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ORIGINAL PAPER

Michelle L. Gumz · David Duda · Robert McKenna · Charles S. Wingo · Brian D. Cain

Molecular modeling of the rabbit colonic (HKα2a) H^+, K^+ ATPase

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Abstract A model of the HKα2a subunit of the rabbit colonic H^+, K^+ ATPase has been generated using the crystal structure of the Ca^{+2} ATPase as a template. A pairwise sequence alignment of the deduced primary sequences of the two proteins demonstrated that they share 29% amino acid sequence identity and 47% similarity. Using O (version 7) the model of HKα2a was constructed by interactively mutating, deleting, and inserting the amino acids that differed between the pairwise sequence alignment of the Ca^{+2} ATPase and HKα2a. Insertions and deletions in the HKα2a sequence occur in apparent extra-membraneous loop regions. The HKα2a model was energy minimized and globally refined to a level comparable to that of the Ca^{+2} ATPase structure using CNS. The charge distribution over the surface of HKα2a was evaluated in GRASP and possible secondary structure elements of HKα2a were visualized in BOBSCRIPT. Conservation and placement of residues that may be involved in ouabain binding by the H^+, K^+ ATPase were considered and a putative location for the β subunit was postulated within the structure.

Keywords HKα2a · ATPase · Kidney

M. L. Gumz · D. Duda · R. McKenna · B. D. Cain (✉)
Department of Biochemistry and Molecular Biology,
University of Florida, 1600 SW Archer Rd., Box 100245 JHMHSC,
Gainesville, FL, 32610, USA
e-mail: bcain@biochem.med.ufl.edu
Tel.: +1 352-392-6473
Fax: +1 352-392-2953

C. S. Wingo
Department of Medicine,
University of Florida, 1600 SW Archer Rd., Box 100245 JHMHSC,
Gainesville, FL, 32610, USA

Introduction

The colonic H^+, K^+ ATPase consists of the HKα2a subunit and an as yet unidentified β subunit. The enzyme is located on the apical membranes of epithelial cells in both the kidney and the colon and is responsible for pumping potassium ions into the cell in exchange for hydrogen ions. The H^+, K^+ ATPase functions in maintaining potassium homeostasis and acid–base balance in the body. Increasing evidence suggests that the colonic isoform of the H^+, K^+ ATPase, or HKα2, plays a critical role in kidney function by conserving potassium. [1, 2, 3]

The H^+, K^+ ATPase is a member of a large family of integral membrane proteins classified as P-type ATPases. Typically, these enzymes consist of a large subunit of about 100 kDa. Unlike the single subunit Ca^{+2} ATPase, the Type II P-type ATPases, such as Na^+, K^+ ATPases and the H^+, K^+ ATPases, also contain smaller β subunits. The β subunits are approximately 30 kDa proteins that are glycosylated to varying degrees. P-type ATPases use the energy of ATP hydrolysis to pump ions against their concentration gradients. Phosphorylation of the α subunit leads to a conformational change from a high-affinity E1 state to a low-affinity E2 state. Changes in ion affinity that accompany the conformation change promote ion translocation. [2]

Recently, a 2.6-Å crystal structure was reported for the rabbit sarcoplasmic reticulum Ca^{+2} ATPase in the E1 conformation. [4] The crystal structure showed the existence of ten transmembrane helices and three cytoplasmic domains, designated A, N and P. The A domain is made up of the N-terminus and the loop between transmembrane helices two and three (L2–3). The P and N domains lie within the large loop between transmembrane helices four and five (L4–5). The N domain, which contains the nucleotide binding site, is the largest of the three cytoplasmic domains. The P domain contains the aspartic acid residue that becomes phosphorylated during the enzyme's catalytic cycle. This aspartic acid residue lies at the C-terminal end of the central β sheet in a Rossman fold.

Following publication of the Ca^{+2} ATPase structure, an 11-Å structure of the duck Na^+, K^+ ATPase was deduced using cryo-electron microscopy. [3] The Na^+, K^+ ATPase structure in the E2 conformation appeared to be similar to that observed for the 8-Å Ca^{+2} ATPase structure in the E2 conformation. [5] In both structures, the three cytoplasmic domains were easily recognizable. Conservation of the structure of this catalytic core provided very strong support for the idea that these enzymes, as well as other P-type ATPases, probably undergo similar E1 to E2 conformation changes that accompany ion translocation. One of the important observations made by Rice et al. was that of an extracellular mass component of the Na^+, K^+ ATPase. This likely corresponds to the location of the β subunit, which was shown to lie near transmembrane helices seven and ten (M7 and M10) of the NaKα1 subunit. Additional evidence for the three-dimensional structure of the Na^+, K^+ ATPase was presented by Hebert et al. [6] in a 9.5-Å structure also deduced from electron micrographs. Densities from within this structure suggested a location for the β subunit near M8 and M10 of the Na^+, K^+ ATPase. This density was taken from a plane closer to the membrane than that used to assign β subunit density in the structure of Rice et al.

