In Vitro Reconstitution and Biophysical Characterization of OEP16, an Outer Envelope Pore Protein of Pea Chloroplasts[†]

Dirk Linke,[‡] Joachim Frank,[§] Josef F. Holzwarth,[§] Jürgen Soll,[||] Christoph Boettcher,[⊥] and Petra Fromme*[,‡]

Max Volmer Institut für Biophysikalische Chemie und Biochemie, Technische Universität Berlin, Strasse des 17, Juni 135, 10623 Berlin, Germany, Fritz-Haber-Institut der Max-Planck-Gesellschaft, Faradayweg 4-6, 14195 Berlin, Germany, Forschungszentrum für Elektronenmikroskopie, Institut für Chemie, Freie Universität Berlin, Fabeckstrasse 36a, 14195 Berlin, Germany, and Botanisches Institut, Universität Kiel, 24089 Kiel, Germany

Received May 5, 2000

ABSTRACT: More than 30% of all proteins in the living cell are membrane proteins; most of them occur in the native membranes only in very low amounts, which hinders their functional and structural investigation. Here we describe the in vitro reconstitution of overexpressed Outer Envelope Protein 16 (OEP16) from pea chloroplasts, a cation-selective channel, which has been purified from *E. coli* inclusion bodies. Reconstitution in detergent micelles was monitored by CD and fluorescence spectroscopy. Electron microscopy showed a homogeneous size distribution of the reconstituted protein, and differential scanning calorimetry gave an estimate of the enthalpy of protein folding. First protein crystals were obtained that have to be further refined for X-ray structural analysis. The described methods of membrane protein reconstitution and biophysical analysis might prove helpful in the study of other membrane proteins.

Chloroplasts are the locus of a wide variety of important biosynthetic pathways in plant cells. The products of these pathways, mainly carbohydrates from photosynthesis, must be exported into the cell cytosol, and there is a steady exchange of other solutes between the chloroplast and the cytosol as well. This solute exchange is mediated across the inner chloroplast membrane by specific transport proteins, like the dicarboxylic acid translocator, the hexose phosphate translocator, or the triosephosphate/phosphate translocator (*1*). In older textbooks, the outer chloroplast membrane was described as completely permeable for solutes of up to 10 000 Da. In contrast, recent publications show the existence of highly specialized transport proteins (*2, 3*). It remains unknown how the transport of solutes across this outer membrane is regulated.

The ancestral relation of plastids with Gram-negative bacteria and mitochondria (*4*) suggests the presence of multiple channel proteins in the chloroplast outer membrane. In mitochondria, several single anion selective channels of high conductance have been discovered, characterized (*5*), and crystallized for low-resolution structural analysis (*6*). In Gram-negative bacteria, different types of high-conductance channels have been found in the outer membrane, which can be classified into porins [rather unspecific diffusion pores, e.g., OmpF from *E. coli* (*7*)], porin-like channels with specific sites that influence the selectivity of the diffusion process (like LamB from *E. coli*) (*8*), and ligand-gated pores, e.g., *E. coli* ferric enterobactin channels (FePA) providing energy-dependent uptake of iron into bacteria (*9*).

Four channel proteins have been identified and functionally characterized in pea chloroplast outer membranes so far. Besides the preprotein conducting channel originating from a prokaryotic ancestor (*10, 11*), three distinct solute channels have been discovered and characterized. They were named according to their molecular weight as OEP16,[1] OEP21, and OEP24 (*2*, *3*, *12*). In pea root plastids, a protein of the porin type has been identified that is not present in green chloroplasts (*13*).

The channel characteristics of the OEP24 protein closely resemble those described for general diffusion pores. Although slightly cation selective, the channel allows the passage of triose phosphates, ATP, PP_i, dicarboxylic acid, and positively or negatively charged amino acids in a reconstituted system (*2*). OEP21 has been shown to form an anion channel which is regulated by ATP and triosephosphates from the intramembrane space (*3*). OEP16, on the other hand, forms a cation-selective high-conductance channel with a remarkable selectivity for amino acids and amines (*12*). Moreover, OEP16 shows homologies to LivH, an amino acid transporter in *E. coli* (*14*).

The amino acid sequence of OEP16 suggests the existence of transmembrane α-helices as well as transmembrane

[†] This work was supported by Deutsche Forschungsgemeinschaft Grant Fr920/3-2.

* To whom correspondence should be addressed at Max Volmer Institut für Biophysikalische Chemie und Biochemie, Technische Universität Berlin, Strasse des 17, Juni 135, D-10623 Berlin, Germany. Phone: +49 30 31426403. Fax: +49 30 31421122. E-mail fromme@phosis1.chem.tu-berlin.de.

[‡] Technische Universität Berlin.

[§] Fritz-Haber-Institut der Max-Planck-Gesellschaft.

[||] Universität Kiel.

[⊥] Freie Universität Berlin.

