Study of Structure and Function of Recombinant Pea Root Plastid Porin by Biophysical Methods†

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ABSTRACT: Pea root plastid porin (Fischer et al. (1994) *J. Biol. Chem. 269*, 25754−25760), which belongs to the family of mitochondrial (eukaryotic) porins, was expressed in *Escherichia coli* in high amounts using the pQE expression system. The recombinant protein was reconstituted into lipid bilayer membranes, and its characteristic properties were compared to those of the native porin isolated from pea root plastids. No significant difference was found between the native and the recombinant form when the protein was preincubated in detergent and sterol. The recombinant porin seems to be a valuable model system for the study of eukaryotic porins by spectroscopic methods, in which high amounts of protein are needed. CD spectroscopy was performed to determine the secondary structure of the porin under different conditions. It was found to have a high degree of β-sheet structure in the nonionic detergent Genapol X-80 and in lipid vesicles. The more polar detergent sodium dodecyl sulfate (SDS) induced a large amount of α-helix structure in the protein. Addition of sterol to the porin in Genapol buffer did not influence its secondary structure to any measurable extent, whereas it had a strong influence on channel forming activity in black lipid bilayers. First refolding experiments performed in decreasing urea concentrations are discussed together with the results of the other measurements with regard to protein folding and channel formation.

Porin has been isolated from the outer membrane of the envelope of non-green plastids of pea root (Fischer et al., 1994). Its immunoreactivity with antibodies against mitochondrial porins, its channel properties, and its primary sequence demonstrate that it is closely related to the eukaryotic porin family. Members of this family are localized in the outer mitochondrial membrane and act as a molecular sieve for hydrophilic solutes (Benz, 1994). Mitochondrial porins (Zalman et al., 1980; Roos et al., 1982) or VDAC (voltage-dependent anion-selective channel)[1] (Schein et al., 1976; Colombini, 1979) form water-filled channels that are slightly anion-selective in the open state and cation-selective in the voltage-induced ion-permeable "closed" states (Benz, 1994). Transport across mitochondrial porins provides a regulated pathway for substrate exchange between mitochondria and the cytoplasm and a tool for dynamic compartmentation of the mitochondrial energy metabolism (for a recent review, see Brdiczka, 1991). The porin of pea root plastids has presumably a similar function (Fischer et al., 1994) and is responsible for the exchange of hydrophilic solutes between the cytosol and the plastid interior.

Mitochondrial porins and probably also plastid porins are synthesized on free cytoplasmic ribosomes as precursors without cleavable presequences and are post-translationally imported into the mitochondria (Mihara et al., 1982; Freitag et al., 1982; Gasser & Schatz, 1983). The import of porin does not require a membrane potential; but metabolic energy in the form of ATP (Pfanner et al., 1988; Kleene et al., 1987) is necessary. Detergents can mimic the function of ATP to a certain extent (Pfaller et al., 1985). However, the exact mechanism of porin import and assembly is not known at present. Water-soluble mitochondrial porin can functionally be reconstituted in artificial lipid bilayers when the protein is preincubated with detergents, such as Triton X-100 or Genapol X-80, and sterols (Popp et al., 1995; Carbonara et al., 1996). Recent experiments have demonstrated that sterols seem to be important for the formation of the channel unit in the aqueous phase before its insertion into the membrane, rather than for targeting to the membrane (Popp et al., 1995).

The primary sequences of many eukaryotic porins are known at present (Mihara & Sato, 1985; Kleene et al., 1987; Troll et al., 1992; Heins et al., 1994; Kayser et al., 1989; Fischer et al., 1994; Blachly-Dyson et al., 1993, 1994; Ha et al., 1993; Elkeles et al., 1995). Despite little identity in the primary sequence, conservation in channel properties such as single-channel conductance, voltage dependence, and

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[1] Abbreviations: ATP, adenosine triphosphate; BSA, bovine serum albumin; DiphPC, diphytanoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine, HTP, hydroxylapatite; EDTA, ethylenediaminetetraacetic acid; Hepes, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; IPTG, isopropyl thiogalactoside; KAc, potassium acetate; LDAO, lauryldimethylamine oxide; RMS, root mean square; SDS, sodium dodecyl sulfate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SUV, small unilamellar vesicle; VDAC, voltage-dependent anion-selective channel.

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selectivity is high among the different eukaryotic porins. Furthermore, there exists a high homology in their predicted secondary structures. Notably, all porins contain stretches of alternating hydrophobic and hydrophilic amino acids, suggesting the formation of amphipathic β-strands (De Pinto et al., 1991; Mannella et al., 1996; for a review, see Benz, 1994). According to secondary structure predictions, the polypeptide chain of mitochondrial porins may be arranged in a cylinder containing either 16 antiparallel, amphiphilic β-strands, which are possibly arranged as a β-barrel similar to bacterial porins (De Pinto et al., 1991; Benz, 1994) or 12 β-strands and the amphiphilic N-terminal α-helix (Blachly-Dyson et al., 1990; Zizi et al., 1995).

To investigate the structure−function relationship of eukaryotic porins, for which in contrast to bacterial porins (Cowan et al., 1992; Schirmer et al., 1995) no high resolution crystallographic data are yet available, the possibility to express these proteins in *Escherichia coli* should be of great importance. Recombinant porin is not only valuable for the analysis of mutants but also for the simple production of large amounts of protein necessary for some spectroscopic techniques, including the study of the secondary structure by CD spectroscopy. In this study, first attempts were made to use this system for the investigation of secondary structures of pea root plastid porin in different environments.