Determination of a high resolution structure for the colonic H^+, K^+ ATPase has remained impractical because the enzyme is present in relatively low abundance in animal tissues and no high yield expression system has been established. Therefore, we have built a molecular model of the colonic H^+, K^+ ATPase structure. The model is based on the atomic coordinates of the Ca^{+2} ATPase. [4] Here we propose a structure for the HKα2a subunit of the rabbit colonic H^+, K^+ ATPase in the E1 conformation. The model indicates possible points of entry for the transported ions, likely secondary structure elements of the transmembrane domain, a putative ouabain binding site, and allows speculation on the position of the β subunit.

Methods

Choice of templates and alignment

The template selected for the modeling exercise was the rabbit sarcoplasmic reticulum Ca^{+2} ATPase. [4] The primary amino acid sequence of the HKα2a subunit (Genbank AAB80941.1) of the rabbit colonic H^+, K^+ ATPase was aligned with that of the Ca^{+2} ATPase (Genbank P04191) and the NaKα1 subunit (Genbank NP_036636) of the Na^+, K^+ ATPase using Clustal W [7] with default settings. The gap penalties used were a gap opening penalty of 10 and a gap extension penalty of 0.05.

Modeling

The PDB coordinates for the Ca^{+2} ATPase (1EUL) were downloaded from the Brookhaven protein database (www.rcsb.org) and loaded into the program O. [8] Individual amino acids of the Ca^{+2} ATPase were mutated to match that of the primary sequence of HKα2a according to the pairwise sequence alignment. Insertions and deletions with respect to the Ca^{+2} ATPase sequence were made interactively and energy minimized locally using the graphics program O, version 7. [8] The transmembrane domain predictor program TMHMM [9] and the program O [8] were used to build an α helix representing the single transmembrane domain of NaKβ2.

Model refinement and validation

The entire model was globally energy minimized using the CNS software package. [10] This minimization was also performed on the model of NaKβ2. The program PROCHECK [11] was used to investigate the stereochemistry of the model. The coordinate files for the HKα2a model and the Ca^{+2} ATPase were loaded into the program and used to generate secondary structure predictions and stereochemical statistics.

Charge distribution

Surface potential energy of the HKα2a model and the Ca^{+2} ATPase were generated using the program GRASP. [12] Parameters were set to −8.566 and 8.501 for HKα2a and −8.965 and 8.493 for the Ca^{+2} ATPase.

Secondary structure prediction

Secondary structure elements of the HKα2a transmembrane domain were visualized using BOBSCRIPT. [13] Data from the Ca^{+2} ATPase crystal structure were used in conjunction with the amino acid sequence alignment to assign the locations of the transmembrane helices in HKα2a.

Results and discussion

Sequence alignments were generated in order to determine if the primary structures of the rabbit HKα2a subunit and the rabbit sarcoplasmic reticulum Ca^{+2} ATPase were sufficiently conserved to consider molecular modeling of the colonic H^+, K^+ ATPase. These alignments revealed that the two proteins share 29% identity and 47% similarity. Other researchers have found similar levels of primary sequence similarity adequate for modeling. [14] HKα2a shares 65% identity with the Na^+, K^+ ATPase compared to 29% with the Ca^{+2} ATPase. The amino acid sequence of rat NaKα1 was included as an added feature of the alignment for this reason and because of the growing body of structural data on the Na^+, K^+ ATPase. Clustal W [7] was used to produce the alignment of the deduced amino acid sequences of the three proteins (Fig. 1). Amino acid conservation was observed within several regions of all three proteins. These included the well-characterized phosphorylation site (position 385 in HKα2a) and surrounding sequence (ICS**D**KTGTLT) and the region near the nucleotide binding pocket (**K**GAPE). The latter contains the conserved lysine (position 517 in HKα2a) that has been shown to lie within the binding pocket of P-type ATPases. Many of the amino acids within the ten transmembrane helices were also found to be conserved. In all three proteins, the large cytoplasmic loop that houses the P and the N domains was located between transmembrane helices four and five (L4–5). In summary, the similarities between the HKα2a subunit and the Ca^{+2} ATPase appeared sufficient to justify molecular modeling of the colonic H^+, K^+ ATPase.