[1] Abbreviations: $C_{12}E_8$, octaethyleneglycol mono-*n*-dodecyl ether; β-DM, *n*-dodecyl-β-D-maltoside; SB12, *n*-dodecyl-*N*,*N*-dimethyl-3-ammoniopropanesulfonate; OG, *n*-octyl-β-D-glucopyranoside; OEP16, outer envelope protein of chloroplasts (16 kDa); kDa, kilodalton(s); CD, circular dichroism; TEM, transmission electron microscopy; MPD, 2-methyl-2,4-pentanediol; PEG, poly(ethylene glycol); CMC, critical micellar concentration; DMPC, dimyristoylphosphatidylcholine.

10.1021/bi001034m CCC: $19.00 © 2000 American Chemical Society
Published on Web 08/12/2000

β-sheet structures (*12*). Thus, there is no structural resemblance of OEP16 to other membrane pores that have been successfully crystallized (for high-resolution analysis) so far.

Here we describe the in vitro reconstitution of recombinant pea OEP16 from *E. coli* inclusion bodies using different detergents. Large amounts of correctly folded protein are essential for extensive structural and functional studies. The described methods of reconstitution and characterization might help in the structural analysis of receptor proteins and other membrane proteins that occur only in very low amounts in vivo.

EXPERIMENTAL PROCEDURES

Materials. The detergents $C_{12}E_8$ and SB12 were bought from Fluka (Buchs, Switzerland); β-DM and OG were from Glycon (Luckenwalde, Germany). All salts and buffers were of analytical grade and were obtained from AppliChem (Darmstadt, Germany). Chromatography columns and media were made by Pharmacia Biotech (Freiburg, Germany).

Purification of Recombinant OEP16. *E. coli* inclusion bodies containing denatured OEP16 were obtained as described (*12*), except that 80 mM MEGA-9 was used instead of Triton X-100 in the washing procedures. OEP16 was isolated in an unfolded state. The whole isolation procedure was performed at room temperature in the presence of 10 mM mercaptoethanol and 6 M urea in order to inhibit disulfide bond formation and to keep the protein unfolded. The salt gradient of 0−250 mM NaCl was slightly modified to improve peak separation.

Reconstitution of Recombinant OEP16. (a) In Liposomes. The reconstitution in liposomes was performed as a control for the quality and functional integrity of the protein preparation. Purified phosphatidylcholine (from soybean, Type S IV, Sigma) or DMPC and 80 mM MEGA-9 were added to the purified OEP16 in 6 M urea, and the suspension was sonicated and dialyzed for 4 h at room temperature against a buffer containing 10 mM Hepes/KOH (pH 7.6) in a dialysis tube with a pore width of 3500 Da (Spectrum). The reconstituted liposomes were used for single-conductance and turbidimetric investigation, done in cooperation with R. Wagner, Osnabrück, Germany. The liposomes were also used as controls in CD and fluorescence spectroscopy.

(b) In Detergent Micelles. Three different procedures were tested for the direct reconstitution of the OEP16 protein in detergent micelles: reconstitution of the protein by dilution in detergent-rich buffer, reconstitution of the protein by gel filtration, and reconstitution by dialysis.

The best results were obtained using the following reconstitution protocol: 0.3 mg/mL OEP16 in 6 M urea, 20 mM Hepes/KOH, pH 7.6, 100 mM NaCl, and 10 mM β-mercaptoethanol was quickly 10-fold-diluted into a buffer containing 10 mM Mops/Tris, pH 7.0 and 5 × CMC of the corresponding detergent (1% for OG). If necessary, the protein was reconcentrated using CENTRIPLUS concentrators (Millipore/Amicon, Eschborn, Germany).

Biophysical Characterization of the Reconstitution. (a) CD Measurements. CD measurements were performed on a Jasco J500 CD spectropolarimeter using 0.2 mm light pathway cells at room temperature while flushing the cuvette chamber with nitrogen gas. Spectra were recorded from 205 to 260 nm with a resolution of 0.1 nm and an acquisition time of 10 s/nm. Five scans were taken for each sample, and the average of these scans was stored for further analysis. Blanks of the respective protein-free samples (only buffer with detergent) showed no CD activity. The protein concentration was 0.3 mg/mL.

(b) Tryptophan Fluorescence Measurements. Tryptophan fluorescence was measured in 10 mm pathway cells in a Shimadzu Fluorescence Spectrophotometer at an excitation wavelength of 280 nm. Emission spectra were taken from 290 to 500 nm. The bandwidth was 5 nm for excitation as well as emission. Pure L-tryptophan was dissolved in alcohols of different chain lengths at a concentration of 1−5 μg/mL and measured with the same parameters to estimate the hydrophobicity (polarity of the microenvironment) of the tryptophan residues in OEP16. The protein concentration was 0.03 mg/mL. For measurements in liposomes, the difference spectra of OEP16-liposomes against protein-free liposome preparations had to be recorded because of the high fluorescence background of the lipid preparations. For measurements in liposomes, protein concentrations ranged from 0.3 to 0.5 mg/mL.

(c) Differential Scanning Calorimetry Measurements. Differential scanning calorimetry (DSC) experiments of OEP16 in different detergent buffers were performed in a MicroCal MC-2 instrument (MicroCal, Northampton, MA) at protein concentrations of 0.55 mg/mL in 20 mM Hepes buffer of pH 7.6 between 20 and 95 °C at a scan rate of 30 °C/h. The heat capacity, C_p (J/K), was measured as a function of temperature (°C). C_p was converted to the molar heat capacity, C_{pm} (kJ mol^{-1} K^{-1}), accounting for the cell volume (V = 1.2249 mL) and the sample concentration. Phase transition temperatures, T_m (°C), of OEP16 were obtained from DSC curves after baseline subtraction at the maximum molar heat capacity C_{pm}. The area under the DSC curve corresponds to the molar enthalpy change, ΔH_{cal} (kJ/mol), for the phase transition.

