Tracking the Unfolding and Refolding Pathways of Outer Membrane Protein Porin from *Paracoccus denitrificans*†

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ABSTRACT: We have investigated outer membrane protein porin from *Paracoccus denitrificans* for its stability against heat and pH. Pathways of unfolding and refolding have been analyzed. Porin incubated at pH 12.5 and above undergoes a slow unfolding into an unordered structure. The unfolded protein could be refolded into a nativelike structure that is functionally active but with distinct deviation from the native protein. This nativelike structure exhibited an entirely different thermal stability. Although aggregation is normally considered a structural "dead-end", the possibility of opening an aggregated porin and forming a functionally active structure was analyzed here. Porin aggregates on heating above 86.2 °C. Incubating the heat-aggregated protein at high pH (≥12.5) leads to a slow opening of the protein into an unordered structure. It was possible to refold this unordered protein into a trimeric nativelike structure which was capable of forming active pores. However, the thermal stability of the refolded porin was unlike that of the native porin. To understand the basic mechanism behind the unfolding processes, the protein was subjected to heating at various pH values. It was observed that at pH ≥12.5 the protein does not aggregate upon heating; instead, it opens into an unordered structure. We conclude that at high pH values, the electrostatic interactions of various amino acid residues are perturbed which leads to unfolding into an unordered structure. This study shows for the first time an entirely new unfolding and refolding pathway for porin.

Porins are the channel-forming proteins found in the outer membrane of Gram-negative bacteria, mitochondria, and chloroplasts. They facilitate the transport of hydrophilic molecules below ≈600 Da in the case of bacterial porins (for reviews, see refs *1*−*3*). The X-ray crystal structure of the *Paracoccus denitrificans* porin shows that the functional unit is a trimer. Each monomer is composed of 16 antiparallel β-strands (*4*). Despite being integral membrane proteins, porins do not form a hydrophobic primary structure or contain hydrophobic sequences typical for transmembrane α-helices (*5*, *6*). Yet the porin trimer is a highly stable structure (*7*−*9*). It is suggested that three structural factors appear to play a pivotal role in the stability of the porin trimer: (i) the extensive hydrogen bonding in the β-barrel, (ii) the rigid and structured loops on both sides of the membrane, and (iii) the strong interaction between the monomers (*10*).

For *P. denitrificans* porin, the calculated pI value is 4.03. It has 17% acidic residues and 7% basic residues with a peculiar arrangement (Figure 1a,b). All the basic residues are positioned inside the channel except two of them which are in the loops in each monomer. All the acidic residues are positioned in the loops except for a few in the barrel wall, which are positioned inside the channel, i.e., to neutralize the effect of the basic residues to keep the pore nonspecific (*4*). A thermodynamically stable protein will exist when the various ionizable residues are electrostatically neutralized. Another point to be noted is that any change in pH will first introduce changes in these acidic and basic residues, which are located in the solvent-accessible regions of the protein.

We have reported the details of the thermal stability of porin from *P. denitrificans* (*8*). We found that porin undergoes temperature-induced aggregation if the protein is in detergent micelles, whereas no change in its secondary structure is observed for the protein reconstituted into liposomes. The reason for this extreme difference in the stability of the protein can be attributed to the difference in the interaction of the protein with lipids and detergents (*7*, *9*). The results with *Paracoccus* porin are not surprising as the thermal stability for most of the membrane proteins such as bacteriorhodopsin and other porins is high, too (*9*−*11*). Aggregation is the fate of most membrane proteins when they are heated to high temperatures (*12*, *13*). It was previously reported for bacteriorhodopsin and cytochrome *c* oxidases that most of the intramembranous secondary structure is maintained even above the denaturation temperatures (*11*−*13*). Numerous other studies have revealed that

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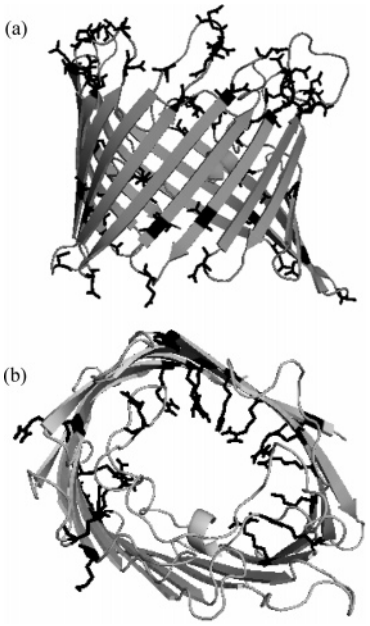


FIGURE 1: Charged residues of porin. The structure coordinates were provided by W. Welte. (a) *P. denitrificans* porin trimer with aspartic acid and glutamic acid residues highlighted as a stick model. (b) *P. denitrificans* porin trimer with arginine and lysine residues highlighted as a stick model.

aggregated proteins still contain a majority of their secondary structure elements (*14*, *15*). This suggests that a structural alteration far more minor than complete unfolding of the secondary structure is sufficient to cause aggregation. In general, it is the minor structural alteration which causes aggregation under in vitro conditions of protein purification. Aggregation is also facilitated by partial unfolding during thermal or oxidative stress and by alteration of the primary structure caused by certain mutations, RNA modification, or translational misincorporation. Aggregates can be considered as oligomeric complexes of non-native conformers that arise from non-native interactions among structures of kinetically trapped intermediates in protein folding or assemblies (*16*, *17*).

It is quite likely, too, that in the case of porin most of the β-sheet secondary structure is maintained after denaturation. In our previous work, aggregation was evident due to the downshift of the absorbance maxima of the β-sheet component (amide I), and it was suggested that formation of intermolecular hydrogen bonds are responsible for the downshift (*8*). Some other interactions may also be involved in aggregation.

