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The Oms66 (p66) Protein Is a *Borrelia burgdorferi* Porin

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In this study we report the purification and characterization of a 66-kDa protein, designated Oms66, for outer membrane-spanning 66-kDa protein, that functions as a porin in the outer membrane (OM) of *Borrelia burgdorferi*. Oms66 was purified by fast-performance liquid chromatography and exhibited an average single-channel conductance of 9.62 ± 0.37 nS in 1 M KCl, as evidenced by 581 individual insertional events in planar lipid bilayers. Electrophysiological characterization indicated that Oms66 was virtually nonselective between cations and anions and exhibited voltage-dependent closure with multiple substates. The amino acid sequence of tryptic peptides derived from purified Oms66 was identical to the deduced amino acid sequence of p66, a previously described surface-exposed protein of *B. burgdorferi*. Purified Oms66 was recognized by antiserum specific for p66 and serum from rabbits immune to challenge with virulent *B. burgdorferi*, indicating that p66 and Oms66 were identical proteins and that Oms66/p66 is an immunogenic protein in infected rabbits. In a methodology that reduces liposomal trapping and nonspecific interactions, native Oms66 was incorporated into liposomes, confirming that Oms66 is an outer membrane-spanning protein. Proteoliposomes containing Oms66 exhibited porin activity nearly identical to that of native, purified Oms66, indicating that reconstituted Oms66 retained native conformation. The use of proteoliposomes reconstituted with Oms66 and other Oms proteins provides an experimental system for determinating the relationship between conformation, protection, and biological function of these molecules.

Lyme disease is caused by a spirochete, *Borrelia burgdorferi* sensu lato, that infects several mammalian species, including humans, via the bite of an infected tick (10, 11). Lyme disease is the most common arthropod-borne infection in the United States and causes a flu-like illness that, if untreated, can develop into a chronic infection with multisystem involvement (25, 31, 37–40). Our interest in pathogenesis and immunity in Lyme disease has centered on the definition and study of the outer membrane-spanning (Oms) proteins of *B. burgdorferi* (7, 28, 30, 35, 36, 43). Since porin proteins are found only in the outer membrane (OM) (17, 18, 24), we have regarded identification and characterization of *B. burgdorferi* porins as an essential foundation for the study of the OM.

Previously, freeze fracture electron microscopy demonstrated that the *B. burgdorferi* OM contains approximately 5- to 10-fold less Oms proteins than enteric gram-negative bacteria (28, 43). Several groups have speculated that the paucity of the Oms proteins in *B. burgdorferi* and other pathogenic spirochetes allows these bacteria to evade the initial innate immune mechanisms of the host (4, 6, 19, 27, 29, 42, 43), thereby resulting in the establishment of an active infection. The low molar abundance of spirochetal surface-exposed proteins has also complicated the identification, purification, and characterization of these molecules. We reasoned that the identification and characterization of a functional Oms protein, like a porin, would provide a valuable marker for the OM of *B. burgdorferi* and a potential surface-exposed vaccine target. Molecular characterization of porin proteins would also provide an important foundation to determine the topological organization of Oms proteins in the OM of *B. burgdorferi* relative to other bacterial porins (12).

To initiate these studies, we isolated the OM from *B. burgdorferi* and characterized porin proteins associated with this material (35). Preliminary electrophysiological characterization indicated that the OM material contained two distinct porin activities with single-channel conductances of 0.6 and 12.6 nS in 1 M KCl (35). We subsequently purified both porin proteins, cloned the gene encoding the 0.6-nS porin, (designated Oms28), and determined its nucleotide sequence (36).

In this study, we report the characterization of the protein associated with the large-channel conductance we had previously identified in our OM preparation (35). We have purified this protein and demonstrate that the observed porin activity is mediated by a 66-kDa protein that we have designated Oms66. Subsequently, we show that Oms66 is the previously described p66 protein (1, 8, 9, 26) and that reconstituted native Oms66 retains porin activity, suggesting that native conformation is also maintained. As such, these results provide the experimental foundation for reconstitution of Oms66 and other Oms proteins, either separately or in combination, into proteoliposomes in order to evaluate their role in pathogenesis and immunity.

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MATERIALS AND METHODS

Bacterial strains. *B. burgdorferi* sensu stricto B31 was grown at 32°C in BSK II medium as previously described (35).

Isolation of OMV. OM vesicle (OMV) preparations were obtained as previously described (35).

Purification of the Oms66 porin protein. OMV preparations derived from 5×10^{10} *B. burgdorferi* B31 passage 3 cells were incubated overnight at 4°C in a 5-ml volume containing 2% hydrogenated Triton X-100 (hTX-100; Calbiochem, San Diego, Calif.) buffered in 50 mM Tris HCl, pH 8.0. Unlike conventional Triton X-100, hydrogenated Triton X-100 does not adsorb at 280 nm. After solubilization, the insoluble material was removed by two successive centrifugations at $13,000 \times g$ for 10 min at 4°C. The resulting protein within the supernatant was separated by using a 1-ml Mono Q column in conjunction with the Pharmacia fast-performance liquid chromatography (FPLC) system (Pharmacia Biotech, Piscataway, N.J.). The column was washed with a 21-ml volume of 0.5% hTX-100 buffered in 50 mM Tris HCl, pH 8.0, and 42 separate 0.5-ml flowthrough fractions were collected. Proteins bound to the column were eluted by using a 0 to 400 mM linear NaCl gradient containing 0.5% hTX-100 buffered in 50 mM Tris HCl, pH 8.0. Forty consecutive fractions were collected, each representing a successive increase of 10 mM NaCl. The fractions were pooled and immediately tested for porin activity in planar lipid membranes as described previously (23, 35, 36). Fractions containing peak porin activity were rechromatographed by using the Mono Q column as described above.

