In Vitro Folding and Oligomerization of a Membrane Protein

TRANSITION OF BACTERIAL PORIN FROM RANDOM COIL TO NATIVE CONFORMATION*

Jean-Luc Eisele and Jürg P. Rosenbusch

From the European Molecular Biology Laboratory, P. O. Box 10.2209, D-69 Heidelberg, Federal Republic of Germany and the Biozentrum, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland

Porin, a channel-forming protein spanning bacterial outer membranes, was denatured in 6 M guanidinium hydrochloride or, alternatively, in sodium dodecyl sulfate at 95 °C. Circular dichroism spectra revealed that this protein, which in its native state consists of β-pleated sheets as the sole detectable secondary structure, is transformed into random coil configuration in the chaotrophic agent, or into α-helical structure in the detergent. From either state, the mature protein refolds in presence of amphiphilic molecules, attaining full structural and functional competence. As structural criteria, the native trimeric state was assessed by analytical ultracentrifugation, gel electrophoresis in sodium dodecyl sulfate, protease resistance, and circular dichroism spectroscopy. Channel formation in planar lipid bilayers reveals that the refolded protein is also functionally competent. It is concluded that the information required for the complete folding of porin is contained within the primary sequence of the mature polypeptide. The study of rapid refolding clearly reveals that this process occurs in the time range of seconds and that preexisting bilayers are not a prerequisite.

SECRETION OF PROTEINS ACROSS AND THEIR INSERTION INTO MEMBRANES-
"trigger" factor (Crooke et al., 1988) have been suggested to participate in this process. In vivo studies as well as in vitro reconstituted systems have indicated that translocation requires partially unfolded precursors, with attached signal sequences (Park et al., 1988). Chaperone proteins (Hemmingsen et al., 1988) such as heat shock proteins (Pehlh, 1989), SecB (Collier et al., 1988), GroEL (Bochkareva et al., 1988), or "trigger" factor (Crooke et al., 1988) have been suggested to maintain precursors in a translocation and folding-competent state. These results are clearly distinct from early studies with soluble proteins (Anfinsen et al., 1961), which showed that the information contained in the protein sequence was sufficient for proper folding. In the work presented here, we have addressed the question whether an integral membrane protein, such as porin spanning Escherichia coli outer membrane (Rosenbusch, 1974; Sass et al., 1989), can be folded from a random coil configuration into its native conformation, with all of its required functional and structural properties.

MATERIALS AND METHODS

Purification and Characterization—Porin was obtained from E. coli B² envelopes, by extraction with octyl-POE, as described previously (Garavito-Navessen and Rosenbusch, 1986). Lipopolysaccharides were purified from the same bacterial strain (Bühler, 1986). Purified porin (Sigma) was added to a final concentration of 10 mg/ml. Incubations were allowed to proceed for 90 min at 37 °C and were stopped by the addition of soybean trypsin inhibitor (Merck) in 3-fold molar excess. Digests were analyzed by SDS-gel electrophoresis. The oligomeric state was assessed by sedimentation equilibration in a model E analytical ultracentrifuge (Beckman) using an An-J rotor. The secondary structure was deduced from circular dichroism spectroscopy (Cary 61 spectrophotopolarimeter) and ellipticity determined as described by Adler et al. (1973). Activity of the protein was assayed by electrical conductance measurements as described previously (Schindler and Rosenbusch, 1978).

Denaturation and Renaturation—For denaturation, porin (1 mg/ml) in standard buffer (20 mM NaPO₄, 0.1 M NaCl, 3 mM NaN₃, 2 mM dithiothreitol, and 1% octyl-POE, pH 7.2) was heated for 5 min at 95 °C in 6 M guanidinium hydrochloride (recrystallized twice from 80% ethanol). The solution was then dialyzed against 1 liter of distilled water for 12 h with one change. The precipitate form was redissolved in 2% SDS to a final protein concentration of 1 mg/ml and used for renaturation experiments. For refolding, 200 μl (0.2 mg of porin) were added to 1 ml of a 0.15 M KCl solution containing soybean lecithin (Sigma) at a concentration of 20 mg/ml in 2% octyl-POE. The clear solution was filtered through Millipore (0.45 pm) and dialyzed against 4 liters of buffer (50 mM NaPO₄, 0.15 M NaCl, 3 mM NaN₃, 2 mM dithiothreitol, and 1 mg of porin) for a total of 48 h with four buffer changes. In rapid refolding experiments, denatured protein was diluted (3-fold) in the phospholipid solution, without subsequent dialysis. Aliquots (40 μl) of the resulting mixture were withdrawn from 10 s to 4 h and subjected to trypsin treatment as described above. SDS-polyacrylamide gel electrophoresis were performed under typical conditions (Laemmli, 1970) with 0.1% SDS in the running buffer. This concentration (3 mM) is well above the critical micelle concentration of 0.5 mM under the conditions used (Reynolds and Tanford, 1970a). The refolding mixture was treated with trypsin and trypsin inhibitor, purified on DEAE-ion exchange chromatography in presence of octyl-POE (Garavito-Navessen and Rosenbusch, 1986) and used for structural tests (see above). For functional tests, vesicles were used and incorporated into bilayers as described (Dargent et al., 1987).

