Transmembrane Topology of $\alpha$- and $\beta$-Subunits of Na$^+\cdot$K$^+$-ATPase Derived from $\beta$-Galactosidase Fusion Proteins Expressed in Yeast*

Bernd Fiedler‡ and Georgios Scheiner-Bobis§

From the Institut für Biochemie und Endokrinologie, Fachbereich Veterinärmedizin, Justus-Liebig-Universität Giessen, Frankfurter Strasse 100, D-35392 Giessen, Federal Republic of Germany

Various models of the transmembrane topology of the Na$^+$,K$^+$-ATPase predict either 8 or 10 membrane spans for the $\alpha$-subunit and one to three membrane spans for the $\beta$-subunit. Structure/function analysis, however, requires precise knowledge about the folding of enzymes. Therefore, the intention of this work was to establish a transmembrane topology model for the subunits of Na$^+$,K$^+$-ATPase. The bacterial enzyme $\beta$-galactosidase was fused to the C termini of truncated $\alpha$- and $\beta$-subunits of Na$^+$,K$^+$-ATPase. Fusions were generated at Arg$^{60}$ (LTTA^60), Glu$^{116}$ (AATGE^116), Ala$^{247}$ (VEGETA^247), Leu$^{311}$ (YTWEL^311), Ala$^{444}$ (VAGDA^444), Ala$^{789}$ (IFIIA^789), Met$^{809}$ (LGTD^809), Asp$^{884}$ (RVTWD^884), Ile$^{946}$ (MKNIK^946), and Arg$^{972}$ (GVALR^972) of the sheep $\alpha$-subunit and at Pro$^{236}$ (LGGYP^236) of the dog $\beta$-subunit. The fusion constructs were expressed in yeast cells for studies on the localization of the fused reporter enzyme. Activity measurements of the reporter enzyme revealed that only intracellular fusion sites lead to active $\beta$-galactosidase. Indirect immunofluorescence microscopy with cells expressing $\alpha$/$\beta$-galactosidase and $\beta$/$\beta$-galactosidase hybrid proteins demonstrated that inactive $\beta$-galactosidase is associated with the yeast plasma membrane and can be detected from the extracellular side. The data obtained suggest that Pro$^{236}$ of the $\beta$-subunit is located on the extracellular surface, corresponding to a model with one transmembrane segment, and that the $\alpha$-subunit of the Na$^+$,K$^+$-ATPase consists of 10 membrane-associated spans. They also suggest that a stretch of the $\alpha$-subunit between membrane spans M7 and M8 might be hidden within the membrane, surrounded by the other hydrophobic spans, in analogy to the P-loop of Na$^+$ or K$^+$ channels and to the “hourglass” structure of water channels.

Na$^+$,K$^+$-ATPase (sodium pump; EC 3.6.1.37) is an enzyme embedded in plasma membranes of animal cells. The enzyme consists of two subunits, $\alpha$ and $\beta$. Various isoforms of both subunits have been described (1–5). Both subunits are required for catalytic activity (6, 7), which consists of the coupling of ATP hydrolysis to the active transport of Na$^+$ and K$^+$ ions against their electrochemical gradients across plasma membranes.

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§ To whom correspondence should be addressed. Tel.: 49-641-702-4841; Fax: 49-641-702-7405.

The structure/function relationships of the sodium pump have been investigated in great detail with biochemical methods and molecular biological techniques. Nevertheless, it is sometimes difficult to define whether the specific amino acids of interest reside within hydrophobic or hydrophilic areas of the protein. Since the exact number of hydrophobic spans of the $\alpha$-subunit is still unknown, it is sometimes controversial whether modified amino acids from hydrophilic areas are localized on the extra- or intracellular sides. A precise investigation of structure/function relationships, however, can be carried out successfully only when the spatial organization of the protein is known. Analysis of the transmembrane topology of $\alpha$- and $\beta$-subunits of the sodium pump by hydrophathy plots (8) does not always produce clear results. While a single transmembrane span can be calculated for the $\beta$-subunit, the folding of the $\alpha$-subunit in the membrane is difficult to predict. Based on current knowledge, the amino-terminal third of the $\alpha$-subunit transverses the membrane four times (9). This is followed by a large hydrophilic third of the protein that has been identified as the ATP-binding domain of the sodium pump.

For the last third of the $\alpha$-subunit, which is localized between the ATP-binding site and the C terminus, hydrophathy analysis has produced conflicting results. The number of the transmembrane spans for this part of the $\alpha$-subunit varies from three to six in different models (10–16). Since the C terminus was shown to be localized on the cytoplasmic side (10, 17), either four or six membrane spans are most likely to be formed by the carboxyl-terminal third of the $\alpha$-subunit. The total number of the hydrophobic spans of the $\alpha$-subunit should therefore be either 8 or 10.

Other methods can help to either correct or supplement the data derived from hydrophathy analysis. In several studies, the detection of single amino acids on either the extracellular or intracellular surface of the plasma membrane was used together with the hydrophathy data as a basis for the analysis of the transmembrane topology. Asn$^{831}$ is the amino-terminal amino acid of a 19-kDa hydrophobic fragment derived from the $\alpha$-subunit that is involved in Rb$^+$-occlusion (18, 19). This fragment can be generated when Na$^+$,K$^+$-ATPase in inside-out vesicles is extensively digested by trypsin. Thus, it was concluded that under physiological conditions, this fragment resides on the cytoplasmic side of the plasma membrane. The detection of Asn$^{831}$ on the intracellular side of the plasma membrane initiated far reaching conclusions about the folding of the $\alpha$-subunit. As the final result, this investigation, a model with 10 transmembrane spans was preferred over one with 8, although the latter could not be excluded (12, 18).

The transmembrane topology of the $\alpha$-subunit has also been analyzed by the use of antibodies directed against either naturally occurring or artificially inserted epitopes (13, 15, 20, 21). Although this approach is most promising for the analysis of transmembrane topology, the results obtained can be contro-
Escherichia coli that is missing the start codon ATG was fused to the cytosolic or on the extracellular side of the yeast cell membrane. A presupposition for this assumption is that the localization of the reporter enzyme is decided by the fusion occurs in frame with the ATG codon of either the α- or β-subunit cDNAs. The hybrid DNA molecules were expressed in the yeast Saccharomyces cerevisiae. Yeast cells contain neither endogenous Na⁺K⁺-ATPase nor β-gal, but they can express both proteins. In all hybrid proteins, β-gal is localized C-terminally. Depending upon the location of the fusion site, it was expected that β-gal would be expressed either on the cytosolic or on the extracellular side of the yeast cell membranes. A presupposition for this assumption is that the expressed hydrophobic parts of the α- or β-subunit are correctly inserted and that the hydrophilic β-gal moiety protrudes out of the plasma membrane.

