Minireview

Location and properties of the digitalis receptor site in Na^+/K^+-ATPase

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Received 12 December 1994

Abstract Since 1985, several research groups have shown that a number of amino acids in the catalytic α-subunit of Na^+/K^+-ATPase more or less strongly modulate the affinity of a digitalis compound like ouabain to the enzyme. However, scrutiny of these findings by means of chimeric Na^+/K^+-ATPase constructs and monoclonal antibodies has recently revealed that the modulatory effect of most of these amino acids does not at all result from direct interaction with ouabain, but rather originates from long-range effects on the properties of the digitalis binding matrix. Starting from this knowledge, the present review brings together the various pieces of evidence pointing to the conclusion that the interface between two interacting α-subunits in the Na^+/K^+-ATPase protodimer $(\alpha\beta)_2$ provides the cleft for inhibitory digitalis intercalation.

Key words: Na^+/K^+-transporting ATPase; Digitalis receptor; Binding cleft; Location; Property

1. Introduction

The Na^+/K^+-ATPase is the biochemical machinery in the Na^+/K^+ pump. The molecular mechanism of the interconversion of scalar chemical energy of ATP and vectorial osmotic energy of Na^+/K^+-gradients over the plasma membrane as well as the molecular mechanism of the allosteric regulation of its pump power appear to be rather well understood [1–3]. Although the medicinally important role of Na^+/K^+-ATPase as the digitalis receptor (target) had already been discovered in the sixties [4–7], the 'microscopic' mechanism of the specific inhibitory interaction of digitalis compounds with the enzyme has remained an open question. The major reason is the fact that the digitalis compounds are not competitive inhibitors, i.e. they do not interrupt the catalytic cycle by displacing an enzyme effector from its activating binding site (cf. [8]). In spite of the absence of distinct spatial knowledge, the digitalis receptor site has repeatedly been tried to compose on the basis of some indirect information [9–13]. As will be reviewed here, the considerations recently have turned out to be untenable and need substitution by a novel concept based on several lines of independent evidence.

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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.

Na^+/K^+-ATPase is a complex of two catalytic α-subunits and two catalytically inert β-subunits, and a number of lipid molecules incorporated into the lipid bilayer of the plasma membrane. The α-subunit contains about 1,012 amino acids. From the primary sequence, hydropathy analysis has been used to compute the local hydrophobicity and predict single-spanning α-helical segments that are long enough to traverse a 40 Å membrane (approximately 20 amino acids). The most recent 'working' model of the membrane topology of the enzyme, presented by Askew and Lingrel [14], comprises ten transmembrane segments (H1–H10) linked by five extracellularly disposed loops. Since the membrane topology models are constantly being revised to accommodate new findings, none of the defensible models (cf. Sweadner and Arystarkhova [15]) will be explicitly invoked here.

2. Outcome of various attempts to identify the amino acids involved in the digitalis receptor site

2.1. *Affinity labeling by use of the* p-*aminobenzyldiazonio-derivative of ouabain*

With this reagent, the NH_2-terminal peptide M_r 41,000 of the α-subunit was labeled via a tryptophan residue. As the reactive substituent was bound to the rhamnosyl residue of ouabain, the digitalis binding site proper could hardly have become labeled by this derivative. The photochemically activatable tryptophan residue utilized in this labeling procedure appears to have been W310 of the H3–H4 loop [16].

2.2. *Information and conclusions derived from chimeric constructs of Na^+/K^+-ATPase*

When the NH_2-terminal 165 amino acid sequence of ouabain-*resistant* rat α-subunit (including the transmembrane segments H1 and H2) is replaced by a sequence from the corresponding region of ouabain-*sensitive Torpedo* α-subunit, the resulting chimeric enzyme is ouabain-sensitive [17]. When the NH_2-terminal amino acids M1 to N162 of the ouabain-*resistant* sarcoplasmic reticulum Ca^{2+}-ATPase is replaced with the corresponding portion of ouabain-*sensitive* Na^+/K^+-ATPase α-subunit (M1 to N200), the chimeric construct shows ouabain-sensitivity [18]. These findings indicate that the NH_2-terminal 165 or 200 amino acids of Na^+/K^+-ATPase α-subunit are sufficient for inhibitory ouabain interaction even in an isolated environment.

The above findings with the two chimeras mean that Y312, W314, T801 and R884, recognized as modulators of ouabain affinity (references in [14]) do not directly interact with digitalis compounds. The amino acids involved in reaction rather appear to be located in the stretch of 165 amino acids forming the

SSDI 0014-5793(95)00020-8

NH_2-terminal of the α-subunit. This knowledge also excludes the validity of the idea [19] that the digitalis compounds may intercalate themselves between the ten transmembrane segments of one α-subunit and interfere in this way with the activity of the enzyme.

