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Subconductance states in OmpF gating

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Abstract

Discrepancies were noted in the published conductance of the *Escherichia coli* porin OmpF. Results from various papers are hard to compare because of the use of different channel preparations, salt types and concentrations, and electrophysiological techniques (black lipid membrane (BLM) vs. patch clamp). To reconcile these data, we present a side-by-side comparison of OmpF activity studied with the two techniques on the same preparation of pure protein, and in the same low salt concentrations (150 mM KCl). The novel aspect of OmpF porin behavior revealed by this comparison is the ubiquitous existence of states of smaller conductance than the monomeric conductance (subconductance states), regardless of the techniques or experimental conditions used, and the drastic enhancement of subconductance gating by polyamines. Transitions to subconductance states have received little attention in previous publications, in particular when BLM electrophysiology was used. Monomeric closures are rare in recordings at clamped potentials, at least at voltages lower than ~ 100–120 mV. Most closing activity is in the form of subconductance gating, which becomes more dominant in the presence of spermine, with a more frequent and prolonged occupation of these substates. A discussion of the molecular basis for this hallmark behavior of porin is presented.

Keywords: Porin; *Escherichia coli*; Bilayer; Channel; Patch clamp; Polyamine

1. Introduction

The channel properties of the pore-forming protein OmpF of the *Escherichia coli* outer membrane have been described by numerous laboratories [1]. A wide variety of protein preparations have been used for these studies, including protein extracted in sodium dodecyl sufate (SDS) [2,3], proteins purified in the detergent *N*-octyl-oligo-oxyethylene (octyl-POE) [4] or Triton X-100 [3], and enriched outer membrane fractions [5,6]. Two types of electrophysiological techniques, namely black lipid membrane (BLM; also known as planar lipid bilayer) and patch clamp, have been employed by various groups, and the concentrations and types of salt used in such experiments have been varied as well [1]. This multiplicity of approaches has made the comparison between results difficult, and this has led to much debate regarding some of OmpF properties, in particular voltage dependence [1,7].

Another feature of OmpF that has received discrepant analysis is the single channel conductance. With OmpF being a trimer with three individual pores [8], a first level of confusion arose with the mere definition of a "single" channel, as either a single pore or a single trimer (see Ref. [1] for further discussion), and the additional complication that the gating of the three pores may not be independent. This aspect was particularly salient in our past publications [1,5,9–11], where we have consistently reported that OmpF and OmpC traces display transitions of various sizes. Our working hypothesis has been that the smallest observed size was that of a monomer, while larger sizes corresponded to monomers gating cooperatively. In addition, at the voltages applied (typically less than 100 mV), and with the recording times used (typically less than 1 min), we rarely found the canonical three-step closing behavior of OmpF [12], which would have given us

Abbreviations: IPTG, isopropyl-β-thiogalactose; octyl-POE, *N*-Octyl-oligo-oxyethylene; LPS, lipopolysaccharide; BLM, black lipid membrane; HEPES, 4-(2-hydrohyethyl0-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sufate

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indication of the size of the current through the monomer. Thus, we have documented single channel conductances for general diffusion porins [1,5,9–11] that have been smaller than those reported by other groups [13–15]. As we were using membrane preparations, where the amount of associated lipopolysaccharide (LPS) was likely to remain substantial, the patch clamp technique, and solutions of much lower ionic strength that those typically used in BLM experiments, we proposed at that time that the low conductance values might be explained by different experimental conditions.

In order to settle the issue of these discrepant conductance values, we present here a thorough side-by-side comparison of OmpF activity studied with patch clamp or the BLM technique, using the same preparation of pure OmpF and the same salt concentrations. We also extend our comparison to other experimental conditions, such as the use of membrane fractions, or the presence of higher salt concentrations in BLM experiments. This type of analysis is allowing us to modify our original interpretation and to present new evidence that OmpF displays gating to states of lower conductance than the single monomeric conductance (subconductance states or substates). Gating to these substates is greatly enhanced by modulation with polyamines, suggesting the involvement of the L3 loop in this activity.

