A Poly(Ethylene Glycol) Water-soluble Conjugate of Porin: Refolding to the Native State†

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ABSTRACT: Porin, from *Rhodabacter capsulatus*, was chemically modified with methoxypoly(ethylene glycol) (m-PEG; molecular mass = 5000 Da) succinimidyl carbonate to yield methoxypoly(ethylene glycol)-porin (m-PEG-SC-Porin), as previously reported for bacteriorhodopsin [Sirokman, G., & Fasman, G. D. (1993) *Protein Sci. 3*, 1101−1170]. The m-poly(ethylene glycol)-porin (m-PEG-SC-Porin 50) conjugate, containing one poly(ethylene glycol) chain, was water soluble. The secondary structure of the conjugate in water was mainly random coil. Circular dichroism spectroscopy showed it was predominantly in the β-pleated sheet structure in 0.6% octyltetraoxyethylene and 0.3 M LiCl, as was porin. A proteoliposome, containing the isolated porin conjugate, was prepared to measure permeability of the sugar stachyose. The m-PEG-SC-Porin 50 proteoliposome of porin maintained the permeability for the sugar, as did the proteoliposome of porin. The swelling rate of the conjugate versus the sugar was lower than it was for porin. This indicated that a pore in the conjugate exists but perhaps with a slightly different pore size. The refolding of the conjugate was studied by stepwise addition of trifluoroethanol (TFE) to lower the dielectric constant, simulating the insertion of porin into the membrane. An α-helical structure that did not exist in the native porin was formed with the m-PEG-SC-Porin 50, upon the addition of TFE, and the helicity increased with increasing concentrations of TFE. The m-PEG-SC-Porin 50 could be stepwise refolded to the native conformation, predominantly in the β-sheet conformation, by the addition of hexafluoro-2-propanol in the 5−10% concentration range. With the addition of HFIP beyond 10%, an α-helical structure was formed. This indicates that the folding of porin requires a highly specific environment, as is found in the membrane.

In a previous study (Sirokman & Fasman, 1993), a water-soluble membrane protein, methoxypoly(ethylene glycol) succinimidyl carbonate bacteriorhodopsin (m-PEG-SC-BR)[1] was synthesized. This was accomplished by covalently linking methoxypoly(ethylene glycol) (m-PEG, 5000 Da) to the exposed sequences of the membrane protein bacteriorhodopsin *in situ*. This water-soluble m-PEG-SC-BR could be refolded into liposomes which pumped protons upon exposure to a 555 nm light source. This conjugate was suggested to be a suitable model for the simulation of refolding of the conjugate as it was inserted into a membrane. The prevailing theory of protein folding (Dill *et al.*, 1993; Freire *et al.*, 1993) suggests this transition is a cooperative process. The closely packed nature of a protein is probably stabilized by a cooperative network of various types of interactions. The alteration of a part of the structure may be propagated to other parts of the molecule. The cooperative process of protein folding is dependent on the characteristic fold of the specific secondary structural attributes of the protein and the specific dielectric of the membrane. Bacteriorhodopsin (BR) contains a seven-helix bundle in the membrane (Henderson *et al.*, 1990). The mechanism of refolding of the α-helical protein (BR) might be different than that of a protein which consists mainly of β-sheets (porin).

Porin is a channel-forming protein of the outer membrane of Gram-negative bacteria (Jap, 1990), through which an influx of nutrients and antibiotics occurs. The structure and function of bacterial porins have been reviewed extensively (Nikaido, 1994; Schulz, 1993). The results from X-ray diffraction studies have indicated that the porin from *Rhodobacter capsulatus* is predominantly in a β-sheet conformation and has only a small fraction of their structure is α-helical (Weiss *et al.*, 1990). It has a defined structure and could serve as a model for studying the folding of a membrane protein that consists mainly of β-sheets.

Unfolding and refolding of porin, from *Escherichia coli*, has been reported by Eisele and Rosenbusch (1990). They found that porin is transformed into a random coil conformation in 6 M guanidine·HCl or into an α-helical structure in SDS. The protein refolds in the presence of amphilic

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[1] Abbreviations: m-PEG, methoxypoly(ethylene glycol) (5000 Da); m-PEG-SC-porin 50, porin covalently modified with one chain of methoxypoly(ethylene glycol)succinimidyl carbonate, having an apparent molecular mass of 50 kDa; m-PEG-SC-porin 66, porin covalently modified with two chains of methoxypoly(ethylene glycol)succinimidyl carbonate, having an apparent molecular mass of 66 kDa; m-PEG-SC-porin 82, porin covalently modified with three chains of methoxypoly-(ethylene glycol)succinimidyl carbonate, having an apparent molecular mass of 82 kDa; m-PEG-SC, methoxypoly(ethylene glycol)succinimidyl carbonate; SDS, sodium dodecyl sulfate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TFE, trifluoroethanol; HFIP, hexafluoro-2-propanol; DNase, deoxyribonuclease; RNase, ribonuclease; EDTA, ethylenediaminetetraacetic acid; LPS, lipopolysaccharides.

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molecules, attaining full structural and functional competence. They suggested that porin folding and insertion might occur in two steps: folding to a conformation close to the native state and its subsequent insertion into the lipid bilayer, the latter being aided by amphiphiles. But it still remains unclear how porin is inserted into the membrane. The reason may be that the normal methods for studying water-soluble proteins cannot be used because of the lack of solubility of membrane proteins in water.

Conjugation of m-PEG with proteins increased the solubility of proteins in organic solvents, and they remained in a partial or full biological active state (Kajihara *et al.*, 1994), extended circulating lifetimes *in vivo*, and had little or no immunogenicity (Ratner, 1990; Berger & Pizzo, 1988; Knauf *et al.*, 1988; Kajihara *et al.*, 1994). Previously, it was reported that m-PEG modified bacteriorhodopsin altered its solvation properties to become water-soluble (Sirokman & Fasman, 1993). It was suggested that m-PEG-SC-BR might be a suitable model for the investigation of refolding of this membrane protein derivative as it was inserted into a membrane. One way to simulate folding in a membrane is to simply mix the water solution with organic solvents of different polarity.