The results presented here confirm this assumption. Based on the localization of the reporter enzyme β-galactosidase in expressed fusion proteins, the data help to derive a topological model for the transmembrane organization of the Na⁺K⁺-ATPase α- and β-subunits. This model might affect our understanding of the functional mechanism of the sodium pump.

MATERIALS AND METHODS

Strains, Vectors, and Media—Strains and vectors are listed in Table I. For subcloning steps with the plasmid pBluescriptII SK⁺ (Stratagene, La Jolla, CA), the E. coli strain HB101 (Life Technologies, Inc.) was used. E. coliSURE2® cells (Pharmacia, Uppsala) were used as a host strain for transformations with the vector pMC1871 (Pharmacia) that contains the complete β-galactosidase gene. Bacteria were grown in LB broth. Antibiotics were supplemented at final concentrations of 50–100 μg/ml for ampicillin and 10–20 μg/ml for tetracycline. Colonies expressing β-galactosidase and β-galactosidase fusion proteins were identified on LB agar plates supplemented with 20–40 μg/ml concentrations of the chromogenic indicator X-gal. S. cerevisiae strain 20B12 (22) was transformed by the method of Ito et al. (23) with vectors based on pCGY1406β (6) containing the fusion constructs described in the next paragraph. The vector pCGY1406β codes for the sheep α-subunit and for the dog β-subunit (6). Yeast cells were grown in YNB, and fusion protein-expressing colonies were visualized on YNB agar plates supplemented with 40–60 μg/ml X-gal.

Construction of Fusion Expression Vectors—Restriction endonucleases and modifying enzymes were purchased from U. S. Biochemical Corp., New England Biolabs Inc. (Beverly, MA), Stratagene, and MBI (Vilnuius, Lithuania). Oligonucleotides, if not otherwise specified, were obtained from Roth (Karlsruhe, Germany). Cloning procedures were performed according to standard protocols (24). The lacZ gene encoding β-galactosidase was fused in frame with the α-gene by various cloning steps.

The fusion expression plasmid pCAL60LZ was constructed by cloning the SsuI fragment of the plasmid pmC1871 (Pharmacia) containing the entire lacZ gene into the XhoI site of the shuttle vector pCGY1406β. Fusion plasmids AL444LZ and AL751LZ were prepared by the following subcloning procedure. The plasmid pBD was used for the insertion of a 10-mer linker containing a BglII site (Stratagene) into the EcoRV site of the plasmid pBluescript II SK⁺ and the subsequent deletion of the BamHI-BgII fragment. This step was required in order to abolish the EcoRI site and to maintain the reading frame for lacZ'p of pBluescript II SK⁺ coding for the α-polypeptide complementing M15 mutants of E. coli cells. The XhoI-ApI fragment of pCGY1406β was isolated by digestion of the plasmid pBD and was designated pBDA. The BstI lacZ cartridge of pMC1871 was ligated to NsiI-cut pBD. The new XhoI-ApI fragment was ligated back into pCGY1406β, resulting in pCAL444LZ. The fusion vector pCAL751LZ was constructed by cloning EcoRI cartridges containing the incomplete lacZ gene into the MunI site of pBD and ligating into the original MunI site of the XhoI-ApI fragment. This construct is missing the 3'-terminal part of the lacZ gene and codes for inactive β-galactosidase. It was used as an internal control.

Fusion AL946LZ was obtained by ligating BglII-PstI adapters to a mobilized BanHI cartridge of the lacZ gene and to BglII-cut pBDA. Fusion AL972LZ was prepared similarly with the use of XhoI-EcoS1I adapters and a lacZ SauI cartridge. Fusion AL247LZ was prepared as follows. A 13-mer linker containing an EcoNI site was inserted into the EcoRV site of pBluescript II SK⁺, resulting in pBE. XhoI- and EcoNI-cut pBE was ligated to the corresponding α1-fragment, resulting in plasmid pBEA. After the insertion of an 8-mer linker containing a BglII site into the Eco72I site within the α1-portion of pBEA, the modified plasmid was digested with BglII and was ligated to the lacZ BamHI cartridge of pMC1871. Mutations were necessary to insert the lacZ gene into sites not accessible by restriction enzymes. The first step consisted of the construction of mutation plasmids (see Table I) containing subcloned portions of the α1-gene. The SnuI-KpnI polynucleotide fragment of pBluescript II SK⁺ was replaced with a new polynucleotide containing sites for BglII, Eco72I, MunI, and XhoI. The new plasmid was designated pBP. XhoI-Eco72I and MunI-BglII fragments of the α1-cDNA were cloned into the new polynucleotide restriction sites. These new plasmids were designated pBPA1 and pBPA2, respectively. Mutagenesis was done by polymerase chain reaction (25) to create new BanHI sites within the SnuI fragment. In addition, pBPA1 was used to introduce a BglII site within the Eco72I-MunI portion of the α1-cDNA. After introduction of the lacZ cartridge into the new restriction sites, the modified α1-genes were re-inserted into pCGY1406β by subcloning steps. The fusion plasmid pCDL236LZ was constructed by deleting the α1-cDNA in pCGY1406β with KpnI, inserting a 10-mer linker containing an Xhol site (Promega, Madison, WI) into the Smal1 site of the resulting plasmid, and then ligating this to the SsoI fragment of the plasmid pCGY1406β. The complete plasmid was recovered by cloning in the XhoI site of the shuttle vector pCGY1406β with a linker. Constructs were analyzed by restriction digests. Correct polymerase chain reaction amplification was controlled by DNA sequencing (26) with the Sequenase™ kit (U. S. Biomedical Corp.).

