Heptameric YggB is a mechanosensitive ion channel (MscS) from the inner membrane of Escherichia coli. We demonstrate, using the patch clamp technique, that cross-linking of the YggB C termini led to irreversible inhibition of the channel activities. Application of Ni$^{2+}$ to the YggB-His$_6$ channels with the hexahistidine tags added to the ends of their C termini also resulted in a marked but reversible decrease of activities. Western blot revealed that YggB-His$_6$ oligomers are more stable in the presence of Ni$^{2+}$, providing evidence that Ni$^{2+}$ is coordinated between C termini from different subunits of the channel. Intersubunit coordination of Ni$^{2+}$ affecting channel activities occurred in the channel closed conformation and not in the open state. This may suggest that the C termini move apart upon channel opening and are involved in the channel activation. We propose that the as yet undefined C-terminal region may form a cytoplasmic gate of the channel. The results are discussed and interpreted based on the recently released quaternary structure of the channel.

Mechanosensitive (MS) ion channels open upon membrane tension, and therefore they represent the simplest mechanosensors. MS channels have been implicated in many physiological processes from growth and cell volume regulation to hearing, blood pressure regulation, and pain sensation (reviewed in Ref. 1). Bacterial MS channels protect these cells against hypoosmotic shock. Two types of MS channels from the cytoplasmic membrane of Escherichia coli, MscL and MscS, play an essential role in the physiology of this bacterium, allowing the efflux of solutes from the cytoplasm when osmolarity of the external medium decreases (2–4). MscL, the large conductance MS channel, has been cloned (5), and a quaternary structure of the channel was recently published (21). The structure reveals that the functional channel is a pentamer, and each subunit consists of two α-helical membrane-spanning domains TM1 and TM2 with both the C and N termini located in the cytoplasm (6). TM1s line the pore, and their hydrophobic residues form the primary, transmembrane gate (6, 14). It is postulated that there are two gates involved in the opening of the channel: the transmembrane and the cytoplasmic gates (7, 8) acting in accordance (15). The transmembrane gate is proposed to act as a pressure sensor, and upon application of pressure, this gate permits initial expansion of the channel without its full opening (7, 8, 10). It is proposed that the other, cytoplasmic gate, which allows full activation of the channel, is composed of five α-helical S1 segments of the cytoplasmic N termini being connected with TM1s via flexible linkers. According to the model, the applied pressure is transmitted to the S1 segments through the flexible linkers and pulls them apart. The channel may fully open when the interactions between the five S1 segments of the cytoplasmic gate break down (7, 8, 10).

The activity of MscS, the E. coli MS channel of a smaller conductance (16), consists of activities of two separate ion channels of very similar conductance encoded by yggB and kefA (3). KefA is a large multidomain protein (1120 amino acids), spanning the inner membrane and possibly having a link to the outer membrane (17), whereas YggB is a small protein of 286 amino acids residing in the inner membrane. Both proteins show amino acid sequence homology in the region corresponding to the entire YggB sequence (3) presented in Fig. 1A. The activities of the KefA and YggB channels recorded directly from the E. coli membrane are kinetically distinct; YggB shows inactivation during sustained pressure, i.e. adaptation (18), whereas KefA does not adapt to pressure (3, 16). The YggB channels are more abundant than those of KefA, and their activities have been recorded after reconstitution of a purified protein in planar lipid bilayers (19, 20), indicating that, similar to MscL, YggB senses membrane stress directly.

The quaternary structure of the YggB channel was recently published (21). The structure reveals that the functional channel is a heptamer and has three transmembrane domains, TM1, TM2, and TM3, in each of seven subunits (Fig. 1B). The TM3s line the channel pore. The cytoplasmic domains are composed mostly of β-sheets and surround the large water-filled chamber with a diameter of ~40 Å. Each subunit of the assembly consists of a middle β domain and a lower α/β domain (Fig. 1B, left view), and all seven subunits are linked together by a β barrel composed of seven strands, which are located at the very ends of the C termini (Fig. 1B, middle view).