With the same purpose in mind, the chemical modification of porin with methoxypoly(ethylene glycol) succinimidyl carbonate is reported in this paper. m-PEG-SC-Porin has been synthesized and purified. This modified water-soluble membrane protein was studied to examine its refolding and its permeability to sugar in liposomes. As with porin, it contains a high content of β-sheet, as has been previously shown by CD spectra in octyltetraoxyethylene and LiCl (Park *et al.*, 1992). m-PEG-SC-Porin also exhibits permeability of a small sugar molecule, stachyose, when it is embedded in a liposome.

The stepwise refolding of m-PEG-SC-Porin 50 was demonstrated by the stepwise addition of trifluoroethanol or hexafluoro-2-propanol to lower the dielectric constant, simulating porin insertion into the bilayer. The results indicated the conformation of porin is *dependent* on the nature of the solvent. In a limited range of low concentration of hexafluoro-2-propanol (5−10%), the conjugate refolded to a structure which was very close to its native state, namely, a β-sheet structure. In the trifluoroethanol, however, the conjugate refolded to a structure which consisted mainly of α-helices. This would suggest that such conjugates require a very specific environment or associate with a particular molecule to be inserted into the membrane to attain its native conformation. It was shown earlier (Dargent *et al.*, 1987) that detergent dialysis was required for the insertion of native porin into preexisting vesicles.

MATERIALS AND METHODS

Isolation of the Cell Wall of R. capsulatus. *R. capsulatus* strain 37b4 was grown and isolated by using the procedures of Schiltz *et al.* (1991). Twenty-five grams of *R. capsulatus* was washed with 10 mM $MgCl_2$ and 20 mM Tris-HCl, pH 8.0. The pellet was resuspended in 15 mL of the same buffer with approximately 5 mg each of DNase and RNase, mixed with glass beads (diameter 0.1 mm; suspension/beads, 1:2, by vol) and disrupted for 20 min at 4 °C in a bead beater (Biospec, No. 11079-00-101). Glass beads were removed on a glass-frit filter. Cell envelopes were washed three times with the same buffer by homogenizing in a Wheaton tissue grinder and centrifuging at 8000 rpm in a JA-20 rotor in a Beckman J2-21 centrifuge for 20 min at 4 °C. The procedure was then repeated twice with 2% Triton X-100 in this buffer, first by stirring for 40 min at 40 °C and centrifugation, and subsequently by stirring for 30 min at 38 °C, followed by centrifugation. Triton was removed by homogenizing and centrifuging the cell walls four times with the same Triton-free buffer. The centrifugation was conducted at 4 °C. The cell envelops were extracted in a buffer containing 2% SDS, 10% glycerol, and 20 mM Tris-HCl (pH 8.0) at 50 °C for 30 min.

To purify the porin, the pellet was resuspended in a buffer containing 2% SDS, 0.5 M NaCl, 0.01% 2-mercaptoethanol, 10% glycerol, and 20 mM Tris-HCl, pH 8.0, at 37 °C for 30 min. After centrifugation (at 45 000 rpm in a 70.1 Ti rotor in a Beckman L7-55 centrifuge, 30 °C, 1 h) the supernant was dialyzed against 0.01% SDS at room temperature for 2 days. The SDS was removed from the protein by passing the solution thru an Extractigel column (Pierce). The protein was eluted with a 10 mM Na_2PO_4 containing 10 mM EDTA buffer, pH 8.0. The porin fractions were collected and concentrated by using a Centricon-10 concentrator (Amicon).

Coupling of the Cell Wall of R. capsulatus with m-PEG-SC To Yield MeO-(CH_2-CH_2-O)$_x$-CO-NH-Porin (m-PEG-SC-Porin). Four grams of the cell wall of *R. capsulatus* was suspended in 16 mL of 0.1 M sodium borate buffer, pH 9.3, with stirring, and 4 g of poly(ethylene glycol)succinimidyl carbonate (PEG-SC) (Zalipsky *et al.*, 1991) was added. The mixture was stirred at room temperature for 60 min and then centrifuged at 45 000 rpm in a 70.1 Ti rotor in a Beckman L7-55 centrifuge, 20 °C, for 1 h. The pellets were washed four times with 10 mM $MgCl_2$ and 20 mM Tris-HCl, pH 8.0.

Purification of m-PEG-SC-Porin. To solubilize the m-PEG-SC-porin, the pellet was resuspended in a buffer containing 2% SDS, 0.5 M NaCl, 0.01% 2-mercaptoethanol, 10% glycerol, and 20 mM Tris-HCl, pH 8.0 at 37 °C for 30 min. After centrifugation at 45 000 rpm in a 70.1 Ti rotor in a Beckman L7-55 centrifuge, 20 °C, for 1 h, the supernant was dialyzed against 0.01% SDS at room temperature for 2 days.

The m-PEG-SC-Porin conjugates were isolated by preparative gel electrophoresis (Huang *et al.*, 1981). Three bands were observed, with apparent molecular masses of 50 kDa (m-PEG-SC-Porin 50), 66 kDa (Di-m-PEG-SC-Porin 66), and 82 kDa (Tri-m-PEG-SC-Porin 82). After the bands were separated by splicing the gel, the protein was isolated by electroelution in a Bio-Rad Model 422 Electro-Eluter.

