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Expression of the 23 kDa protein from the oxygen-evolving complex of higher plants in *Escherichia coli*

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Abstract

The 23 kDa protein from the oxygen-evolving complex of higher plants regulates the binding of one Ca^{2+} ion, which is an essential cofactor of water splitting. In this report its expression in *Escherichia coli* is described. The 23 kDa protein was expressed and secreted to the periplasm where it accumulated in a soluble form. After purification by cation exchange chromatography the recombinant protein was bound to NaCl-washed Photosystem II. Detailed analysis of oxygen-evolving activity demonstrates its function in Ca^{2+} binding identical to the spinach 23 kDa protein. This expression system opens the way for mutational analysis and isotopic labelling in order to study its function in water splitting.

Key words: Photosystem II; Calcium; Oxygen evolution; Water splitting

1. Introduction

Photosynthetic oxygen-evolution takes place on the lumenal side of Photosystem (PS) II (see Ref. [1] for a recent review). Four successive charge separations are needed to oxidize two molecules of water resulting in one molecule of oxygen and four protons. A cluster of four manganese ions is involved in the storage of positive charge equivalents created by the four charge separations, undergoing five different redox states. The S-states are called S_0–S_4, where the S_0-state is the lowest and the S_4-state is the highest oxidized state.

Three extrinsic proteins with apparent molecular masses of 33, 23 and 16 kDa have been shown to be closely related to oxygen evolution in plants. Their function has been widely studied by dissociation and reconstitution experiments [2]. Treatment of PS II with 1–2 M NaCl releases the 23 and 16 kDa proteins along with one Ca^{2+} ion [3–5]. The activity is drastically decreased but can be restored by adding 5 mM Ca^{2+} and 30 mM Cl^- to the assay medium or by rebinding of the 23 kDa protein in the presence of Ca^{2+}. This indicates that binding of Ca^{2+} to PS II is modulated by the 23 kDa protein. It was shown that upon the release of Ca^{2+} the S_3 to S_0 transition is inhibited [6,7]. If the NaCl treatment is carried out in the presence of mM concentrations of Ca^{2+} chelators (EGTA, citrate etc.) at least one S-state becomes modified: in Ca^{2+}-depleted PS II the S_2 state is stable at room temperature and the EPR multiline signal has an altered shape [8]. These EPR data and recent ESEEM data suggest that the chelators bind directly to the manganese cluster [9,10].

With EPR spectroscopy no differences could be detected in the S_2-state of Ca^{2+}-depleted PS II in the presence or absence of the 23 kDa protein [7]. Some differences on the split S_3-signal which appears in Ca^{2+}-depleted PS II [8] and which was attributed to an oxidized histidine [11] were observed. This signal is wider and more stable in the presence of the 23 and 16 kDa proteins [11]. Recent publications of thermoluminescence measurements on Ca^{2+}-depleted PS II have concluded that the 23 kDa protein modulates the stability of the $S_2Q_A^-$ state [12,13]. However, the magni-

Abbreviations: PS II, Photosystem II; EPR, electron paramagnetic resonance; ESEEM, electron spin echo envelope modulation; NMR, nuclear magnetic resonance; MES, 2-(*N*-morpholino)ethane sulphonic acid; Q_A, primary electron acceptor of PS II; OmpA, outer membrane protein A; BCIP, 5-bromo-4-chloro-indolyl-phosphate 4-toluidine salt; NBT, Nitro blue tetrazolium; DIFP, diisopropyl fluorophosphate; SDS-PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

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tude of the temperature shifts differs greatly in the two reports and the attribution of the high temperature thermoluminescence band has been questioned [14].

In cyanobacteria the protein composition on the lumenal side is different from that in plants: in the bacteria only the 33 kDa protein is present but not the 23 and the 16 kDa proteins [15].

