Biochemical and Biophysical Characterization of the Cell Wall Porin of *Corynebacterium glutamicum*: The Channel Is Formed by a Low Molecular Mass Polypeptide†

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ABSTRACT: The cell wall of the Gram-positive bacterium *Corynebacterium glutamicum* contains a channel (porin) for the passage of hydrophilic solutes. The channel-forming protein was identified, by lipid bilayer experiments, in the cell envelope fractions isolated by sucrose-density centrifugations and in organic solvent of whole cells. It was purified to homogeneity by fast-protein liquid chromatography across a Mono-Q column. The pure protein had a rather low molecular mass of about 5 kDa as judged by SDS−PAGE, which suggested that the cell wall channel is formed by a protein oligomer. The monomer has according to partial sequencing no significant homology to known protein sequences. The purified protein formed large ion-permeable channels in lipid bilayer membranes from phosphatidylcholine/phosphatidylserine mixtures with a single-channel conductance of 5.5 nS in 1 M KCl. Experiments with different salts suggested that the cell wall channel of *C. glutamicum* was highly cation-selective caused by negative charges localized at the channel mouth. The analysis of the single-channel conductance data using the Renkin correction factor suggested that the diameter of the cell wall channel is about 2.2 nm. Channel-forming properties of the cell wall channel of *C. glutamicum* were compared with those of mycobacteria. These channels share common features because they form large and water-filled channels that contain point net charges.

In Gram-positive bacteria, the cytoplasmic membrane is surrounded by a thick murein sacculus, which contains up to 100 peptidoglycan layers. It prevents the cells from osmotic lysis and shields it against the attack of enzymes. In many Gram-positive bacteria the murein layer does not represent a permeability barrier for hydrophilic solutes up to a molecular mass of 100 kDa (*1*). Therefore, it has been widely accepted that Gram-positive bacteria do not possess pore-forming proteins in the cell wall. Members of the group of actinomycetes such as mycobacteria, nocardia, and corynebacteria represent presumably an exception among Gram-positive bacteria because they contain, besides the peptidoglycan layer, a large amount of lipids in form of mycolic acids in their cell wall (*2*−*4*). The mycolic acids are linked to the arabinogalactan attached to the murein of the cell wall (*5*−*8*). The length of the mycolic acids varies within these bacteria. They are especially long in mycobacteria (60 and 90 carbon atoms) and in Nocardia (46−90 carbon atoms). The mycolic acids of corynebacteria are in comparison relatively short and have only a length of 22−38 carbon atoms (*4*−*6*, *9*−*13*). The permeability of the cell wall of mycobacteria is unusually low (*14*), presumably because the mycolic acids represent a second permeability barrier surrounding the peptidoglycan layer (*15*). Similarly, the mycolic acid layer of other members of the group of actinomycetes acts probably also as a permeability barrier similar to that of mycobacteria (*14*, *16*). This means that the mycolic acid layer has probably the same function as the outer membrane of Gram-negative bacteria, which contains channel-forming proteins, the porins, for the passage of hydrophilic solutes (*1*, *17*). In analogy to the situation in the outer membrane of Gram-negative bacteria, channels are present in the mycolic acid layer of the mycobacterial cell wall (*16*, *18*, *19*).

Channels have recently been shown to exist also in the cell wall of *Mycobacterium chelonae* (*16*, *18*) and *Mycobacterium smegmatis* (*19*). Similarly, channel-forming activity has been detected in the cell wall fraction of the envelope of *Corynebacterium glutamicum* (*20*). Corynebacteria belong to the same group of bacteria, the actinomycetes. Like mycobacteria, they have a peptidoglycan layer that is probably covalently bound to arabinogalactan and they possess mycolic acids linked to the polysaccharides in a similar way as in mycobacteria (*2*, *3*, *7*). In this study, we further studied the channel from the cell wall fraction of the envelope of *C. glutamicum* and identified the protein responsible for channel-formation layer in *C. glutamicum* as a small protein with a molecular mass of about 5 kDa,

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which forms probably an oligomer. The protein has according to partial sequencing no significant homology to known proteins. The properties of the cell wall porin were investigated using the lipid bilayer assay. According to the results of the electrophysiology, the cell wall channel of *C. glutamicum* is wide and water-filled. It is mainly permeable for cations, which is caused by the presence of negatively charged groups at the channel mouth.

MATERIALS AND METHODS

Bacterial Strain and Growth Conditions. *C. glutamicum* strain MH 20-22B, which is a leucine auxotroph derivative of the wild-type strain ATCC13032 (*21*), was grown in continuous fermentation at 30 °C, pH 7.0, at a dilution rate of 0.05 h^{-1} in a medium that has been described previously in detail (*20*).

Isolation of the Cell Wall. The cells were harvested by centrifugation and washed once in 10 mM Tris-HCl[1] (pH 8). From part of the cells, the cell wall was isolated by sucrose density centrifugation. For this, the cells were passed three times through a French pressure cell at 900 psi gage pressure (13 000 psi cell pressure). Unbroken cells were removed by centrifugation at 5000*g* for 15 min. The cell envelopes (cytoplasmic membrane and cell wall) were obtained by centrifugation of the supernatant at 170000*g* for 90 min (rotor 70.1 Ti). The pellet was resuspended in 2 mL of 10 mM Tris-HCl (pH 8) and applied to a sucrose step gradient of 30% (3 mL), 40% (4 mL), and 70% (3 mL) sucrose similar to that used previously to separate cytoplasmic membrane and cell wall of *M. chelonae* (*16, 18, 22*) and of *C. glutamicum* (*20*). The gradient was centrifuged at 170000*g* for 16 h in a Beckman Optima 90 XL ultracentrifuge (rotor SW40Ti). Eight fractions were collected. Fraction 3 contained most of the cytoplasmic membrane [as assessed by the NADH oxidase activity (*23*)] and fraction 7 most of the cell wall and most of the channel-forming activity (*20*).