2. Materials and methods

2.1. Strains, chemicals and media

The strains used were *E. coli* K12 strains AW738 [16] and AD102/pNLF10 for preparation of membrane fractions and pure wild-type OmpF protein, respectively. The construction of AD102/pNLF10 was done as follows. The strain AW741 (*ompF*, *ompA*) was obtained from AW739 [16] by selecting for a spontaneous resistant clone to phage K3 that uses OmpA as a receptor [17]. AW741 was transformed with the *ompF*-containing plasmid pNLF10 [18], and subjected to P1 transduction with a P1 lysate grown on AW738 (Δ*ompC zei*::*Tn*10) to knockout the *ompC* gene. The resulting strain, AD102/pNLF10, does not express *ompC* or *ompA*, and expresses *ompF* from the plasmid only in an isopropyl-β-thiogalactose (IPTG)-dependent manner, as confirmed by resistance to phages SS4 (which uses OmpC as a receptor) and K3, and an IPTG-dependent sensitivity to colicin A (which uses OmpF as a receptor). Cells were grown in Luria-Bertani broth (1% tryptone, 1% NaCl and 0.5% yeast extract) with appropriate antibiotics as required (kanamycin at 100 μg/ml, and tetracycline at 15 μg/ml). IPTG (1 mM) was used to induce *ompF* expression from pNLF10. Tryptone and yeast extract were from Difco laboratories. Octyl-POE was purchased from Alexis Biochemicals. Other chemicals were from Sigma or Fisher.

2.2. Protein purification

Purification of OmpF from AD102/pNLF10 was essentially done as described for *Vibrio cholerae* porins in a previous publication [19], except that protein extraction with the detergent octyl-POE was done at 37 °C instead of 4 °C, and four extractions at 3% were performed instead of two. Purification of OmpF was performed by anion exchange chromatography (Mono Q, Pharmacia), and the protein eluted between 250 and 400 mM NaCl (in 0.5% Octyl-POE, 10 mM Na phosphate buffer, pH 7.6). Protein visualization and purity were assessed by silver staining after SDS-PAGE. Samples were either left at room temperature or heated at 96 °C for 10 min prior to electrophoresis. Non-heated samples demonstrated that all the recovered proteins exist as a stable trimer. No contaminant band was detected by silver staining, testifying of the high purity of the sample. Pure OmpF was kept at − 80 °C in 1% octyl-POE, 10 mM Na phosphate buffer, pH 7.6, and 50 mM NaCl, prior to use in electrophysiology. Protein concentration was determined with the bicinchoninic assay (Pierce).

2.3. Preparation of membrane fractions

OmpF-enriched outer membrane fractions were prepared according to published procedures from strain AW738 [5].

2.4. Reconstitution in BLMs

BLMs were formed with azolectin, a lipid preparation containing essentially phosphatidylcholine (Sigma), as described [19]. Reconstitution of channels was performed by adding 1 to 2 μg of pure porin into ~ 4 ml of buffer in one chamber (*cis* compartment). Buffers were 10 μM $CaCl_2$, 0.1 mM K-EDTA, 5 mM HEPES, pH 7.2, with either 1 M KCl or 150 mM KCl.

2.5. Reconstitution in liposomes

Patch-clamp experiments were performed on blisters induced from giant liposomes containing the reconstituted pure porin [5]. Reconstitution of pure protein or outer membrane fraction into azolectin multilamellar liposomes was performed as described for *Vibrio* porins [19] and for *E. coli* preparations [5], respectively. Protein/lipids ratios of 1:6,000 to 1:10,000 (w/w) (pure protein) or 1:1,600 to 1:1,750 (membrane fractions) were typically used. Patches were obtained as described with 10 MΩ pipettes [5]. Patch clamp experiments were performed with the same buffer in the pipette and/or the bath (150 mM KCl, 5 mM HEPES, 0.1 mM K-EDTA, 10 μM $CaCl_2$, pH 7.2).

2.6. Data recording and analysis

Currents were recorded with an Axopatch-1D amplifier (Axon Instruments), using the CV-4 headstage for patch

clamp experiments, and the CV-4B headstage for bilayer experiments. The current was first filtered at 1 kHz. Continuous recordings were digitized (VR-100, Instrutech), and data acquisition was done at 10 or 11.8 kHz. Analysis was done with programs developed in the laboratory. For bilayer experiments, the *cis* side of the membrane was defined as ground, as documented by others [20].