SDS-PAGE and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were conducted essentially as described previously (35). Briefly, the protein in fractions containing the peak porin activity was concentrated by precipitation with either a 10-fold excess of ice-cold acetone or 5% trichloroacetic acid. The precipitated protein was pelleted by centrifugation at $16,000 \times g$ for 15 min, washed once with ice-cold acetone, and resuspended in SDS-PAGE sample buffer (21). The unboiled samples were resolved by SDS-PAGE, electroblotted to a polyvinylidene difluoride (PVDF) membrane (Millipore Corp., Bedford, Mass.), and stained with either 0.1% amido black or colloidal gold (Aurodye forte; Amersham Corp., Arlington Heights, Ill.) to determine the purity of the given porin-active fractions. To determine whether the 66-kDa porin was the previously described p66 protein (8), 2 μl from porin-active fractions was spotted onto nitrocellulose (Schleicher and Schuell, Keene, N.H.) and probed with antiserum specific for p66. SDS-polyacrylamide gel-resolved samples or dot blots were immunoblotted with either serum from rabbits immune to challenge (15), e.g., immune rabbit serum, or antiserum specific for p66, both diluted 1:1,000. Immobilized immune complexes on the PVDF membrane were detected with either a 1:2,500 dilution of donkey anti-rabbit immunoglobulin conjugated to horseradish peroxidase (Amersham Corp.) or a 1:500 dilution of protein A conjugated to horseradish peroxidase (Zymed Corp., South San Francisco, Calif.). Immunoblots were developed with the enhanced chemiluminescence (ECL) system (Amersham Corp.) and exposed to Kodak XAR-5 film.

Antiserum. Antiserum specific for p66 was generated as described by Bunikis et al. (8). Immune rabbit serum was obtained essentially as described elsewhere (15, 35).

Protein sequencing. The amino acid sequence of purified Oms66 (approximately 5 μg) was conducted essentially as described elsewhere (5, 36, 41). Briefly, the FPLC-purified Oms66 protein was resolved by SDS-PAGE, immunoblotted to nitrocellulose, stained with 0.1% amido black, and destained with distilled, deionized H_2O. The Oms66 band was cut from the nitrocellulose membrane and incubated with trypsin as previously described (5, 36, 41). The resulting peptides were purified by reverse-phase high-performance liquid chromatography, and four of these peptides were sequenced via Edman degradation (5, 36, 41).

Planar lipid bilayer assays. Assays to detect porin activity using diphytanoyl phosphatidylcholine (DPPC) or asolectin (both purchased from Avanti Polar Lipids, Alabaster, Ala.) as the lipid substrate were conducted essentially as described elsewhere (23, 35, 36). DPPC is a synthetic phospholipid, and asolectin is a crude mixture of soybean-derived phospholipids, of which approximately 50% are phosphatidylcholine. Fractions with peak activity were diluted from 10^{-5} to 10^{-6} and assayed at membrane potentials between 10 to 20 mV. Voltage-dependent closure of Oms66 was monitored at membrane potentials ranging from −120 to +120 mV as previously described (23, 35).

Reconstitution of Oms66 into liposomes. Proteoliposomes containing Oms66 were generated as follows. Twenty milligrams of asolectin (see above for description) was hydrated in 10 ml of 1 M KCl–10 mM Tris HCl (pH 7.5) and sonicated in an ice bath until the sample was completely clear. The lipid suspension was then subjected to three freeze-thaw cycles using alternating dry-ice–ethanol bath and room temperature incubations. The liposome suspension was then diluted to a final volume of 100 ml with 1 M KCl–10 mM Tris HCl (pH 7.5), and the sample containing partially purified Oms66, in a 400-μl volume, was added such that the final concentration of Triton X-100 was less than 0.01%. Liposomes with no protein added were also prepared as controls. This suspension was then incubated, with mixing, at room temperature for 1 h. The proteoliposomes were pelleted at $40,900 \times g$ for 45 min, and the supernatant was discarded. The pellets were resuspended in 0.15 M KCl–10 mM Tris HCl (pH 7.5)–20 μM EDTA to a final volume of 100 ml, and the sample was recentrifuged at $40,900 \times g$ for 45 min. The supernatant was discarded, and the final pellet was resuspended in 0.5 ml of 0.15 M KCl–10 mM Tris HCl (pH 7.5)–20 μM EDTA. To determine whether Oms66 had been incorporated into the liposomes, the final proteoliposomes, as well as control liposomes with no added Oms66, were solubilized in 2.5% Triton X-100 for 30 min on ice. The detergent-solubilized samples were diluted 10^{-3} to 10^{-4} and then tested for porin activity on planar lipid bilayers as described above.

