The EMBO Journal vol.9 no.6 pp.1743–1748, 1990

Expression in *Escherichia coli* of the *psbO* gene encoding the 33 kd protein of the oxygen-evolving complex from spinach

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Communicated by H.Michel

The cDNA for the 33 kd protein from the oxygen-evolving complex of spinach together with the coding region for the hydrophobic C-terminal part of the transit sequence was cloned into the expression plasmid pDS12/33Ex. The 33 kd protein precursor was expressed in *Escherichia coli*, secreted into the periplasm and correctly processed to the mature 33 kd protein. Thus the hydrophobic domain of the transit sequence, preceded by a methionine and two lysine residues, can function as a bacterial signal peptide. The periplasmic proteins were released from the cells by osmotic shock and the expressed protein was purified by anion exchange chromatography. The protein was identified by SDS–PAGE and Western blotting. N-terminal sequence analysis showed that the cleavage of the signal peptide occurred at the correct position. The expressed protein could be rebound to $CaCl_2$-washed PSII particles and oxygen evolution was restored in equal amounts by the 33 kd protein from both *E.coli* and spinach.

Key words: expression in *E.coli*/oxygen evolution/photosystem II/33 kd protein/protein targeting

Introduction

In the thylakoid membranes of chloroplasts, photosynthetic oxygen evolution takes place on the lumenal side of photosystem II (PSII). A cluster of four manganese ions in PSII plays a central role in accumulating positive charges for the oxidation of water to yield molecular oxygen. Three extrinsic proteins with apparent molecular masses of 33, 23 and 16 kd have been shown to be closely involved in the process of oxygen evolution (for review see Murata and Miyao, 1985). All three proteins are located on the lumenal side of the thylakoid membrane (Åkerlund and Jansson, 1981). They can be removed by various treatments with concomitant loss of manganese and oxygen-evolving activity (Yamamoto *et al.*, 1981; Kuwabara and Murata, 1982). In contrast, NaCl-treatment releases the 23 and 16 kd proteins but not the 33 kd protein or manganese (Åkerlund *et al.*, 1982). $CaCl_2$ (Ono and Inoue, 1983) and urea/NaCl treatments (Miyao and Murata, 1983) release all three extrinsic proteins but manganese is retained in PSII.

By washing PSII particles with NaCl, oxygen evolution capability is partially lost. Most of it can be restored in the presence of nonphysiological concentrations of Ca^{2+} and Cl^- (Ghanotakis *et al.*, 1984a; Miyao and Murata, 1984), indicating that the 23 and 16 kd protein do not play a catalytic role. It has been concluded that the 23 kd protein creates a calcium binding site at the membrane (Ghanotakis *et al.*, 1984b).

A more dramatic effect is observed with $CaCl_2$-washed PSII particles. In the absence of calcium, the oxygen evolution activity is completely lost and two of the four manganese ions are released from the membrane (Ono and Inoue, 1984). The manganese cluster in these particles is only stable in the presence of Ca^{2+} and high concentrations of Cl^-. In contrast to the NaCl-washed particles, oxygen-evolving activity remains low. Most of it can be restored by rebinding the 33 kd protein (Kuwabara *et al.*, 1985; Ono and Inoue, 1986). These results indicate that one of the functions of the 33 kd protein is the stabilization of the manganese cluster. The existence of a manganese binding site on the 33 kd protein is a matter of debate. There are two reports of the isolation of the protein with bound manganese (Abramovicz and Dismukes, 1984; Yamamoto *et al.*, 1984). In contrast to these findings, electron paramagnetic resonance data indicate that the 33 kd protein can be replaced by Cl^- and is therefore not necessary for the catalytic process of water splitting (Miller *et al.*, 1987; Styring *et al.*, 1987). Also, the location of the protein binding site at the membrane is under discussion. One binding site seems to be located near the reaction center complex of $D_1/D_2/cytb_{559}$ (Gounaris *et al.*, 1988; Brody, 1988; Mei *et al.*, 1989) but other candidates are the chlorophyll *a* binding proteins CP 47 (Bricker *et al.*, 1988; Enami *et al.*, 1989) and CP 43 (Isogai *et al.*, 1985).