(d) Transmission Electron Microscopy (TEM). A droplet (5 μL) of the protein solution was placed on hydrophilized carbon-filmed grids (60 s plasma treatment at 8 W using a BALTEC MED 020 device), the excess fluid was blotted off, and the sample was air-dried. A droplet of phosphotungstic acid (2% w/v, pH 7.0) was then added for 45 s and again removed by blotting. The dried sample was inserted into a Philips CM12 TEM and imaged at a primary magnification of 58300× respectively using the microscopes low-dose protocol to avoid unnecessary irradiation.

Crystallization Experiments. A first screening of possible crystallization conditions was performed using OEP16 reconstituted in the presence of the detergent $C_{12}E_8$. The protein solution was brought into the super-saturated state by vapor diffusion using the method of hanging drops. Experiments were performed at 4 and 18 °C in parallel. Four microliters of the protein solution, containing a start concentration of 5 mg/mL OEP16 and 0.3% $C_{12}E_8$, was mixed with 1 μL of the precipitant solution. The hanging drops were equilibrated against a reservoir of 1 mL of the precipitant solution. A wide varity of possible precipitants (different salts, additives as PEG or alcohols) were tested in a pH range between 4.6 and 9.5.

Preliminary Electrophoretic and X-ray Characterization of the Crystals. The crystals and the supernatant were analyzed by SDS gel electrophoresis as described below,

using SDS HD Phast Gels and silver staining in a Pharmacia Biotech Phast System. The crystals could be harvested without loosing their ability to change the plane of polarized light by adding 30 μL of the reservoir solution with 0.3% $C_{12}E_8$. They were mounted in cryo-loops (Hampton Research) at room temperature for the first test of X-ray diffraction. A wide glass capillary was put around the cryo-loop. In the top of the glass capillary a filter tip with reservoir buffer was placed to prevent drying of the crystal during X-ray exposure.

RESULTS

OEP16, the cation-selective channel of the outer chloroplast membrane, was overexpressed in *E. coli* in the form of inclusion bodies. The protein was resolubilized in the presence of 6 M urea and purified as described under Experimental Procedures. Reconstitution into liposomes lead to an active channel with a conductivity of 1.2 nS and a selectivity for cations and amino acids as described previously (*12*). In parallel to the reconstitution into liposomes, a large set of experiments was undertaken for the direct reconstitution of the protein into detergent micelles. With respect to further structural analysis, the direct reconstitution has several advantages over the reconstitution into liposomes: the reconstituion can be performed in the absence of lipids, which can hinder the three-dimesional crystallization of the protein. Larger amounts of the protein can be reconstituted in the same batch, and mild detergents with low CMC can be used for the direct reconstitution, whereas these detergents are not optimal for the reconstitution into liposomes.

During reconstitution, urea is removed and the protein is folded into the native state in the form of protein−detergent micelles. Different methods such as gel filtration or dialysis were evaluated for the reconstitution; the best results were obtained by 10-fold dilution of the OEP16 solution into a detergent-rich buffer.

Different types of detergents were used for these experiments; as examples, the results obtained with three nonionic detergents (β-DM, OG, and $C_{12}E_8$) and the zwitterionic detergent SB12 are presented here.

The reconstitution of the protein was monitored by different spectroscopic methods. As controls, liposomes containing reconstituted (and functionally active) OEP16 were tested with CD and fluorescence spectroscopy.

(a) CD Spectroscopy. First insights into the quality of the reconstitution depending on the different types of detergents came from CD spectroscopic measurements. The experiments could only be perfomed from 205 to 260 nm due to the high UV absorption of the buffers used. The spectra of the reconstituted protein were compared to the spectra of the completely unfolded protein in the presence of 6 M urea. The results are shown in Figure 1. The shape of the spectra is similar to the spectra observed with the functional intact OEP16 reconstituted into liposomes (taken from ref *12*), represented in Figure 1, line F. Secondary structure predictions from the primary sequence predicted three transmembrane α-helices and four β-sheets per monomer (*12*). Even if secondary structure predictions from the protein sequence should be taken with care, these values give a rough estimate of the main structural elements in the native folded state.

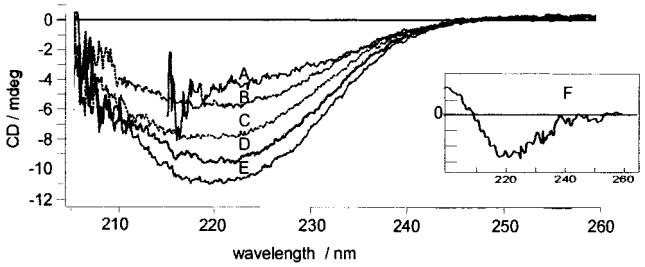

FIGURE 1: CD spectroscopy. CD spectra of 0.3 mg/mL OEP16. A, in 6 M urea; B, in 1% OG; C, in 1% SB12; D, in 0.5% β-DM; E, in 0.3% $C_{12}E_8$. Different concentrations of the same detergents did not affect the CD signal strongly. Spectrum F shows the CD signal of OEP16 functionally reconstituted in phosphatidylcholine vesicles as peviously published (*12*).

