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Elucidation of the Na^+, K^+-ATPase digitalis binding site

Susan M. Keenan [a], Robert K. DeLisle [b], William J. Welsh [a,*], Stefan Paula [c], William J. Ball Jr. [c]

[a] *Department of Pharmacology, University of Medicine and Dentistry, Robert Wood Johnson Medical School, Piscataway, NJ 08854, USA*
[b] *Pharmacopia, Box 5350, Princeton, NJ 08543, USA*
[c] *Department of Pharmacology and Cell Biophysics, University of Cincinnati, College of Medicine, Cincinnati, OH 45267, USA*

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Abstract

Despite controversy over their use and the potential for toxic side effects, cardiac glycosides have remained an important clinical component for the treatment for congestive heart failure (CHF) and supraventricular arrhythmias since the effects of *Digitalis purpurea* were first described in 1785. While there is a wealth of information available with regard to the effects of these drugs on their pharmacological receptor, the Na^+, K^+-ATPase, the exact molecular mechanism of digitalis binding and inhibition of the enzyme has remained elusive. In particular, the absence of structural knowledge about Na^+, K^+-ATPase has thwarted the development of improved therapeutic agents with larger therapeutic indices via rational drug design approaches.

Here, we propose a binding mode for digoxin and several analogues to the Na^+, K^+-ATPase. A 3D-structural model of the extracellular loop regions of the catalytic α1-subunit of the digitalis-sensitive sheep Na^+, K^+-ATPase was constructed from the crystal structure of an E_1Ca^{2+} conformation of the SERCA1a and a consensus orientation for digitalis binding was inferred from the in silico docking of a series of steroid-based cardiotonic compounds. Analyses of species-specific enzyme affinities for ouabain were also used to validate the model and, for the first time, propose a detailed model of the digitalis binding site.
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Keywords: Digitalis binding site; Na^+, K^+-ATPase structure; Congestive heart failure (CHF); Arrhythmia; Cardiac glycosides

1. Introduction

The Na^+, K^+-ATPase is a heterodimeric (designated, α- and β-subunits) transmembrane protein complex that serves as the receptor for cardiotonic steroids such as digitalis. Na^+, K^+-ATPase and other P_2-type ATPases use the hydrolysis of ATP to drive ion transport across cell membranes [1]. Alteration of intracellular sodium and potassium ion concentrations as a result of Na^+, K^+-ATPase regulation contributes to many essential physiological processes including maintenance of the membrane potential for muscle contraction. Cardiac glycoside binding inhibits the enzyme's ATPase and cation transport activity which, by raising Na^+ levels, indirectly results in the increase of intracellular Ca^{2+} levels, thus producing the drug's inotropic action. The inotropic effects of digitalis were first identified more than 200 years ago and, despite the development of newer pharmacological agents such as angiotensin-converting enzyme (ACE) inhibitors, angiotensin II receptor antagonists and β-blockers, the cardiac glycosides digoxin and digitoxin remain first-line agents in the treatment of CHF and supraventricular arrhythmias. There are two lingering concerns over the use of cardiac glycosides as therapeutic options: first, there remains controversy over their exact role in treatment [2] and, second, they have very narrow therapeutic ranges and therefore the high potential for considerable toxic side effects which include ventricular arrhythmias that can result in patient death [3–6].

Despite the long history of use and extensive body of literature on cardiac glycosides, a molecular-level understanding of the mechanism of drug–receptor binding and enzyme inhibition has remained elusive. The catalytic or α1-subunit of the enzyme contains the ATP and cation binding

* Corresponding author.
E-mail address: welshwj@umdnj.edu (W.J. Welsh).

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sites as well as the drug-binding site. It is now generally accepted that the α-subunit is topologically arranged into 10-transmembrane (TM) helices with the majority of its mass constituting the ATP binding and phosphorylation sites located on the intracellular side. The cardiac glycoside binding site is presumably located within the five-extracellular loops (TM1–TM2, TM3–TM4, etc.). Studies utilizing chimeras of individual Na^+, K^+-ATPase loop regions inserted into either the skeletal muscle sarcoplasmic reticulum/endoplasmic reticulum Ca^{2+}-ATPase (SERCA1a) [7,8] or the gastric H^+, K^+-ATPase [9,10] have identified the TM1–TM2, TM3–TM4 and TM5–TM6 extracellular loops as important for binding of the cardiac glycoside, ouabain. More precisely, mutagenesis studies have identified several amino acid residues (C104, Y108, Q111, P118, D121 and N122) found in the TM1 segment and TM1–TM2 loop as critically important for the high affinity binding of ouabain [11–13]. Furthermore, significant reduction in the binding affinity of ouabain [13–18] has been demonstrated subsequent to the alteration of individual residues in the TM5–TM6 loop (e.g., L793, T797); in the TM7–TM8 loop (e.g., R880); and other residues in the TM4, TM7 and TM10 segments [13–18]. Repke et al. [19] and Cerri et al. [20] have proposed as an alternative hypothesis that ouabain binds within the membrane along the TM1 and TM2 segments of two juxtaposed α-subunits of a dimeric $(\alpha\beta)_2$ protomer, but this model is inconsistent with much of the mutational data, and with both our recent 3D-quantitative structure–activity relationship (3D-QSAR) model developed using comparative molecular field analysis (CoMFA) of cardiotonic steroid inhibition of ATPase activity [21], and solid-state NMR results obtained using ouabain derivatives [22].

In the present study, we have aligned the sequences of the α1-subunit isoform of the digitalis-sensitive sheep Na^+, K^+-ATPase and SERCA1a and have developed a three-dimensional structural model of the extracellular loop regions by applying homology modeling techniques. Detailed analysis of published reports on species-specific inhibitor affinities and on the effects of site mutations on ouabain affinity has enabled us to propose the putative drug-binding site within the extracellular surface. By docking a series of cardiac glycosides onto the modeled extracellular surface of the α1-subunit, we were able to pinpoint a consensus drug-binding site that accommodates the lactone ring moiety oriented towards the TM1–TM2 loop and the sugar moieties directed towards the TM9–TM10 loop, with two to three likely hydrogen-bond interactions between the drug and receptor easily identifiable. Additional structural models of the cardiac glycoside-insensitive rat enzyme α1-subunit and mutated variants of sheep α1 reveal the possible loss of one or two hydrogen-bond interactions present in normal sheep and human α1-subunit models that consequently produced higher calculated drug–receptor binding energies. Results that are consistent with experimental data show diminished ouabain affinities for the rat α1 and these specific sheep α1 mutated variants. Furthermore, our work has identified two previously unrecognized amino acid residues as potentially important constituents of the digitalis binding site. These structural modeling and drug-docking studies provide the basis for future experimental testing of the effects of site-specific modifications of the α1-subunit and will enable the development of pharmacophore models that define the structural and spatial requisites for molecular recognition and binding of the drug by this receptor. Knowledge of the pharmacophore will offer guidance towards the rational de novo design of a new generation of inotropic agents with improved therapeutic indices.