Isolation of Yeast Membranes—Yeast cells were grown in YNB at 28°C to late log phase (A600nm = 1.0–1.5). The culture was centrifuged at 1500 × g at 4°C and washed twice with ice-cold deionized water. At this point, pellets can be stored frozen at −80°C. All following steps were carried out at 0–4°C. Cells were suspended in freshly prepared breakage buffer (0.2 M Tris-HCl, pH 7.5, 10 mM MgCl₂, 10% (v/v) glycerol, 2 mM dithiothreitol, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, and 0.01 mg/ml leupeptin) and were stirred with glass beads in a bead beater apparatus six times for 20 s with 1-min intervals on ice. Cell debris was pelleted by a 10-min centrifugation at 6000 × g. The supernatant was centrifuged for 30 min at 200,000 × g. The resulting membrane pellet was suspended in 1–2 ml of 25 mM imidazole and 1 mM Na₂EDTA, pH 7.4, and was stored at −20°C.

SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting—Yeast membranes were suspended in sample buffer (27) and heated at 70°C for 7 min before loading onto SDS gels (4% stacking gel, 6% separating gel) according to Laemmli (27). A total of 20 μg of protein was loaded on each lane. After electrophoresis, proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) at 100 mA for 1.5 h (28). Blots were probed with a mouse monoclonal antibody against the fusion protein of β-galactosidase of the pBluescript II SK⁺ plasmids (29313 and 29314) was loaded on each lane. After electrophoresis, proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) at 100 mA for 1.5 h (28). Blots were probed with a mouse monoclonal antibody against the fusion protein of β-galactosidase of the pBluescript II SK⁺ plasmids (29313 and 29314). The resulting membranes were incubated for 2 h at room temperature with the monoclonal antibody, washed with 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% Tween-20, and blocked with 5% nonfat dried milk. The blots were incubated with the peroxidase-conjugated secondary antibody and processed with the appropriate substrate to visualize the reaction.

1 The abbreviations used are: β-gal, β-galactosidase; X-gal, 5-bromo-4-chloro-3-indolyl β-galactopyranoside; ONPG, o-nitrophenyl β-D-galactopyranoside; C₆F₇DGC, 5-dodecanoylaminofluorescein di β-galactopyranoside; CPCQ, 2-(5-chloro-2-phosphoryloxyphenyl)-6-chloro-4'-Hquinazolinolone; NΑT, axidodeoxine 5'-triphosphate.

2 The abbreviations used are: β-gal, β-galactosidase; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; ONPG, o-nitrophenyl β-D-galactopyranoside; C₆F₇DGC, 5-dodecanoylaminofluorescein di β-galactopyranoside; CPCQ, 2-(5-chloro-2-phosphoryloxyphenyl)-6-chloro-4'-Hquinazolinolone; NΑT, axidodeoxine 5'-triphosphate.
mg of protein was diluted with 900 μl of Z-buffer (60 mM NaH₂PO₄, 40 mM Na₂HPO₄, 10 mM KCl, 1 mM MgSO₄, and 0.27% (v/v) 2-mercaptoethanol), and 60 μl of 0.1% SDS and 90 μl of chloroform were added. The mixture was vortexed vigorously for 20 s. After equilibration at room temperature for 5 min, ONPG was added to a final concentration of 0.6 mg/ml. The reaction was stopped at various times by adding 0.5 ml of 1 M Na₂CO₃. Specific activity was monitored by measuring the absorbance at 420 nm and comparing this with a standard curve established using β-galactosidase of known activity (Boehringer).

Fluorescence Microscopy—Substrate fluorescence imaging for the detection of intracellularly localized β-galactosidase was performed using the fluorogenic β-galactosidase substrate C₄-FDG (Molecular Probes, Inc., Eugene, OR) as described by Zhang et al. (31). Yeast cells in late logarithmic phase growth were suspended in Z-buffer supplemented with 0.3 mM chloroquine (Sigma) to reduce background fluorescence (31) and were incubated at room temperature for 30 min. After centrifugation at 6000 × g for 10 min, the cells were resuspended in Z-buffer containing 25 mM C₁₂FDG. Fluorescence was observed after incubation for 1–2 h by using a Leitz fluorescence microscope with an excitation filter in the blue range of 350–450 nm and an emission filter open above 520 nm.

Indirect immunofluorescence for the detection of extracellularly localized β-galactosidase was investigated by use of ELF™ (enzyme amplification fluorescence; Molecular Probes, Inc.). First, yeast cells were fixed by incubation for 1.5 h in 0.1 M phosphate buffer, pH 6.5 (with KOH), containing 4% paraformaldehyde. Cells were washed in 0.1 M phosphate buffer, pH 6.5; suspended in 1 M sorbitol and 1 mM EDTA, pH 8.0; and treated with 50 units of glucuronidase (Sigma) and 10 units of Zymolyase®-20T (Seikagaku Corp., Tokyo) for 1 h at 30°C to prepare spheroplasts. Cells were centrifuged at 5000 × g for 3 min and were washed twice with 1 ml of 1 M sorbitol and 1 mM EDTA. Afterwards, cells were suspended in 100 μl of the same buffer, and 20 μl of the spheroplast suspension was transferred to poly-L-lysine (Sigma)-coated slides and fixed in methanol for 5 min and acetone for 30 s at −20°C (32). After incubation for 30 min in blocking buffer (30 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1% bovine serum albumin), a polyclonal antibody (Sigma) against the β-galactosidase of the fusion proteins (1 μg/ml after dilution in wash buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, and 50% (v/v) ethanol) was added, and cells were incubated for an additional 12 h. Thereafter, slides were washed three times in wash buffer, and a biotinylated goat anti-mouse antibody (10 μg/ml after dilution; Molecular Probes, Inc.) was applied for another 2 h. Fluorescence was achieved by adding streptavidin, biotinylated alkaline phosphatase, and the substrate CPCQ of ELF. Between additions, washing steps were included. The enzyme reaction was stopped by 25 mM EDTA, 1 mM levamisol, and 0.01% Triton® X-100 in phosphate-buffered saline, and slides were visualized by microscopy as described above.

