Purification and molecular properties of 3 polypeptides released from a highly active O_2-evolving photosystem-II preparation by Tris-treatment

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Tris-treatment of a highly active O_2-evolving photosystem-II preparation induced release of 3 polypeptides (M_r 33000, 24000 and 18000), concomitant with inhibition of O_2 evolution [FEBS Lett. (1981) 133, 265–268]. The 3 polypeptides were purified with the use of electrofocusing. Isoelectric points of the proteins were 5.1, 6.5 and 9.2 in order of decreasing M_r value. Only a trace amount of histidine, cystein and methionine were detected in these proteins. Based on the amino acid compositions, polarity indexes of the proteins were calculated to be 47–49%, suggesting the 3 proteins to be hydrophilic.

O_2 evolution *Membrane protein* *Photosystem II* *Chloroplast* *Photosynthesis*

1. INTRODUCTION

In spite of efforts to unveil structure of the enzyme complex functioning in the water-splitting process of photosynthesis, little is known about the protein components of the complex. Recent advance in the technique of isolating highly active O_2-evolving PS-II preparations from blue-green algae [1] and spinach chloroplasts [2–5] facilitated the study of the functional and structural aspects of the O_2-evolution enzyme complex. Tris-treatment (0.8 M, pH 8.4) of the O_2-evolving PS-II subchloroplasts from spinach, induced an inhibition of O_2-evolution and a concomitant release of 3 polypeptides (M_r 33000 24000 and 18000) [2]. Polypeptides with almost the same M_r as above were released from inside-out thylakoid membranes obtained by two-phase partition, upon washing them by alkaline Tris [6] or by a high [NaCl] solution [7]. Rebinding of a M_r 23000 polypeptide to the NaCl-treated inside-out membrane vesicles resulted in a successful reconstitution of O_2-evolution activity [8]. These results suggest that all or some of the 3 polypeptides are involved in the water-splitting process, probably as constituents of the O_2-evolution enzyme complex.

Here, we have purified these 3 polypeptides and determined some of their molecular properties.

Abbreviations: PS, photosystem; Tris, tris(hydroxymethyl)aminomethane; Mes, 2-(*N*-morpholino)-ethanesulphonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrohporesis; A_{280}, absorbance at 280 nm; pI, isoelectric point; TMBZ, 3,3′,5,5′-tetramethylbenzidine; CF_1, chloroplast coupling factor 1; atrazine, 2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine

2. MATERIALS AND METHODS

2.1. *Preparation of a highly active O_2-evolving PS-II subchloroplasts*

A highly active O_2-evolving PS-II preparation was obtained from spinach as in [3], with some modifications: instead of phosphate, 10 mM Mes (pH 6.2) was used in the buffer medium, and *p*-benzoquinone was replaced by a combination of 0.2 mM phenyl-*p*-benzoquinone and 1 mM potassium ferricyanide as in the PS-II electron acceptor system. O_2-evolution activity of the PS-II prepara-

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tion thus obtained, was usually 150–400 μmol O_2.mg chl^{-1}.h^{-1}.

2.2. *Tris-treatment*

The PS-II preparation (0.1 mg chl/ml) was treated with 0.8 M Tris–HCl (pH 8.4) for 10 min at 4°C. The suspension was centrifuged at 30000 × *g* for 1 h and the supernatant was separated from the pellet. After a dialysis overnight against 10 mM Mes (pH 6.2), the supernatant was concentrated with the use of an Amicon ultrafiltration cell, model 52.

2.3. *Electrofocusing*

Electrofocusing was carried out at 4°C in an Ampholine column (110 ml), connected to a thermostated water bath circulator. Samples were introduced as a narrow zone at the center of the column which contained 1% Ampholine (pH 3.5–10) in sucrose with a density gradient of 0–50%). Constant voltage of 500 V was applied to the column for 48 h.