3. Results

All of our previous work on the electrophysiological properties of porins has been performed with membrane fractions reconstituted in liposomes and analyzed with the patch clamp technique at relatively low ionic concentrations. Analysis of OmpF activity in BLM, however, has typically been done in 1 M KCl or 1 M NaCl. In order to make comparisons with our results in patch clamp experiments, it is first essential to determine the trimeric conductance in the same solutions as those used for patch clamp experiments. The trace of Fig. 1A shows the successive sharp current jumps that correspond to insertion events of pure OmpF trimers at +50 mV in buffer A (150 mM KCl, 10 μM $CaCl_2$, 0.1 mM K-EDTA, 5 mM HEPES, pH 7.2). In order to obtain enough events to generate reliable amplitude histograms, we pooled the data from 10 experiments, and we display 268 conductance measurements of such insertion events. The first two peaks were reliably fitted with the Igor multi-peak routine, and gave the values of 930 ± 187 and 1908 ± 195 pS, representing the conductance of one and two trimers, respectively, in these relatively low salt solutions. The large value of the standard deviations is reflective of the broad width of the histograms, for which we do not have an explanation. The average value for one trimer (~ 940 pS) allows us to deduce a single monomer conductance of ~ 313 pS in these conditions. This conductance value is ~ 22% of the 1.4 nS value reported in 1 M KCl [13,21], and is in agreement with the calculated value obtained from computational studies [22].

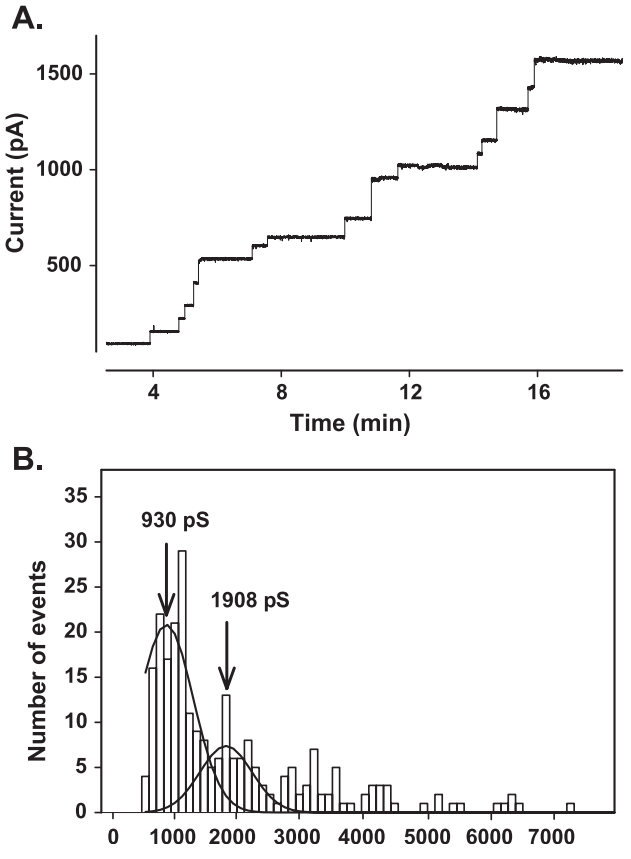

Fig. 1. Measurement of OmpF trimeric conductance in 150 mM KCl in BLM. (A) Recording of successive insertion events of pure OmpF trimers into an azolectin bilayer at +50 mV. Protein was added to the *cis* of the membrane. (B) Amplitude histogram of current derived from insertion events at +50 mV. Results from 10 experiments were pooled. The smooth lines represent the Gaussian fit of the first two peaks obtained with the "multi peak" routine of the Igor program. Buffer composition was 150 mM KCl, 10 μM $CaCl_2$, 0.1 mM K-EDTA, 5 mM HEPES, pH 7.2.

The knowledge of the trimeric conductance was crucial in assigning gating events to those of monomers or other states in traces recorded at maintained voltages. Most of the BLM recordings presented in the literature show the sequential closure of porins at high voltage, and attribute these sharp transitions to the closures of single monomers. These traces are often distinct from the patch clamp traces we reported in our papers, as they show little or no flickering activity in between the sharp closures. In addition, patch clamp traces rarely show the canonical three-step closures of monomers, in part because the high voltages required to trigger this behavior are not easily maintained across membrane patches. In order to determine whether these differences were due to material preparation, techniques or other experimental conditions (such as salt concentration), we present in Fig. 2 a three-way comparison of OmpF activity. Care was taken to use only BLM experiments where a single trimer had been inserted. The conductance obtained from monomeric closures in 1 M KCl was 1.4 nS, identical to values reported by others in this salt concentration [13,21]. It is essential to clamp the potential at values close to the critical voltage of ~ ± 130 mV to observe the three-step closure of OmpF. Although it would be ideal to display the three traces at the same voltage, we have found it difficult to maintain a healthy recording at high voltages in patch clamp experiments. In addition, it appears that the slow three-step gating occurs more favorably at positive potentials in BLM than at negative ones in patch clamp. This may be due to an opposite orientation of the channels in the two systems. Thus, the displayed patch clamp trace was recorded at the highest voltage attained of − 120 mV, at which this type of gating was observed.