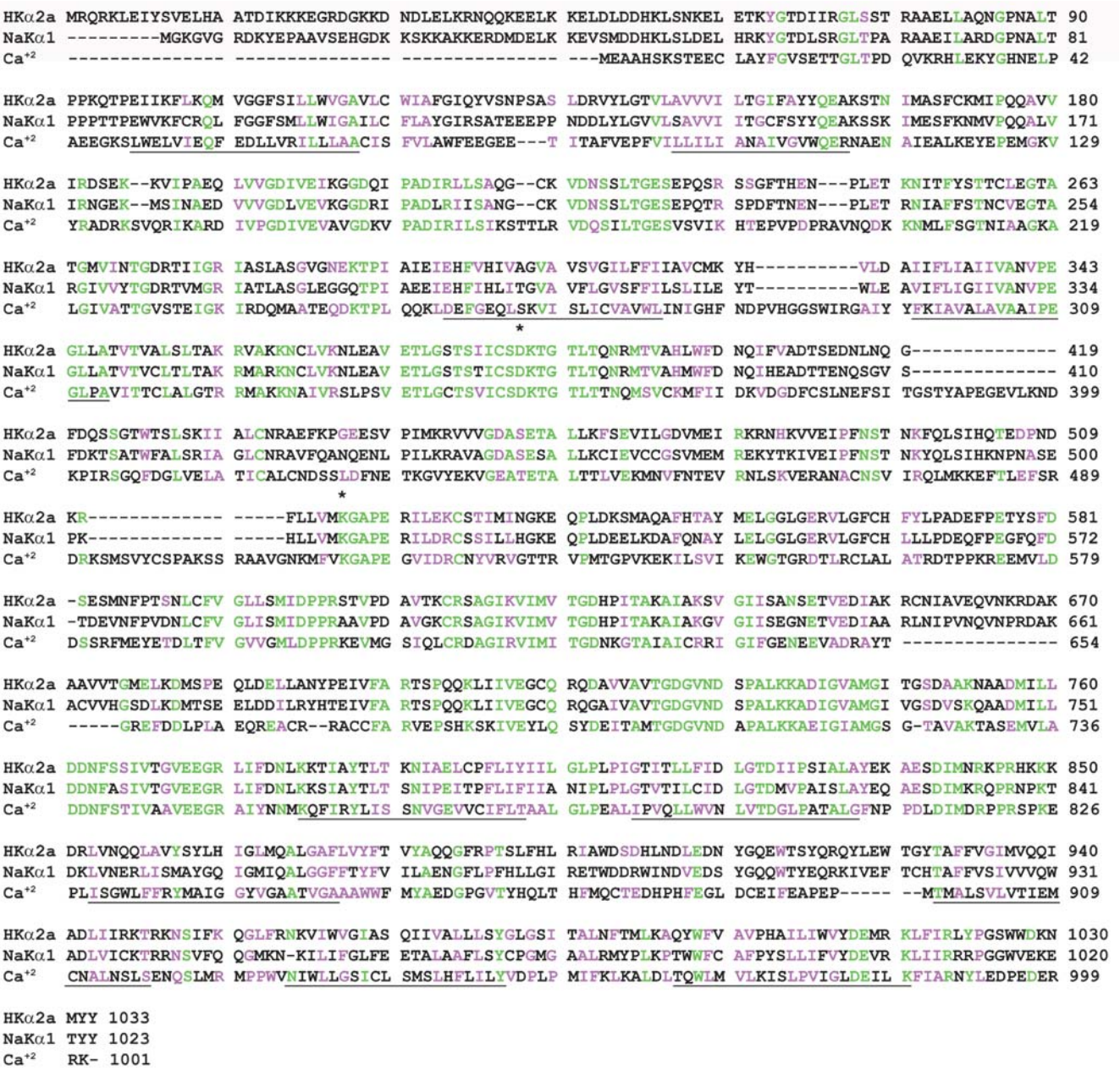

Fig. 1 Primary structure alignment of HKα2a, Na^+, K^+ ATPase α1, and Ca^{+2} ATPase. Amino acid sequences were aligned using Clustal W [7] with gap penalties. The numbering system at the right corresponds to each individual amino acid sequence. Identical amino acids between HKα2a (*top*) and Ca^{+2} ATPase (*bottom*) are shown in *green*. Similar amino acids are shown in *pink*. When applicable, similar and identical amino acids of the Na^+, K^+ ATPase (*middle*) were highlighted as well. *Underlined* regions correspond to the ten transmembrane domains of the Ca^{+2} ATPase. Aspartic acid 385 and lysine 517 are denoted by *.

