Functional Expression of the PorAH Channel from Corynebacterium glutamicum in Cell-free Expression Systems

**IMPLICATIONS FOR THE ROLE OF THE NATURALLY OCCURRING MYCOLIC ACID MODIFICATION**

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PorA and PorH are two small membrane proteins from the outer membrane of Corynebacterium glutamicum, which have been shown to form heteromeric ion channels and to be post-translationally modified by mycolic acids. Any structural details of the channel could not be analyzed so far due to tremendous difficulties in the production of sufficient amounts of protein samples. Cell-free (CF) expression is a new and remarkably successful strategy for the production of membrane proteins for which toxicity, membrane targeting, and degradation are key issues. In addition, reaction conditions can easily be modified to modulate the quality of synthesized protein samples. We developed an efficient CF expression strategy to produce the channel subunits devoid of post-translational modifications. 15N-labeled PorA and PorH samples were further characterized by NMR and gave well resolved spectra, opening the way for structural studies. The comparison of ion channel activities of CF-expressed proteins with channels isolated from C. glutamicum gave clear insights on the influence of the mycolic acid modification of the two subunits on their functional properties.

Corynebacterium glutamicum belongs to the suborder of Corynebacterineae, having an unusual cell wall composition and architecture in comparison with other Gram-negative bacteria. C. glutamicum is widely used in fermentation industry for production of L-glutamate and L-lysine. Moreover, C. glutamicum genes are orthologues to a number of human pathogens of related pathogenic bacteria possessing an outer membrane, i.e. M. tuberculosis, Neisseria meningitides, N. gonorrhoeae, and Salmonella typhii, are associated with host-cell recognition and invasiveness during the early stage of infection and thereby contribute to pathogenicity and virulence (10-12). Both PorA and PorH lack signal sequence at their N termini and must be post-translationally modified for trafficking to the cell wall. Recently it was found for the first time in bacteria that these proteins are O-acylated by mycolic acids and that this modification plays a critical role in the channel-forming ability of these porins (13). However, many questions on the role and nature of these post-translational modifications still remain unsolved, and structural data are clearly required to fully address these issues. It is also unclear whether the modification is required on both partners or on simply one of them.

**In vitro** studies on the function and structure of the PorAH channel require large amounts of proteins as well as efficient labeling procedures for the production of samples with incorporated stable isotopes suitable for NMR spectroscopy. However, the recombinant production of membrane proteins (MPs) in heterologous hosts is notoriously difficult as a result of (i) toxic effects due to insertion of MPs into the host cellular membrane, (ii) improper translocation or inefficient transport.

3 The abbreviations used are: MP, membrane protein; CF, cell-free; CECF, continuous exchange CF; RM, reaction mixture; FM, feeding mixture; P-CF, precipitate-forming mode; D-CF, direct soluble expression mode in the presence of detergent; Tricine, N-[2-hydroxy-1, 1-bis(hydroxymethyl)methyl]glycine; BLM, black lipid membrane; TROSY, transverse relaxation optimized spectroscopy; MALDI-TOF, matrix-assisted laser-desorption/ionization time-of-flight; LMPG, 1-myristoyl-2-hydroxy-sn-glycero-3-phosphocholine; LPPG, 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine; DPC, dodecylphosphocholine; DDM, n-dodecyl-β-o-maltoside; LDAO, lauryldimethylamine oxide; β-OG, n-octyl-β-D-glucopyranoside.
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of overexpressed MPs to the outer membrane destination, (iii) proteolytic cleavage by cytoplasmic protein degradation machines, and (iv) lack of stability during in vivo MP extraction from the cell membrane. Indeed, our preliminary attempt to express PorAH channels in Escherichia coli failed due to toxic effects upon induction. Cell-free (CF) expression system has emerged as an efficient and multidimensional technique in the rapid production of integral MPs for structural and functional studies as it overcomes most sorts of difficulties (14–19). CF expression reactions can be operated in the batch configuration or in the more efficient continuous exchange CF (CECF) configuration in which the reaction mixture (RM) containing the translation machinery and other high molecular weight compounds is separated from a feeding mixture (FM) containing low molecular weight precursors by a semi-permeable membrane (20). MPs can be CF-produced in different modes. In the precipitate-forming mode (P-CF), no hydrophobic compounds are added into the RM and the synthesized MPs instantly precipitate. Those precipitates often consist of reversible aggregates that can be resolubilized with suitable detergents without preceding refolding procedures. In the direct soluble expression mode in the presence of detergent (D-CF), detergents are provided directly into the RM, and freshly translated MPs can instantly form proteomicelles (21, 22).