In this paper we have studied a possible role for C termini in functioning of the YggB channels. We demonstrate that cross-linking of the YggB C termini yields inactive channels. We also show that intersubunit coordination of Ni$^{2+}$ in the YggB-His$_6$ channels prevents the channels from opening. The Ni$^{2+}$ coordination leading to the inhibition of activities occurs in the
channel closed state, and we did not observe it in the channel open conformation. This may suggest that the C termini move apart upon channel opening and are involved in the process of the channel gating.

EXPERIMENTAL PROCEDURES

**Chemicals**—Nonpolymerized, microscopy grade glutaraldehyde (GDH) (Grade I) was purchased from Sigma and stored at -80 °C in small aliquots. Bis(sulfosuccinimidyl)suberate (BS3) (Sigma) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (Sigma) were dissolved in the bath solution shortly before use. SDS and isopropyl-1-thio-D-galactopyranoside were from Biomol.

**Bacterial Strains, Constructs, and Overexpression**—All of the electrophysiological experiments were performed on E. coli strains: MJF379, Frag1 kefA::kan; MJF429, Frag1 kefA::kan yggB. Frag1 is a wild-type strain, a derivative of E. coli K-12. All of the strains were kindly provided by I. R. Booth (University of Aberdeen, Aberdeen, UK).

The construct pYggB-His6 with a histidine tag added to the end of its C terminus was obtained as follows. The yggB open reading frame was amplified from genomic DNA of E. coli wild-type DH5α strain with proofreading Pfu polymerase (Promega) using the following primers:

- **Upper primer**, 5'-TAGCCCAATGGAGATTTGAATGTC-3' (underlined bases represent the NcoI restriction endonuclease recognition site, and the sequence of yggB open reading frame is in italics), and
- **Lower primer**, 5'-GGAATCCTTAAGATTGATGGTGATGGTGACGCGCAGCTTTGTCTTCTTT-3' (underlined bases represent the KpnI restriction endonuclease recognition site; the sequence in italics represents the yggB open reading frame, and the bold sequence codes the six-histidine tag followed by a stop codon). The resulting PCR product was digested with NcoI and KpnI (Promega), purified using QIAex II gel purification kit (Qiagen), and ligated into fragment of pTrc99A vector (Amersham Biosciences) digested with NcoI and KpnI. The resulting plasmid is carrying YggB-His6 under control of isopropyl-1-thio-D-galactopyranoside-inducible trc (trp/λlac) promoter. The pYggBHis6 construct was verified by restriction digestion and sequencing of the insert.

The pYggBHis6 construct was expressed in the MJF429 strain. The bacteria were grown at 37 °C overnight in liquid LB broth containing 10 g/liter bacto-tryptone, 5 g/liter yeast extract, 5 g/liter NaCl, and supplemented with ampicillin (100 µg/ml). The overnight culture was diluted to A600 = 0.2, and the bacteria were next grown to A600 ~ 0.6. Expression was induced by adding 1 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h. The cells were spun down and frozen in liquid nitrogen. Lysate was obtained by treatment of cells with lysozyme (25 µg/ml) (Sigma) in the buffer C containing 50 mM HEPES, 100 mM NaCl, 10 mM MgCl₂, 0.5 mM EDTA, and 0.1% Triton X-100 (all at pH 7.4).
Movement of the YggB C Termini

pH 7.4, in the presence of 1 mM phenylmethylsulfonyl fluoride (Merck). The lysate was treated with DNase (5 μg/ml) (Sigma) and spun down in Sorvall SS-34 rotor at 10 000 rpm for 10 min.

Western Blotting—An insoluble fraction of E. coli cells containing membranes was resuspended in buffer C and divided equally into Eppendorf tubes. NiCl$_2$ or MgCl$_2$ (as indicated) was added to the final concentration of 50 mM, and the samples were incubated for 30 min at room temperature. The membrane fraction was spun down in a microcentrifuge at 14,000 rpm and washed once with buffer C. The pellet was solubilized in 0.5% Laemmli sample buffer and incubated for 5 min at the indicated temperature.