Isolation and Purification of the Channel-Forming Protein from the Cell Wall. The cell wall fraction of the sucrose density centrifugation contained a channel-forming protein (*20*). The isolation of the cell wall by sucrose density centrifugation was always accompanied by a substantial loss of material. Therefore, we used an alternative method for the extraction of the channel-forming activity from whole cells. For this extraction, 100 mL of cell suspension was washed twice in 10 mM Tris-HCl (pH 8). The final pellet (5 mL) was extracted two times with a 1:2 mixture of chloroform:methanol in a proportion of 1 part cells and 5−8 parts chloroform/methanol. The duration of the extraction was about 10 h at room or slightly elevated temperature (30 °C) under stirring in a closed container to avoid loss of the chloroform. Cells and chloroform/methanol solution were centrifuged for about 15 min (10 000 rpm in Beckman LH20). The pellet (cells) was discarded. The supernatant contained the channel-forming activity. It was mixed in a ratio of 1 part supernatant to 9 parts ether and was kept overnight at −20 °C. The precipitated protein was dissolved in a solution containing 0.4% LDAO and 10 mM Tris-HCl (pH 8) and inspected for channel-forming activity. The protein was subjected to fast-protein liquid chromatography (FPLC) across a Mono-Q column. The column was washed first with a buffer containing 0.4% LDAO and 10 mM Tris-HCl (pH 8) and the protein was eluted with 0.4% LDAO in 10 mM Tris-HCl (pH 8) using a linear gradient between 0 and 1 M NaCl.

SDS−PAGE. SDS−PAGE was performed according to ref *24* or according to Schägger and von Jagow (*25*) with tricine containing gels because of the low resolution of the former gel system for low molecular mass proteins. The gels were stained with Coomassie brilliant blue or with silver staining (*26*).

Peptide Sequencing. The purified polypeptide with a molecular mass of about 5 kDa was precipitated using trichloroacetic acid to remove the detergent. The amino acid sequence of the peptide was determined by the Edman degradation method using a gas-phase sequenator (470A, Applied Biosystems) with on-line detection of the amino acids.

Peptide Digestion. The purified polypeptide with a molecular mass of about 5 kDa was treated for 5 min with 50 units/mL proteinase K (EC 3.4.21.64; Sigma, St. Louis, MO) in a buffer containing 0.4% LDAO and 10 mM Tris-HCl (pH 8).

Immunological Techniques. Polyclonal antibodies against the 5 kDa protein were raised in rabbits following the protocol of Harlow and Lane (*27*) using the MPL+TDM+CWS Adjuvant System (Sigma, St. Louis, MO). The ELISA experiments were carried out as described. Different amounts of cells were coupled per well, and the preimmune serum was used as negative control.

Lipid Bilayer Experiments. The methods used for black lipid bilayer experiments have been described previously (*28*). The experimental set up consisted of a Teflon cell with two water-filled compartments connected by a small circular hole. The hole had an area of about 0.4 mm^2. Membranes were formed across the hole from a 1% solution of diphytanoyl phosphatidylcholine (PC) phosphatidylserline (PS) and mixtures of both lipids (Avanti Polar Lipids, Alabaster AL) in *n*-decane. The temperature was maintained at 20 °C during all experiments. All salts were obtained from Merck (Darmstadt, Germany, analytical grade). They were used unbuffered. The single channel records were performed using calomel electrodes (with salt bridges) connected in series to a voltage source and a current-to-voltage converter made with a Burr Brown operational amplifier. The amplified signal was monitored on a storage oscilloscope (Tektronix 7633) and recorded on a strip chart or tape recorder. Zero current membrane potentials were measured with a Keithley 610 C electrometer 5−10 min after a 10-fold salt gradient was established across the membranes (*28*).

Estimation of the Channel Diameter. The calculation of the channel size is possible from the conductance data when the ions move inside the channel similar as in the aqueous phase. It is based on the same assumptions that have been used previously for the derivation of the Renkin correction factor (*29*) for the diffusion of neutral molecules through

[1] Abbreviations: LDAO, lauryl dimethylamine oxide; nS, nanosiemens; $P(G)$, probability of occurrence of the conductance step with the conductance G°; Tris, tris(hydroxyaminomethane); SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; FPLC, fast-protein liquid chromatography; PC, diphytanoyl phosphatidylcholine; PS, phosphatidylserine.

porous filters and porins. The Renkin correction factor can be applied to the channel conductance when the entering of a hydrated ion into the effective area, *A*, of the channel mouth is the rate-limiting step (and not the diffusion of the hydrated ion through the channel itself). This means that the Renkin correction factor can only be used when the ions or the other solutes studied (*30*) do not interact with the channel interior.

The permeability of a cylindrical channel (radius *r*) for solutes is proportional to the aqueous diffusion coefficient, *D*, multiplied with the Renkin correction factor given by

$$A/A_0 = [1 - (a/r)]^2[1 - 2.104(a/r) + 2.09(a/r)^3 - 0.95(a/r)^5] \quad (1)$$

where *A* is the effective area of the channel mouth, A_0 is the total cross sectional area of the channel, and *a* is the radius of the hydrated ions or substrates passing through the channel. To apply the Renkin correction factor for the calculation of the channel size, we have to know the radii of the hydrated ions and their diffusion coefficients, *D*, in the aqueous phase. The radii, *a*, of the hydrated monovalent ions can be calculated from the Stokes equation by using their limiting molar conductivity, λ_i, in the aqueous phase according to

$$a = \frac{Fe}{6\pi\eta\lambda_i} \quad (2)$$

F ($F = 96\,500$ As/mol) is the Faraday constant, *e* ($e = 1.602\ 10^{-19}$ As) is the elementary charge, and η ($= 1.002\ 10^{-3}$ kg/ms) is the viscosity of the aqueous phase. The diffusion coefficient of the hydrated ions in the aqueous phase is implicitly also given by the limiting molar conductivity, λ_i, of the different ions. It is not necessary to calculate it (although this is possible) since the Renkin correction factor times the diffusion coefficient and the single-channel conductance may both be given relative to a given ion. Using this method the relative conductance of the different ions with respect to Rb^+ is given as a function of the hydrated ion radius, which corresponds to the dependence of the relative rate of permeation on solute radius in the liposome swelling assay with solutes of different size (*30*). This method has nothing to do with the estimation of the channel size from the absolute conductance by assuming an aqueous cylinder with a given aqueous conductivity, which has been heavily criticized (*31*). It is noteworthy that the use of this method is not affected by whether the conductive unit contains one channel or a bundle of channels similar to the situation with Gram-negative bacterial porins where the trimers contain three individual channels (*30*). The validity of the method has previously been assessed by comparing the size of the cell wall channel of the *M. chelonae* as estimated from the method described above (i.e., from the single-channel conductance) and from the vesicle-swelling assay (*18, 19*).