RESULTS

Denaturation of Porin—Circular dichroism spectra of native porin (Fig. 1) showed a minimum in mean residue weight ellipticity ([θ] = -8050 degrees-cm²-dmol⁻¹) at 217 nm, typ-
activity in preparation purified from cells grown in presence of \[^{32}P\]phosphates (Bühler, 1986). If the protein was reconstituted with added lipopolysaccharides, a ladder consisting of several bands exhibiting slightly lower mobility was observed (lane b). This is indicative of tightly associated glycolipids (Garavito et al., 1983; Bühler, 1986). If native trimers were treated with trypsin (lane c), no decrease in intensity of the band corresponding to the protein was detected, confirming that native porin is completely protease-resistant (Schindler and Rosenbusch, 1984). When protein denatured in guanidinium hydrochloride was applied to SDS-gel electrophoresis after removal of the chaotropic agent by extensive dialysis (Fig. 2B), the protein migrated as monomer regardless whether it was heat-treated or not. Treatment of this material with trypsin (lane c) completely degraded the polypeptide to small fragments that could not be detected in SDS gels. Lipopolysaccharide binding to the protein was no longer observed (lane b).

**Refolding of Porin**—Detergent was removed from the solution containing protein and phospholipids by extensive dialysis (4–72 h), yielding a turbid solution of vesicles. Gel electrophoresis of this material, resolubilized in SDS sample buffer, produced the patterns shown in Fig. 2C. Samples not heated above 75 °C yielded two bands (Fig. 2C, lane a). Quantification of the two bands indicated that, on average, 40% of the material was in the trimeric state. Recoveries up to 80% have been observed. More significant, this value never fell below 20%. The assignments of the native trimeric conformation was validated by the following criteria. First, the mobility of the band was the same as that observed with trimeric porin (Fig. 2A, lane a). When lipopolysaccharides were added, the characteristic bands with mobility slightly lower than that of pure protein were clearly visible (compare lanes b in Fig. 2, A and C). Moreover, it can be seen that the lower band (denatured protein) was fully sensitive to trypsin, whereas the upper one was not (lane c). Protease resistance of the refolded porin was also tested with other several proteases, all yielding the same result (not shown).

This protease resistance provided a simple method for the purification of trimers. Refolded porin was treated sequentially with trypsin and trypsin inhibitor and then chromatographed on the same anion exchange column as used for the purification in the presence of the detergent octyl-POE. Thus, removal of fragments, protease and inhibitor was achieved simultaneously with delipidation. Circular dichroism spectroscopy of this purified material (Fig. 1) yielded a spectrum that was typical of \(\beta\)-pleated sheet structure and similar to that of denatured porin. Analysis of the same preparation by sedimentation equilibrium analysis yielded a mass of 109 ± 4 kDa, characteristic of trimers and indistinguishable from measurements of non-denatured porin (Rosenbusch, 1974). Finally, the purified, renatured trimers formed channels in reconstituted planar bilayers, with activation occurring in single steps and a conductance of 0.8 nS in 1 M KCl, as observed with porin extracted from *E. coli* membranes (Schindler and Rosenbusch, 1978).

In the standard procedure used for refolding, the precipitated porin was resolubilized in SDS after guanidinium hydrochloride had been removed by extensive dialysis. No resolubilization or refolding was observed in octyl-POE alone. We therefore tested the SDS properties that are essential for the recovery of native state. If nonionic or zwitterionic detergents were used, reproducible renaturation was observed but the yield was consistently low (10–20%). Cationic detergents such as decyl trimethylammonium bromide did not allow renaturation in detectable amounts. Dodecyl sulfate yielded
recoveries comparable to those in SDS. Both sulfate and sulfonate detergents were less effective if their alkyl groups were octyl rather than dodecyl chains. When the short chain phospholipid diheptanoyl phosphatidylcholine (Eisele and Rosenbusch, 1989) was used, as the single amphiphile to substitute for both octyl-POE and soy bean lecithin, no recoveries comparable to those in SDS. Both sulfate and sulfonate detergents were less effective if their alkyl groups were octyl rather than dodecyl chains. When the short chain phospholipid diheptanoyl phosphatidylcholine (Eisele and Rosenbusch, 1989) was used, as the single amphiphile to substitute for both octyl-POE and soy bean lecithin, no recoverying was observed.