MATERIALS AND METHODS

Materials. The plasmid pQE60 was obtained from Quiagen (Hilden, Germany). HTP was bought from Biorad (München, Germany); β-sitosterol (cholest-5-en-24-ethyl-3β-ol) and stigmasterol (cholest-5,22-dien-24-ethyl-3β-ol) were bought from Sigma (Deisenhofen, Germany). Genapol X-80 was obtained from Fluka (Buchs, Switzerland). Media were purchased from Difco (Augsburg, Germany). All salts and buffers were of analytical grade and were obtained from Merck (Darmstadt, Germany); ultrapure urea was from Roth (Karlsruhe, Germany). Dioleoylphosphatidylcholine (DOPC) and diphytanoylphosphatidylcholine were purchased from Avanti Polar Lipids, Alabaster, AL.

Purification of Native Porin. Envelope membranes were isolated from pea root plastids and separated on a sucrose density step gradient as described before (Fischer et al., 1994). These membranes were treated with buffer containing the detergent Genapol X-80 (2% w/v), and hydroxylapatite (HTP) was then added to the detergent-solubilized proteins. Using this batch procedure, most of the proteins, but not porin, are adsorbed to the HTP material (Schmid et al., 1992). The HTP was pelleted by centrifugation and the supernatant used for studies in black lipid bilayer membranes.

Cloning of Porin from Pea Root Plastids into an E. coli Expression System. The cDNA insert of clone pspor 8 (Fischer et al., 1994) was digested with *Xho*I, treated with T4 DNA polymerase, cut with *Nco*I, and cloned into the *Nco*I-cut/*Bgl*II-filled bacterial expression vector pQE60 (Diagen, Hilden, Germany). Transformed *E. coli* TG1 cells harboring the resulting construct pQE60-pspor were grown to an OD_{260} of 1.0 and induced by the addition of 2 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 5 h. Cells were disrupted by the addition of 0.5 volumes of phenol, and the protein in the organic phase was precipitated with 2 volumes of EtOH. After centrifugation, the pellet was resuspended in 1/10 volumes of 50 mM Tris-HCl (pH 6.8), 5% SDS, 2% β-mercaptoethanol, and 10% glycerol, and an aliquot (10 mL) was loaded on SDS−PAGE (Laemmli, 1970) to check for expression.

Expression, Purification, and Reconstitution of the Recombinant Porin. An overnight culture in DYT medium (16 g Bacto-Tryptone, 10 g of yeast extract and 5 g of NaCl dissolved in 1 L of distilled water) of the *E. coli* strain TG1 containing the vector pQE60-pspor was diluted and grown to an A_{600} of 0.7−0.9. Expression was induced by adding IPTG to a final concentration of 2 mM, and the culture was grown for an additional 4−5 h before harvesting the cells by centrifugation. The cell pellet was washed once with 30 mM Tris-HCl (pH 7.5) and resuspended in the same buffer, and cells were broken by three passages through a French pressure cell at 1000 psi. The inclusion bodies were pelleted by centrifugation for 5 min at 3000*g*, and the pellets were washed three times with 2% LDAO, 10 mM K-phosphate (pH 7), and 1 mM EDTA. The resulting pellets were stored at −20 °C. For further purification or for direct use in CD measurements, porin pellets were thawed and solubilized at a concentration of 5−10 mg/mL in 1% SDS, 10 mM K-phosphate (pH 7.0), and 0.1 mM EDTA. Further purification of the porin protein was achieved by preparative SDS−PAGE. The concentrated stock was loaded onto a SDS−PAGE and was run at low voltage (100 V) to prevent heating. The band corresponding to the 30 kDa protein was excised from the gel, and the protein was eluted overnight in buffer containing 2% (v/v) Genapol X-80, 10 mM K-phosphate, and 0.1 mM EDTA at 4 °C.

Preincubation with Sterol. Sterol suspensions were prepared by evaporating a solution of the respective sterol in chloroform and then suspending the sterol at a concentration of 1% (w/v) in 2% Genapol, 10 mM K-phosphate, pH 7.0, and 0.1 mM EDTA. One volume of porin was mixed with 1 volume of a 1% (w/v) sterol suspension and vortexed for 10 s. The samples were stored on ice until use.

Lipid Bilayer Experiments. The methods used for the "black" lipid bilayer experiments have been described previously in detail (Benz et al., 1978). In brief, membranes were formed from a 1% (w/v) solution of diphytanoylphosphatidylcholine in *n*-decane across circular holes (surface area about 0.1 mm^2) in the thin wall of a Teflon cell separating the two aqueous compartments. The aqueous solutions were either unbuffered and had a pH around 6, or were buffered with 10 mM Hepes at a pH of 7. Porin was added from the stock solutions to the aqueous phase of the cis compartment (the compartment to which the voltage was applied) after the membranes had turned optically black in reflected light. Zero-current membrane potentials were measured 5−10 min after the application of a salt gradient across the membrane (Benz et al., 1979).

CD Measurements. CD measurements were performed on a Riber Jobin Yvon CD6 spectropolarimeter using 0.1 mm light pathway cells at room temperature while flushing the cuvette chamber with nitrogen gas. Spectra were recorded from 190−260 nm with a resolution of 1 nm and an acquisition time of 1 s/nm. Ten scans were taken for each sample, and the average of these scans was smoothed and stored for further analysis. Blanks of the respective protein-free sample were recorded under the same conditions and subtracted from the protein spectrum before analysis. Only parts of the spectra with an absorption below 1.5 were used for analysis. Analysis of the CD spectra was performed

by expressing the spectra as a linear combination of 4 reference spectra. The coefficients were calculated by a least-squares method as described elsewhere (de Jongh & de Kruijff, 1990) using a computer program kindly provided by H. de Jongh. Data for $(Lys)_n$ (Greenfield & Fasman, 1969) were taken as reference spectra for α-helix, β-sheet, and random coil, and the reference spectrum for β-turns was calculated from 15 proteins of known structure (Chang et al., 1978). This set of reference spectra gave RMS values, as defined by Brahms and Brahms (1980), up to 10.