The model of HKα2a was built using the program O [8] and was based on the published atomic coordinates of the Ca^{+2} ATPase. [4] Using the amino acid sequence alignment as a guide (Fig. 1), amino acids of the Ca^{+2} ATPase were changed to match the primary sequence of HKα2a. A total of 739 individual amino acid substitutions were made; of these, 204 changes were to similar amino acids. HKα2a is 48 amino acids longer than the Ca^{+2} ATPase at its N-terminus. Secondary structure prediction using Gor II [15] suggested that these amino acids would predominantly form a helix (data not shown). Since there was no counterpart for these in the Ca^{+2} ATPase structure they were necessarily omitted from the model. As might be expected, all insertions and deletions with respect to the Ca^{+2} ATPase occurred in both HKα2a and NaKα1. In total, 33 amino acids were added and 50 amino acids removed with respect to the Ca^{+2} sequence.

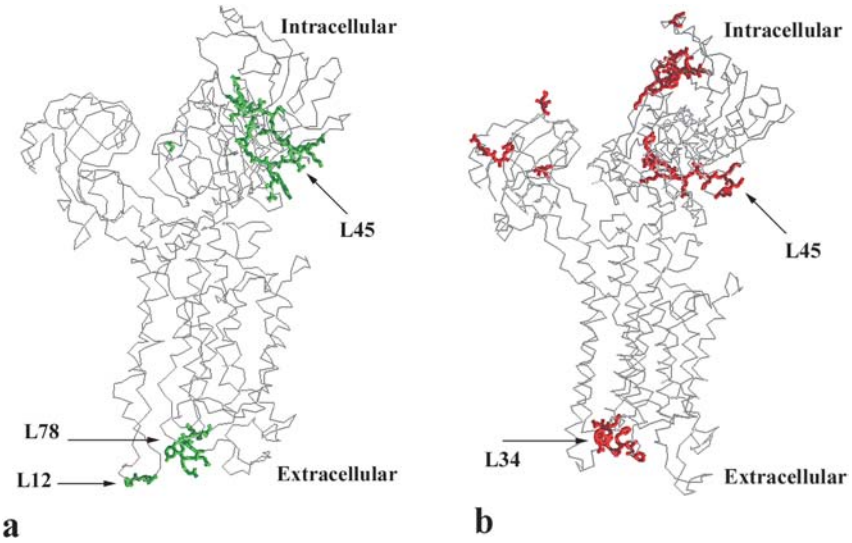

Fig. 2a–b Comparison of the HKα2a model with the Ca^{+2} ATPase.**a** The Cα trace of the HKα2a subunit model is shown with insertions labeled in *green*. For emphasis, the side chains of the added amino acids are highlighted as well. **b** The Cα trace of the Ca^{+2} ATPase is seen with amino acids that are absent in HKα2a displayed in *red*. Figure was prepared using O, Version 7 [8]

The insertions and deletions in HKα2a as compared to the Ca^{+2} ATPase are highlighted in Fig. 2. Areas of insertion or deletion occurred only in extra-membraneous loop regions such as the extracellular loops and the P domain (Fig. 2a). Examples of insertions contributing to changes in the extracellular domain include a three amino acid addition in L1–2 and a seven amino acid addition in L7–8. A more substantial area of addition is a 20 amino acid insertion in L4–5 that occurs in the P domain. Since this insertion is also present in the Na^+, K^+ ATPase, it may represent the "protuberance" from the P domain discussed by Rice et al. [3] This mass appeared as a slight protrusion in the P domain that was distinctly absent in the Ca^{+2} ATPase.

Both the major insertions into HKα2a appeared to fill space vacated by deletions of amino acids present in the Ca^{+2} ATPase (Fig. 2). For example, ten Ca^{+2} ATPase amino acids were absent from L3–4 in HKα2a. This space in the extracellular domain was occupied by the insertions in L1–2 and L7–8 in the model. Similarly, the space vacated by the deletion of material from L4–5 of the Ca^{+2} ATPase was filled by the large 20 amino acid insertion. Smaller areas of deletion were not obviously replaced by additions in HKα2a but these areas were located on the surfaces of the N and A domains (Fig. 2b).

