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REVIEW ARTICLE

MODELING OF THE THREE-DIMENSIONAL STRUCTURE OF THE DIGITALIS INTERCALATING MATRIX IN Na^+/K^+-ATPase PROTODIMER

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Based on the knowledge that the digitalis receptor site in Na^+/K^+-ATPase is the interface between two interacting α-subunits of the protodimer $(\alpha\beta)_2$, the present review makes an approach towards modeling the three-dimensional structure of the digitalis intercalating matrix by exploiting the information on: the primary structure and predicted membrane topology of the catalytic α-subunit; the determinants of the secondary, tertiary and quaternary structure of the membrane-spanning protein domains; the impact of mutational amino acid substitutions on the affinity of digitalis compounds, and the structural characteristics in potent representatives. The designed model proves its validity by allowing quantitative interpretations of the contributions of distinct amino acid side chains to the special bondings of the three structural elements of digitalis compounds.

KEY WORDS: Na^+/K^+-transporting ATPase, digitalis intercalating matrix, three-dimensional structure, digitalis affinity determinants

WAY OF LOOKING AT THE PROBLEM

The goal of the present review includes, in parallel, modeling of the three-dimensional structure of the digitalis binding site in the receptor enzyme Na^+/K^+-ATPase and the search for the relationship between the essential structural features of both the digitalis binding matrix and the potent enzyme inhibitors of the digitalis type. The difficulties to be overcome emerge from the long prehistory of this endeavour.

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Abbreviations: Na^+/K^+-ATPase, Na^+/K^+-transporting adenosine triphosphate phosphohydrolase (EC 3.6.1.37); digitalis, generic name of steroids inhibiting Na^+/K^+-ATPase by intercalation in the digitalis binding cleft.

In 1973, we deduced the hypothesis that Na^+/K^+-ATPase works in the membrane in the form of a dimer of two functionally identical subunits.[1] In 1983, Askari and coworkers[2] confirmed by chemical cross-linking studies that Na^+/K^+-ATPase exists in the plasma membrane as a noncovalent dimer of the α, β-protomer, i.e., as the protodimer $(\alpha\beta)_2$. Their findings that enzyme phosphorylation from ATP favoured the formation of the cross-linked α, α-dimer, and enzyme ligation with K^+ and ATP reduced cross-linking, indicated function-linked changes in the juxtapositions of the appropriate reactive groups at the α, α-domain of two associated α, β-protomers. In 1985, our group[3] derived from various findings that the digitalis binding matrix in Na^+/K^+-ATPase protein is the cleft between two neighboring lobes of the catalytic α-chain that communicates with the extracellular space and is approximately 20 Å deep. We further concluded that the interactive energy surface becomes locked on intercalation of cardiac glycoside so as to envelope it: its lactone side chain lying at the bottom of the cleft, its steroid nucleus, and its sugar residue bound next to the steroid carrier, lying near the mouth of the cleft.

Remarkably, the newer sets of information published between 1989 and 1994 have uncovered that the deduced cleft for inhibitory digitalis intercalation is provided by the interface between two interacting α-subunits in the Na^+/K^+-ATPase protodimer $(\alpha, \beta)_2$ (for a recent review cf.[4]). Specifically, it has been shown that ATP, through enzyme phosphorylation, favours protomer-protomer association and that ouabain, through interprotodimeric intercalation, makes the protomer association much stronger and enormously stabilizes the $(\alpha, \beta)_2$ protodimer structure.