RESULTS

Construction of Vectors—Ten α₅lacZ fusions and one βlacZ fusion were generated by direct fusion of lacZ carboxidases to the 3′-termini of the α₅- and β-genes of the Na⁺,K⁺-ATPase. This cloning strategy included subcloning procedures like insertion of linkers and adapters and polymerase chain reaction mutation (Table I). All constructs were verified by restriction analysis and sequencing of the fusion sites. Fusion locations on the α₅-subunit were chosen on the basis of the theoretical models of Shull et al. (11) and Karlish et al. (12) predicting either 8 or 10 membrane spans, respectively (Fig. 1). When required, single restriction sites were introduced by mutations. Depending upon which of the two models is correct, fusion proteins of the carboxy-terminal third that follows the ATP-binding domain should be expressed either extra- or intracellularly (Fig. 1, A

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**TABLE I**

| Strains and vectors | Genotype or relevant properties | Supplier or Ref. |
|---------------------|--------------------------------|-----------------|
| **Strains** | | |
| E. coli HB101 | F− DmacR-rovr leu supE44 ara14 galK2 lacY1 proA2 repL20 (str+) xyl-5 mil-1 recA13 | Life Technologies, Inc. |
| S. cerevisiae 20B12 | trp1 pep4-3 Shuttle vector carrying sheep α, and dog β CUP1 PKG (amp′, TRP1) | 22 |
| pCM1871 | Vector carrying lacZ′ (laccamp′) lacZ fusion vector without promotor (tet′) | Stratagene |
| pCALLZ series | αlacZ fusions in pCG1406β | This study |
| pCABL236LZ | βlacZ fusion in pCG1406β | This study |

**FIG. 1.** Cloning sites for the construction of fusion proteins between Na⁺,K⁺-ATPase subunits and β-galactosidase. CDNA regions coding for possible transmembrane spans of the α₅-subunit (A and B) and the β-subunit (C) are shown by small numbered rectangles. A represents an eight-transmembrane span model (11). B shows a model with 10 transmembrane segments (12). Naturally occurring or polymerase chain reaction-generated (M) restriction sites for the fusion of the lacZ gene were chosen in a way that would allow one to distinguish between the two models. Thus, the extra- or intracellularly localized fusion sites of the carboxy-terminal third of the α₅-subunit are on opposite sides in A and B. The construction of a fusion protein between the Na⁺,K⁺-ATPase β-subunit and β-galactosidase (C) served as a positive control for extracellularly fused β-galactosidase. The lacZ gene from plasmid pMC1871 that was used for fusion constructs (D) differs from the gene found in wild-type E. coli in that the first eight codons are altered. It also lacks a start codon. Thus, β-galactosidase will be expressed only if the lacZ gene is fused in frame with the initiation codon of the α₅- or β-cDNA.
Underlined codons represent the gene of the sheep \(\alpha_1\)-subunit or the gene of the dog \(\beta\)-subunit. The DNA strand is extended by the lacZ gene of the plasmid pMC1871. Due to the lacZ cartridges and adaptors used, the junction codons differ from each other. Adaptor bases are in italics, and mutated bases are in boldface. Measurement of reporter enzyme activity using ONPG as substrate (last column) was carried out with crude yeast cell membranes from cells carrying pCGY1406\(\beta\) or each of the fusion vectors. The total amount of protein for the microsomal fractions was determined, and the activity of \(\beta\)-galactosidase was assayed with ONPG as described under “Materials and Methods.” The mean values ± S.E. of triplicate experiments are given.

TABLE II

| Fusion vector | Sequence junction (5' → 3') | Fusion site | Fusion protein | \(\beta\)-gal activity |
|---------------|-----------------------------|-------------|----------------|-----------------------|
| pCAL60LZ      | ACT GCC CGA CGG ATC CC GGA ATT | Arg^{60}    | a60/\(\beta\)-gal | 254 ± 17             |
| pCAL161LZ     | ACA GAA GAG GAT CCC GTC GGT TTA | Glu^{116}   | a116/\(\beta\)-gal | 0                     |
| pCAL247LZ     | GCC ACT CGA GCA GAT CCC GTC GGT GGT | Ala^{247}  | a247/\(\beta\)-gal | 60 ± 8                |
| pCAL311LZ     | ACC TGG GTA CCC GTC GGT TTA | Leu^{311}   | a311/\(\beta\)-gal | 0                     |
| pCAL444LZ     | GTG GAT GCA GGT CGA GGA GGT ATT | Ala^{444}   | a444/\(\beta\)-gal | 7 ± 2                  |
| pCAL751LZ     | GCC TCA ATT CCC GGT GAT CCC GTC | Ile^{751}   | a751/\(\beta\)-gal | 0                     |
| pCAL789LZ     | ATT ATT GCA AAA GAT CCC GTC GGT | Ala^{789}   | a789/\(\beta\)-gal | 38 ± 4                |
| pCAL809LZ     | ACC GAC ATG GAT CCC GTC GGT TTG | Met^{809}   | a809/\(\beta\)-gal | 0                     |
| pCAL841LZ     | ACC TGG GAT CCC GTC GGT TTA | Asp^{841}   | a841/\(\beta\)-gal | 30 ± 3                |
| pCAL944LZ     | ACC TGG ATT CCC GTC GGT TTA | Ile^{944}   | a944/\(\beta\)-gal | 15 ± 2                |
| pCAL972LZ     | GTA GCC CGG AGG ACT GCA CGG ATC | Arg^{972}   | a972/\(\beta\)-gal | 0                     |
| pCBL236LZ     | GCC GCC TAC CCC CCT CGA CGG ATC | Pro^{236}   | \(\beta\)236/\(\beta\)-gal | 2 ± 1                |

In the case of yeast cells transformed by the constructed fusion vectors (Fig. 2A), only fusions at Arg^{60}, Ala^{247}, Ala^{789}, Asp^{841}, and Ile^{944} resulted in markedly blue clones. Yeast cells transformed with pCAL444LZ showed only a weak blue color that grew darker with time. Clones bearing fusion vectors pCAL116LZ, pCAL311LZ, pCAL809LZ, and pCAL972LZ remained white. Measurements of the \(\beta\)-gal activity within isolated yeast membranes (Table II) revealed the highest activity for the shortest fusion protein (a60/\(\beta\)-gal), although the observed enzymatic activity did not correlate with the length of the truncated \(\alpha_1\)-chain. Membranes isolated from yeast cells transformed with fusion vectors pCAL116LZ, pCAL311LZ, pCAL809LZ, and pCAL972LZ did not display any \(\beta\)-gal activity (Table II). The same applies for membranes isolated from cells transformed with pCAL751LZ. This vector codes for the expression of an inactive \(\beta\)-gal with a truncated carboxyl terminus (Table II).