One possible method to investigate the role of the 23 kDa protein would include isotopic labelling and/or site-directed mutagenesis of specific amino acid residues. This would require an expression system for the 23 kDa protein. After purification, isotopic labelled or mutagenized 23 kDa protein would have to be bound to NaCl-treated PS II. Modifications of such complexes could be investigated by EPR, ESEEM, NMR and thermoluminescence. Extensive mutational analysis is under way for the 33 kDa protein [16,17] but no mutagenesis has been reported for the 23 kDa protein. It has been shown that *E. coli* is a well suited host for expression of wild type [18,19] and mutagenized [16,20] 33 kDa protein. In this communication the expression of the 23 kDa protein and its functional reconstitution to NaCl-washed PS II is reported.

2. Materials and methods

2.1. Materials

Restriction enzymes were purchased from New England Biolabs, Yeast extract and tryptone from Difco, IPTG, BCIP and NBT from Sigma, SP-Sepharose from Pharmacia, PVDF-membrane from Millipore and goat anti-rabbit IgG-alkaline phosphatase conjugate from Promega.

2.2. E. coli strains

During the construction of the expression plasmid pASK6023 the strain DH5a (endA1, recA1, hsdR17 ($r_k^- m_k^+$), supE44, thi-1, l^-, gyrA, relA1, F^-, Φ80dlacZΔM15, Δ(lacZYA-argF)U169) [21] was used. For the expression of the 23 kDa protein the strains KS272 (F^-, ΔlacX74, galE, galK, thi, rpsL (strA), ΔphoA) [22], KS474 (KS272 degP41(ΔPstI-Kan^r)) [23], SF110 (KS272 ΔompT degP41(ΔPstI-Kan^r) [24], SF120 (KS272 ptr-32::ΩCm^r degP41(ΔPstI-Kan^r) ΔompT) [24] were used.

2.3. Construction of the expression plasmid

The coding region of the mature 23 kDa protein from spinach was excised from a pBluescript derivative containing the 3′ part of the psbP cDNA [25] with *Fsp*I and *Sal*I. *Fsp*I cleaved the plasmid exactly at the 5′ end of the 23 kDa protein coding region. The expression plasmid pASK60 (essentially identical to pASK60-strep [26] but without strep-tag) was cleaved with *Bsa*I and the single strand overhangs were filled in with T4-DNA polymerase. This recreates the coding region for the *E. coli* OmpA signal peptide with a 3′ blunt end. This vector fragment was then cleaved with *Sal*I and ligated with the psbP cDNA fragment.

2.4. Expression and purification of the 23 kDa protein

An overnight culture of KS272/pASK6023 was diluted 50-fold in 5.6 l LB medium or 2.8 l tryptone-phosphate medium (2% tryptone, 1.5% yeast extract, 0.8% NaCl, 0.3% Na_2HPO_4, 0.1% KH_2PO_4) [27] containing 100 mg/l ampicillin. The cultures were shaken in 8×2 litre flasks with 200 rpm at 22°C until the optical density of the culture at 600 nm reached 0.6 (Shimazu UV160 Spectrophotometer). Expression was induced by adding IPTG to a final concentration of 0.25 mM. 4 h after induction, the cells were harvested by centrifugation (JA 10 rotor, 8000 rpm/15 min/4°C). The periplasmic fraction was isolated using a modified method described in [16]. The cells of 850 ml culture were resuspended in 20 ml lysis buffer (20% sucrose/20 mM sodium phosphate (pH 6.5)/10 mM EDTA). After 10 min incubation at 0°C, 20 ml of 25% lysis buffer containing 0.1 mg/ml lysozyme was added. After 20 min incubation at 0°C, the spheroplasts were sedimented (conditions as above). The supernatant was again centrifuged (JA20 rotor, 20000 rpm/15 min/4°C) and stored at −20°C until further use. Further fractionation of cellular protein was carried out as described in [19].

Thawed periplasmic fraction was loaded onto an SP-Sepharose column (2.6×10 cm, flow rate 3 ml/min) preequilibrated with 20 mM sodium phosphate (pH 6.5)/1 mM EDTA/0.5 mM DIFP (buffer A). The column was washed with the same buffer until the absorption at 280 nm of the eluate had decreased to zero. The 23 kDa protein was eluted with a NaCl gradient of 0–300 mM in 250 ml of buffer A. A part of the fractions containing the 23 kDa protein also contained a contamination at about 60 kDa. These fractions were pooled, dialysed against buffer A (without DIFP) and purified further using an SP-Sepharose column of 1×12 cm with a flow rate of 1 ml/min. Elution of the 23 kDa protein was carried out with 50 ml buffer A (without DIFP) and a NaCl gradient of 50–300 mM.