If in aggregated proteins a large fraction of the secondary structure is maintained and most of the aggregated fractions are stabilized by hydrogen bonds and electrostatic interactions, then to disaggregate an aggregated protein, these interactions between the aggregate fractions have to be broken. Heating the aggregated porin is not sufficient to cause disaggregation. Another approach to disaggregating a protein is to change its environment by perturbing its ionic properties at an altered pH value.

In this report, we have addressed the pH-dependent stability, unfolding, and refolding of porin. Another major question we have explored is whether it is possible to "open up" and "refunctionalize" a heat-aggregated porin.

EXPERIMENTAL PROCEDURES

The porin gene cloned in vector pJC 40 was obtained from B. Ludwig, Institut für Biochemie, Johann Wolfgang Goethe-Universität Frankfurt (*18*, *19*). The structure coordinates of *P. denitrificans* porin were provided by W. Welte, Fakultät für Biologie, Universität Konstanz, Konstanz, Germany (*4*). All the chemicals required in this study were procured from Roth Germany, Merck Germany, and Sigma Germany.

Protein Purification. Purification of the protein was carried out according to the method of Saxena et al. (*18*).

FTIR[1] *Spectroscopy.* The measurements were carried out in transmission mode. For the protein in detergent, the sample (20 μL, 2 mg/mL) was dried in a gentle stream of N_2 and resuspended in 3 μL of ^2H_2O. Approximately 1.5 μL of sample was loaded in the center of a CaF_2 microcell with an optical path length of 7.4 μm (*20*). IR spectra were recorded with a Bruker VECTOR 22 FTIR spectrometer. For each spectrum, 20 interferograms were averaged, apodized with a Blackman-Harris four-term function, zero-filled, and Fourier-transformed to yield a nominal spectral resolution of 2 cm^{-1} with a data point interval of 1 cm^{-1}. The spectra were processed and visualized using OPUS version 3.1. The built-in program for calculating second derivatives of the spectra in OPUS was used to identify the minute changes in band positions and intensity.

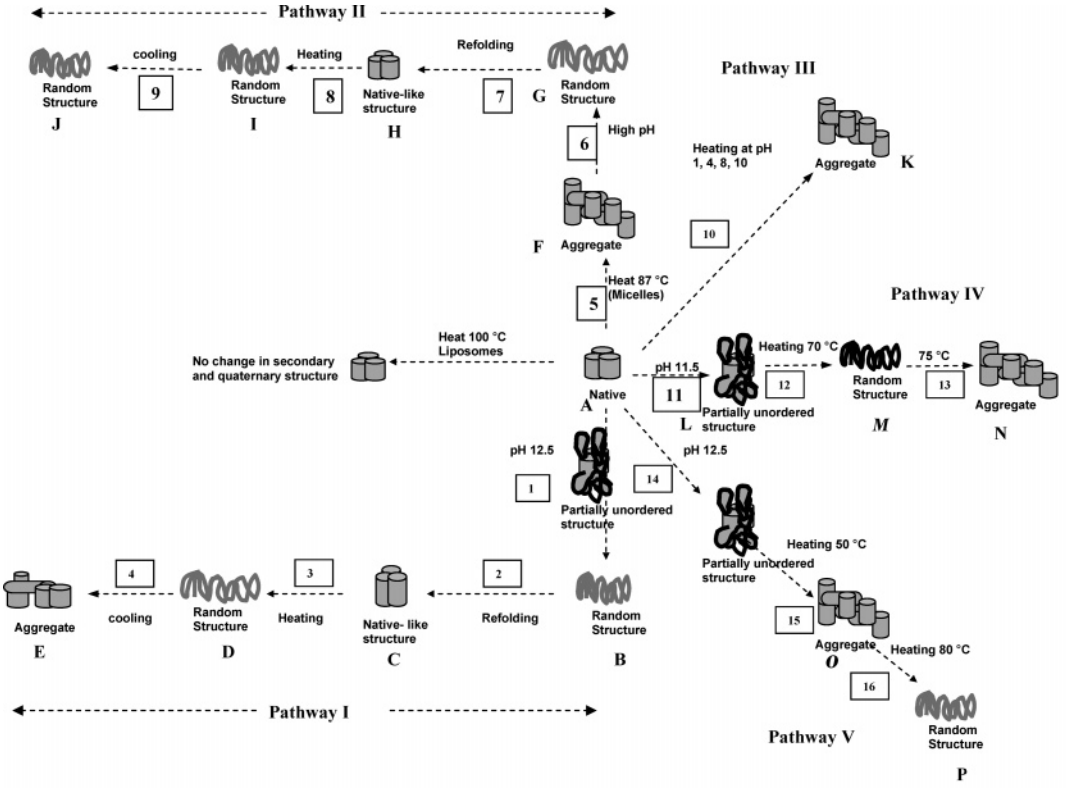
CD Spectroscopy. A JASCO J 720 spectropolarimeter was used to record the CD spectra in the range of 195−260 nm. The spectral bandwidth used was 1 nm with a response time of 1 s. A quartz sample cell with a path length of 0.01 mm was filled with 20 μL of sample (1 mg/mL). Before the measurement of the sample spectra, reference spectra of air and buffer were also recorded.