Immunological Detection of \(\beta\)-Galactosidase Fusion Proteins in S. cerevisiae—To determine whether all fusion constructs and B. Additionally, fusion proteins on the amino-terminal end (XhoI site) and the ATP-binding domain (NsiI site) were formed as an internal control for intracellularly expressed \(\beta\)-galactosidase. Fusion proteins within the ouabain-binding site of the \(\alpha_1\)-subunit localized between membrane spans M1 and M2 (Fig. 1A) and fusion proteins with the \(\beta\)-subunit (Fig. 1C) were formed as a control for extracellular localization of fusion sites. Other fusion proteins within the amino-terminal third of the \(\alpha_1\)-subunit (Fig. 1A) were formed in order to verify whether the theoretical predictions of both models for four membrane spans between the N terminus and ATP-binding domain are indeed correct.

**Functional Expression of \(\beta\)-Galactosidase Fusion Proteins in E. coli and S. cerevisiae**—The fusion constructs (Table II) were transformed into E. coli and S. cerevisiae cells. On X-gal-supplemented agar plates, all bacteria were blue in color except clones bearing pCGY1406\(\beta\) without any fusion proteins and pCAL751LZ lacking the EcoRI-3' terminus fragment of the entire lacZ gene (Fig. 2A). These negative controls indicate that at least the lacZ portion of all constructed fusion genes is expressed in E. coli. It is unlikely, however, that correct insertion of \(\alpha\beta\)-complexes containing \(\alpha_1/\beta\)-gal and \(\beta/\beta\)-gal fusion proteins takes place within the bacterial plasma membrane since functional expression of sodium pumps in E. coli was never achieved.

In the case of yeast cells transformed by the constructed fusion vectors (Fig. 2B), only fusions at Arg^{60}, Ala^{247}, Ala^{789}, Asp^{841}, and Ile^{944} resulted in markedly blue clones. Yeast cells transformed with pCAL444LZ showed only a weak blue color that grew darker with time. Clones bearing fusion vectors pCAL116LZ, pCAL311LZ, pCAL809LZ, and pCAL972LZ remained white. Measurements of the \(\beta\)-gal activity within isolated yeast membranes (Table II) revealed the highest activity for the shortest fusion protein (a60/\(\beta\)-gal), although the observed enzymatic activity did not correlate with the length of the truncated \(\alpha_1\)-chain. Membranes isolated from yeast cells transformed with fusion vectors pCAL116LZ, pCAL311LZ, pCAL809LZ, and pCAL972LZ did not display any \(\beta\)-gal activity (Table II). The same applies for membranes isolated from cells transformed with pCAL751LZ. This vector codes for the expression of an inactive \(\beta\)-gal with a truncated carboxyl terminus (Table II).

**Fig. 2. Expression of fusion proteins in E. coli and S. cerevisiae.** The number above or below each clone indicates the respective expressed protein. A, bacteria were transformed with various fusion vectors and were allowed to grow overnight on an LB plate containing X-gal. Under these conditions, cells turn blue, thus demonstrating the expression of catalytically active \(\beta\)-galactosidase. In contrast, cells transformed with either pCGY1406\(\beta\) (denoted \(\alpha\beta\)) or pCAL751LZ (denoted 751) remain white. While pCGY1406\(\beta\) does not contain the lacZ gene, pCAL751LZ codes for an inactive form of \(\beta\)-galactosidase. B, a YNB plate supplemented with X-gal and phosphate buffer, pH 7.0, was used to grow S. cerevisiae transformed with various vectors. After 5 days of incubation at 28 °C, yeast cells expressing fusion proteins with active \(\beta\)-galactosidase take on a blue coloration. Analysis of the data verifies that active \(\beta\)-galactosidase is expressed only when fused to intracellular sites of the \(\alpha_1\)-subunit.
are expressed in full length, immunoblotting was performed with preparations of crude yeast cell membranes. The stable expression of shuttle vector pCGY1406αβ in yeast cells was shown earlier (6, 7, 33). Proteins were detected by a monoclonal antibody directed against the β-galactosidase. The visualized protein bands correspond to the expected molecular masses of the fusion proteins as calculated from their respective amino acid composition (Fig. 3). No immunoreaction was present in membranes obtained from cells transformed with pCGY1406αβ (Fig. 3). The fusion proteins α789/β-gal, α884/β-gal, and α972/β-gal appear to be unstable in this heterologous expression system. Although proteins with the expected molecular masses can be detected by the antibody, immunoreactivity is also obtained at lower molecular masses (Fig. 3).

Localization of Fused β-Galactosidase in the Cytosol of S. cerevisiae—To determine whether the fusion sites Ala237, Ala544, Ala586, Asp934, and Ile946 are located in the cytosol of whole, undisrupted, fusion protein-expressing yeast cells, the hydrolysis of the fluorogenic β-galactosidase substrate C21FDG was measured. Cells carrying fusion vector pCAL60LZ, which was expected to produce a cytosolic fusion protein, served as a control for an intracellular signal. As expected from the experiments with X-gal (Fig. 2) and ONPG (Table II), cells expressing α60/β-gal produce a strong fluorescence signal upon incubation with C21FDG (data not shown). As can be seen in Fig. 4, expression of the fusion proteins α247/β-gal, α444/β-gal α789/β-gal, α884/β-gal, and α946/β-gal resulted in brightly fluorescent yeast cells, indicating that the reporter enzyme is located in the cytosol. In the case of cells bearing no fusion protein and cells containing the fusion proteins α116/β-gal, α311/β-gal, α809/β-gal, α972/β-gal, and β236/β-gal, only background fluorescence was obtained.

Localization of Fused β-Galactosidase on the Cell Surface of S. cerevisiae—The localization of β-galactosidase within the fusion proteins α116/β-gal, α311/β-gal, α809/β-gal, α972/β-gal, and β236/β-gal was performed by indirect immunofluorescence using the ELF kit of Molecular Probes, Inc. The principle of this detection method is based on the production of a fluorescent precipitate, in this case, on the extracellular surface of the yeast cells. Yeast cells that express the proteins α116/β-gal, α311/β-gal, α809/β-gal, α972/β-gal, and β236/β-gal were incubated with a polyclonal antibody against β-galactosidase and afterwards with a biotinylated secondary antibody. The successive addition of streptavidin, biotinylated alkaline phosphatase, and the substrate of alkaline phosphatase (CPCQ) leads to the production of a fluorescent product that precipitates on the surface of the yeast plasma membrane and helps to visualize the yeast cells expressing the proteins α116/β-gal, α311/β-gal, α809/β-gal, α972/β-gal, and β236/β-gal (Fig. 5). A comparable result was not obtained when the primary antibody was omitted or with cells expressing α60/β-gal or α946/β-gal (data not shown).