One method for the investigation of the role of the 33 kd protein in manganese binding and the determination of its binding site in PSII involves the substitution of amino acid residues in the 33 kd protein by site-directed mutagenesis of the cDNA and subsequent measurement of the binding of expressed protein to PSII. This would require an expression system for the 33 kd protein and its mutants. Here we report the over-expression of the 33 kd protein from spinach in *E.coli*, and its re-binding to PSII particles which have been depleted of the extrinsic proteins.

Furthermore, we wanted to investigate the relationship between the mechanism of translocation of proteins across the thylakoid membrane and the bacterial plasma membrane. Some proteins, such as the 33 kd protein are nuclear-encoded and synthesized in the cytoplasm as larger precursors (Dobberstein *et al.*, 1977). They carry N-terminal extensions called transit sequences which enable the proteins to cross three membranes, the two chloroplast membranes and the thylakoid membrane. During protein import, the transit sequence is removed in two steps (Smeekens *et al.*, 1986; Hagemann *et al.*, 1986). The first processing is done by a soluble stroma protease (Smith and Ellis, 1979; Robinson and Ellis, 1984). Processing to the mature form of the protein takes place after translocation across the thylakoid membrane by a membrane-bound protease (Hagemann *et al.*, 1986).

The C-terminal part of the transit sequence shows some

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Fig. 1. A: Transit sequence of the 33 kd protein. **B**: Signal peptide of the 33 kd protein expressed in *E.coli*. The hydrophobic domain is underlined; the recognition site of the signal peptidase of *E.coli* is underlined twice.

similarities with bacterial signal peptides which direct precursor proteins to the periplasm. The transit sequence of the 33 kd protein (Figure 1A) contains at the C-terminus 18 mostly hydrophobic amino acids. The last three (A-N-A) form a recognition site for the signal peptidase I from *Escherichia coli* (Perlman and Halvorson, 1983). Also, the positively charged amino acid (lysine) before the hydrophobic domain is found in many bacterial signal peptides. In this work we used the C-terminal part of the transit sequence for transport of the 33 kd protein across the plasma membrane of *E.coli*.

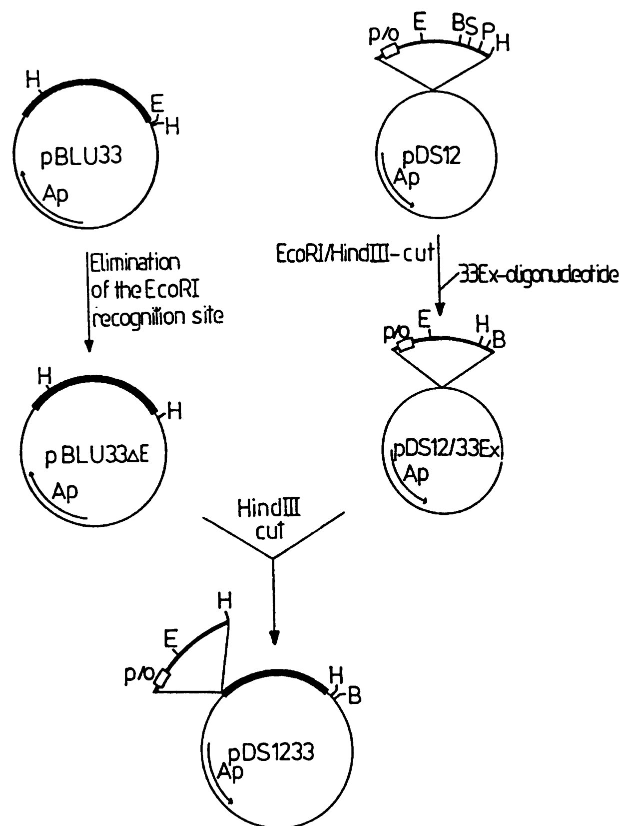

Fig. 2. Construction of the expression plasmid pDS1233. Abbreviations: E = *Eco*RI, H = *Hin*dIII, B = *Bam*HI, S = *Sal*I, P = *Pst*I, p/o = promoter/operator, Ap = ampicillin resistance gene.