For Western blot, the proteins were separated in 9% SDS-PAGE mini-gels and electroblotted on a polyvinylidene difluoride membrane using semi-dry transfer apparatus (Bio-Rad). The membrane was blocked by overnight incubation in 2% bovine serum albumin (SERVA) in TBST buffer (25 mM Tris, 100 mM NaCl, 0.05% Tween 20, pH 7.4). The YggB-His$_6$ protein was detected by monoclonal anti-His C-terminal antibodies (Invitrogen) in 1:7500 dilution in TBST. The antibodies did not react with any protein from a lysate of a wild-type E. coli cells (M1P429) not carrying pYggB-His$_6$ (not shown). As secondary antibodies, the anti-mouse IgG conjugated to alkaline phosphatase antibodies (Promega) in dilution 1:5000 were used. For detection of alkaline phosphatase, 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium color substrate (Promega) was used according to manufacturer’s instructions. The protein masses and the band intensities were estimated according to prestained protein standards (Bio-Rad) and measured from Western blot scans using Image Quant software (Molecular Dynamics).

Electrophysiological Recordings and Data Analysis—All of the experiments were performed on E. coli giant protoplasts prepared as described previously (22). Single channel recordings were obtained from inside-out excised membrane patches, and the experimental procedure, including the equipment used and the application and measuring of suction, was the same as described earlier (18). Bath solution was 150 mM KCl, 400 mM sorbitol, 4 mM CaCl$_2$, 1 mM MgCl$_2$, 5 mM HEPES, pH 7.2, whereas the pipette solution was the same except that the sorbitol concentration was 300 mM. 60-s intervals or longer were maintained between applications of suction. The data were acquired (with a sampling rate of 2.5 kHz), filtered at 1 kHz, and analyzed using pCLAMP6 software. The mean single-channel open probability, $P_o$, during the pressure pulse (not shorter than 12 s) was calculated by integrating the current passing through all active channels $I$ during the pulse and dividing this integral by the current through a single open channel $I$ and the number of active channels $N$ according to the formula $P_o = \frac{I}{NI}$. 

RESULTS

Conservation of YggB Domains among Its Bacterial Homologues—Homologues of the E. coli YggB can be found in a wide range of bacteria and archaea (3). In Fig. 2 we compared 36 amino acid sequences of bacterial YggB homologues. In this figure each bar represents a single residue in the YggB sequence and its length is proportional to the identity of amino acids estimated for this residue. Among three membrane domains TM3 shows the highest level of homology as a whole (44% identity comparing to 30 and 23% estimated for TM1 and TM2, respectively), but there are also numerous short spans of C terminus whose identities are higher than 50%, implicating the importance of that region.

Cross-linkers Inhibit YggB Activities in Membrane Patches—The model of the MscL gating postulates that the cytoplasmic gate composed of five N termini occludes the pore of the channel until a mechanical force pulls them apart and the channel opens (7, 8). If a cytoplasmic gate exists in the YggB channel, it should be composed of its C termini. In the amino acid sequence of the cytoplasmic region of YggB all lysines but one (Lys$^{107}$) are situated in the C terminus (Fig. 1A). We assumed that cross-linking of lysines by different C termini of the channel would hamper or prevent the channel opening providing its C termini being pulled apart during opening. We therefore applied lysine-specific reagents to the cytoplasmic side of the membrane patches containing the YggB channels. Fig. 3 shows effects of GDH, EDC, and highly lysine-specific BS$_3$ on the channels. Each reagent, at concentration 2 mM, was applied to the closed channels, and the recordings were obtained at postive pipette voltages (+15 mV). Nonpolymerized GDH of undefined cross-linking distance and BS$_3$ with a spacer arm of 11.4 Å inhibited the YggB activity irreversibly, whereas EDC with a spacer arm of 0 did not affect it. Each set of traces in Fig. 3 represents a single experiment, but similar effects of GDH, BS$_3$, and EDC were observed in at least three other experiments in which these cross-linkers were used. The difference in control traces arises from a different number of channels in each patch and also from the different pressure applied in each case. The rate of adaptation is inversely proportional to the suction (18), and accordingly, the highest rate of adaptation is observed in the control trace of experiment with the lowest suction applied (Fig. 3B).