To address the challenge of producing sufficient quantities of PorA and PorH and to reveal the impact of the covalent mycolic acid modification on their function, we produced both proteins in an E. coli-based CF expression system. After systematic protocol and template optimization, milligram quantities per milliliter of RM as well as of 15N-labeled samples for NMR analysis could be produced within 2 days. CF-synthesized PorA and PorH were completely non-modified in contrast to the proteins purified from their natural host C. glutamicum. The proteins were produced in the P-CF and in the D-CF mode, and the channel activity was analyzed with the single proteins, with and without the C-terminal Poly(His)6 tag and thrombin cleavage site. Moreover, XbaI-XhoI fragments of PorANhis and PorHChis from pET28a were ligated, respectively, into restricted pIVEX2.3d vector resulting in pIVEX2.3d-PorANhis and pIVEX2.3d-PorHChis with Factor Xa cleavage sites. XbaI-EcoRI fragments of plVEX2.3d-PorAChis were ligated into restricted pXMJ19 to have pXMJ19-PorAChis. Unless and otherwise mentioned all clones were sequenced and analyzed. DNA templates used for CF expression were isolated from maxi-preparation of resulting constructs in pET28a and plVEX2.3d using commercial kits (Qiagen).

Expression and Purification—An established CECF expression system based on E. coli extracts was used for the in vitro expression of PorA and PorH (18, 19). CF S30 (sedimentation, 30,000 × g) extract were prepared from E. coli strain A19 (23). Analytical scale reactions were performed in Mini-CECF-reactors for optimization and screening steps in which the RM:FM ratio of 1:14 was maintained with a reaction volume of 60 μl (23). Preparative scale expressions with an RM of 2 ml and an RM:FM ratio of 1:17 were carried out in Maxi-CECF-reactors with commercial Slide-A-Lyzer devices (Pierce) as RM container. Unless otherwise mentioned, the RM was separated from the FM by a semi-permeable membrane of cut-off size 10 kDa, and all reactions were carried out at 30 °C for ~20 h with gentle shaking. For MP solubilization either in the P-CF or in the D-CF mode, the following detergents with suitable concentration (indicated under "Results and Discussion") were used: Brij-35, Brij-58, Brij-72, Brij-98, LDAO, and Triton X-100 (Sigma-Aldrich); LMPG, LMPC, LPPG, DHPC, and DPC (Avanti Polar Lipids, Alabaster, AL); β-OG (Glycon Biochemicals, Luckenwalde, Germany); DDM (AppliChem, Darmstadt, Germany); and SDS (Roth, Karlsruhe, Germany).

The P-CF proteins were obtained almost pure after re-solubilization in 20 mM Tris-Cl, pH 7.7, 150 mM NaCl (Buffer A) supplemented with appropriate detergent. The D-CF-expressed proteins were purified in one step by using immobilized metal-chelated affinity chromatography. 2 ml of RM was mixed with 600 μl of Ni2+-loaded NTA resin (Qiagen) for overnight at 4 °C. The resin-bound proteins were washed by gravity flow with 10 ml of buffer B (buffer A plus 0.4% LDAO) followed by sequential washings with 10 ml of 20 mM imidazole in buffer B and finally eluted with 4 ml of 500 mM imidazole. The pure PorA or PorH fractions were concentrated to 2 ml using Vivaspine devices (3-kDa cut-off, Sartorius Biolab, Goettingen, Germany), and imidazole was removed by passing through a 2.5-ml PD-10 column (Sephadex G25, GE Healthcare). Protein samples were analyzed by using 16% Tris-Tricine SDS-PAGE followed by Coomassie Blue staining and/or further detected by immunoblotting using anti-His antibodies (24, 25). Purified proteins were quantified according to their molar extinction coefficient (PorA: 8250 cm−1M−1; PorH: 1250 cm−1M−1) by measuring UV absorbance at 280 nm. Furthermore, NMR samples were prepared in 2-ml preparative scale reactions either in the P-CF mode or in the D-CF mode. The unlabeled amino acid mixture in the RM and FM was replaced by a 98% U-15N-labeled amino acid mixture (Cambridge Isotope Laboratories Inc., Andover, MA).

