Cell-Free Synthesis of a Functional Ion Channel in the Absence of a Membrane and in the Presence of Detergent†

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Received May 11, 2004; Revised Manuscript Received July 29, 2004

ABSTRACT: We have investigated the possibility of cell-fee synthesis of membrane proteins in the absence of a membrane and in the presence of detergent. We used the bacterial mechanosensitive channel MscL, a homopentamer, as a model protein. A wide range of nonionic or zwitterionic detergents, Triton X-100, Tween 20, Brij 58p, *n*-dodecyl β-D-maltoside, and CHAPS, were compatible with cell-free synthesis, while *n*-octyl β-D-glucoside and deoxycholate had an inhibitory effect. In vitro synthesis in the presence of Triton X-100 yielded milligram amounts of MscL per milliliter of lysate. Cross-linking experiments showed that the protein was able to oligomerize in detergents. When the purified protein was reconstituted in liposomes and studied by the patch-clamp technique, its activity at the single-molecule level was similar to that of the recombinant protein produced in *Escherichia coli*. Cell-free synthesis of membrane proteins should prove a valuable tool for the production of membrane proteins whose overexpression in heterologous systems is difficult.

A bottleneck in the study of membrane proteins resides in the difficulties that are encountered in overexpressing them. While prokaryotic membrane proteins can usually easily be expressed in a host such as *Escherichia coli*, the expression of eukaryotic membrane proteins has proven far more problematic. Although successes have been reported for the expression of some proteins in *E. coli* (*1*) and yeast or insect cells (*2*), failures are more common, even if they go unreported. The interest in obtaining high yields of membrane proteins is particularly obvious in the case of structural studies, but is also important for functional studies. In ion channel studies, for instance, purification of the relevant protein can be bypassed most of the time by expression of the gene coding for a putative channel in a cell (e.g., frog oocyte) that can be studied by electrophysiological methods. However, in some cases, the corresponding protein does not migrate to the plasma membrane, thus rendering its study impossible with a patch-clamp electrode or microelectrode. Moreover, and evidently, ion channels of organelles cannot be studied by this approach. In such cases, the protein has to be overexpressed, purified, and reconstituted in artificial lipid bilayers for electrophysiological study.

Overexpression of membrane proteins is difficult because, unlike soluble proteins, these proteins have to be targeted and inserted into the membrane as soon as their synthesis begins. We therefore reasoned that cell-free synthesis of membrane proteins, in the absence of a membrane, but in the presence of detergent, might be an interesting alternative. This implies that the membrane protein is able to fold in the absence of the insertion machinery. Although the study of membrane protein folding has been limited, experiments, mostly performed with bacteriorhodopsin and diacylglycerol kinase, indicate that an α-helical membrane protein can be denatured and refolded after transfer to detergents or lipids (*3*).

We chose to produce the mechanosensitive channel MscL from *E. coli*. The channel, which was purified and cloned by Kung and co-workers (*4*), is a homopentamer with each subunit (14 kDa) consisting of two transmembrane α-helices (*5*). The protein is easily overexpressed in *E. coli* and is functional, as assayed by the patch-clamp technique, after purification and reconstitution in a pure lipid bilayer (*6*, *7*). It is therefore possible to compare in vivo and in vitro production. We recently reported that the N- and C-halves of the MscL channel can be reassembled in detergent to yield a functional mechanosensitive channel (*8*).

For cell-free expression we used a commercial system developed by Roche (*9*). It consists of an *E. coli* lysate containing the machinery to drive coupled transcription and translation in one reaction vessel in the presence of a DNA template. Following the principles outlined by Spirin and co-workers (*10*), a semipermeable membrane allows a continuous supply of substrates and removal of inhibitory byproducts, thus extending the duration of expression and the protein yield. The system, which avoids codon usage bias,

† This work was supported in part by a grant from CNRS (DRAB03/36).

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10.1021/bi049049y CCC: $27.50
Published on Web 09/11/2004

is used extensively for the synthesis of eukaryotic and prokaryotic soluble proteins.