Black Lipid Bilayer Activity Measurements. The methods used for black lipid bilayer experiments have been described previously (*21*, *22*). The experiments were performed at room temperature. The instrumentation consisted of a Teflon chamber with two compartments separated by a thin wall. The membranes were formed from a 1% (w/v) solution of diphytanoylphosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) in *n*-decane. Bilayer formation was indicated when the membrane appeared optically black in the reflected light. The porin was added from the 0.1 mg/mL protein stock solution. Single-channel pore conductance was measured after application of a fixed membrane potential with a pair of Ag/AgCl electrodes with salt bridges inserted into the aqueous solutions on both sides of the membrane. The current through the membrane was measured with a current−voltage converter. It was monitored on a storage oscilloscope and recorded on a strip chart recorder.

Selectivity Measurements. Zero-current membrane potentials were measured as previously described (*21*, *22*). The membranes were formed in a 10 mM KCl solution containing a defined protein concentration so that the membrane conductance increased ~100−1000-fold within 10−20 min of membrane formation. At this time, the instrumentation was switched to the measurements of the zero-current potentials, and the salt concentration on one side of the

[1] Abbreviations: FTIR, Fourier transform infrared; CD, circular dichroism; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; OmpF, outer membrane protein F.

Scheme 1: Folding Pathways of Porin[a]



[a] Pathways are indicated in numerals. Each step is specified by a number and each state of the protein by a letter.

membrane was increased by adding small amounts of concentrated salt solutions. The zero-current membrane potential reached its final value within 2−5 min.

RESULTS AND DISCUSSION

The folding pathways derived from the studies presented here are summarized in Scheme 1. It is evident that the pathway of unfolding and refolding depends on the destabilizing conditions. In the following sections, results which lead to these pathways are discussed.

Opening of the Protein into Unordered Structure

To observe the changes in the secondary structure of the natively folded protein, CD spectra were analyzed for porin incubated between pH 1 and 13. Figure 2a depicts the CD spectra of porin recorded after incubation for 4−5 min at pH 1 and 4 in comparison to that recorded at pH 8. It is evident from the spectra that porins incubated at pH 1 and 4 have spectra similar to that of the native one at pH 8. A typical β-sheet structure in a CD spectrum is characterized by a negative lobe at 217 nm and a positive lobe below 207 nm. The spectra obtained are very similar to those obtained for other porins (*23*, *24*). Figure 2b represents the spectra of porin incubated at pH 10, 12.5, and 13. In comparison to that at pH 8, the spectrum at pH 12.5 shows an intermediate state which in an additional 50 min unfolds into unordered structure (pathway 1, step 1). At pH ≥13, it unfolds into unordered structure in a short time, which could not be resolved in this study. Incubating native porin at acidic pH values for a longer period did not bring about any further change in the spectral characteristics.

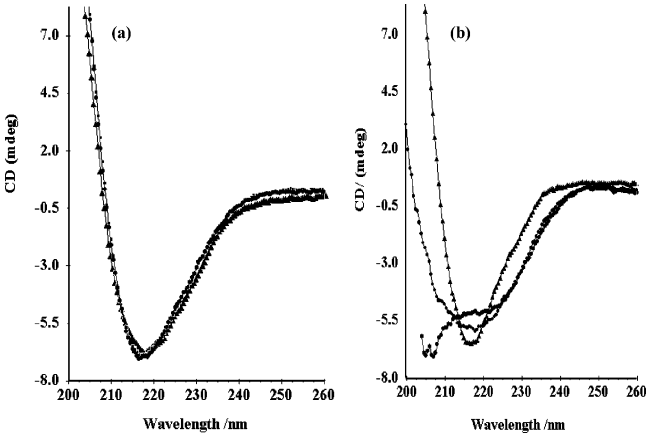


FIGURE 2: CD spectra of the secondary structure conformation of porin after incubation for 4−5 min at various pH values: (a) (●) pH 1, (▲) pH 4, and (■) pH 8 and (b) (▲) pH 10, (●) pH 12.5, and (■) pH 13.

The time-dependent unfolding of the porin at pH 12.5 was measured after incubation for 4−50 min (Figure 3). The spectra obtained after 10 and 50 min are compared with that of the native protein at pH 8. After 50 min, no further change was observed in the spectra. The changes observed between 0 and 10 min were drastic, while further changes were slow and gradual, indicating that the initial denaturation of the solvent-exposed region is faster than that of the β-sheet region. One of the mutants that was analyzed (E81Q) exhibited a very similar unfolding pattern (data not shown). Hence, it can be concluded that increasing the pH to ≥12.5 destabilizes the protein. At the same time, decreasing the pH to the extreme acidic range at pH 1 does not bring about significant changes in the secondary structure. SDS−PAGE

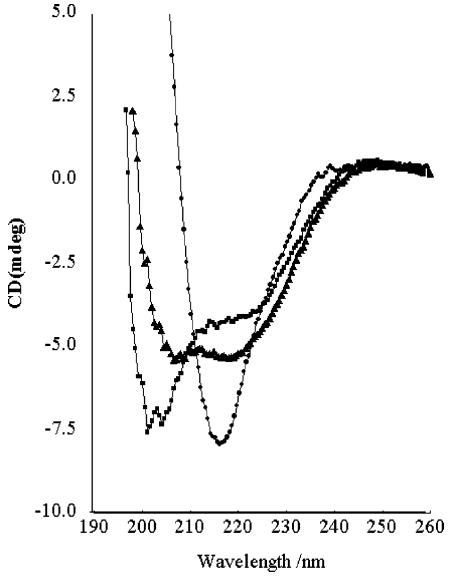


FIGURE 3: Time-dependent unfolding of porin: (●) native protein at pH 8, (▲) native protein at pH 12.5 and after incubation for 10 min, and (■) native protein at pH 12.5 and after incubation for 50 min.