Inhibition of YggB-His$_6$ Channel Activities by Ni$^{2+}$ Coordination—The previous set of experiments showed that cross-linking of lysines by BS$_3$ on the cytoplasmic side of the closed YggB channel reduces the probability of the channel being open upon application of pressure. An important question, however, arises: which lysines were those involved in the cross-linking preventing the channel from opening. BS$_3$ could bind lysines situated within a single C terminus or lysines from C termini of different subunits. Alternatively, Lys$^{107}$ located in the linker between TM1 and TM2 and situated in the cytoplasm (Fig. 1) could be also involved. Cross-linking studies performed with disuccinimidyl suberate showed that lysine-specific cross-linkers react with groups from different subunits (19). We also confirmed this observation using BS$_3$ (a water-soluble analogue of disuccinimidyl suberate). Therefore, we assumed that cross-linking of lysines from different subunits of the YggB closed channel irreversibly prevented the opening of the channel. However, we wanted to verify this assumption with a different experimental procedure. We noticed that three of all eight lysines of the C terminus are situated within the stretch of the last nine amino acids (Fig. 1A) (the entire sequence of the C terminus consists of ~160 amino acids), and accordingly the highest probability of cross-linking occurs in this region. Therefore, we added a His$_6$ tag to the end of the C terminus and studied the effect of Ni$^{2+}$ binding to their imidazole groups. It

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2 P. Koprowski and A. Kubalski, unpublished observation.
was shown previously that the addition of His6 tag to the hemolysin channel renders a channel that could be inhibited by Ni2+ (23). It is worthwhile to note at this point that there are no other histidines in the entire amino acid sequence of YggB. We reasoned that, if in the closed state of the YggB-His6 channel its tagged C termini are close enough to coordinate Ni2+, the gating should be altered. This indeed proved to be the case.

The response to pressure of the YggB channels was essentially the same whether or not they had the attached C-terminal histidine tag (not shown). Fig. 4 shows the effect of exposure of the YggB-His6 channels to 0.25, 0.5, 1, 2, 5, and 10 mM. Each point represents the channel open probability \( P_o \) at various Ni2+ concentrations, and each recording was performed within 1 min after the end of the preceding pressure pulse. The most severe reduction in activity was observed at 1 mM Ni2+, and the lowest \( P_o \) was at 5 and 10 mM Ni2+. In three experiments of similar procedure but at positive pipette voltage (+15 mV), we did not observe this effect, and the data from one of these experiments are presented in Fig. 5 (top trace). Control experiments of identical procedure were performed on the channels without the His6 tag (three cases). The activities of these channels were not affected by the addition of Ni2+, and one of the control experiments is shown in Fig. 5 (bottom trace).

We applied the antiHis6 antibodies to three membrane patches containing YggB-His6 channels. We did not observe any inhibiting effect of the antibodies on the channel activities (not shown).

Ni2+ Coordination in YggB-His6 in Vitro—Inhibition of channel activities of YggB-His6 in the presence of Ni2+ suggested that histidines from different subunits are involved in coordinating Ni2+. We looked, therefore, for confirmation of this hypothesis in vitro.

Western blot of YggB-His6 (Fig. 6) in the absence of Ni2+ and at 25 °C revealed a ladder pattern of six bands (left panel, first lane) indicated by numbers. Under our conditions of electrophoresis, the lowest band migrated close to the front of the gel. It represents the monomeric polypeptide of 28 kDa. The dimer, trimer, tetramer, pentamer and hexamer are present at 47, 93, 190, and 210 kDa, respectively. The oligomers were stable in SDS buffer at 25 °C. At higher temperatures they dissociated; at 55 °C the bands of monomers and dimers are very well resolved, and the binding seems to be stable from trimer to hexamer. The patterns yielded at 55 °C and at 75 °C are very similar to that observed at 25 °C, indicating that the Ni2+...
bound oligomers are stable at higher temperatures. This is in a marked contrast to the YggB oligomers existing in the absence of Ni$^{2+}$. We used Mg$^{2+}$ as a control for the effect of Ni$^{2+}$, and the ladders obtained in the presence of Mg$^{2+}$ (third lane in each panel) were very similar to the control ones (first lane in each panel) at all of the temperatures tested.