MATERIALS AND METHODS

Plasmids, Strains, and Growth Conditions. The *mscL* gene was cloned under T7 promoter control into pIVEX plasmids (Roche). *Nco*I and *Xma*I restriction sites were introduced upstream and downstream of the gene by PCR, and the *Nco*I and *Xma*I fragments were subcloned into the vector pIVEX 2.4b or pIVEX 2.3, which allows addition of a six-histidine tag at the N-terminus or the C-terminus, respectively. pIVEX 2.4b carries a factor Xa protease cleavage site which allows elimination of the tag. Both constructs were checked by sequencing.

E. coli DH5α (Invitrogen) and BL21 (λDE3) (Novagen) strains were used as the host strain and as expression strains for the pIVEX−*mscL* plasmids. Cultures were grown under aerobic conditions at 37 °C in LB medium. Where appropriate, ampicillin (100 μg/mL, Fluka) and chloramphenicol (30 μg/mL, Appligene) were added to the medium. The expression of the recombinant MscL in BL21 cells was induced for 3 h by 1 mM IPTG (Sigma).

In Vitro Expression of the Recombinant MscL. In the RTS system 100 HY (high yield), the low-scale protein production system, 0.5 μg of plasmid DNA was incubated for 6 h at 30 °C in the presence of an *E. coli* lysate containing 0.1% Triton X-100 and 10 μg/mL *E. coli* lipids in a final reaction volume of 50 μL. In a comparative study, a range of different detergents were used, as indicated in the caption of the corresponding figure. The expression of the protein was detected by immunoblotting, using an antibody against the His_6 tag. When the RTS system 500 HY was used for preparative experiments, 15 μg of recombinant plasmid was incubated at 30 °C for 20−24 h, in a final reaction volume of 1 mL with 0.2% Triton X-100 and 18 μg/mL *E. coli* lipids.

Recombinant MscL Protein Purification and Biochemical Analysis. In vivo and in vitro produced recombinant MscL proteins were purified in one step, using a Ni−NTA[1] column. In the first case, transformed BL21 (λDE3) cells (2 g wet weight) were French-pressed, and the membranes were isolated by ultracentrifugation (300000*g*, 4 °C, 25 min). The membrane pellet was solubilized at 4 °C overnight in 5 mL of extraction phosphate saline buffer (PBS) (50 mM Na_2HPO_4, pH 7.5, 300 mM NaCl) containing 10 mM imidazole and 2% Triton X-100. The extract was cleared by a second ultracentrifugation (300000*g*, 4 °C, 25 min) and incubated for 1 h at 4 °C with 500 μL (bed bead volume) of Ni−NTA agarose beads (Quiagen) equilibrated in the previous buffer. The column was then packed in a 2.5 mL shell, extensively washed four times with 4 bed volumes of the PBS buffer containing 20 mM imidazole and 1% Triton X-100. The bound proteins were eluted in four steps with 200 μL of PBS buffer supplemented with 250 mM imidazole and 0.1% Triton X-100. In vitro expressed MscL was purified in the same way except that 1 mL of RTS lysate was incubated with 1.2 mL of Ni−NTA and that 0.2% Triton X-100 was used throughout the purification. After purification, protein samples were analyzed by 15% SDS−PAGE, and concentrations were determined using the bicinchoninic acid method (Pierce Chemical).

Reconstitution in Liposomes and Formation of Giant Proteoliposomes. A few microliters of purified recombinant MscL was added to 1 mL of 10 mM HEPES−KOH, pH 7.4, 100 mM KCl, 1% octylglucoside containing 1 mg of lipids (azolectin from soybean, type IV-S, Sigma) to achieve lipid-to-protein ratios of 2−200. SM-2 (Bio-Rad) Bio-Beads were then added at a concentration of 160 mg/mL. After 3 h, the Bio-Beads were discarded, and the suspension was centrifuged for 25 min at 300000*g* (*11*). To obtain giant proteoliposomes amenable to patch-clamp recording, the pellet was resuspended in 20 μL of 10 mM HEPES−KOH, pH 7.4, and the resulting suspension was subjected to a dehydration/rehydration cycle as previously described (*12*). Rehydration was performed in 10 mM HEPES−KOH, 100 mM KCl, pH 7.4. A 2 μL sample of the giant proteoliposome suspension was deposited in a patch-clamp chamber and diluted with 2 mL of bath solution (10 mM HEPES−KOH, 100 mM KCl, pH 7.4).