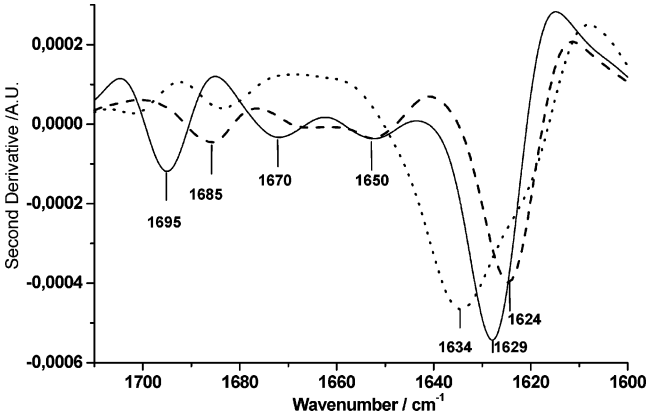


FIGURE 4: Second derivatives of FTIR spectra showing the native refolded protein (state C, - - -) and the aggregated refolded protein (state H, ···) in comparison to the native protein (—).

results showed monomerization at high pH (data not shown), which is similar to that reported for porin OmpF (*25*).

Refolding of the Protein to Nativelike Structure

Unfolded protein (state B) obtained from denaturation of porin at high pH was refolded by altering the pH of the buffer from 12.5 to 8 (step 2). FTIR spectra of the refolded protein (state C) are compared to that of the native one in Figure 4. The amide I spectral region shows that the protein has characteristic β-sheet structure as evident from the peaks at 1624 and 1685 cm^{-1} (*26*, *27*). The bands representing the helix and loops were also seen at 1650 and 1670 cm^{-1}, respectively (*8*). Unfolding of porin may lead to a stronger exposure of amide protons and thus result in an extended $^1H-^2H$ exchange. Consequently, shifts of amide I components can be expected, although the original structure was fully restored upon refolding. In the case of β-sheet segments, for example, unfolding in ^2H_2O followed by refolding may lead to a shift by approximately 10 cm^{-1} as can be seen for the high-frequency β-sheet component in Figure 4. However, the β-sheet structure is retained. It can thus be argued that

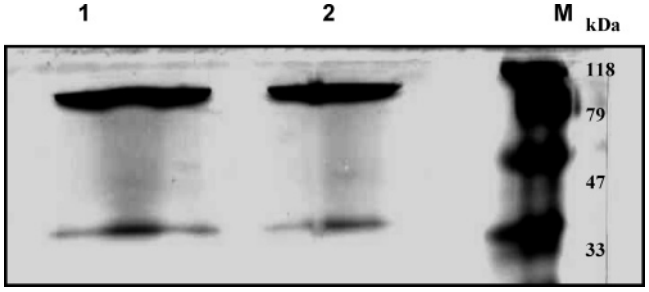


FIGURE 5: SDS−PAGE gel profile of the refolded protein. Lane 1 contained refolded aggregated porin (state H), lane 2 refolded native porin (state C), and lane M protein molecular mass markers.

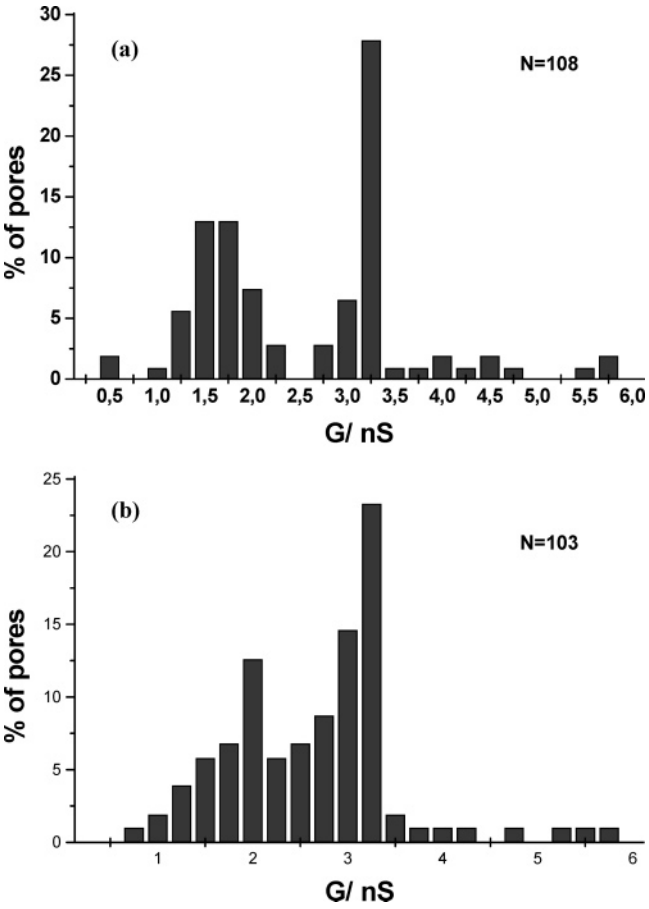


FIGURE 6: Frequency distribution of measured pore conductivity for refolded porin recorded by the black lipid membrane technique: (a) native refolded protein (state C) and (b) aggregated refolded protein (state H). *N* is the number of pores counted in each measurement.

nativelike structures are formed once refolding has occurred. In support of this finding, these structures were also characterized by trimers in SDS−PAGE (Figure 5).

Activity of the Nativelike Structure

To characterize the nativelike structure (state C), activity and selectivity have been analyzed and compared to those of the native structure (state A). Single-channel experiments revealed that the nativelike refolded porin reconstituted into bilayers as efficiently as the native porin.