Removal of SDS from m-PEG-SC-Porin. EDTA was added to the m-PEG-SC-Porin samples obtained by electroelution, to a final concentration of 10 mM. The SDS was removed from m-PEG-SC-Porin samples by passing each over an Extractigel column (Pierce). The protein eluted with 10 mM Na_2PO_4 containing 10 mM EDTA buffer, pH 8.0. The m-PEG-SC-Porin fractions were collected and concentrated by using a Centricon-10 concentrator (Amicon). The clear supernatant was used for protein and detergent analysis. At least 99.5% of the SDS was removed. The final aqueous solution contained only six molecules of SDS per protein molecule. The content of the SDS in the protein solution was determined by the method of Hayashi (1975). The

protein concentration was determined by BCA assay (Pierce) (Smith *et al.*, 1985).

Preparation of Proteoliposomes and Measurement of Their Osmotic Swelling Rate. A mixture of 2.4 μmol (1.68 mg) of egg phosphatidylcholine (type IX, Sigma) and 0.2 μmol (0.11 mg) of dicetyl phosphate were dried under a stream of N_2 in a test tube. The lipids were dissolved in anhydrous benzene, dried by blowing N_2 over it to remove traces of water, and then dissolved in ethyl ether and dried on a laboratory rotatory evaporator to produced a thin even film. The drying process was completed by leaving the tubes over silica gel for at least 2 h in an evacuated desiccator, using a water aspirator, and then the lipid film was resuspended in 0.2 mL of water or water containing the purified protein (for proteins, 40 μg of SDS were introduced/1 mol of phospholipid). The suspension was mixed by vortexing and finally by a brief (1 min) sonication in a bath-type sonicator. The lipid/protein mixture was then dried using a water aspirator, while rotating the tubes in a water bath at 45 °C. The drying was then continued by leaving the test tubes in a vacuum dessicator, containing silica gel, evacuated using a water aspirator, for at least 1 h. The dried protein/lipid film was resuspended in 5 mM Tris-HCl buffer, pH 8.0, containing 15% of Dextran T-40 (Pharmacia), first by wetting the film by the gentle rotation of the test tube, followed 30 min later by hand shaking of the tubes. To measure the swelling rate, 0.68 mL of an isotonic solution of a test solute and 0.02 mL of the liposome suspension were added. The content is mixed by shaking rapidly, for about 15 s. The absorbance is recorded at 400 nm on a Aviv 14DS spectrophotometer as a function of time.

Other Methods. UV and visible spectra were recorded on a Aviv 14DS spectrophotometer. CD spectra were recorded on a computer-operated Jobin Yvon Auto-Dichrograph Mark V instrument (Prevelige & Fasman, 1987). CD spectra of the porin and m-PEG-SC-Porin 50 were recorded at a protein concentration of 2 mg/mL in a 0.1 mm cell (Helma) under various conditions. Apparent molecular masses were determined on 9% polyacrylamide gels by SDS−PAGE (Laemmli, 1970). The m-PEG content of the conjugate was determined by the method of Skoog (1979). Analytical ultracentrifugation was performed on an F rotor in a Beckman model E analytical ultracentrifuge (at 32 000 rpm for 60 min, 26 000 rpm for 90 min at 10 °C), using short columns and overspeeding (Perez-Ramirez *et al.*, 1994). The distribution of the monomer and trimer of porin or m-PEG-SC-Porin 50 was monitored by nondenaturing gel electrophoresis (Hames, 1990).

Stepwise Refolding of m-PEG-SC-Porin in TFE and HFIP. The samples of the m-PEG-SC-Porin 50 were prepared for CD spectroscopy by mixing the appropriate volume of the water solution with the specified amount of TFE or HFIP.

RESULTS

Chemical Modification of Porin. Porin was transformed into three products by the reaction of m-PEG-SC with the cell wall of *R. capsulatus* (Figure 1). The three products were separated by SDS−PAGE electrophoresis (Laemmli, 1970), into three major bands with apparent molecular masses of 50, 66, and 80 kDa. The bands were cutout and electroluted separatly in a Bio-Rad apparatus. SDS was successfully removed from these products by chromatography on Extractigel columns. The bound SDS was determined by Hayashi's method (Hayashi, 1975). A small amount of SDS remains bound to the protein (about six molecules per protein molecule). Clear solutions were obtained for each product. These solutions were stable at room temperature for at least a month. SDS−PAGE electrophoresis was used to identify the purity of these products (Figure 2).

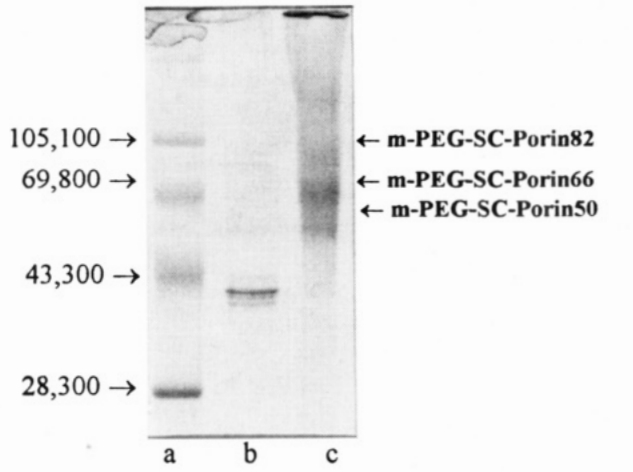

FIGURE 1: SDS−PAGE profiles of a mixture of m-PEG-SC-Porin conjugates. (Lane a) Molecular weight standards (Gibco BRL, No. 26041-020; phophorylase B, 105 100; bovine serum albumin, 69 800; ovalbumin, 43 300; carbonic anhydrase, 28 300). (Lane b) Porin. (Lane c) Mixture of m-PEG-SC-Porin conjugates.