Patch-Clamp Recording. Single-channel activity was recorded using standard patch-clamp methods (*13*). Patch electrodes were pulled from Pyrex capillaries (Corning code 7740) using a P-2000 laser pipet-puller (Sutter Instruments Co.) and were not fire polished before use. Micropipets were filled with a buffer similar to that of the patch-clamp chamber consisting of 2 mM $MgCl_2$ and 5 mM $CaCl_2$. A negative pressure (suction) was applied to the patch pipet with a syringe and monitored with a piezoelectric pressure transducer (Bioblock Scientific). Unitary currents were recorded using a Biologic RK-300 patch-clamp amplifier with a 10 GΩ feedback resistance and stored on digital audio tape (Biologic DTR 1200 DAT recorder). Records were subsequently filtered at 1 kHz (−3 dB point) through a four-pole Bessel low-pass filter, digitized off-line at a rate of 2 kHz, and analyzed on a personal computer, with a program developed by G. Sadoc (Gif sur Yvette). Data were plotted on a Hewlett-Packard LaserJet printer, using Sigmaplot software (Jandel). The membrane potential refers to the potential in the bath minus the potential in the pipet.

RESULTS

Detergents Compatible with Cell-Free Synthesis. The *mscL* gene was inserted into plasmids pIVEX 2.3 and pIVEX 2.4b. These two plasmids, which allow synthesis of the product of the gene under the control of a T7 promoter, are optimized for in vitro synthesis. They are designed to yield a protein with a His_6 tag on its C-terminus or its N-terminus, respectively.

Both constructs allowed the synthesis of MscL protein in the absence of detergent, as detected by immunoassay, using an antibody against the His_6 tag. We next studied the compatibility of different detergents with the coupled transcription/translation reaction. For these experiments we used a batch system (RTS system 100 HY). We chose detergents widely used in membrane protein studies and belonging to the existing different classes: nonionic detergents with a low critical micellar concentration (CMC) such as Triton X-100, Tween 20, Brij 58p, *n*-dodecyl β-D-

[1] Abbreviations: CMC, critical micellar concentration; OG, *n*-octyl β-glucoside; CHAPS, 3-[(3-cholamydopropyl)dimethylammonio]-1-propanesulfonate; Ni−NTA, Ni^{2+}-nitrilotriacetic acid.

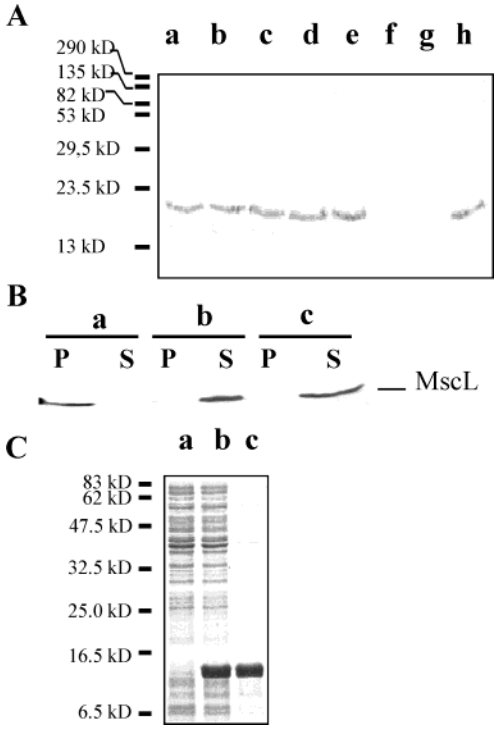

FIGURE 1: In vitro synthesis and purification of tagged MscL. (A) Effect of different detergents on the cell-free expression of MscL. The tagged protein was synthesized by using the RTS 100 HY system in the absence of detergent (lane a) or in the presence of 0.12 mM Tween 20 (lane b), 0.14 mM Brij 58p (lane c), 0.2 mM *n*-dodecyl β-D-maltoside (lane d), 1.5 mM Triton X-100 (lane e), 30 mM *n*-octyl β-glucoside (lane f), 4 mM desoxycholate (lane g), and 12 mM CHAPS (lane h). After synthesis, 1 μL of total extract was analyzed by 15% SDS−PAGE, and the tagged protein was detected by immunoblotting. (B) Localization of in vitro synthesized MscL. The tagged protein was synthesized in the absence (a, b) or in the presence (c) of 0. 2% Triton X-100. The lysate was diluted 5 times in the absence (a) or in the presence (b, c) of 0.2% Triton X-100. In each case, after centrifugation at 20000*g* for 10 min, the tagged protein was detected by immunoblotting in the pellet (P) and the supernatant (S). (C) One-step purification of in vitro synthesized MscL tagged on its C-terminus. The synthesis was carried out using the RTS 500 HY system, in the presence of 0.2% Triton X-100. Extracts of lysate before expression (lane a) and after expression (lane b) and purified protein after purification on a Ni−NTA column in the presence of 0.2% Triton X-100 (lane c) were analyzed by 10% SDS−PAGE after Coomassie blue staining.