1.1. Results and discussion

The propensity for toxicity resulting from the narrow therapeutic index associated with cardiac glycoside administration remains a considerable clinical concern. Unfortunately, delineation of the molecular mechanism of enzyme action and the design of novel, safer therapeutic agents has been thwarted by the lack of structural information for the digitalis receptor. While low-resolution structures (11 and 9 Å) of an E_2 form of Na^+, K^+-ATPase in tubular and 2D crystals by electron microscopy have been obtained [23,24], attainment of a high-resolution structure has proven exceedingly difficult due in part to the highly hydrophobic nature of the enzyme. Fortunately, the recently published crystal structure of an E_1Ca^{2+} conformation of the related P_2-type ATPase SERCA1a at 2.6 Å resolution [25] provides a structural template with which to build a three-dimensional model of the Na^+, K^+-ATPase α1-subunit using homology modeling techniques and, thereupon, to conduct a molecular-level investigation of the digitalis-Na^+, K^+-ATPase interactions. The E_1Ca^{2+} SERCA1a conformation rather than the more recently solved E_2 conformation [26] was employed to construct the homology model as cardiac glycosides under physiological conditions initially bind enzyme with moderate affinity as with the E_1Mg^{2+}, E_1Mg^{2+}ATP and $E_1Na^+Mg^{2+}$ATP conformations and then induce conformational changes that result in the stable, cardiac glycoside inhibited E_2 conformation [27,28].

1.2. Sequence alignment and model development

The first and inarguably the most important step in the structure modeling process involves alignment of the query protein's sequence to that of the template, thereby enabling structural alignment of specific residues in three-dimensional space. As the overall sequence identity between the Na^+, K^+-ATPase α1-subunit and SERCA1a is ∼25%, initial global alignments of the sheep Na^+, K^+-ATPase α1-subunit and SERCA1a were performed using PAM250 and Blosum35 matrices with heavy penalties for gap opening. While the alignments were significant and indeed improved upon the alignment previously reported [12] that used a Blosum62 matrix [29] and generated >18 gaps of three or more residues, the alignments did introduce gaps into several of

the putative transmembrane domains [13] and adjacent to essential functional domains. Therefore, strategies beyond standard residue-to-residue matching were undertaken. We employed the generalized alignment scheme known as "threading" that aligns the sequence of interest to libraries of known protein folds from resolved structures [30] to exploit both the known functional and structural similarities of the family of P_2-type ATPases. Alignment of the sheep Na^+, K^+-ATPase α1-subunit with SERCA1a (Fig. 1) in this manner revealed striking similarities in the local alignment of essential functional domains and generated only nine gaps of three or more residues, with the majority occurring in proposed loop regions. In particular, the threading technique conserves alignment of the sheep α1 regions such as the 369-DKTGTLT sequence that contains residue D369 which is the site for phosphoenzyme intermediate formation, the 501-KGAPE site of FITC-labeling within the nucleotide binding domain as well as the latter 701-TGDGVNDSPALKKA stretch of the P domain that is required for Mg^{2+} binding [31].

Similarities and differences in the transmembrane helical segments obtained by the present threading alignment and those predicted by others employing secondary structure predictors [32], hydrophobicity analysis [13,33,34] and Modeler4 [35] are compared in Table 1. While there is a high level of consistency among the methods, our analysis suggests a lengthened and slightly shifted TM1–TM2 loop and TM2 segment as well as a lengthened TM8 helix.

As the cardiotonic steroids are presumed to bind the extracellular side of the α1-subunit, we removed the intracellular mass of the protein and focused on five structurally conserved segments, which collectively form the extracellular portions of the 10 transmembrane helices and their connecting loops. In these regions, the corresponding sequences of sheep Na^+, K^+-ATPase α1 and rabbit SERCA1a are ∼33% identical. The coordinates of the structurally conserved regions of the sheep α1-subunit were assigned those of E_1Ca^{2+}SERCA then, where necessary, loops were generated that were sterically acceptable and resulted in reasonable phi-psi angles for the spliced regions. More details of the modeling and refinement procedures are given in Section 2. Ribbon representations of the cross-section and the extracellular side views of the final sheep model are shown in Fig. 2A and B, respectively. The resulting structural arrangements, as well as the locations of the transmembrane domains, are very similar to those proposed from the low-resolution structures of the Na^+, K^+-ATPase [23,24].

The plethora of available biological data allows for model validation. For example, our model places residues P118, T309, L793, M973 into extracellular loop regions, consistent with the results of Hu and Kaplan [36] who concluded on the basis of cysteine residue mutational studies that these four residues are solvent exposed on the extracellular surface. Furthermore, in agreement with patterns found for other transmembrane proteins, our model locates a majority of tryptophan and lysine residues close to the proposed membrane surfaces with most of the tryptophan residues on the extracellular surface and lysine residues on the intracellular surface (Fig. 2A) [37,38].

1.3. Elucidation of the cardiac glycoside binding mode

Having constructed a 3D-model of the extracellular arrangement of the sheep α1-subunit, we next sought to elucidate the site and possible mode(s) of cardiac glycoside binding. We systematically docked digoxin and eight other cardiac glycoside steroids onto the surface of the α1-subunit using Genetic Optimization for Ligand Docking (GOLD) [39]. GOLD is a ligand-docking application that utilizes a genetic algorithm (GA) to explore ligand conformation and orientation, and satisfy ligand-binding requirements. One advantage of GOLD over many other docking algorithms is that it allows for both unconstrained ligand flexibility and partial flexibility of the binding pocket, thus, affording a more realistic environment for ligand–receptor associations. Specifically, the geometrical position of S-, T- and Y-hydroxyl groups and $K–NH_3^+$ hydrogen atoms of the protein are optimized during the GOLD run. Furthermore, as compared to deterministic algorithms, the stochastic nature of GOLD ensures that the search space is well explored and local energy extremes are less influential.