Cleavage of Affinity Tags—The purified PorA and PorH fractions were dialyzed in cleavage buffer containing 50 mM Tris-Cl, pH 8.0, 100 mM NaCl, 5 mM CaCl2, and 0.4% LDAO. Restriction grade thrombin (Novagen) at 1 unit/mg of protein and
Factor Xa (Qiagen) at 1 unit/50 μg of protein were used to cleave the C-terminal poly(His) tags from PorA and PorH, respectively. Cleavage was performed at 20 °C for 16 h, and proteases were removed by specific removal resins provided by commercial suppliers. Cleavage with >90% efficiency was observed at protein concentration of 0.8 mg/ml, and furthermore the cleaved protein samples were verified by matrix-assisted laser-desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry and SDS-PAGE analysis. The N-terminal tag used to promote CF expression of PorH resisted to proteolytic cleavage (PreScission protease) and was used as such, the protein being called T-PorH.

Peptide Sequences after Cleavage of Respective C-terminal Histidine Tags—The sequences used were: PorA (molecular mass = 5146 Da): MENYYELGNLVDGSGLGYFYFDFLGAISKWAGAYADLGLG-LVPR; PorH (molecular mass = 6617 Da): MDLLKETLGYYTFGGNIGTALQSIPTLLDSILNFDNFDLADTTGENDNFSSEGR; and T-PorH (molecular mass = 9124 Da): MKYKYYKKYKLEVFQGGMDLLKETLGYYTFGGNIGTALQSIPTLLDSILNFDNFGLADTTGENDNFSSEGR. (Bold letters represent extra residues after cleavage of tags.)

Mass Spectrometry Analysis—MALDI-TOF spectra of in vivo- and in vitro-expressed and purified proteins were acquired on a Voyager-DE STR mass spectrometer (PerSeptive Biosystems) fitted with a pulsed nitrogen LASER emitting at 337 nm, and all spectra were analyzed in linear mode using an extraction delay of 100 ns with accelerating voltage of 25 kV. Protein samples were mixed with sinapinic acid as matrix and were loaded onto a metal plate; 2500 shots were accumulated for each sample in a positive ion mode, and all data were acquired with default calibration for the instrument. To reduce problems caused by detergents in measurements, either the ethyl acetate method (26) or the simple-dilution method in water were used to obtain an improved signal-to-noise ratio.

Channel Activity Measurements—Planar lipid bilayers (so-called black lipid membranes (BLMs)) were formed across a 250-μm diameter hole by presenting a bubble of asolectin lipids (Sigma) dissolved in n-decane (30 mg/ml). Recordings were performed in symmetric media containing an unbuffered solution of 1 M KCl (as in Ref. 8). Currents were amplified using an Axon 200B patch-clamp amplifier, filtered at 1 kHz through an a-pole Bessel filter, and digitized at 2 kHz. Data were analyzed with pCLAMP software. Unless otherwise mentioned the protein samples were diluted in 1 M KCl to obtain appropriate concentrations for measurements. Control experiments using detergent alone were performed systematically and did not show any characteristic signal.

RESULTS AND DISCUSSION

Optimization of CF Expression Protocols for the Preparative Scale Production of PorA and T-PorH—CECF reaction protocols described before (18–20) were used as the basis to optimize the expression of PorA and PorH. At first the expression of PorA and PorH was evaluated in analytical scale P-CF reactions with the constructs pET28-PorA, pIVEX2.3-PorA, pET28-PorH, and pIVEX2.3-PorH as templates. Good expression levels of PorA from both constructs were observed. The location of the poly(His)$_6$ tag at either the N or C terminus had no detectable effect on expression of PorA. However, no expression of PorH was observed from both templates after analysis by either Coomassie Blue-stained SDS-PAGE or by Western blot analysis with anti-His antibodies. Considering the reduced complexity of protein production in CF systems, complex mRNA secondary structures preventing efficient translation initiation at the translational start codon are primary reasons of insufficient expression yields. The 5’-end of the PorH coding region was therefore modified by fusion with a long AT-rich sequence encoding for 10 additional amino acids. The addition of the small expression tag improved production of PorH to high levels. The expression yields of PorA and T-PorH were further maximized by optimizing Mg$^{2+}$ concentrations in the CF reaction. After Mg$^{2+}$ screening in the range of 8–22 mM, a broad optimum between 10 and 18 mM was identified for PorA, whereas T-PorH showed a rather narrow optimum between 14 and 16 mM (Fig. 1). These identified conditions were used for subsequent preparative scale productions in the P-CF and D-CF modes.