maltoside, a nonionic detergent with a high CMC, *n*-octyl β-D-glucoside (OG), a zwitterionic detergent, CHAPS, and an ionic detergent, deoxycholate. All detergents were assayed at a minimum concentration corresponding to 2−5 times their CMC, except for OG (30 mM for a CMC of 19−25 mM). These are the conditions normally used in membrane biochemistry to keep membrane proteins in a soluble state. As shown in Figure 1A, under these conditions, all tested detergents were compatible with in vitro protein synthesis except OG and deoxycholate. These two detergents inhibited synthesis totally.

If protein synthesis is very efficient, it might be necessary to use some detergents at concentrations well above the CMC. At a protein concentration of several milligrams per milliliters, binding of detergent to the protein is no longer negligible, in particular for low-CMC detergents. When higher detergent concentrations were used, we observed that low-CMC detergents (Tween 20, Brij 58p, *n*-dodecyl β-D-maltoside) were compatible with protein expression at 10 times the CMC, and even 50 times for Triton X-100 (not shown). However, at the highest concentration a decrease in protein production was noted.

When MscL was synthesized in the absence of detergent, it was found in the insoluble fraction which sedimented upon centrifugation at 20000*g* for 10 min (Figure 1B, a), whereas when synthesized in the presence of 0.2% Triton X-100, it remained in the soluble fraction (Figure 1B, c). Interestingly, when MscL was synthesized in the absence of detergent, it could be recovered in the soluble fraction upon addition of 0.2% Triton X-100 (Figure 1B, b).

Cell-Free Synthesis of MscL and Functional Assay. Previous experiments have shown that a functional MscL channel can be recovered from *E. coli*, after solubilization and purification in OG (*4*) or Triton X-100 (*14*). In view of the above results, we chose to produce MscL in vitro in the presence of Triton X-100. The plasmid pIVEX 2.3 carrying the *mscL* gene was added to the lysate containing 0.2% Triton X-100. It is well-known that complete membrane protein delipidation usually leads to protein inactivation, and it is also possible that the lipid requirement may be necessary for folding (*15*). We therefore supplemented the RTS 500 HY lysate with *E. coli* lipids (18 μg/mL). Synthesis was performed for 24 h at 30 °C. The MscL protein was purified using a Ni−NTA column (Figure 1B). The protein yield was variable (four experiments): in most of the cases 1−1.3 mg of protein was recovered, but in one case the yield was 3.6 mg. In one case a fraction (30%) of the pure protein later formed aggregates and sedimented as a deposit at the bottom of the protein solution.

We also performed two syntheses in the absence of detergent. In one case the lysate was supplemented with 1 mg of liposomes obtained by sonication of *E. coli* lipids. Triton X-100 was added to the lysate after synthesis. Purification on the Ni−NTA column yielded 1.1 mg of pure MscL when the synthesis was performed in the presence of liposomes and 300 μg in the other case.

The functionality of the purified protein was assayed by the patch-clamp technique. The MscL protein was reconstituted in liposomes by withdrawal of detergent, and the resulting proteoliposomes were subjected to a cycle of dehydration−rehydration to yield giant proteoliposomes amenable to patch-clamp recording. As shown in Figure 2, mechanosensitive channels similar to the native MscL channel could be detected in these preparations. Application of a negative pressure to the patch resulted in the opening of channels that closed upon pressure release. The conductance of the channels (1500 pS in 100 mM KCl symmetrical solutions) was identical to that found for MscL channels in the native membrane of *E. coli*, or after reconstitution of channels in liposomes, under identical ionic conditions (*16*). The relationship between open probability and pressure was similar to that previously documented for the MscL channel (*17*). No other channel activity than that of MscL was observed in these preparations. Therefore, at the single-molecule level, functional MscL channels produced in vitro cannot be distinguished from channels purified from *E. coli*. These results were obtained with MscL synthesized in the presence as well as in the absence of detergent.