**DISCUSSION**

A reporter enzyme was fused at certain locations along the α-subunit of sheep kidney Na⁺,K⁺-ATPase, and its activity was localized after expression in the yeast S. cerevisiae. The reporter enzyme in our investigation is the β-galactosidase from E. coli. The lacZ gene of E. coli coding for β-galactosidase was ligated to specific fragments of cDNA coding for either the α-subunit from sheep kidney or the β-subunit from dog kidney Na⁺,K⁺-ATPase. The fusion sites were chosen based on published models (Fig. 1). Suitable vectors that contained the fusion constructs (Table II) were used for yeast transformations. All the expected fusion proteins (Table II) are expressed in full length in the yeast cells as demonstrated by immunodetection in a Western blot using monoclonal antibodies against β-galactosidase (Fig. 3).

β-Galactosidase has often been used as a reporter enzyme in fusions with soluble enzymes. This knowledge provides a basis for our investigation, but there are no examples in the literature of the expression of hybrids between mammalian membrane proteins and β-galactosidase in S. cerevisiae. It was therefore necessary to ascertain that our approach could deliver reliable results. This was achieved by the construction and investigation of several hybrids that served as internal controls for the applicability of the method.

To investigate the correctness of our approach and to verify the proposed topology of the first third of the α-subunit that predicts four transmembrane passes between the amino termi-
Fig. 5. Detection of β-galactosidase on the surface of yeast cells by indirect immunofluorescence. β-Galactosidase fused to extracellular sites of the α- or β-subunits of Na\(^{+}\),K\(^{+}\)-ATPase can be detected on the surface of the yeast cells by an antibody against β-gal and the ELF system, which includes the fluorogenic substrate CPCQ. Fluorescence is not obtained when the primary antibody is omitted or when β-galactosidase is expressed in the cytosol.

nus and the ATP-binding domain, we constructed the vectors pBDLZ60, pBDLZ116, pBDLZ247, pBDLZ311, and pBDLZ444 and used them to transform yeast. The vectors code for the fusion proteins α60/β-gal, α116/β-gal, α247/β-gal, α311/β-gal, and α444/β-gal, respectively.

The fusion protein α60/β-gal, in which the β-galactosidase is attached to Arg\(^{260}\) of the α-subunit, does not contain any hydrophobic areas and should therefore be expressed on the intracellular side (Fig. 6A). In the hybrid α444/β-gal, the β-galactosidase is adjacent to Ala\(^{444}\) of the α-subunit. This amino acid is localized between the phosphorylation site Asp\(^{346}\) (2, 3, 34) and the peptides that become labeled by the ATP analogs 8-N\(^{3}\)ATP and 2-N\(^{3}\)ATP and fluorescein isothiocyanate (35–37). Therefore, the fusion protein α444/β-gal should also be expressed on the cytosolic side. The β-galactosidase of the fusion protein α116/β-gal is adjacent to Glu\(^{116}\). This amino acid is localized between Gln\(^{111}\) and Asn\(^{262}\), an area that participates in ouabain binding and that was shown by the use of a sequence-specific antibody to be localized on the extracellular side of the α-subunit (21). Finally, according to the hydropathy-derived predictions, the fusion site Ala\(^{247}\) of the fusion protein α247/β-gal should be localized on the cytosolic side, and the fusion site Leu\(^{311}\) of the fusion protein α311/β-gal should project toward the extracellular space.

Yeast colonies that express the fusion proteins α60/β-gal, α247/β-gal, and α444/β-gal turn blue when allowed to grow on YNB plates containing the chromogenic β-galactosidase substrate X-gal (Fig. 2). Whole single cells from the same colonies fluoresce when incubated with the fluorogenic β-galactosidase substrate C\(_{12}\)FDG (Fig. 4), indicating that active β-galactosidase is indeed expressed intracellularly and that the blue coloration shown in Fig. 2 is not due to lysed cells. This leads to the conclusion that Arg\(^{260}\), Ala\(^{247}\), and Ala\(^{444}\) are localized intracellularly (Fig. 6A).

In contrast, yeast colonies expressing the fusion proteins α116/β-gal and α311/β-gal do not display blue coloration on YNB plates with X-gal (Fig. 2). Single cells remain invisible upon incubation with C\(_{12}\)FDG. Thus, the fusion proteins α116/β-gal and α311/β-gal contain inactive β-galactosidase. The same applies for the fusion protein β236/β-gal, which consists of β-galactosidase attached to Pro\(^{236}\) of the β-subunit. This amino acid is localized between the glycosylation sites Asn\(^{159}\) and Asn\(^{267}\) (38) and therefore should be extracellular (Fig. 6A). Our finding is in good agreement with the results obtained by Yoon and Guidotti (15). These authors were able to detect an artificially inserted epitope after Glu\(^{226}\) on the extracellular side of the rat β-subunit.

When β-gal enzymatic activity is measured in crude membrane preparations, ONPG hydrolysis can be detected only if membranes are derived from cells expressing intracellular β-galactosidase (Table II). Taken together with the Western blot data (Fig. 4), this result indicates that although the fusion proteins α116/β-gal, α311/β-gal, and β236/β-gal are expressed, they are inactive. Similar observations were made for hybrid
proteins between the MalF protein and β-galactosidase (39), where fusion of the lacZ gene to potentially periplasmic sites always resulted in expression of inactive β-galactosidase. Froshauer et al. (39) assumed that the amino-terminal area of the β-galactosidase is pulled by its periplasmic attachment point through the membrane, but that the remaining molecule does not penetrate. In agreement with this hypothesis, β-galactosidase from yeast spheroplasts expressing the fusion proteins α116/β-gal, α311/β-gal, and β236/β-gal can be extracellularly detected by a polyclonal antibody against β-galactosidase, a secondary antibody coupled to alkaline phosphatase by biotin/streptavidin, and the fluorogenic substrate of alkaline phosphatase (CPCQ). Fig. 5 demonstrates that whole cells expressing the previously mentioned hybrid proteins become fluorescent on their extracellular surface under these conditions, thus indicating that parts of the β-galactosidase are exposed to the antibody. A fluorescent cell surface was never obtained when the primary antibody was omitted or when yeast cells expressing active (intracellularly localized) β-galactosidase were used. Therefore, the proposed insertion of β-galactosidase in the plasma membrane when fusion sites are localized extracellularly (39) seems indeed to take place. The presence of parts of the amino terminus of the β-galactosidase within the membrane, however, apparently distorts the conformation and results in an inactive enzyme (Table II). It should be mentioned here that the catalytic activity of β-galactosidase is maintained by a tetrameric enzyme (39, 40), and it might be that this structure cannot be formed if parts of the amino- terminal sequence are hidden within the membrane.