Following completion of the primary structure changes, CNS [10] was used to perform a global energy minimization on the model. PROCHECK [11] was then used to evaluate the stereochemical properties of the model. The CNS minimization and PROCHECK evaluation were also performed on the structure of the Ca^{+2} ATPase using the published coordinates. Table 1 lists pertinent stereochemical parameters of the model as compared to the Ca^{+2} ATPase crystal structure. Importantly, the root mean square deviations (RMSD) listed for the HKα2a model were strikingly similar to those observed for the Ca^{+2} ATPase for the great majority of the parameters. The bond lengths and angles values both fell within accepted limits. The omega torsion angle, representing rotation around the peptide bond, and the zeta torsion angle, giving a measure of Cα tetrahedral distortion, were similar to that observed in the Ca^{+2} ATPase structure. As a measure of stability of side chains, the chi angles were also very similar between the two structures. An important parameter for judging the overall quality of a structure is the G factor, used to estimate the "normalcy" of the structure. PROCHECK calculates an average overall G factor by evaluating phi–psi and chi1–chi2 values for each residue. Accepted G values are typically greater than –0.5 and any value that falls below –1.0 requires further investigation, according to the program. Notably, the overall G factor for the HKα2a model was –0.28, compared to a value of –0.2 for the Ca^{+2} ATPase structure.

Table 1 Stereochemical statistics

Statistic	HKα2a[a]	Ca^{+2a}
Bond length	0.0569	0.0481
Bond angles	1.9344	1.7945
Omega angle	1.3	1.2
Zeta angle	1.8	1.9
H bond energy	0.7	0.8
Bad contacts	2.6	1.3
Chi-1 *gauche* minus	19.5	17.6
Chi-1 *trans*	18.1	17.6
Chi-1 *gauche* plus	18.5	17
Chi-1 pooled	19.4	17.8
Chi-2 *trans*	14.9	15.6
Overall G-factor	–0.28	–0.2

[a] Values displayed are root mean square deviations (RMSD) between the actual and most likely values

The program GRASP was used to visualize the charge distribution over the surface of the HKα2a model (Fig. 3). The area surrounding the phosphorylation site is rich in positively charged amino acids that probably interact with the phosphate group (Fig. 3a). A concentration of basic amino acids was found in the region linking the P domain to the transmembrane domain (arrow in Fig. 3b). Acidic

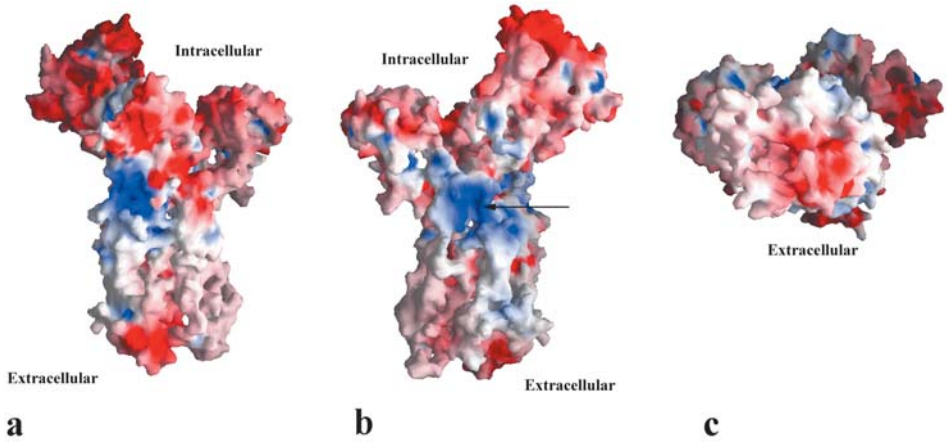

Fig. 3a–c Charge distribution over surfaces of HKα2a. Areas of negative charge are designated in *red* and areas of positive charge are indicated in *blue*. **a** A view of the charge distribution over the entire model is seen here. **b** Figure 3a was rotated 180° in the *x* direction. **c** Figure 3b was rotated 90° in the *y* direction. Figures were prepared with GRASP [12]

amino acids clustered at the extracellular face of the molecule (Fig. 3c). Substitutions in the extracellular loops of HKα2a actually resulted in a net increase of eight positive charges with respect to the comparable region in the Ca^{+2} ATPase. In fact, the overall charge of the Ca^{+2} ATPase is −28 compared to −11 for HKα2a. This disparity in charge may represent the difference between the two pumps for association–dissociation of a proton as opposed to a divalent calcium ion, or it may reflect the requirement of the H^+, K^+ ATPase to transport cations in both directions. Nevertheless, the negatively charged regions at the extracellular face of HKα2a probably represent the location of the entrance to the ion channel.