Rapid refolding experiments (Fig. 3), involving dilution without dialysis, showed that renaturation occurred as soon as the protein was exposed to the phospholipids present in mixed micelles with detergent. Analysis of aliquots removed 30 s after mixing revealed the presence of protease resistant trimers, whereas digestion of denatured material occurred within seconds, as shown by the sample at time 0 (Fig. 3). Variations in the octyl-POE concentration in the refolding mixtures showed that high detergent concentrations (>5%) slowed down the kinetics and final yields of refolding to less than 10% after 4 h.

DISCUSSION

Porin is fully denatured either in guanidinium hydrochloride or in SDS. In the chaotropic agent, the protein dissociates quantitatively into its constituent polypeptide chains which, according to circular dichroism spectra, exist in random coil configuration. Due to unfavorable signal to noise ratios at wavelengths shorter than 205 nm, the presence of less than 5% α-helical configuration cannot be ruled out. Since no indication of even very limited quantities of α-helix is observed in the native protein, it seems highly unlikely that a small segment of the polypeptide could assume such a configuration or retain such secondary structure in guanidinium hydrochloride. In SDS, less than 1% of incompletely denatured protein would be detected, due to the sensitivity of the gel system and the resistance of the undenatured protein to enzymatic hydrolysis. Moreover, as recoveries of refolded protein reach values as high as 80%, the probability of porin molecules escaping denaturation seems negligible.

Refolding, either by rapid dilution or after extensive dialysis, was demonstrated to be complete by the full recovery of the native structure of porin and of its channel conductance. This renaturation requires an amphiphile, preferentially SDS, although others such as zwitterionic detergents were effective. Whether these amphiphiles are critical in the formation of intermediates cannot presently be determined. Earlier experiments (Dargent et al., 1987) showed that amphiphiles are required for the insertion of native porin into preexisting membrane (planar bilayer), although concentrations well below their critical micelle concentration were sufficient. Membrane protein folding and insertion might therefore be viewed to 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. This proposal seems compatible with recent evidence obtained in vivo with lactose permease (Roepe and Kaback, 1989). Furthermore, earlier attempts to refold porin in the absence of amphiphiles (Rosenbusch, 1974; Markovic-Housley and Garavito, 1986) have shown that, although the β-sheet structure was recovered, no other characteristics of native porin were present.

How could the pathway of the folding and insertion of porin into the membrane be visualized? Bacteriorhodopsin has been shown to reassume its native structure and functional properties after complete unfolding (Huang et al., 1981). In that case, insertion was proposed to occur by the partitioning of overall hydrophobic helical hairpins, or corresponding helical domains (Popp et al., 1987), a concept of micro-assembly in accordance with the proposal advanced by Engelman and Steitz in 1981. Though this may be a valid hypothesis for bacteriorhodopsin, it seems unlikely to account for membrane insertion of porin, with its many polar residues dispersed over the entire sequence (Paul and Rosenbusch, 1985). Even if minor α-helical domains that would have escaped detection were present, they alone could hardly account for membrane insertion of the entire protein. As in β-pleated sheets, the hydrogen bonds occur between, rather than within peptide strands, at least four segments would be necessary for thermodynamically favorable insertion (Tanford and Reynolds, 1976), a reasoning which does not even include the large number of polar side chains in porin.

By analogy to the situation in situ, preexisting membranes have been tacitly assumed to be a prerequisite for membrane protein assembly. To address the question of whether such an assumption was justified, we have allowed refolding of porin with phospholipids in presence of detergent in excess of its critical micelle concentration. The presence or absence of structures larger than 50 Å, typical of mixed micelles (Zulauf and Rosenbusch, 1983), was ascertained by quasi-elastic light scattering studies. As no structures larger than that size were found in conditions under which refolding occurred with significant yields, the presence of membranes seems not to be required for the proper folding of porin.

Are such observations limited to porin only, or can they be extended to other proteins such as membrane receptors? It may be provocative to investigate the applicability of spontaneous folding to other membrane proteins at a time when overexpression of receptors appears potentially so fecund. Irrespective of such considerations, our results clearly show that all the information necessary for the proper folding of the protein to its native state is contained within the mature segment of the polypeptide and that the signal peptide, as well as other components, may affect the targeting of the proteins and the kinetics of their folding process.

