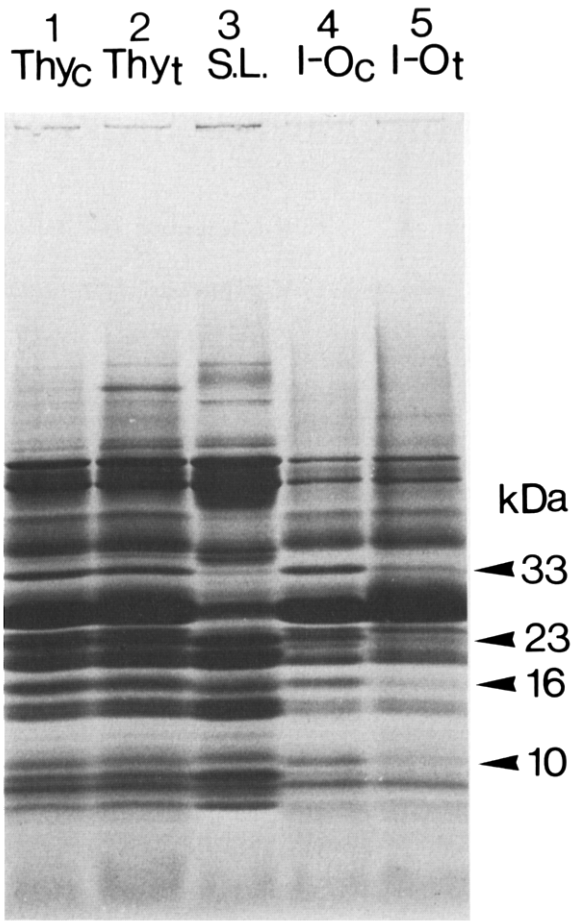

Fig.1. SDS–urea PAGE of: (1) control thylakoids (Thy_c), (2) Tris-washed thylakoids (Thy_t), (3) stroma lamellae (S.L.), (4) inside-out vesicles ($I\text{-}O_c$), (5) Tris-washed inside-out vesicles ($I\text{-}O_t$).

(cf. [1]). Therefore, the release of the 10-kDa polypeptide in such a photosystem II preparation [13,14] was examined. As can be seen in fig.2, lanes 1 and 4, Tris washing of these particles released the 10-kDa polypeptide to a large extent (60%). In contrast, washings with 1 M NaCl or $CaCl_2$ were ineffective (fig.2, lanes 2 and 3). In this respect, the 10-kDa polypeptide differs from the 33-kDa protein, which is released by $CaCl_2$, and the 23- and 16-kDa proteins, that are released by both $CaCl_2$ and NaCl (fig.2, lanes 2 and 3) [1,5]. As judged from the gel electrophoresis no wash treatment removed all 10-kDa polypeptide from the photosystem II membranes (figs 1 and 2). Whether this means that the polypeptide is quite