Results

In order to express the 33 kd protein, the *Eco*RI–*Hin*dIII fragment of the expression plasmid pDS12/RBSII was replaced by a synthetic oligonucleotide, forming the plasmid pDS12/33Ex (Figure 2). The oligonucleotide carries codons for a methionine and two lysines 9 bp downstream from the Shine–Dalgarno sequence. A *Hin*dIII cleavage site was located within the second lysine codon whereas the original *Hin*dIII cleavage site of the plasmid was lost. After deletion of the *Eco*RI recognition site in the plasmid pBLU33, the 0.9 kb *Hin*dIII fragment from the plasmid pBLU33ΔE, carrying the coding region of the 33 kd protein and the hydrophobic domain from the transit sequence, was cloned into pDS12/33Ex forming the final expression plasmid pDS1233 (Figure 2). The resulting signal peptide is shown in Figure 1B.

pDS1233 was transformed into *E.coli* DH5α cells carrying the plasmid pDMI,1. Expression was induced by adding isopropyl-β-D-thiogalactoside (IPTG) to the cell culture. The expressed protein was detected by SDS–PAGE (Figure 3, lanes 9 and 10). The expressed protein represents ~7% of the total cell protein, as estimated by gel scans of Coomassie blue stained gels. From the total protein concentration of 1.3 mg/ml cell culture, a concentration of 90 μg/ml was calculated for the expressed protein.

In order to determine the subcellular localization of the expressed protein, periplasm, cytoplasm and membranes were isolated from the cells. The protein contents of these fractions were investigated by SDS–PAGE (Figure 3). The periplasmic and the membrane fractions from cells treated with IPTG contained an additional protein of ~33 kd. Nearly 20% of the expressed protein was found in a soluble form in the periplasm, the remainder was membrane-associated. It could be released by washing the membrane with 0.1 M NaOH or 2 M guanidinium hydrochloride.

The expressed protein was identified by Western blotting (Figure 4). In both the periplasmic and total cell protein fractions, the antibody bound to a protein of the same molecular weight as a protein in the thylakoid membrane of spinach. The minor bands at lower molecular weights in lanes 4 and 5 may well be degradation products.

In order to verify the correct processing of the precursor, the N-terminus of the expressed protein isolated from total cell protein by SDS–PAGE was sequenced by automated Edman degradation. The first 15 amino acids correspond to the N-terminus of the mature 33 kd protein from spinach (Oh-oka *et al.*, 1986).

In order to purify the expressed protein, the periplasmic proteins were first released by osmotic shock. A single DEAE column step yielded 10 mg of protein from one liter of *E.coli* culture at a purity of at least 95% (Figure 5).

We then tested the functional competence of the expressed 33 kd protein by rebinding to $CaCl_2$-washed PSII particles from spinach. Figure 6 shows the removal of the 16 and 23 kd proteins by NaCl treatment (lane 3) and the removal of all three extrinsic proteins by $CaCl_2$ treatment of PSII particles (lane 4). By adding an excess of the 33 kd protein, 75% of the original amount could be rebound. No significant difference was found between the 33 kd protein isolated from *E.coli* and that isolated from spinach as a control (Figure 6, lanes 5 and 6). Oxygen evolution capabilities of these complexes are shown in Table I.