RESULTS

Purification of a 66-kDa porin protein from the OM of *B. burgdorferi*. We had previously observed a large-channel-forming activity in detergent-solubilized OMV preparations derived from *B. burgdorferi* (35) and therefore sought to purify the protein species associated with this porin activity. OMV preparations derived from *B. burgdorferi*, corresponding to approximately 200 μg of total protein, were solubilized in detergent. Subsequent FPLC purification indicated that the majority of the large-channel-forming activity, with an approximate 10-nS conductance (in 1 M KCl), was present in the flowthrough fractions just prior to the start of the NaCl gradient. While the majority of the flowthrough proteins (including the OspA lipoprotein [3] [data not shown]) were immediately collected early in the 21-ml wash (between 1 to 7 ml), the 10-nS channel-forming protein came off the column late in the flowthrough fractions (between 11 and 16 ml, with a peak between 13 and 14 ml) (Fig. 1, solid arrow). The amount of protein in this peak corresponded to approximately 6 to 10% (or approximately 12 to 20 μg) of the total protein in the OMV preparation. These porin-active fractions were reapplied to the Mono Q column (to effectively eliminate residual OspA contamination [data not shown]) and recovered at exactly the same volume range (peak between 11 to 16 ml of the wash [data not shown]). SDS-PAGE analysis of these active fractions, followed by transfer to a PVDF membrane and staining with either colloidal gold or amido black, indicated that these samples contained a single 66-kDa protein (Fig. 2). Based on the channel-forming activity observed, the presence of this protein in our OMV preparation, and the knowledge that porin proteins are associated exclusively with the OM, we designated this protein Oms66, for outer membrane-spanning 66-kDa protein (Fig. 2, lane 3). Porin assays of the rechromatographed fractions indicated that these samples had a high specific activity since dilutions to 10^{-5} yielded 581 individual stepwise insertional events (Fig. 3A) over a total period of approximately 15 min. The mean (with standard deviation) single-channel conductance observed for the 581 individual events was 9.62 ± 0.37 nS (Fig. 3B).

Protein assays of fractions containing purified Oms66 indicated that 12 to 20 μg of Oms66 was recovered from OMV preparations derived from 2.5×10^{10} *B. burgdorferi* B31 passage 3 cells. Individual fractions with peak activity contained approximately 5 to 10 μg of Oms66 per ml. Therefore, dilutions of the active fractions out to 10^{-5} yielded samples with a final Oms66 concentration of 50 to 100 pg/ml, an amount of porin protein that is within the detectable range of activity for the assay conditions utilized (23, 35, 36).

Amino acid sequence of tryptic fragments derived from purified Oms66. In order to determine the amino acid sequence of the purified Oms66 protein, approximately 75 pmol (5 μg) of purified Oms66 was digested with trypsin, the resulting tryptic peptides were purified by reverse-phase high performance liquid chromatography, and the peptides were sequenced via Edman degradation as previously described (5, 36, 41). The amino acid sequence of four independently isolated peptides was identical to the deduced amino acid sequence of the surface-exposed p66 protein derived from the nucleotide sequence of the *p66* gene (8). The sequences of the four Oms66

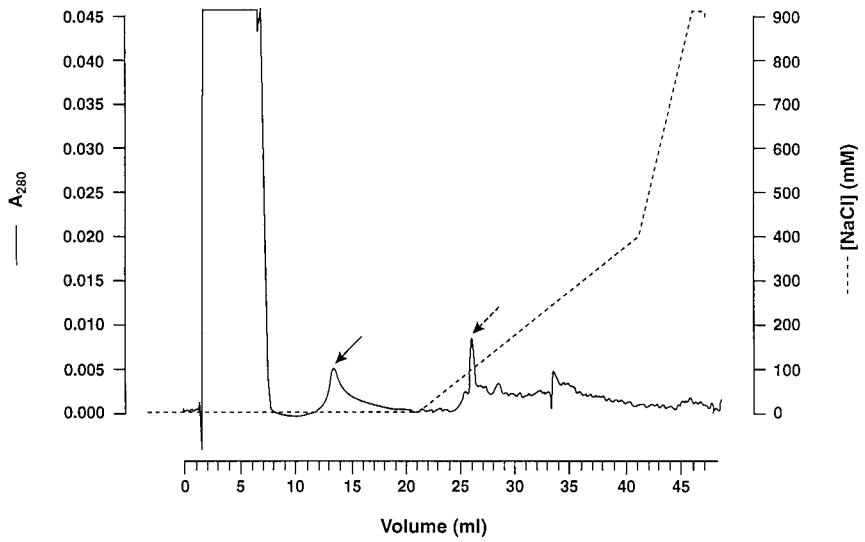

FIG. 1. Chromatogram of FPLC-purified OMV proteins derived from *B. burgdorferi*. Detergent-solubilized OMV proteins were loaded onto a 1-ml Mono Q column and eluted as described in Materials and Methods. Solid arrow, fractions containing maximal porin activity; dashed arrow, fractions containing Oms66 that exhibited residual large-channel-forming porin activity (see Discussion). Solid line, absorbance at 280 nm (A_{280}); dashed line, concentration of NaCl for a given fraction volume (indicated on the *x* axis).

peptides (from the amino terminus of Oms66/p66) are LDLT FAIGGTGTGNR, YKLGLTK, INDKNTYLILQMGTDFGI DPFAS, and DTGEKESWAIK; the second and third peptides are directly adjacent to one another in the deduced amino acid sequence (8).

