J Membrane Biol (2010) 235:63–72
DOI 10.1007/s00232-010-9256-3

ORIGINAL ARTICLES

Investigation on Stability of Transporter Protein, Glucuronide Transporter from *Escherichia coli*

Noriyuki Ishii

Received: 26 November 2009 / Accepted: 29 April 2010 / Published online: 19 May 2010

Abstract The glucuronide transporter GusB, the product of the *gusB* gene from *Escherichia coli*, is responsible for detoxification of metabolites. In this study, we successfully expressed GusB homologously in *E. coli* and investigated its oligomeric state in *n*-dodecyl-*β*-D-maltoside (DDM) detergent solution. Evidence for a pentameric state with a Stokes radius of 57 ± 2 Å for the purified GusB protein in DDM solution was obtained by analytical size-exclusion HPLC. The elution peak corresponding to pentameric GusB is commonly seen in elution profiles in the different buffer systems examined over a wide pH range. Hence, it is likely that GusB resides in the membrane as a pentamer. Stability studies with different incubation periods with the typical lipids, such as dimyristoylphosphatidylcholine, and total *E. coli* phospholipids, as the representatives of both phosphatidylcholine and phosphatidylethanolamine, show some clues to two-dimensional crystallization of GusB with lipids.

Keywords Membrane protein · Glucuronide transporter GusB · Two-dimensional crystallization · Stability · Size-exclusion HPLC

N. Ishii
Biological Information Research Center, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba Central-6, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8566, Japan

N. Ishii
Skirball Institute of Biomolecular Medicine, Department of Cell Biology, New York University School of Medicine, 540 First Avenue, New York, NY 10016, USA

N. Ishii
Life Science Division, Donner Laboratory, Lawrence Berkeley National Laboratory, Molecular and Cell Biology Department, University of California, Berkeley, CA 94720, USA

N. Ishii (✉)
Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba Central-6, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8566, Japan
e-mail: ishii@ni.aist.go.jp

Introduction

Membrane transport is a process that is vital for both the capture of nutrients and the excretion of waste products, toxins and antibiotics. Nearly 15% of genes in bacteria are predicted to encode membrane proteins related to transport (Ward et al. 2000). The energy required for these processes is derived from the electrochemical gradients of ions across the membrane and/or from the hydrolysis of ATP. Drug metabolism is classified into phase I and phase II reactions (Zamek-Gliszczynski et al. 2006). Phase I metabolism usually does not result in a large change in molecular weight or water solubility of substrate but is of great importance because oxidative reactions add or expose sites where phase II metabolism can subsequently occur. In contrast, phase II conjugation typically results in an appreciable increase in molecular weight and water solubility. The classes of metabolic enzymes involved in this field, such as the cytochrome P-450 family, are responsible for the biotransformation of the majority of drugs. That is why understanding their modulation involving important therapeutic and toxic implications is required. Glucuronidation is the major conjugation process in mammals and other vertebrates. It is quantitatively the most important phase II biotransformation reaction in the liver. Endogenous metabolic wastes, vitamins, steroid hormones,

animal- and plant-derived secondary metabolites, xenobiotics and pharmaceuticals are often conjugated with glucuronic acid. The reaction is catalyzed by many isomers of glucuronyltransferase in the lumen of the endoplasmic reticulum, together with UDP-glucuronic acid (Burchell et al. 1995). Those glucuronides once conjugated are transported out of the mammalian cells by ATP-dependent transport processes. The conjugated compounds are typically much more water-soluble than the respective aglycones and are often biochemically and biologically inactive. The glucuronidation and excretion of such compounds have thus often been described as "detoxification."

The enterobacterium *Escherichia coli* colonizes all known vertebrates. Most *E. coli* strains living in natural environments possess β-D-glucuronidase (EC 3.2.1.31) and are able to acquire glucuronides as nutrients. GusB, the product of the *gusB* gene, is predicted to possess 12 transmembrane α-helices and is a member of the major facilitator superfamily (MFS), the largest secondary transmembrane family known in the genomes sequenced to date (Saier et al. 1999; Henderson 1993). Although genetic investigation on *gusBC* of *E. coli* has been reported (Liang et al. 2005), the molecular mechanism for substrate transport by GusB is unclear due to the lack of structural information. As for three-dimensional detailed structure, only a few MFS members have been determined. This is due to difficulties in obtaining a high yield of such multispan integral membrane proteins, purifying them to homogeneity and maintaining their stability once removed from the natural lipid environment (Grisshammer and Tate 1995). Larger quantities of pure detergent-solubilized proteins are required for attempting structural analysis using X-ray crystallography, NMR spectroscopy and electron cryomicroscopy, as well as biochemical and biophysical characterization. The stability information, including which kind of coexisting lipid enhances the stability, would provide an ideal strategy for obtaining stable two-dimensional crystals of the membrane protein, which would ultimately lead to the final goal of three-dimensional structural reconstitution of the transporter by electron crystallography in combination with cryogenic methods.

In the three-dimensional structural determination of proteins, thousands of structures for soluble proteins have been reported. However, only several kinds of membrane protein structures have been determined at atomic resolution. This paucity will continue for a while since the elucidation of membrane protein structure using physical techniques is an extremely challenging task. Many membrane proteins are expressed in a live cell at low level, frequently corresponding to <0.1% of total proteins in a cell. Therefore, construction of an amplification expression system for the gene of interest is a necessary and practical approach to obtain sufficient protein for structural studies. Usually, high hydrophobicity makes it difficult to determine the structure for the majority of membrane proteins since they have to be removed from the membrane, then treated so as to monodisperse and studied in the presence of detergents. We assessed the stability of GusB over a wide pH range and in several remarkable lipids commonly used, assuming two-dimensional crystallization of the membrane protein for later electron crystallography using TEM with cryo-methods. This information may be used for structural biochemical and biophysical studies of the transporter protein, including two- and three-dimensional crystallization trials.