The addition of small amounts of the refolded porin to lipid bilayer membranes allowed the resolution of stepwise conductance increases. Figure 6a depicts the single-channel conductance recorded from the refolded porin (state C). The

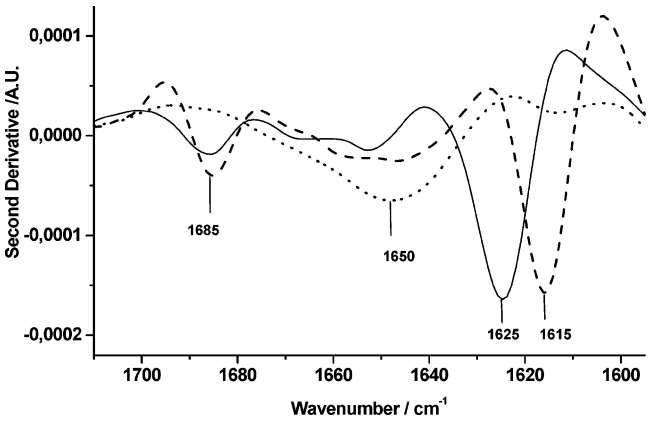


FIGURE 7: Second derivatives of FTIR spectra showing thermal stability analysis of the refolded protein at pH 7: room temperature (—), 75 °C (•••), and 30 °C (cooling) (- - -).

bar indicates that the conductance exhibited by a maximum number of pores is 3.25 nS. The frequency distribution shows a second peak at 1.75 nS which is observed in the wild-type refolded protein, too. This is interpreted as a species of conducting pores of the same protein as the distribution pattern is exactly similar to that of the native (state A) (data not shown).

It was evident from FTIR data that the nativelike porin (state C) had minute differences in interactions. To clarify whether these changes had altered the selectivity of the porin, selectivity measurements were carried out. These measurements were performed by applying 10-fold gradients of KCl across the bilayer containing at least 100 pores. The net charge inside the channel determines whether the pore prefers the passage of anions or cations. The nativelike porin (state C) shows no significant changes in selectivity when compared to the wild-type porin (state A). The potential of the nativelike porin was always found to be negative on side of the bilayer containing a lower ion concentration, indicating a slightly preferential movement of anions through the pores. The potentials were quantified using the Goldman−Hodgkin−Katz equation (*22*). The resulting ratio R [the cation permeability, P_{cat}, divided by the anion permeability, P_{an} (P_{cat}/P_{an})] of the nativelike porin was ∼0.3, similar to those of the wild type reported previously (*20*). Hence, even though refolding had introduced certain alterations into the porin structure, these changes altered neither the selectivity nor the activity of the porin.

Thermal Stability of Nativelike Porin

The black lipid bilayer activity measurements report the functional characteristics of single trimers, whereas the spectroscopic measurements reproduce the entire population. Thus, specific conformers could be favored upon insertion into the black lipid bilayer membrane, and functionally inactive molecules could be lacking in the measured signal. The study of the thermal stability is an additional approach that probes the entire population and characterizes the nativelike structure with respect to the native one. The thermal stability profile of nativelike porin (state C) is shown in Figure 7. It is evident that upon being heated to 75 °C the protein unfolds into unordered structure characterized by the appearance of a broad band centered around 1650 cm^{-1} (step 3). Cooling the protein to room temperature results in aggregation which is evident from the bands at 1615 and 1685 cm^{-1} (step 4). It should be noted that in the second derivative spectra at 75 °C, where the majority of the secondary structure is unordered (dotted line), a small band is seen at 1615 cm^{-1}, which possibly arises from a small aggregated region (*27*). It can be observed in the second derivative spectra that upon cooling no bands arise from any ordered secondary structure. The thermal denaturation is irreversible. It is evident from the spectra that the unfolding pattern of the refolded protein does not match the unfolding of the native protein at pH 8 (see pathway II, step 5) or at pH 12.5 (see pathway V). The unfolding at pH 12.5 shows the presence of an aggregated intermediate which opens up into random structure, but in this case, it directly unfolds into unordered structure and maintains the unordered structure when heated to 90 °C. Once it has cooled, the native protein at pH 12.5 maintains the unordered structure, but the refolded protein (state C) aggregates. Consequently, native porin (state A) and nativelike porin (state C) occupy different minima in the folding landscape. The refolded protein differs from the native protein only slightly in the secondary structure, but the thermal stability is entirely different. There are probably some key interactions which are modified as a result of high-pH-induced unfolding.

Opening of Aggregated Porin

Porin aggregated by heating above its transition temperature for 5 min (pathway II, step 5) was subjected to an increase and decrease in the pH value. It was observed that the turbid aggregated protein solution turned clear if the pH was increased above 12.5 and became more turbid when the pH was decreased to the acidic range. When the secondary structure changes were analyzed in CD spectra (Figure 8a), it was evident that there is a gradual opening of the aggregated protein into unordered structure if it is incubated at pH ≥12.5 (step 6). Figure 8a shows the comparison among native, aggregated, and unfolded porin. Aggregated porin cannot be analyzed in a CD spectrum because of light scattering. To cross-validate the results, similar experiments were carried out by FTIR spectroscopy. Figure 8b depicts the second derivative spectra of the amide I spectral region for aggregated porin (state F) and for pH-unfolded aggregated porin (state G). The bands at 1685 and 1615 cm^{-1} clearly indicate that the protein is aggregated (*8*, *27*). In contrast to that, the amide I band of the protein represents that of an unordered structure if the pH of the aggregated protein is increased to ≥12.5. The bands representing the aggregate disappeared, and a broad band appeared at 1645 cm^{-1}, clearly indicating the presence of unordered protein. Hence, results of CD and IR spectroscopy complement each other. This proves that it is possible to "open up" an aggregated protein, irrespective of what the basic mechanisms could be. Heat and pH changes in the same experiment were used to study their effect on protein destabilization.