2.5. Purification of the 23 kDa protein from spinach

PS II-enriched membranes were prepared according to Ref. [28]. The membranes were treated in SMN buffer (50 mM MES (pH 6.0), 0.4 M sucrose, 15 mM NaCl) containing 1 M of $CaCl_2$ (0.3 mg/ml chloro-

phyll). After incubation for 30 min in the dark the membranes were sedimented in 20 min at 40000 × *g* and 4°C. The supernatant was dialysed twice against 20 volumes of 5 mM MES (pH 6.0) and once against 20 volumes of 10 mM sodium phosphate (pH 6.0)/40 mM NaCl (buffer B). The solution was passed through a CM-Sepharose column (1 × 10 cm, flow rate 1 ml/min) preequilibrated with buffer B. The column was than washed with approx. 100 ml of the same buffer. Whereas the 33 kDa protein was not retarded, the 23 and 16 kDa proteins were eluted with a NaCl gradient of 40–600 mM in 100 ml buffer B. The main part of the 23 kDa protein was eluted at a NaCl concentration of about 200 mM.

2.6. Reconstitution of the 23 kDa protein to PS II

NaCl-treatment of PS II-enriched membranes was carried out essentially as described in Ref. [7]. Briefly, the membranes were stirred in a buffer containing 0.3 M sucrose/25 mM MES (pH 6.5) (SM-buffer) and 1.2 M of NaCl at a chlorophyll concentration of 0.5 mg/ml at room light and 4°C. After 30 min EGTA was added to a final concentration of 50 μM. The membranes were sedimented at 40000 × *g* and 4°C in 20 min. They were washed once in SM-buffer containing 10 mM of NaCl and 50 μM EGTA and resuspended in the same buffer.

Reconstitution was carried out in 1 ml SM-buffer containing 10 mM $CaCl_2$ or 20 mM NaCl and 50 μM EGTA with NaCl-treated PS II (0.25 mg chlorophyll) and different amounts of 23 kDa protein (0.5–10-times the molar concentration of PS II). After incubation for 30 min on ice in the dark the membranes were sedimented, washed once with 1 ml of SM-buffer containing 10 mM of NaCl and 50 μM EGTA in order to remove residual amounts of Ca^{2+}, and resuspended in the same buffer at a final volume of 0.5 ml.

2.7. SDS-PAGE, Western blotting and protein concentration

SDS-PAGE was carried out as described in Ref. [19].

For Western blotting (modified after Ref. [29]), PVDF-membrane and Whatman 3MM filter papers were incubated in blotting buffer (25 mM Tris (pH 8.3), 150 mM glycine, 10% methanol). The SDS-gel and membrane were sandwiched between two Whatman filters. After incubation for 5 min the filter was blocked in TBST (10 mM Tris (pH 8.0), 150 mM NaCl and 0.05% Tween-20) containing 5% dry milk powder. After 1 h the filter was washed twice with TBST and then incubated for another hour with a 1:3000 dilution of the anti-23 kDa protein antibody in TBST. The membrane was than washed twice with TBST and incubated for 1 h with a 1:7500 dilution of the goat anti-rabbit IgG-alkaline phosphatase conjugate in the same buffer. After washing the membrane twice with TBST and once with AP-buffer (100 mM Tris (pH 9.5), 100 mM NaCl, 5 mM $MgCl_2$), staining was carried out in AP-buffer containing BCIP and NBT, each 0.125 mg/ml.

Protein concentration was determined using the BioRad protein assay reagent with bovine serum albumin, fraction V as standard.

3. Results

The constructed expression plasmid pASK6023 is shown in Fig. 1. From this plasmid the 23 kDa protein is expressed as a precursor carrying the N-terminal signal peptide of *E. coli* outer membrane protein A. The expression is controlled by the *lac* promoter/operator in conjunction with the *lac* repressor gene. The plasmid carries also the origin of replication of the f1-phage. This allows single-stranded DNA formation of the plasmid and therefore mutagenesis directly in the expression plasmid.