Other Methods. Small unilamellar vesicles (SUVs) were prepared by taking aliquots of a chloroform solution of the lipid DOPE, evaporating the solvent, and drying the pellet overnight under vacuum. The lipid was then suspended by several freeze/thaw cycles in the respective buffer, and small unilamellar vesicles were formed by sonication. Protein digestion was performed at room temperature and a trypsin concentration of 2 mg/mL. Digestion was stopped after 30 min by adding a 5-fold excess of trypsin inhibitor. Protein concentrations were determined using a commercial BCA assay purchased from Pierce (Rockford, IL).

RESULTS

Expression and Purification of Plastid Porin. The cDNA of porin from non-green plastids has been sequenced in a previous study (Fischer et al., 1994). This allowed us to construct an expression system for the eukaryotic porin. A *Nco*I/*Xho*I-filled fragment of clone pspor 8 representing the entire coding region was subcloned into the bacterial expression vector pQE60 (Diagen, Hilden, Germany). *E. coli* clones harboring the plasmid pQE-pspor were analyzed for expression as described in Materials and Methods. Positive clones expressed the eukaryotic porin having a molecular mass of bout 30 kDa with high efficiency when its expression was induced by the addition of IPTG (Figure 1, lane 4). Control experiments demonstrated that the 30 kDa protein was absent when the *E. coli* cells contained only the expression plasmid pQE60 without the gene of the pea root porin (Figure 1, lane 2) or when the expression of the protein was not induced by the addition of IPTG (Figure 1, lane 3). In fact, in both cases we observed only a faint band in the 30 kDa region (see Figure 1). The recombinant protein was found to be deposited in inclusion bodies in *E. coli* cells, which consisted mainly of the 30 kDa protein. Further purification of the inclusion bodies was achieved by washing them with the LDAO buffer (see Figure 1, lane 5).

The inclusion bodies were subsequently solubilized in 1% SDS solution. The soluble protein was diluted into 2% Genapol and after addition of sterol added to the aqueous phase bathing a black lipid bilayer membrane. Spontaneous insertion of single channels occurred, which had single-channel conductances of about 1.5, 2, and 4 nS in 1 M KCl. After insertion of at least 50 channels, we applied different voltages between 10 and 80 mV to check for voltage-dependent closure, which is a characteristic property of all eukaryotic porins studied to date including the plastid porin (Benz, 1994; Fischer et al., 1994). Only a small part of the inserted channels closed under these conditions (see Figure 2A). This result could be explained either by incorrect folding of the recombinant porin or by its contamination with bacterial porins. The latter porins have a higher channel-forming activity in lipid bilayer membranes than eukaryotic

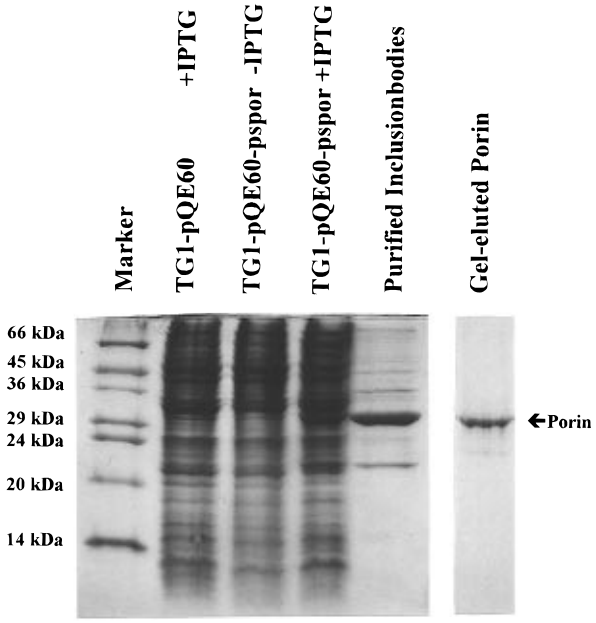

FIGURE 1: Expression and purification of recombinant porin from non-green plastids. Lane 1: Molecular mass markers. Lanes 2−4: *E. coli* cells containing the plasmids indicated in the figure and grown for an additional 4 h in the presence or absence of 2 mM IPTG as indicated. Whole cell pellets were solubilized in SDS buffer. Lane 5: Inclusion bodies purified by 3 wash steps in 2% LDAO buffer dissolved in SDS buffer. Lane 6: Recombinant porin purified by gel elution from preparative SDS−PAGE. Aliquots of the resulting samples were analyzed by SDS−PAGE and staining with Coomassie Blue.

porins and can interfere with their voltage dependence because *E. coli* porins do not close in the considered voltage range (Lakey, 1987). Control experiments with *E. coli* cultures, which habor the pQE60 plasmid without the gene of the pea root porin, confirmed the presence of bacterial porins because they exhibited high channel-forming activity with a single-channel conductance of 1.5 nS in 1 M KCl, but did not show any voltage dependence (Figure 2B).

To remove all bacterial porins, we further purified the eukaryotic 30 kDa porin by elution from preparative SDS−PAGE under nondenaturating conditions. Under these conditions, most of the bacterial porin migrated as trimers with an apparent molecular mass of 66 kDa, which is typical for bacterial porin trimers folded in β-strands. These trimers have in SDS−PAGE a much higher mobility than the unfolded 36 kDa monomers (Benz et al., 1978; Surrey et al., 1996). However, it was also possible to separate the 36 kDa bacterial porin monomers from the 30 kDa eukaryotic porin. The eluted protein (Figure 1, lane 6) showed after the addition of sterol very high channel-forming activity in the lipid bilayer membranes. Furthermore, the voltage dependence was very similar to that of native plastid porin and of other eukaryotic porins (Figure 2C). Thus, the recombinant plastid porin purified by gel elution did not contain any detectable contamination of bacterial porins. These samples were used for all further experiments in the black lipid bilayer system.