Materials and Methods

Construction of the Expression Plasmid pGEX-4T2-GUSB and Purification of GusB

The DNA encoding the *gusB* gene from the genomic DNA of wild-isolate *E. coli* strain K-12 was amplified by PCR using the oligo-nucleotides 5′-CGCGGATCCAATCAACAACTCTCCTGGCGCACC-3′ for forward and 5′-GCGCGCGACGCGGCCGCATTAGTGATATCGCTGATTAATTGCTGCTG-3′ for reverse. The PCR products were digested by *Bam*HI and *Not*I and ligated into pGEX-4T2 comprising tac promoter, glutathione *S*-transferase tag, thrombin cleavage motif and a gene coding for ampicillin resistance, following standard molecular biology protocols (Sambrook et al. 1989). In cloning for plasmid construction, *E. coli* DH5α treated with calcium chloride was used as a competent cell for transformation. The constructed plasmid was confirmed by restriction enzyme digestion and DNA sequencing and finally named "pGEX-4T2-GUSB." In DNA sequencing, the following oligonucleotides were used: 5′-GGGCTGGCAAGCCACGTTTGGTG-3′, 5′-TTCCTTATGGTTCACTTGC-3′, 5′-TGGTGCTTTACTTCATCTGC-3′ and 5′-ATCGCTTCAATTGGTCAGG-3′ for forward and 5′-GGAGTTCTTAATGCTCGG-3′, 5′-TGCCTGACCGCATTTACG-3′ and 5′-CCTCTGACACATGCAGCTCCCGG-3′ for reverse. The constructed vector was purified and then transfected into *E. coli* BL21(DE3)/pLysS. The single colony of *E. coli* BL21(DE3)/pLysS carrying the plasmid of interest, pGEX-4T2-GUSB, on an LB amp^{100} agar plate was inoculated to 4 ml of LB medium containing amp^{100} and cultured at 37°C with shaking at 180 rpm through the night. One milliliter of the culture medium containing the cells was put into 2 l of LB amp^{100} medium, with continued cultivation until the optical density at 600 nm reached 1.2. For overexpression of GusB fusion protein, cells were induced with 0.2 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) for 4–5 h. Then, the cells were harvested by centrifugation for 30 min at

3,500×*g*. After washing the cells with 40 ml of 50 mM potassium phosphate buffer (pH 7.5), they were gathered by centrifugation. About 5 g of wet pellets of the cells were available at this stage, and those were suspended in 20 ml of 20 mM potassium phosphate buffer (pH 7.5) containing 500 mM NaCl and 1 mM ethylenediaminetetraacetic acid (EDTA); then, the cells were disrupted through a French press chamber at 12,000 psi. The precipitant of membrane vesicles was gathered after ultracentrifugation for 1 h at 150,000×*g* and 4°C (HitachiKoki, Tokyo, Japan). The membrane portion was resuspended in 2.5 ml of 20 mM potassium phosphate buffer (pH 7.5) containing 500 mM NaCl and 1% Triton X-100. After incubation for 15 min on ice with occasional gentle shaking, the supernatant of Triton X-100 extract was gathered after centrifugation (at 150,000×*g*). The Triton X-100 extract was applied onto a GST affinity column (GSTrap HP; GE Healthcare UK, Buckinghamshire, UK), then eluted with the buffer containing 50 mM Tris–HCl and 10 mM glutathione (pH 7.9) according to the manufacturer's recommendation. The fractions containing GST–GusB fusion protein were gathered and incubated for digestion with thrombin at room temperature through the night using five units of thrombin (GE Healthcare). GusB protein was isolated from other proteinaceous components using an HPLC gel permeation column (Shodex protein KW804; Showa Denko, Kanagawa, Japan); meanwhile, the buffer was exchanged so as to contain 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesuffonic acid (HEPES)-NaOH, 100 mM NaCl, 20% glycerol, 3 mM NaN_3, 0.5 mM EDTA and 1 mM β-mercaptoethanol (pH 7.9); stored at 4°C; and used for stability study within 2 weeks.

Prediction of Membrane-Spanning Segments

The protein sequence was submitted to a transmembrane protein topology prediction algorithm, SOSUI, using default input parameters. SOSUI evaluates amino acid hydrophobicity and amphiphilicity for its predictions and complements the hidden Markov models as it is not dependent on training sets. The program predicts the topology of transmembrane proteins spanning the membrane with α-helices (Hirokawa et al. 1998; Mitaku et al. 2002).

Determination of Stokes Radius of GusB by Size-Exclusion Chromatography

The oligomeric state of GusB in DDM detergent was determined by analytical size-exclusion chromatography. Purified GusB was loaded onto a Shodex protein KW804 size-exclusion column using HPLC system CCPS connected with UV detector UV8020 (Tosoh, Tokyo, Japan). The protein solutions were applied without prefiltration treatment in order to monitor all events at wavelength of 280 nm, and a running buffer containing 10 mM HEPES–NaOH, 200 mM NaCl and 0.02% DDM (pH 7.9) was used. The Stokes radius of the GusB–DDM (detergent) complex was determined using six soluble proteins with known Stokes radii as standards: ferritin (63 Å), catalase (52 Å), aldorase (46 Å), bovine serum albumin (35 Å), ovalbumin (28 Å) and chymotrypsinogen (22 Å). Following a modified protocol of the reference (Harlan et al. 1995; Taylor et al. 1999), a 10-fold excess of the buffer was added to the protein solution in order to examine the stability of the protein at various pHs. After the desired incubation period at the indicated temperature, 25°C or 28.9°C, the sample was analyzed using a Shodex protein KW804 size-exclusion chromatography column on HPLC. The association state and monodispersity of GusB in other buffer salts at different pHs, with or without a certain lipid and after different incubation periods, were analyzed by size-exclusion HPLC after the protein concentration was adjusted to 0.9 mg/ml.

The desired buffer systems were employed as follows: *acetate buffer*, 20 mM acetate buffer containing 50 mM NaCl, 3 mM NaN_3, 0.5 mM EDTA and 20% glycerol; *citrate buffer*, 10 mM citrate buffer containing 50 mM NaCl, 3 mM NaN_3, 0.5 mM EDTA, 1 mM β-mercaptoethanol and 20% glycerol; *2-morpholinoethanesulfonic acid (MES) buffer*, 20 mM MES buffer containing 50 mM NaCl, 3 mM NaN_3, 0.5 mM EDTA, 1 mM β-mercaptoethanol and 20% glycerol; *HEPES buffer*, 20 mM HEPES buffer containing 50 mM NaCl, 3 mM NaN_3, 0.5 mM EDTA, 1 mM β-mercaptoethanol and 20% glycerol.

Electron Microscopy

Purified GusB was applied to specimen grids covered with a thin carbon support film which had been made hydrophilic by the ion bombardment device (JEOL HDT-400; JEOL Ltd., Akishima, Tokyo, Japan), then negatively stained with 1% uranyl acetate for 1 min. Images were taken on Kodak electron image films (Kodak SO-163; Eastman Kodak Company, Rochester, NY) under low-electron dose conditions at a magnification of 50,000× in the transmission electron microscope (Tecnai F20; FEI Company, Eindhoven, the Netherlands) operated at anode voltage of 120 kV, which were developed in full-strength Kodak developer D19 for 11 min at 20°C and fixed for 10 min in Kodak fixer.