Clearly, the channel activity is essentially identical with the three methodologies presented, i.e. a combination of slow kinetics leading to three-step closures and fast kinetics events. A high flickering activity, as seen at the end of trace A, is relatively rare, both in BLM (trace A) and in patch clamp (trace C), and should not be taken as repre-

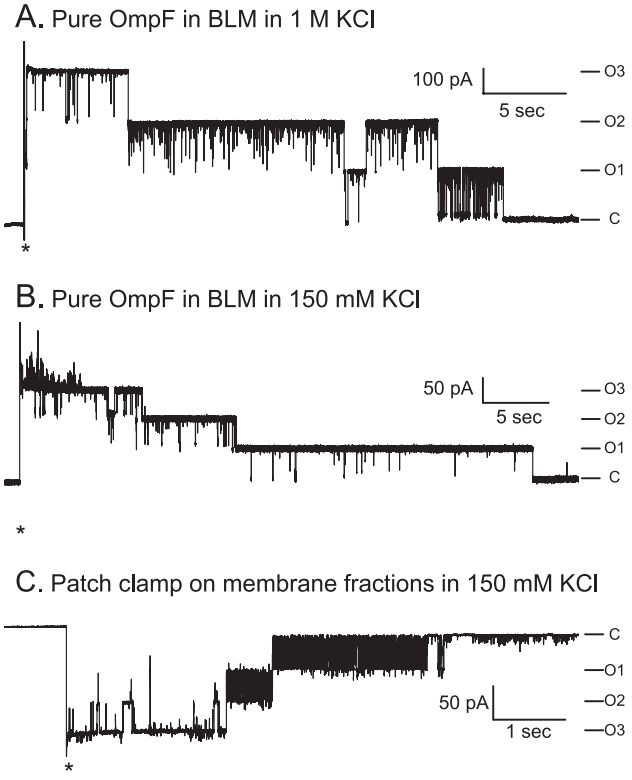

Fig. 2. Three-step closing behavior of monomers. The displayed current traces were obtained in the conditions given in the panel titles at voltages of +140 mV (panels A and B) and −120 mV (panel C). In each case, only one trimer was present in the bilayer or the patch. Current levels corresponding to all monomers closed (C) or to one to three monomers open (O1, O2, O3) are indicated. The time at which the voltage step was given is indicated by an asterisk under the trace.

sentative. However, the frequency of flickers at the beginning of these traces and in trace B is representative of our recordings. It is important to point out that the appearance of the traces is heavily influenced by the filtering frequency and the number of points plotted. We use a filtering frequency of 1000 Hz and plot every sample points, and hence the traces reflect a lot of kinetic details. With high enough voltage, it is also possible to detect sequential monomeric closures of pure OmpF when recorded with patch clamp (data not shown), and thus the nature of the biological material used in liposomes does not appear to qualitatively affect this phenomenon. In addition, the following observations can be made: (1) flickering activity does exist when channels are studied with the BLM technique, (2) the three-step closure behavior can be seen in patch-clamp experiments as long as the recordings are made at sufficiently high voltages and for a prolonged time, (3) the conductance of the largest transitions is close to a third of the trimeric conductance, and thus represents the monomeric conductance.

Once the monomeric conductance is established, it becomes imperative to address the issue of the flickering events. To our knowledge, attention has been drawn to these fast kinetics events only in experiments that have used the patch-clamp technique [5,6,9,23]. Although long-lived substates have been documented in *Haemophilus influenzae* type b porin [24], fast gating flickering activity has been typically undetected or ignored in BLM experiments, except for a recent report of the pH-dependence of OmpF conductance and selectivity [25]. Fig. 3 shows that many of the flickering events are resolved regardless of the methodologies used, and are often prolonged enough to allow a conductance determination. All three traces originate from single-trimer experiments. On the right hand size, the lower tick mark indicates the current level of the baseline (three open monomers), and upper tick mark indicates the current level observed when one monomer has closed. The patch clamp traces of Fig. 3A and B are similar to those shown in our numerous previous publications [1,5,9–11], as they show conductance levels of various sizes. In the absence of the canonical three-step closures, and the trimeric conductance in our patch clamp buffers unknown, we had made the working hypothesis that the transition of smallest