Effect of Sterols on Channel Formation by Recombinant Plastid Porin. The influence of sterols on channel insertion was investigated using sterols, which occur in plants (stigmasterol and sitosterol). Similar to the situation in a previous study with water-soluble porin before (Popp et al., 1995), we observed at low salt concentrations of up to 150 mM

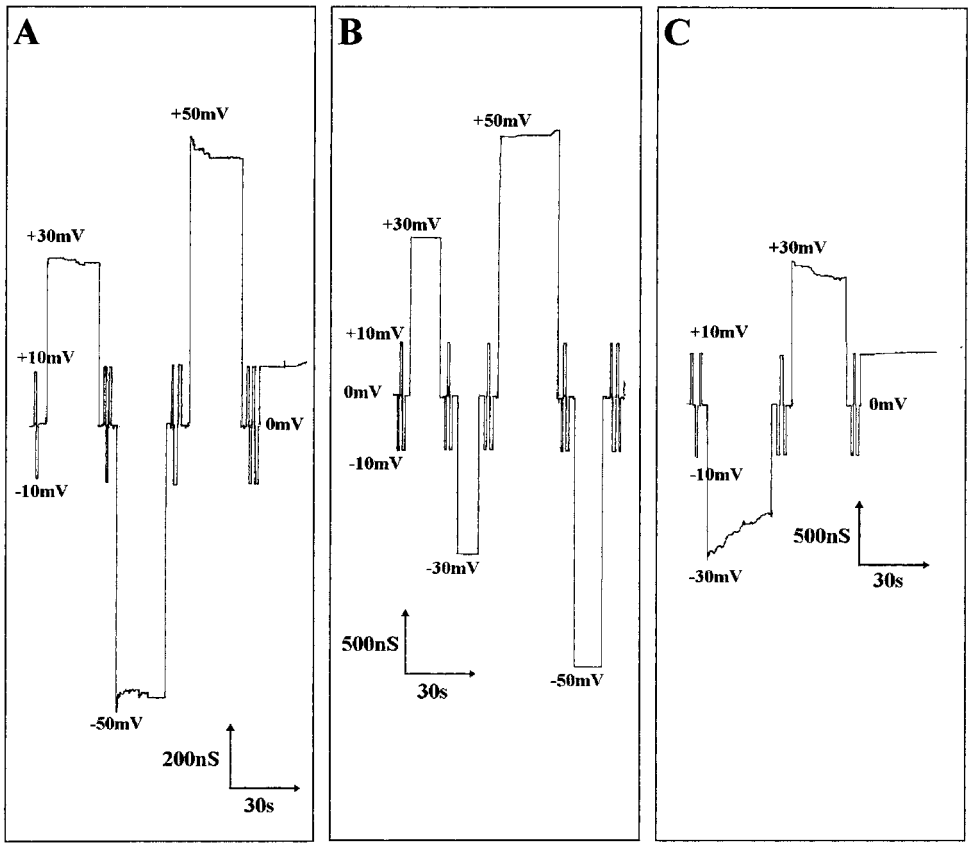

FIGURE 2: Degree of contamination with bacterial porins indicated by the voltage-dependent closure of multiple channels obtained from different preparations. The indicated voltages were applied to a membrane containing at least 50 channels and the membrane conductance is plotted versus time in the figure. Note that the voltage dependence and not the conductance level is typical for the presence of eukaryotic porin. (A) Porin reconstituted from whole inclusion bodies (Figure 1, lane 5). (B) Control to (A) Porin reconstituted as for A, but from *E. coli* cells containing the vector pQE60 without the pspor insert.(C) Recombinant porin from non-green plastids purified by elution from SDS−PAGE (Figure 1, lane 6).

KCl no channel-forming activity without the addition of sterol. Only at very high salt concentration (3 M KCl) did single channels occur without of the addition of sterol. In 1 M KCl, channel-forming activity was much lower (about 100-fold) as compared to that with the addition of sterol. Thus stigmasterol or sitosterol was usually added. The preincubation with sterol yielded the same channel-forming activity as observed with native plastid porin.

Comparison to the Native Porin. To see whether the expression and reconstitution of the recombinant plastid porin had any influence on the channel properties, we compared the channels formed by the recombinant porin in black lipid bilayer experiments to those of the native porin from pea root plastids (Figure 3). Single-channel conductances were measured in different salts and showed characteristic steps for both the native and the recombinant porin. Single-channel distributions of both proteins showed two characteristic maxima at about 2 and 4 nS in 1 M KCl. It is noteworthy that we did not observe the 1.5 nS channels that are typical for bacterial porins. The results of these experiments are summarized in Table 1. Both preparations had single-channel properties very similar to each other and to those observed for mitochondrial porins. The only significant difference between the native and the recombinant porin was the frequent appearance of larger steps in the case of the recombinant porin. These larger steps were probably multiples of the 4 nS channel and indicated the insertion of several channels at once.

When voltages higher than 10 mV were applied to membranes containing many channels, a similar behavior was observed for the native and the recombinant porin. Both channels closed into ion-permeable substates for both negative and positive voltages higher than about ±20 mV in an almost symmetrical manner. The ratio of the conductance G_U at a voltage U divided by the conductance at very low voltage (G_0) resulted for both forms of the porin in a bell-shaped curve that is characteristic for all eukaryotic porins described to date (Benz, 1994; see Figure 4). Only the degree of closure was somewhat smaller for the native porin as compared to the recombinant one and to other eukaryotic porins. The reason for this may be caused by the influence of contaminant proteins, which were not completely removed by the purification procedure of the native porin (described in Materials and Methods).