We selected a subset of nine glycoside Na^+, K^+-ATPase inhibitors whose potencies have been previously determined in our laboratory (Table 2; Fig. 3) [21]. We chose these molecules for three reasons: (1) they are amongst those most commonly used in the clinic and for research; (2) they exhibit a reasonable degree of structural diversity; (3) they all possess high but varying binding affinities. Each inhibitor was docked a total of 25 times independently within a 12-Å radius binding sphere. Similar results were obtained using a 10-Å radius sphere, but a 15-Å radius sphere proved excessively large and yielded unrealistic orientations in which the molecules docked perpendicular to the surface of the enzyme. From the results generated with the 12-Å radius sphere, we determined the consensus binding mode for each inhibitor from the ensemble of 25 independent dockings. GOLD scores each binding mode using a fitness function that accounts for the steric and electrostatic complementarity between the ligand and receptor. For each of the nine ligands, we first determined the consensus binding conformation for each ligand, and then selected the conformation from each consensus binding operation corresponding to the highest fitness score for further analysis. Remarkably, from these independent docking procedures, a single consensus binding mode emerged. Our docking studies show that all the ligands fully occupy a groove in the surface of the α1-subunit and in fact would appear to essentially block the occlusion site. Consistent with the orientation proposed by Lingrel et al. [13], our consensus binding mode places the lactone ring oriented toward TM1–TM2, the steroid moiety between TM2 and

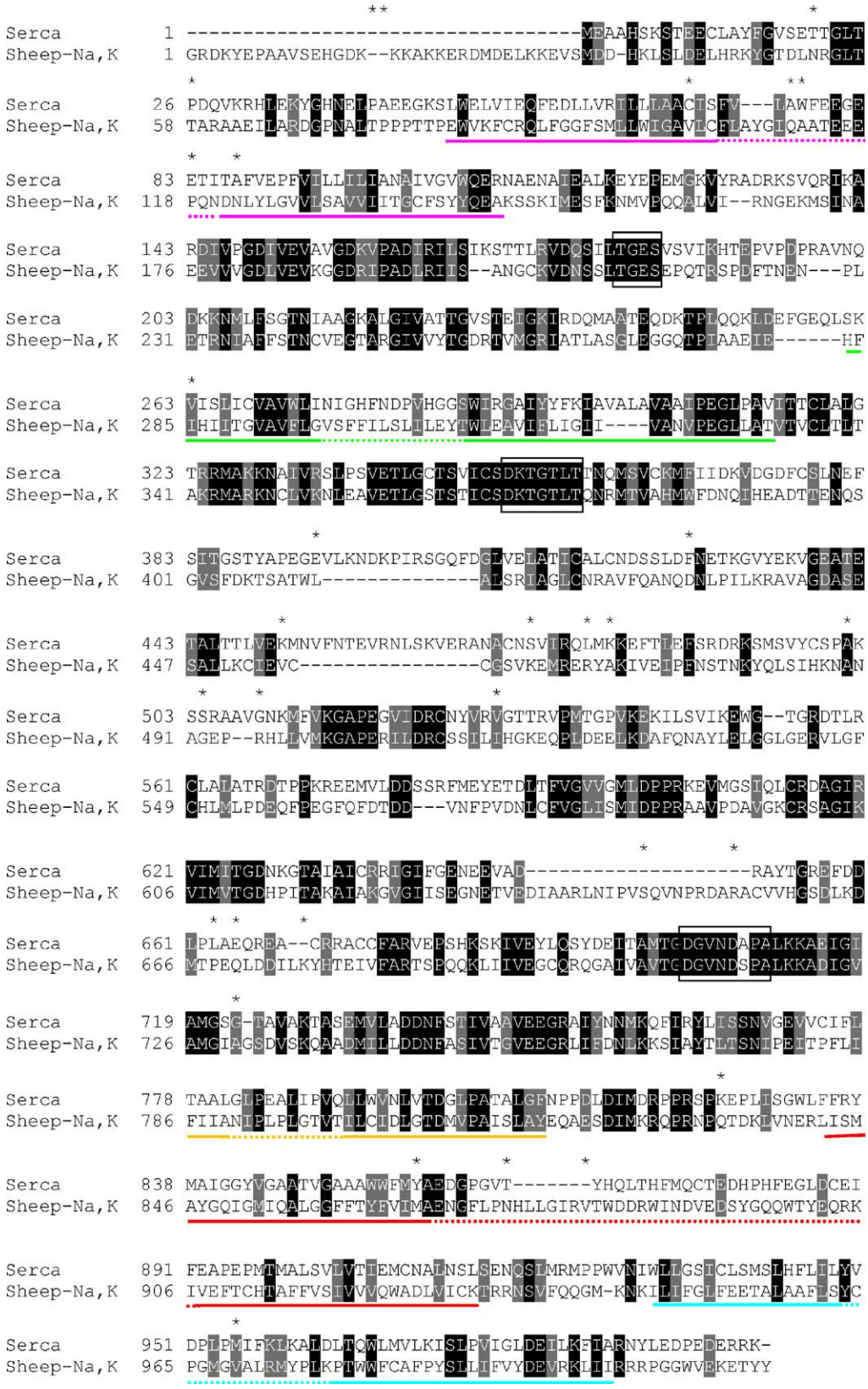

Fig. 1. Alignment of the sheep 1α-subunit sequence of Na^+, K^+-ATPase with SERCA1a. The alignment shown is the result of "threading" the Na^+, K^+-ATPase sequence to that of the template. Identical residues between the two sequences are illustrated with filled black boxes, while similar residues correspond to the filled gray boxes. Regions modeled are noted by colored lines and the colors correspond to the helices and loops depicted in Fig. 2A and B. The solid lines represent helical structures, while the broken lines indicate loop regions. Local areas of high identity correlating with functional motifs are shown by open black boxes and described in the text. Positions of sequence changes between the sheep and rat Na^+, K^+-ATPases are denoted with an asterisk (*).

Table 1
Delineation of the extracellular portions of the transmembrane domains and the extracellular loops

Loops	Threading	Hydropathy	Structure	Modeler4
1–2	105–121	110–123	110–123	
3–4	296–308	304–311	305–314	–313
5–6	789–799	791–796	791–800	795–802
7–8	867–906	865–909	866–914	866–914
9–10	963–977	970–975	964–978	970–

Our threading alignment is compared to the hydropathy predictions by Lingrel et al. [13], secondary structure-based predictions [32] and Modeler4 [35].