Production of PorA and T-PorH in Micelles, Using P-CF and D-CF Mode Expressions—In P-CF reactions, PorA and T-PorH instantly precipitate after translation in the RM. However, many P-CF-produced MPs can efficiently be resolubilized in a variety of detergents, whereas the solubilization efficiency of a particular detergent depends on the individual MP (21, 22, 27). We therefore performed a resolubilization screen to identify detergents efficient for the resolubilization of P-CF-generated PorA and T-PorH. The precipitates were centrifuged at 15,000 × g for 15 min and washed with Buffer A followed by immediate resolubilization in various detergents without any denaturation/renaturation steps. For resolubilization screening, the following detergents were evaluated: LMPG (1-myristoyl-2-hydroxy-sn-glycero-3-[phosphor-rac-(1-glycerol)]) (1%), LMPC (1-myristoyl-2-hydroxy-sn-glycero-3-phosphocholine) (1%), LPGG (1-palmitoyl-2-hydroxy-sn-glycero-3-[phosphor-rac-(1-glycerol)]) (1%), DHPC (1,2-dihexanoyl-sn-glycero-3-phosphocholine) (1%), DPC (dodecylphosphocholine) (1%), DDM (n-dodecyl-β-D-maltoside) (1%), LDAO (lauryldimethylamine oxide) (1%), SDS (1%), and β-OG.
n-octyl-β-D-glucopyranoside) (2%). The choice of these detergents was based on a long term experience in solubilizing P-CF-expressed membrane proteins (18). A panel of non-ionic detergents (DDM and β-OG) and zwitterionic (LMPC, DHPC, DPC, and LDAO), and negatively charged (LMPG, LPPG, and SDS) ones was chosen. Many of them display typical phospholipid polar heads (LMPC, LMPG, LPPG, DHPC, and DPC). A specific focus was placed on detergents typically used for solution state NMR spectroscopy and x-ray crystallography of membrane proteins. LDAO was a common detergent throughout this work, because it was efficient in extracting and purifying "in vivo" PorAH from C. glutamicum membranes, and it gave well resolved NMR spectra on these samples. After incubation for 2 h at 30 °C with gentle shaking, the supernatant and residual precipitates were again separated by centrifugation and analyzed by SDS-PAGE. In comparison with controls, PorA precipitates completely solubilized in LPPG, LMPG, LPPG, DHPC, and DPC and SDS to a minor extent but still up to 50% in DHPC, LDAO, DDM, and β-OG. Solubilization of P-CF-produced T-PorH was complete in LPPG, LMPG, and SDS and up to 50% in DPC, LDAO, and LMPC. Only minor fractions of PorH were solubilized in DHPC, DDM, Triton X-100, and β-OG (Fig. 2).

Alternatively to the resolubilization of P-CF precipitates, PorA and T-PorH were further produced in soluble form in the presence of detergents by using the D-CF expression mode. Suitable detergents that do not inhibit the CF expression system were screened in analytical scale reactions (21). Detergents selected at initial final concentrations were Brij-35 (polyethylene-(23)-laurylether) (0.5%), Brij-58 (polyoxyethylene-(20)-cetylether) (1%), Brij-72 (polyoxyethylene-(2)-stearylether) (1%), Brij-98 (polyoxyethylene-(20)-oleylether) (1%), digitonin (0.4%), and Triton X-100 (0.1%). All Brij derivatives could completely solubilize PorA in the D-CF mode, and no residual PorA precipitate was detectable. The presence of different detergents also modulated the expression efficiency to some extent while highest yields were obtained with Brij-72 (Fig. 3). In contrast, the D-CF solubilization of T-PorH was generally much less effective, and the protein was not fully solubilized in any of the analyzed detergents (Fig. 3). The highest solubilization efficiency was obtained with Brij-72, and complete solubilization was obtained after further increase of the final detergent concentration to 1.2%.