Effect of Negatively Charged Groups Attached to the Channel Mouth. Negative charges at the pore mouth result in substantial ionic strength-dependent surface potentials at the pore mouth that attract cations and repel anions. Accordingly, they influence both single-channel conductance and zero-current membrane potential. A quantitative description of the effect of the point charges on the single-channel conductance may be given by the treatment, which has been proposed by Nelson and McQuarrie (*32*) and has been used by Menestrina and Antolini (*33*) for the description of the ionic strength dependent conductance of the hemocyanin channel. It describes the effect of point charge on the surface of a membrane. The potential Φ created by a point charge *q* (in As), on the mouth of a channel with a radius *r* is given by

$$\Phi = \frac{2qe^{(-r/l_D)}}{4\pi\epsilon_0\epsilon r} \quad (3)$$

ϵ_0 ($=8.85 \times 10^{-12}$ F/m) and ϵ_r ($=80$) are the absolute dielectric constant of vacuum and the relative constant of water, respectively, and l_D is the so-called Debye length, which controls the decay of the potential (and of the accumulated positively charged ions) in the aqueous phase:

$$l_D^2 = \frac{\epsilon\epsilon_0/RT}{2F^2c} \quad (4)$$

c is the bulk aqueous salt concentration, and *R*, *T*, and *F* ($RT/F = 25.2$ mV at 20 °C) have the usual meaning.

The concentration of the monovalent cations near the point charge increases because of the negative potential. Its concentration c_0^+, at the channel mouth is given by

$$c_0^+ = c_0 e^{-(\Phi F)/(RT)} \quad (5)$$

The cation concentration at the mouth of the pore can now be used for the calculation of the effective conductance-concentration curve:

$$G(c) = G_0 c_0^+ \quad (6)$$

G_0 is the concentration independent conductance of the channel.

RESULTS

Identification of the Channel-Forming Cell Wall Protein. To identify the protein, which is responsible for the channel-forming activity of the cell wall of *C. glutamicum*, the cell wall fraction of the sucrose-density gradient was subjected to preparative SDS−PAGE (data not shown). Different fractions of the gels were eluted overnight with a buffer containing 1% Genapol. The different molecular mass fractions were examined for channel-forming activity in the lipid bilayer assay. Interestingly, some small channel-forming activity was smeared out over most molecular masses of the gel, which could not be related to defined bands. Very high activity (membrane conductance about 10^3−10^4-times higher), however, was obtained in the low molecular mass region of the gel. This result suggested that the channel-forming protein of the cell wall had a low molecular mass and that its channel-forming activity was resistant to SDS treatment. Therefore, we used an alternative method, which avoided the substantial loss of cell wall protein during sucrose density centrifugation. The washed and centrifuged cells were extracted with organic solvent, a 1:2 mixture of chloroform:methanol in a proportion of 1 part cells and 5−8 parts chloroform/methanol. Cells and organic solvent were centrifuged at 10000*g*, and the cells were