The amount of fraction applied to each lane was the same; however, in control at room temperature the overall intensity of the bands representing dimers and higher oligomers is higher than in the other lanes. The overall intensity of the bands in the lanes with Ni$^{2+}$ (normalized to the intensity at room temperature) increases with an increase of temperature (1.0, 2.5, and 3.1), indicating that more protein could enter the gel at higher temperatures as dimers and higher oligomers. In contrast, the higher the temperature the lower the intensity of the bands in the control lanes (1.0, 0.13, and 0.10) and in the lanes with Mg$^{2+}$ (1.0, 0.38, and 0.47), suggesting that more protein can exist in a monomeric state. The monomeric band migrates at the front of the gel, and an estimation of its intensity is difficult. This explanation applies as well to the apparent difference in intensity between the bands in the presence and in the absence of Ni$^{2+}$ at room temperature (Fig. 6, first panel, second and first lanes, respectively).

The observed stability of the Ni$^{2+}$-bound oligomers allows us to conclude that histidines from different subunits of YggB-His$_6$ channel participate in Ni$^{2+}$ coordination. An additional confirmation of this conclusion comes from the YggB crystal structure, which revealed that the $\beta$ strands located at the ends of C termini form a parallel $\beta$-barrel (Fig. 1B). The ends of the strands from the $\beta$-barrel are in a close proximity, and after addition of His$_6$ epitopes, Ni$^{2+}$ coordination by histidines from different subunits is very likely to occur.

**Fig. 4.** Effects of 0.5 and 5 mM Ni$^{2+}$ applied to the cytoplasmic side of the YggB-His$_6$ channels with a histidine tag added to end of the C terminus. Ni$^{2+}$, added 5 min prior to recording, inhibits the channel activities reversibly. The arrows indicate application (downward arrow) and release (upward arrow) of suction (180 mm Hg). C marks the closed level. All of the recordings were obtained at −15 mV.

**Fig. 5.** Effects of Ni$^{2+}$ at various concentrations added to the cytoplasmic side of the YggB-His$_6$ channels at +15 mV and −15 mV. Ni$^{2+}$ did not affect the channel activities at +15 mV (top plot). The inhibiting effect of Ni$^{2+}$ occurred at −15 mV when applied to the YggB-His$_6$ channels (middle plot) but not to the YggB channels (bottom plot). The channel activities were measured as a ratio $P_o/P_{oc}$ where $P_o$ is the open probability and $P_{oc}$ is the open probability in control. Five to six suction pulses were applied at each Ni$^{2+}$ concentration. The increases in the Ni$^{2+}$ concentration are marked with increasingly darker shades of gray. The beginning and the end of each trace indicates $P_o/P_{oc}$ in control and after washout, respectively. The suction pulse was kept constant in each experiment, and it was 240 mm Hg in experiments presented in the top and the middle plot (both experiments were performed on the same membrane patch) and 245 mm Hg in the experiment shown in the bottom plot.
Ni\(^{2+}\) Coordination in the Open State of the YggB Channels—We exposed the C termini of the open YggB-His\(_6\) channels to Ni\(^{2+}\), assuming that the Ni\(^{2+}\) coordination between the His\(_6\)-tagged C termini might not occur. The control experiment was basically similar to that in which we had investigated Ni\(^{2+}\) coordination in the closed YggB-His\(_6\) channels, but we introduced a time scale. In this experiment we measured channel activity by a short pressure pulse, and then 5 mM NiCl\(_2\) was applied to the closed channels (Fig. 7). After exposure to Ni\(^{2+}\) for 2 min, a second pulse of identical pressure was applied. A change in the channel activities in both pulses was measured as a ratio: \(I_{\text{max2}}/I_{\text{max1}}\), where \(I_{\text{max1}}\) and \(I_{\text{max2}}\) represent peak currents (corresponding to the number of active channels) during the first and the second pulse, respectively. Fewer channels open in response to the second pulse because of the Ni\(^{2+}\) coordination and the ratio \(I_{\text{max2}}/I_{\text{max1}} = 0.46 \pm 0.01\) (\(n = 3\)). After washout of the chamber with the bath solution devoid of NiCl\(_2\) and with 2 mM EDTA similar two-pulse procedure was performed. The response to both pulses was very similar, \(I_{\text{max2}}/I_{\text{max1}} = 0.99 \pm 0.02\) (\(n = 3\)), and it returned to its initial level.