Results and Discussion

The amplified expression system of GST–GusB fusion protein was constructed with *E. coli*. Figure 1a shows the

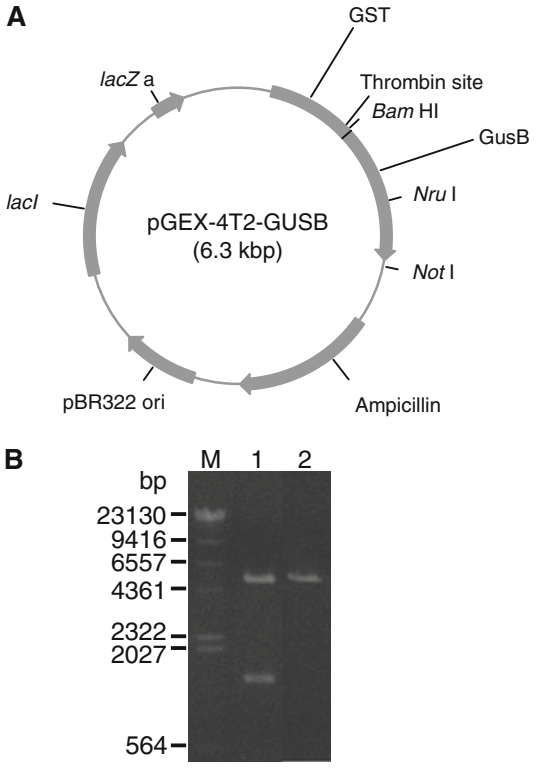

Fig. 1 **a** Schematic diagram of pGEX-4T2-GUSB. The *gusB* gene was inserted between the *Bam*HI and *Not*I sites. **b** Analysis of pGEX-4T2-GUSB by restriction enzyme. *Lane 1*, digested by *Nru*I and *Bam*HI, generating 5.2- and 0.8-kb fragments; *lane 2*, digested by *Nru*I and *Not*I, generating 5.5- and 0.5-kb (not shown on the gel) fragments; *lane M*, molecular weight marker. Agarose gel 1% was used

physical map of the plasmid with the relevant sites for restriction enzymes. In the plasmid pGEX-4T2-GUSB, one can predict a digestion site for a restriction enzyme, *Nru*I, at around the midpoint of the *gusB* gene and each site for *Bam*HI and *Not*I in pGEX-4T2. The construction of pGEX-4T2-GUSB was confirmed using the restriction enzymes in combination with *Nru*I and *Bam*HI and with *Nru*I and *Not*I. In the combination with *Nru*I and *Bam*HI, two fragments of 5,257 and 863 bp in size were theoretically estimated, and two fragments of 5,593 and 527 bp in size were expected for the case with *Nru*I and *Not*I. As shown in Fig. 1b, the cleaved bands of 5,257 and 863 bp, guessed from theoretical estimates, were clearly observed when the constructed plasmid was digested with *Nru*I and *Bam*HI; however, only one band of 5,593 bp could be seen, and the other smaller counterpart appeared to have electrophoretically eluted out from the front end for the digestion with *Nru*I and *Not*I. Although the predicted smaller band for the case of digestion with *Nru*I and *Not*I was eluted out, the size of the larger–molecular weight band was in good agreement with the predicted size. From these results, we concluded the plasmid pGEX-4T2-GUSB was constructed preferably.

When the protein of the final yield was electroeluted from the gel onto a polyvinylidene difluoride membrane, its N-terminal sequence was found to be WRTIVGYSLGD-VAN, corresponding to the amino acid sequence of GusB protein predicted from the DNA sequence of the *gusB* gene. Based on the DNA sequence analyzed in this study, the *gusB* gene from *E. coli* is predicted to encode 457 amino acid residues with a theoretical size of 49,693 daltons. By careful examination of the sequence in comparison with the reported one, we found replacements of amino acids at the N-terminal region, GSNPTL instead of MNQQLS. In addition, three amino acid residues were found to be replaced: Ile-235 was replaced with Ser, Val-324 with Ala and Val-335 with Ala. It is not clear at this moment whether those are mutations due to original diversity or artifacts in PCR. Although there were unexpected mutations in the protein obtained from this amplified expression system, we continued further stability studies using this product as GusB. Optimization of the purification procedure allowed us to produce homogeneous GusB protein in milligram quantities. Optimization applied to the cell culture and purification processes led to a yield of $\sim$1.8 mg of homogeneous GusB per liter of *E. coli* culture.

Thus, characterization of the oligomeric state and the stability of purified GusB were examined. The oligomeric state and stability of detergent-solubilized GusB were characterized by analytical size-exclusion HPLC (Harlan et al. 1995; Taylor et al. 1999; Boulter and Wang 2001). Judged from its retention time on the size-exclusion column, GusB appeared pentameric in DDM detergent solution (Fig. 2). The Stokes radius of the GusB–DDM complex was 57 $\pm$ 2 Å using soluble proteins as standards. The inset shows an electron microscopic image of negatively stained GusB protein. A typical appearance in dispersed membrane proteins is clearly recognizable (Sato et al. 1994). They often tend to make aggregates and stacks. The characterization of the oligomeric state and stability of purified GusB are used to identify pH with buffer salt system and evaluate lipids and detergents capable of maintaining the membrane protein in a monodisperse state for later two-dimensional crystallization experiments.