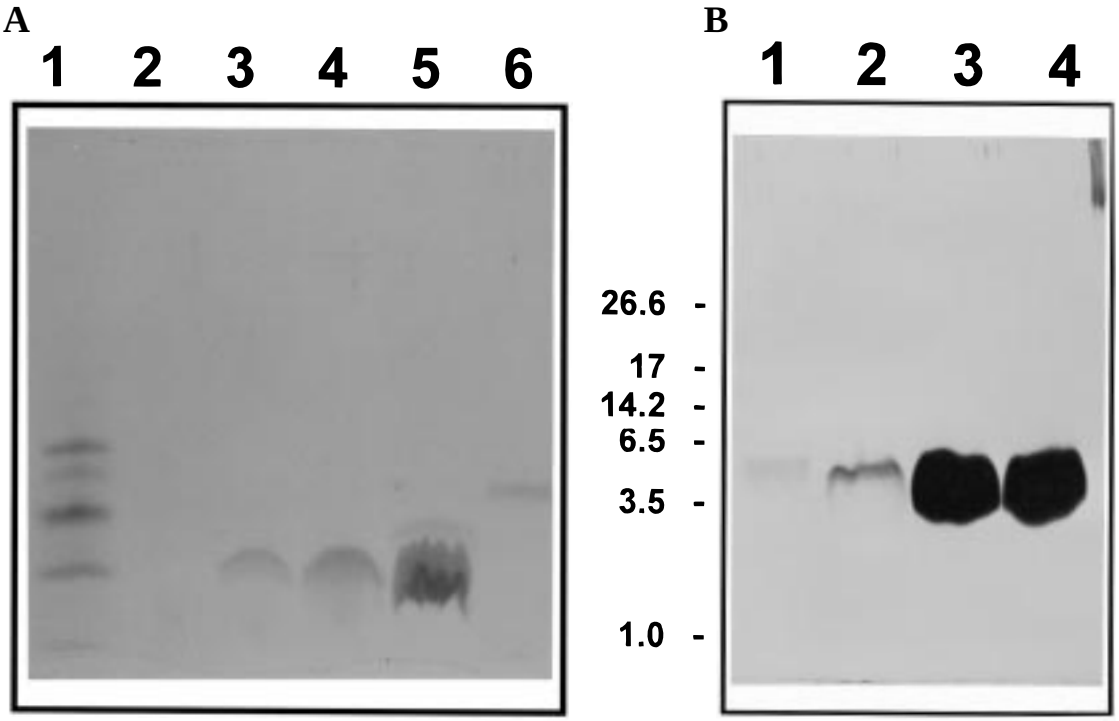

FIGURE 1: (A) Tricine (10%) containing SDS−PAGE according to ref *24* of the purification procedure of the cell wall channel protein of *C. glutamicum*. The gel was stained with Coomassie. Lane 1: molecular mass markers 16.9, 14.4, 10.7, 6.2, and 2.5 kDa. Lane 2: 12 μL of fraction 9 of the Mono-Q FPLC column, solubilized at 40 °C for 30 min in 5 μL of sample buffer. Lane 3: 12 μL of the fraction 11 of the Mono-Q FPLC column, solubilized at 40 °C for 30 min in 5 μL sample buffer. Lane 4: 12 μL of the fraction 16 of the Mono-Q FPLC column, solubilized at 40 °C for 30 min in 5 μL sample buffer. Lane 5: 12 μL of the fraction 25 of the Mono-Q FPLC column, solubilized at 40 °C for 30 min in 5 μL sample buffer. (B) 10% tricine containing SDS−PAGE according to ref *24* of the purification procedure of the cell wall channel protein of *C. glutamicum*. The gel was stained with silver. Lane 1: 5 μL of fraction 9 of the Mono-Q column, solubilized at 40 °C for 30 min in 5 μL sample buffer. Lane 2: 5 μL of fraction 13 of the Mono-Q column, solubilized at 40 °C for 30 min in 5 μL sample buffer. Lane 3: 5 μL of fraction 16 of the Mono-Q column, solubilized at 40 °C for 30 min in 5 μL sample buffer. Lane 4: 5 μL of fraction 17 of the Mono-Q column, solubilized at 40 °C for 30 min in 5 μL sample buffer.

discarded. The supernatant of this extraction, the chloroform/methanol mixture, contained most of the channel-forming activity as judged from lipid bilayer experiments. After precipitation of this phase with ether, the protein pellet was subjected to lipid bilayer studies.

Purification of the Channel-Forming Protein from the Cell Wall of C. glutamicum. The final purification of the channel-forming protein from the cell wall of *C. glutamicum* was achieved by FPLC. The pellet from the ether precipitation of the chloroform-methanol phase was dissolved in 4 mL of 0.4% LDAO in 10 mM Tris-HCl (pH 8) and was applied to a Mono-Q FPLC column. The column was washed with 25 mL of the same buffer and then eluted with a linear NaCl gradient between 0 and 1 M NaCl. 75 Fractions of 2 mL were taken. From fraction 11 on the channel-forming protein was obtained with high purity as judged by SDS−PAGE (see lane 3 of Figure 1A). It had an apparent molecular mass of about 5 kDa. Lipid bilayer experiments revealed that it was the channel-forming component of the cell wall of *C. glutamicum*. Another low molecular mass band (lane 5 of Figure 1A) had low channel-forming activity. To test whether the 5 kDa protein was really pure, we subjected some of the fractions of the Mono-Q FPLC column to silver staining (see Figure 1B). Again, we did not find any indication for impurities in several different fraction, i.e., the 5 kDa polypeptide appeared to be pure.

Partial Sequencing of the 5 kDa Protein. We subjected the 5 kDa protein to partial sequencing starting from the N-terminal end using Edman degradation. Nineteen amino acids were resolved, which had the sequence ENVYE-FLGNLDVLXGXXLI (X = unidentified amino acid). No side sequences were observed, which indicated that the protein was essentially free of major amounts of contaminant protein, which was consistent with the results of the SDS−PAGE. So far, no significant homology of the partial sequence with other protein sequences was found in different databases.

Effect of Proteolytic Digestion of the Cell Wall Protein on Its Membrane Activity. To check whether the channel-forming activity was caused by a protein component and not by an unidentified lipid component, a solution of the purified 5 kDa protein was subjected to proteolytic degradation with proteinase K. After treatment of the polypeptide for 5 min, the channel-forming activity was completely abolished.

Immunological Detection of the 5 kDa Protein. We raised polyclonal antibodies against the 5 kDa protein to check for the possible protein oligomers on SDS−PAGE. This search was unsuccessful because only the 5 kDa band could be observed in Western blot (data not shown). In a next step, we performed ELISA with whole cells. Different amounts of cells were coupled per well and the preimmune serum was used as negative control. The results of the ELISA experiments (see Figure 2) demonstrated that the antigenic structure is indeed localized on the surface of the *C. glutamicum* cells, i.e., the 5 kDa protein is a cell wall component.

Interaction of the Cell Wall Protein with Lipid Bilayer Membranes. The interaction of the 5 kDa cell wall protein

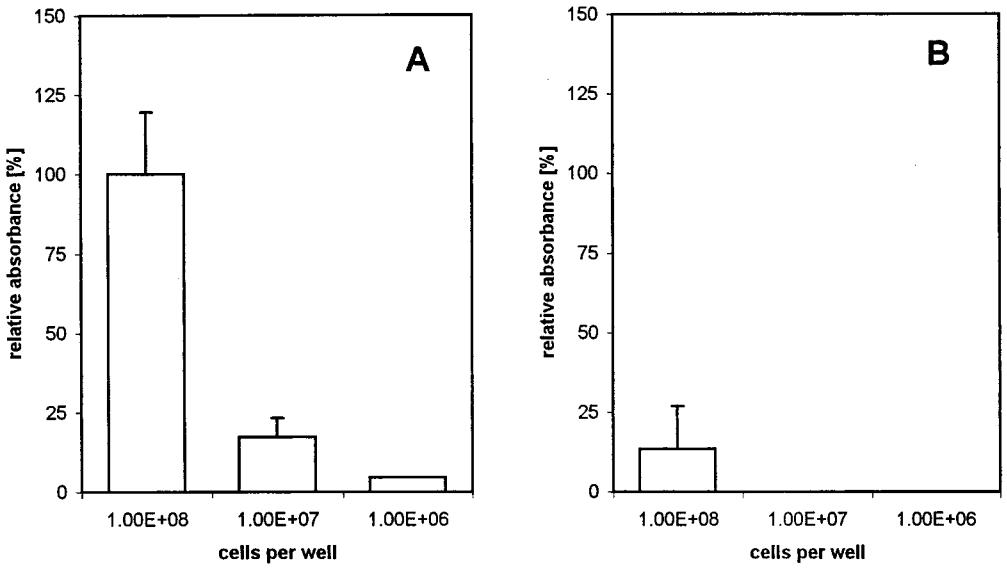

FIGURE 2: Immunological detection of the cell wall porin of *C. glutamicum* on the cell surface. (A) Polyclonal antibodies against the 5 kDa cell wall protein. (B) Corresponding preimmune serum. The maximum binding was set to 100% (corresponding to A_{405} between 0.5 and 0.8) and the cell number per well is indicated. The bars show the results ± SD of at least four experiments.