The building of these structural elements during the reconstitution can be monitored most clearly by following the change in the spectra in the region between 220 and 230 nm (*15*). In the presence of 6 M urea, the protein is completely unfolded, and the spectrum shows nearly no signal in this region in the control (Figure 1, line A). The spectra of OEP16 reconstituted in the presence of different detergents are shown in Figure 1, lines B to E. The largest change in the CD spectrum was observed for the OEP16 reconstituted in the presence of $C_{12}E_8$ (E). Similar secondary structural elements are also formed in the presence of β-DM, whereas the reconstitution in the presence of OG and SB12 led to a weaker signal at the same protein concentration, which can be interpreted as an incomplete or inhomogeneous reconstitution of OEP16.

(b) Tryptophan Fluorescence. Tryptophan fluorescence can be used as an indicator for the hydrophobicity of the medium sourrounding the tryptophan residues. OEP16 contains two tryptophan residues. In the denatured state, these two tryptophans are exposed to the hydrophilic medium. The tryptophan fluorescence spectrum of the protein in 6 M urea is shown in Figure 2A, spectrum 1: The spectrum shows a maximum tryptophan fluorescence at 355 nm, which corresponds to the value of a very hydrophilic surrounding (for comparison: the maximum of the fluorescence of isolated L-tryptophan in water is at 360 nm). It is expected that the native structure containing α-helices and β-sheets is formed during reconstitution. The absorption maximum is then shifted to smaller wavelengths, in correlation with a hydrophobicity increase surrounding the tryptophan residues in the native protein (*16*). The tryptophan fluorescence was determined for the reconstitution in the presence of $C_{12}E_8$, β-DM, OG, and SB12. During reconstitution, the maximum of the tryptophan fluorescence (excited at 280 nm) shifted from 355 to 330 nm. The same shift was observed for all four detergents under investigation. Nevertheless, the spectrum of the OEP16 protein reconstituted in the presence of OG shows a shoulder, which can be interpreted as an incomplete reconstitution of the protein. The results are shown for the detergents OG and $C_{12}E_8$ in Figure 2A, spectra 2 and 3. The spectra of the protein reconstituted in the presence of $C_{12}E_8$, β-DM, and SB12 differ only slightly in the intensity of the peaks at equal protein concentrations. The reconstitution in $C_{12}E_8$ led to the largest intensities, in good agreement with the results observed with CD spectroscopy mentioned above. The shift corresponds to a very hydrophobic surrounding of the tryptophan residues in the native, reconstituted protein.

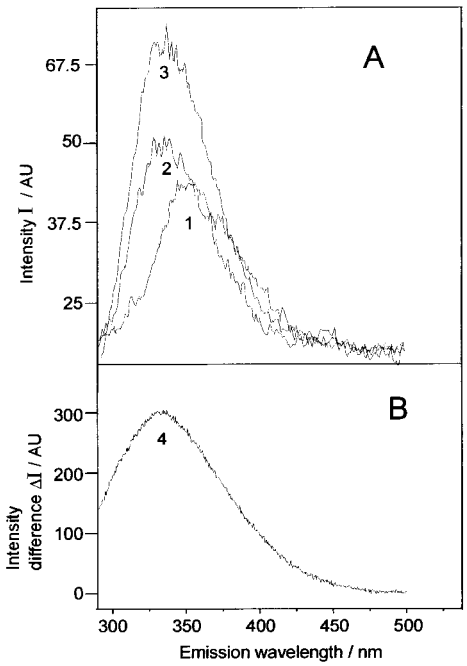

FIGURE 2: Fluorescence spectroscopy. (A) Tryptophan fluorescence spectra of 0.03 mg/mL OEP16: 1, in 6 M urea; 2, in 1% OG; 3, in 0.03% $C_{12}E_8$. As with the CD spectra, different detergent concentrations did not significantly affect the shape or intensity of the spectra. (B) Difference spectrum of the tryptophan fluorescence of OEP16 (0.3 mg/mL) functionally reconstituted in liposomes.

For comparison, the fluorescence of L-tryptophan in alcohols of different chain lengths was determined (data not shown). The tryptophan fluorescence maximum at 330 nm in the reconstituted OEP16 corresponds to the fluorescence of L-tryptophan in *n*-decanol. To demonstrate that the fluorescence shift of the detergent- reconstituted OEP16 corresponds to the native conformation of the protein, difference spectra with phosphatidylcholine vesicles containing functionally active OEP16 were recorded. The results are shown in Figure 2B, spectrum 4. OEP16 in lipid vesicles showed the same shift to 330 nm as the detergent-reconstituted protein, indicating that both proteins are in the same conformation.