Different *E. coli* strains were tested for the expression of the 23 kDa protein. Among them, KS272 was shown to be most suitable. In this strain the 23 kDa protein accumulates to approximately 8% of total periplasmic protein (Fig. 2A). This strain had to be used, since in strains used successfully for the expression of the 33 kDa protein like DH5α [19] or JM83 only traces of 23 kDa protein could be detected in the periplasmic protein fraction by Western blotting (data

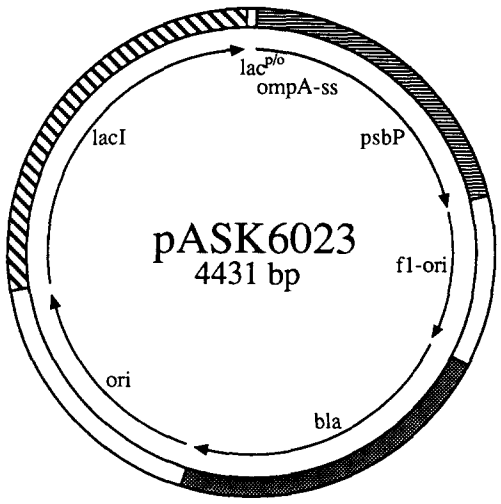

Fig. 1. Expression plasmid pASK6023. lac$^{p/o}$ = Lac promoter/operator element; ompA-ss = signal sequence of the outer membrane protein A; psbP = 3′ part of the cDNA encoding the mature part of the 23 kDa protein; f1-ori = origin of replication of the f1-phage; bla = β-lactamase gene; ori = origin of replication of the plasmid; lacI = lac repressor gene.

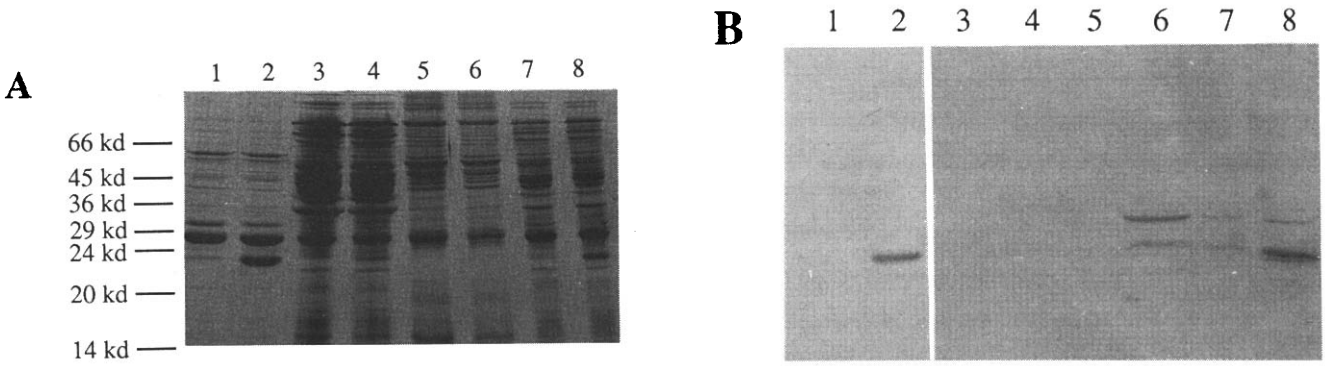

Fig. 2. SDS-PAGE (A) and Western blot (B) of cell fractions of *E. coli* expressing the 23 kDa protein (50 ml culture, tryptone-phosphate medium). 15 h after induction, the cells were fractionated and the fractions were analysed on a 12.5% SDS-polyacrylamide gel. Lanes 1 and 2, periplasmic protein fractions of cells from 50 μl culture; lanes 3 and 4, cytoplasmic protein fractions of cells from 25 μl culture; lanes 5 and 6, insoluble protein fraction of cells from 50 μl culture; lanes 7 and 8, total cellular protein of cells from 10 μl culture. Lanes 1, 3, 5 and 7, protein fractions from uninduced culture; lanes 2, 4, 6, 8, protein fractions from induced culture.

not shown). The use of strains with periplasmic proteinase deficiencies (KS474, SF110, SF120) led to no further increase in soluble expression product (data not shown).