with lipid bilayers was studied by the addition of small amounts of the protein to the aqueous phase bathing black membranes formed from either a variety of pure lipids, such as phosphatidyl choline (PC) and phosphatidyl serine (PS), or a mixture of both lipids in a molar ratio of 4:1. In experiments with membranes formed of pure PC and PS and with the PC:PS-mixture, we observed a substantial increase of the specific membrane conductance in the presence of the purified cell wall protein, and up to 10^6 channels/cm^2 could be formed in a membrane. However, the cell wall protein-induced conductance increase needed a certain time until it reached a plateau value for membranes from the PC:PS mixture (data not shown). About 2 min after addition of the protein, the membrane conductance started to raise and increased by several orders of magnitude in approximately 30 min. Only a small further increase, as compared to the initial one, occurred after that time. The time course of the conductance increase was similar irrespective of whether the protein was added to one or both sides of the membranes. However, the resulting conductance was somewhat smaller when the protein was added to only one side of the membrane. It is noteworthy that similar experiments with pure PC membranes showed a time delay of about 20−30 min between the addition of the protein and the start of the conductance increase. The reason for this delay is presumably caused by slow formation of high molecular mass oligomers from the protein monomers, which may be enhanced in the presence of negatively charged lipids.

Single-Channel Analysis. Single-channel experiments revealed that the membrane activity described above for the cell wall protein of *C. glutamicum* was caused by the formation of ion-permeable channels. Figure 3 shows a single-channel record of a membrane from the 4:1 PC:PS mixture in the presence of the 5 kDa protein, which was added to a black membrane in a concentration of about 10 ng/mL. The single-channel recording demonstrates that the cell wall protein formed defined channels. The single-channel conductance of most channels formed by the cell wall protein was 5.5 nS in 1 M salt. Only a minor fraction of channels had approximately half the conductance of this

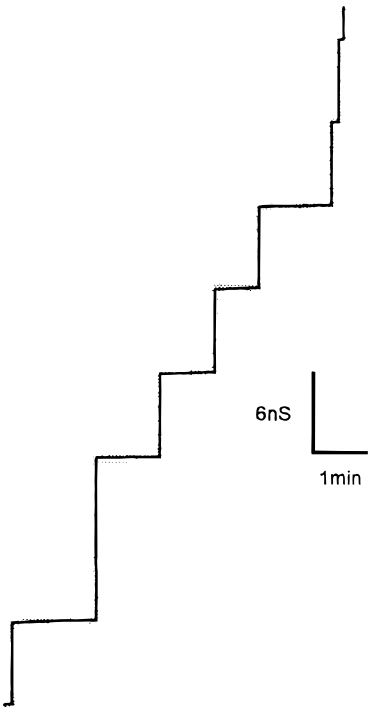

FIGURE 3: Single-channel record of a PC/PS (molar ratio 4:1)/*n*-decane membrane in the presence pure 5 kDa protein of the cell wall of *C. glutamicum*. The aqueous phase contained 1 M KCl (pH 6) and 10 ng/mL cell wall protein. The applied membrane potential was 20 mV; $T = 20$ °C.

value, indicating that different channels could be formed by the cell wall protein. This could indicate that the channels are formed by oligomers of different numbers of monomers or that we also observed substates of the open channel (see the histogram of Figure 4A). The histograms of channel distributions observed in the presence of detergent-solubilized cell wall extracts and those in that of the 5 kDa monomer were very similar (compare panels A and B of Figure 4), indicating that the channels formed by both preparations were identical. It is noteworthy that the channels observed in this study had the same single-channel conductance as has been

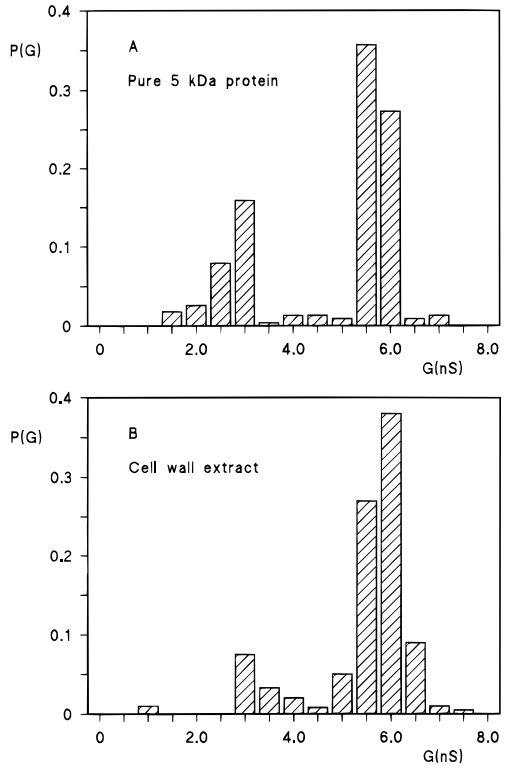

FIGURE 4: Histogram of the probability P(G) for the occurrence of a given conductivity unit observed with membranes formed of PC/PS (molar ratio 4:1)/*n*-decane in the presence of the cell wall extracts and pure 5 kDa protein of *C. glutamicum*. P(G) is the probability that a given conductance increment G is observed in the single-channel experiments. It was calculated by dividing the number of fluctuations with a given conductance increment by the total number of conductance fluctuations. The aqueous phase contained 1 M KCl. The applied membrane potential was 20 mV; $T = 20$ °C. The average single-channel conductances were 5.5 nS for 227 single-channel events (pure 5 kDa protein, panel A) and 5.7 nS for 335 single-channel events (detergent-solubilized cell wall fraction, panel B).

observed in a previous study (*20*). In general, the cell wall channel of *C. glutamicum* showed substantial similarities to those that have been detected previously for cell wall proteins (porins) of *M. chelonae* (*16, 18*) and *M. smegmatis* (*19*).