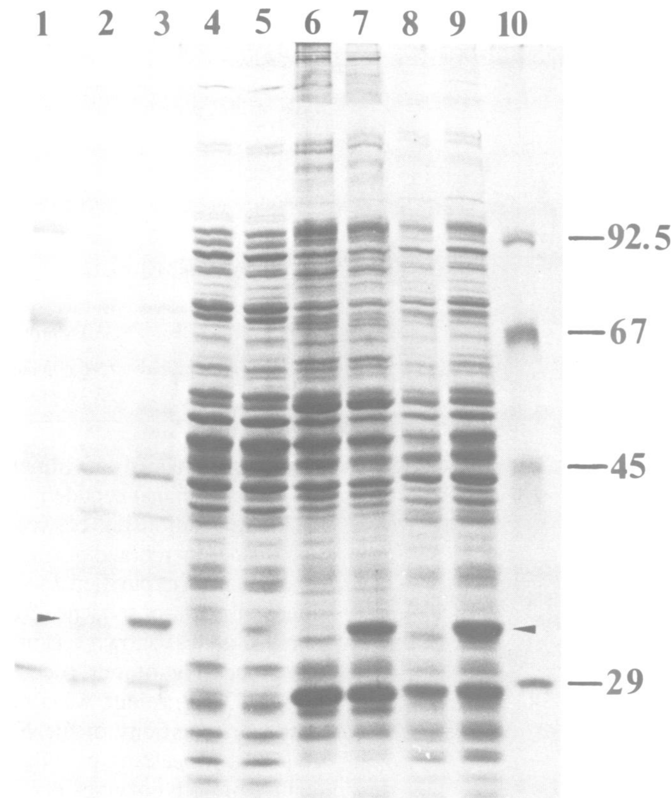

Fig. 3. SDS–PAGE of subcellular compartments from *E.coli* after 12 h expression. Lanes 1 and 10, protein mol. wt marker. Lanes 2 and 3, periplasmic proteins; lanes 4 and 5, cytoplasmic proteins; lanes 6 and 7, membrane-bound proteins, lanes 8 and 9, total cell protein from *E.coli* cultures without (lanes 2, 4, 6 and 8) and with induction by IPTG (lanes 3, 5, 7 and 9).

The NaCl treatment of PSII particles results in a decrease of oxygen evolution activity to 74%. The $CaCl_2$-washed particles which contained no extrinsic proteins show a decreased activity of 9%. Rebinding of the 33 kd protein from spinach and *E.coli* increased the activity at the same rates. This suggests that both proteins have the same conformation.

Discussion

Expression of foreign genes in *E.coli* has become a widely used tool for obtaining large amounts of protein and/or for obtaining mutants produced by site-directed mutagenesis of the corresponding (c)DNA. The major problems with this technique are the instability of foreign proteins due to attack by proteases and the difficulty of achieving correct folding of the protein, particularly of those proteins containing disulfide bridges. Disulfide bridges can only be formed under the oxidizing conditions of the periplasm and not in the cytoplasm of *E.coli* (Duffaud *et al.*, 1987).

For translocation to the periplasm, the proteins have to be expressed as precursors with N-terminal extensions of 18–27 amino acids. These 'signal peptides' show only very low sequence homologies. Common features are one or more positively charged amino acids prior to a stretch of hydrophobic amino acids and a signal peptidase recognition site, usually A-X-A (Inouye and Halegoua, 1980; von Heijne, 1985). These features are all present in the C-terminal part of the transit sequence of the 33 kd protein, and are generally taken as an indication of the (cyano-)bacterial origin of the chloroplast in the framework of the endosymbiotic theory. In such a (cyano-)bacterial ancestor, the C-terminal part of the transit sequence may have functioned as a signal peptide. Due to the possibility of coevolution of the 33 kd protein and its signal peptide, we left the C-terminal part of the transit sequence unchanged with the following exception. Because *E. coli* signal peptides

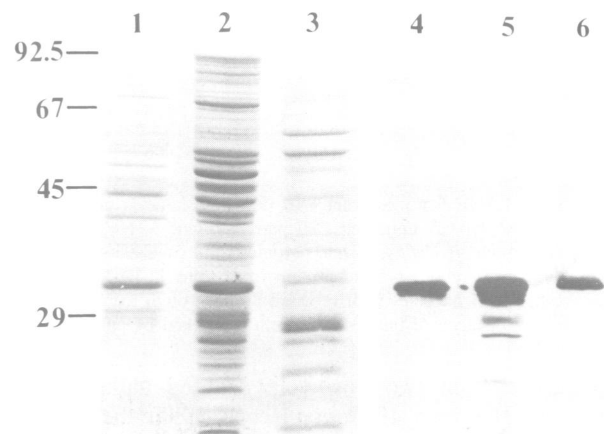

Fig. 4. SDS–PAGE and subsequent Western blot for identification of the expressed protein. Lanes 1 and 4, periplasmic proteins; lanes 2 and 5, total cell proteins from induced *E.coli* cultures. Lanes 3 and 6, thylakoid membrane proteins. After electrophoresis, protein bands were visualized with Coomassie staining (lanes 1–3) or blotted onto a PVDF membrane and incubated with an antibody against the 33 kd protein (lanes 4–6). The antibody binding was visualized by the enzymatic activity of alkaline phosphatase conjugated to an anti-rabbit IgG.