The expression experiments described above were carried out at 22°C. At this temperature almost all of the recombinant protein appears in a soluble form. In Western blotting, faint bands appear at around 23 and 30 kDa in the insoluble fraction (Fig. 2B). These bands appear in induced and non-induced cells, although in the latter to a lower extent, because of a slight leak of the *lac* promoter. The 30 kDa band is related to the expression of the 23 kDa protein, since it does not appear in a Western blot with *E. coli* carrying the plasmid pASK60 (data not shown). This band might be the precursor protein where the signal peptide was not cleaved off, although the molecular mass is higher than expected.

The same concentrations of the 23 kDa protein in the periplasm were obtained at 25 and 28°C. At 37°C, the amount of soluble 23 kDa protein obtained was much lower and the protein could not be definitely detected after SDS-PAGE of the periplasmic protein fraction (data not shown).

The time course of appearance of soluble 23 kDa protein in the periplasm was studied. In LB medium, the maximum enrichment was reached after 2 h of expression. The level remained constant for another 2 h. Subsequently, the concentration of the expressed protein in the periplasmic protein fraction declined slowly. In tryptone-phosphate medium, the expression could be continued for 15 h. After 4 h of expression in LB medium, a concentration of the 23 kDa protein in the periplasmic protein fraction of approximately 8% and a cell density (A at 600 nm) of 1.9 was obtained. However, after 15 h expression in tryptone-phosphate medium the concentration of the 23 kDa protein increased to approximately 14% and the A_{600} to 4.1. (Note that lane 2 in Fig. 2A and lane 1 in Fig. 3 are not directly comparable, since the culture media and volumes were different. For unknown reasons, the concentration of the 23 kDa protein in the periplasmic protein fraction is higher in small cultures than in big ones. However, the amount of recombinant protein per litre culture is almost identical in all cultures.)

No difference in mobility in the SDS gel could be observed between the protein isolated from *E. coli* (Fig. 3, lane 3) and from spinach (Fig. 3, lane 4). N-terminal sequencing of the recombinant protein reveals that the OmpA signal peptide was cleaved at the expected site, leaving the N-terminus identical to the wild-type protein.

The 23 kDa protein contains a single cysteine residue [25]. SDS-PAGE under oxidizing conditions of the periplasmic protein fraction and subsequent Western blotting revealed that no dimerization via the cysteine residue occurred (data not shown).

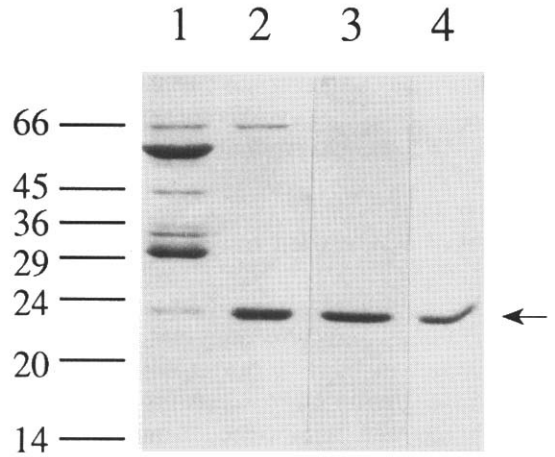

Fig. 3. Purification of the 23 kDa protein from spinach and *E. coli*. Samples from different stages of the purification were analyzed on a 12.5% SDS-polyacrylamide gel. Lane 1, periplasmic protein fraction from cells grown in 700 ml LB medium 4 h after induction by IPTG; lane 2, pooled fractions of the eluate of the first SP-Sepharose column (without the fractions already containing pure 23 kDa protein); lane 3, 23 kDa protein eluted from the second SP-Sepharose column; lane 4, 23 kDa protein isolated from spinach.