Size of the Cell Wall Channel. We performed also single-channel experiments with salts other than KCl to obtain some information on the size of the cell wall channels of *C. glutamicum* and its ion selectivity. The results are summarized in Table 1. The replacement of chloride by the less mobile acetate had only a little if any influence on the conductance of the channels. The influence of the cations on the single-channel conductance in different 1 M salt solutions was more substantial (see Table 1), which suggests that the cell wall channel was cation-selective. The mobility sequence for the different cations within the cell wall channel was $Cs^+ \approx Rb^+ \approx K^+ > Na^+ > Li^+ > Tris^+$ and $NH_4^+ > N(CH_3)_4^+ > N(C_2H_5)_4^+$, which means that the permeability through the channel followed the mobility sequence of these ions in the aqueous phase. Table 1 shows also the average single-channel conductance, *G*, as a function of the KCl concentration in the aqueous phase. Similarly, as in the case of the cell wall channel of *M. chelonae* (*18*), the relationship between conductance and KCl concentration was not linear. Instead, the slope of the conductance versus concentration curves on a double logarithmic scale was approximately 0.5,

Table 1: Average Single-Channel Conductance, *G*, of the Cell Wall Channel of *C. glutamicum* in Different Salt Solutions[a]

salt	concentration (M)	*G* (nS)
LiCl	1.0	2.6
NaCl	1.0	3.5
KCl	0.03	0.60
	0.10	1.1
	0.3	2.0
	1.0	5.5
	3.0	14.5
KCH_3COO (pH 7)	1.0	4.8
RbCl	1.0	6.3
CsCl	1.0	5.6
NH_4Cl	1.0	5.0
$N(CH_3)_4Cl$	1.0	2.2
$N(C_2H_5)_4Cl$	1.0	1.0
TrisCl	1.0	0.6

[a] The membranes were formed of PC/PS (molar ratio 4:1) dissolved in *n*-decane. The aqueous solutions were unbuffered and had a pH of 5 unless otherwise indicated. The applied voltage was 10 mV, and the temperature was 20 °C. The average single-channel conductance, *G*, was calculated from at least 80 single events.

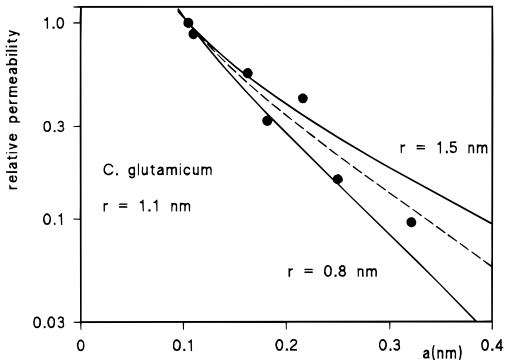

FIGURE 5: Fit of the single-channel conductance data of the cell wall channel by using the Renkin correction factor times the aqueous diffusion coefficients of the different cations. The values were normalized to 1 for $a = 0.105$ nm (Rb^+). Single-channel conductances were normalized to the ones of Rb^+ and plotted versus the hydrated ion radii taken from Table 3. The single-channel conductances (full points) correspond to Li^+, Na^+, K^+, $NH4^+$, Cs^+, $N(CH_3)_4^+$, $N(C_2H_5)_4^+$, and $Tris^+$, which were all used for the pore diameter estimation (see Discussion). The fit (solid lines) is shown for the cell wall channel of *C. glutamicum* with $r = 1.5$ nm (upper line) and $r = 0.8$ nm (lower line). The best fit of all data was achieved with $r = 1.1$ nm (diameter = 2.2 nm), which corresponds to the broken line.

which indicated the influence of point net charges localized in or near the channels (see also Discussion and Figure 6). It is noteworthy that the charge effect was caused by charges attached to the channel and not by the negatively charged lipids present in the membrane. This was judged from the observation that the cell wall channel had the same conductance in neutral and in negatively charged membranes.

Selectivity of the Cell Wall Channel of C. glutamicum. Zero-current membrane potential measurements allow the calculation of the permeability ratio P_{cation}/P_{anion} in multi-channel experiments. PC/PS membranes (molar ratio) were formed in 50 mM salt solution and concentrated cell wall protein was added to the aqueous phase when the membranes were in the black state. After incorporation of 100−1000 channels into a membrane, 10-fold salt gradients were established by addition of small amounts of concentrated salt solution to one side of the membrane. For all salts (KCl, LiCl, and KCH_3COO) tested in experiments with the cell

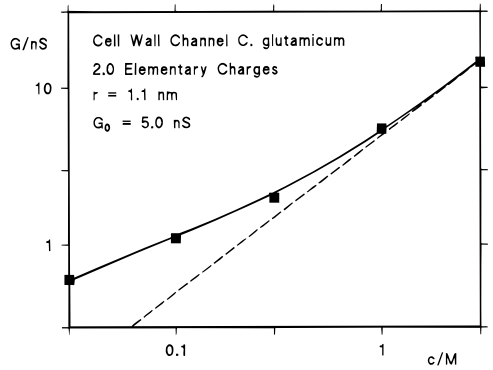

FIGURE 6: Single-channel conductance of the cell wall channel of *C. glutamicum* as a function the KCl-concentration in the aqueous phase (full squares). The solid line represents the fit of the single-channel conductance data with eqs 3−5 assuming the presence of negative point charges (2.0 negative charges; $q = -3.2 \times 10^{-19}$ As) at the channel mouth on both sides of the membrane and assuming a channel diameter of 2.2 nm (radius $r = 1.1$ nm). *c*, concentration of the KCl-solution in molarity; *G*, average single-channel conductance in nanoSiemens (10^{-9} S). The broken (straight) line shows the single-channel conductance of the cell wall channel without the effect of point charges and corresponds to a linear function between channel conductance and bulk aqueous concentration.