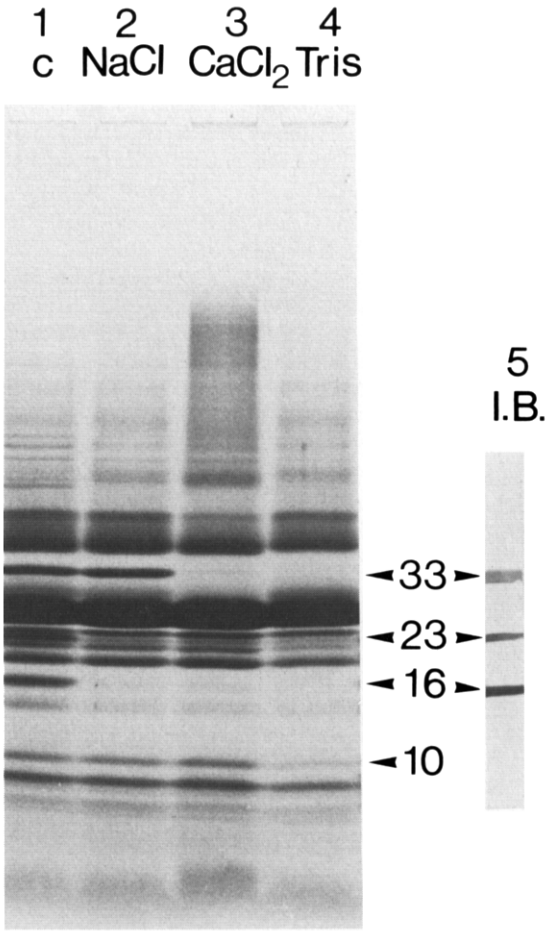

Fig.2. SDS–urea PAGE of photosystem II particles: (1) control, and washed in (2) 1 M NaCl, (3) 1 M CaCl, (4) 0.8 M Tris–HCl, pH 8.4. (5) shows an immunoblotting of the photosystem II particles against a mixture of antisera against the 16-, 23- and 33-kDa proteins.

firmly bound to the membrane or if there is a comigrating polypeptide has to be clarified by immunological quantification.

The possibility that the 10-kDa polypeptide is a degradation product from any of the 33-, 23- and 16-kDa proteins was tested by Western blotting, using antibodies against each of the 3 proteins. As shown in fig.2, lane 5, none of these antisera reacted with the 10-kDa polypeptide. Furthermore, in some experiments the protease inhibitor PMSF (2 mM) was included in the wash media without any changes in the release patterns. Coelectrophoresis of the photosystem II particles with pure cytochrome *b*-559 revealed that the 10-kDa polypeptide is not identical to this cytochrome (not shown).

Previous studies (cf. [1]) and the data of table 1 show that manganese is released from the photosystem II particles by Tris, but not by $CaCl_2$ [5] or NaCl. Thus, compared to the 33-, 23- and 16-kDa proteins, the release of the 10-kDa polypeptide corresponds better to the release of manganese. This could be demonstrated by washing the photosystem II particles with 1 M $CaCl_2$ prior to the Tris washing. In that way the 33-, 23- and 16-kDa proteins were first released while the 10-kDa polypeptide and manganese remained on the membrane, to be released by the subsequent Tris wash (fig.2 and table 1). In this context it is of interest to note that a low molecular mass mangano protein has been isolated from a blue-green algae [17]. There are several other indications in the literature of a small molecular mass polypeptide involved in photosynthetic oxygen evolution. In an early mutant study on *Chlamydomonas* it was shown that a mutant devoid of oxygen-evolving capacity lacked 3 extrinsic proteins of 21-, 18-, and 6-kDa [18]. Authors in [3] showed that the restoration of oxygen evolution with their 24- and 17-kDa proteins in cholate–NaCl-extracted thylakoids could be enhanced by an unknown component of less than 15 kDa. Inactivation of oxygen evolution by treatment of thylakoids with lauroyl choline chloride released among several polypeptides one at 10 kDa

Table 1

The amount of 10-kDa polypeptide and Mn remaining on the photosystem II particles after various treatments

Photosystem II particles	Amount of 10-kDa polypeptide (%)	Amount of Mn (%)
Control	100	100[a]
1.0 M NaCl-washed	100	100
1.0 M $CaCl_2$-washed	98	101
1.0 M $CaCl_2$ and 0.8 M Tris-washed (subsequent washing)	56	9
0.8 M Tris	40	3

[a] 100% = 21 Mn/1000 Chl

[19]. In a recent immunoprecipitation study [20] it was shown that the 10-kDa polypeptide, in addition to a 22- and a 24-kDa polypeptide, is structurally in close association with the 23- and 33-kDa proteins of the oxygen-evolving complex.

In conclusion, we regard the present 10-kDa polypeptide as an extrinsic photosystem II polypeptide which, in addition to the 33-, 23- and 16-kDa proteins, is located at the inner grana thylakoid surface and with a likely role in photosynthetic oxygen evolution.

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