TM4, and TM5 and TM6, and the sugar units directed toward TM9–TM10 loop. For compounds with multiple (bis- and tri-) sugar units, these moieties appear to interact with the TM7–TM8 loop. The overall alignment of the nine ligands representing the consensus binding mode is illustrated in Fig. 4A and B and, as an example for an individual compound, the alignment of the 25 independent docking conformations of ouabain is shown in Fig. 4C. The increased bulk of thevetin B that arises from its particular arrangement of the tri-sugar, thevetose and gentiobiose can be observed in Fig. 4A and B and it should be noted that even this molecule's lactone ring and α-sugar maintains the consensus binding orientation.

The results from our docking studies lead to several key observations. First, the consensus binding mode reveals a highly conserved orientation in which the lactone ring and α-sugar alignments are highly restricted and remains consistent across this series of inhibitors. Second, there is appreciable variation in the orientations of the steroid backbones. While the hydrophobic nature of the steroid moiety is crucial for drug binding, the model does not show any obvious aromatic groups (Y, W or F) located within the binding groove about the hydrophobic steroid. A plausible explanation is that these results illustrate the inherent flexibility of this region of the inhibitor. Ligand flexibility as well as that of the enzyme may be essential to allow for the enzyme to proceed through a series of ligand-induced conformational changes. In fact, a wide variety of modifications to the steroid rings have little impact upon drug binding affinities and ATPase activity inhibition [21 and unpublished data]. These data suggest that it should be possible to design a new class of inhibitors containing an alternative (e.g., non-steroid) group that mimics the size, shape and hydrophobicity of this steroid moiety. Third, only α-sugars appear to influence the consensus binding mode as broad flexibility in the positioning of the β- and γ-sugars is observed. For example, digoxin (three sugars) and digoxigenin bisdigitoxose (digoxin, except with two sugars) possess identical binding orientations (Fig. 4D). These data are consistent with the knowledge that these sugars, while adding considerable bulk, actually have little or no effect on enzyme inhibition [21]. Finally, our ligand binding mode indicates that the bound cardiac glycosides are clearly in a position to alter the

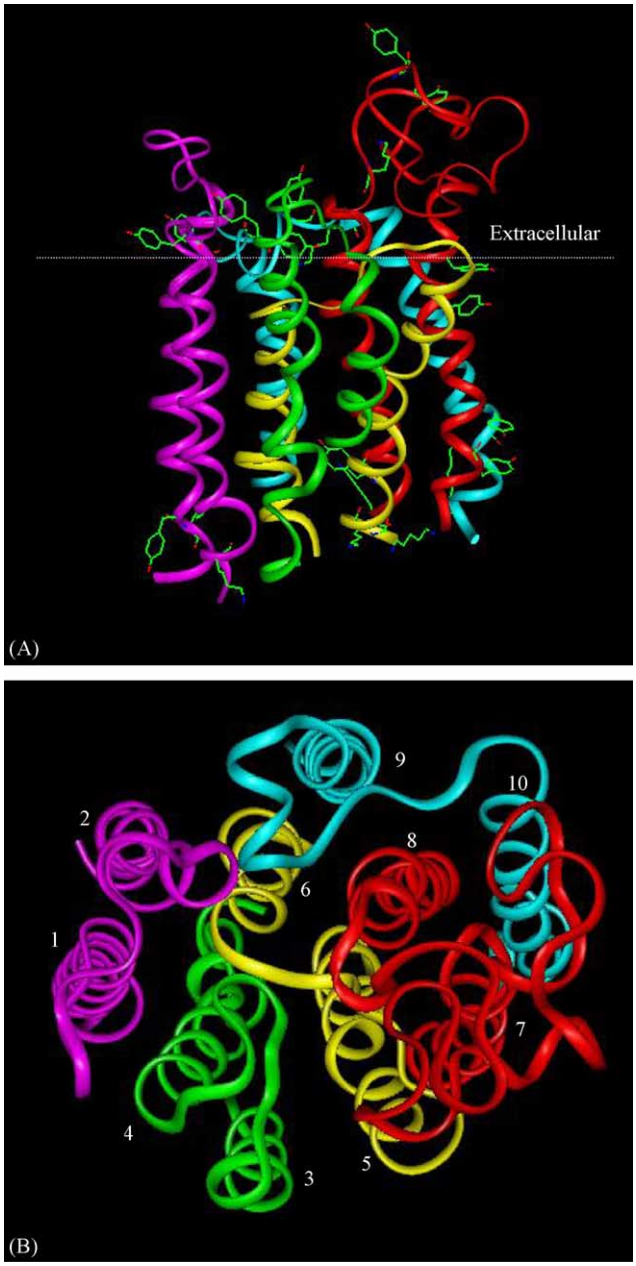

Fig. 2. Representation of the extracellular loops and the transmembrane domain regions of the sheep α1-subunit of Na^+, K^+-ATPase. Visual representation of the three-dimensional structure as obtained by homology modeling (A), and representation of the α-helices shown from the extracellular domain (B). Only alpha carbons are depicted. The numbering and orientation of the helices are consistent with the information obtained from low-resolution crystal structures. Tryptophan and lysine residues are displayed (A).

conformational relationship between TM helices that have been proposed to generate the Na^+ and K^+ binding and occlusion sites within the membrane. Furthermore, cardiac glycoside binding in this mode would also be expected to interfere and/or compete with the extracellular K^+ binding site but not the intracellular Na^+ sites, a phenomenon which has been demonstrated experimentally [27].

Table 2
Cardiac glycosides selected for present docking study

Cardiac glycoside	Na^+, K^+-ATPase relative inhibition[a]
Digoxin	1.0 ± 0.1
Digoxigenin bisdigitoxose	0.63 ± 0.06
Digoxigenin monodigitoxose	0.44 ± 0.02
Digitoxin	0.24 ± 0.04
Digitoxigenin bisdigitoxose	0.15 ± 0.01
Digitoxigenin monodigitoxose	0.22 ± 0.02
Ouabain	1.0 ± 0.1
Proscillaridin A	0.13 ± 0.002
Thevetin B	0.81 ± 0.05

[a] Relative inhibition values were determined previously in our laboratory [21].