At the beginning, the candidate membrane proteins were selected as those predicted to have transmembrane helices by the application SOSUI. In this investigation of hydrophobicity we used the reported sequence of GusB. Figure 3a shows the hydropathic profile. From the hydropathic profile of the predicted amino acid sequence, GusB is predicted to comprise 12 membrane-spanning α-helices. Figure 3b shows a cartoon of the occurrence of structures

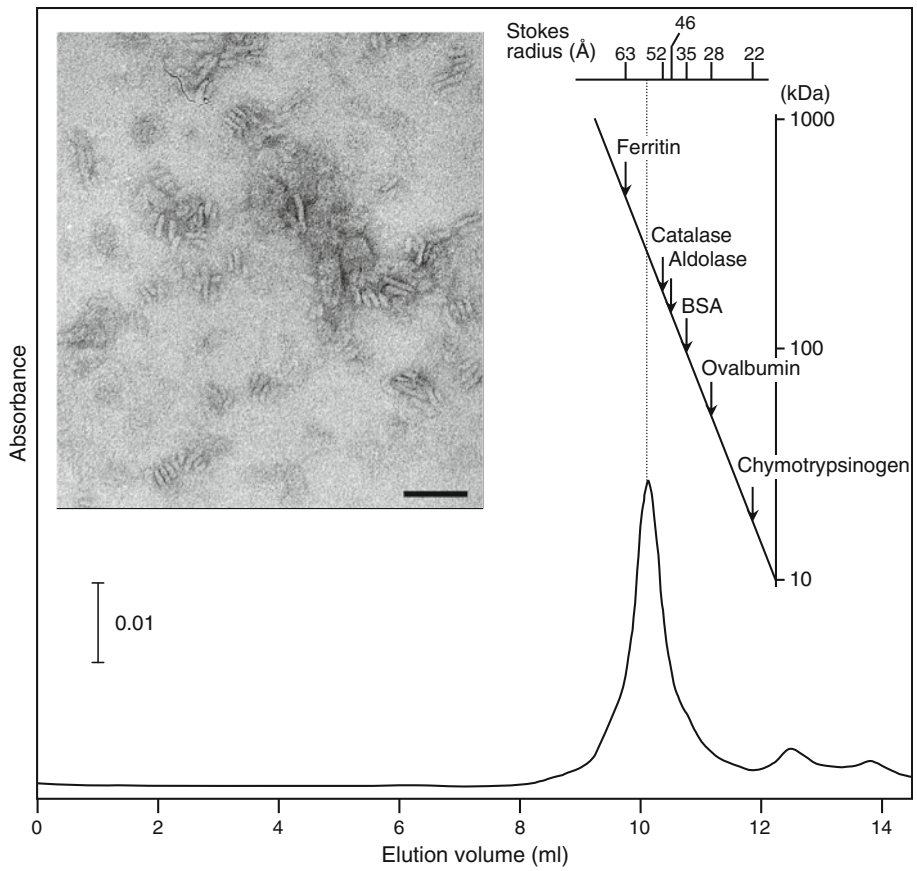

Fig. 2 Size-exclusion HPLC elution profile of GusB with molecular size marker standards. The GusB–DDM complex was found to have a Stokes radius of 57 Å. Six soluble proteins of known Stokes radius were used for calibration: ferritin (63 Å), catalase (52 Å), aldorase (46 Å), bovine serum albumin (35 Å), ovalbumin (28 Å) and chymotrypsinogen (22 Å). Inset is a negatively stained transmission electron microscopic image of the peak fraction. Scale bar, 500 nm. Based on the elution profile and TEM image, GusB in the presence of DDM most probably stays as a heptameric oligomer

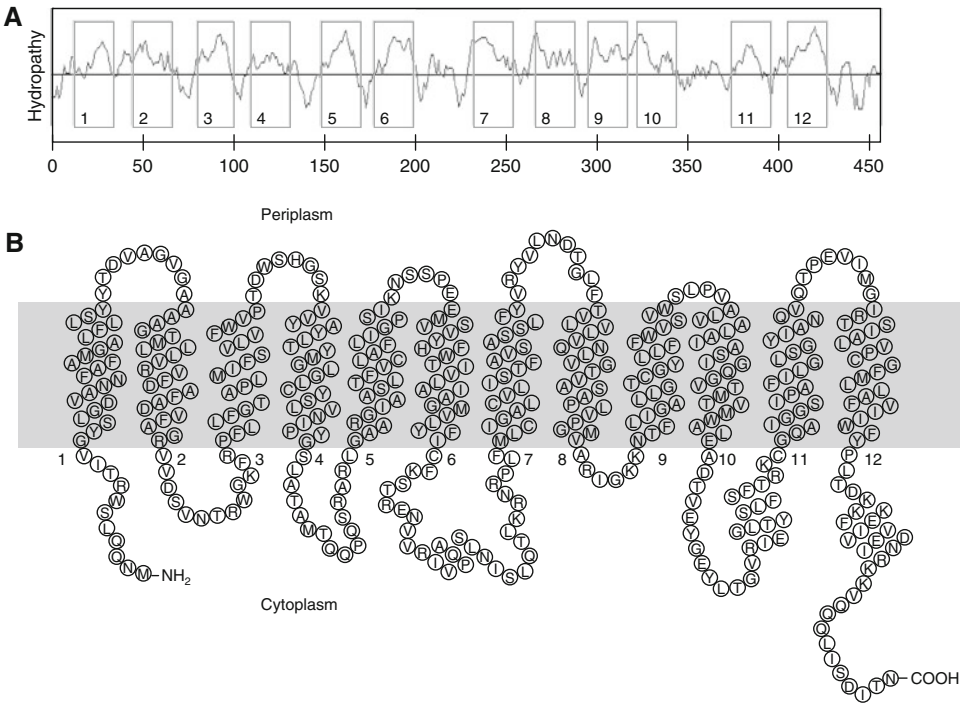

Fig. 3 **a** Hydrophobicity profile of GusB with predicted membrane-spanning domains *boxed*. **b** Schematic drawing of the predicted second structure of GusB

with GusB topology, i.e., the number of transmembrane α-helices and the localization of the N terminus, based on predictions with SOSUI. This clearly shows that GusB consists of 12 transmembrane α-helices. The predicted membrane-spanning domains are shown as a schematic drawing with amino acid sequences. The GusB protein has

457 amino acids, and its topology is predicted to consist of 12 transmembrane α-helices with both N and C termini in the cytosol.

The effects of pH and buffer salt species on the stability and oligomeric state of the protein were also monitored by size-exclusion HPLC. As seen in Fig. 4, judging from the shape, height and integration of the peak, GusB in citric buffer at pH 5.5 appeared to keep its initial state up to 2 days of incubation at 25°C. The integration areas of the peak after 1-day and 2-day incubation at 25°C were 80–85% of the control. That of the peak after 5-day incubation reduced to 60% of the control. Whereas the protein in acetate buffer at pH 4.5 and MES buffer at pH 6.5 showed a small shoulder in the lower–molecular weight region after 1-day incubation at 25°C, the peak area corresponded to 80% and 70% of control, respectively. Those reduced to 50% in acetate buffer and 25% in MES buffer after 5-day incubation at 25°C compared to each control.