In summary, the results derived from the investigation of the fusion proteins between β-galactosidase and parts of the amino-terminal third and the ATP-binding domain of the α-subunit indicate that detection of β-galactosidase activity is a useful tool for the investigation of the transmembrane topology of the sodium pump. Our findings confirm the results or predictions of others concerning the extracellular localization of Arg39, Glu116, and Ala444 (9, 11, 14, 21, 41, 42) and verify experimentally an intracellular localization for Ala47 (39) and an extracellular localization for Leu47 (47). Thus, in good agreement with all current models (49), we also have to assume four membrane spans between the cytotoxic amino terminus and the ATP-binding site of the α-subunit of the sodium pump (Fig. 6A).

The determination of the transmembrane topology of the last third of the α-subunit has been a controversial subject in numerous reports. We have investigated this structure with the vectors pBDLZ789, pBDLZ809, pBDLZ884, pBDLZ946, and pBDLZ972, which code for the fusion proteins α789/β-gal, α809/β-gal, α884/β-gal, α948/β-gal, and α972/β-gal, respectively. All of these fusion proteins are successfully expressed in yeast (Fig. 3).

In the fusion protein α789/β-gal, the β-galactosidase is attached to Ala789 of the α-subunit. According to the transmembrane span model of Karlish et al. (12), Ala789 should be localized near the extracellular part of their proposed M5 span. Since the fusion protein α789/β-gal exhibits β-galactosidase activity (Figs. 3 and 5 and Table II), however, we must conclude that Ala789 is not far away from the cytosolic side of the M5 span. In the eight-transmembrane span model proposed by Shull et al. (11) and in one of the models proposed by Mohraz et al. (20), this amino acid is on the cytosolic third of the M5 span. In fusion proteins with lactose permease, it was shown earlier that alkaline phosphatase is expressed on the periplasmic side if fusion sites were toward the periplasmic half of membrane spans and toward the cytosolic side if the location of the fusion sites was toward the cytosolic half of the same spans (44, 45). If we make a similar assumption, then localization of Ala789 near the cytosolic side of M5 allows the attached β-galactosidase to fold correctly and to display enzymatic activity (Fig. 6A). Therefore, we favor an M5 span similar to the M5 span (Ile789–Ile803) proposed in the older eight-transmembrane span model of Shull et al. (11) or in the recent investigation of Xie et al. (M5 = Ile789–Ile803) of the rat α-subunit (46). This conclusion is supported by the fact that the β-galactosidase of the fusion protein α809/β-gal is inactive (Fig. 2 and Table II) and extracellularly localized (Fig. 5). The amino acid Met830 is localized within the sequence 809MOVPAISLAYEQAESDIM925, which was shown previously by the use of a specific antibody to be at least in part extracellular (13, 20). This result, taken together with results showing that Lys828 (here Lys826) (15) and Asn831 (12) are localized on the cytosolic side, supports the conclusion that there must be a membrane span between Met830 and these amino acids. In our interpretation, the area between Met830 and Asn831 would include the M6 part of the α-subunit (Fig. 6A). Although this possibility has been described in one of the transmembrane topology models proposed by Mohraz et al. (20), the recent investigation of Xie et al. (46) proposes that M6 does not penetrate the plasma membrane, but is rather embedded within the membrane in a U-form.

The β-galactosidase moiety of the fusion protein α884/β-gal is attached to Asp864 of the α-subunit. In various theoretical models, this amino acid is localized within the sequence Glu826–Glu830, either between membrane-spanning sequences M6 and M7 (11) or M7 and M8 (12). Various attempts to localize amino acids or epitopes between these two membrane spans either were unsuccessful or produced conflicting results. In two independent investigations, the epitope 877DYSQQUVYQR904 was localized using the same IIC9 antibody on either the intracellular (13) or extracellular (20) side. In another investigation, the attempt to localize an artificial epitope inserted after Glu830 was unsuccessful, although in the same work, there was no difficulty in localizing Lys826, Val830, and the carboxyl terminus on the cytosolic side using the same epitope as a marker (15). If the loop between M7 and M8 is on the extracellular surface, the Asp864 fusion site of the β-galactosidase should also be located on the extracellular side. Therefore, yeast cells expressing the fusion protein α884/β-gal should remain uncolored on X-gal-containing media and should not be able to hydrolyze C14-FDG. Our experiments, however, demonstrate just the opposite (Figs. 2 and 4). How might this result be explained? One possibility would be that the predicted transmembrane spans M7 and M8 do not exist. This would mean, however, that the hydropathy predictions are completely wrong. Although this seems quite unlikely, hydrophobic structures do not necessarily need to be localized within a membrane. The lowest free energy state of a protein is determined by protein/protein interactions and by interactions with the solvent. Thus, consideration only of interactions of protein with lipid might be insufficient to define membrane spans (47). The necessity to consider cooperativity in order to determine membrane spans is demonstrated by the findings of Kyte and Doolittle (8), who calculated a 19-amino acid sequence from the water-soluble dogfish lactate dehydrogenase as the most hydrophobic peptide (47). Apparently, this hydrophobic domain of the dogfish lactate dehydrogenase is kept away from the water surface by interactions with other areas of the enzyme protein. Our results could be explained by an analogous assumption. Nevertheless, it was shown earlier that the sequence Glu826–Glu830 between M7 and M8 also contains 26 amino acids that are essential for the interaction of the α-subunit with extracellular sequences of the β-subunit (48, 49). Therefore, M7 and M8 must penetrate the membrane.
In a recent model that was derived from the topogenic properties of hydrophobic segments of the α-subunit investigated by the insertion of fusion proteins into endoplasmic reticulum membranes, the M6 span (Leu842–Ala869 in our investigation) is proposed to be bent within the plasma membrane (Fig. 6C) due to the presence of two adjacent glycines in the middle of this hydrophobic sequence (46). If this assumption is correct, then one can imagine that the fused β-gal in our experiments does not allow the bent membrane span to protrude out of the membrane. This could explain why the extracellularly expected fusion site Asp884 is found here intracellularly.