The consensus binding mode is consistent with our three-dimensional quantitative structure–activity relationship models [21] constructed from a much larger series of cardioactive steroid hormones. Our model is also compatible with that proposed by Lingrel et al. [13], but differs from those proposed by Middleton et al. [22] and Antolovic et al. [40] which position ouabain in an essentially reverse orientation with its α-sugar oriented to toward the TM1–TM2 loop and in close proximity to C104 of the TM1 segment and the lactone ring adjacent to the extracellular edges of the TM5 and TM7 segments. Middleton et al. [22] acknowledge, however, that their model is at odds with their own 2D-NMR data that argues for only a loose association between the enzyme and terminal sugars that would not place the α-sugar into the membrane near C104.

1.4. Specific interactions between ouabain and the ATPase binding site

Besides shape complementarity, another important factor that influences the Gibb's free energy (ΔG) of small molecule–receptor complex formation is hydrogen bonding. To explore the possible role of hydrogen bonds in cardiac glycoside–receptor binding, we systematically measured the distances of all potential hydrogen bond donor and acceptor atoms of the docked ligands and residues comprising the α1-subunit binding pocket using the standards adopted by Kabsch and Sander [41]. This analysis locates a potential hydrogen bond between the keto-group of the lactone ring and the side-chain of α1 residue Q111, consistent with the ∼10-fold loss in sensitivity to ouabain observed for the Q111R mutant versus the wild-type sheep enzyme [42]. Our analysis also identifies a hydrogen bond between the α-sugar hydroxyl group and D121, consistent with the 1000-fold loss in sensitivity to ouabain found for the D121N mutant versus the wild-type sheep α1-subunit [43,44]. The indication that residue D121 of the TM1–TM2 loop may hydrogen bond with the α-sugar appears to be in conflict with the interpretation by O'Brien et al. [45] whose results following an investigation of ouabain and ouabagenin inhibition of sheep α1 mutant enzymes' activity indicated that the α-sugar does not interact with TM1–TM2 loop residues. Their work, however, is inconclusive since ATPase activity inhibition shows little sensitivity to the presence of the sugars. Indeed, recent studies in our laboratory show insignificant differences in the IC_{50} values of several aglycones and their monodigitoxoses.

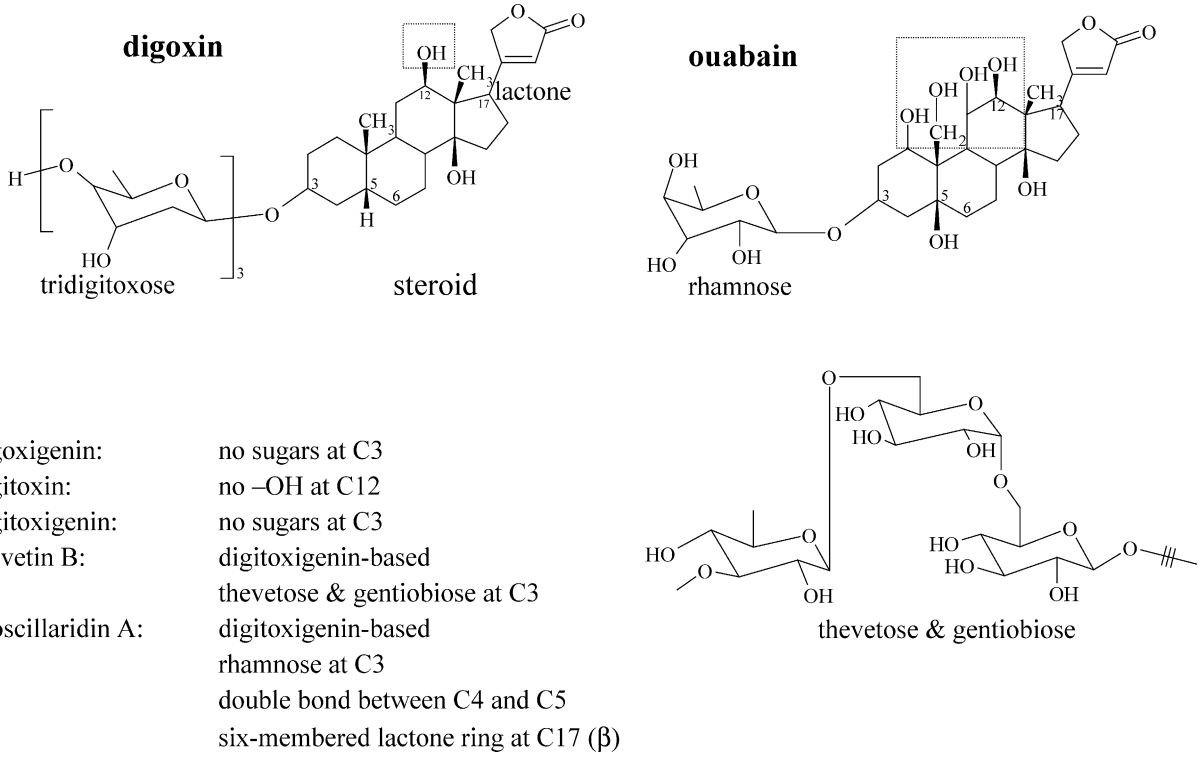

Fig. 3. Cardiac glycoside structures. Structural drawings of the cardiac glycosides discussed in this report.