Antigenicity of Oms66. To further verify that the purified porin Oms66 was the previously described p66 protein (1, 8, 26), we conducted immunoblot analysis with antiserum specific for the *B. burgdorferi* p66 protein (8). The Oms66 porin was specifically recognized by the p66 antibodies (data not shown).

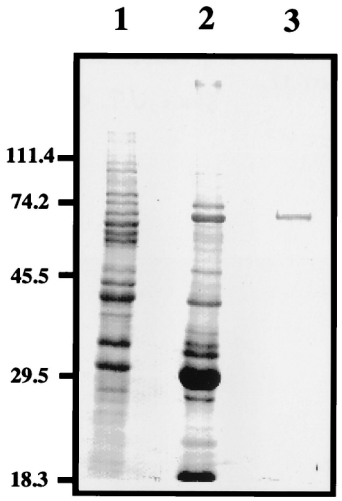

FIG. 2. Purification of the large channel isolated from the *B. burgdorferi* OMV preparation. SDS-PAGE analysis of passage 3 whole cells of *B. burgdorferi* B31 and fractionated samples derived from *B. burgdorferi*. Samples were separated by SDS-PAGE, and the protein was transferred onto a PVDF membrane and stained with 0.1% amido black. Lane 1, 1×10^8 *B. burgdorferi* B31 passage 3 whole cells; lane 2, OMV preparation derived from 5×10^9 *B. burgdorferi* B31 passage 3 whole cells; lane 3, 500 ng of FPLC-purified and porin-active Oms66 derived from a solubilized *B. burgdorferi* OMV preparation. The molecular masses of the protein standards (in kilodaltons) are indicated.

In addition to the porin-active Oms66 detected in the flowthrough fraction, some Oms66 bound to the Mono Q column. When Mono Q column fractions were spotted onto nitrocellulose and immunoblotted with antiserum specific for p66, Oms66 (p66) was also observed in samples that were eluted with 90 to 100 mM NaCl (data not shown). However, when these fractions were tested for porin activity, they were approximately 100-fold less active (data not shown) relative to the Oms66-containing flowthrough fractions (Fig. 1, dotted arrow; see Discussion).

We also probed purified Oms66 with serum from rabbits exhibiting infection-derived immunity to reinfection with as many as 4×10^7 *B. burgdorferi* B31 passage 4 cells (15). Immune rabbit sera recognized purified Oms66, indicating that Oms66 was immunogenic in infection-immune rabbits (data not shown).

Purified Oms66 is non-ion selective and voltage dependent. Oms66 channels exhibited a symmetric voltage dependence similar to that observed for the mitochondrial voltage-dependent anion-selective channel (for a review, see reference 2) and the PorB channel from pathogenic *Neisseria* (particularly in the absence of nucleoside triphosphates [2, 32]). All Oms66 channels were open at voltages near 0 mV and began closing with increasing voltages of either polarity, starting at approximately ±30 mV (Fig. 4A). Approximately half the channels are open at ±70 mV, whereas more than 90% of the open-state conductance was turned off at voltages greater than ±100 mV. Voltage-dependent behavior of Oms66 appeared symmetric with respect to positive and negative voltages.

Figure 4B shows a typical example of the voltage-dependent behavior of a single Oms66 porin channel. At +20 to −23 mV, the Oms66 channel is open virtually all the time, as indicated by the straight line of current without fluctuations. At ±80 mV, multiple substates of Oms66 are visualized mostly as stepwise decreases in conductivity until the channel partially closes such that, over time, the steady-state conductance is reduced to approximately 40% of the initial observed conductivity (Fig. 4A). The fact that the Oms66 channel tended to close slowly (over periods of seconds to minutes) whereas openings were