(*c) Denaturation Heat Determination of the Recombinant OEP16 by Differential Scanning Calorimetry (DSC).* Differential scanning calorimetry was used to obtain a qualitative impression of the energy content of the structure, built up by the renaturation of the protein. A detergent solution without the protein was used as a reference. The reference and protein samples were heated in a highly sensitive calorimeter, and the heat capacities of the protein sample and the reference were compared. When the protein reaches its denaturation temperature, energy is used for the breakage of the specific intermolecular interactions that hold the protein in its native conformation (*17*, *18*). The difference between sample and reference can be used to estimate the energy that is needed for the unfolding of the protein and to determine the denaturation temperature. The experiment was done with the protein reconstituted in $C_{12}E_8$ and SB12 exclusively, because the measurements are very protein-consuming. The molar heat capacity for OEP16 in SB12 is shown as a function of temperature in Figure 3. The denaturation temperature was 84 °C for both protein samples independent of the detergent type. The enthalpy of folding/unfolding was 580 kJ/mol of monomer for samples in SB12

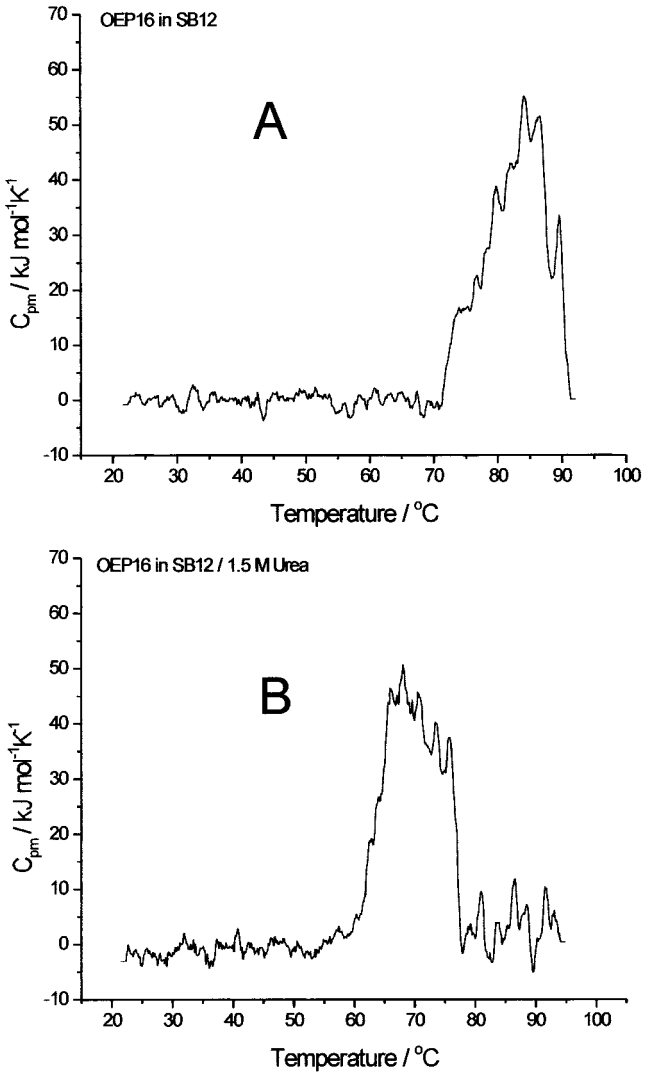

FIGURE 3: DSC. (A) Differential scanning calorimetry measurement of 0.55 mg/mL OEP16 reconstituted in 1% SB12. The temperature of heat denaturation is 84 °C; the molar heat capacity for the OEP16 monomer is 580 kJ/mol. Slightly higher values (800 kJ/mol) were obtained for 0.3% $C_{12}E_8$, but as the measurement was strongly hindered by a phase transition of the detergent itself at about 80 °C, these values have to be taken with care. (B) DSC measurement of 0.55 mg/mL OEP16 in 1% SB12 and 1.5 M urea. The heat denaturation temperature shifted down to 67 °C in this experiment, while the molar heat capacity (per monomer) remained unchanged.

(Figure 3A). The energy content for the samples with $C_{12}E_8$ could be estimated only qualitatively (data not shown, $\Delta H \approx 800$ kJ/mol), because the detergent without protein shows a phase transition at 80 °C at the given concentration of 0.3% (*19*). The denaturation energy in this case not only reflects the breakage of specific interactions in the protein itself, but also includes the denaturation energy of the whole protein−detergent micelle, so that the detergent micelle itself might influence the denaturation behavior of OEP16. Thus, absolute values for the molar heat capacity of denaturation for OEP16 are difficult to compare. However, as the denaturation temperature is constant for both detergents tested, we chose $C_{12}E_8$ for further experiments because of the spectroscopic results. To show that the phase transition temperature really is a protein effect, we added 1.5 M urea, a chaotropic reagent well-known to destabilize protein secondary structure, to the samples reconstituted in SB12. The denaturation temperature