A procedure of similar time scale was used to the open YggB-His\(_6\) channels. In this experiment, the first pressure pulse was not released, and 5 mM NiCl\(_2\) was applied to the open channels. After ~3 min the pressure was released, and the second short pulse was applied. The activities during the second pulse were reduced (\(I_{\text{max2}}/I_{\text{max1}} = 0.67 \pm 0.13\) (\(n = 5\))) (Fig. 8, middle trace); however, the degree of the decrease was similar to the control experiment (Fig. 8, top trace), in which no nickel ions were added and \(I_{\text{max2}}/I_{\text{max1}} = 0.67 \pm 0.08\) (\(n = 4\)). This suggests that the decline of activities was due to the lack of recovery of the adapted channels from their inactive state. We found previously that the recovery rate from an inactive state of the YggB channels was 72 and 95% following intervals of 60 and 120 s, respectively (18). In the experiment presented in Fig. 8, the bottom recording was obtained 2 min after the middle recording, and all of the channels were able to recover from their inactive state. Over this time Ni\(^{2+}\) was present in the chamber; however, nickel ions were not coordinated by the closed channels as seen from \(I_{\text{max1}}\) of the lower recording (\(I_{\text{max1}}\) from the bottom recording/\(I_{\text{max1}}\) from the middle recording = 1.074). We applied additional 5 mM NiCl\(_2\); so over the next 2 min the closed channels were exposed to 10 mM NiCl\(_2\), and still we did not observe any effect of coordinating Ni\(^{2+}\) by histidines. As shown in Fig. 5, the most severe reduction of the YggB-His\(_6\) activity occurred at 1 mM Ni\(^{2+}\), suggesting that the large number of nickel ions must be coordinated by many histidines from different subunits to have an effect on the channel activity. Single histidine coordinates nickel ion with a low affinity (millimolar range) (24); however, nickel ion is coordinated within a stretch of histidines with a high affinity (micromolar range) (25). This implies that in the experiment presented in Fig. 8, nickel ions bound to histidines when the channels were open and probably saturated the imidazole groups within a single histidine tag (with a high affinity), preventing the coordination between subunits (with a low affinity) even after exposing the channels to the high Ni\(^{2+}\) concentration.

The set of the two experiments described above shows that the closed channels were able to coordinate Ni\(^{2+}\) between their subunits, and it resulted in a reduction of the number of active channels. In the open channels, although they were exposed over similar time period to the same concentration of Ni\(^{2+}\), intersubunit binding of Ni\(^{2+}\) did not occur because there was no inhibition of the channel activities.

**DISCUSSION**

The existing model predicts that MscL, the large conductance MS channel from *E. coli* may be gated in a two-step manner (7, 8, 10). In this model there are two gates involved in the channel opening: the transmembrane gate that consists of the intramembraneous channel domains and the cytoplasmic one that is composed of the parts of the N termini. MscS, the small conductance MS channel from *E. coli*, is different from MscL in its amino acid sequence, membrane topology, and...
channel activities. The most striking difference is that MscL functions as pentamer and has two membrane-spanning domains, TM1 and TM2, whereas functional YggB is a heptamer and has three membrane domains, TM1, TM2, and TM3. The YggB TM3 domains line the channel pore and, similar to the TM1 domains of MscL, form the transmembrane gate of the channel (21). It has been proposed that both channels, although different, might have a common evolutionary origin (12). The hypothesis was based on a high sequence identity between the MscL TM1 and the YggB TM3 helices. The YggB TM3 helix shows the highest level of conservation among all three membrane domains in various YggB homologues in bacteria (3) (Fig. 2) and eukaryotes (13). There are also short spans of C terminus that are highly conserved among YggB bacterial (Fig. 2), archean (12), and eukaryotic homologues (13).

In the model of the MscL functioning, the role of its C terminus is not clear. The large portion of it (the last 27 amino acids of the total 41) can be deleted, and the truncated channel still remains active; however, greater deletions result in an inactivation of the channel (26, 27). Within the portion of C terminus that can be deleted without affecting channel activities there is an a-helical S3 domain. It has been found recently that S3 helices form a pentameric coiled-coil structure and separate upon channel opening. It is suggested that they may not be directly involved in the channel gating (28). The role of the conserved portion of C terminus adjacent to TM2 and indispensable for the channel proper function has yet to be established.