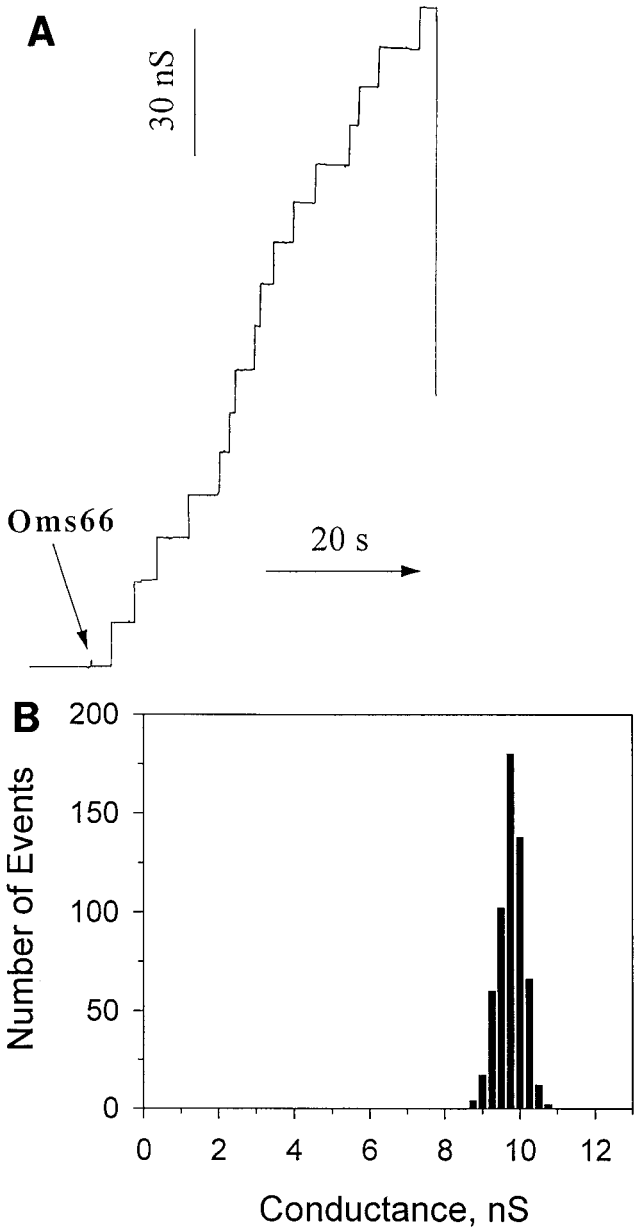

FIG. 3. Porin activity associated with FPLC-purified Oms66. (A) Single-channel conductance observed for Oms66. Fractions containing FPLC-purified Oms66 (identical to that in Fig. 2, lane 3) were added to a lipid bilayer composed of DPPC bathed in 1 M KCl buffered in 10 mM Tris HCl, pH 7.5. The time point at which the purified Oms66 was added to the bilayer is indicated. (B) Histogram of the individual single-channel conductance events observed for the purified Oms66 porin (n = 581).

extremely fast (occurring in milliseconds [Fig. 3A]), is typical of porins.

Reconstitution of Oms66 into liposomes. To further confirm that Oms66 was an outer membrane-spanning protein, we sought to determine whether this protein would be incorporated into liposomes. We initially utilized an Oms66-containing fraction that had been FPLC separated only once over a Mono Q column. This partially purified form of Oms66 was then added to liposomes composed of asolectin. Incorporation of Oms66 was evaluated by both SDS-PAGE and planar lipid bilayer assays. SDS-PAGE analysis of the crude Oms66-containing sample prior to and after incubation with the liposomes indicated that Oms66 was preferentially incorporated into the proteoliposomes as evidenced by both Coomassie blue staining and immunoblot analysis (Fig. 5A and B, respectively). SDS-PAGE analysis of liposomes prepared without any added protein had no detectable protein species (data not shown). Note that the 31-kDa OspA protein present in the crude sample is apparently not observed in the final proteoliposomal preparation (Fig. 5A). Colloidal gold stains of the proteoliposomes indicated that residual OspA was present (data not shown), although Oms66 was clearly enriched for in these samples. Therefore, the ability of Oms66 to be preferentially incorporated into proteoliposomes functioned as a purification step.

To determine whether Oms66 retained native channel-forming activity following incorporation into proteoliposomes, detergent-solubilized samples were tested for porin activity and compared with liposomes with no added *B. burgdorferi* protein. The average channel-forming activity of the reconstituted Oms66, 9.86 ± 0.47 nS based on 119 individual insertional events (Fig. 6), was nearly identical to the porin activity of purified native Oms66 (9.62 ± 0.37 nS) obtained from the solubilized *B. burgdorferi* OMV preparation (compare Fig. 3 and 6). Samples containing liposomes alone had no detectable porin activity at dilutions that yielded stepwise increases in conductance in the Oms66-containing proteoliposomes (data not shown). The reconstituted Oms66 also exhibited the same voltage dependence as native Oms66 (data not shown). These results further confirm that Oms66 is an outer membrane-spanning protein.

DISCUSSION

Porin proteins allow for the passive diffusion of low-molecular-weight compounds across the OM. We initiated studies to identify OM porin proteins in order to establish a paradigm for the study of the additional *B. burgdorferi* surface proteins, particularly virulent-strain-associated outer membrane-spanning proteins (35). The further characterization of porin proteins in *B. burgdorferi* could also provide information pertaining to the physiology of the spirochete and, more importantly, yield a protein that could function as a potential vaccine candidate.

In this study we have purified, using FPLC, a 66-kDa protein, which we have termed Oms66, that is associated with the 12.6-nS porin activity we previously reported (35). However, following separation from other OMV proteins, we found that the average single-channel conductance observed was reproducibly reduced from 12.6 to 9.62 nS (Fig. 3). Electrophysiological data indicated that the voltage dependence of channel opening/closing, the voltage-dependent closure to multiple substates with nearly identical amplitudes, and the relative lack of cation/anion selectivity of both the 12.6- and the 9.62-nS channels (Fig. 4 and data not shown) were nearly identical, suggesting that these two activities were most likely the result of the same molecule. One possible explanation for the differences in the large-channel conductance observed in this study could be due to the purification of the porin protein away from other contaminating proteins present in the OMV preparation (35). These proteins may have modified the conformation of the large channel, thereby resulting in a different average single-channel conductance for Oms66.