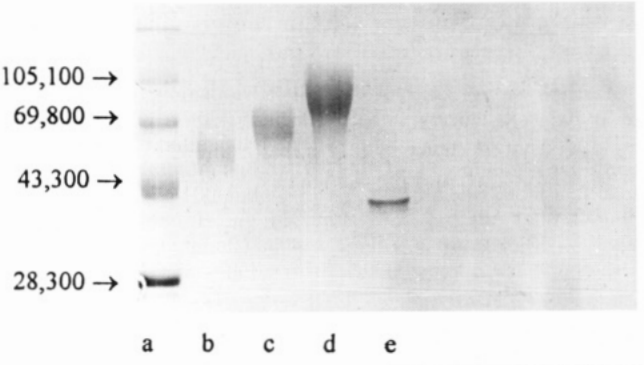

FIGURE 2: SDS−PAGE profiles of porin, m-PEG-SC-Porin 50, m-PEG-SC-Porin 66, and m-PEG-SC-Porin 82. (Lane a) molecular weight standard (same as in Figure 1). (Lane b) m-PEG-SC-Porin 50. (Lane c) m-PEG-SC-Porin 66. (Lane d) m-PEG-SC-Porin 82. (Lane e) porin.

Samples of the conjugate were analyzed for protein and m-PEG to determine the stoichiometry of the coupling reaction. m-PEG-SC-Porin 50 was found to contain 1.1 PEG group per molecule of the conjugate. The m-PEG content was determined by Skoog's method (Skoog, 1979) and protein content by BCA assay (see Material and Methods).

Conformation of m-PEG-SC-Porin 50 in H_2O or Detergents: CD Spectroscopy. The CD spectrum of m-PEG-SC-Porin 50 in H_2O indicated a random coil structure (Greenfield & Fasman, 1969) of the conjugate (Figure 3), as did porin in H_2O. There is a slight difference between the spectra of porin and m-PEG-SC-Porin 50; however, the difference is minor. In 0.6% octyltetraoxyethylene and 0.3 M LiCl, both porin and m-PEG-SC-Porin 50 contained high contents of β-sheet structures based on their CD spectra (Figure 4). Separately, in octyltetraoxyethylene or LiCl, neither porin nor m-PEG-SC-Porin 50 folded into their native structure.

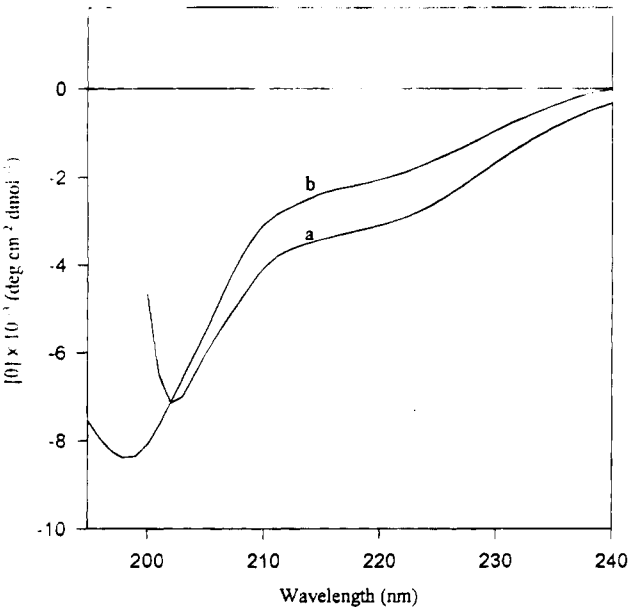

FIGURE 3: Circular dichroism spectra of porin (curve a) and m-PEG-SC-Porin 50 (curve b) in water. Protein concentration: m-PEG-SC-Porin 50 = 2.208 mg/mL; porin = 4.85 mg/mL. The cell path length was 0.01 cm. The spectra represent the average of four scans recorded at 20 °C.

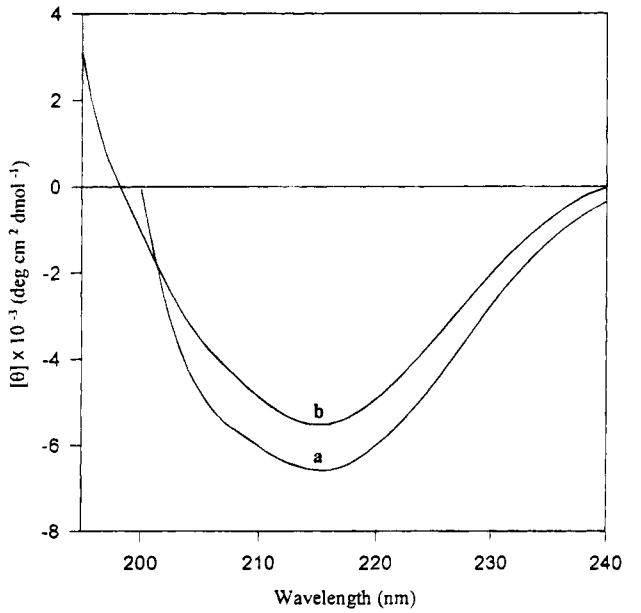

FIGURE 4: Circular dichroism spectra of porin (curve a) and m-PEG-SC-Porin 50 (curve b) in the 0.6% octyltetraoxyethylene and 0.3 M LiCl. Protein concentration: m-PEG-SC-Porin 50 = 2.208 mg/mL; porin = 4.24 mg/mL. The cell path length was 0.01 cm. The spectra represent the average of four scans recorded at 20 °C.

Other detergents such as SDS and β-octylglucoside also failed to cause porin and m-PEG-SC-Porin 50 to fold correctly. When PEG-5000 was added to porin with a ratio of PEG/Porin = 1, the CD spectrum of porin was unchanged. Similarly, when PEG was added to porin in 0.6% octyltetraoxyethylene and 0.3 M LiCl, no change occurred. Similarly, when lipopolysaccharides (LPS) were added to porin at different ratios, it did not change the CD spectra of porin in H_2O or in the 0.6% octyltetraoxyethylene and 0.3 M LiCl. Both PEG-5000 and LPS, when added to porin, did not change the CD spectra significantly.