The quaternary structure of YggB reveals that extramembranous C-terminal domains are composed mostly of $\beta$-sheets and surround a large water-filled chamber with eight openings with diameters of 8–14 Å (Fig. 1B, middle panel). The YggB structure may reflect an open configuration of the channel, and it is suggested that the movement of solutes to the water chamber occurs via the openings. The entire cytoplasmic domain would serve as a screening filter against large molecules (21). At the end opposite to the membrane of this assembly, there is a parallel $\beta$-barrel structure composed of seven strands, which, in our experiments, were tagged with the hexahistidine tags.

From the experimental results presented in this study we can conclude that the C termini of the YggB channel move apart upon channel opening. The intersubunit Ni$^{2+}$ coordination between the histidine tags added to the ends of the $\beta$-barrel strands leads to inhibition of the channel activities, and it occurs only when the channel is closed. This result suggests that these parts are close to each other in the channel closed state, and they are more distant to each other when the channel is open. It is likely that they move apart during opening of the channel when mechanical force is applied. We have also shown that YggB channels become inactive when their cytoplasmic parts were exposed to BS3 and the nonpolymerized GDH in the closed state of the channel. We failed to observe this phenomenon when EDC was used. Our interpretation of these data is that after the cross-linking of lysines from the YggB C termini, the cytoplasmic domain becomes more rigid, and therefore the closed conformation of the channel is stabilized.

These results suggest that the entire cytoplasmic domain of the channel is a dynamic structure. It is likely that the movement of the ends of C termini may occur during activation of the channel, and this is due to conformational changes within each subunit and/or due to a change of interactions between subunits. There are several examples in the literature showing that large cytoplasmic domains directly or indirectly participate in the channel gating. Activation of the voltage-gated K+ channel Kv is coupled to the conformational changes in its cytoplasmic domains T1 (29, 30), and the conformational changes induced by Ca$^{2+}$ binding to the cytoplasmic domains of the bacterial calcium-activated K+ channel MthK are linked to the membrane domains, resulting in the channel activation (31). Cytoplasmic C termini of KcsA, pH-activated bacterial K+ channel (21), suction step, bottom trace, compare with Fig. 7, arrows. The YggB TM3 domains line the channel pore and, similar to the TM1 domains of MscL, form the transmembrane gate of the channel.

FIG. 8. Ni$^{2+}$ (5 mM) applied for 2 min to the open YggB-His$_6$ channels did not change their activities. In control recording (top trace), the suction pulse was applied for ~3 min. After release of suction, the second, short pulse of identical pressure was applied. Fewer channels open because of the inactive state of the adapted channels. A similar procedure was performed, and the channels were exposed to 5 mM Ni$^{2+}$ (middle trace). The obtained recording was very similar to the control one. The bottom trace was obtained 2 min after recording the middle one. Over this time, the closed channels were exposed to 5 mM Ni$^{2+}$, and it did not result in inhibition of the channel activities (compare with Fig. 1, upper trace). Additional application of 5 mM Ni$^{2+}$ (total 10 mM) also did not affect the channel activities (response to the second suction step, bottom trace). We conclude that the coordination of Ni$^{2+}$ occurred within a channel single subunit. The bars above the middle and bottom traces show changes of Ni$^{2+}$ concentration in the chamber. The arrows indicate application (downward arrow) and release (upward arrow) of suction (225 mm Hg). All of the traces were recorded at −15 mV. C marks a closed level.
channel, do not directly participate in gating; however, they contribute to modulating of the pH gating and stabilize the closed conformation of the channel (32).