Investigation of the Secondary Structure of Recombinant Porin in Different Environments by CD Measurements. The recombinant porin showed after reconstitution in lipid bilayer membranes the characteristic properties of eukaryotic porins. Therefore, we considered it a good model system for studies, in which the folding characteristics and the structure of this protein under different conditions were investigated. If not mentioned otherwise, for these measurements we used porin with a purity greater than 95% from inclusion bodies (Figure 1, lane 5). The protein was solubilized in 1% SDS. Its concentration was high enough to obtain a reasonable signal to noise ratio in the CD spectra even after a 1:10 dilution of

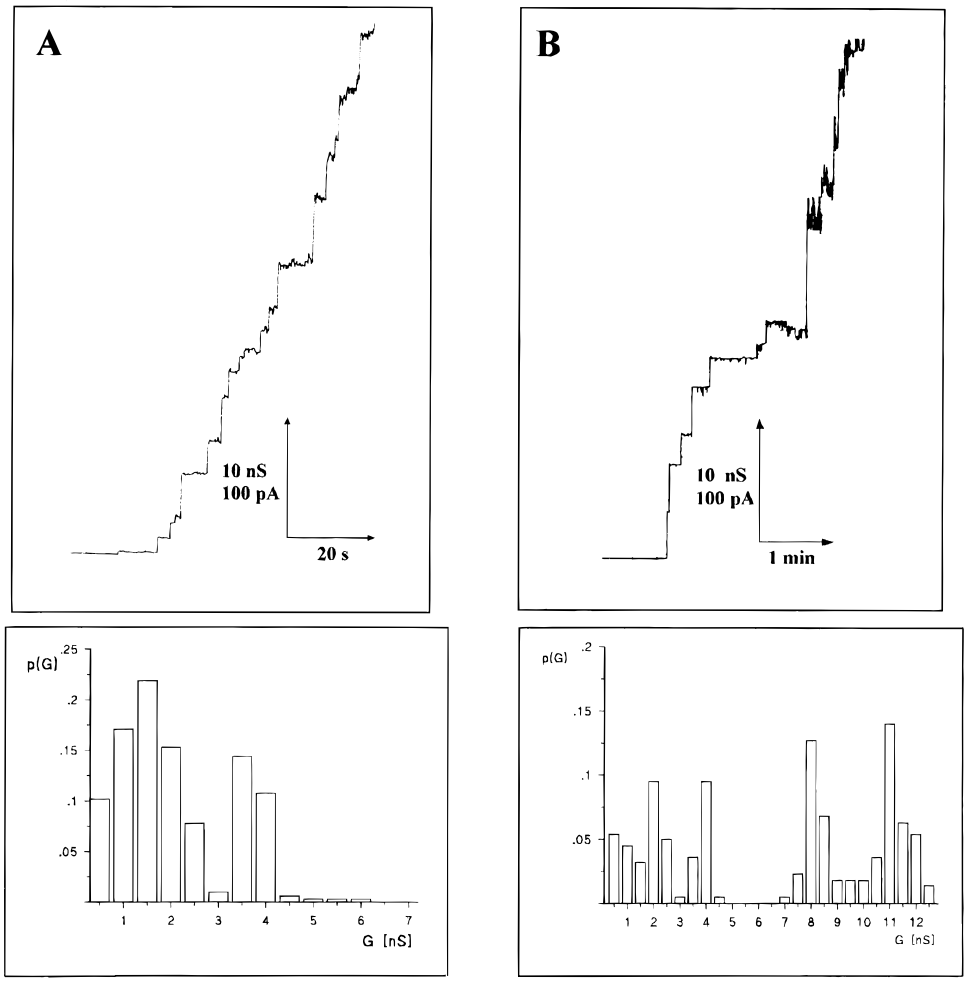

FIGURE 3: Single-channel recordings of native (A) and recombinant (B) porin from non-green plastids inserted into DiphPC/*n*-decane membranes and corresponding histograms of the observed conductance fluctuations. In (A), 5 ng/mL native porin purified as outlined in Materials and Methods; in (B), 1 ng/mL recombinant porin purified by gel elution was added to the aqueous phase bathing a black lipid bilayer. $P(G)$ is the probability that a given conductance increment G is observed in the single-channel experiments. $P(G)$ was calculated by dividing the number of fluctuations with a given conductance increment by the total number of conductance fluctuations. The number of observed conductance increments was 333 for the native porin (A) and 221 for the recombinant porin (B). The aqueous phase contained 1 M KCl. The applied membrane potential was +10 mV; $T = 20$ °C.

Table 1: Single-Channel Conductances of Recombinant and Native Porin of Non-green Plastids in Different Salts[a]

	1 M KCl (nS)	1 M LiCl (nS)	1 M KAc (nS)
native porin	1.5; 3.7	1.5; 3.0	1.6; 2.0
recombinant porin	2.1; 3.9; (8.2; 11.2)	1.5; 2.5; (5.0; 7.8; 9.8)	2.0; (6.4)

[a] The single-channel conductances given in the table represent the peaks of the single-channel histograms. Numbers in parentheses represent the multiples of the larger right side peak observed for the recombinant porin. The applied membrane potential for the determination of the single-channel conductances was +10 mV; $T = 20$ °C.

the protein into the respective buffers (protein concentrations 0.5−1 mg/ml). Protein that precipitated during the dilution was removed by centrifugation, and the supernatant was used to record the spectra.

When the recombinant porin was diluted in 1% SDS, the resulting spectrum showed a peak at 208 nm and a shoulder at 220 nm (Figure 5A). This spectrum is characteristic for a high content of α-helical structure. This was confirmed by the fit procedure of the CD spectrum that resulted in an estimate of about 40% α-helix, 40% random coil, 10% β-sheet, and 5% turn structure. By dilution into water instead of the 1% SDS buffer, the CD spectrum changed slightly (Figure 5A) and the fit resulted in a decrease of the α-helical content to about 35% and an increase of the β-sheet content to about 25%. When the porin was diluted into 2% Genapol buffer, the characteristic α-helical shape of the CD spectrum disappeared and a peak at 216 nm, which is characteristic for β-sheet structure, appeared (Figure 5A). According to this picture, the fit procedure yielded an increased β-sheet content of about 70% and decreased parts of α-helix (20%) and random coil (10%) configurations, while the percentage of β-turns was almost zero. Thus, only in the presence of the nonionic detergent Genapol X-80, the porin showed the expected predominant β-sheet conformation, which agrees with secondary structure predictions (Fischer et al., 1994). No difference in the secondary structure of the protein in different detergents was observed for BSA from which we took spectra as controls (Figure 5B).