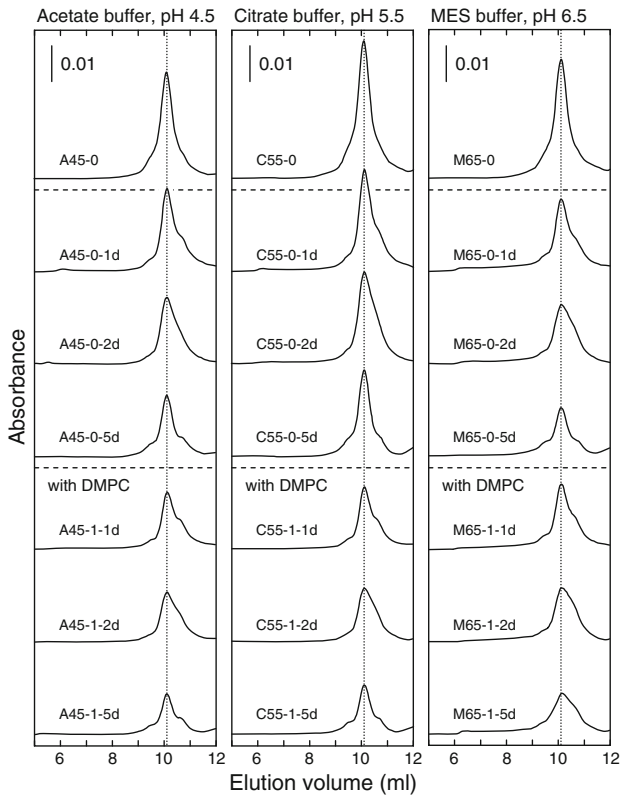

Fig. 4 Oligomeric state and monodispersity of GusB under different buffers with pH conditions. Uppermost elution profiles (A45-0, C55-0 and M65-0) show freshly prepared protein at 4°C; the remainder are profiles after desired incubation time at 25°C. A45-0-1d, C55-0-1d and M65-0-1d, after incubation for 1 day; A45-0-2d, C55-0-2d and M65-0-2d, after incubation for 2 days; A45-0-5d, C55-0-5d and M65-0-5d, after incubation for 5 days; A45-1-1d, C55-1-1d and M65-1-1d, after incubation with DMPC for 1 day; A45-1-2d, C55-1-2d and M65-1-2d, after incubation with DMPC for 2 days; A45-1-5d, C55-1-5d and M65-1-5d, after incubation with DMPC for 5 days

When examined using GusB of the same preparation, after the corresponding incubation time, along with the similar condition in the presence of DMPC, a decrease in the height of the peak became dominant. The peaks appeared to become wider and the peak area reduced to about 50% of each control after 1-day incubation with DMPC. It seems that coexistence of DMPC during incubation at 25°C no longer helps to stabilize GusB to maintain the initial condition. Although DMPC often has been used for reconstitution of proteoliposomes and functional assay for membrane proteins, the reason why DMPC did not help to stabilize GusB might be due to the solid state-like behavior at 25°C because its transition temperature is 28.9°C.

We next investigated the effects of pH with different buffer systems after 1- and 2-h incubation at 28.9°C (Fig. 5), followed by similar experiments in the presence of DMPC (Fig. 6). As seen in Fig. 5, although the appearances of the peaks in citrate buffer were almost similar to each other even after 2-h incubation at 28.9°C, small aggregations were recognized in void region, as was the case with the buffer at pH 5.0 and 5.5, after 1- and 2-h incubation. When examined with MES buffer, a small shoulder peak at around 32 kDa emerged, and this property appeared enhanced after 2-h incubation, which might be a dissociated GusB monomer if we assume that the dissociated component resides in a compact form. The dissociation in the smaller–molecular weight region was remarkable with HEPES buffer. Judging from the appearance of elution profiles, it seems that GusB is allowed to stabilize in the buffer incubated for 1 h at pH 7.0 and 7.2. It seems that GusB cannot stay stable under higher basic pH, beyond pH 7.4. The decrease in peak height indicates that the environment is unfavorable for GusB to maintain its initial conformation (assembly). As shown in Fig. 6, when DMPC coexisted with GusB during incubation, acetate buffer at pH 4.5–6.0 appeared to contribute to the monodispersion of GusB even after 2-h incubation at 28.9°C and citrate buffer at pH 5.0–6.5 also moderately helped the protein to maintain its initial state even after 2-h incubation at 28.9°C. Detailed examination of the elution profiles with citrate buffer revealed a small shoulder peak corresponding to the conspicuous presence of GusB monomer after 1-h incubation. The tendency is seen in common at pH 5.0–6.5. In MES buffer, although the peak height is relatively small compared to the other buffer systems, the small shoulder peak that presumably corresponds to the GusB monomer appears remarkable. The feature is enhanced as the incubation gets longer and is seen both with and without DMPC. When examined in HEPES buffer system, GusB appeared to be stable up to pH 7.2, while it appeared