Table 2: Zero-Current Membrane Potentials, V_m, of PC/PS (Molar Ratio 4:1) Membranes in the Presence of the Cell Wall Channel of *C. glutamicum* Measured for a 10-fold Gradient of Different Salts[a]

salt	V_m (mV)	P_{cation}/P_{anion}
KCl	39	8.1
LiCl	31	4.9
KCH_3COO (pH 7)	43	11.6

[a] V_m is defined as the difference between the potential at the dilute side (50 mM) and the potential at the concentrated side (500 mM). The pH of the aqueous salt solutions was 6 unless otherwise indicated; $T = 20$ °C. The permeability ratio P_{cation}/P_{anion} was calculated with the Goldman−Hodgkin−Katz equation (*27*) from at least three individual experiments.

wall channel, the more diluted side of the membrane became positive, which indicated preferential movement of cations through the channel. The zero-current membrane potentials for the salts mentioned above were between 31 mV (LiCl) and 43 mV (KCH_3COO). Analysis of the zero-current membrane potentials of Table 2 using the Goldman−Hodgkin−Katz equation suggested that anions also could have a certain permeability through the cell wall channels because the ratios of the permeability coefficients were between 4.9 and 11.6. On the other hand, the permeability ratios were rather high in favor of the cations, which means that anions have a much smaller permeability than cations through the cell wall channels if they have any (see Discussion).

DISCUSSION

The Cell Wall of C. glutamicum Contains an Ion-Permeable Channel. In this study, we confirmed our previous observation that the cell wall of *C. glutamicum* contains an ion-permeable channel. This channel has been discovered in the cell wall fraction of *C. glutamicum*, isolated by sucrose-density centrifugation of the cell envelope (*20*). Immunological detection suggests that it is localized in the mycolic acid layer of this bacterium. It is responsible for the cell wall permeability for hydrophilic solutes. This means that the channel has the same function in the cell wall as those of *M. chelonae* (*16*, *18*) and *M. smegmatis* (*19*), which limit the cell wall permeability properties of these fast-growing mycobacteria. All these proteins form high-conductance channels in lipid bilayer membranes with a long lifetime at small transmembrane potential (mean lifetime at least 5 min). The analogy to the mycobacterial cell wall channels suggests that the cell wall channel of *C. glutamicum* has a function similar to those of Gram-negative bacterial porins. Their channel-forming proteins are responsible for the permeability properties of bacterial outer membranes (*1*, *17*), which definitely have a different structure than the mycolic acid layer of actinomycetes but represent also a permeability barrier. The name porin for the cell wall channel protein used in different studies may be therefore be justified although the structures of both membrane channels are probably completely different (see below).

Using the lipid bilayer technique, very small channel-forming activity was identified in different molecular mass regions of SDS−PAGE, indicating that these regions contain also small amounts of the 5 kDa protein. This could mean that the channel is formed by a protein oligomer, because a monomer of 5 kDa cannot form such a wide water-filled channel. It is also possibe, however, that the 5 kDa band smeared across a considerable molecular mass range. In any case we were not able to detect oligomers on SDS−PAGE or in Western blots. It is noteworthy, that channel formation by the 5 kDa protein was not a rare event. Up to 10^6 channels/cm^2 were formed when 1 μg/mL cell wall channel protein was added to the aqueous phase. The subunit of the channel was purified to homogeneity using ion exchange chromatography across a Mono-Q column, which was demonstrated by SDS−PAGE using tricine-containing gels (see Figure 1). The 5 kDa protein is a cell wall component because antibodies raised against the 5 kDa protein bound to the surface of whole cells. Their penetration through the cell wall seems to be impossible even in Gram-positive bacteria that do not contain mycolic acids. This can be deduced from the high molecular mass of the antibodies and the cell wall exclusion limit (Nikaido, 1994).

The Subunit of the Cell Wall Channel Has an Unusually Small Molecular Mass. The channel subunit had the extremely small molecular mass of about 5 kDa, as derived from SDS−PAGE. This is the smallest subunit of a channel observed to date in a biological membrane. The porins from the outer membrane of Gram-negative bacteria that are analogous in function have a molecular mass of at least 30 kDa (*17*) and monomers in the trimers contain one channel. There existed still the possibility that lipids and/or glycolipids from the corynemycolic acid layer formed the large channels in reconstitution experiments. However, we could demonstrate that the channel-forming activity was completely abolished when the 5 kDa protein was treated for a short time with proteinase K. Furthermore, we performed N-terminal sequencing of the channel-forming protein. Nineteen amino acids could be resolved in these experiments without any indication for the presence of contaminating protein. We found no significant homology to the sequence of known proteins in different databases (Swiss Prot and Gene EMBL). These results clearly indicated that the channels formed in the reconstitution experiments were

Table 3: Radii of the Cations and Average Single-Channel Conductance, *G*, of the Cell Wall Channel of *C. glutamicum* in Different Salt Solutions[a]

cation	limiting molar conductivity λ_i (mS/M)	hydrated ion radius *a* (nm)	*G* (nS)
Li^+	38.68	0.216	2.6
Na^+	50.10	0.163	3.5
K^+	73.50	0.110	5.5
NH_4^+	73.55	0.110	5.0
Rb^+	77.81	0.105	6.3
$(CH_3)_4N^+$	44.92	0.182	2.2
$(C_2H_5)_4N^+$	32.66	0.250	1.0
$Tris^+$	25.50	0.321	0.6

[a] The data for the limiting conductivities of the different ions were taken from ref *38*. The limiting conductivity for $Tris^+$ was calculated from the specific conductivity of 10 mM Tris-HCl in the aqueous phase. The single channel conductance of the cell wall channel was taken from Table 1.

formed by a polypeptide and not by a lipid component of the mycolic acid layer.