To estimate how close the secondary structure in Genapol buffer is to the structure in the native conformation in a lipid environment, we reconstituted the recombinant porin into SUVs from DOPC. Insertion of porin into the vesicles was controlled by treatment of the vesicles with trypsin. The results of tryptic digestion suggested that about 50% of the porin was resistant to proteolysis after incubation with the

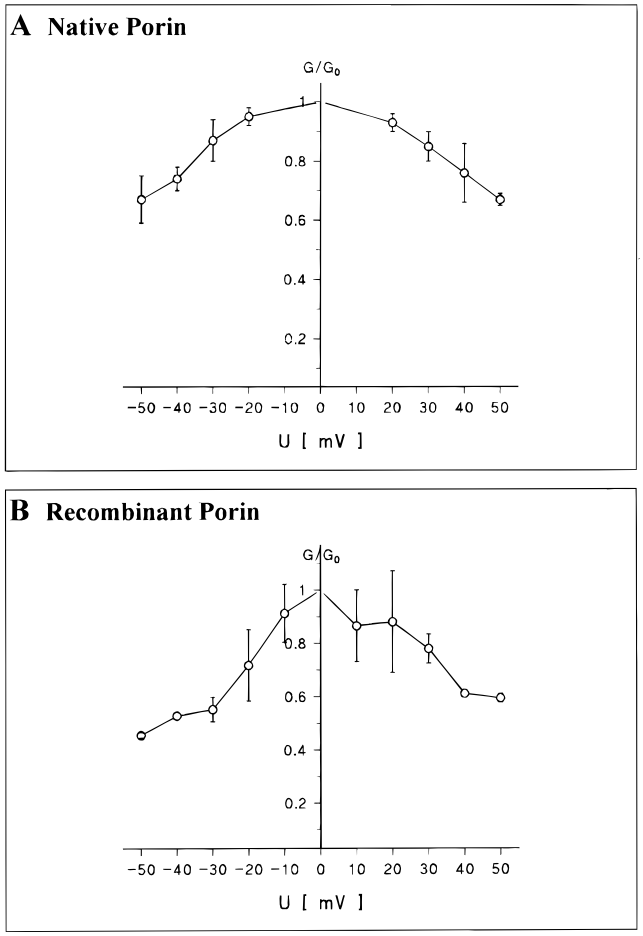

FIGURE 4: Voltage dependence of native and reconstituted recombinant porin from non-green plastids. The ratio of the conductance, G_U, at a given voltage, U, and G_0 at 10 mV is shown as a function of voltage. The membranes were formed of DiphPC/*n*-decane. The aqueous phase contained 1 M KCl (pH 6.0); $T = 20$ °C.

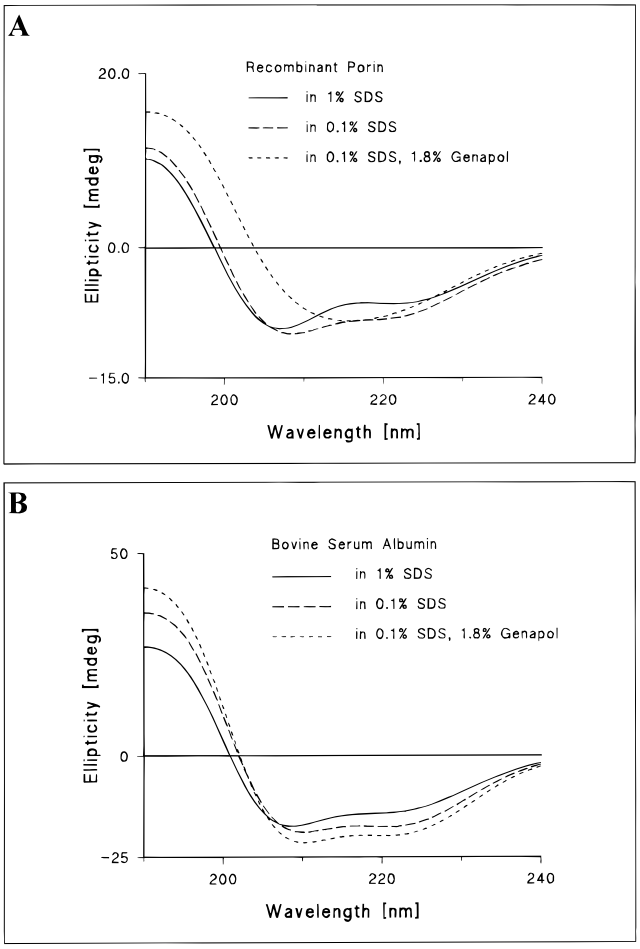

FIGURE 5: CD spectra of recombinant eukaryotic porin (A) and bovine serum albumin (B) under different conditions. The recombinant porins from inclusion bodies and BSA were solubilized at a concentration of 10 mg/mL in 1% SDS and diluted 1:10 into the respective buffer.

SUVs for at least 1 h. The spectra recorded from the vesicle/porin mixture indicated a secondary structure of the porin, which was very similar to that observed in 1.8% Genapol buffer but with a slight, but presumably not significant, increase of the β-sheet content (up to 78%) at the cost of the α-helical part (Figure 6).

Here and in previous studies it has been shown (Pfaller et al., 1985; Popp et al., 1995) that sterols play an important role for the successful reconstitution of unfolded or water-soluble eukaryotic porins in lipid bilayer membranes. Therefore, we wanted to see whether the addition of sterols to the recombinant porin has any influence on its secondary structure. We compared CD spectra of the porin in a suspension of the plant sterols stigmasterol or β-sitosterol in Genapol buffer to spectra recorded in detergent buffer without any sterol. Within the limits of experimental variations, no difference was observed between the spectra with and without sterol, which indicated that the addition of sterol to the porin did not induce distinct changes in the secondary structure.