The Ni\(^{2+}\) coordination between YggB subunits occurred with a \(K_e\) in a millimolar range. This affinity is similar to that estimated for a single imidazole group in solution (24). Coordination of Ni\(^{2+}\) between multiple histidines on adjacent subunits of the cyclic nucleotide gating channel was reported to occur in the range of micromolar concentrations of Ni\(^{2+}\) (33); however, histidines from the center of α-hemolysin polypeptide from Staphylococcus aureus were coordinated by Zn\(^{2+}\) at a concentration of 0.1–0.6 mM (23). Even higher concentrations of Ni\(^{2+}\) (0.1–50 mM) were reported to inhibit epithelial sodium channel by coordination of histidines from two extracellular subunits α and γ (34). In our case a histidine tag was added to the end of a very long C terminus (−160 amino acids) whose mobility is not known. High concentration of Ni\(^{2+}\) yielding the inhibiting effect on the channel activities is probably due to the requirement of coordination of many nickel ions by multiple subunits. This suggestion is also supported by another result from this report: a lack of the channel inhibition in the presence of antiHis\(_6\) antibodies. Specific antibodies can block ion channels providing the targets are located on external parts of the channels (35). Based on the channel crystal structure, we assume that the histidine tags added to the ends of the β-barrel strands (Fig. 1B) are easily accessible to the antibodies. However, they may not bind a sufficient number of the antibody molecules to block the channel. The external diameter of the β-barrel is estimated to be 8 Å (20), and the external one is −20 Å (our estimation). Because of the flexible hinge present in the antibodies, these molecules bivalently bind epitopes separated by 40–100 Å (36, 37). Using the crystal structure of the Fab antibody fragment with six histidines bound, we estimated the minimal distance for bivalent binding to be −37 Å. Because the external diameter of the β-barrel is −20 Å, seven histidine tags can bind monovalently only one anti-His\(_6\) molecule. One monovalently bound antibody molecule does not suffice to inhibit the channel.

The inhibition of YggB-His\(_6\) activities in the presence of Ni\(^{2+}\), was voltage-dependent. The open probability of MscS activities are modulated by voltage (16), and it is likely that voltage induces conformational changes of the channel. Based on the crystal structure of YggB, such changes within the transmembrane regions are suggested (21, 38), and they might be transmitted to the cytoplasmic domains of YggB similarly to how it was shown to the Kv channel and its T1 domains (31). Even small changes in the position of the β-barrel strands may affect Ni\(^{2+}\) binding to the histidine tags.

We demonstrated previously that exposure of the cytoplasmic side of the MscS channels to 1 mg/ml Pronase almost entirely abolished their activities (18). We concluded that Pronase removed responsiveness of the channels to the membrane tension by disrupting the tension transmission mechanism responsible for the opening of the channel. Now, knowing the amino acid sequence of YggB, the topology in the membrane, and its quaternary structure, we assume that Pronase could digest the C termini and/or the cytoplasmic linker between the TM1 and TM2 domains (Fig. 1A). In the case of the latter, the entire structure of the channel would be destroyed, and we could not expect the channel to be active. In the case of the C terminus digestion, a question arises as to which part of it was removed because this region was crucial for the proper channel function. In our preliminary studies leading to localization of this region, we wanted to delete a part of C terminus and obtain an inactive channel. We removed the last 89 amino acids from the C terminus (the last amino acid in the remaining portion was Asp\(^{397}\), indicated in Fig. 1A) and examined membrane patches of the mutant. We did not detect any YggB activity in any of the examined patches (two preparations, 20 patches of each). At present we cannot entirely rule out a possibility that the truncated C termini make the YggB channels unable to assembly in the membrane. However, if taken together with our former observation regarding the effect of Pronase on the assembled channels, the lack of activity in the mutant suggests strongly that the C termini may indeed be involved in activation of the YggB channel.

Based on the results summarized and discussed above, we propose that the YggB C termini are involved in the gating of YggB. It means that in addition to the channel transmembrane gate composed of its TM3a, the other cytoplasmic gate may be formed by as yet undefined fragments of C termini. As calculated, the heptameric symmetric transmembrane gate of YggB cannot make a tight constriction resulting in the closed conformation of the channel because the helices cannot come closer than at a distance of −9 Å (38). It is suggested that to close the conducting pathway, the symmetry of the gate might be broken or that the periplasmic linker between the TM2 and TM3 helix should become a part of the gate (38). Alternatively, a part of the C terminus may contribute to gating by composing a part of the transmembrane gate or by forming a separate gate. Which fragments of C termini might be involved in gating? We suggest that the gate may exist within the removed stretch of 89 amino acids. The fragment proved to be crucial for gating, and there are several highly conserved regions within it. More deletion mutations are needed to localize the region of the hypothetical gate.

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