These results suggest indeed that the cell wall channel of *C. glutamicum* is formed by a small molecular mass polypeptide. Only the subunits of the channels formed by certain peptide antibiotics, such as gramicidin A [molecular mass 2138 Da (*34*)], alamethicin [molecular mass about 1964 Da (*35*)], and the bee venom melittin [molecular mass 2846 Da (*36*)] are smaller. It is noteworthy that these compounds are at least to a certain extent water soluble and show an association-dissociation equilibrium in membranes, which means that the channels are transient and induced by high membrane voltage (*34*−*37*). The subunit of the cell wall channel of *C. glutamicum* comprises probably only about 50 amino acids, but the oligomer is stable within membranes because the lifetime of the cell wall channels is rather long as compared to the lifetime of the alamethicin, gramicidin A, and melittin channels. The subunits of the cell wall channels have obviously a high aggregation tendency, which leads rapidly to a defined oligomer, when negatively charged lipids are present. The prospective oligomer itself is SDS sensitive and dissociates on SDS−PAGE. The number of subunits in a conducting channel represents an open question. It varies only little as suggested by the rather uniform histogram of the conductance steps. Only a limited number of channels with smaller single-channel conductances were observed, which indeed may indicate that, besides the main state of the channel, also a channel with substates may be formed by the 5 kDa channel-forming protein.

Estimation of the Diameter of the Cell Wall Channel (Porin). The estimation of the channel size was possible using the Renkin correction factor (*29*) similar as it has been performed with the porin channels of *E. coli* (*30*) and the cell wall channel (porin) of *M. chelonae* (*18*). Table 3 contains the limiting molar conductivity (taken from ref *38*) and the hydrated ion radii calculated according to eq 2. The fit of the single-channel data with eq 2 is possible because the channel is preferentially permeable for cations as demonstrated by the selectivity measurements and the single-channel conductance experiments. Figure 5 shows the best fit of the single-channel conductance of the cell wall channel from *C. glutamicum* with the Renkin correction factor (eq 1) times the aqueous diffusion coefficient of the corresponding cation. The permeability is given relative to that for Rb^+ (relative permeability equal to unity), and the best fit of the single-channel conductance as a function of hydrated ion radius was obtained with $r = 1.1$ nm, which means that the diameter of the channel is approximately 2.2 nm. The data lie within the range from $r = 0.8$ to 1.5 nm as shown in Figure 6. A diameter of 2.2 nm is very similar to the diameters of the cell wall channels of *M. chelonae* [2.0 nm (*16, 18*)] and *M. smegmatis* [2.6−3.0 nm (*19*)] as have been derived from the liposome swelling assay and from a similar procedure as was used here (see Table 4).

Table 4: Comparison of the Cell Wall Channel Properties of *M. chelonae, M. smegmatis* and *C. glutamicum*

cell wall channel	*G* (nS) in 1 M KCl	selectivity P_c/P_a in KCl	negative point charges at the channel mouth	channel diameter (nm)	ref
M. chelonae	2.7	14	2.5	2.0	*17*
M. smegmatis	4.1	9.7	4	2.6, 3.0	*18*
C. glutamicum	5.5	8.1	2	2.2	This study

The single-channel conductances of the cell wall channels of the two mycobacteria and *C. glutamicum* are also similar, which agrees with the estimation of their diameters. On the other hand, the cell wall channel of *C. glutamicum* has the highest single-channel conductance of all three channels considered here. This does not seem to be consistent with the diameters calculated here and in other studies (*18, 19*). However, we have to keep in mind that the mycolic acid layers of the cell walls of mycobacteria and corynebacteria have not the same thickness because of the different length of the mycolic acids. The mycolic acids of corynebacteria are in comparison relatively short and have only a length of 22−38 carbon atoms (*4*−*6, 8*−*13, 39, 40*), which has to be compared with a length of the mycolic acids of mycobacteria of 60−90 carbon atoms. This means also that the channel is presumably shorter than those of mycobacteria, which in turn leads according to Ohm's law to a higher conductance for a channel of the same size.

Effects of Point Net Charges. The single-channel conductance was not a linear function of the bulk aqueous concentration (see Table 1). Instead, we observed a dependence of the single-channel conductance on the square root of the salt concentration in the aqueous phase. This means (i) that the cation specificity of the cell wall channel is not related to the presence of a binding site and (ii) that point charges are involved in ion selectivity as we and others have demonstrated previously for a variety of membrane channels (*33, 41, 42*), including mycobacterial porins (*18, 19*). These charges are attached to the channel itself. When we apply eqs 3−5 to the cell wall channel we receive also a reasonable fit of the data of Table 1 if the channel has a diameter of about 2.2 nm and that two negative point charges ($q = -3.2 \times 10^{-19}$ As) are attached to the channel mouth. The results of this fit are shown in Figure 6. The solid line represents the fit of the single-channel conductance versus concentration by using the Nelson and McQuarrie (*32*) treatment and the parameters mentioned above together with a single-channel conductance, $G_0 = 5.0$ nS at 1 M salt. The broken line corresponds to the single-channel conductance of the cell wall channel without point net charges, i.e., it shows a linear relationship between the cation concentration in the aqueous phase and single-channel conductance. Our results indicate that the two methods used here for the

calculation of the channel diameter (Renkin correction factor and point charges) lead to satisfactory agreement.

Comparison to Mycobacterial Cell Wall Channels. Table 4 shows a comparison of the properties of the cell wall channels from the group of actinomycetes that are known to date. All channels are cation-selective caused by negatively charged groups attached to the channel opening. The number of charges varies between 2.5 and 4. All channels appear to be wide and water-filled, and their diameter ranges between 2 nm (*M. chelonae*) and 3 nm (*M. smegmatis*). The channel-forming protein of *C. glutamicum* has a low molecular mass of only about 5 kDa, and the channel is formed by an oligomer. From the other members of the group of actinomycetes, only the channel-forming protein of *M. chelonae* is known which has a molecular mass of 62 kDa (*18*). However, it is not known whether it is also composed of subunits as we showed here for the cell wall channel of *C. glutamicum*. Further investigation into the cell wall protein of actinomycetes is necessary to understand the structure and function of the cell wall channels and will allow to deduce their primary structure from the cloning of the corresponding gene.

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