In a last set of experiments, we investigated the folding process of the recombinant porin during the dilution process in urea. The recombinant porin was dissolved for these experiments in 8 M urea. Then it was diluted in solutions with lower urea concentration but which always contained 2% Genapol. The Genapol was added to prevent precipitation of the porin and to mimic an environment close to the native one in lipid. CD spectra in 8, 6, 4, and 0.5 M urea were taken and analyzed (Figure 7). While the porin appeared to be in a mostly unordered conformation in 6 and 8 M, urea it seemed to gain a more α-helical structure in 4 M urea. At smaller urea concentration, the recombinant

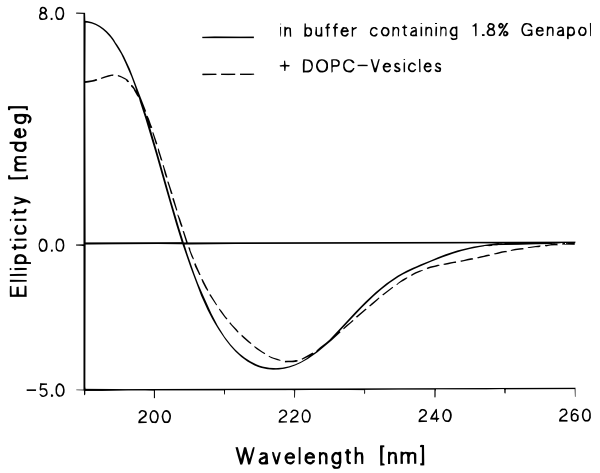

FIGURE 6: CD spectra of the recombinant eukaryotic porin solubilized from inclusion bodies at a concentration of 10 mg/mL in 1% SDS and diluted 1:20 into 2% Genapol buffer (10 mM K-phosphate, pH 7.0, 0.1 mM EDTA) or into a solution containing the following final concentrations: 5 mM DOPC vesicles, 10 mM K-phosphate, pH 7.0, and 0.002% Genapol.

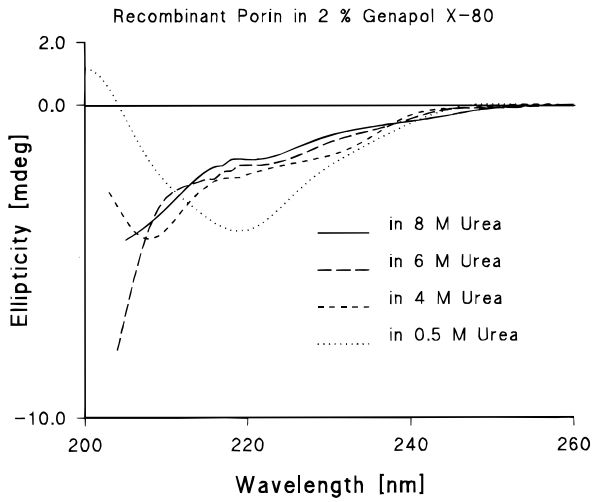

FIGURE 7: CD spectra of the recombinant eukaryotic porin solubilized from inclusion bodies at a concentration of 8 mg/mL in a buffer containing 8 M urea, 2% Genapol, 10 mM K-phosphate, pH 7.0, and 0.1 mM EDTA and diluted 1:15 into buffers containing final concentrations of urea as indicated in the figure and 2% Genapol, 10 mM K-phosphate, pH 7.0, and 0.1 mM EDTA.

porin assumed a structure which was very close to that of the protein in 1.8% Genapol X-80 or in SUVs. This means that it contained a large amount of β-sheet structure when the urea became more diluted. However, these spectra have to be interpreted with care because urea reduces the resolution of the CD spectra at low wavelength because of its high absorbency in the UV range.

DISCUSSION

Functional Properties of Recombinant Pea Root Plastid Porin Reconstituted in Lipid Bilayers. In this work we studied structural and functional properties of recombinant pea root plastid porin expressed in *E. coli*. The basic advantage of this system is that it allows the expression of large amounts of a eukaryotic porin from non-green plastids and its subsequent reconstitution. Pea root plastid porin in its native and its recombinant forms had the same characteristic biophysical properties as other eukaryotic porins and thus presented a good model for the investigation of the structure and the folding process of eukaryotic porins to channel-forming components. The porin from non-green plastids was expressed very efficiently in *E. coli* cells as control experiments revealed (see Figure 1) and was deposited in inclusion bodies. The aggregated porin could be partially purified by washing the protein pellets with a buffer containing the detergent LDAO. However, for complete removal of bacterial porins, which was necessary before lipid bilayer experiments could be performed, further purification steps such as elution from a preparative SDS−PAGE were necessary. The recombinant porin could be dissolved in SDS, or alternatively in somewhat lower concentration in 8 M urea. By its dilution into 2% Genapol buffer and addition of sterol it folded into a form that had the same channel-forming activity as native porin. The requirement for sterol for channel formation has been observed before (Pfaller et al., 1985; Popp et al., 1995; Carbonara et al., 1996) and was confirmed here for salt concentrations in the physiological range. It is noteworthy that it has been observed previously that eukaryotic porins loose their channel-forming properties when they are solubilized in ionic detergents such as SDS or cholate (De Pinto et al., 1989; Popp et al., 1995), but not in nonionic detergents such as Genapol, Triton, or LDAO.