However, these experiments, based on single-channel recording, cannot document whether all MscL proteins that are produced in vitro are functional, or whether only a

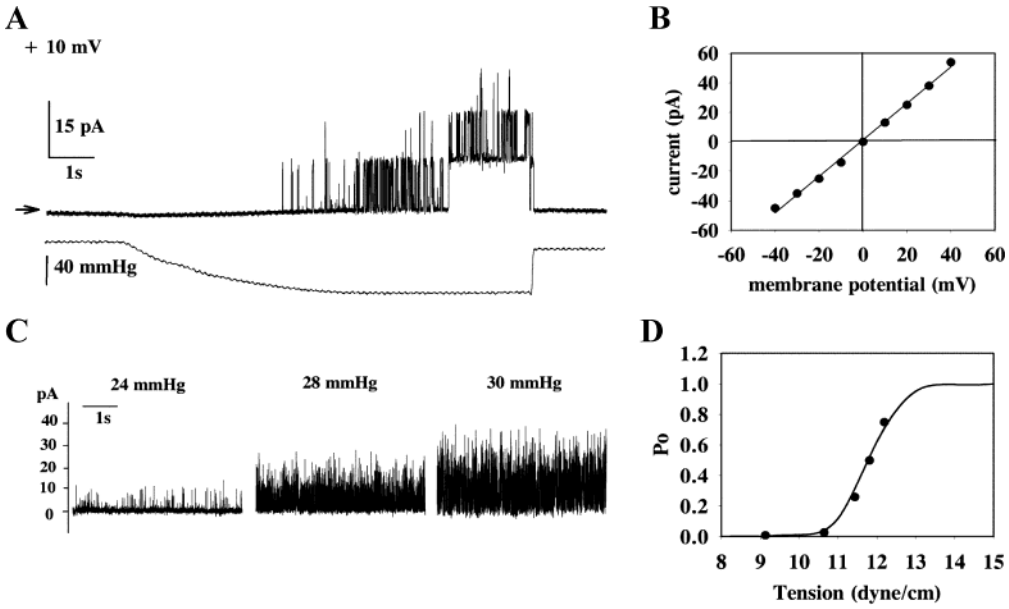

FIGURE 2: Electrophysiological activity of the MscL tagged on its C-terminus and produced in vitro. (A) Patch-clamp experiments were performed on giant proteoliposomes in the inside-out excised configuration. The upper trace shows the current, and the lower trace shows the pressure applied to the membrane patch. Application of suction in the pipet resulted in the opening of channels that closed upon release of suction. Time and current scales are indicated by small bars, and the arrow indicates the closed level of the channels. The bath and pipet medium are as indicated in the Materials and Methods. (B) Current−voltage relationship corresponding to the channels recorded in (A). (C) Channel activity recorded at differents levels of suction. Pressures are indicated for each trace. The membrane potential was +10 mV. (D) Open probability (P_o) versus the applied tension, at a fixed membrane potential of +10 mV, of the channels whose activity is shown in (C). The data, obtained from 20 s segment recordings at each pressure, were first fitted with a Boltzmann distribution of the form $P_o = (1 + (\exp \alpha)(p_{1/2} - p))^{-1}$, where P_o is the open probability, p is the applied pressure, $p_{1/2}$ is the pressure at which the open probability is 0.5, and α is the sensitivity. The $P_o(p)$ curve was converted to a $P_o(T)$ curve by rescaling to a midpoint $T_{1/2}$ of 11.8 dyn/cm as described (*17*), and fitted to the equation $P_o = (1 + \exp \beta(T_{1/2} - T))^{-1}$ with $1/\beta = 0.4$ dyn/cm.