**Purification of C. glutamicum and CF-expressed Proteins—In vivo** expression and purification of PorA or PorH out of C. glutamicum were carried out as described previously to obtain both proteins with mycolic acid modification (8). Because the in vivo-expressed samples were analyzed in LDAO (0.4%), we kept the same detergent for sample preparation of CF-expressed PorA and T-PorH. The direct resolubilization of P-CF-expressed PorA and T-PorH in LDAO resulted in relatively pure protein, and hence, those samples were directly used for further characterization. As a second option, the two proteins were expressed in the D-CF mode in the presence of Brij-72, and the detergent was then exchanged to LDAO upon immobilization of the proteins at Ni-NTA affinity columns. The immobilized proteins were washed with 20 mM imidazole and finally eluted in LDAO micelles with 500 mM imidazole. The purity and homogeneity of both in vivo and CF-expressed proteins were analyzed by SDS-PAGE followed by Coomassie Blue staining (Fig. 4). After cleavage of the C-terminal poly(His)_6 tags the samples were further characterized by mass spectrometry and with respect to their pore-forming activities. The final expression yield of 200 μM/liter of culture for in vivo expression and 200 μM/ml of RM for in vitro expression were obtained.

**Biochemical Characterization of PorA and PorH by Mass Spectrometry**—The presence or absence of mycolic acid modification on in vivo- and in vitro-expressed PorA and PorH were characterized by MALDI-TOF mass spectrometry. The theoretical masses of native PorA and PorH without modification are, for (M + H^+), 4681 Da and 6162 Da, respectively (13). In the case of the recombinant PorA and PorH constructs, after cleavage of the affinity tags used for purification of PorA and
PorH, the predicted (M + H+) masses without mycoloylation are 5147 Da for PorA, 6618 Da for PorH, and 9125 Da for T-PorH modified with the N-terminal expression tag. As shown in Fig. 5 (A and C), MALDI-TOF spectra of purified PorA expressed in *C. glutamicum* and CF synthesis confirmed the presence of mycolic acid modification with a difference of 506 Da corresponding to C34:0 naturally occurring corynomycolic residues, as was described previously (13). Similarly, we observed the absence of mycolic acid modification on CF-expressed T-PorH according to the theoretical molecular weight. The increase in molecular mass of CF-expressed PorH resulted from the N-terminal modification with the AT-rich expression tag. The mycolic acid post-translational modification has been described as an esterification reaction; hence, it is susceptible to alkaline treatment. This was further confirmed by treatment of *in vivo*-expressed PorA and PorH with 0.1 M NaOH, and the subsequent MALDI-TOF spectra analysis confirmed the loss of 506 Da.

**NMR Spectroscopy**—Two-dimensional, $^{15}$N,$^{1}$H transverse relaxation optimized spectroscopy (TROSY) spectra (28, 29) of PorA and T-PorH were obtained after preparative scale expression of the proteins in the presence of labeled amino acids. NMR samples (0.25 mM) were prepared in 20 mM Tris-Cl, pH 6.7, in LDAO (~2%), and the spectra were recorded at 25 °C. For P-CF-expressed proteins, TROSY spectra of good resolution could be obtained for both proteins (Fig. 6). The spectra of both proteins were identical regardless whether the proteins were directly resolubilized in LDAO, or first LPPG-resolubilized and then exchanged to LDAO upon Ni-NTA chromatography (data not shown). Furthermore, $^{15}$N-labeled D-CF-produced samples of PorA and T-PorH were generated and measured under identical conditions. The dispersion and signal intensity of P-CF- or D-CF-produced samples of PorA and T-PorH did not show significant differences (Fig. 6). In addition, other detergents besides LDAO were analyzed for their effects on the quality of NMR spectra. PorA and T-PorH were exchanged into DHPC upon affinity chromatography, and recorded NMR spectra were identical to those in LDAO (data not shown). Hence, it can be concluded that P-CF- or D-CF-expressed PorA and T-PorH maintain their structural fold in LDAO and in DHPC. Moreover, the good resolution and spectral diversity gave evidence that the P-CF- and D-CF-expressed samples are structurally homogenous in LDAO or DHPC. This result opens the way for structural studies of PorA and T-PorH using NMR spectroscopy provided that the activities of these CF-expressed proteins can be demonstrated (30–33).

**Ion Channel Activity of in Vivo- and in Vitro-expressed PorA and PorH**—In recent years the functionality of PorA and PorH has been studied with protein extract from the cell wall of *C.
glutamicum, and results indicate the formation of a functional complex out of both proteins (8), in addition to the requirement of mycolic acid modifications (on position Ser-15 in the case of PorA) for pore-forming activity (13).