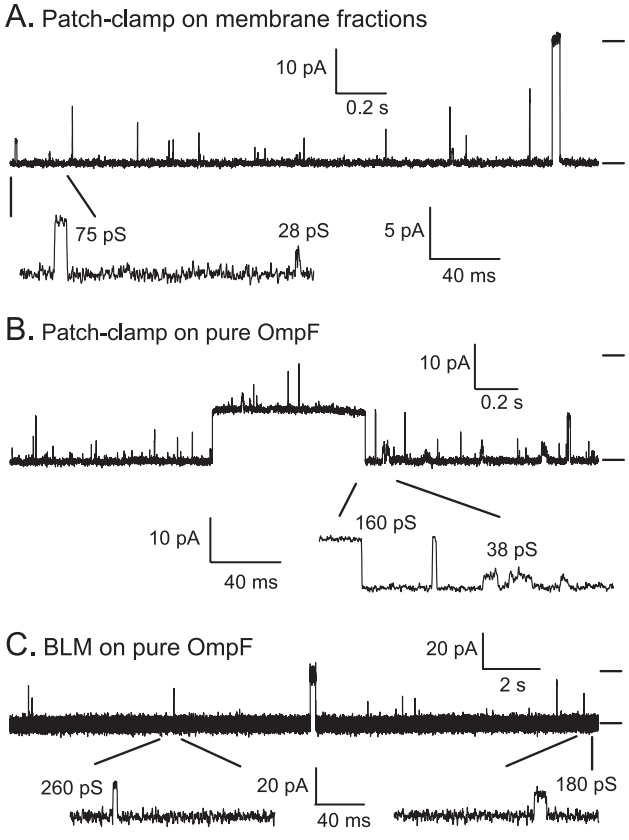

Fig. 3. Subconductance levels are detected in BLM and patch clamp traces. The displayed current traces were obtained in the conditions given in the panel titles, in buffer A (150 mM KCl, 10 μM $CaCl_2$, 0.1 mM K-EDTA, 5 mM HEPES, pH 7.2), and at voltages of −70 mV (panels A and B), and +70 mV (panel C). In each case, only one trimer was present in the bilayer or the patch. For each panel, one or two short segments of the top trace are displayed at higher time resolution to illustrate that the transitions to substates are well resolved. The current step corresponding to the monomeric conductance of 380 pS is indicated by tick marks. Note the different current scales and the higher noise level of the BLM trace.

conductance (~ 30–50 pS) represented the closure of a single monomer. Larger events were interpreted as the cooperative closures of multiple monomers. In light of the results presented in the previous two figures, we need to reassess this interpretation. Transitions of ~ 320–350 pS are assigned as monomeric closures (such as the large event shown in Fig. 3A), based on the trimeric conductance measured in Fig. 1. Smaller transitions must then represent subconductance states or substates. It is quite remarkable that the channel can gate to a variety of substates in a single kinetic step, i.e. without stopping at smaller levels (see, for example, the 75 pS state of Fig. 3A or the 160 pS state of Fig. 3B). Possible mechanistic explanations for this phenomenon are provided in Discussion.

Some of these substates have rather small conductance values (< 50 pS) and are obscured within the noise of BLM trace (Fig. 3C). This is probably in part the reason why sublevels have not typically been reported in BLM experiments. Larger substates, however, are clearly distinct in BLM traces, as shown in Fig. 3C. Although some variability exists from experiment to experiment, it is often the case that substate gating and three-step monomeric closures are more pronounced at negative potentials in patch-clamp experiments than at positive potentials in BLM experiments (Figs. 2 and 3). This is probably due to differences in the channel orientation in the membrane during reconstitution.