fraction yield mechanosensitive channels. Since the measurement of a global activity is not possible here, we chose to compare the number of active channels per patch obtained after reconstitution of MscL channels produced in vitro, in the presence of detergent, and in vivo. The MscL protein with a polyhistidine tag on its C-terminus was produced in vivo in an *E. coli* strain expressing the T7 polymerase which was transformed with the pIVEX 2.3 plasmid carrying the *mscL* gene. After lysis of the *E. coli* cells in a French press cell, the membrane fraction was recovered, and MscL was purified from this fraction. Proteins produced in vitro, in the presence of detergent, and in vivo, were reconstituted at a similar lipid-to-protein ratio of 40. In each patch, pressure was applied until rupture of the patch, and the number of channels that opened was recorded. In some cases this procedure allowed the opening of all the channels (a maximum of 30 channels) in the patch, while in other cases the patch broke before all channels could be opened. The mean number of observed channels per patch was similar in both cases: 8.3 ± 1.8 channels per patch out of 30 patches for the protein synthesized in vitro in the presence of detergent, versus 9.3 ± 5.5 channels per patch out of 26 patches for the protein synthesized in vivo. We conclude that a major fraction of the protein synthesized in vitro is functional.

We also performed the synthesis of MscL tagged on its N-terminus. From the RTS 500 HY lysate, 1.2 mg of MscL protein/mL could be recovered. When the protein was reconstituted in giant liposomes and assayed for activity by patch-clamp experiments, we observed channels that opened upon application of pressure, and closed upon release of pressure. The conductance of the channels was similar to that of MscL, but the kinetics were completely abnormal (Figure 3B), and the number of channels per patch was low. When the same protein was produced in vivo, the same abnormal behavior was observed (data not shown). We therefore suspected that the modified kinetics was due to the extension at the N-terminus carrying the tag, and not to misfolding introduced by in vitro synthesis. Indeed, when the His tag was excised, using factor Xa, the corresponding protein exhibited normal kinetics after reconstitution in giant liposomes (Figure 3C). It is worth noting that when a different construct was used for the in vivo synthesis of N-terminus histidine-tagged MscL, this abnormal behavior was not observed (*18*, *19*). The difference lies in the length of the linker between the tag and the native protein which comprises the factor Xa specific site. Thus, a small modification can influence the folding or the activity of the protein.

MscL Can Oligomerize in Detergent. Since our functional assay involved necessarily the reconstitution of the membrane protein in a lipid bilayer, it cannot be deduced from these experiments whether refolding and oligomerization of the protein took place in the detergent, in the presence of lipids, or whether it needed the environment of the lipid bilayer to be completed. The oligomerization of KcsA, the tetrameric bacterial channel, was recently studied by MacKinnon and co-workers (*20*). The recombinant KcsA channel was denatured by a mixture of trifluoroethanol and trifluoroacetic acid, and its oligomerization was monitored by SDS−PAGE. Indeed KcsA is so stable that, unless boiled, it migrates as a tetramer on SDS−PAGE. This study has shown that insertion of the protein in a lipid bilayer, and not the mere presence of lipids, is necessary for tetramerization. When the protein, after denaturation, was reconstituted in liposomes,