Table 1
Oxygen-evolving measurements of different PS II preparations

PS II preparation	Additions during rebinding	Additions to the O_2 measurement	Relative activity
PS II		10 mM $CaCl_2$	100%
PS II, NaCl-washed		–	20%
PS II, NaCl-washed		10 mM $CaCl_2$	69%
PS II, NaCl-washed + spinach 23 kDa protein	10 mM $CaCl_2$	–	61%
PS II, NaCl-washed + spinach 23 kDa protein	–	–	23%
PS II, NaCl-washed + spinach 23 kDa protein	10 mM $CaCl_2$	10 mM $CaCl_2$	72%
PS II, NaCl-washed + *E. coli* 23 kDa protein	10 mM $CaCl_2$	–	58%
PS II, NaCl-washed + *E. coli* 23 kDa protein	–	–	19%
PS II, NaCl-washed + *E. coli* 23 kDa protein	10 mM $CaCl_2$	10 mM $CaCl_2$	65%

The oxygen-evolving activity of PS II equivalent to 20 μg chlorophyll was measured in a total volume of 1 ml SM buffer containing either 20 mM of NaCl or 10 mM of $CaCl_2$. The activity of intact PS II (410 μmol O_2/h mg chlorophyll, no difference in the presence or absence of Ca^{2+} was observed) was set as 100%. Each sample was measured in triplicate and each reconstitution experiment was carried out three times, except for the reconstitution in the absence of Ca^{2+} which was carried out twice. The standard deviation was between 7% and 13% of the absolute activity of each sample.

Periplasmic proteins were released by spheroplast formation and purified by cation exchange chromatography (Fig. 3). By this procedure, 0.5–1 mg 23 kDa protein per litre *E. coli* culture in LB medium (5 experiments) and 2.5–3.1 mg per litre in tryptone-phosphate medium (2 experiments) could be obtained.

In order to prove the functional conformation of the recombinant protein, different amounts of the 23 kDa protein isolated from spinach and *E. coli* were bound to NaCl-washed PS II and oxygen-evolving activity was measured in the absence of exogenous Ca^{2+} (Fig. 4). For both proteins, a 3-fold molar excess over NaCl-treated PS II was necessary to reconstitute maximum activity. In Table 1, reconstitution experiments are summarized. NaCl-treatment in the light decreases the activity to 20% or less (note that the residual activity declines rather rapidly, indicating that the active PS II complexes have lost the 23 kDa protein but not the Ca^{2+}). 70% of the original activity could be restored by adding 10 mM $CaCl_2$ to the assay buffer. The reconstituted systems both show an activity of about 60% in the absence and 70% in the presence of exogenous Ca^{2+}. As a control the 23 kDa proteins were reconstituted in the absence of Ca^{2+}. In this case, no reconstitution of activity could be observed. This demonstrates that the recombinant 23 kDa protein can replace the spinach 23 kDa protein in function, and has therefore the same conformation as the spinach 23 kDa protein.

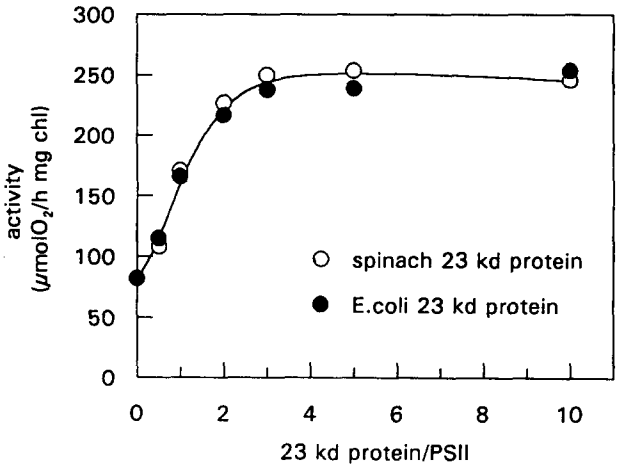

Fig. 4. Saturation of binding of the 23 kDa protein isolated from spinach and *E. coli* to NaCl-washed PS II monitored by oxygen-evolving activity. The amount of PS II was calculated assuming 220 chlorophyll per reaction centre. Rebinding was carried out in the presence of 10 mM $CaCl_2$. The oxygen-evolving activity was measured in SM buffer containing 20 mM NaCl. Each sample was measured in triplicate and each reconstitution was carried out twice. The standard deviation was between 4% and 13%.