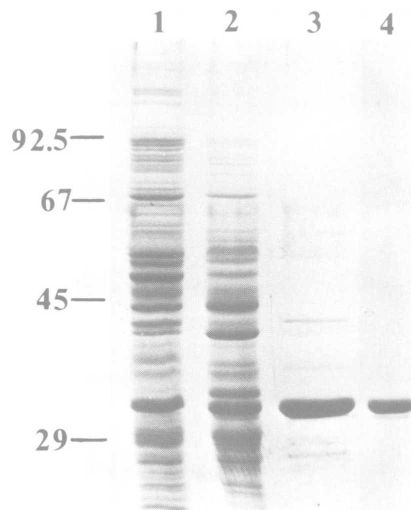

Fig. 5. SDS–PAGE of the purification of the expressed 33 kd protein. Lane 1, total *E.coli* protein after expression; lane 2, released periplasmic proteins from *E.coli*; lane 3, 33 kd protein after DEAE column; lane 4, purified 33 kd protein from spinach.

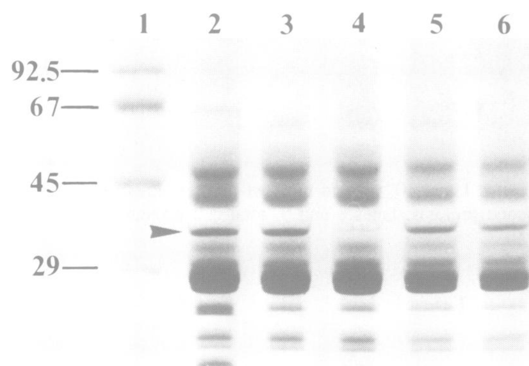

Fig. 6. SDS–PAGE from different PSII particles. Lane 1, protein mol. wt marker; lane 2, untreated PSII particles; lane 3, NaCl-washed PSII particles; lane 4, $CaCl_2$-washed particles; lane 5, $CaCl_2$-washed PSII particles with rebound 33 kd protein from spinach; lane 6, $CaCl_2$-washed PSII particles with rebound 33 kd protein from *E.coli*. The migration distance of the 33 kd protein is marked by an arrow.

frequently contain two positively charged amino acids (Inouye and Halegoua, 1980; von Heijne, 1985), the plasmid pDS1233 was constructed with an additional lysine codon behind the translation start codon. We did not know whether the part of the transit sequence which we used in the expression experiment was the whole thylakoid transfer domain or only part of it. It was assumed that the thylakoid transfer domain contains 27–42 amino acids (Smeekens and Weisbeek, 1988; Ko and Cashmore, 1989) but no direct evidence, such as an N-terminal sequence of the observed import intermediate in chloroplast import assays (Smeekens *et al.*, 1986; Kirwin *et al.*, 1989), is available.

We have achieved expression of the 33 kd protein in *E.coli* at high levels. Twelve hours after induction with IPTG, the expressed protein represented 7% of the total cell protein. Subcellular localization and N-terminal sequencing showed that export and correct processing of the precursor occur. This is the first demonstration that the C-terminal part of

Table I. Oxygen evolution measurements of different PSII particle preparations

Type of PSII particles	Addition	O_2 evolution [μmol O_2 (h mg Chl)$^{-1}$]	Relative O_2 evolution
Control	none	268	100%
2.0 M NaCl-washed	none	198	74%
1.2 M $CaCl_2$-washed	none	24	9%
1.2 M $CaCl_2$-washed	isolated spinach 33 kd protein	76	28%
1.2 M $CaCl_2$-washed	recombinant 33 kd proten	72	27%

a transit sequence of lumenal chloroplast proteins can function as a signal peptide in *E.coli*. The results demonstrate the close relationship between the translocation apparatus of the inner membrane of *E.coli* and the thylakoid membrane of spinach. The purified *E.coli* signal peptidase I is able to remove the transit sequence from the 23 kd precursor protein *in vitro*, as was shown recently (Halpin *et al.*, 1989). With the processing of our precursor protein in *E.coli* to the mature 33 kd protein, we confirm the similar if not identical substrate specificity of the *E.coli* signal peptidase and the thylakoid processing peptidase *in vivo*. A further common feature of both enzymes is that they are integral membrane proteins with their active sites at that membrane surface to which the proteins are translocated (Wolfe *et al.*, 1983; Kirwin *et al.*, 1988).