2.4. *SDS–polyacrylamide gel electrophoresis*

Samples were solubilized in a solution containing 65 mM Tris–HCl (pH 6.8), 10% sucrose, 1% β-mercaptoethanol, 1% SDS and 1‰ Coomassie blue R-250 for 30 min at room temperature. Gel electrophoresis was carried out in the discontinuous buffer system as in [9]. Either 12.5% acrylamide or 15–7.5% gradient acrylamide was used as the resolving gel, and 5% acrylamide as the stacking gel. Where indicated, 0.5 M urea was included both in the solubilizing medium and in the acrylamide gel. M_r was determined with the following M_r standard: phosphorylase *b* (94000), albu-

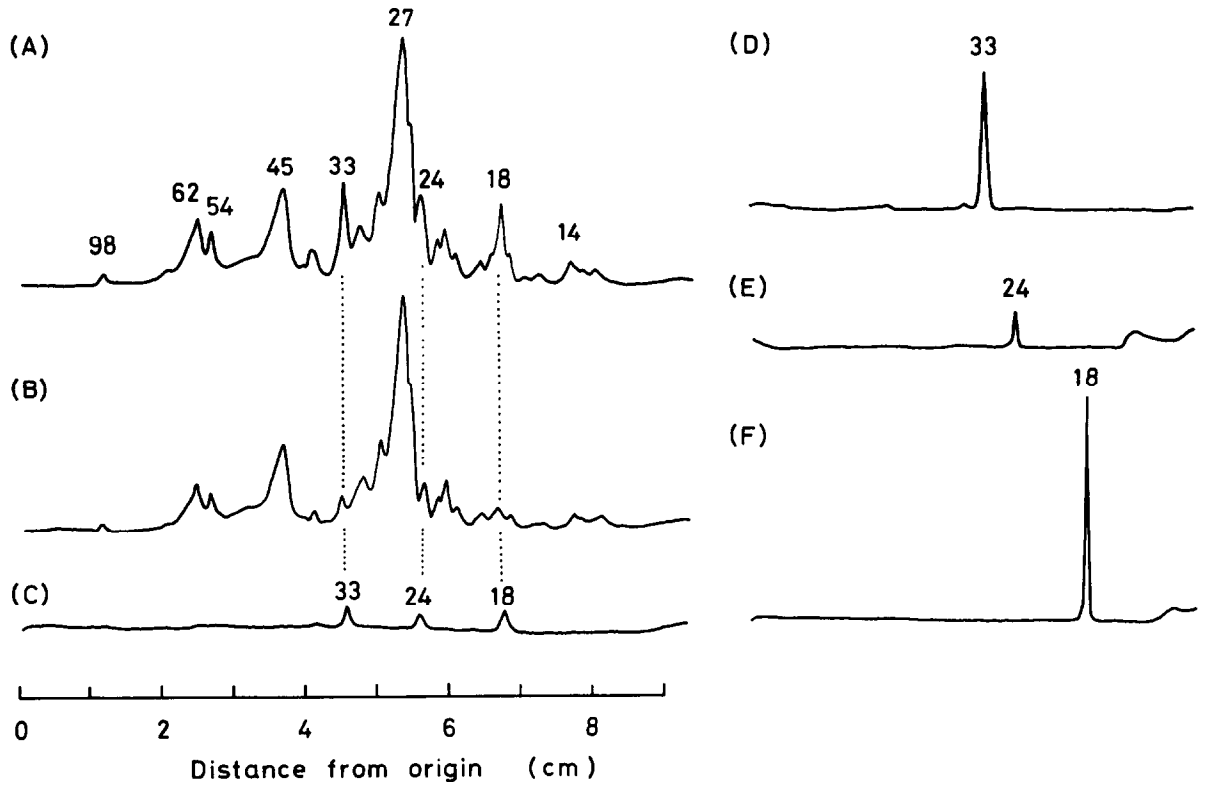

Fig. 1. Densitograms of SDS–polyarcrylamide slab gel showing Coomassie blue-stained polypeptides in the purification steps of the proteins I, II and III: (A) O_2-evolving PS-II preparation; (B) PS-II preparation washed by 0.8 M Tris (pH 8.4); (C) supernatant fraction obtained by centrifugation of 30000 × *g* for 1 h after Tris-treatment of the PS-II preparation; (D) protein I; (E) protein II; (F) protein III. SDS–PAGE was carried out with the use of 15–7.5% gradient acrylamide as the resolving gel. Small absorbance near the front of the gel in (E) and (F) was due to Ampholine.

min (67000), ovalbumin (43000), carbonic anhydrase (30000), trypsin inhibitor (20000) and α-lactalbumin (14400). After the electrophoresis for 5 h at constant power of 4 W, the gel was stained by Coomassie blue R-250 and analyzed at 565 nm by a Toyo digital densitometer DMU-33c. Heme-staining was done after SDS–PAGE as in [10].

2.5. *Amino acid analysis*

The proteins isolated by electrofocusing were dialyzed against distilled water for 5 days to remove Ampholine and sucrose. After liophilization, the samples were hydrolyzed in 5.7 N HCl at 110°C for 24 h. A Hitachi amino acid analyzer, model 835, was used for the amino acid analysis. Tryptophan content was determined spectrophotometrically as in [11]. Polarity indexes of the proteins were calculated as in [12].