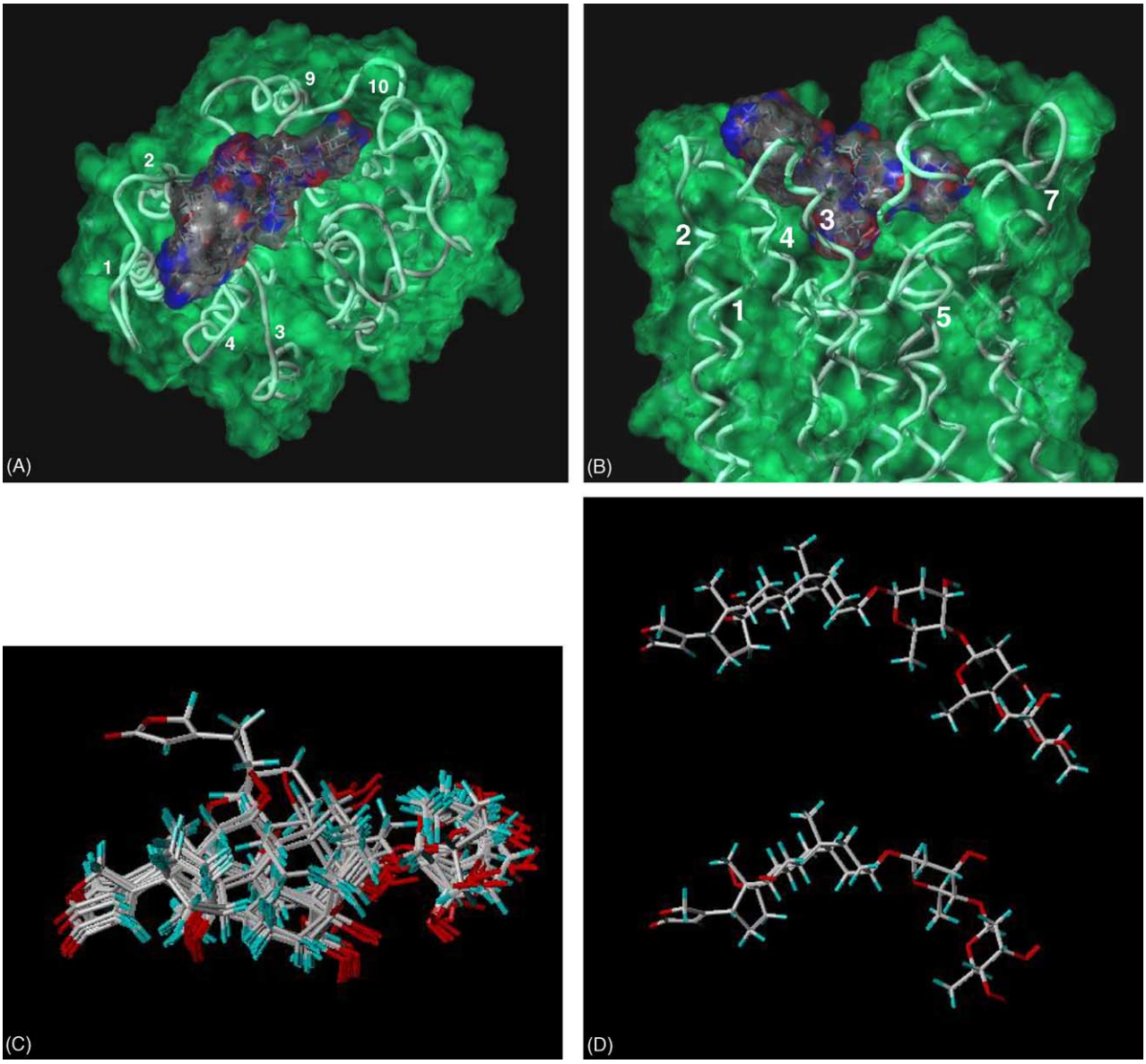

Fig. 4. Binding orientation of cardiac glycosides. The extracellular (A) and cross-section (B) representation of the consensus orientation for all nine docked cardiac glycosides. Both Na^+, K^+ATPase (green) and ligands are depicted with Connelly surfaces. Hydrogen bond donor (blue) and acceptor (red) atoms/groups are highlighted. Consensus orientation of 25 individual dockings of ouabain to the 1α-subunit of the sheep Na^+, K^+-ATPase (C). Orientation of digoxin (D, upper) and digoxigenin bisdigitoxose (D, lower) illustrating the identical conformation regardless of the number of sugar moieties.

Interestingly, our binding model suggests that the side chain of E908 and the backbone nitrogen of M973 interact with the α-sugar and are, respectively, hydrogen bond acceptors and donors, and thus potentially contribute to ouabain's high binding affinity (Fig. 5A).

1.5. Effects of α1 mutations on sensitivity to ouabain

Sheep, human and rat sequences for the 1α-subunit of the Na^+, K^+-ATPase differ sequentially by only ∼3%, yet modest single or double residue substitutions can result in extraordinary variability in the enzyme's sensitivity to ouabain inhibition. For example, the wild-type sheep- and human α1-subunits are highly sensitive to ouabain inhibition while the rat α1-subunit is essentially ouabain insensitive (∼1000-fold less sensitivity than the sheep or human subunits). Two key residues, Q111 and N122, are invariant in the sheep and human subunits but are, respectively, mutated to arginine (R) and aspartic acid (D) in the rat. As mentioned earlier, incorporating the single mutation Q111R into the wild-type sheep 1α results in an ∼10-fold decrease in ouabain inhibition, while expression of the D121N or the Q111R, N122D double mutation diminishes ouabain inhibition by ∼1000 fold (reviewed in [13]; Table 3)

To explore these observations within the context of our computational analysis, we constructed structural models of