At first overlooking this progress, we developed in 1993 alternative considerations[5] on the potential nature of the digitalis recognition and binding matrix. They were guided by tentative interpretations of the impressive changes in inhibitory digitalis potency associated with: primary amino acid sequence variations in some isoforms of Na^+/K^+-ATPase, site-specific mutational amino acid substitutions, or various chimeric constructs of the enzyme (for a recent review cf.[6]). As a word of caution, rationalizing our 1993 endeavour,[5] we added then that all interpretations derived from these types of information are severely hampered by the knowledge that localized sequence changes can exert long-range effects on the global behaviour of enzymes including delocalized structural reorganizations, which render topological interpretations difficult.[5] In the same vein, Lingrel[7] summarizing in 1993 the excellent work done by him and his group as well as by several others, reservedly concluded that any amino acid substitution could have a subtle effect on digitalis affinity to Na^+/K^+-ATPase, which would be difficult to predict or explain without knowledge of the three-dimensional structure of the enzyme. Indeed, this has also been our belief[3,8] behind our present endeavor in that direction. It has been rendered possible through the significant progress reached in the elucidation of the topological determinants of the tertiary and quaternary structure of membrane-spanning proteins.[9–13]

MODELING THE THREE-DIMENSIONAL STRUCTURE OF THE DIGITALIS BINDING CLEFT

Since 1979, various types of analysis have supported oligomeric models for Na^+/K^+-ATPase-driven Na^+/K^+ transport with an $(\alpha, \beta)_2$ diprotomeric structure being favored.[14–20]

More recently, studies with a series of Na^+/K^+-ATPase-H^+/K^+-ATPase chimeras[21] have shown that a cytoplasmic mid region in the α-subunit of Na^+/K^+-ATPase (arginine 350 – proline 785) is necessary for specific, stable α, α-association, whereas the N-terminal and C-terminal transmembrane regions alone are incapable in supporting stable α, α-association. Chemical cross-linking studies with proteolytic fragments of Na^+/K^+-ATPase have likewise indicated α, α-interaction in the C-terminal side of alanine 439.[22] The process of oligomerization appears to be completed by the specific association of complementary surfaces of adjoining intramembrane helices from the N-terminal portions of two dimerized α-subunits.[23] Since the bordering helices termed H1 and H2 (H stands for hydrophobic), are pairwise linked by a short extramembraneous amino acid sequence ("loop"),[24] the interface cleft in the dimer would be lined by the surfaces of four helix segments from the two H1·H2 pairs. Based on the hydropathy profile of the amino acids involved, it has been concluded that H1 could stretch from Q88 to T114 and H2 from N122 to Q143 in the α1 subunit from human Na^+/K^+-ATPase,[25] in which these intramembrane segments are connected by an extracellularly disposed loop as specified in Figure 1. Raman spectroscopic data demonstrated that the intramembrane peptide sequences are in the α-helical configuration.[26] Transition of enzyme conformation from E1 to E2, characterizing major changes in the functional behavior of the enzyme,[27,28] does not alter the α-helical configuration of the intramembrane sequences which remains unaltered.[29] Apparently, the E1 and E2 enzyme conformations differ only in the orientational relationships of the α-helices such that this transition could produce opening and closing of the extracellularly disposed digitalis binding cleft as well as alter (see[29]) the conformation of the intracellular domains of the catalytic protein.

In an approach to define the surroundings of the intramembrane helices of the H1·H2 pairs, we have chosen a model, which divides the surface of each helix into four differently disposed regions (cf.[30]). This model, visualized in Figure 1, is admittedly simplistic, but in the absence of definite structural information, a more detailed model would be unjustified. Four regions with different surroundings are to be specified.

First, the regions, suggested to be in contact with the hydrophobic core of the lipid bilayer, appear to include all amino acids usually regarded as hydrophobic (A, V, L, I, F, W) plus serine and tyrosine. Serine can satisfy its hydrogen bonding potential by bonding within the helix chain and thus can be on its lipid-facing surface. Tyrosine residues are known to occur on lipid-facing surfaces mainly between the non-polar and the polar parts of the membrane.

Second, the regions of H1 and H2, putatively involved in the interaction with neighboring helix bundles, would be thought to be located in the outer boundaries of the H1·H2 pairs as marked in Figure 1. In line with this suggestion appears to be the absence of highly polar groups and the preponderance of amino acids with hydrophobic side chains such that their precise packing interactions between the helices can provide the energy for the stable interaction of the helix bundles in the α-subunit (cf.[31]).