An analysis of the distribution and frequency of substates has been given in our previous publications [5,10,26], and will not be duplicated here. The analysis is based on the working hypothesis that the substate of lowest conductance (30–50 pS) represented 1 "unit" of conductance (i.e. a monomer). Larger conductance events were then categorized as "multiples" of this unit conductance (2, 3, 4,. . ., units). The frequency of appearance of substates goes down as the size of the substate goes up (i.e. 1-unit conductance events are more frequent than 2-unit conductance events, etc.) [5,10,26]. However, a statistical analysis revealed that the frequency of multiple conductance events is always greater than expected if these units of conductance represented independently gating pores [5]. It is important to point out that we note quite a bit of variability in the frequency of these events, as some patches seem more biased towards one or the other subconductance states without apparent reason. This variability can also be observed within the same patch, when different traces recorded at different voltages are analyzed. A strict correlation between the type of substate and voltage has not be been revealed, except for the fact that large substates are less often encountered at positive pipette potentials than at negative ones in patch clamp traces. Except for the fact that lower conductance levels are difficult to distinguish among the noise of the bilayer system, there does not appear to be any significant difference between the frequency distribution of substates when channels are studied with the BLM or the patch clamp technique.

We have previously documented that polyamines, such as spermine, exert a strong modulatory effect on OmpF and OmpC gating kinetics [11,26]. We showed that spermine was interacting with residues of the L3 loop and the barrel to promote increased closing activity and stabilization of closed states [18]. These experiments were performed with OmpF-containing membrane fractions examined with patch clamp. Here, we have extended our comparison to preparations of pure OmpF studied with both the patch clamp and the BLM techniques (Fig. 4). In all cases, a greatly increased activity is observed. With the knowledge of the monomeric conductance in these conditions (highlighted by the solid line through the modulated traces in Fig. 4), we can interpret the increased activity to gating to the substates described above. The presence of spermine leads to a higher frequency of transitions to these substates, to a prolongation of dwell times in these substates, and to the multiplicity of

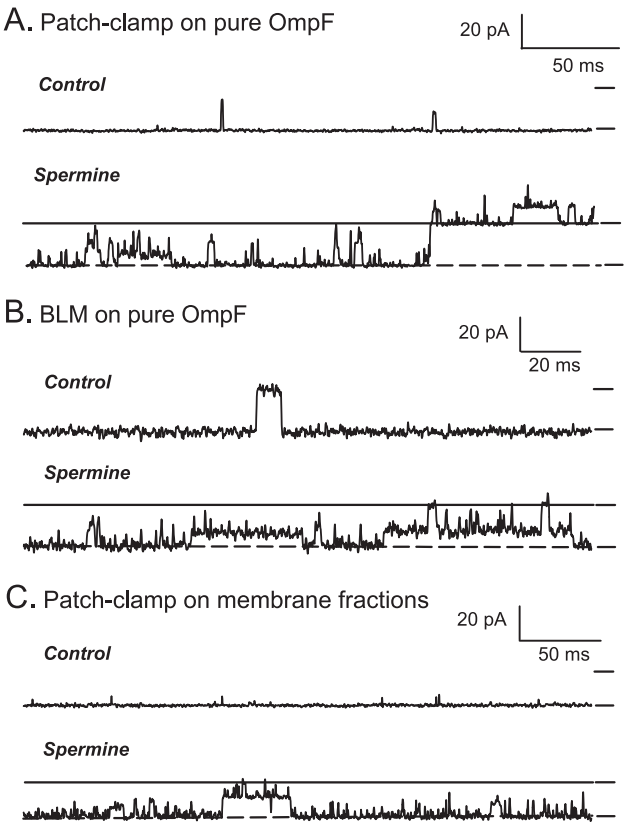

Fig. 4. Subconductance gating is enhanced by spermine modulation. The displayed current traces were obtained in the conditions given in the panel titles at voltages of − 70 mV (panels A and B), and − 60 mV (panel C). In each case, only one trimer was present in the bilayer or the patch. After the current was recorded in control conditions, the activity of the same channel was recorded in 100 μM spermine applied either to the bath solution (patch clamp) or to the *cis* side (BLM). In all cases, buffer A (150 mM KCl, 10 μM $CaCl_2$, 0.1 mM K-EDTA, 5 mM HEPES, pH 7.2) was present on each side of the membrane. The current step corresponding to the monomeric conductance of ~ 350 pS is indicated by tick marks in the control trace. For traces obtained in spermine, the current flowing through all open monomers is given by a dashed lined, while the monomeric conductance is highlighted by a solid horizontal line.

types of states visited, in particular the more frequent appearance of substates of higher conductance. This electrophysiological signature is observed in all three traces of Fig. 4, and thus the phenomenon is not an idiosyncrasy of the biological material or the electrophysiological technique used. We do not wish to present here a quantitative analysis of these patterns, as this would duplicate already published data. However, we need to point out that what was published as cooperative closures of many monomers is reinterpreted here as closures to substates of varying conductance values. Monomeric closures are also more frequent in the presence of spermine than in control conditions, but they are not the dominant type of transitions. In addition, the observation that spermine enhances substate gating and acts at the level of the L3 loop [18] allows us to gain some mechanistic insights in substate gating, as discussed below.