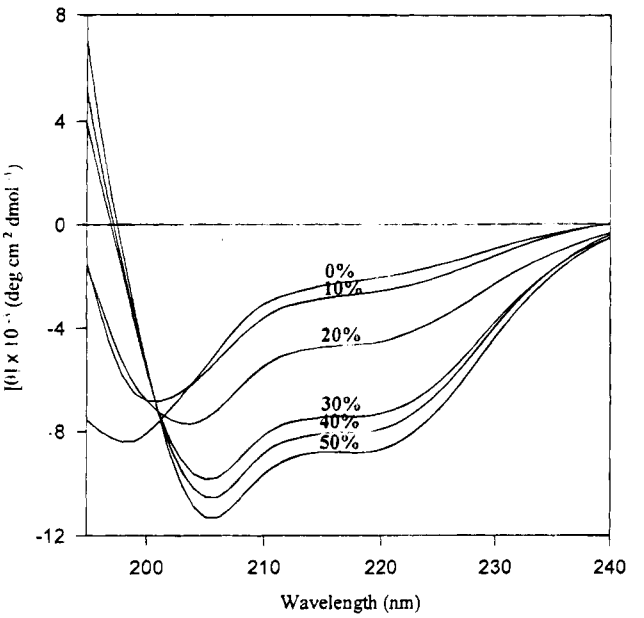

FIGURE 5: Circular dichroism spectra of m-PEG-SC-Porin 50 as a function of trifluoethanol concentration. Protein concentration: m-PEG-SC-Porin 50 = 2.208 mg/mL. The cell path length was 0.01 cm. The spectra represent the average of four scans recorded at 20 °C.

Folding of Porin and m-PEG-SC-Porin 50 in Trifluoroethanol. By the stepwise addition of TFE to an aqueous solution of porin or m-PEG-SC-Porin 50, a solvent known to induce secondary structure, the change in secondary structure was followed by utilizing CD spectroscopy (Figure 5). A gradual increase in the helical content was observed upon increasing the TFE concentration from 0% to 50% for both porin and the porin conjugate.

Folding of Porin and m-PEG-SC-Porin 50 in Hexafluoro-2-propanol. By the stepwise addition of hexafluoro-2-propanol (HFIP) to an aqueous solution of porin or m-PEG-SC-Porin 50, the change in secondary structure, as measured by CD, showed that there was a high content of β-sheet structure induced with low concentrations of HFIP, in the range of 5−10% HFIP (Figure 6). In the lower concentrations of <5% HFIP, both porin and m-PEG-SC-Porin 50 are in the random structure. At higher concentrations of HFIP (>10%), both porin and m-PEG-SC-Porin 50 fold to α-helical structures. The β-sheet structure, a structure close to the native state of the protein, looks similar to the folding intermediate in the folding by HFIP. The transition appears to be from random coil to β-sheet and then β-sheet to α-helix. This indicates that the β-sheet structure is not the final stable state in these mixed solvent solutions, as is found in the membrane environment.

Permeability of Porin and m-PEG-SC-Porin 50 to Stachyose in Proteoliposomes. Porin and m-PEG-SC-Porin 50 were embedded in proteoliposomes, and the swelling rates were determined upon the addition of stachyose to the suspensions. The changes in the optical density traces of proteoliposome-containing porin (Figure 7) or m-PEG-SC-Porin 50 (Figure 8) diluted into hyper- (50 mM stachyose for proteoliposome-containing porin, more than 37.1 mM stachyose for proteoliposome-containing m-PEG-SC-Porin 50) and hyposomotic (35.7 mM stachyose for both) stachyose solution, were obtained. (Hyper- or hyposomotic is the osmotic concentration outside of liposome compares to the osmotic concentra-

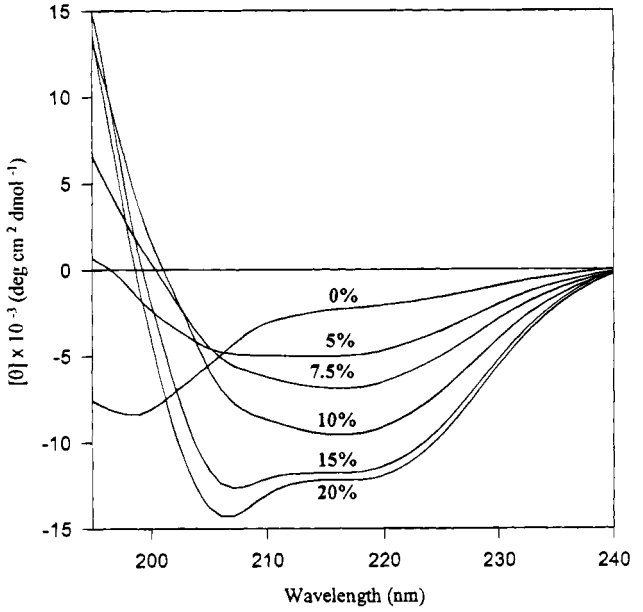

FIGURE 6: Circular dichroism spectra of m-PEG-SC-Porin 50 as a function of hexafluoro-2-propanol concentration. Protein concentration: m-PEG-SC-Porin 50 = 2.208 mg/mL. The cell path length was 0.01 cm. The spectra represent the average of four scans recorded at 20 °C.