In a first set of experimental conditions, we checked whether the expression and reconstitution procedure had any influence on the functional properties of the porin, namely, on its channel-forming properties in the black lipid bilayer system. The reconstituted, recombinant protein did not show any significant difference in single-channel conductance and voltage dependence as compared to its native form and a number of eukaryotic porins studied to date (review: Benz, 1994). The appearance of larger steps, which indicated the insertion of multiples of single channels, could be explained by a strong aggregation tendency of the highly concentrated recombinant porin. The insertions of multiple channels could be minimized by further dilution of the reconstituted samples in Genapol buffer for a longer time. It is noteworthy that the eukaryotic porin as purified by preparative SDS−PAGE did not contain any bacterial porin contaminant.

CD Spectroscopy of Different Forms of the Recombinant Porin. The recombinant eukaryotic porin could be folded into a protein with native channel properties. Thus, it seemed to be a good system for the study of its secondary structure and the folding process of a polypeptide chain into a membrane channel. Similar studies have until now only been performed with Gram-negative bacterial porins (Markovic-Housley & Garavito, 1986; Eisele & Rosenbusch, 1990, Surrey & Jähnig, 1992; Wei & Fasman, 1995; Surrey et al., 1996; Schmid et al., 1996), which probably have a high structural similarity with eukaryotic porins (De Pinto et al., 1991; Benz, 1994; Mannella et al., 1996). In a first set of experiments, we measured the secondary structure of the recombinant porin under different conditions, by using far-UV-CD spectroscopy. The eukaryotic porin solubilized in 1% SDS buffer contained a high amount of α-helical structure together with some random coil and almost no indication for β-sheet structure. Similar observations have been made previously for bacterial porins, which have also a high content of α-helical structure in SDS buffer (Eisele & Rosenbusch, 1990). By dilution into water (final SDS concentration: 0.1%) the amount of α-helix slightly decreased and some β-sheet structure appeared. When the SDS solution was diluted by 2% Genapol (SDS concentration 0.1%), the β-sheet content of the protein strongly increased, while the a-helical part sank below 20%. This means that the protein now had a secondary structure that was close to that of the secondary structure predictions (Fischer et al., 1994) and to that of native porin of *Neurospora crassa* (Shao et al., 1996). It is noteworthy that the eukaryotic porin has under these conditions a CD spectrum that is very close to that of bacterial porins (Markovic-Housley & Garavito, 1986).

Mixed secondary structure similar to our results has been found also for OmpA after dilution into water (Surrey & Jähnig, 1992), although this protein does not represent an outer membrane porin, and predominantly β-sheet structure was measured in a buffer containing nonionic detergent for porin from *Rhodobacter capsulatus* in octyltetraoxyethylene (Park et al., 1992) and for *E. coli* porin in β-octyl glucoside and octylpentaoxyethylene, respectively (Markovic-Housley & Garavito, 1986; Eisele & Rosenbusch, 1990). The structure of the eukaryotic porin in Genapol containing about 70% β-sheet was found to be very similar to the presumably

native structure in DOPC vesicles (Figure 6). Addition of sterol to the porin in 2% Genapol did not cause any change in the secondary structure, not even the slight increase in β-sheet content accompanied with a decrease in α-helix that was observed after addition of the vesicles. This is a very interesting result, when we consider the strong influence of sterols on the reconstitution rate of recombinant porin. This means probably that sterol is not essential for the formation of the β-strands, which we originally thought (Popp et al., 1995), i.e., it does not seem to work by marked changes in the structure caused by the association with the sterol. Sterol acts either by a change in the tertiary structure, which could not be observed in the CD spectra, or by a charge shielding effect of the channel-forming structure through associated sterol molecules. The observed dependence of the sterol-mediated reconstitution rate on the salt concentration argues for the latter mechanism.

Under all conditions, in which the eukaryotic porin was not completely denatured, it retained at least some small amount of α-helix structure, in agreement with predictions. The N-terminal part of the protein forms according to secondary structure predictions, and comparison to mitochondrial presequences an α-helix (Mihara & Sato, 1985; Kleene et al., 1987; Kayser et al., 1989), which was confirmed by CD spectroscopy of peptides with a sequence corresponding to residues 1−20 of the *N. crassa* mitochondrial porin (Guo et al., 1995).

Although our results concerning the structure formation of the porin are still preliminary, some things can be learned about the folding process. The eukaryotic porin seems to exist in quite different conformations depending on the environment of the protein as observed before for bacterial porins (Markovic-Housley & Garavito, 1986; Eisele & Rosenbusch, 1990; Wei & Fasman, 1995). It shows a probably native channel conformation in vesicles and in nonionic detergent, as concluded from channel formation in the artificial bilayer and the expected high β-sheet content in the CD spectra (Figures 5A and 6). As concluded from the native-like structure in Genapol buffer, the importance of the presence of nonionic detergents for channel formation is possibly not only caused by a disturbance of the bilayer structure through the detergent, that facilitates channel insertion (Dargent et al., 1987), but also by a supporting role of the nonionic detergent in structure formation, which for the relatively polar porins might occur already in the aqueous phase before insertion into the lipid phase takes place. In 2% SDS the eukaryotic porin adopts a presumably misfolded state, which results in the inability to form channels in the bilayer and is characterized by a high amount of α-helix in its CD spectrum (Figure 5A). The same increase in α-helix coming from a mostly denatured state in 8 M urea was observed after dilution into intermediate urea concentrations (4 M). At further dilution into 0.5 M urea, a high content of β-sheet appeared (Figure 7). Hence the dominantly α-helical conformation could also play a role as a folding intermediate similar to the molten globule postulated in the folding process of soluble proteins. This molten globule is believed to arise from a hydrophobic collapse in which the hydrophobic core of water-soluble proteins forms, but not their detailed final structure (Christensen & Pain, 1991). Hydrophobic effects might also play a role in the aggregation of the eukaryotic porin, which easily occurs if no detergents, vesicles, or chaotropic agents are present, and which is also accompanied by an increase in the content of α-helix (data not shown).

Further experiments will be necessary to elucidate the folding and insertion process of eukaryotic porins. In particular, a closer look at vesicle systems, using different lipids under different conditions, and time resolved folding studies should be of great value.

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