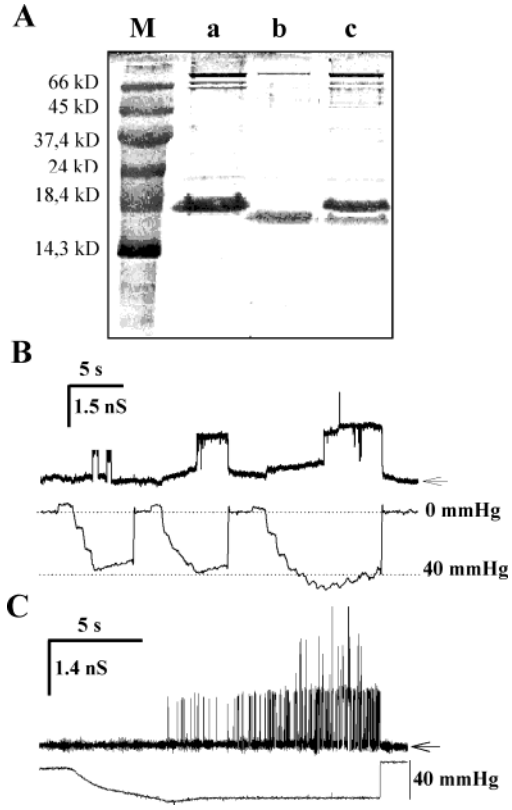

FIGURE 3: Effect of the N-terminal tag on the MscL channel activity. (A) Excision of the histidine tag from the N-terminal extension of the MscL synthesized in vitro. After in vitro expression and purification, the protein extract was dialyzed and incubated in the presence of the biotin-labeled factor Xa protease. Proteolyzed extract was then loaded onto a streptavidin column to remove the protease. Protein extracts before (lane a) and after (lane b) tag excision were analyzed by 15% SDS−PAGE. A mixture of equivalent quantities of the uncleaved and cleaved proteins was deposited in lane c. M indicates molecular weight markers. (B) Electrophysiological activity of the in vitro produced MscL, tagged on its N-terminus. (C) Electrophysiological activity of the same protein after excision of the tag.

a fraction of the protein was able to oligomerize. However, the presence of detergent under similar conditions inhibited the refolding process.

We tested whether oligomerization of MscL also requires a lipid bilayer. The pentameric structure of MscL is not stable enough to be detected by SDS−PAGE. Even at relatively low SDS concentration (0.5%), only the monomer is observed. We used cross-linking experiments and SDS−PAGE to address this issue. We compared the in vivo synthesized protein in detergent, which is pentameric, the in vitro synthesized protein in detergent, and the in vitro synthesized protein after reconstitution in a lipid bilayer (Figure 4). As previously reported (*21*), when MscL solubilized and purified from *E. coli* is incubated with a cross-linking agent in detergent and later subjected to SDS−PAGE, five bands corresponding to the different levels of oligomerization can be observed. When cross-linking was performed with the MscL protein synthesized in vitro, purified and kept in detergent, the same pattern was observed. Finally, when the protein synthesized in vitro was cross-linked after reconstitution in liposomes, the pattern was again similar. Interestingly, the same pattern was also observed for the MscL protein synthesized in vitro in the absence of detergent and lipids and purified in the presence of Triton X-100 (not shown).

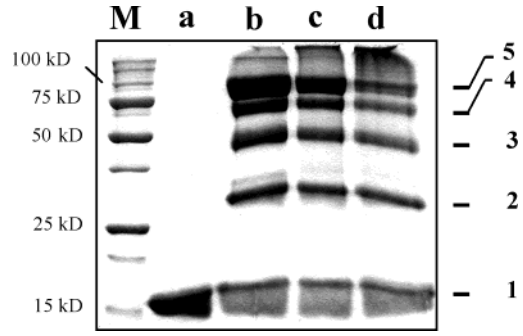

FIGURE 4: Cross-linking of MscL channels synthesized in vivo and in vitro. Proteins from pIVEX 2.3−*mscL* were produced in *E. coli* (lanes a and b) or were in vitro synthesized (lanes c d). After purification on Ni−NTA beads, 20 μg of each protein in detergent was incubated (lanes b and c) or not (lane a) with 2% formaldehyde for 1 h, then supplemented with the loading SDS−PAGE buffer, and analyzed directly by gel electrophoresis without boiling. In vitro synthesized protein was reconstituted in liposomes and then solubilized with Triton X-100 in the presence of formaldehyde (lane d). The different oligomers are indicated by small bars.

As an alternative approach, we also used blue native PAGE (*22*). MscL purified from *E. coli* did not migrate as a single band. Instead, several bands were observed, presumably corresponding to the different oligomers. The pattern was less clear than for the cross-linked protein, as the lower band, presumably the monomer, was predominant and the higher bands were blurred. However, no difference in the pattern could be observed between MscL synthesized in vivo and MscL synthesized in vitro.

DISCUSSION

We show here that a functional oligomeric membrane protein can be synthesized in vitro, in the absence of a membrane. While this work was in progress, two reports indicated that EmrE, a small multidrug resistance transporter from *E. coli*, and other related transporters can be synthesized in vitro (*23*, *24*). When the EmrE protein was reconstituted in liposomes, a proton motive force driven methyl viologen uptake could be observed (*23*). In the present case, patch-clamp experiments indicate that the electrophysiological activity of the protein synthesized in vitro cannot be distinguished from that of the protein synthesized in vivo, at the single-molecule level.