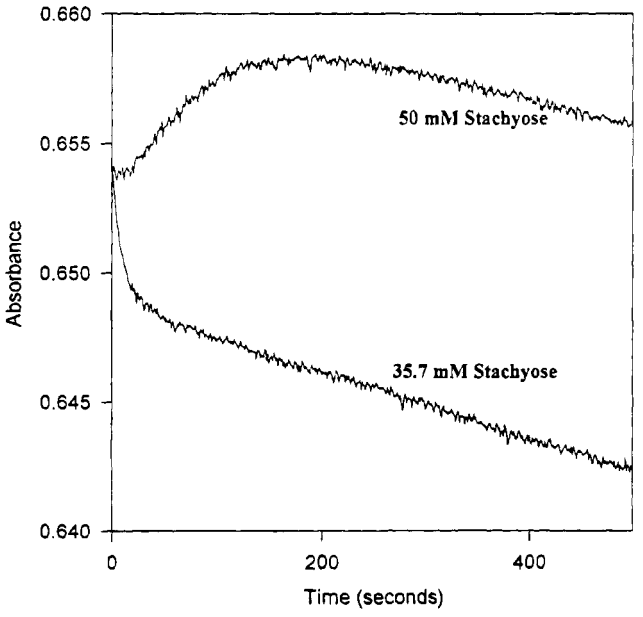

FIGURE 7: Optical density (A_{400}) changes in proteoliposomes containing porin diluted into stachyose solutions. Liposomes containing 8 μg of porin/μmol of lipids were made as described under Material and Methods. Proteoliposome were diluted into different concentration of stachyose as indicate in the figure.

tion inside the liposomes.) It is difficult to determine the osmotic concentration of stachyose because porin and m-PEG-SC-Porin 50 showed there was a continuous influx from the outside to the inside of the liposome. The rate of continuous influx of stachyose from outside to inside of the liposome containing m-PEG-SC-Porin 50 is lower than found in porin, which may be due to a different pore size (narrower) in the m-PEG-SC-Porin 50 than in porin.

DISCUSSION

Protein folding is probably a multistep process (King, 1993). There may be one or more intermediates in the

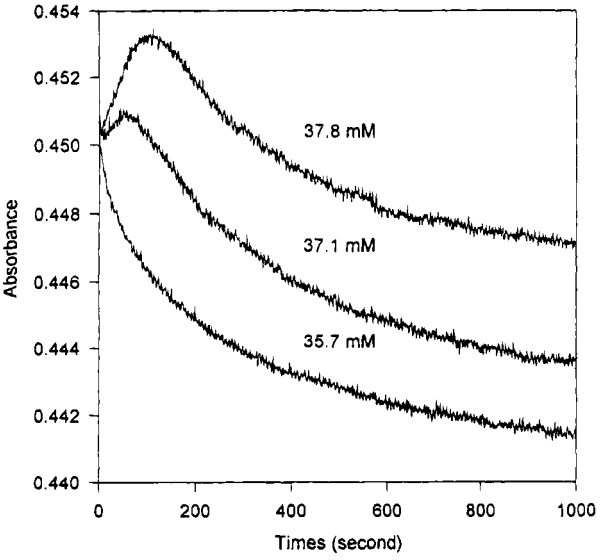

FIGURE 8: Optical density (A_{400}) changes in proteoliposomes containing m-PEG-SC-Porin 50 diluted into stachyose solutions. Liposomes containing 8 μg of m-PEG-SC-porin/μmol of lipids were made as described under Material and Methods. Proteoliposome were diluted into different concentrations of stachyose as indicate in the figure.

pathway of the protein folding process. Some intermediates may aggregate easily. Chaperones have been shown to prevent the aggregation of proteins during the process of protein folding (Craig, 1993; Agard, 1993; Mendoza *et al.*, 1994). Similarly, chaperones may bind to membrane proteins and assist in their insertion into the membrane.

Certain functions of PEG have shown that it may behave similarly to chaperones (Cleland *et al.*, 1992a,b; Cleland & Randolph, 1992). As a cosolvent, PEG could enhance the refolding of some proteins and inhibit the aggregation of intermediates without changing the characteristics of the proteins. Bacteriorhodopsin has been converted to a water-soluble membrane protein, m-PEG-SC-BR, previously (Sirokman & Fasman, 1993). This was accomplished by covalently linking methoxypoly(ethylene glycol) (m-PEG, 5000 Da) to the exposed sequences of the membrane protein bacteriorhodopsin in *situ*. This water-soluble m-PEG-SC-BR could be refolded into liposomes, and, upon exposure to a 555 nm light source, it pumped protons. It was shown that the derivatization of this membrane protein with PEG causes it to become water-soluble and prevented its aggregation during the folding process. It was proposed that it is possible to simulate the insertion of membrane proteins into a membrane environment by changing the polarity of the solution by the addition of organic solvents (Sirokman & Fasman, 1993). Herein, porin, a β-sheet protein, has been successfully conjugated with m-PEG (5000 Da). As indicated previously (Sirokman & Fasman, 1993), the m-PEG reagents are known to couple to the ϵ-amino group of the lysine side chains. There are 10 lysines in porin, with six buried within the membrane. According to the Weiss model obtained by X-ray diffraction studies (Weiss *et al.*, 1991), Lys 69, Lys 198, and Lys 230 are buried deep in the membrane bilayer, and Lys 3, Lys 169, and Lys 191 are located near the ends of the membrane-spanning β-sheets. Lys 46 and Lys 138 are part of interconnecting loops and very likely exposed to water. Lys 298 and Lys 300 are located in the C-terminal of the porin and are exposed in the periplasma. Because