Acknowledgments—We wish to thank L. Bühler for the conductance measurements, Ariel Lustig for analytical ultracentrifugation, Dr. M. Zulauf (Hofmann-La Roche) for quasi-elastic light scattering experiments, and Dr. Anthony P. Fuglsey for critically reading the manuscript.

REFERENCES

Adler, A. J., Greenfield, N. J., and Fasman, G. D. (1973) Methods Enzymol. 27, 675-735
Anfinsen, C. B., Haber, E., Sela, M., and White, F. H., Jr. (1961) Proc. Natl. Acad. Sci. U. S. A. 47, 1309-1314
Refolding of Porin

Bochkareva, E. S., Lissin, N. M., and Girshovich, A. S. (1988) Nature 336, 254-257
Bühler, L. (1986) Reconstitution of Matrix Protein. Diploma Thesis, Basel University
Collier, D. N., Rankaitis, V. A., Weiss, J. B., and Bassford, P. J., Jr (1988) Cell 53, 273-283
Crooke, E., Guthrie, B., Lecker, S., Lill, R., and Wickner, W. (1988) Cell 54, 1003-1011
Dargent, B., Rosenbusch, J. P., and Pattus, F. (1987) FEBS Lett. 220, 136-142
Eisele, J.-L., and Rosenbusch, J. P. (1989) J. Mol. Biol. 206, 209-212
Engelmann, D. M., and Steitz, T. A. (1981) Cell 23, 411-422
Garavito, R. M., and Rosenbusch, J. P. (1986) Methods Enzymol. 125, 309-328
Garavito, R. M., Jenkins, J. A., Jansonius, J. N., Karlsson, R., and Rosenbusch, J. P. (1983) J. Mol. Biol. 164, 313-327
Greenfield, N., and Fasman, G. D. (1969) Biochemistry 8, 4108-4116
Hemmingsen, S. M., Woolford, C., van der Vries, S. M., Tilly, K., Dennis, D. T., Georgopoulos, C. P., Hendrix, R. W., and Ellis, R. J. (1988) Nature 333, 350-334
Huang, K.-S., Bayley, H., Liao, M.-J., London, E., and Khorana, H. G. (1981) J. Biol. Chem. 256, 3802-3809
Laemmli, U. K. (1970) Nature 227, 680-685
Markov-Housley, Z., and Garavito, R. M. (1986) Biochim. Biophys. Acta 869, 158-170
Park, S., Liu, G., Topping, T. B., Cover, W. H., and Randall, L. L. (1988) Science 239, 1033-1035
Paul, C., and Rosenbusch, J. P. (1985) EMBO J. 4, 1593-1597
Pehlman, R. B. (1989) EMBO J. 8, 3171-3176
Popot, J.-L., Gerchman, S.-E., and Engelman, D. M. (1987) J. Mol. Biol. 198, 655-676
Revnolds, J. A., and Tanford, C. (1970a) Proc. Natl. Acad. Sci. U. S. A. 66, 1007-1007
Revnolds, J. A., and Tanford, C. (1970b) J. Biol. Chem. 245, 5161-5165
Riese, P. D., and Kaback, H. R. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 6087-6091
Rosenbusch, J. P. (1974) J. Biol. Chem. 249, 8019-8029
Saier, M. H., Jr., Werner, P. K., and Muller, M. (1989) Microbiol. Rev. 53, 333-366
Saier, M. H., Jr., Werner, P. K., and Muller, M. (1989) Microbiol. Rev. 53, 333-366
Sass, H. J., Buldt, G., Beckmann, E., Zemlin, F., van Heel, M., Zeitler, E., Rosenbusch, J. P., Dorset, D. L., and Massalski, A. (1989) J. Mol. Biol. 209, 171-175
Schindler, H., and Rosenbusch, J. P. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 3701-3705
Schindler, M., and Rosenbusch, J. P. (1984) FEBS Lett. 173, 85-89
Tanford, C., and Reynolds, J. A. (1976) Biochim. Biophys. Acta 487, 133-170
Verner, K., and Schatz, G. (1988) Science 241, 1307-1313
Wickner, W. (1989) Trends Biochem. Sci. 14, 280-283
Zulauf, M., and Rosenbusch, J. P. (1983) J. Phys. Chem. 87, 855-862
In vitro folding and oligomerization of a membrane protein. Transition of bacterial porin from random coil to native conformation.

J L Eisele and J P Rosenbusch

J. Biol. Chem. 1990, 265:10217-10220.

Access the most updated version of this article at http://www.jbc.org/content/265/18/10217

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/265/18/10217.full.html#ref-list-1