3. RESULTS

Three polypeptides were released from the O_2-evolving PS-II preparation by 0.8 M Tris-treatment (fig. 1A–C). These 3 polypeptides were subsequently purified by electrofocusing. A typical profile of the polypeptide fractions after electrofocusing showed 3 peaks of A_{280}, at pH 4.8, 6.5 and 9.2 (fig. 2). By subjecting each fraction to SDS–PAGE, proteins responsible for the A_{280} were identified (fig. 2, inset). In the fraction with pI 4.8, a small amount of chlorophyll–protein existed, which could not be precipitated by centrifugation of 30000 × *g* for 1 h after the Tris-treatment. A M_r 33000-protein was detected in a fraction with pI 5.1 and could be isolated without contamination of the chlorophyll-containing component. A M_r 24000, and a M_r 18000 protein were found in fractions corresponding to pI 6.5 and 9.2, respectively (hereafter the 3 proteins with pI 5.1, 6.5

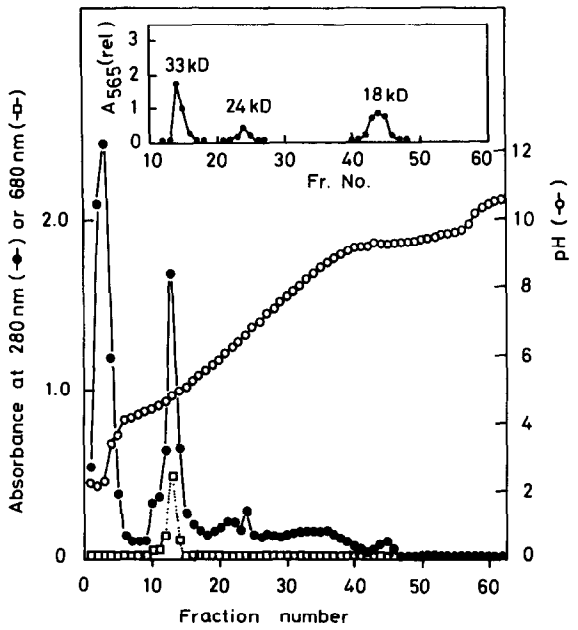

Fig. 2. A typical profile of polypeptide fractions after electrofocusing of the supernatant of Tris-washed PS-II preparation. pH of each fraction was measured at 4°C (○) pH; (●) A_{280}; (□) A_{280}; inset shows A_{565} of Coomassie blue-stained polypeptides after SDS–PAGE of the fractions.

Table 1

Molecular properties of proteins I, II and III

	Protein I	Protein II	Protein III
M_r ($\times 10^{-3}$)[a]			
− urea	32.8 ± 0.2	24.2 ± 0.2	17.8 ± 0.2
+ urea (0.5 M)	32.6 ± 0.5	24.1 ± 0.2	17.7 ± 0.2
Isoelectric point[b]	5.06 ± 0.05	6.52 ± 0.07	9.15 ± 0.13
Absorption max. in the UV region	275 nm	264 nm	266 nm
Heme-staining	not stained	not stained	not stained

[a] Determined in 12.5% gel in the presence and absence of 0.5 M urea; each datum is the average of 5 measurements ± SD