Dot blot analysis of column fractions reactive with antiserum to p66 (data not shown) indicated that native Oms66 was present in both the low-salt, late flowthrough fractions (Fig. 1, solid arrow) and fractions that were eluted off the column at a concentration of NaCl between 90 to 100 mM (Fig. 1, dashed arrow). However, the Oms66 present in the flowthrough frac-

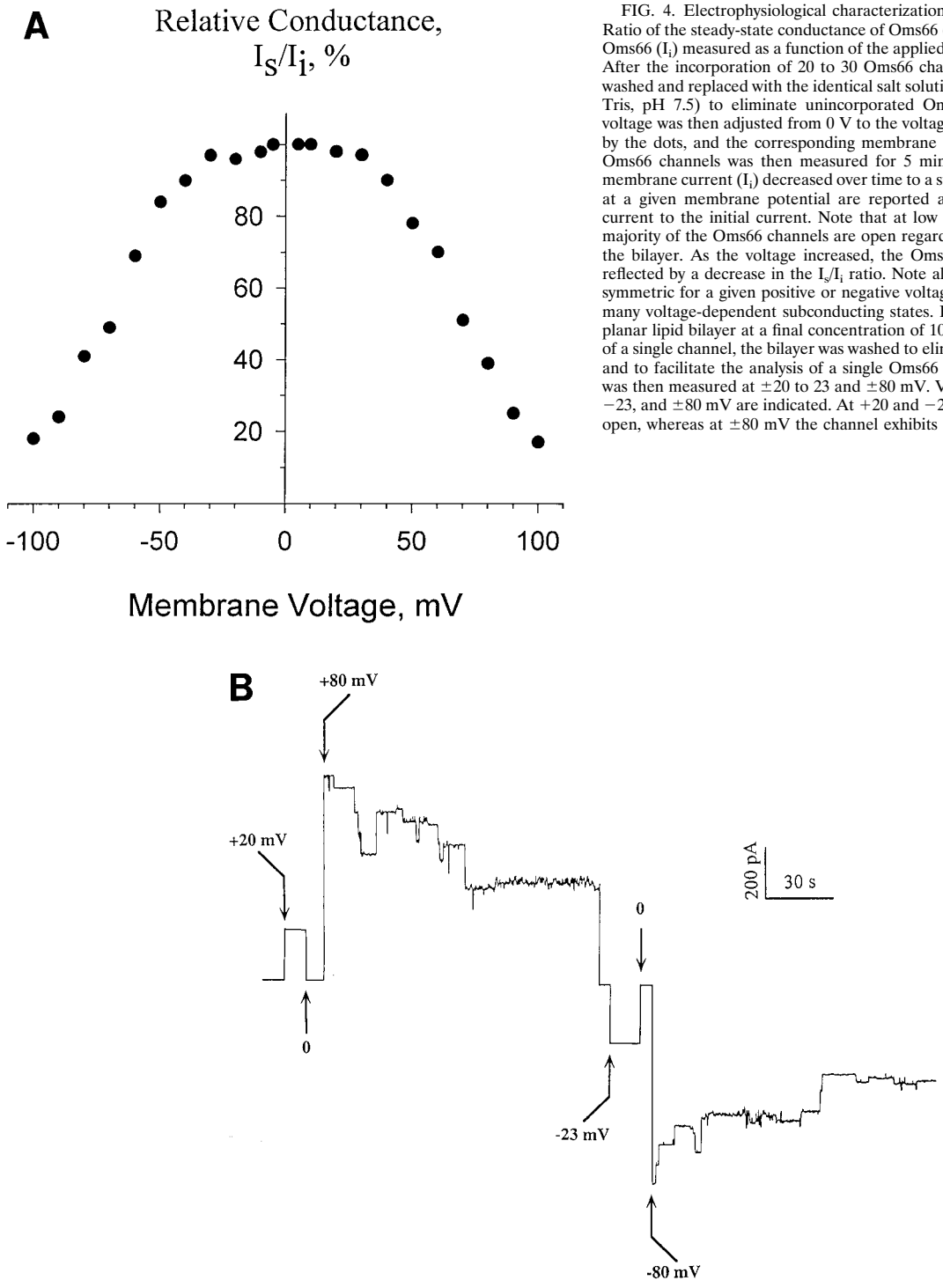

FIG. 4. Electrophysiological characterization of FPLC-purified Oms66. (A) Ratio of the steady-state conductance of Oms66 (I_s) to the initial conductance of Oms66 (I_i) measured as a function of the applied membrane potential over time. After the incorporation of 20 to 30 Oms66 channels, the bilayer chamber was washed and replaced with the identical salt solution (1 M KCl buffered in 10 mM Tris, pH 7.5) to eliminate unincorporated Oms66 channels. The membrane voltage was then adjusted from 0 V to the voltages (at both polarities) indicated by the dots, and the corresponding membrane current elicited by the inserted Oms66 channels was then measured for 5 min. During the 5 min the initial membrane current (I_i) decreased over time to a steady-state level (I_s). The values at a given membrane potential are reported as the ratio of the steady-state current to the initial current. Note that at low membrane potentials the great majority of the Oms66 channels are open regardless of the polarity imposed on the bilayer. As the voltage increased, the Oms66 channels began to close, as reflected by a decrease in the I_s/I_i ratio. Note also that the degree of closure is symmetric for a given positive or negative voltage. (B) The Oms66 channel has many voltage-dependent subconducting states. Purified Oms66 was added to a planar lipid bilayer at a final concentration of 100 pg/ml, and after the insertion of a single channel, the bilayer was washed to eliminate unincorporated channels and to facilitate the analysis of a single Oms66 channel. The degree of closure was then measured at ±20 to 23 and ±80 mV. Voltage shifts between 0, +20 to −23, and ±80 mV are indicated. At +20 and −23 mV the channel is completely open, whereas at ±80 mV the channel exhibits multiple substates.