The relative positions of the phosphorylation site and the nucleotide binding site were maintained in the HKα2a model. The two amino acids used to indicate the locations of the P and N domains of the enzyme were the phosphorylation site aspartic acid at position 385 and the lysine at position 517 in the nucleotide binding pocket (Fig. 4). The distance between these two residues was measured to be 29 Å. This value was very close to that determined for the same two amino acids using the reported coordinates for the Ca^{+2} ATPase [4] and in a model of L4–5 of the Na^+, K^+ ATPase. [14]

The amino acid sequence alignment, together with data from the crystal structure of the Ca^{+2} ATPase, were used to assign putative secondary structure elements to the model of HKα2a (Fig. 4). While there is little direct evidence about the secondary structure of the H^+, K^+ ATPase, it has been determined that the Ca^{+2} ATPase, the *N. crassa* H^+ ATPase, and the duck and pig kidney Na^+, K^+ ATPases all contain ten transmembrane helices. [4] Ten transmembrane domains were readily apparent in the HKα2a model. Each occupied a position comparable to transmembrane domains in other P-type ATPases for which structural information is available.

It has been reported that the colonic H^+, K^+ ATPase has partial sensitivity to the cardiac glycoside ouabain, [16] while the Na^+, K^+ ATPase has been long recognized as highly sensitive to the drug. Extensive mutagenesis studies with the Na^+, K^+ ATPase have revealed the participation of several key residues in conferring ouabain

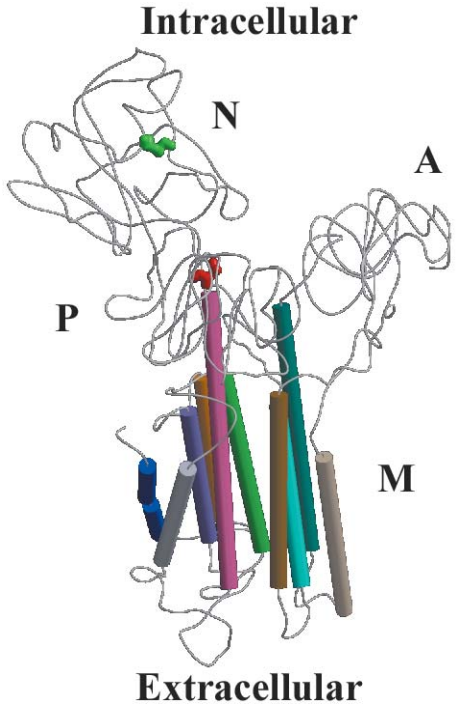

Fig. 4 Possible architecture of the HKα2a subunit. The residue in *green* is the lysine (position 517, Fig. 1) that lies in the nucleotide binding pocket and the residue in **red* is the aspartic acid at the phosphorylation site (position 385). Based on an alignment with the Ca^{+2} ATPase, ten transmembrane helices were modeled into HKα2a. The ten transmembrane helices are drawn as rods and shown in different colors for clarity. From left to right, the transmembrane helix designations are M10 (*blue*), M7 (*gray*), M8 (*purple*), M9 (*orange*), M5 (*pink*), M6 (*green*), M3 (*brown*), M4 (*cyan*), M2 (*teal*), and M1 (*almond*)

sensitivity to the pump (for a review see [17]). In an attempt to glean information from the model about the ouabain sensitivity of our enzyme, we used the alignment in Fig. 1 and our model of HKα2a to look at the conservation of these key residues as well as their placement in the H^+, K^+ ATPase. Of the 18 residues that have been defined as contributing to ouabain sensitivity in the Na^+, K^+ ATPase, [17] 12 were conserved in the

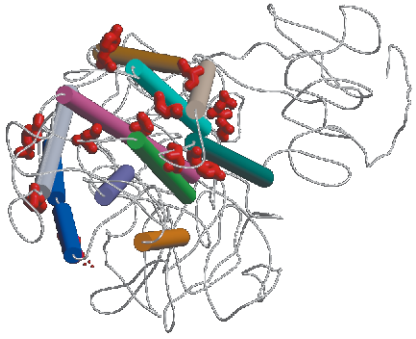

Fig. 5 Ouabain binding residues in HKα2a. Ouabain binding residues from the Na^+, K^+ ATPase that are conserved in HKα2a are highlighted in *red*. The model from Fig. 4 was rotated to give a view of the extracellular face of the enzyme and the clustering of the putative ouabain binding residues in this area. Helix colors and designations are the same as described for Fig. 4

colonic H^+, K^+ ATPase. Of the 12 conserved amino acids, nine were located at the extracellular face of the molecule where ouabain binding occurs (Fig. 5). The remaining residues appeared to be too far from the extracellular binding site to participate in an interaction with the drug and might be involved with functions outside the ouabain binding site.

