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Structure–Function Correlation of Outer Membrane Protein Porin from *Paracoccus denitrificans*

Abstract: Porins from outer membrane of Gram negative bacteria have a highly stable structure. Our previous studies on porin from *Paracoccus denitrificans* showed that the outer membrane protein porin is extremely stable toward heat, pH, and chemical denaturants. The major question we have addressed in this paper is whether the high stability of porin is a consequence of the β barrel structure and whether it is required for its function. To explain this we have analyzed two cases: first, we used porin wild type and mutants and compared their structure and function; second, we compared the activity of porin preheated to different temperatures. Structural changes were monitored by infrared spectroscopy. We observed that the structural stability of porin is not equivalent to functional activity as minor alteration in the structure can result in drastic differences in the activity of porins.

Keywords: porin; structural stability; functional activity; infrared spectroscopy

INTRODUCTION

Porins are the channel-forming proteins found in the outer membrane of Gram-negative bacteria, mito-

chondria, and chloroplasts. They facilitate the transport of hydrophilic molecules below ~ 600 Da in the case of bacterial porins.¹ The X-ray crystal structure of the *P. denitrificans* porin shows that the functional

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unit is a trimer. Each monomer is composed of 16 anti-parallel β -strands.² Despite being an integral membrane protein, porins do not have a hydrophobic primary structure nor do they contain hydrophobic sequences typical for transmembrane α -helices. Yet the porin trimer is a highly stable structure.³ 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.³ Various groups have shown that outer membrane proteins from Gram-negative bacteria have very high thermal stability (for review see 3, 4). Our previous studies with *P. denitrificans* porin showed, apart from very high thermal stability, a high stability toward pH and chemical denaturants.^{5,6}

The question arises whether having the highly stable structure is a functional requirement. Since *P. denitrificans* survives at temperatures close to 30°C, such a highly stable structure in its outer membrane is not a functional requirement. On the other hand a structurally stable protein is significant from an evolutionary point of view, as *P. denitrificans* is also a Gram-negative bacterium, most of which survive in extremely harsh environmental conditions, from soil to stomach.⁴ In this paper we have compared the structure and function of porin in different sets of experiments involving (1) the wild-type and four site-directed mutants and (2) the wild-type porin heated to different temperatures. We have investigated porin for its structural stability using FTIR spectroscopy and correlated it to its activity by analyzing function in black lipid bilayer measurements.

EXPERIMENTAL

Porin gene cloned in vector pJC 40 was obtained from B. Ludwig, Institut für Biochemie, Universität Frankfurt am Main, Germany.^{7,8} The coordinates of the *P. denitrificans* porin X ray structure were provided by W. Welte, Fakultät für Biologie, Universität Konstanz, Germany.² All of the chemicals required in this study were procured from Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany) and Sigma (Munich, Germany).

Protein Purification and Mutant Selection

Purification of protein was carried out according to Saxena et al.⁸ The selection of mutants was based on the comparison of protein sequences of *Rhodobacter blastica*, *Rhodobacter capsulatus*, and *P. denitrificans* porin. Residue D148 is conserved in all compared proteins. W74 is conserved in *R. blastica* and *P. denitrificans* and is also structurally important as it is located in the monomer interface. Residue E81 was selected based on the study by Phale et al.⁹ on *Escherichia coli* OmpF porin, where replacing an equiva-

lent residue located in the extra cellular loop reduced the stability significantly.⁹ A PCR based mutation of the wild type porin gene was carried out using Quick change site directed mutagenesis kit[®] from Stratagene (Heidelberg, Germany). The DNA of the wild type and the mutant constructs were sequenced and were found to be error free and containing the required base pair changes for the mutation. Reconstitution of porin into liposomes was carried out according to a standard freeze thaw protocol.

FTIR Spectroscopy

The measurements were carried out in transmission mode. For the protein in detergent, the sample (20 μ L of 2 mg/mL) was dried in a gentle stream of N₂ and resuspended in 3 μ L D₂O. Approximately 1.5 μ L of sample was loaded in the center of a demountable CaF₂ microcell with an optical path length of 7.4 μ m.^{10,11} The cuvette was sealed by coating the outer ring of one of the windows with a thin layer of an ethanol oil mixture to prevent loss of sample by evaporation. IR spectra were recorded with a Bruker (Ettlingen, Germany) VECTOR 22 FTIR spectrometer equipped with a pyroelectric DTGS detector. For each spectrum, 20 interferograms were averaged, apodized with a Blackman Harris 4 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 the windows software OPUS version 3.1, Bruker (Ettlingen, Germany). The built in program for calculating second derivatives of the spectra in the OPUS software was used to identify the minute changes of band positions and intensities.

Black Lipid Bilayer Activity Measurements

The methods used for black lipid bilayer experiments have been described previously.^{12,13} The membranes were formed from a 1% (w/v) solution of diphytanoyl phosphatidylcholine (Avanti Polar Lipids, Alabaster, Alabama, USA) in *n* decane. Bilayer formation was indicated when the membrane appeared optically black in the reflected light. The porin was added from the protein stock solution of 0.1 mg/mL. The single channel conductance of the pores 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 by a current/voltage converter and amplified. It was monitored on a storage oscilloscope and recorded on a strip chart recorder. Activity of porins were measured using an equal amount of protein in the chamber. The measurements were recorded continuously for 30 min. Data shown are the average of three measurements.