Third, a quarter of each helix wheel appears to contribute to form the interprotodimeric cleft in which cardiac glycosides intercalate to interrupt the catalytic cycle of Na^+/K^+-ATPase. This hypothesis is supported by the direct involvement of cysteine 104 and tyrosine 108 as well as glutamic acid 117 in the digitalis binding process (see next section). The depth of the cleft would then span about 19 Å (cf. Figure 2) and stretch between E117 and C104.

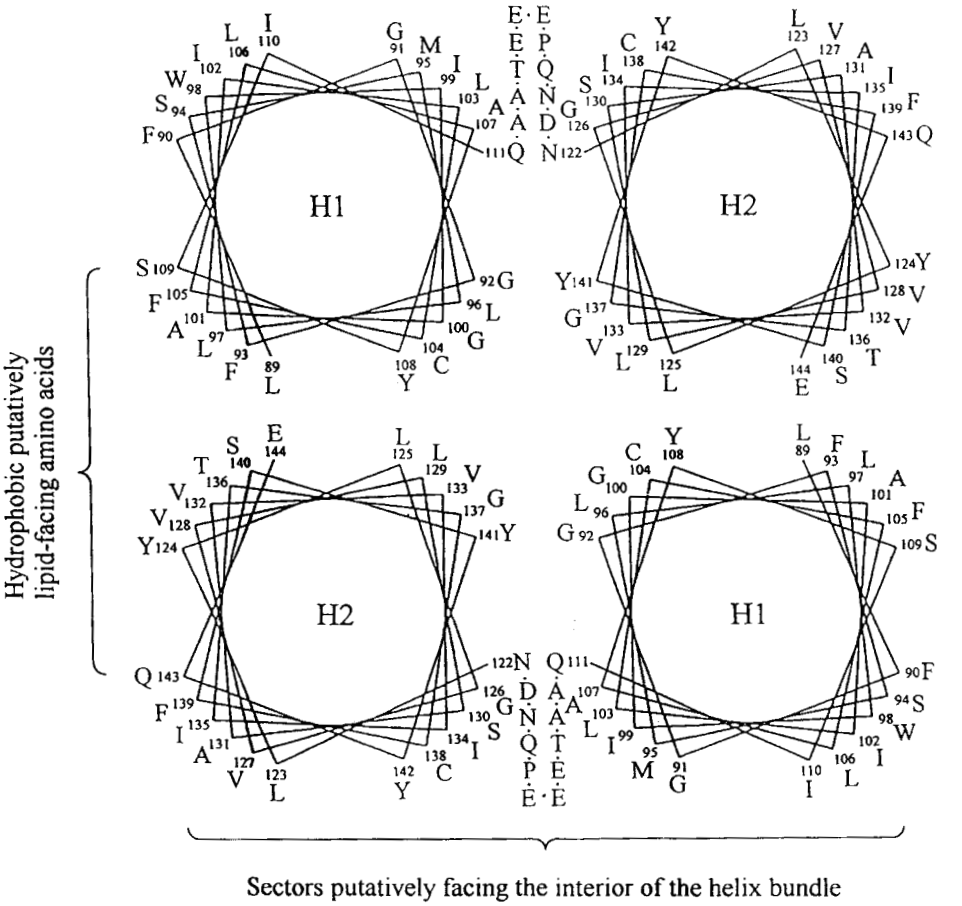

FIGURE 1 Presumed topological arrangement of first and second transmembrane helix (H1 and H2) in two dimerized α1 subunits of human Na^+/K^+-ATPase visualized by helical wheel projection of C_α positions, assuming a periodicity of 3.9 residues per turn. L89 at the beginning of H1 and E144 at the end of H2 thus are at the inner surface of the plasma membrane, whereas the loop between the helices protrudes from the outer membrane surface. This method of projection can be misleading since the position of the side-chains may deviate from the periodicity. In numbering the amino acids, the highly conserved cysteine 104[6] has been chosen for standardization throughout this paper. The pictorial design of the helical wheel follows the plot given for the transmembrane helix of glycophorin.[30]

Fourth, if this applies, the interprotodimeric interaction forces would evolve beyond C104, i.e., between the side chains of G100, L96, G92, V133, G137, Y141 in the corresponding two H1 and two H2 helix sections. The amino acids with relatively hydrophobic side chains (leucine, valine, tyrosine) could contribute to interprotodimeric hydrophobic bonding. Due to the phenolic OH's, the two tyrosines would be able to form interprotodimeric hydrogen bonds.