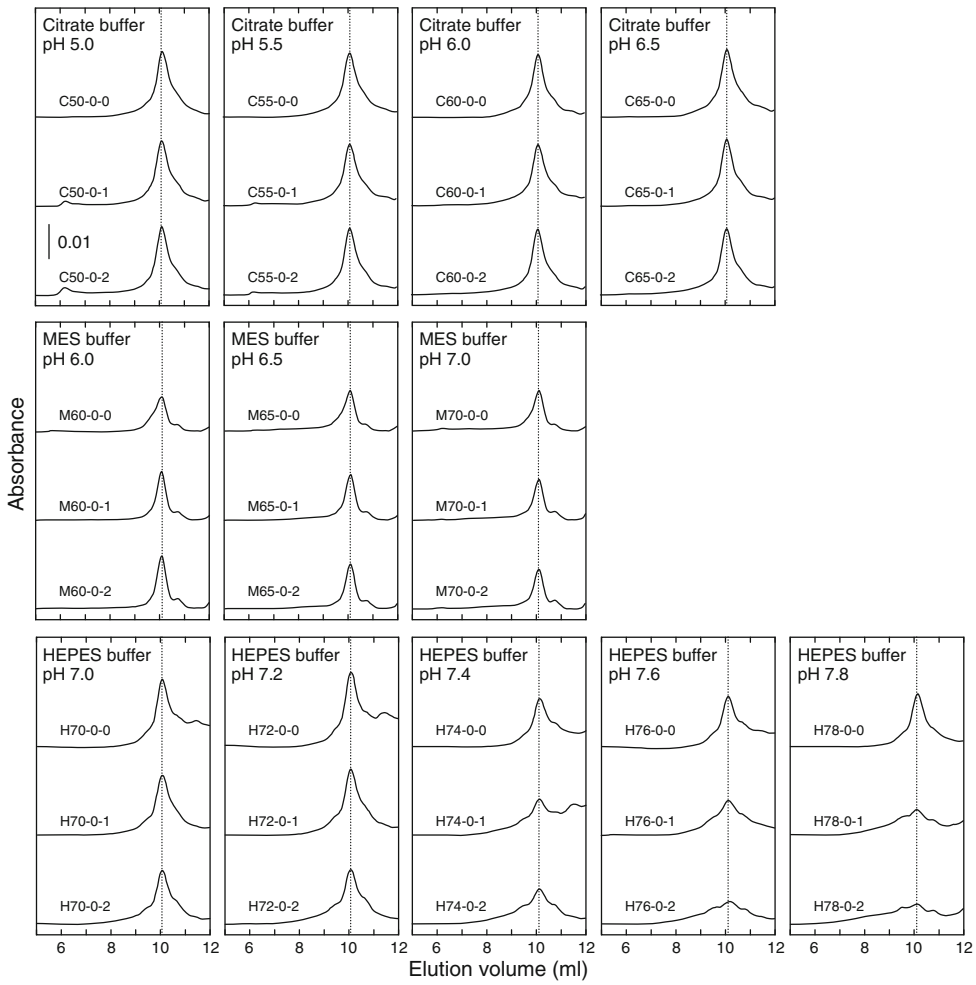

Fig. 5 Oligomeric state and monodispersity of GusB under different buffers and pH conditions. Incubation was performed at 28.9°C for 1 h (*middle profile* in each panel), for 2 h (*lowest profile* in each panel) and control at 4°C (*uppermost profile* in each panel)

unstable and dissociation might have been promoted at higher pH beyond 7.2. Interestingly, it seems to have recovered the appearance of the initial peak just after 1-h incubation at 28.9°C in the presence of DMPC.

Since GusB is originated from the *E. coli* membrane, how effective *E. coli* phospholipids were in the stability of GusB was examined. As shown in Fig. 7, the citrate buffer system was employed. The elution profiles of GusB after 2-h incubation at 25°C in the presence of total *E. coli* phospholipids at three different pH values (5.0, 5.5 and 6.0) showed almost similar shape, as shown in Fig. 7a. Aggregates at the void region could be seen along with a remarkable shoulder peak on the slope at the relatively high–molecular weight region of the peak of pentameric GusB. After 1-day incubation at 25°C, although a relatively small shoulder peak emerged in the smaller–molecular weight region of the GusB sample incubated with total *E. coli* phospholipids, the peak that corresponded to the pentameric GusB appeared to have maintained almost similar height (Fig. 7b). The area of deconvolusion of the peak corresponding to the pentameric GusB in the presence of *E. coli* phospholipids changed to almost 94% after 1-day, 83% after 5-day, 82% after 9-day, 79% after 16-day and 80% after 30-day incubation at 25°C compared to control, while those in the absence of any phospholipids changed to 84% after 1-day, 65% after 5-day, 48% after 9-day, 38% after 16-day and 27% after 30-day incubation at 25°C compared to control.

The zwitterionic phospholipids, mainly phosphatidylcholine (PC) and/or phosphatidylethanolamine (PE), together comprise the majority of the membrane phospholipids of eukaryotic cells, gram-negative and gram-positive bacteria. Due to the more favorable physical properties of PC in forming vesicles and the defined structure in solution, PC has been preferentially used in many in vitro studies. However, there are significant differences in chemistry and properties between these two lipids. PE has a smaller head group and thus can form hydrogen bonds through its ionizable amine. The head group composition of the phospholipids of *E. coli* is relatively invariant under a broad