Thermal Stability at Various pH Values

pH 1.5, 4, 8, and 10. The thermal stability of porin was analyzed at pH 1.5, 4, 8, and 10 (pathway III). Figure 9 shows the amide I maximum as a function of temperature. The temperature profile was analyzed for the transition temperature (T_M) as explained previously (*8*, *28*). The T_M

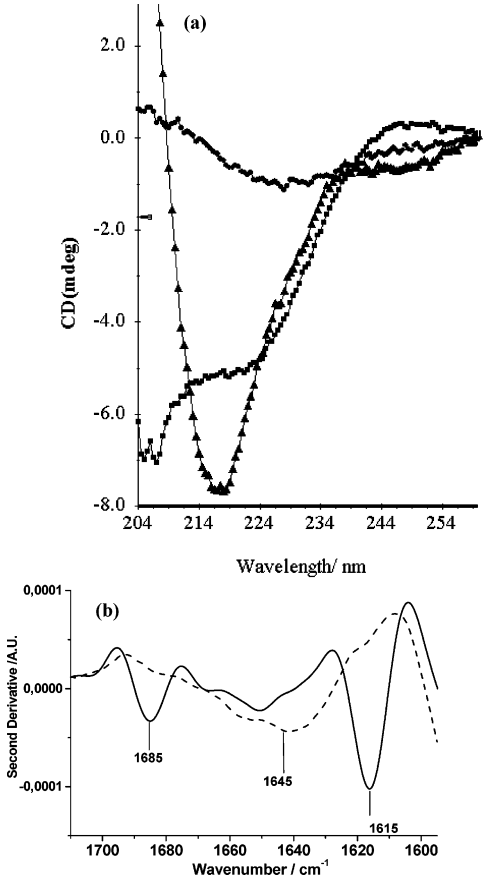


FIGURE 8: (a) CD spectra of porin in the native (▲), aggregated (●), and unordered state (■). (b) Second derivatives of FTIR spectra of the aggregated protein (—) and the protein opened into unordered structure (- - -).

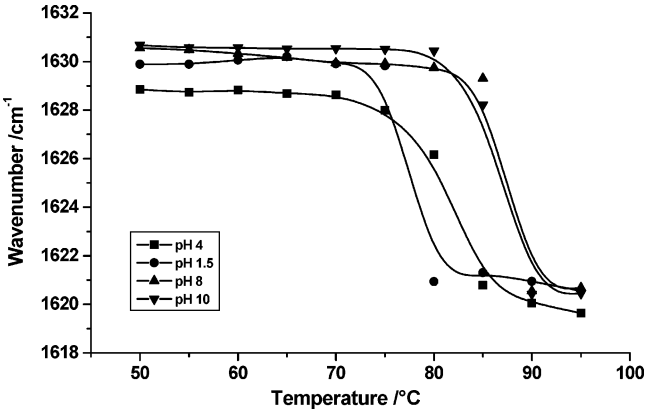


FIGURE 9: Temperature-dependent position of the amide I band for porin at pH 1.5 (T_M = 78.5 °C), 4 (T_M = 82.6 °C), 8 (T_M = 86.2 °C), and 10 (T_M = 86.2 °C).

value for porin was found to be 78.2 °C at pH 1.5 and 82.6 °C at pH 4. The T_M value observed at pH 4 is lower than that at pH 8 and 10 (T_M = 86.2 °C). However, the thermal stability is quite similar, and thermal unfolding results in aggregation. A notable feature of *P. denitrificans* porin is that the protein maintains extreme stability even in the presence of the cumulative effect of two denaturing conditions, heat and ionic strength.

pH 11.5 and 12.5. Figure 10a shows the spectra of porin incubated at pH 11.5 and heated from room temperature to 95 °C (pathway IV). It is observed that upon incubation at room temperature there is a large band characteristic of

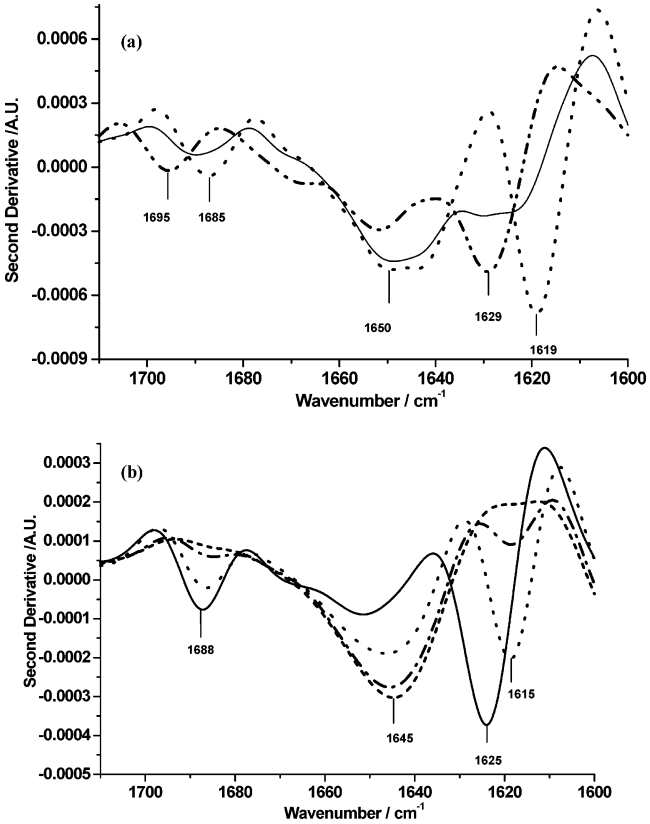


FIGURE 10: (a) Second derivatives of FTIR spectra showing porin at pH 11.5 at room temperature (-••-), 70 °C (—), and 75 °C (- - -). (b) Second derivatives of FTIR spectra showing porin at pH 12.5 heated to various temperatures: room temperature (—), 50 °C (•••), 70 °C (-•-), and 80 °C (- - -).