In vitro synthesis was compatible with a wide range of detergents used in membrane biochemistry. Several low-CMC nonionic detergents and a zwitterionic detergent, CHAPS, could be used for the synthesis of MscL at concentrations well above their CMC. Deoxycholate, an ionic detergent, inhibited protein synthesis, presumably because of its charge. OG, which is widely used in membrane protein studies, also inhibited synthesis completely. In the study of Elbaz and co-workers (*23*) 0.4% OG was reported to have no significant effect on the synthesis of EmrE, but this concentration is lower than the CMC. In the study of Klammt et al. (*24*), OG at 1.5-fold its CMC totally inhibited the synthesis of EmrE. Since all detergents appear to inhibit synthesis when their concentration is raised, it is probable that the inhibitory effect of OG is simply due to the fact that, because of its high CMC, it must be used at high concentrations.

The MscL protein can also be synthesized in the absence of detergent, but at a lower level. After solubilization in detergent and purification, this protein is functional. Elbaz et al. (*23*) also reported that EmrE could be synthesized in a milligram amount in the absence of detergent in the RTS system, and that the protein was active after solubilization in detergent. These authors speculated that the in vitro synthesized protein had inserted into a membrane fraction, present in the lysate, from which it can be recovered by addition of detergent. We attempted to estimate the amount of plasma membrane still present in the RTS lysate. Using an antibody against the membrane protein DjlA, we found that the amount of this protein in 1 mL of RTS lysate is 4% of that found in a crude lysate of BL21 *E. coli* cells containing the same amount of total proteins. From the correspondence between total proteins in a crude extract and plasma membrane lipids (*25*, *26*), we calculate that 1 mL of RTS lysate should contain about 50 μg of plasma membrane lipids. This is certainly insufficient for the insertion of 300 μg of MscL or 1−2 mg of EmrE. Another explanation is simply that the precipitated protein can be solubilized by the detergent. Clearly this will not apply to all proteins, and in future application it is probably desirable to perform the synthesis in the presence of detergent.

The currently accepted model for the folding of helical membrane proteins is that of Popot and Engelman (*27*) and Engelman et al. (*28*), which involves two main steps: insertion in the membrane of the helices and association of the helices to give the tertiary fold in the membrane. Importantly, this model predicts that membrane protein folding is to some extent independent of the insertion process. Indeed, experiments performed with bacteriorhodopsin, diacylglycerol kinase (*3*), and the KcsA channel (*20*) indicated that these proteins can be denatured and renatured. The semisynthesis and folding of KcsA (*29*) and very recently the total chemical synthesis of MscL (*30*) have been reported. The cell-free synthesis of EmrE and related transporters (*23*, *24*) and of the MscL channel (this paper) are also indications of the fact that folding of membrane proteins is largely independent of the insertion machinery.

Little is known of the folding of oligomeric membrane proteins. Studying the biogenesis of the KcsA prokaryotic potassium channel, van Dalen et al. (*31*) have shown that conversion from the monomeric form to the tetrameric form occurs in the membrane of *E. coli* and that this step is faster in the presence of a proton motive force. Recently, Mackinnon and co-workers in their study of the folding of the tetrameric KcsA potassium channel have obtained evidence that this channel is unable to oligomerize in the absence of a membrane (*20*). Our results indicate that this is not a general rule since for the pentameric MscL channel, oligomerization can occur in detergent.

The possibility of obtaining milligram amounts of membrane proteins is promising for structural studies. Our experiments indicate that most, if not all, of the in vitro synthesized protein is functional and therefore properly folded. It remains to be seen whether the preparations are homogeneous enough for the growth of crystals. In any case, this approach should prove extremely valuable for the biochemical and functional study of membrane proteins, in particular those whose putative genes are known, but whose activity has not been detected. As a caveat, we included in this paper our experiments showing how a tag can modify the activity of a protein. Whenever possible, it should be desirable to excise tags, or use different tags for the same protein, to characterize its genuine activity.

Because the cell-free synthesis system is an open system, it will be particularly valuable for the incorporation of labeled amino acids or for the coexpression of several proteins. Moreover, it should prove helpful for the characterization of mutant membrane proteins (e.g., channels that are permanently open) whose expression is toxic to a cell.

ACKNOWLEDGMENT

We thank Marc le Maire for discussions and Margret Ogmundsdottir for technical help. We are grateful to Emmett Jonhson for critically reading the manuscript.

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BI049049Y