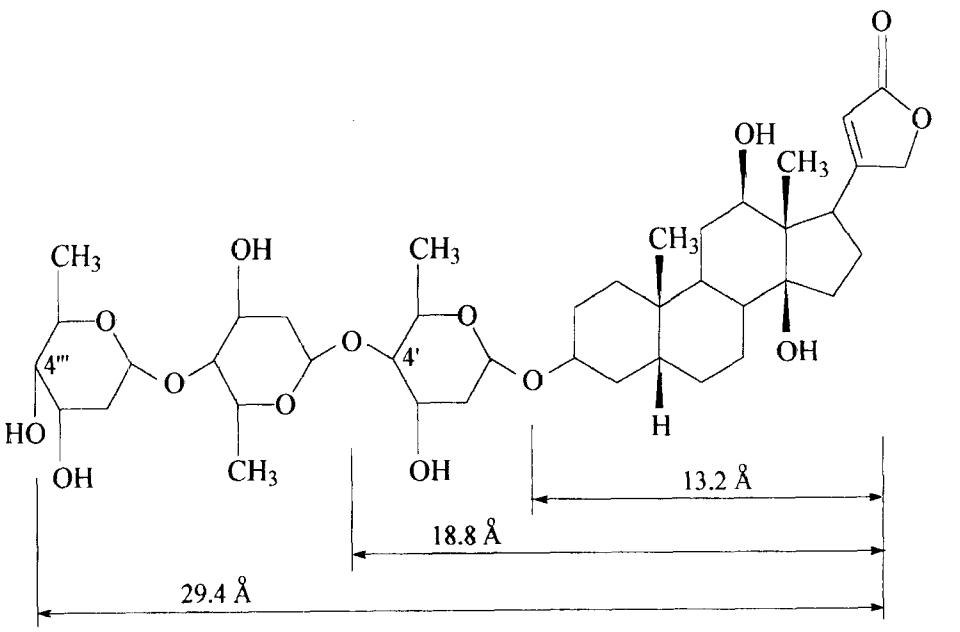

FIGURE 2 Structural formula and dimension of digoxigenin tridigitoxoside (digoxin) used to measure the length of the digitalis intercalating cleft between two dimerized α-subunits. The distances are derived from X-ray crystallographic analysis.[32]

INTERPRETATION OF THE INHIBITORY DIGITALIS INTERACTION WITH Na^+/K^+-ATPase IN TERMS OF THE THREE-DIMENSIONAL STRUCTURE MODEL OF THE DIGITALIS BINDING CLEFT

As reasoned by us for many years, based on various arguments, one of the first steps in the formation of the inhibitory digitalis-enzyme complex should be hydrogen bonding between the partners (for recent reviews see[5,8]). The capacity of the carbonyl and bridge oxygens in the butenolide and pentadienolide side chains of digitalis compounds to accept two hydrogen bonds is well supported by the presence of deep negative potential walls around these oxygens and their marked electronegativity (reviewed in[5,8]). The above deduction has generally been ignored as yet, since the hydrogen donors were not identified. Fortunately, the outcome of mutational amino acid substitutions published recently has opened the way to their identification.