[b] Average of 5 expt ± SD

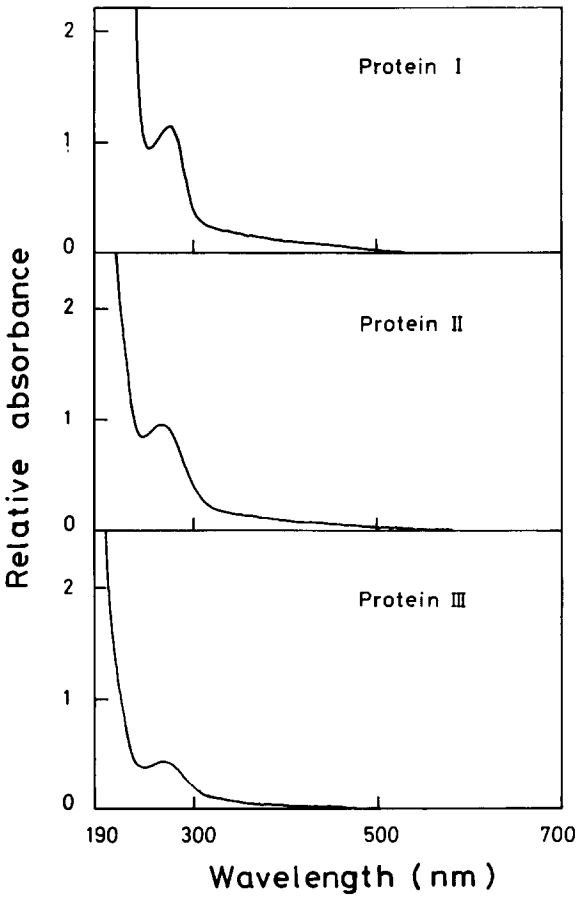

Fig. 3. Absorption spectra of proteins I, II and III. Each protein separated by electrofocusing was dialyzed against distilled water for 5 days to remove Ampholine and sucrose, and then lyophilized. The proteins were dissolved in 0.02 M phosphate buffer (pH 6.5) at appropriate concentrations.

and 9.2 are referred to as protein I, II and III). Judging from the densitogram of SDS–PAGE, each protein was purified to a reasonable degree (fig. 1D–F). M_r or the each protein was ascertained in the presence of 0.5 M urea in SDS–PAGE where resolution of the lower M_r region should be improved (table 1). The 3 proteins showed no absorption in the visible region (fig. 3). Not being stained by TMBZ after SDS–PAGE, the 3 proteins do not contain heme *c* (table 1).

Polarity indexes of the proteins calculated from their amino acid composition suggest their hydrophilic nature (table 2). It should also be mentioned

Table 2

Amino acid compositions and polarity indexes of proteins I, II and III (mol%)

Amino acid	Protein I	Protein II	Protein III
Lys	9.14	10.01	9.01
His	trace	trace	trace
Arg	2.75	8.39	4.85
Asp	9.25	9.41	9.75
Thr	6.60	4.84	4.90
Ser	7.35	7.19	8.73
Glu	11.64	8.70	10.78
Pro	7.12	8.30	8.99
Gly	10.65	9.30	6.56
Ala	6.14	7.08	8.69
Cys	trace	trace	trace
Val	6.92	6.62	4.28
Met	0.69	trace	trace
Ile	3.28	3.43	4.21
Leu	7.09	5.89	10.18
Tyr	3.32	2.35	2.85
Phe	4.89	4.89	2.18
Try	2.78	3.25	3.53
Polarity index	47%	49%	48%

that only a trace amount of histidine, cysteine and methionine was detected in these proteins.

4. DISCUSSION

Proteins I (M_r, 33000), II (24000) and III (18000) are associated with the O_2-evolving PS-II preparation and released from membrane by several treatments which inhibit O_2 evolution [2,5]. As reconstitution of O_2 evolution could be accomplished simply by adding these proteins to the depleted inside-out membrane [8], they are expected to be hydrophilic. The polarity indexes of the proteins purified here were shown to be >40%, thus they might be included in a group of water-soluble proteins such as CF_1 [13] and ferredoxin-NADP oxidoreductase [14]. Proteins I, II and III did not contain Mn, although >70% of Mn associated with the PS-II preparation was released, accompanied by the release of the 3 proteins upon Tris-treatment [15]. Probably, Tris-washing was too harsh for the O_2-evolution enzyme complex to retain Mn in its protein moiety. It is known that an

Mn–protein is stable only around pH 6.5 [16].

Protein I is quite similar in its molecular nature to the M_r 33000 protein obtained in [17]. They suggested that the M_r 33000 protein was not the atrazine herbicide-binding protein [18] which was reported to have M_r of 32000–34000 [19,20]. The 32000–34000 protein was characterized in [21] to be hydrophobic, based on the evidence that the protein could be extracted quantitatively from thylakoid by chloroform–methanol. In [22] however, the herbicide binding protein does not contain lysine, which is similar to the characteristics of our protein I. Recent results on the DNA and therefore the amino acid sequence of this protein, also showed that the protein did not contain lysine (submitted). Therefore, there is still a possibility that the two M_r 32000–34000 proteins are identical.

A polypeptide with M_r 32000–34000 was also reported to be lost in some algal and maize mutants which have deletions in PS-II [23,24]. Significant decrease in the amount of Mn in the mutant suggests that the protein is an Mn-carrying molecule [23]. Although the proteins I, II and III obtained here did not have any Mn in their purified form, the possibility that these proteins carry Mn in situ is not excluded. Further study is being undertaken to explore the specific role of these proteins in O_2 evolution.

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