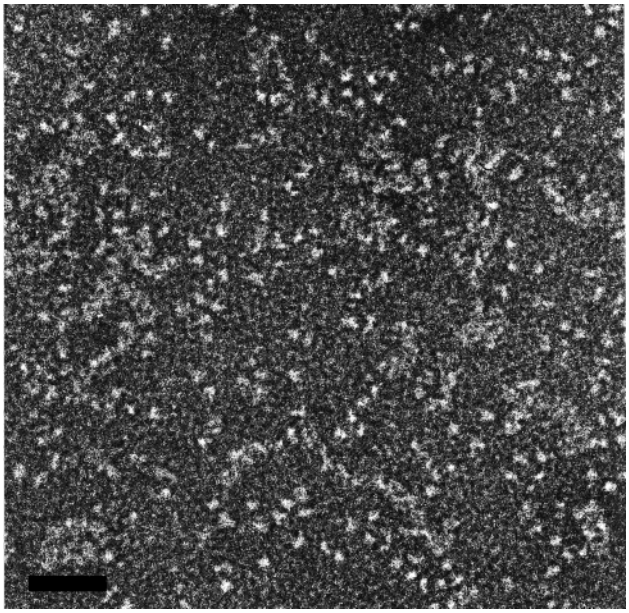

FIGURE 4: Electron microscopy. Transmission electron microscopy picture of OEP16 reconstituted in $C_{12}E_8$. Bar = 30 nm. The particle size can be estimated to be 5 nm in diameter.

in this sample shifted down to 67 °C (Figure 3B). Again, SB12 without protein in 1.5 M urea showed no heat capacity changes in the temperature range tested.

Electron Microscopy. Transmission electron microscopy images of the negatively stained sample using phosphotungstic acid at pH 7 show an even size distribution of the protein particles, which is vital for protein crystallography. The size (Figure 4) can be estimated to be ~5 nm in diameter. Future investigations should provide more accurate structural details by employing image analysis and three-dimensional reconstruction techniques (*20*).

Crystallization Experiments. For a first, rough screening of possible crystallization conditions for the OEP16 protein, the method of vapor diffusion was used. The experiments were carried out at 4 and 18 °C in parallel. Here we give a short summary of the influence of the different parameters on the solubility of OEP16:

(A) pH. Microcrystalline precipitation can be observed in the pH region of 4.6 up to 9.5. The size of the crystallites increases with pH up to the isoelectric point of OEP16 at pH 9. As expected, the solubility of OEP16 is lowest in the region between pH 8.5 and 9.5.

(B) *Crystallization Additives*. In the presence of small poly(ethylene glycol) molecules (like PEG 400), phase separation was observed, whereas the addition of long-chain PEG species (>PEG 6000) lead to the formation of amorphous precipitation. With PEG of medium chain length (like PEG 4000), phase separation and amorphous precipitation were both observed in parallel. The protein solubility in the presence of PEG is decreased by increasing the pH. Nevertheless, no crystal growth was observed in the presence of PEG. In the presence of alcohols, like MPD, the protein remained soluble at 4 °C, whereas amorphous precipitation was observed at 18 °C. There is some indication that in this case denaturation of the protein takes place.

(*C) Ionic Strength.* The solubility of proteins in general is highest at medium salt concentrations and decreases with both the decrease ("reverse of salting in") and the further increase of ionic strength ("salting out"). Dialysis of OEP16 at a protein concentration of 1 mg/mL against a buffer with low ionic strength (containing no salt, 10 mM buffer, and 1.5 × CMC of $C_{12}E_8$) did not decrease the solubility of the protein to supersaturation.

In contrast, the increase of the ionic strength lead to a dramatic decrease of the solubility of OEP16. The solubility of OEP16 is lower at 4 °C than at 18 °C under conditions of high ionic strength. Microcrystals were observed in the presence of different salts. The solubility of the protein does not depend on the ionic strength exclusively, but also on the nature of the salt. It could be shown that the influence of anions on the solubility of the protein is higher than the influence of cations. The influence on the solubility was rather low for chloride and rather high for phosphate and sulfate, whereas the presence of citrate strongly decreased the solubility of OEP16. Crystalline material could be observed in the presence of citrate in the pH range of 6.5−9.5. The size of the crystals increased with increasing pH. After 48 h, phase separation could be observed in the hanging drop experiments. One phase consisted of small droplets of detergent (without protein as observed by silver-stained SDS gel electrophoresis, see below), with a second, more solid phase (crystals) containing most of the OEP16. The supernatant was essentially protein-free. Citrate concentrations below 1 M did not give phase separation at 4 °C. This limit shifted to 800 mM at 18 °C.

Figure 5A shows initial small crystals of OEP16. Figure 5B shows the crystals under polarized light. The crystals are very thin plates with a length of 100−200 μm and a width of 50−100 μm, but the height of the plates is not larger than 10 μm. SDS gel electrophoresis (Figure 6) shows that most of the OEP16 can be found in these plates, which change the plane of the polarized light, whereas the round phase separation drops consist of the detergent $C_{12}E_8$. Only very small amounts of protein can be found in these drops or in the supernatant after 1 week of crystal growth. Only the washed crystals dissolved in sample buffer showed the strong 16 kDa band characteristic for OEP16 (Figure 6, lane B). No protein degradation could be detected.

X-ray Characterization of the OEP16 Crystals. X-ray diffraction was observed to 20 Å using a rotating anode as X-ray source. One unit cell constant was estimated to be in the range of 57 Å, which is reasonable and similar to the size of the protein detergent micelle estimated from the EM pictures (see above). The X-ray diffraction was obviously mainly limited by the small size of the crystals (thin plates with a height of the plates of about 10 μm).