In 1992, Rossier and colleagues[33] discovered that the replacement of cysteine 104 in H1 of the wild type enzyme by tyrosine (C104Y) or by phenylalanine (C104F) much increased the inhibition constant K_i of ouabain. The dissociation rate constants with the glycoside ouabain and the aglycone strophanthidin were much alike so that the authors hypothesized that the mutations alter the binding of the genin moiety. In 1993, Schultheis and Lingrel[7]

reported that the mutations C104A and C104F confer graded losses of ouabain sensitivity to the genuine enzyme. The I_{50} value for the wild-type enzyme increased from 0.032 μM to 0.2 μM for C104A substitution and to 4.8 μM for C104F substitution. The surprisingly large increase by phenylalanine substitution could be due to steric hindering of mutual interaction between its bulky side chain and the lactone residue (cf. below). The smaller increase by alanine substitution, corresponding to a decrease of $\Delta G^{0'}$ from −44.5 kJ/mol to −38.8 kJ/mol, appears to us to indicate the loss of two hydrogen bonds (the value for one bond lies between −1.2 and −4.3 kJ/mol).[34]

In 1994, Askew and Lingrel[35] discovered that the amino acid substitution C104Y conferred different sensitivity for digitoxin and digoxin. They proposed that the differential resistance of the C104Y receptor enzyme for these ligands is a consequence of altering two features of the ligand-receptor interaction; one, a disruption of a common hydrogen bond with C14 hydroxyl resulting in general loss of affinity for cardiac glycosides and the other, after reorientation of the ligand-receptor interface, formation of a new H-bond between the C12 hydroxyl of digoxin and the receptor, thus specifically augmenting the stability of this ligand receptor complex. In the light of the above-mentioned H-bond acceptor capacities of the oxygens in the lactone side chain of both glycosides we suggest here that the common hydrogen bond is formed between the lactone oxygens of either cardiac glycoside and the SH of cysteine 104, and the new H-bond evolves between the C12 hydroxyl of digoxin and the phenolic OH of tyrosine 104. The exceptional role of cysteine 104 also emerges from the findings that the exchanges of cysteine 138 – C138F[7] and C138S[33] – do not alter ouabain affinity.

The strongly bent shape of the lead structure in digitalis compounds, i.e., 5β,14β-androstane-14,17β-diol, that is due to the cis-junction of the rings A and B as well as C and D, implies that the digitalis recognition matrix is primarily a wide open cleft, the closure of which is elicited only after slippage of the lead into the cleft.[5] This possibly explains the report that *N*-hydroxysuccinimidyl-digoxigenin-3-methylcarbonyl-ε-amino-caproate would form a thioester with cysteine 104.[36,37] Regularly, however, cysteine 104 does not play a role in binding of the usual side chain at C3 of cardiac glycosides, i.e., their sugar moiety. This emerges from the observation that the I_{50} ratio of ouabagenin to ouabain is not changed by replacement of cysteine 104 by phenylalanine.[38] Normally, instead, the digitalis compounds enter into the binding cleft not with the side chain at C3 but with the lactone ring at C17 ahead. This derives from the findings that linkage of the sugar chain of ouabain or digoxin with a large protein or sepharose via a long polyamide or hydrocarbon bridge allows the development of the inhibitory action of the digitalis derivatives.[39,40]

The steroid binding subsite of the cleft binds 5β,14β-androstane-14,17β-diol, 5α,14α-androstane, estradiol-17β and progesterone with a similar Gibbs energy of interaction.[5] The subsite thus displays a surprisingly great adaptability to the moulding interaction with the very different molecular surfaces of the various steroids. It appears to involve a change of the rotational relationship of the intramembrane helices between the local minima in the unliganded and liganded state as referred to below. This renders it at first difficult to locate and identify the finally interacting amino acid residues. Interestingly, the exchange Y108A in H1 increases the I_{50} value of ouabain from 0.032 μM to 0.29 μM[7] corresponding to a loss of Gibbs interaction energy by −5.7 kJ/mol. For comparison, the $\Delta G^{0'}$ value of the lead structure in digitalis compounds (5β,14β-androstane-3β,14-diol)[3] amounts to