4. Discussion

The 23 kDa protein was expressed as a precursor carrying the signal peptide of the *E. coli* protein OmpA at its N-terminus. The signal peptide directs the protein to the periplasm where it is cleaved off. This strategy has been successfully applied for the 33 kDa protein of PS II [18,19] and plastocyanin [30]. Plastocyanin could not be expressed in the periplasm of *E. coli*, although it does not contain a disulfide bridge which can only be formed in the periplasm but not in the cytoplasm of *E. coli*. The reason for this might be the lower proteinase content in the periplasm [31].

The use of the thylakoid transfer domain of the transit peptide of the 23 kDa protein as bacterial signal peptide, as done in the expression of the 33 kDa protein, did not seem to be advisable in this case, since this domain contained some features normally not present in *E. coli* signal peptides, e.g., charged amino acid residues near the cleavage site (see Ref. [25]).

Although the export was successful, in the first expression experiments the amount of expression prod-

uct obtained was low. The 23 kDa protein only accumulated in the periplasm of KS272 and its derivatives. DegP and OmpT are periplasmic proteinases known to be involved in degradation of some abnormal proteins [22–24]. However, strains deficient in these proteinases (KS474, SF110, SF120) do not accumulate more 23 kDa protein than KS272. Therefore, the accumulation of the 23 kDa protein in a soluble form might not to be limited by proteolysis.

Problems in protein folding are thought to be one of the main limits in obtaining soluble recombinant protein [32]. The temperature during protein expression is known to be an important factor for protein folding. Generally temperatures below 30°C increase the solubility of the expression product [32]. Indeed, expression at temperatures between 22°C and 28°C result in a much higher yield of the 23 kDa protein than 37°C. On the other hand, almost no formation of insoluble aggregates could be observed. This might be due to rapid proteolytic degradation of any misfolded 23 kDa protein. Another possible explanation would be a slow processing of the precursor protein. The appearance of small amounts of putative precursor protein might indicate this. The signal peptide possibly inhibits a tight, proteinase-resistant, folding of the protein. However, an increase in recombinant protein in the proteinase-deficient strains may then be expected. Another argument against this possibility is that in expression experiments with the 33 kDa protein, precursor protein was observed under some conditions (A.S. and H. Michel, unpublished data), although the yield of soluble mature protein was limited by protein folding [19].

The yield of soluble recombinant protein is probably not limited by the expression plasmid itself, since a modified form of the 33 kDa protein with the OmpA signal peptide could be expressed in strain JM83 from this plasmid, resulting in an enrichment of 20% of the periplasmic proteins (A.S., submitted).

The periplasmic proteins were released by spheroplast formation and could be directly purified by cation exchange chromatography. Due to the relatively high abundance in the periplasmic fraction and the tight binding to the cation exchange column, this procedure results in up to 3.1 mg/l *E. coli* culture of pure 23 kDa protein.

Correct processing was verified by N-terminal sequencing of the recombinant protein, indicating that the *E. coli* signal peptidase I is able to recognize its recognition site Ala-Xaa-Ala [33] even in the chimeric construct. This is not trivial, since it is believed that a secondary structure in addition to the Ala-Xaa-Ala motive is required for correct processing [34].

The functional conformation was verified in reconstitution experiments. The recombinant protein could be bound to NaCl-washed/Ca^{2+}-depleted PS II in stoichiometric amounts. When the rebinding was carried out in the presence of Ca^{2+}, oxygen-evolving activity was restored as with the protein isolated from spinach, demonstrating the functional equivalence and therefore the identical conformation to the spinach protein.

With this system it will be possible to produce, isotopically or otherwise, labelled 23 kDa protein which can be investigated after binding to NaCl-washed PS II by EPR, NMR and related techniques. It is especially well suited to investigate the function of the protein by site-directed mutagenesis. Since the expression plasmid contains the origin of replication for the f1-phage, single-strand DNA formation of and therefore site-directed mutagenesis directly in the expression plasmid is possible. This fact combined with the fast purification allows the screening of a large amount of mutated 23 kDa proteins.

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