Nearly 20% of the expressed protein occurs in a soluble form in the periplasm; the remaining 80% is associated with the membrane, although it is processed to the mature form. The reason for this remains to be clarified. We have assumed that the membrane-bound 33 kd protein has a different conformation. One possibility is that intra- or intermolecular disulfide bridges are formed before correct folding occurs. These proteins may not be able to take up normal conformation and hydrophobic parts of the protein which are normally inside the protein may then be located at the surface and interact with the *E.coli* membrane. Another possibility is that formation of periplasmic aggregates of incorrectly folded proteins occurs; these may interact poorly with the membrane but cosediment upon centrifugation. These views are supported by the observation that removal of the protein by treatment of the membrane with guanidine hydrochloride and subsequent dialysis is not sufficient to obtain a homogeneous protein preparation.

To confirm that the soluble 33 kd protein had the correct conformation, the purified protein was rebound to $CaCl_2$-washed PSII particles. The restoration of oxygen-evolving activity indicates the functionality of the protein. The percentage of restoration of activity was lower than in other reports (Kuwabara *et al.*, 1985; Ono and Inoue, 1986), but the same values of oxygen evolution were reached for both the expressed 33 kd protein and the native 33 kd protein isolated from spinach. The reason for the lower activity seems to be associated with our PSII preparation and the procedure used to remove the native 33 kd protein. We hope to overcome this problem in the near future.

We have constructed an expression system for the 33 kd protein. With this system we can now start to express mutant proteins produced by site-directed mutagenesis to investigate

the interactions of the 33 kd protein with the other components of photosystem II.

Materials and methods

Materials

Restriction enzymes, T4 DNA ligase, RNase A, S1 nuclease, lysozyme and proteinase K were obtained from Boehringer Mannheim or Bethesda Research Laboratories. Polyvinylidene difluoride (PVDF) membrane for Western blotting was purchased from Millipore. The antibody against the 33 kd protein was a gift of C.Jansson, Stockholm. Anti-rabbit IgG alkaline phosphatase conjugate was obtained from Sigma. DEAE−Sephacel was obtained from Pharmacia LKB, CM-Fractogel from Merck. IPTG was purchased from Biomol. All other chemicals were of analytical grade.

Construction of the expression plasmid

pBLU33 was a gift of R.G.Herrmann, Munich; it consists of the pBluescript KS $M13^+$ with the cDNA for the 33 kd protein (Tyagi *et al.*, 1987) cloned into the *Bam*HI restriction site. Before cloning the cDNA into the expression plasmid, the *Eco*RI recognition site was deleted by cutting with *Eco*RI, removing the single strand DNA with S1-nuclease and religation, forming the plasmid pBLU33ΔE.

The expression plasmid pDS12/RBSII, a gift from H.Bujard, Heidelberg, belongs to the pDS-family (Bujard *et al.*, 1987) and contains a promoter/operator element consisting of the P_{N25} promoter of the bacteriophage T5 which was controlled by the *lac* operator. The 25 bp of the polylinker behind the synthetic ribosome binding site RBSII was replaced by the following oligonucleotide which adapts the vector for expression:

AATTCATTAAAGAGGAGAAATTAACTATG-
AAGAAGCTTGGATCCG

The first 29 bp containing the ribosome binding site and the translation start codon are identical with the original sequence. The last 16 bp which replaced the original polylinker contain a *Hin*dIII and a *Bam*HI recognition site.

The *Hin*dIII fragment of pBLU33ΔE was cloned into the new *Hin*dIII cleaving site of this plasmid forming the plasmid pDS1233.

To prevent expression in an early state of cell growth, a second plasmid DMI,1 (Certa *et al.*, 1986) was present in *E.coli*. This plasmid carries the $lacI^q$ gene which codes for the lac repressor.

Plasmid amplification and protein expression was carried out in *E.coli* K12 strain DH5α (endA1, recA1, hsdR17 ($r_k^- m_k^+$), supE44, thi-1, λ^-, gyrA, relA1, F^-, Φ80d*lac*ZΔM15, Δ(lacZYA-argF)U169).

Expression, localization and purification of the 33 kd protein

The *E.coli* cells were grown to an optical density of 0.6 at 600 nm as measured with a Perkin-Elmer lambda 15 UV/VIS spectrophotometer. Expression was induced by adding IPTG to a final concentration of 0.3 mM. After shaking for 12−15 h the cells were collected by centrifugation.