−22.6 kJ/mol. Clearly, further amino residues are involved in binding of the steroid nucleus (cf. below). Anyhow, tyrosine 108 appears to participate therein since its substitution by phenylalanine does not decrease the I_{50} value. Phenylalanine is, as is tyrosine, known to form hydrophobic bonds especially to other extended molecules. Additional hydrophobic bondings to interact with the apolar steroid skeleton could be provided by the leucines L125 and L129 in H2. Indeed, the involvement of H2 in ouabain binding was proven by the findings that mutations Y124C, I135V or S140C cause ouabain resistance.[41]

The location of the sugar binding subsite of cardiac glycosides was clearly marked through two independent analytical approaches. First, the Gibbs energies of interaction with Na^+/K^+-ATPase were found to decrease in the order genin-monosaccharide > genin-disaccharide > genin-trisaccharide.[5] This gradation indicates that only the proximate sugar provides groupings for an attractive interaction with the binding subsite. The decrease of the $\Delta G^{0'}$ values by the second and third sugar would result from the increase of rotational and translational entropies of the glycosides by the unbound extreme ends of the sugar chain. Second, photoaffinity labeling of Na^+/K^+-ATPase with 4′-ethyldiazomalonyl-digitoxigenin monodigitoxoside or with 4‴-ethyldiazomalonyl-digitoxigenin tridigitoxoside revealed that either the α-subunit or the β-subunit becomes exclusively labeled.[42] Taken together with the above findings, the labeling data indicate that the proximate sugar is immobilized in the mouth of the cleft, whereas the remote sugar is free to rotate and thus able to allow the photolabel to react with the β-subunit.

Concerning the chemical identity of the sugar binding subsite, the following conclusions could be reached. As shown by Lingrel and coworkers,[38] the mutational substitution of C104, Q111, A112, D121 or N122 does not change the I_{50} ratio for ouabagenin to ouabain. Apparently, these amino acids do not participate in the binding of the rhamnose moiety of ouabain so that it should bind to residues in the H1·H2 region which have not been tested. The most likely candidates appear to be the three glutamates (E115, E116, E117) in the H1 helix cap of the α1 isoenzyme (cf. Figure 1) about 19 Å away from C104 (cf. Figure 2). This derives from our following findings.[5] All highly potent cardenolide monosaccharides have α-L-rhamnose or its 4′-amino-4′-deoxy derivative as the sugar component. Since $-NH_2$ serves better than -OH at C4′ in generating high interaction energy, a hydrogen bond-accepting reaction with an acid proton likely underlies the maximum efficacy of the carrier cardenolides. Because this involves an acid partner, that linkage would be a particularly strong hydrogen bond.[43] The involvement of carboxyl side-chains in the sugar binding subsite is also suggested by the findings that, through electrostatic repulsion, acid substituents at C3β-OH of cardenolides as glucuronide or dicarbonic acid esters as well as sulphate esters decrease or eliminate the activity of the parent genin.[5]

Between the above three potential partners of sugar residue binding, glutamates 115 and 116 are replaceable by serine[44] or glutamine,[45] respectively, with only modest or no loss of ouabain affinity. However, replacement of glutamate 117 by asparagine is accompanied by a strong drop of ouabain sensitivity.[46,47] The Gibbs energy of inhibitory interaction drops from −42.1 kJ/mol to −30.5 kJ/mol such that the $\delta\Delta G^{0'}$ value even surpasses the usual decrement effected by removal of the α-L-rhamnosyl side chain from ouabain amounting to ~ −10 kJ/mol.[5] Apparently then, in the α1 isoenzyme glutamate 117 is the binding partner of the rhamnose side chain of ouabain. In line with this conclusion, the substitution of glutamate 117 by aspartate, being the unique sequence difference between the rat α2

and $\alpha 3$ isoenzymes, increases fivefold the ouabain affinity (cf.[48,49]). Rhamnose, as other sugars, are in themselves without inhibitory effect; that is the sugar binding subsite does not pre-exist but is disposed in the process of cleft closure induced by cardiac glycoside binding.[5]