m-PEG cannot penetrate the hydrophobic membrane bilayer, and the pore in porin is probably too small to allow a 700 Da or larger molecule to penetrate it, only Lys 46, Lys 138, Lys 298, and Lys 300 of the protein could possibly react with m-PEG when the cell wall of *R. capsulatus* is used. Those four lysines can, theoretically and with a high probability, react with m-PEG because those are located in a hydrophilic environment. In the SDS−PAGE electrophoretic profile (Figure 1), three bands can be seen, which indicated reaction with m-PEG. These conjugates could be correlated with one, two, or three m-PEG molecules per protein molecule in each conjugate, respectively. Chemical modification of lysines in the matrix of porin, from *E. coli*, has been reported by Schindeler and Rosenbusch (1982), and three lysyl residues were derivatized in the native state. The results herein are similar to that reported even though porin was modified with isothiocynate and citriconic anhydride in a β-octylglucoside solution. The apparent molecular masses of these conjugates herein from SDS−PAGE is much higher than those obtained by summing the molecular mass of the porin and PEG. These conjugates have 50, 66, and 82 kDa apparent molecular masses. Kurfurst (1992) reported a method for molecular mass determination of PEG-modified hirudin by SDS−PAGE. It was found that the pegylated proteins moved very slowly during nongradient PAGE. This indicated that the pegylated proteins have a lower mobility than the native proteins with the same molecular mass. It was suggested that entanglement of PEG chains is not the only mechanism for the observed low mobility but that, in addition, there exists a charge shielding effect of the hydrophilic PEG shell surrounding the protein (McGoff *et al.*, 1988). For this reason, it is assumed that the m-PEG-SC-Porin 50, 66, and 82 are related to one, two, or three m-PEG molecule per protein molecule in each conjugate.

Pfaller *et al.* (1985) have reported to have obtained a water-soluble porin by a different method that used herein. Small amounts of detergents and phospholipids were used (0.5 and 0.08 mol/mol of porin, respectively). Triton X-100 was used as the detergent to solubilize porin, which was later removed easily from the protein. To obtain this water-soluble protein, a brief alkaline treatment was used. No evidence was shown for a structural change of the porin, and they reported that the channel conductance was recovered. A water soluble form of porin was obtained herein without any chemical treatment. Porin at a concentration of 4.85 mg/mL yielded a water-soluble form (see Figure 3).

m-PEG-SC-Porin 50 has been shown to be soluble in aqueous solution. Porin, a membrane protein, has a hydrophobic surface to interact with the phospholipid in the membrane. It easily aggregates in aqueous solution. Porin could be solubilized in detergents as shown in previous studies (Eisele & Rosenbusch, 1990; Nikaido *et al.*, 1991; Park et al., 1992; Markovic-Housley & Garavito, 1986). The effect of covalently attaching the hydrophilic PEG to porin apparently causes a substantial change in the hydrophobic properties of porin. The rearrangement of the apolar groups induced a random coil like structure, as indicated by the CD spectra (Figure 3). The interactions of the hydrophobic surfaces, which cause aggregation between porin monomers, are probably prevented, and thus the aggregation of porin was prevented.

The random coil structure of m-PEG-SC-Porin 50 and the soluble form of porin in water has been shown by the CD spectra (Figure 3) (Greenfield & Fasman, 1969). SDS and β-octylglucoside failed to cause a conformational change to the native structure. Ultracentrifugation was used to determine the average molecule mass of the porin in aqueous solution, and it was found to be a mixture of monomer and trimer in aqueous solution. The distribution of the monomer and trimer were also monitored by nondenaturing gel electrophoresis (Hames, 1990). The trimer-like form of porin has been shown to contain high contents of the β-sheet structure. Markovic-Housley and Garavito (1986) have reported that refolded matrix porin (Omp F) in SDS solution was predominantly in the α-helical conformation, while in 1% octylglucoside it was in the β-conformation. m-PEG-SC-Porin 50 and porin are predominantly in the β-sheet conformation when dissolved in 0.6% octyltetraoxyethylene and 0.3 M LiCl (Figure 4), as was previously shown by Park *et al.* (1992). When PEG-5000 was added in the mol ratio PEG/Porin = 1 or lipopolysaccharide (LPS) was added individually, or together, there was no change in the CD spectra of porin in water or in the 0.6% octyltetraoxyethylene and 0.3 M LiCl. This indicates that the structure of m-PEG-SC-Porin 50 is almost the same as porin, although PEG is covalently bound to it.

Trifluoroethanol (TFE) was added stepwise to an aqueous solution of m-PEG-SC-Porin 50 to simulate the process of insertion of the protein into a membrane. The m-PEG-SC-Porin 50 in 10% TFE still has the random structure as measured by CD, with only a small trough shift, from less than 200 nm in water, to slightly above 200 nm (Figure 5). In 20% TFE, an α-helical structure was induced with a peak at 203 nm and a shoulder at 220 nm. In 30−50% TFE, the CD spectra of m-PEG-SC-Porin 50 is that typical of an α-helical structure (Greenfield & Fasman, 1969), with a peak at 205 nm that is slightly lower than standard α-helical CD spectra (Figure 5). Porin gave the same behavior in TFE with PEG present at a ratio of PEG/porin = 1. An increased helical content was obtained for both porin and m-PEG-SC-Porin 50 on increasing the TFE concentration. TFE has been used to induce helical structures in peptides (Nelson & Kallenbach, 1986; Sönnichsen *et al.*, 1992; Barrow *et al.*, 1992). Previous experiments have shown that the CD spectra of randomly coiled or partially structured polypeptides, including some polypeptides in the β-sheet conformation, generally exhibit a sharp transition to an α-helix as the TFE concentration is increased above a critical value (Fan *et al.*, 1993; Barrow *et al.*, 1992). Bovine monellin, which is predominantly in a β-sheet conformation, has been studied by CD and NMR in TFE (Fan *et al.*, 1993). In the native state of monellin, the A-chain is predominately in the β-structure, and the B-chain contains both α- and β-structure. Upon addition of 50% TFE, the native structure of monellin is disrupted, resulting in a state with a far-UV CD spectrum that indicated a higher helical content than was found in the native state. Preliminary 2D NMR assignments also showed that in TFE monellin undergoes a β-sheet to α-helix conversion. Bovine myelin (Liebes *et al.*, 1975; Fan *et al.*, 1993), which is in the random coil structure in aqueous solution, upon the addition of TFE is converted to a higher helical content. This result is in agreement with that reported herein. The TFE first acts as a denaturant, unfolding the hydrophobic core of the protein. The interaction between the protein and TFE became increasingly weaker with increasing concentrations of TFE. TFE stabilizes secondary