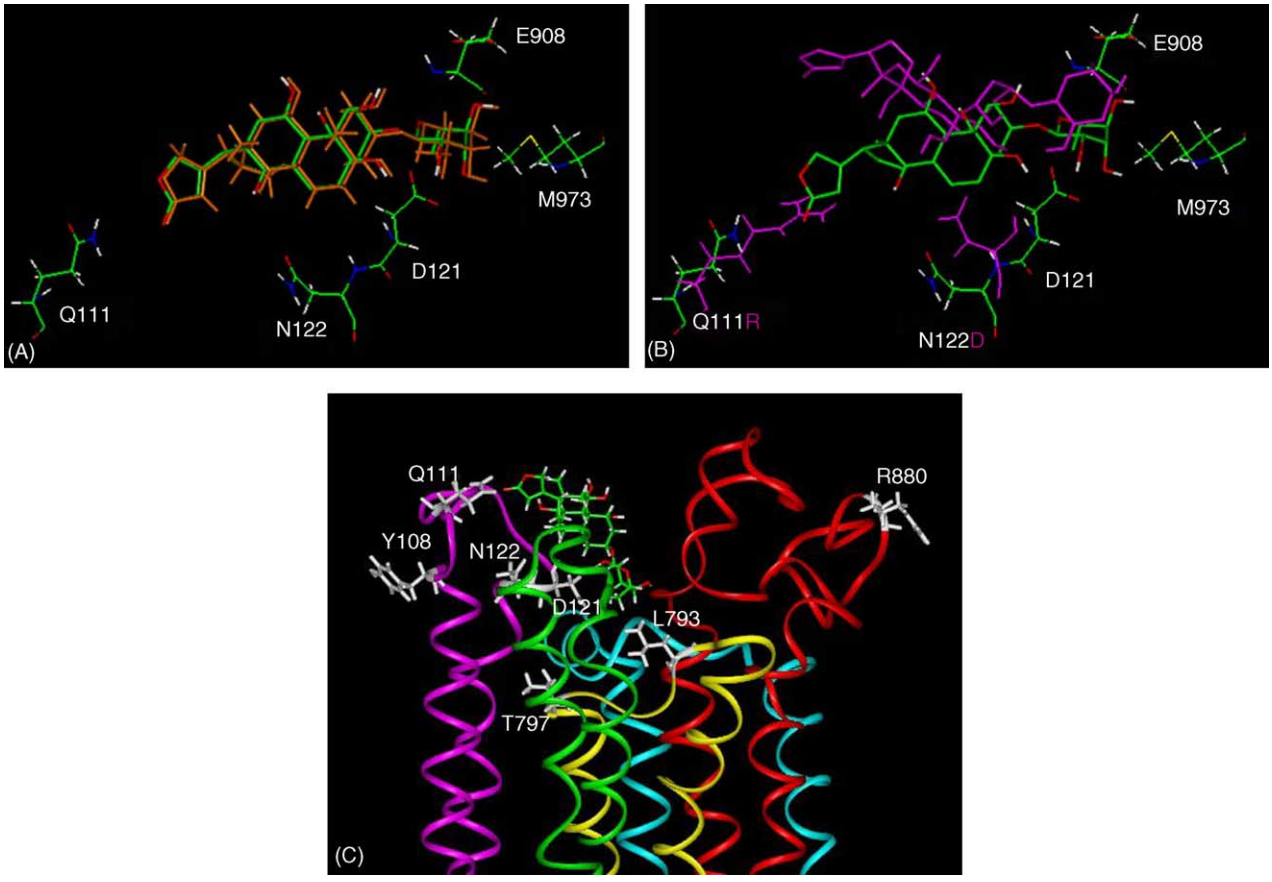

Fig. 5. Comparison of docking orientations of ouabain. Consensus orientation of ouabain docked to the sheep α1-subunit of Na^+, K^+-ATPase (colored by atom-type) and human α1-subunit of Na^+, K^+-ATPase (orange) (A); orientation of ouabain docked to the sheep α1-subunit of Na^+, K^+-ATPase (colored by atom-type) and the rat α1-subunit of Na^+, K^+-ATPase (magenta) (B). The residues identified in both figures correspond to the sheep α1-subunit Na^+, K^+-ATPase sequence. In the rat α1-subunit, Q111 is mutated to arginine and D122 to aspartic acid; summary of the location of mutations discussed in this report (C).

the human- and rat α1-subunits. Similarly, we constructed models of the sheep 1α-subunit individually expressing the point mutations described above. Docking studies with ouabain were then repeated for the human, rat and mutant sheep structures. The consensus binding orientation of ouabain to the human 1α-subunit model was identical to that observed for the sheep ATPase. In contrast, incorporation of the single-point mutation Q111R in the sheep 1α-subunit model resulted in reorientation of the glycoside's substituted lactone moiety from the TM1–TM2 loop toward the TM3–TM4 loop and the loss of at least one hydrogen bond.

Table 3
Calculated relative (Rel.) changes in calculated binding energies ($\Delta E_{binding}$)

Species/mutant[a]	Rel. inhibition	Rel.[b] $\Delta E_{binding}$ (Kcal/mol)
Sheep	1	0
Rat	1000	14
Human	1	1.5
Q111R	10	4.2
Q111R, N122D	1000	11.4
E908L	Unknown	7.0
M973A	Unknown	5.6

[a] Mutation of sheep structure.
[b] Relative to sheep structure.

Furthermore, mutated residues in both the modeled rat α1-subunit and the Q111R, N122D mutant sheep, result in ouabain being completely dislodged from the consensus orientation observed with the human and sheep wild type subunits, consistent with the ∼1000-fold decrease in the inhibitory effects of ouabain found by experiment [13]. For the sake of clarity, we show only the orientations obtained for ouabain docked to the sheep 1α-subunit versus either the human-ouabain sensitive (Fig. 5A) or rat-ouabain insensitive α1-subunits (Fig. 5B).

The degree of consistency between the present computational findings and experimental evidence as to the species specificity and influence of mutations on ouabain sensitivity provides compelling support for the validity of the proposed Na^+, K^+-ATPase α1-subunit structural model and, more precisely, the putative digitalis binding site.

1.6. Calculated binding energies reflect mutational effects on ouabain sensitivity

The mutational effects with respect to ouabain sensitivity, described above, can be further explored computationally since a decrease in receptor sensitivity to an inhibitor often

reflects diminished binding affinity. As such, changes in binding energies ($\Delta E_{binding}$) associated with various ouabain–enzyme interactions resulting from the docking studies were calculated and then compared with available inhibition data. Values of $\Delta E_{binding}$ were computed as the difference in calculated energy between the ouabain–receptor complex ($E_{complex}$) and the sum of the energies calculated separately for the free ligand (E_{ligand}) and the free receptor ($E_{receptor}$). Details of the calculations are provided in Section 2. The molecular mechanics calculations included non-bonded van der Waals (vdW) and Coulombic energy terms and the electrostatic component of solvation. The $\Delta E_{binding}$ data (Table 3) show a strong statistical correlation with the experimentally derived inhibitory effects of the mutants (Pearson correlation $r = 0.96$). Calculated values of $\Delta E_{binding}$ for the wild-type sheep- and human α1-subunits are consistent with their nearly equivalent ouabain affinity. Furthermore, introduction of the Q111R mutation which is known to lower ouabain's affinity for the sheep enzyme by ∼10-fold [42] resulted in a corresponding decrease in the calculated binding affinity. Calculations of ouabain $\Delta E_{binding}$ values for the rat α1 or the double mutant (Q111R, N122D) [43,44] form of the sheep α1 model yielded results consistent with their dramatically lowered sensitivities to ouabain inhibition [13].

As the results of our molecular modeling studies have implicated two additional residues as important components of the digitalis binding site, we sought to further investigate the role of these amino-acid residues with respect to ouabain binding. While in vitro mutation studies are beyond the scope of the present study, we simulated these mutations in silico to determine the influence of these residues on the affinity of ouabain. Mutant sheep α1-subunits were constructed incorporating either a E908L or M973A mutation and docking analyses with ouabain were performed. Then the consensus orientation of ouabain binding to each of these models was obtained as described above and selected for further analysis. Interestingly, the consensus orientation observed from the docking ensemble differed from the consensus orientation of ouabain bound to the wild-type sheep enzyme suggesting that these residues each independently play a role in ouabain binding. Values of $\Delta E_{binding}$ were calculated (Table 3) and compared with the $\Delta E_{binding}$ values calculated for the wild-type and mutant sheep-, rat- and human enzymes. The results indicate that both mutants could have a large impact on ouabain binding affinity.