DISCUSSION

We have presented here a method to reconstitute small eukaryotic membrane proteins from inclusion bodies. OEP16 could be useful as a model system for the functional und structural investigation of other rare membrane proteins. Even today, with more than 4000 structures of soluble proteins solved by X-ray structure analysis, the structural and functional investigation of membrane proteins is still very difficult. Less than 20 different membrane protein structures have been solved so far. There are several reasons for the lack of structural and functional information.

1. The membrane proteins are quite insoluble in water. They have to be solubilized by the use of amphiphilic

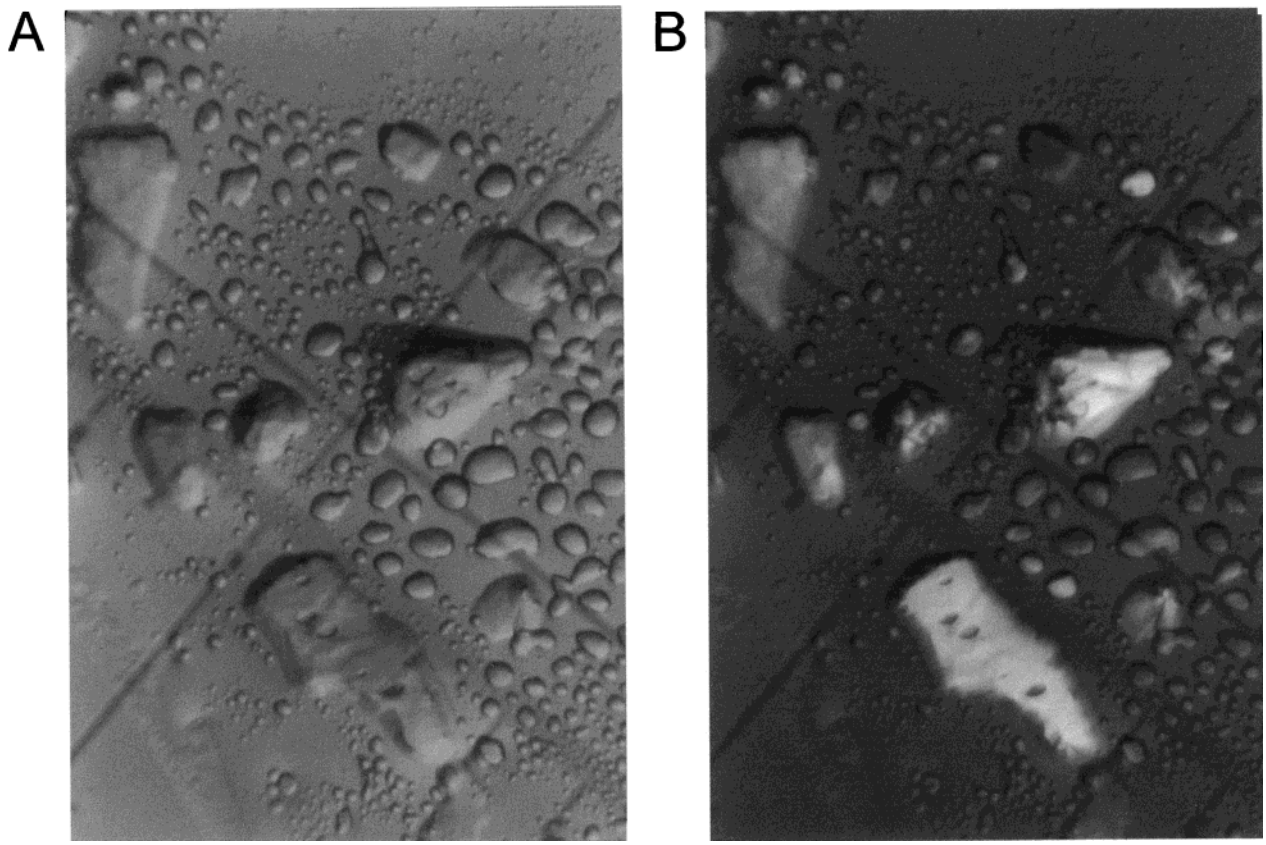

FIGURE 5: Crystals. Crystals of OEP16 obtained with 1 M sodium citrate as a precipitant. (A) Picture taken with white light. (B) Picture taken with polarized light. The smaller, nonpolarizing "droplets" contained mainly the detergent $C_{12}E_8$.

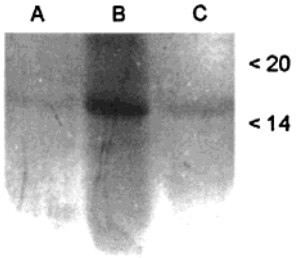

FIGURE 6: SDS gel (silver-stained) of the different crystallization phases. The different phases in the crystallization batches could be separated mechanically. The smaller, nonpolarizing droplets became very viscous when heated. Practically no protein was found in these droplets (lane C) as well as in the supernatant of the crystallization experiment (lane A). The OEP16 band of 16 kDa shows clearly in lane B, where the polarizing crystals, washed with 1 M sodium citrate and dissolved in sample buffer, were applied to the gel.

molecules (as detergents) or organic solvents, which can lead to the partial or complete denaturation of the protein. The spectroscopic methods used for the measurement of the refolding of OEP16 can therefore be very useful to test the folding status of membrane proteins solubilized from native membranes.