In the present study, we investigated ion channel activity, using expressed and purified protein samples with (proteins expressed from C. glutamicum) and without mycolic acid modification using the advantage of E. coli-based CF expression. In the latter case, we are in a position to use purified proteins, without any chemical treatment and after mass spectrometric characterization. When a mixture of in vivo PorA and PorH was added to the chamber, porin-like channel activity could be observed for a concentration as low as 1 ng/ml. Fig. 7A shows the profile characteristic of multiple channel insertion in the

FIGURE 6. $^{15}$N,$^{1}$H TROSY spectra of CF-expressed U-$^{15}$N-labeled PorA and T-PorH in 20 mM Tris-Cl, pH 6.7, containing 2% LDAO at 25 °C. A, PorA from P-CF; B, PorA from D-CF; C, T-PorH from P-CF; D, T-PorH from D-CF.

FIGURE 7. Porin channel activity of a mixture of in vivo-expressed PorA and PorH. The aqueous phase contained 1 M KCl. A, multiple insertions in the membrane upon addition of the mixture in the bilayer chamber. B, slow and fast kinetics of closure of the channels upon application of $+40$ mV. C, application of $-100$ mV to a bilayer containing a large number of channels resulted in the closure of channels that could be re-opened upon return to 0 mV. The channels closed, but with a different time course, upon application of $+100$ mV.

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bilayer with time. The channels that were opened around 0 mV closed upon application of a membrane potential, positive or negative. Fig. 7B shows that opening and closing of the channels occurred with two distinct kinetics, one with a millisecond time scale, the other with an approximate time scale of seconds. Different conducting states could be clearly observed. As shown in Fig. 7C the tendency to close at high membrane potentials was greater at one polarity. When in vivo-expressed PorA and PorH were added to the chambers separately, no such channel activity could be recorded. At very high concentrations (0.5–1 µg/ml) noisy perturbations of the membrane could be detected, indicating that some proteins could insert in the bilayer. Our results confirm the requirement of both PorA and PorH to form a proper ion channel (8). They show in addition that the PorA-PorH channel shares the characteristic fingerprints of channels such as bacterial porin (34) and also mitochondrial porin (35): closure at positive and negative potential with fast and slow kinetics, asymmetric voltage dependence, and multiple conductance states. Similar porin channels can be found in related families of bacteria, *C. diphtheria*, *C. efficiens*, and *N. farcinina* (8, 36, 37).

To understand further the importance of mycolic acid modification on channel activity, we repeated the BLM measurement with CF-expressed PorA and T-PorH lacking any mycolic acid modification. Insertion of PorA or T-PorH alone lead to noisy profiles and membrane destabilization. When using a 1:1 mixture (w/w) of PorA and T-PorH, progressive insertions were observed with P-CF-expressed samples (Fig. 8A) at high concentrations (500 ng/ml) and with D-CF-expressed samples (Fig. 8B) already at 25-fold lower concentrations (20 ng/ml). However, these insertions persisted only for a short time period (typically less than a minute) leading to perturbation and breakage of the membrane and did not result into the voltage-dependent characteristics as observed with a PorA and PorH mixture with mycolic acid modification. This indicates that CF-expressed proteins, when added to the measurement chamber as a PorA:T-PorH mixture, are able to insert into the membrane, but they create a rapid membrane perturbation instead of a well-defined voltage-gated membrane channel. This behavior of CF-expressed proteins in BLM is similar to pore formation as observed in the case of outer membrane-spanning proteins, Oms38 from different *Borrelia* species (38) and also in the pore formation of *Clostridium difficile* toxin B (39). The same experiment has been performed using “in vivo”-produced and -purified proteins, chemically treated to remove the mycolic acid modification, using the protocol described before (13). In this case, the 1:1 mixture of PorA and PorH gave no signal at all and no sign of insertion. This may indicate that the chemical treatment makes more than just the expected deacylation in terms of the proteins primary or tertiary structure.