tions was at least 100-fold more active than the Oms66 fractions that were eluted off the column in 90 to 100 mM NaCl. Surprisingly, the Oms66 present in the flowthrough proteins did not wash out with the other flowthrough proteins. This observation suggested that Oms66 was separated from the other flowthrough proteins due to a weak interaction between either Oms66 or Oms66-detergent micelles and the column matrix. The observation that two sets of column fractions contained Oms66 yet had disparate specific activities suggested that these forms of Oms66 had different conformations and that the conformation of the low-salt form of Oms66 was optimal for activity in the planar lipid bilayer assay. The Oms66 conformation required for maximal pore-forming activity correlated with its inability to bind the anion-exchange column (Fig. 1 [solid arrow] and Fig. 3), and conversely, the ability of Oms66 to bind the column implied that the conformation had

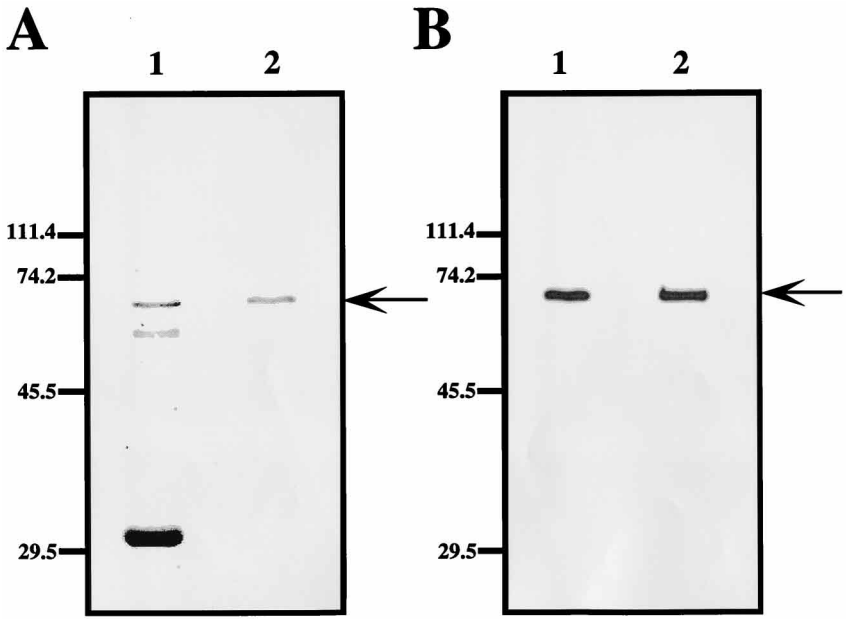

FIG. 5. Reconstitution of native Oms66 into liposomes. (A) Coomassie blue stain of the SDS-PAGE analysis of samples prior to and after their addition to liposomes. Lane 1, partially purified fractions containing a porin-active form of Oms66; lane 2, proteoliposomes composed of asolectin phospholipids obtained after incubation with the Oms66-containing porin-active sample in lane 1. (B) Immunoblot of samples identical to those shown in panel A probed with antiserum specific for p66.

been altered, resulting in its subsequent binding and concomitant decrease in porin activity (Fig. 1, dashed arrow).

There are several lines of evidence indicating that Oms66 is an outer membrane protein in *B. burgdorferi*. First, Oms66 is present and enriched for in our OMV preparation relative to whole cells (35) and, most importantly, exhibits a porin activity consistent with its OM location. Additionally, a 66-kDa protein, presumably Oms66 (p66) is also observed in *B. burgdorferi* OM preparations obtained by alternative and independent methodologies (7, 30). Second, Bunikis and coworkers have shown, using thin-section immunoelectron microscopy, that antibody specific for p66 recognizes a protein on the surface of *B. burgdorferi* sensu lato (8) and, more recently, have identified a surface-exposed immunogenic domain of p66 (9). Third, previous results have shown that the addition of proteinase K to intact cells resulted in the preferential proteolysis of the surface-exposed p66 protein to a 50-kDa form (1, 8, 26) under conditions where subsurface endoflagella and a putative periplasmic 83-kDa protein were not degraded (26). Additionally, a previously reported 66-kDa protein of *B. burgdorferi*, with an acidic pI, was labeled with ^{125}I; by analogy with the other data presented above, this protein is most likely the Oms66 porin (22).

The abundance of Oms66 and its large pore diameter place it in the class of spirochetal porin proteins with large pore diameters. The major outer membrane protein of *Spirochaeta aurantia* (20) and the two major surface proteins of *Treponema denticola*—Msp, a 53-kDa species (13, 14), and a 64-kDa species (45)—form channels with diameters from 2.3 to 3.4 nm, a range that comprises the predicted 2.6-nm diameter of the Oms66 pore based upon its average single-channel conductance (17). In addition to their porin activities, the *T. denticola* 64-kDa protein functions as an adhesin when added to cultured gingival fibroblasts (44) and the 53-kDa Msp protein has also been shown to bind to various extracellular matrix proteins (16). By analogy with the *T. denticola* porins, it is tempting to speculate that Oms66 may also function as an adhesin, although there is no data at present to support this contention. Other known spirochetal porins from *B. burgdorferi* (Oms28 [23 and 36] and Oms45 [34], *Treponema pallidum* (Tromp1 [5]), and *Leptospira kirschneri* (OmpL1 [33]) have smaller single-channel conductances and, by analogy, smaller pore diameters. No putative link to pathogenesis has yet been established for these smaller spirochetal porins.