structure by favoring hydrogen bonds within the protein chain at the expense of intermolecular hydrogen bonds between the protein and solvent. Under these condition, α-helices are stabilized by TFE. By comparison, the β-sheet structure involves the bringing together of noncontiguous regions of the chain and is thought to be stabilized by packing of nonpolar residues in the interior of the sheet. But TFE apparently destabilizes the hydrophobic core of the protein and denatures the β-sheet structure of the protein. Porin and m-PEG-SC-Porin 50 are predominantly in a β-sheet conformation in the native state. Their structures probably undergo the same transition as does monellin in TFE. The β-sheet conformation is disrupted and the α-helical structure is formed.

m-PEG-SC-Porin 50 and porin were also examined in HFIP. HFIP was likewise added stepwise to simulate the process of insertion of the protein into the membrane. The structure of m-PEG-SC-Porin 50 in less than 10% HFIP undergoes a change from a random coil structure to form β-pleated sheets, with an increasing magnitude of the negative trough at 215 nm (Greenfield & Fasman, 1969) (Figure 6). A 208 nm shoulder appeared, probably due to the two short α-helices in the native state of the porin. Upon addition of more than 10% HFIP, the α-helical structure was induced, which had a trough at 206 nm and a shoulder at 220 nm in the CD spectra. A clear conversion from a β-sheet conformation to an α-helical one was observed. It is known that HFIP is a much stronger α-helical inducer than is TFE (Barrow *et al.*, 1992; Safar *et al.*, 1993). The CD spectra of synthetic amyloid β-peptides have been reported (Barrow *et al.*, 1992). The peptide was predominantly in a β-sheet conformation in aqueous solution. In HFIP, the α-helical structure was induced. The structure of the peptide undergoes a transition from a β-structure to an α-helical conformation. The conformational transition of scrapie amyloid protein, which is predominantly in a β-sheet conformation in the native state, was also observed to change from a β-sheet to an α-helical conformation by the addition of HFIP. All these results strongly suggest that HFIP is a stronger α-helical inducer than is TFE. To our knowledge, this is the first time that HFIP is reported to induce the β-sheet structure. These results indicate that low concentrations of HFIP can be used to simulate the membrane environment for studying the refolding of membrane proteins which are predominantly in the β-sheet conformation.

The permeability of porin is a very important feature of the membrane. The pore made by porin allows the influx of nutrients to the bacteria and efflux of waste products. Several methods have been used to measure the permeability of porin (Nikaido, 1994; Schulz, 1993). The swelling rate of porin versus sugar, called the "liposome swelling assay" (Nikaido *et al.*, 1991), was used herein. Porin and m-PEG-SC-Porin 50 were embedded in proteoliposomes, and the swelling rate was determined upon the addition of stachyose by measuring the change of light scattering (absorbance) of the proteoliposomes at 400 nm. For a comparison of the result with m-PEG-SC-Porin 50 (Figure 8), the swelling rate of porin was measured as shown in Figure 7. In the first few minutes, the change of absorbance is due to an adjustment of osmotic activity of the diluent. It is difficult to determine the isosomotic concentration of stachyose because both porin and m-PEG-SC-Porin 50 show a continuous influx from the outside to the inside of the liposome. The influx into liposomes containing m-PEG-SC-Porin 50 is lower than that found for porin (Figures 7 and 8). Thus the pore in m-PEG-SC-Porin 50 may be slightly narrower than that of porin, or the pore in the m-PEG-SC-Porin 50 could be partially covered by the long chain of the PEG. LPS (lipopolysaccharides) has been shown to possibly affect the pore in the porin opening (Ishii & Nakae, 1993). Accordingly, LPS have been added when the proteoliposome of the m-PEG-SC-Porin 50 was being made. There was no improvement in the influx of sugar. The exclusion limit would be expected to change for smaller sugar molecules. However, a decreased permeability of the proteoliposome containing the m-PEG-SC-Porin 50 versus stachyose was found. It has been suggested that a charge shielding effect exists by the hydrophilic PEG shell surrounding the protein (McGoff *et al.*, 1988). This might cause a change in the selection of charged solutes.

Microcrystals of m-PEG-SC-Porin 50 were obtained which were not satisfactory for X-ray diffraction studies (Fasman & Wei, 1994). This technique might develop into a new, much-easier technique for crystallization of membrane proteins.

Future studies will be aimed at determining the kinetics of the refolding of the m-PEG-SC-Porin 50. Stop-flow CD, UV, and fluorescence will be used. As reported herein, m-PEG-SC-Porin 50 could be correctly refolded in HFIP, yielding the β-conformation. Thus, HFIP will be used to simulate the polarity of the membrane. Perhaps this model system for the simulation of the insertion of a membrane protein into a membrane may reveal new insights into this interesting biological phenomena.

Conclusions. (1) Porin, a membrane protein from *R. capsulatus*, has been modified by reaction with methoxypoly(ethylene glycol) succinimidyl carbonate to yield a water-soluble m-PEG-SC-Porin 50 conjugate. (2) The conjugate can be refolded in the 0.6% octyltetraoxyethylene and 0.3 M LiCl to restore the native β-sheet structure. (3) The conjugate was embedded into a proteoliposome, and the swelling rate was determined versus stachyose. Thus the pore of porin can be reestablished with m-PEG-SC-Porin 50. (4) Stepwise refolding of m-PEG-SC-Porin 50 by continuous addition of TFE yielded an α-helical structure. (5) Stepwise refolding of m-PEG-SC-Porin 50 to yield the native β-sheet structure can be achieved by stepwise addition of HFIP (at ≃ 10%).

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