unordered protein at 1650 cm^{-1} (step 11, state L). When further heated to 70 °C, the protein unfolds into unordered structure, which is evident from the band at 1650 cm^{-1} and from the reduction of the amplitude of the bands at 1629 and 1695 cm^{-1}. However, this state (state M) seems to be highly unstable as when it is further heated to 75 °C bands at 1619 and 1685 cm^{-1} start to appear, which indicates aggregation of the protein (state N). The band indicative of aggregation becomes very prominent with heating to 95 °C. There is no detectable change in the aggregate bands upon cooling (data not shown). The observation here supports the possibility that initial unfolding of the protein is fast and the regions probably involved are the loops and helices which are the easily accessible hydrophilic surfaces (see the explanation about step 1). Even though a large fraction of the protein attains unordered structure at 70 °C, a further increase in temperature causes it to aggregate. This result is crucial as it indicates that an immediate effect on the protein in the presence of denaturing conditions causes it to unfold, but the thermodynamic threshold is not crossed; instead, the protein moves into a thermodynamically stable aggregate (*16*, *17*).

The protein incubated at pH 12.5 had a shift in the β-sheet band position indicating extended $^1H-^2H$ exchange; it is observed to be shifted to 1625 and 1687 cm^{-1} at room temperature (Figure 10b) (pathway V). When the protein is heated to 50 °C, the 1625 cm^{-1} band shifts to 1615 cm^{-1}, but there seems to be no shift in the 1687 cm^{-1} band. As there is an increase in the area of the band around 1650 cm^{-1}, it can be argued that some part of the protein unfolds, too.

With further heating to 80 °C, the protein unfolds into unordered structure as seen by the further increase in the 1645 cm^{-1} band component and the disappearance of bands at 1615 and 1688 cm^{-1}. This indicates that as the first step the protein stabilizes its residues from unfolding by forming an aggregate, but due to the presence of the destabilizing ionic environment, the protein tends to unfold. It is evident that at high pH the protein tends to unfold completely to an unordered structure rather than denaturing to an aggregate. The thermal stability of porin incubated at pH 11.5 and 12.5 showed an entirely different unfolding pattern when compared to that of denaturation in the acidic range.

On the basis of the experiments described above, we conclude that aggregation of porin is pH-dependent. Similarly, for heat-aggregated porin incubated at pH >12, the various interactions which stabilize the aggregate are broken, resulting in exposure of the highly protected residues and hence in unfolding.

Refolding of Heat-Aggregated Porin

Second derivative spectra of the native protein and the protein refolded from porin which was aggregated and subsequently unfolded are shown in Figure 4 (pathway II, state H). The refolded protein has the β-sheet structural features. In comparison to the native protein, the amide I β-sheet components are shifted to 1634 and 1685 cm^{-1}, which was possibly induced by extended deuteration. The bands representing loops and helices are not well-defined. It has to be considered that the helix and loop regions are unfolded upon heating as previously observed (*8*), so the possibility that these local regions are irreversibly denatured by heat cannot be ruled out. The protein refolds back into nativelike structures which also form trimers (Figure 5). Nevertheless, this study shows that it is possible to refold a heat-aggregated protein to nativelike structure without the presence of any chaperones.

Activity and Thermal Stability of the Refolded Protein

The refolded protein was characterized for its activity and selectivity and compared to the native one. Figure 6b shows the single-channel conductance. It indicates that the conductance shown by maximum pores is 3.25 nS. However, the activity is substantially reduced as 5-fold concentration of the protein was required to record the single-channel conductance. The reason can be highly unstable trimers or changes in the loop structure. Our studies with various mutants have shown that mutations in loops drastically reduce the activity (unpublished data).

Zero-current membrane potential measurements were carried out to study the selectivity of the nativelike porins (state H). The nativelike porin exhibited slight changes in the selectivity compared to the native porin. The resulting ratio R (P_{cat}/P_{an}) of the refolded protein (state H) was 0.54. The refolded protein showed increased selectivity for cations compared to that of the native protein ($P_{cat}/P_{an} = 0.3$).

Figure 11 depicts the thermal stability analysis of the refolded protein (state H). The spectra of the refolded protein show that when it is heated to 90 °C, there is a gradual shift of the band at 1634 cm^{-1} toward 1641 cm^{-1}, and when the protein is cooled, the band does not shift to the original position but only to 1637 cm^{-1}. There is a small shoulder at

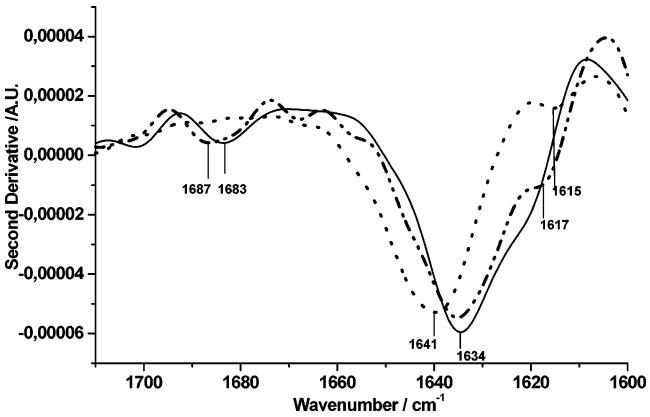


FIGURE 11: Second derivatives of FTIR spectra denoting thermal stability analysis of refolded porin at room temperature (—), 90 °C (•••), and 35 °C (-••-).