2. Only membrane proteins that are very common in nature (as the complexes of photosynthesis and respiration as well as the very abundant porins) have been successfully crystallized for high-resolution structure determination so far. Most of the membrane proteins, however (for example, receptors, special transporters, etc.), occur in the cells only in very low amounts and are therefore difficult to isolate. Today, soluble proteins are very frequently produced in large amounts by overexpression in *E. coli*.

Unfortunately, the overexpression of membrane proteins in *E. coli* usually leads to the formation of inclusion bodies, aggregates of unfolded protein. The refolding of a membrane protein into its native state is difficult to investigate, because the function of the membrane protein is often a transport or signal transduction process. This can be assayed only by reconstitution of the protein into liposomes. On the other hand, lipids and liposomes hinder 3D crystallization (2D crystals can be formed in some cases). Here we have shown that this problem can be avoided by following the direct reconstitution in detergent micelles using different spectroscopic methods. The best reconstitution procedure for our protein was the direct dilution into a detergent-rich buffer. This method is simple, quick, and easy to handle, allowing a large-scale screening for optimal detergents and buffer conditions.

From the reconstituted protein, crystals were gained that have to be further improved for X-ray analysis. Larger crystals might be gained with seeding techniques, and the use of Synchrotron radiation (for example, using the strong highly focused beam on the microfocus beamline at ESRF in Grenoble) will be considered. Experiments for freezing the crystals are under way. Very recently similar approaches have also been successfully used in the biophysical and structural analysis of other membrane channels (*6*). We hope that the method of in vitro protein reconstitution in detergent micelles can help to resolve more structures of small membrane proteins in the future.

ACKNOWLEDGMENT

We thank Prof. R. Wagner, Osnabrück, Germany, for single-conductance measurements and helpful discussions.

REFERENCES

1. Flügge, U. I. (1998) *Curr. Opin. Plant Biol. 1* (3), 201−206.
2. Pohlmeyer, K., Soll, J., Grimm, R., Hill, K., and Wagner, R. (1998) *Plant Cell 10*, 1207−1216.
3. Bölter, B., Soll, J., Hill, K., Hemmler, R., and Wagner, R. (1999) *EMBO J. 18* (20), 5505−5516.
4. Martin, W., and Müller, M. (1998) *Nature 392*, 37−41.
5. Xu, X., Decker, W., Sampson, M. J., Craigen, W. J., and Colombini, M. (1999) *J. Membr. Biol. 170*, 89−102.
6. Dolder, M., Zeth, K., Tittmann, P., Gross, H., Welte, W., and Wallimann, T. (1999) *J. Struct. Biol. 127*, 64−71.
7. Schirmer, T., Keller, T. A., Wang, Y. F., and Rosenbush, J. P. (1995) *Science 267*, 512−514.
8. Keller, T. A., Ferenci, T., Prilipov, A., and Rosenbush, J. P. (1994) *Biochem. Biophys. Res. Commun. 199*, 767−771.
9. Jiang, X. Q., Payne, M. A., Cao, Z. H., Foster, S. B., Feix, J. B., Newton, S. C., and Klebba, P. E. (1997) *Science 276*, 1261−1264.
10. Hinnah, S. C., Hill, K., Wagner, R. Schlicher, T., and Soll, J. (1997) *EMBO J. 16*, 7351−7360.
11. Bölter, B., Soll, J., Schulz, A., Hinnah, S., and Wagner, R. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 15831−15836.
12. Pohlmeyer, K., Soll, J., Steinkamp, T., Hinnah, S., and Wagner, R. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 9504−9509.
13. Popp, B., Gebauer, S., Fischer, K., Flügge, I., and Benz, R. (1997) *Biochemistry 36*, 2844−2852.
14. Rassow, J., Dekker, P. J. T., van Wilpe, S., Meijer, M., and Soll, J. (1999) *J. Mol. Biol. 286*, 105−120.
15. Saxena, V. P., and Wetlaufer, D. B. (1971) *Proc. Natl. Acad. Sci. U.S.A 66*, 969−972.
16. Godik, V. I., Blankenship, R. E., Causgrove, T. P., and Woodbury, N. (1993) *FEBS Lett. 321*, 229−232.
17. Thompson, L. K., Blaylock, R., Sturtevant, J. M., and Brudvig, G. W. (1989) *Biochemistry 28* (16), 6686−6695.
18. Kholodenko, V., and Freire, E. (1999) *Anal. Biochem. 270*, 336−338.
19. Mitchell, D. J., Tiddy, J. T., Waring, L., Bostok, T., and McDonald, M. P. (1983) *J. Chem. Soc., Faraday Trans. 79*, 975−1000.
20. van Heel, M., Harauz, G., Orlova, E. V., Schmidt, R., and Schatz, M. (1996) *J. Struct. Biol. 116*, 17−24.

BI001034M