There are a number of observations on the outcome of amino acid replacements which cannot be interpreted in terms of an involvement in a direct, attracting or repulsing interaction with digitalis structure components. For example, in the C-terminal of the loop between H1 and H2 the substitution of glutamine 111 by arginine conferred ouabain resistance to the enzyme.[50] The initial interpretation that glutamine is somehow involved in ouabain binding[51] could not be maintained because its replacement by alanine[52] or leucine[53] did not produce a ouabain-resistant enzyme. Further in the C-terminal of the said loop, the tried exchanges of aspartate 121 – conservative (glutamate), isosteric (asparagine) and nonconservative (alanine or serine) – all decreased the ouabain affinity to the enzyme nearly 100 fold.[54] The authors suggested an involvement of the carboxyl residue of D121 in ouabain binding and the necessity for its precise positioning. However, it does not relate to rhamnose binding since the D121E substitution decreases the inhibitory potency of ouabagenin and ouabain by the same ratio.[38] The substitution of asparagine 122 by alanine[52] does not reduce ouabain affinity at all.

Double substitutions such as N120D and N122H,[55] Q111D and N122R,[48,50,52] Q111R and N122D,[50,53] or Q111K and N122K[50,52] produce the highest decreases in ouabain potency, i.e., either excessive[48,50,52,53] or complete[55] resistance. Remarkably, the combined exchange Q111A and N122A,[52] involving uncharged amino acids, does not confer ouabain resistance. From these findings, Lingrel and associates have drawn the following conclusions (reviewed in[6]). Substitution of glutamine 111 and asparagine 122, which have uncharged side chains, by amino acids with charged side chains, positive or negative, invariably produces a resistant enzyme. The nature and combination of charges does not appear to be particularly important. These findings have led to the postulate that the introduced charges would alter the ouabain-induced conformational change which otherwise effectively prevents rapid ouabain dissociation from a sensitive enzyme.[38,50]

The substituted amino acids, listed above, do not directly participate in ouabain binding. Interestingly enough, they are all located in the border positions of the H1-H2 loop. The puzzling mechanism of the impact of changes in its amino acid composition on the 'conformational change', apparently defining the shape of the protodimer interface cleft, may be deduced from the knowledge that extramembraneous loops can constrain the spatial relationship between the ends of transmembrane helices and thus determine their orientational relationship.[9,11] The closure of the digitalis binding cleft upon ouabain binding could parallel the response of the aspartate receptor in which the binding pocket residues are brought closer together upon ligand complexation by a rigid body rotation of the helix bundles about an axis perpendicular to the dimer interface (cf. reviews).[9,11] In this way, the transmembrane helices may undergo a transition from one local energy minimum (in the unliganded state) to another (in the liganded state), resulting in both an altered relationship between the *intracellular* domains of the receptor and an effective transmembrane signaling by such receptors.[9,11] These interconnections help to realize the inhibitory action of a digitalis compound on Na^+/K^+-ATPase via its attack from the extracellular space and its interprotodimeric intercalation followed by loss of the affinity of the intracellularly disposed

catalytic center to ATP.[56] Reciprocally, phosphate binding to the catalytic phosphorylation site, aspartate induces a conformational change in the enzyme, which increases its affinity to ouabain. Thus, catalytic ATPase activity and ouabain binding are linked through this phosphorylation site.[57]

CONCLUSIONS

Due to the high conformational adaptability of the digitalis intercalating matrix in Na^+/K^+-ATPase, documented above, its three-dimensional structure could only roughly be designed here. The task of rational design of novel drugs when based on this knowledge offers a daunting prospect (cf.[58]). The existence of the three isoenzymes in human heart muscle and human brain[59–62] appears to open a new avenue for the discovery of novel drugs[63] which would inhibit only the one or the other isoenzyme and thus could limit toxic side reactions.[60] What is clearly needed now is the in-depth analysis of the fundamental connection between configuration of steroidal compounds and predicted specificity of their inhibitory interaction with the $\alpha 1$-, $\alpha 2$- and $\alpha 3$-isoforms of human Na^+/K^+-ATPase as comprehensively derived elsewhere.[64,65]

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