As pointed out in our previous publications, polyamines, such as cadaverine, spermidine and spermine, act as inhibitors or porin activity by enhancing gating to closed states and by promoting long-lived occupancy of these states [11,26]. The data presented here clarify that these closed states represent essentially substates, along with some monomeric closures. This enhanced gating activity can lead to substantial decrease in total current and in open probability [11,26]. Antibiotic flux assays in live cells indeed confirmed that polyamines can drastically decrease the overall permeability of the outer membrane [27,28]. Thus, this ability of porin subconductance gating to be strongly modulated by polyamines represents a highly significant phenomenon for the physiology of the cell. We envision that polyamines can act as natural modulators of outer membrane permeability. For example, we have shown that the endogenous production of cadaverine by cells exposed to acidic pH leads to a reduced permeability through the outer membrane, and confers onto these cells a selective advantage for survival at acidic pH [29]. There is also indication that the polyamine-induced inhibition of porin can lead to decreased antibiotic penetration and increased resistance [30].

4. Discussion

The present report was spearheaded by our desire to reconcile the single monomeric conductance values we reported in our previous publications with those presented by other laboratories. For this, we compared side-by-side the same preparation of pure OmpF in BLM and patch clamp experiments in the same buffer solutions, and extended our comparison to our previous results obtained with OmpF-containing membrane fractions studied with patch clamp. We believe that there is merit in presenting this comparison, but the truly novel aspect to be reckoned with this report is the demonstration that an essential signature of OmpF channel kinetics is the gating to subconductance states.

The existence of substates in porin gating has been mentioned in a few other publications. Three conductance values were reported for OmpC and OmpF at low ionic strength, leading the authors to suggest that porins display quite a bit of "plasticity" [13]. Dahan et al. [24] documented that the voltage-dependent gating of porin from *H. influenzae* type b (Hib) often involved the occupancy of long-lived substates of varying conductance. Berrier et al. [6] reported that OmpF and OmpC porins display fast and slow gating kinetics, and described multiple conductance states, including one of 50–70 pS in 100 mM KCl, which is similar to the lowest conductance we describe in 150 mM KCl [5,11]. In another paper, they present a thorough description of PhoE activity in patch clamp experiments [23], and show that the porin displays a fast gating kinetics to conductance steps ranging from 50 to 200 pS (in 100 mM KCl) superimposed on a slow kinetics of presumed monomeric closures. They propose that the absence of documentation of the fast transitions in BLM studies is not due to a different behavior of porin reincorporated in BLM, but stems from the high filtering of the data often used with the BLM technique. Here we confirm their proposal, as we show that substate gating of OmpF does exist when the porin is studied with BLM. Because of the multiplicity of conductance levels, Berrier and colleagues acknowledged the difficulty in assigning the full monomeric conductance in patch clamp traces. Indeed, we believe that only the side-by-side comparison of channel behavior in BLM and patch clamp, combined with the measurement of trimeric conductance, is the only reliable way to assign monomeric conductances in patch clamp traces, as we have done here.

What is the molecular nature of these substates? This is an important question to resolve, not only from a protein dynamics point of view as this mode of gating is hallmark of porin activity, but since enhanced gating to substates underlies inhibition of porin activity by natural metabolites, such as polyamines [11,26–28]. We have previously shown that spermine and spermidine interact with two pore-exposed residues of the L3 loop, D113 and D121, and proposed that these molecules might bring about inhibition by saddling over the L3 loop [18]. A complete pore plugging by the polyamines would result in current flickers whose size corresponds to the monomeric conductance, as seen for the antibiotic ampicillin [21]. This is not the case here. These molecules are too small to completely occlude the pore, but they might partially interrupt current flow as they move through the pore or tumble within the pore. Although we should not completely rule out some partial block, this interpretation does not provide a satisfactory explanation for the multiplicity of subconductance values and the discreet single-step transitions to any of those substates. In addition, as shown here and previous reports [5,6,9,23], substate gating exists in the absence of polyamines, and we need to consider an intrinsic mechanism for this phenomenon.