1.7. Concluding remarks

The results of our structural modeling and docking studies provide strong and consistent evidence that the cardiac glycosides bind in a shallow groove between TM1–TM2, TM5–TM6 and TM9–TM10 loops of the α-subunit of Na^+, K^+-ATPase. The consensus docking model identifies several residues that appear essential for cardiac glycoside binding: Q111, D121, E908 and M973. The latter two residues have not thus far been identified as important for high-affinity digitalis binding; therefore, we conducted molecular modeling studies to investigate the structural effects of mutating E908 and M973. The results of this study offer credence to our hypothesis, and encourage in vitro mutation analyses for confirmation. Our binding-site model represents an excellent starting point for further investigations of the receptor's structure and drug binding. To understand the effects of conformational changes of the enzyme on digitalis–enzyme interactions, we are currently modeling the digitalis binding site based on the recently solved E_2-thapsigarin Ca^{+2} free form of SERCA1a [26]. Another important avenue of interest is to analyze the binding mode of aglycones and compare the results to the present study.

The results presented here provide insight into the mode of cardiac glycoside binding and, therefore, offer guidance for the design of novel inhibitors of the Na^+, K^+-ATPase. Efforts are currently underway to use the knowledge gained of the proposed digitalis binding site to construct three-dimensional pharmacophore models that delineate the structural prerequisites for molecular recognition and high affinity binding of the drug by the ATPase receptor. These models will hopefully permit the structure-based drug design of a new generation of therapeutic Na^+K^+-ATPase inhibitors possessing an improved pharmacological profile as compared with digoxin and digitoxin.

2. Methods

2.1. Sequence slignment

The sequences of the α1-subunits of the human, sheep, and rat Na^+, K^+-ATPases were obtained from GenBank (Accessions nos. P05023, P04074 and P06685, respectively). The SERCA1a crystal structure (1EUL) was obtained from RCSB-PDB and the sequence was extracted using Insight II (Accelrys, Inc., San Diego, CA). The sequence alignment between SERCA1a and the α1-subunit of the sheep Na^+, K^+-ATPase was deduced from matrix-based (PAM250 and Blosum35) approaches (www.ebi.ac.uk/clustalw/) and by threading the sheep sequence to the structural features of SERCA1a using THREADER (http://bioinf.cs.ucl.ac.uk) from University College London and the resulting alignment was further manually refined. The amino acid identity resulting from this alignment is 25.6% and all local areas of structural and functional importance are well conserved. The extracellular gaps resulting from the alignment are localized to loop regions (TM1–TM2 and TM7–TM8) and are thus easily accommodated.

2.2. Homology modeling

All molecular modeling operations were carried out using InsightII and related modules (Accelrys, Inc.). The three-

dimensional coordinates of the extracellular and transmembrane domains of the α1 subunit of sheep Na^+, K^+-ATPase were assigned. Undefined loops and gaps in the aligned sequences were accommodated by random generation of intervening sequences while conserving the integrity of backbone orientations in structurally defined, flanking residues. Inclusion of the randomly modeled regions required splice site relaxation in order to allow the protein backbone to adopt appropriate psi/phi angles. This operation was completed by relaxing the backbone atoms of those residues involved in splice sites and subjecting the molecule to a localized energy minimization procedure. After generation of the model, the side chain residues were checked for acceptable rotomer positioning using the internal rotomer library and steric clashes were resolved by preliminary energy minimization of the entire molecule (250 iterations, steepest descent). Finally, the model was subjected to a full energy minimization through a series of independent steps in which hydrogen atoms, side chains, backbone atoms, and ultimately, the entire molecule were successively allowed freedom of movement. Similarly, the human and rat sequences were aligned to the sheep sequence, and then modeled and refined as described above. Point mutations as described in the text were incorporated into the fully refined sheep structure, and the resulting mutants were again energy minimized through the steps discussed above. This procedure implemented the CVFF force field with a distance dependent dielectric function ($\varepsilon = \varepsilon_o r$, with $\varepsilon_o = 4$) until the convergence criterion of 0.04184 kJ/mol (0.01 kcal/mol) change in energy between successive iterations was achieved. The dielectric constant $\varepsilon_o = 4$ was chosen as it corresponds to that of water in a fast-moving dynamic field.

2.3. GOLD docking

The 3D structure of each cardiotonic compound was built using the molecular fragment library of Sybyl 6.6 (Tripos, Inc., St. Louis, MO) and based on the structure of digoxin as determined by X-ray crystallography [46]. The conformation of the β- and γ-sugars was obtained by global energy minimization determinations. As previously described [21], the final structural models were energy minimized using the Tripos force field with the Gasteiger–Marsili method for assigning partial atomic charges. The GOLD operator usage probabilities for crossover, mutation and migration were set to 95%, 95% and 10%, respectively. The maximum distance between hydrogen bond donors and fitting points was set to 5 Å, and the non-bonded cutoff for the van der Waals energies was adjusted to the developer's recommended settings ($10k_{ij}$ = well depth of the van der Waals energy of the atom pair i, j). The ligand orientation accepted was the top scoring consensus orientation of 25 independent genetic algorithm (GA) runs, each with a maximum number of 1000 GA operations performed on a single population of 100 individuals. The selection pressure was 1.1. The centroid of the binding sphere was determined as the intersection of the vectors connecting TM1–TM2 with TM5–TM6, TM1–TM2 with TM9–TM10 and TM5–TM6 with TM9–TM10.

2.4. Binding energy calculations

We define the change in binding energy ($\Delta E_{binding}$) as the difference between the potential energy of the ouabain–receptor complex ($E_{complex}$) and the sum of potential energies of the ligand (E_{ligand}) and receptor ($E_{receptor}$).

$$\Delta E_{binding} = E_{complex} - (E_{ligand} + E_{receptor})$$

Each energy calculation represents the summation of non-bonded energy terms, and the effects of solvation.

$$\Delta E_{total} = E_{Coulombic} + E_{van\ der\ Waals} + E_{solvation}$$

Energy values represent the minimum potential energy resulting from the optimization procedure described above. Ouabain was allowed full freedom of movement throughout the process. Various orientations of ouabain with respect to the ATPase were obtained by combining the consensus orientations of ouabain as determined by the GOLD docking, with the appropriate homology modeled enzyme. The linear Poisson–Boltzmann equation was solved to obtain the electrostatic contributions of the solvation energy (reaction field energy). All energy values were calculated using DelPhi [47,48].

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