An alternative explanation that would account for our results and the discrepant conclusions of other investigations would be that the hydrophilic loop Glu868–Glu908 between M7 and M8 is not freely accessible, but rather is hidden within the membrane, possibly surrounded by membrane spans (Fig. 6B). Such a structure would correspond to the so-called P-loop of membrane, possibly surrounded by membranes spans (Fig. 6). The model proposed here makes it and is not accessible to the antibody. Under these circumstances, it would be detected on neither the extracellular nor the intracellular side (15). The model proposed here makes it also possible to understand why the sequence 884–WIND-VEDSYGQWTEQR904 of the α-subunit can be detected on the cytosolic side with the IIC9 antibody after permeabilization of the cells in one investigation (13) and in an other investigation on the extracellular side after extended incubation of Na+-K+-ATPase-containing membranes with the same antibody and subsequent labeling with immunogold (20). It should be mentioned, however, that the membranes used in the latter experiment were isolated after SDS extraction; therefore, it cannot be excluded that parts of the α-subunit might unfold in a way that makes them accessible to the antibodies. Consistent with this, the detection of the sequence 887–WINDVEDSYG-QQWTEQR904 was not possible when Na+-K+-ATPase was inserted in right-side-out vesicles (20). Additionally, the insertion of the α-subunit in the membrane beyond the M7 span seems to be rather labile. Reducing agents and incubation at 50 °C cause an unfolding of this region out of the plasma membrane (53, 54).

As mentioned before, the loop Glu868–Glu908 between M7 and M8 also contains a sequence that is essential for the interaction of α- and β-subunits (48). Evidence is accumulating that the β-subunit might be involved in recognition of cations (55) and that it influences the properties of Na+-K+-ATPase toward the transported Na+ or K+ ions (56, 57). Since P-loops of Na+ or K+ channels seem to participate in the gating of the channel and in ion conductance (50, 51), it would be interesting to investigate a possible involvement of the loop proposed here in the transport of ions through the sodium pump. At this stage, we cannot know whether the entire Glu868–Glu908 sequence or parts of it are required for the formation of the proposed hidden loop. The presence of 8 carboxyl and 16 other polar groups within the sequence, however, provides a basis for further investigation.

There are not many reports about the exact number of membrane spans after M8. Although Ovchinnikov et al. (13) and Bayer (58) assigned the C terminus to the extracellular surface, it is currently generally accepted that both N and C termini of the catalytic subunit are localized intracellularly (10, 15–17, 59). Epitope tagging studies additionally revealed an intracellular localization of Val929 (here Val857) (15). Since both Val839 and the carboxyl-terminal amino acid Tyr1015 are localized on the cytosolic side, the sequence Val938–Tyr1015 could either be entirely cytosolic or form two paths through the plasma membrane (15). The fact that the sequence Val938–Tyr1015 is resistant to the action of proteases, however, has led to the conclusion that the sequence must be largely hidden within the membrane (12). Nevertheless, resistance to proteases is a rather indirect proof for membrane insertion.

The demonstration of an extracellular loop within the sequence Val938–Tyr1015 would clarify the transmembrane topology of this part of the α-subunit. Therefore, we constructed the vectors pBDLZ946 and pBDLZ972, which code for the fusion proteins α946/β-gal and α972/β-gal, respectively. In the fusion protein α946/β-gal, the β-galactosidase molecule is attached to Ile946 and is therefore in the vicinity of Val938. Yeast cells expressing this fusion protein display a catalytically active β-galactosidase (Figs. 2 and 4), consistent with Ile946 being localized on the cytosolic side and confirming a cytoplasmic location of Lys842 (60) or Ser838 (here Ser840) (46). In contrast, the β-galactosidase of the α972/β-gal fusion protein remains inactive (Table II) and is localized on the extracellular surface of the plasma membrane (Fig. 5). Therefore, there must be an M9 span between Ile946 and Arg972 and an M10 span between Arg972 and the cytosolic C terminus. In a recent investigation, Arg974 (here Arg972) was also shown to be extracellular by analyzing the location of the chloroamphenicol acetyltransferase fused to the α-subunit of the sodium pump (46). An extracellular location for Met937 was also proposed by Lutsenko and Kaplan (61) based on the analysis of peptides that remain associated with the plasma membrane after extensive proteolytic digestion. Based on their and our findings, models postulating an intracellular localization of Arg972 and a total number of eight membrane spans appear inadequate.

Using various analytical methods, 10 membrane spans have been predicted for the α-subunit of gastric H+K+-ATPase (62–64) and for the Ca2+-ATPases from sarcoplasmic reticulum (64, 65). Ten transmembrane segments have been recently determined also for the H+-ATPase of the fungus Neurospora crassa through the use of an in vivo translation system (66), although the number of segments was formerly predicted to be eight (67). A point of interest is that the authors (66) suggested that transmembrane segments are inserted in pairs according to the helical hairpin hypothesis (68). This hypothesis is strongly supported by our observation that all fusion sites between a pair of inserted transmembrane segments lead to inactive fused β-galactosidase. Taking into consideration the close homology between sodium, calcium, and proton pumps (for instance, the sheep Na+-K+-ATPase shares 64% identity with the rat H+-ATPase (69), these data together support a common 10-transmembrane span model for all P-type ATPases. Although our results could fit into such a model (Fig. 6A) (12), our findings are in a good agreement with and could also be explained by the recent model of Xie et al. (46) that proposes nine membrane spans for the α-subunit of Na+-K+-ATPase, with one of them, M6, reflecting partially the M6/M7 spans of 10-transmembrane span models. This possibility is demonstrated in Fig. 6C, which essentially reproduces the model of Xie et al. (46), except that it also includes the loop between M7 and M8 proposed here. Interestingly, Xie et al. (46) also proposed the existence of a peptide loop between M7 and M8 (M8 and M9 in Fig. 6C). Since similar structures from the intracellular side of Na+ or K+ channels are thought to interact with their P-loops on the “extracellular” side, and since the hourglass structure of aquaporin consists of such a structure, it will be of great interest to investigate a possible interaction of the