Periplasmic proteins were released from the cells by osmotic shock (Neu and Heppel, 1965). The cells were incubated in a buffer containing 20% sucrose, 20 mM Tris−HCl, pH 8.0, and 2 mM EDTA at 25°C for 10 min. They were then collected by centrifugation and resuspended in distilled water. The supernatant obtained upon centrifugation contained the periplasmic proteins. To separate the cytoplasmic and the membrane fractions (Libby *et al.*, 1987), the spheroplasts were incubated with a buffer containing 50 mM Tris−HCl, pH 8.0, 20 mM EDTA and 100 μg/ml lysozyme for 15 min at 37°C. The suspension was frozen three times in a methanol/dry ice bath and pressed several times through a thin needle. The membranes were precipitated by centrifugation at 100 000 *g* for 30 min.

The proteins released from the cells by osmotic shock were loaded onto a DEAE column (5 × 10 cm) equilibrated with 50 mM 4-morpholinoethanesulfonic acid (MES), pH 6.5. 0.1 M phenylmethylsulfonyl fluoride was added routinely to all solutions. The column was washed with 600 ml 50 mM MES, pH 6.5, 25 mM NaCl. When no further protein was eluted under these conditions, the NaCl concentration was increased to 75 mM. The 33 kd protein was eluted with 250 ml 50 mM MES, pH 6.5 and 75 mM NaCl.

Rebinding assay

PSII preparation was carried out according to the method of Ghanotakis *et al.* (1984c). The PSII particles were washed several times with SMN buffer (400 mM sucrose, 50 mM MES, pH 6.0, 15 mM NaCl). $CaCl_2$ washing was carried out similarly to the method of Kuwabara *et al.* (1985). PSII particles with a chlorophyll concentraton of 1.5 mg/ml were diluted with four volumes of a buffer containing 1.5 M $CaCl_2$, 400 mM sucrose and 50 mM MES, pH 6.0. After incubation on ice for 20 min in the dark, PSII particles depleted of the extrinsic proteins were collected by centrifugation at 40 000 *g* for 30 min. The pellet was washed three times with SMNC buffer (400 mM sucrose, 50 mM MES, pH 6.0, 200 mM NaCl, 10 mM $CaCl_2$).

The supernatant was dialyzed against 50 mM MES, pH 6.5 and loaded onto a CM cation exchange column (0.5 × 5 cm). Under these conditions, the 33 kd protein did not bind to the column. The proteins running through this column were loaded onto a DEAE column (0.5 × 5 cm) equilibrated with 50 mM MES, pH 6.5 and the 33 kd protein was eluted with a salt gradient of 0−200 mM NaCl in 50 mM MES pH 6.5. For the rebinding assay the purified 33 kd protein from both spinach and *E.coli* was dialyzed against SMNC buffer. Rebinding to the $CaCl_2$-washed PSII particles was carried out at a chlorophyll concentration of 0.3 mg/ml with an excess of 33 kd protein. Oxygen evolution activity was determined with a Clark type electrode and 2,6-dichloro-*p*-benzoquinone as electron acceptor after standing on ice for 1 h. PSII-particles were resuspended in SMN buffer, salt-washed and reconstituted in 400 mM sucrose, 50 mM MES, pH 6.0, 30 mM NaCl and 10 mM $CaCl_2$.

Other methods

SDS−PAGE was carried out as described by Laemmli (1970). For electrophoresis of PSII particles, 6 M urea was present in the gel.

Protein concentration was determined as described by Lowry *et al.* (1951) and for chlorophyll determination the method of Arnon (1949) was used.

Acknowledgements

We thank R.G.Herrmann for the cDNA clone of the 33 kd protein and C.Jansson for the antibody. We thank also Dr E.Kojro and Dr F.Fahrenholz for sequencing the N-terminus of the expressed protein. The oligonucleotide was synthesized on an Applied Biosystems 3A DNA synthesizer by Prof. Dr J.Engels. This work was supported by the Deutsche-Forschungs-Gemeinschaft (Leibniz Programm to H.M.) and the Max-Planck-Gesellschaft.

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Received on January 22, 1990; revised on March 12, 1990