Because naturally occurring PorA and PorH are modified by mycolic acids, and because it was not clear whether both modifications on PorA and on PorH are required for the formation of a functional channel, we decided to observe the activity of mixed samples, containing one protein purified from *C. glutamicum* (“in vivo”) and the other one CF-expressed (“in vitro”) (Fig. 9). The PorA in vivo-T-PorH in vitro sample displays the complete set of characteristic of a voltage-dependent ion channel (Fig. 9A). By contrast, the PorA in vitro-PorH in vivo mixture displayed membrane insertion, but none of the other two typical features, i.e. slow and fast conductance and voltage dependence (Fig. 9B). Several important conclusions may be drawn from these experiments. First, the CF-expressed T-PorH is fully functional when mixed with mycoloylated PorA. It shows that the small AT-rich N-terminal expression tag does not prevent the formation of a functional channel and that the CF-expressed T-PorH is capable of acquiring rapidly the right fold in the black lipid membranes. Although T-PorH differs from PorH by an N-terminal extension, it is hardly possible that this hydrophilic tail compensates for the absence of mycoloyla-
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tion, and it is therefore reasonable to conclude that the mycoloylation on PorH is not a strict requirement for activity. The *vice versa* mixture PorA in vitro-PorH in vivo gave also channels in BLM experiments, but these did not possess the entire expected functional characteristic. The behavior of PorA in complex with PorH in vivo is therefore clearly different from that of PorA in vitro alone or from the complex of PorA in vitro-T-PorH in vitro. The membrane stays stable, and the detected channel activity most likely reflects some stabilization of PorA in vitro by interaction with the cotranslocated PorH in vivo.

In summary, the results indicate for the first time that the post-translational modification by mycolic acids is not an absolute requirement for the function of PorH as part of a voltage-dependent ion channel, whereas it is so in the case of PorA. Furthermore, CF expression has been shown to be a valuable tool for the study of these ion channels as (i) large amounts of pure and functionally active protein can be synthesized in less than 3 days, (ii) it allows the straightforward analysis of the role of post-translational modifications by the production of nonmodified protein derivatives, and (iii) labeled and structurally folded proteins can be produced for studies by NMR spectroscopy. Approaches by NMR spectroscopy will further be supported by the fast production of combinatorially labeled samples or by the preparation of PorA-PorH complexes in which only one protein will be labeled. The comparison of activities between PorA/PorH alone or in complex clearly shows that one protein will be labeled. The comparison of activities between PorA/PorH alone or in complex clearly shows that one protein will be labeled. The comparison of activities between PorA/PorH alone or in complex clearly shows that one protein will be labeled.