RESULTS AND DISCUSSION

Structure–Function Correlation of Porin Wild-Type and Mutants

Figure 1 represents the FTIR spectra of the amide I region of the wild-type and three mutants E81Q, W74C,

FIGURE 1 FTIR transmission spectra of protein reconstituted in liposomes. Spectra are shifted by an offset for easier comparison. From top to bottom: wild type (—), E81Q (···), W74C (---), and E81Q/D148N (- · - ·).

and E81Q/D148N. The protein was reconstituted into liposomes. The characteristic peaks at 1630 cm^{-1} and 1695 cm^{-1} point out that the secondary structure of the wild-type is predominantly composed of β -sheet,¹⁴ which is in line with the structural information available from the X-ray crystallography.² The mutants E81Q, W74C, and E81Q/D148N had similar IR spectra, thus suggesting similar secondary structure compared to the wild-type. CD spectroscopy (data not shown) also revealed that the secondary structure of the wild-type and the mutants are the same. The minor differences seen in the spectral region between 1700 cm^{-1} and 1600 cm^{-1} are due to different protein-to-lipid ratios in each sample and varying content of H_2O in each sample. In this study, the activity of porin is defined as maximum conductivity (in nS) observed through a single membrane in 30 min for unit concentration of protein. Figure 2A shows the

lipid bilayer activity assays for the wild-type and the mutants. The amount of protein added was kept the same in each activity reaction (Figure 2B). The activity of wild-type was defined as 100%; mutant activity is given relative to it. The triple mutant EDW (data not shown) did not show any characteristic IR band for secondary structure and hence no activity. The ratio of trimeric to monomeric porin was also similar for three of the mutants and the wild-type (see Figure 2B). It is clearly evident that there is a significant reduction in activity for the mutants. This result is surprising as the wild-type and the mutants show very similar structures and our thermal stability studies showed that the deviation of the transition for these mutants was within 5°C .⁵ The question thus arises whether the protein has lower conductance or whether the reconstitution of porin into the bilayer is affected by mutation. The single-channel experiments suggest that the mutants have a single-channel conductance of 3.5 and 1.5 nS (Figure 3). Yet, the distribution pattern varied for the mutants compared to wild-type. The distribution around the 1.5 nS peak was much more dominant in the mutants compared to the wild-type. This suggests that the population of porins producing 1.5 nS conductance is increased due to mutations. These could be porins in which the trimer assembly is modified slightly in a way that only one or two of the pores are conducting at a time. The reason for this distortion in the trimer symmetry could be the mutation of conserved residues. This distortion in the secondary structure and trimer assembly could not be identified in the FTIR spectra. Our repeated attempts to obtain the triple mutant EDW in natively folded form were not successful, suggesting that, even though porins have very few conserved residues,⁴ mutations in these conserved residues could lead to drastic changes in structure and function. Hence it can be concluded that, even though *P. deni-*

FIGURE 2 (A) Lipid bilayer activity profile for wild type and mutants. (B) SDS PAGE gel with equal amounts of wild type and mutant proteins. M, standard protein molecular weight marker.

FIGURE 3 Single channel conductance measured for wild type and mutants.

trificans porin has a highly stable structure, alteration in conserved residues can cause minor changes in its structure but drastic changes in the function.

Structure–Function Correlation of Porin Wild-Type at Different Temperatures

Our previous heating studies showed that *P. denitrificans* porin undergo a change from β -sheet structure

to an aggregated form with a transition temperature of 86.2°C when the protein is in detergent micelles.⁵ Figure 4 shows the amide I region of the protein at 25 and 50°C. It is evident that no significant changes are detectable in the secondary structure of the protein at 25 and 50°C. Infrared spectroscopy, in particular, reaction-induced difference spectroscopy, has proven to be extremely sensitive for conformational changes of proteins down to a level of individual bonds. Absorption spectra of large proteins as shown here

FIGURE 4 Second derivative spectra of the amide I region of the wild type protein at 25°C (—) and 50°C (---).

FIGURE 5 Lipid bilayer activity profile for wild type porin at various temperatures.

are lower in sensitivity, yet allow for the detection of alterations on a level of a few residues. The activity of porin preheated to 50°C is reduced by 90% compared to porin at room temperature (Figure 5). The conformational alterations associated with the loss of activity, however, correspond to minute changes in the secondary structure and/or in the side chain geometry that are too small to be detected here. Another factor to be considered is that the distribution of monomers, dimers, and trimers also changes continuously at different temperatures.⁵ The small changes in the loops of porin may cause a problem for the protein to insert into the bilayer. A noticeable result from these experiments is that the protein preheated to 50°C undergoes some irreversible change, which is surprising as the transition temperature of the protein is much higher. Our previous studies showed that changes in the tyrosine environment in the aromatic "girdles" of porin can be observed along with a small increase in temperature.⁶ These changes are not reversible. Thus a minor alteration in the conformation of porin can result in substantial changes in its function.

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