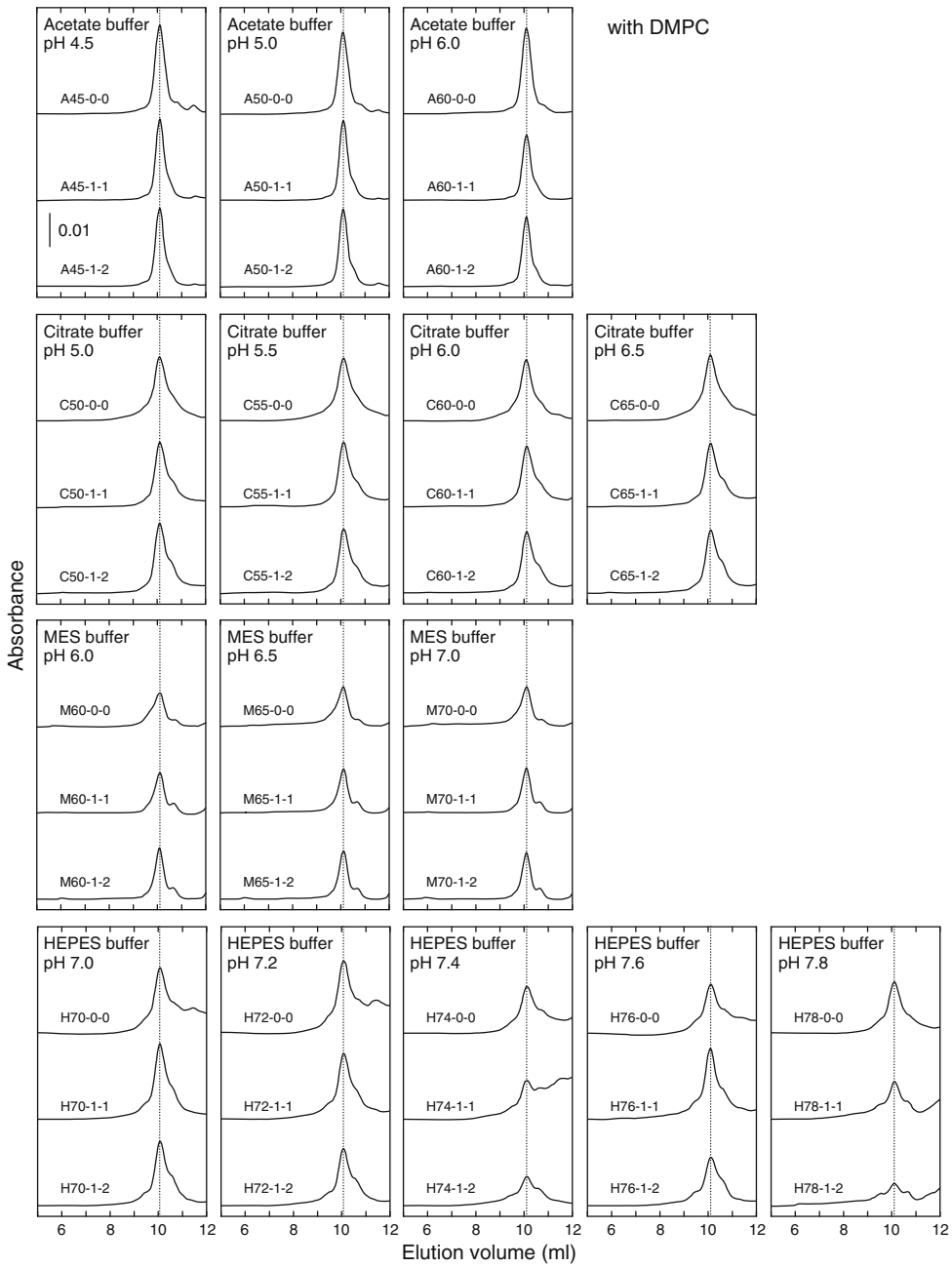

Fig. 6 Oligomeric state and monodispersity of GusB under different buffers and pH conditions. Incubation was performed at 28.9°C in the presence of DMPC for 1 h (*middle profile* in each panel), for 2 h (*lowest profile* in each panel) and control (without DMPC) at 4°C (*uppermost profile* in each panel)

spectrum of growth conditions, i.e., PE at 70–80%, phosphatidylglycerol (PG) at 20–25% and cardiolipin (CL) at 5% or less. Chen and coworkers (Chen and Wilson 1984; Seto-Young et al. 1985) have reported that PE has a specific involvement in supporting active transport by the lactose permease of *E. coli* in reconstituted proteoliposomes. Interestingly, PC could not substitute for PE in supporting active transport. Because the membrane phospholipid matrix replaces the cytoplasm as the solvent for integral membrane protein, it should not be surprising that phospholipids play a large role in the folding assembly and stabilizing of membrane proteins. It is needless to mention that ancillary proteins such as chaperones are required during the assembly of membrane proteins, to prevent the initiation of dead-end folding pathways prior to membrane insertion.

It is known that the fatty acid composition of PE and PG in *E. coli* membranes changes according to the growth temperature. It is also generally assumed that membrane lipids may undergo thermotropic phase transitions and/or lateral phase separation (Nishihara et al. 1976). Therefore, treatment of GusB purified in DDM solution with

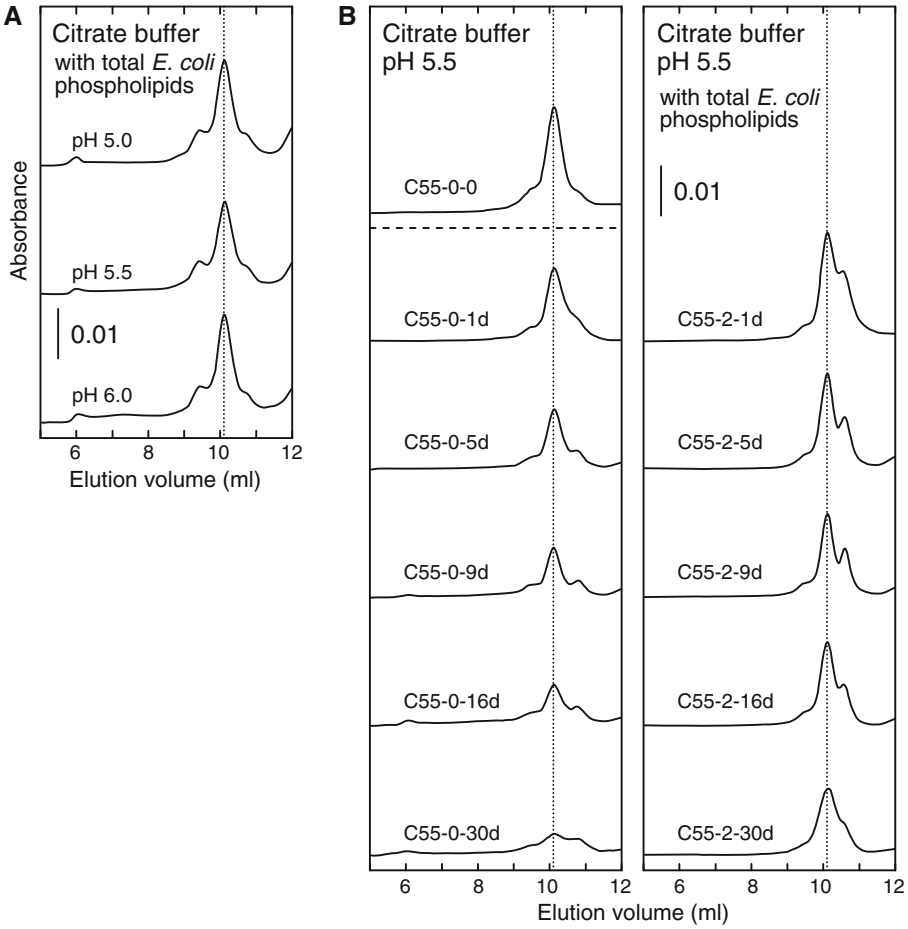

Fig. 7 **a** Stability study of GusB with different pH conditions in the presence of *E. coli* total lipid extract at 25°C. **b** Comparison of stability study of GusB with and without *E. coli* total lipid extract and different incubation time at 25°C. Incubation times were 1 day (C55-0-1d, C55-2-1d), 5 days (C55-0-5d, C55-2-5d), 9 days (C55-0-9d, C55-2-9d), 16 days (C55-0-16d, C55-2-16d) and 30 days (C55-0-30d, C55-2-30d). C55-0-0 is control at 4°C. The area of deconvolusion of the peak corresponding to the pentameric GusB in the presence of *E. coli* phospholipids changed to almost 94% after 1-day, 83% after 5-day, 82% after 9-day, 79% after 16-day and 80% after 30-day incubation compared to control, while those in the absence of any phospholipids changed to 84% after 1-day, 65% after 5-day, 48% after 9-day, 38% after 16-day and 27% after 30-day incubation compared to control

additional DMPC or total *E. coli* phospholipids (70–80% of which is PE) and by changing the incubation temperature from that in vivo may affect the lipid environment surrounding GusB such that the fluidity and the proper protein–lipid interaction become disturbed. Considering the viscotropic regulation by the lipid components in total *E. coli* phospholipids, the data of this report suggest that GusB is associated with a boundary or an annular lipid so as to stabilize itself, although it should be noted that the lipid close to GusB may easily exchange with adjacent lipid.