1617 cm^{-1} in the room temperature spectra of the protein which disappears upon heating. Instead, a small band appears at 1615 cm^{-1} probably indicating aggregation. The small bands at 1683 cm^{-1} are not seen in the spectra of the heated protein, but the band at 1687 cm^{-1} reappears upon cooling. It is evident from the amide I region that changes occur in the secondary structure which are irreversible. The results also indicate that the refolded protein has an entirely different unfolding pathway compared to the native one.

Contributions to Stability

Hydrogen Bonds and Electrostatic Interactions. Hydrogen bonds and electrostatic interactions play a very crucial role in protein stability. Porins are antiparallel β-sheet structures, and the stability of the sheet itself depends on the formation of hydrogen bonds (*29*). Most of the acidic residues, Asp and Glu, are located in the loops which form the hydrophilic part of porin (Figure 1a) and thus should have a pK close to that in solution. Most of the basic residues, Arg and Lys, are found in the barrel wall (Figure 1b). In the native state (pH 8), most of them are probably involved in hydrogen bond interactions. Their deprotonation with an increase in pH should lead to destabilization and unfolding of the protein. Heating should promote deprotonation of buried residues.

In general, aggregates are thermodynamically stable species (*16*, *17*), and they tend to become unstable due to the changes in electrostatic interactions. Our studies show that the change in pH induces minor instability in the porin β-barrel structure in the low-pH range. At pH 11.5, it is observed that the loop and helix regions are unfolded into unordered structure before heating (pathway IV). When the protein is heated, the fraction of unordered structure increases, but a major portion of the protein still aggregates. This probably indicates that the thermodynamic instability caused by changed electrostatic interactions is not sufficient to unfold the protein completely, only partially. This partially unfolded protein is a highly unstable species which further aggregates to protect some of its hydrophobic residues. When further heated, the porin aggregate opens up into an unordered structure. Hence, a slight change in electrostatics causes the changes in the structural intermediates in the unfolding pathways (III−V).

Tyrosines. The ionic strength of the solution will affect other residues besides the charged ones. Tyrosines are located

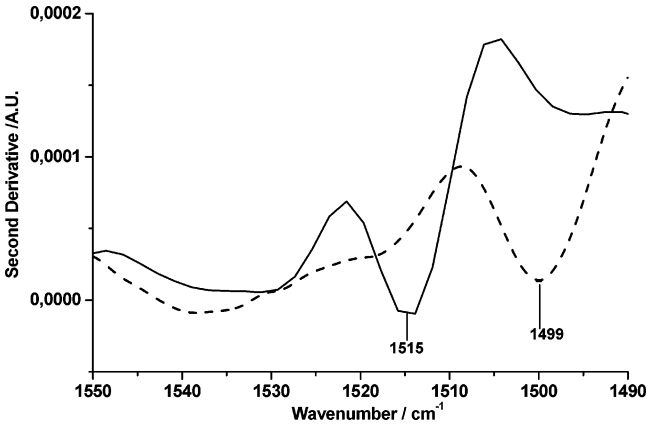


FIGURE 12: Second derivatives of FTIR spectra showing the tyrosine C−C mode at pH 8 (—) and pH 12.5 (- - -).

in the hydrophobic, aromatic girdles of the porin barrel wall and may function as H-bond donors and acceptors. IR spectra can clearly identify the C−C ring mode of Tyr and hence provide access for observation of the changes in Tyr deprotonation. The band of the protonated Tyr side chain is positioned around 1515 cm^{-1}, whereas in the deprotonated form, the band shifts to 1498 cm^{-1} (*30*, *31*). Figure 12 represents the second derivative spectra of porin at pH 12.5 in comparison to those at pH 8. The Tyr ν(C−C) stretching mode for porin at pH 8 is 1515 cm^{-1}, whereas it shifts to 1499 cm^{-1} in the completely deprotonated state at pH 12.5. It is evident from these results that the deprotonation of Tyr occurs at a very high pH of $\geq$12.5. Tyr deprotonation may play a role in destabilizing the porin, but unfolding and the opening up of porin are probably the cumulative effects of the tyrosines and the various charged residues. Other studies have shown that tyrosines play a key role along with lysines in unfolding of spectrin from human erythrocytes and barstar (*32*, *33*).

It can be suggested that protein aggregation is the result of intermolecular interactions which occur to protect the hydrophobic regions of the protein. A major amount of those intermolecular interactions are probably caused by H-bonds of tyrosines, as also indicated by the downshift of the Tyr band position in the study of lipid−protein interaction (*7*). Thus, the deprotonation of the Tyr seems to play a key role in the opening of aggregated porins. However, more experimental evidence from similar membrane proteins and from soluble proteins may be needed for a conclusive explanation.

Porin undergoes a slow unfolding at pH $\geq$12.5. The unfolding is not completely reversible as the refolded structure deviates from the native one spectroscopically. For the first time, we report the opening, refolding, and "refunctionalization" of heat-aggregated porin without the presence of any chaperones. The structural stability of porin is highly maintained by various interactions within the protein.

The key points to the structural stability of porin are various evolutionary aspects associated with the evolution of membrane proteins. In particular is the fact that porins are found in the outer membrane of Gram-negative bacteria which survive in extreme environments, from soil to the human gastrointestinal tract (*34*). Consequently, it is very crucial for these organisms to have outer membrane proteins stable over a wide range of ionic strengths, pH, and temperature variations. The various pathways explored in this study will form the basis for future investigations of the energy levels of the local minima of the folding funnel. Kinetic studies on these pathways will explain the step-by-step opening of the β-barrel structure.

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