As proposed for the molecular basis of voltage dependence [31,32], we are faced here with two alternative

hypotheses: one that proposes that subconductance levels are the manifestations of a pore of smaller size due to the steric occlusion by a protein segment, such as the L3 loop (“steric hypothesis”), or one that surmises that transient fluctuations in the intrinsic electrostatic field of the constriction zone without protein motion lead to abrupt changes in the permeation rates, and hence changes in the conductance (“electrostatic hypothesis”). A combination of both mechanisms is also not excluded.

In favor of the “steric hypothesis”, we have previously proposed that subtle distortions in L3 positions might underlie the spontaneous gating activity of porin channels. This proposal was supported by our observations that mutations that disrupt the interactions of L3 with the adjacent barrel wall lead to increased gating activity in OmpC [33]. Karshikoff et al. [34] proposed that a hydrogen bond network exists between the tip of L3 and the adjacent barrel wall, and that residue D312 in OmpF (D315 in OmpC) plays an important role in this interaction. Interestingly, we found that the D315A mutation in OmpC leads to a distinct spontaneous gating pattern from wild type, characterized by an increased closing activity. Similarly, the mutations of residues at the root of the L3 loop that are likely to participate in salt bridges tethering L3 to the barrel also led to increased activity, albeit with a very different kinetic signatures [33]. An interesting proposal for the discreet substate levels is that L3 might act as a ratchet by taking on various defined positions across the pore. However, a large motion of the L3 loop has not been revealed in computational studies [35–37]. Alternatively, we might speculate that spontaneous local L3 movements, such as slight tip shifts as proposed by some computational models [31,37], or conformational changes at the level of the short α-helix present in L3 might cause a change in the pore configuration and thus sudden perturbations in ion permeation, which would be manifested as abrupt transitions to sub-conducting states. Fluctuations in pore size have been detected in computer simulations, and appear related to local shifts in the position of the L3 tip, in particular at residue Pro116, as water molecules flux between L3 and the adjacent barrel wall [36,37].

In favor of the “electrostatic hypothesis”, we have also documented that the spontaneous gating activity is affected by mutations of charged residues that participate in the intrinsic electrostatic potential of the constriction zone of OmpC [38]. These mutations (K16Q, E109Q, D118Q) are unlikely to cause drastic structural changes, but would have a profound impact on the electrostatic properties of the eyelet. Many, but not all, of these mutants had increased closing activity with distinct kinetic signatures from wild type. These observations highlight the fact that the spontaneous gating activity to subconductance states is quite sensitive to the electrostatic configuration of the pore. Since substate gating is still observed at 0 mV (when ion flow is driven by a concentration gradient across the membrane), it is unlikely that it is caused by fluctuations in the intrinsic electrostatic field due to the imposed voltage. Possibly, subtle changes in the intrinsic field might be produced by ion permeation itself. In a recent study, Nestorovich et al. [25] reported a drastic increase in OmpF open channel noise at acidic pH, with well-resolved transient closures to subconductance levels. These substates, which are rare at neutral pH and enhanced as the pH becomes more and more acidic, are attributed to the reversible protonation of some residues of the eyelet. Whether these pH-dependent substates and the substates described here are the same remains to be confirmed. Interestingly, Nestorovich and colleagues elegantly demonstrated that the effect of pH on conductance is likely to be electrostatic rather than steric in nature.

Substate gating also appears to be modulated by voltage, as the frequency of transitions to substates is increased as the voltage approaches Vc [5,11,39]. Interestingly, many of the mutations that affect voltage gating [14,32,40–42] also affect the spontaneous gating to substates and its voltage modulation [38,43]. Therefore, an interplay might exist between these two forms of gating, i.e. voltage gating (the drastic decrease in open probability observed at voltages greater than Vc) and the spontaneous gating to substates highlighted here.

In conclusion, we have shown here that OmpF porin displays spontaneous gating to subconductance states, that this behavior is observable in patch clamp and BLM techniques regardless of the salt concentration used or the nature of the biological material, and that this activity is greatly enhanced by spermine. Obtaining more mechanistic information on this activity will require computational and biophysical studies, in order to delineate whether conformational changes and/or electrostatic changes in the pore underlie this interesting channel behavior.

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