The formation of a pentameric assembly is rare in nature, but there are several examples, such as the acetylcholine receptor consisting of hetero subunits (Miyazawa et al. 2003), glycine (Langosch et al. 1988), GABAA and serotonin ($5HT_3$) receptors (Lester 1992), the MscL mechanosensitive ion channel (Chang et al. 1998; Maurer et al. 2000; Yoshimura et al. 2008) and the CorA transporter (Lunin et al. 2006). In addition to these common pentameric organizations reported for receptors and transporter proteins, clarification of the molecular structures as well as assembly on GusB together with membrane elements is expected.

Conclusion

We successfully constructed an expression system of GusB in *E. coli* and recovered much of the membrane protein. From the hydropathic profile of the amino acid sequence, GusB is predicted to comprise 12 membrane-spanning α-helices. Although DMPC destabilizes GusB at 25°C, which might be due to a deficiency of DMPC in softness and flexibility at this temperature, it appears to enhance the stability of GusB when the temperature is raised to around 28.9°C, at which DMPC acquires fluidity. On the other hand, nearly 80% of GusB protein is stabilized with total *E. coli* phospholipids (70–80% of which is PE) even after incubation for 30 days at 25°C. The two-dimensional crystallization of GusB in the presence of lipids is under study using the information on stability discussed here.

Acknowledgements The author is grateful to those who have encouraged him to promote the study, especially Dr. G. Sano for his assistance in the construction of the amplified expression system and Dr. Y. Sadakane for DNA sequence analysis and discussions. He thanks the individuals who have advised him at the laboratories on both sides of the Pacific Ocean and the East Coast. He expresses his thanks to Dr. K. Kim for critical reading of the manuscript. A part of

the research was financially supported by an Overseas Research Fellowship from the Japan Science and Technology Corporation.

References

Boulter J, Wang DN (2001) Purification and characterization of human erythrocyte glucose transporter in decylmaltoside detergent solution. Protein Expr Purif 22:337–348

Burchell B, Brierley CH, Rance D (1995) Specificity of human UDP-glucuronosyltransferases and xenobiotic glucuronidation. Life Sci 57:1819–1831

Chang G, Spencer RH, Lee AT et al (1998) Structure of the MscL homolog from *Mycobacterium tuberculosis*: a gated mechanosensitive ion channel. Science 282:2220–2226

Chen CC, Wilson TH (1984) The phospholipid requirement for activity of the lactose carrier of *Escherichia coli*. J Biol Chem 259:10150–10158

Grisshammer R, Tate CG (1995) Overexpression of integral membrane proteins for structural studies. Q Rev Biophys 28:315–422

Harlan JE, Picot D, Loll PJ et al (1995) Calibration of size-exclusion chromatography: use of a double Gaussian distribution function to describe pore sizes. Anal Biochem 224:557–563

Henderson PJF (1993) The 12-transmembrane helix transporters. Curr Opin Cell Biol 5:708–721

Hirokawa T, Boon-Chieng S, Mitaku S (1998) SOSUI: classification and secondary structure prediction system for membrane proteins. Bioinformatics 14:378–379

Langosch D, Thomas L, Betz H (1988) Conserved quaternary structure of ligand-gated ion channels: the postsynaptic glycine receptor is a pentamer. Proc Natl Acad Sci USA 85:7394–7398

Lester HA (1992) The permeation pathway of neurotransmitter-gated ion channels. Annu Rev Biophys Biomol Struct 21:267–292

Liang WJ, Wilson KJ, Xie H et al (2005) The *gusBC* genes of *Escherichia coli* encode a glucuronide transport system. J Bacteriol 187:2377–2385

Lunin VV, Dobrovetsky E, Khutoreskaya G et al (2006) Crystal structure of the CorA Mg^{2+} transporter. Nature 440:833–837

Maurer JA, Elmore DE, Lester HA et al (2000) Comparing and contrasting *Escherichia coli* and *Mycobacterium tuberculosis* mechanosensitive channels (MscL): new gain of functional mutations in the loop region. J Biol Chem 275:22238–22244

Mitaku S, Hirokawa T, Tsuji T (2002) Amphiphilicity index of polar amino acids as an aid in the characterization of amino acid preference at membrane–water interfaces. Bioinformatics 18:608–616

Miyazawa A, Fujiyoshi Y, Unwin N (2003) Structure and gating mechanism of the acetylcholine receptor pore. Nature 423:949–955

Nishihara M, Ishinaga M, Kato M et al (1976) Temperature-sensitive formation of the phospholipid molecular species in *Escherichia coli* membranes. Biochim Biophys Acta 431:54–61

Saier MH Jr, Beatty JT, Goffeau A et al (1999) The major facilitator superfamily. J Mol Microbiol Biotechnol 1:257–279

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning, a laboratory manual, vol 1, 2nd edn. CSHL Press, New York

Sato MH, Kasahara M, Ishii N et al (1994) Purified vacuolar inorganic pyrophosphatase consisting of a 75-kDa polypeptide can pump H^+ into reconstituted proteoliposomes. J Biol Chem 269:6725–6728

Seto-Young D, Chen CC, Wilson TH (1985) Effect of different phospholipids on the reconstitution of two functions of lactose carrier of *Escherichia coli*. J Membr Biol 84:256–267

Taylor AM, Boulter J, Harding SE et al (1999) Hydrodynamic properties of human erythrocyte band 3 solubilized in reduced Triton X-100. Biophys J 76:2043–2055

Ward A, Sanderson NM, O'Reilly J (2000) The amplified expression, identification, purification, assay, and properties of hexahistidine-tagged bacterial membrane transport proteins. In: Baldwin SA et al (eds) Membrane transport. Oxford University Press, Oxford, pp 141–166

Yoshimura K, Usukura J, Sokabe M (2008) Gating-associated conformational changes in the mechanosensitive channel MscL. Proc Natl Acad Sci USA 105:4033–4038

Zamek-Gliszczynski MJ, Hoffmaster KA, Nezasa K et al (2006) Integration of hepatic drug transporters and phase II metabolizing enzymes: mechanisms of hepatic excretion of sulfate, glucuronide, and glutathione metabolites. Eur J Pharm Sci 27:447–486