Our methodology for producing proteoliposomes is similar to the planar lipid bilayer assay in that the protein is added to a preformed lipid bilayer, but in the form of vesicles, in the presence of 1 M KCl. These two factors eliminate the possibility of trapping protein inside the proteoliposomes and wash away peripherally bound proteins, respectively. Current methodologies employed to produce proteoliposomes do not ensure that proteins are not present inside or associated with the outside of the vesicles and are generally more time-consuming or have other caveats (17). Furthermore, the incorporation of Oms66 into liposomes serves as a further purification step, enriching for Oms66 (Fig. 5A).

The ability to reconstitute native Oms66 into liposomes and the retention of native porin activity indistinguishable from the activity observed for the FPLC-purified Oms66 (compare Fig. 3 and 6) indicate that the native conformation of Oms66 can be simulated in proteoliposomes and suggest that surface-exposed epitopes of Oms66, presumably important for both native function and binding by borreliacidal antibodies, may also be preserved in these proteoliposomes. Due to the abundance of Oms66 on the surface of *B. burgdorferi* and its immunogenicity in rabbits and humans, it is tempting to speculate that Oms66 may function as an effective target for borreliacidal-antibody binding and, as such, may be an effective vaccine candidate for Lyme borreliosis.

Based on the ease of this proteoliposomal procedure and the indication that native conformation of Oms66 can be preserved, it may now be possible to generate proteoliposomes

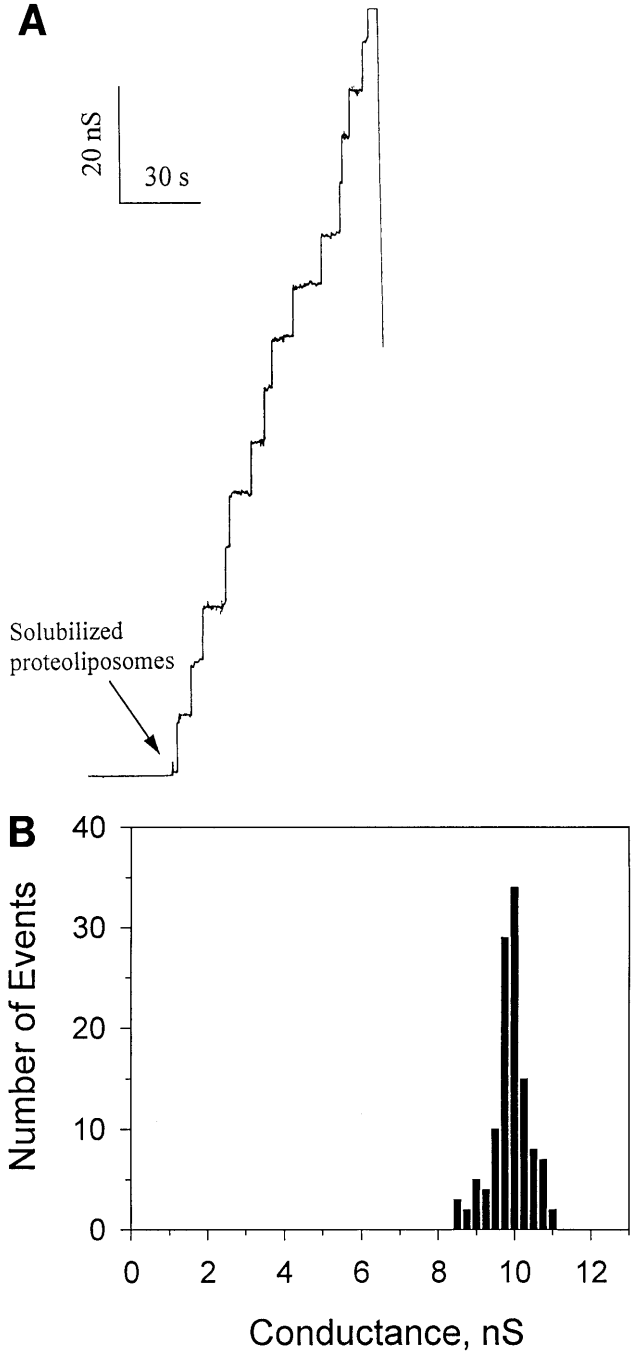

FIG. 6. Porin activity of Oms66-containing proteoliposomes. (A) Single-channel conductance of protein solubilized from proteoliposomes containing Oms66. Protein was solubilized from the proteoliposomes as outlined in Materials and Methods and was added to a planar lipid bilayer composed of DPPC in a salt solution containing 1 M KCl buffered in 10 mM Tris HCl, pH 7.5. Note that the channels are identical to those in Fig. 3. (B) Histogram of single-channel conductances from the detergent-solubilized proteoliposomes containing Oms66 (n = 119).

containing various combinations of other purified Oms proteins, including Oms28 (36), Oms66, and other virulent-strain-associated Oms proteins (35), either individually or in combination, to determine their roles in both pathogenesis and protective immunity. We are currently pursuing this experimental approach to determine which Oms proteins are necessary to confer protection against *B. burgdorferi* experimental infection.

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