Both the H^+, K^+ ATPase and the Na^+, K^+ ATPase require a β subunit to function. Structural as well as biochemical data have revealed several important interactions between the α and β subunits of Type II P-type ATPases. Together with our model of HKα2a, the data concerning α and β interactions were used to consider a possible location for the β subunit. Although the identity of the β subunit that pairs with the HKα2a subunit has not yet been determined, four possible partners are known. These are Na^+, K^+ ATPase β1, β2, β3 and gastric H^+, K^+ ATPase β; all share similar molecular architectures. The probable topology consists of a short intracellular region, a single α-helical transmembrane domain, and a large glycosylated extracellular domain that contains six well-conserved cysteines thought to form three disulfide bridges. Studies in Xenopus oocytes have demonstrated that, of the four possible β subunit partners, NaKβ2 is an effective partner for HKα2. [18] We have recently cloned and expressed rabbit NaKβ2 (Genbank AY069937). We have built an α helix representing the single transmembrane domain of NaKβ2 (Fig. 6). Figure 6c shows the model of the transmembrane helix of NaKβ2. The positions of several phenylalanines were evident along one face of the helix, consistent with the helical wheel prediction. Two of these are conserved across all four β subunits, as illustrated in the alignment in Fig. 6d.

Our generation of a three-dimensional model of HKα2a allows consideration of a location for the β subunit that accounts for all known α/β interactions in Type II P-type ATPases. The 11-Å structure of the duck Na^+, K^+ ATPase deduced from electron micrographs showed a large extracellular mass in the vicinity of M7 and M10. [3] Placement of the β subunit in this area