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REFERENCES

1. Zuber, B., Chami, M., Houssin, C., Dubochet, J., Griffiths, G., and Daffe, M. (2008) *J. Bacteriol.* 190, 5672–5680
2. Hoffmann, C., Leis, A., Niederweis, M., Plitzko, J. M., and Engelhardt, H. (2008) *Proc. Natl. Acad. Sci. U.S.A.* 105, 3963–3967
3. Daffe, M. (2005) in *Handbook of Corynebacterium glutanicum* (Lothar Egging, M. B., ed) pp. 121–148, Taylor and Francis group A, CRC Press Book, Boca Raton, FL
4. Brennan, P. J., and Nikaido, H. (1995) *J. Mol. Biol.* 253, 25–36
5. Lichtinger, T., Burkovsky, A., Niederweis, M., Krämer, R., and Benz, R. (1998) *Biochim. Biophys. Acta* 1795, 25–36
6. Hoffmann, C., Leis, A., Niederweis, M., Plitzko, J. M., and Engelhardt, H. (2008) *Proc. Natl. Acad. Sci. U.S.A.* 105, 3963–3967
7. Lichtinger, T., Burkovsky, A., Niederweis, M., Krämer, R., and Benz, R. (1998) *Biochim. Biophys. Acta* 1795, 25–36
8. Costa-Riu, N., Maier, E., Burkovsky, A., Krämer, R., Lottspeich, F., and Benz, R. (2003) *Mol. Microbiol.* 50, 1295–1308
9. Barth, E., Barceló, M. A., Kläcka, C., and Benz, R. (2010) *J. Bacteriol.* 192, 786–800
10. Ziegler, K., Benz, R., and Schulz, G. E. (2008) *J. Mol. Biol.* 379, 482–491
11. Nikaido, H. (2003) *Microbiol. Mol. Biol. Rev.* 67, 593–656
12. Svetliková, Z., Skovierová, H., Niederweis, M., Gaillard, J. L., McDonnell, G., and Jackson, M. (2009) *Antimicrob. Agents Chemother.* 53, 4015–4018
13. Huc, E., Meniche, X., Benz, R., Bayan, N., Ghazi, A., Trops, M., and Daffe, M. (2010) *J. Biol. Chem.* 285, 21908–21912
14. Savage, D. F., Anderson, C. L., Robles-Colmeneros, Y., Newby, Z. E., and Stroud, R. M. (2007) *Protein Sci.* 16, 966–976
15. Klamm, C., Lühr, F., Schäfer, B., Haase, W., Dötsch, V., Ruterjans, H., Glaubitz, C., and Bernhard, F. (2004) *Eur. J. Biochem.* 271, 568–580
16. Berrier, C., Park, K. H., Abes, S., Bironne, A., Betton, J. M., and Ghazi, A. (2004) *Biochemistry* 43, 12585–12591
17. Klamm, C., Schwarz, D., Lühr, F., Schneider, B., Dötsch, V., and Bernhard, F. (2006) *FEMS J.* 273, 4141–4153
18. Reckel, S., Sobhanifar, S., Dürst, F., Lühr, F., Shiroyko, V. A., Dötsch, V., and Bernhard, F. (2010) *Methods Mol. Biol.* 607, 187–212
19. Schwarz, D., Junge, F., Dürst, F., Flößich, N., Schneider, B., Reckel, S., Sobhanifar, S., Dötsch, V., and Bernhard, F. (2007) *Nat. Protoc.* 2, 2945–2957
20. Shiroyko, V. A., Kommer, A., Kolb, V. A., and Spira, A. S. (2007) *Methods Mol. Biol.* 375, 19–55
21. Käi, L., Kaldenhoff, R., Lian, J., Zhu, X., Dötsch, V., Bernhard, F., Cen, P., and Xu, Z. (2010) *PLoS One* 5, e12972
22. Klamm, C., Schwarz, D., Fedner, K., Haase, W., Dötsch, V., and Bernhard, F. (2005) *FEBS J.* 272, 6024–6038
23. Schneider, B., Junge, F., Shiroyko, V. A., Dürst, F., Schwarz, D., Dötsch, V., and Bernhard, F. (2010) *Methods Mol. Biol.* 601, 165–186
24. Schägger, H. (2006) *Nat. Protoc.* 1, 16–22
25. Sambrook, J., and Russell, D. W. (2000) *Molecular Cloning: A Laboratory Manual*, 3rd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
26. Yeung, Y. G., Nieves, E., Angeletti, R. H., and Stanley, E. R. (2008) *Anal. Biochem.* 382, 135–137
27. Wu, J. I., and Swartz, J. R. (2008) *Biochem. Biophys. Acta* 1778, 1237–1250
28. Pervushin, K. V., Wider, G., and Wüthrich, K. (1998) *J. Biomol. NMR* 12, 345–348
29. Renault, M., Saurel, O., Czaplicki, J., Demange, P., Gervais, V., Lörhr, F., Réat, V., Pirotto, M., and Milon, A. (2009) *J. Mol. Biol.* 385, 117–130
30. Abidine, A., Verhoeven, M. A., Park, K. H., Ghazi, A., Guittet, E., Berrier, C., Van Heijenoort, C., and Warschawski, D. E. (2010) *J. Magn. Reson.* 204, 155–159
31. Maslennikov, I., Klamm, C., Hwang, E., Kefala, G., Okamura, M., Esquivies, L., Mörs, K., Glaubitz, C., Kwiatkowski, W., Jeon, Y. H., and Choe, S. (2010) *Proc. Natl. Acad. Sci. U.S.A.* 107, 10902–10907
32. Koglin, A., Klamm, C., Trbovic, N., Schwarz, D., Schneider, B., Schafer, B., Lühr, F., Bernhard, F., and Dotsch, V. (2006) *Magn. Reson. Chem.* 44 Spec No, S17–23
33. Klamm, C., Trbovic, N., Schwarz, D., Lühr, F., Bernhard, F., and Dotsch, V. (2006) *Mol. Microbiol.* 59, 159–168
34. Schiffer, B., Barth, E., Daffe, M., and Benz, R. (2007) *J. Bacteriol.* 189, 7709–7719
35. Kläcka, C., Knörzer, P., Riess, F., and Benz, R. (2011) *Biochim. Biophys. Acta* 1808, 1601–1610
36. Thein, M., Bunikis, I., Denker, K., Larsson, C., Drancourt, M., Schwan, T. G., Mentele, R., Lottspeich, F., Bergström, S., and Benz, R. (2008) *J. Bacteriol.* 190, 7035–7042
37. Genisyeruèk, S., Papanikolou, P., Guttenberg, G., Schubert, R., Benz, R., and Aktories, K. (2011) *Mol. Microbiol.* 79, 1643–1654