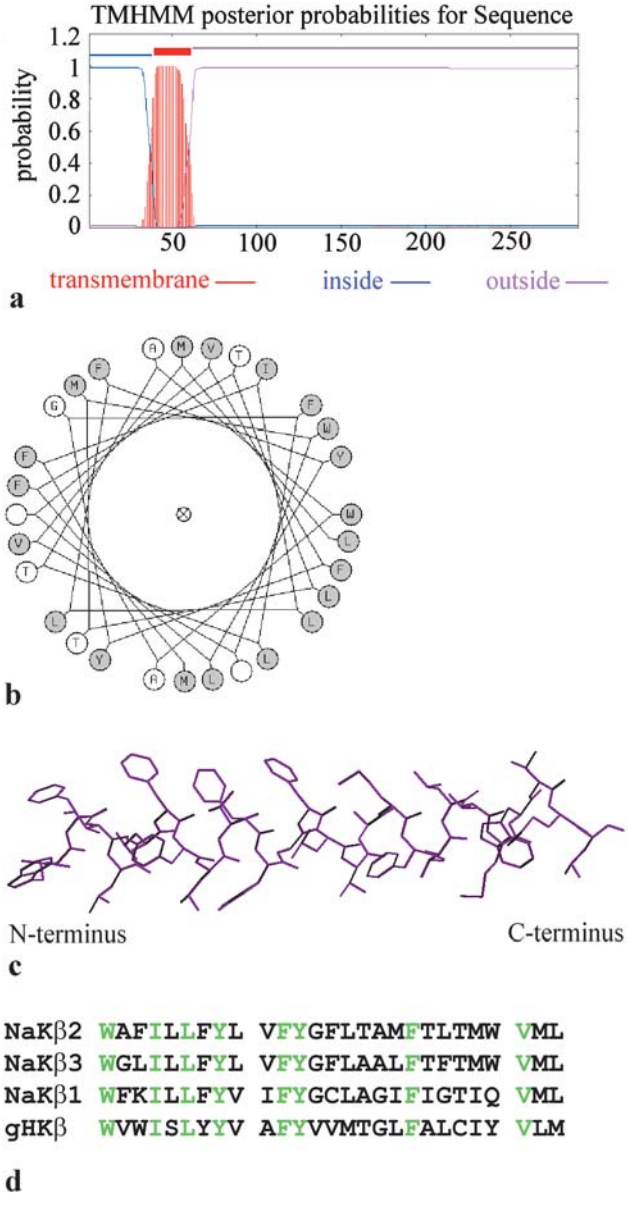

Fig. 6a–d Modeling of the transmembrane segment of NaKβ2. **a** The program TMHMM [9] was used to predict the transmembrane segment of NaKβ2, spanning amino acids 37 through 63. **b** Helical Wheel Custom Images and Interactive Java Applet (http://marqusee9.Berkeley.edu/kael/helical.htm) was used to draw a helical wheel plot for the 27 amino acids that span the membrane domain of the NaKβ2 subunit. **c** The program O [8] was used to build a model of the transmembrane helix of NaKβ2. In this view, the helix is seen from the side, with the intracellular end on the left and the extracellular end on the right. **d** Clustal W alignment of NaKβ2, NaKβ3, NaKβ1, and gHKβ. Conserved residues are highlighted in *green*

would allow for possible contact of the extracellular region of β with L7–8 of the α subunit, an interaction for which there is direct biochemical evidence. [19] A yeast-two-hybrid system coupled with alanine scanning mutagenesis was used to identify four particular amino acids, SYGQ, in L7–8 that were necessary for α and β interaction in the Na^+, K^+ ATPase. [19] The correspond-

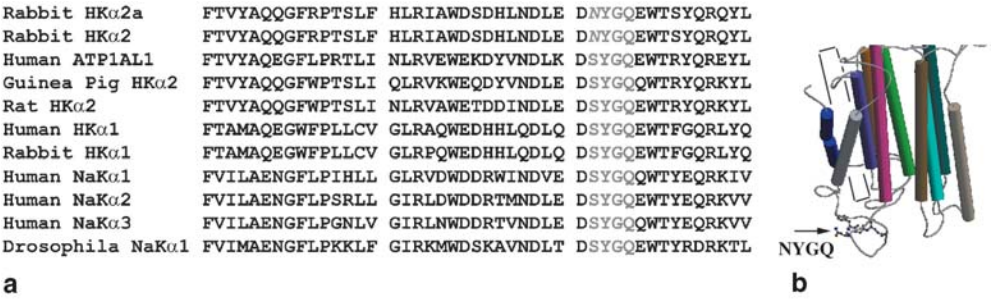

Fig. 7a–b A putative location for the β subunit of the H^+, K^+ ATPase. **a** Clustal W alignment of the L7–8 amino acid sequence of eleven Type II P-type ATPase α subunits. The conserved four amino acids are highlighted. **b** The four amino acids that have been shown to be necessary for interaction with the β subunit are highlighted in L7–8. The model of HKα2a pictured in Fig. 4 is shown here with a possible location for the β subunit indicated with *dashed lines*. Helix colors and designations are the same as described for Fig. 4

ing amino acids in the rabbit colonic H^+, K^+ ATPase are NYGQ as determined by our laboratory [20] and independently by Fejes-Toth et al. [21] (Fig. 7a). In contrast, the 9.5-Å resolution structure of the pig Na^+, K^+ ATPase by Hebert et al. [6] revealed density attributable to β near M8 and M10. Interaction of the transmembrane domain of the β subunit with M8 in the Na^+, K^+ ATPase was supported by cross-linking studies. [22] A model of NaKβ1 was recently built and used to place the β subunit at either of two places- near M7 and M10 or M8 and M10. [23] Importantly, the cross-section of electron density used to suggest proximity of β to M7 and M10 in the duck Na^+, K^+ ATPase structure was taken closer to the extracellular side of the membrane than the cross-section used to show β near M8 and M10 in the pig structure. We propose that the β subunit lies near M7 and M10 at the extracellular face and near M8 and M10 closer to the cytoplasmic surface in the HKα2a model (Fig. 7b).

Conclusions

We have generated a structural model for the HKα2a subunit of the rabbit colonic H^+, K^+ ATPase. The gross structural features and specific locations of functional features of the enzyme strongly resemble those of the P-type ATPases for which structures have been determined. The model of the colonic H^+, K^+ ATPase provides a basis for an understanding of the position of features such as the β subunit, the likely ouabain binding site, and the major intracellular domains, including the nucleotide binding site and phosphorylation site. A direct test of the model will require establishment of a functional expression system for the rabbit colonic H^+, K^+ ATPase.

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