2.3. Observations with monoclonal antibodies

Because of its location, the extracellularly disposed H3–H4 loop has often been thought to be involved in digitalis binding via Y312 and W314 (references in [14]). The loop, however, lies outside the N-terminal 165 amino acid stretch recognized as at least partially involved in forming the digitalis binding domain (see above) and thus appears to be unable to directly interact with digitalis compounds. This conclusion is supported by the finding that an antibody, interacting with the region encompassing H3, H4 and the H3–H4 loop, enhances rather than blocks ouabain binding [20].

The amino acids P122, D125 and N126 localized in the H1–H2 loop have been recognized as modulators of ouabain affinity and thus supposed to serve directly in digitalis binding (references in [14]). Remarkebly enough, however, the monoclonal antibody VG_4, known to interact with the first extracellular loop, enhanced ouabain binding. If one accepts the premis that an antibody, which binds to residues in a ligand binding site, should sterically impede binding, the conclusion is that the H1–H2 loop is not part of the cardiac glycoside binding site [21].

2.4. Modulating effects of amino acids on digitalis affinity

As deduced above, the amino acids P122, D125, N126, Y312, W314, T801 and R884 do not directly interact with the digitalis compounds. The detrimental effect on digitalis affinity arising from their substitution by non-equivalent amino acids (references in [14]) does apparently result from long-range effects on the global behavior of the enzyme including delocalized structural reorganizations. This renders topological interpretations difficult or impossible (cf. [22,23]).

Most remarkably, only the amino acids C108, Y112, Q115, and A116 located in the high ouabain-affinity maintaining H1 transmembrane sequence (references in [14]) appear to be able to interact directly with the digitalis compounds. This conclusion will be extended below to the location and structure of the digitalis receptor site in Na^+/K^+-ATPase.

3. Corollaries of the protodimeric structure of Na/K-ATPase for the search on the digitalis binding matrix

3.1. Structure of the dimer interface

It can be inferred from the association constant obtained for the self-association equilibrium $2\alpha\beta \rightleftharpoons (\alpha\beta)_2$, that the solubilized enzyme exists only as $(\alpha\beta)_2$ protodimer in situ and works in this form in the membrane [24,25]. In the α–β assembly, the aminoacyl residues between membrane domains H7 and H8 (possibly also from the H9–H10 domains in the carboxyl-terminal portion of α-subunit are involved [26], while in the α–α dimer formation the transmembrane segments in the amino-terminal portion of α-subunit are responsible [27]. Since the segments H1 and H2 are associated in pairs [28], the interface in the dimer could be formed by four transmembrane segments. According to the principles of protein–protein association [29–31], the reduction of the water-accessible surface areas ensuing from monomer–monomer association corresponds to large entropy losses and appreciable steric strain. Since this 'hydrophobic bonding' dominates the association, relatively small contact areas already guarantee stability such that the assembly of subunits can be described as separated by clefts [31]. The nonpolar character of the interface between the two interacting H1 segments results from the amino acid composition of the subunit surfaces involved in contacts.

3.2. Observations favoring the protodimeric interface as the digitalis intercalating cleft

The analysis of Na^+/K^+-ATPase crystals by using electron microscopy and computer image processing techniques showed that two $(\alpha\beta)$ protomers, contacting in their centre parts, are linked by a two-fold rotation axis normal to the membrane plane and have a contact area which is about 20 Å in height [32]. This roughly corresponds to the dimensions of the interface and is more than enough to accommodate a potent digitalis glycoside (cf. [13]).

The following findings invite to relate the properties and responses of the interface cleft with the 'microscopic' mechanism of digitalis interaction with Na^+/K^+-ATPase. First, the denominator of the inhibitory digitalis action on the enzyme is the digitalis-elicited relaxation of negentropy strain [33] which, as deduced above, partially resides in the intersubunit interaction. Second, the inability of the ouabain-complexed enzyme to perform the reaction cycle is not because of blockade of binding sites for the effectors ATP, Mg^{2+}, Na^+ and K^+, but is most likely due to ouabain-produced disruption of interprotomer-mediated effector site–site interactions [8]. Third, the protodimeric enzyme shows the expected coexistence of two ATP binding sites but only one ouabain binding site [34,35], which is understandable in the present context. Fourth, ouabain bound to the solubilized enzyme modifies the interface behavior such that the association between the two protodimers becomes 22-fold stronger [36]. Ouabain traps the enzyme in the tightly associated $(\alpha\beta)_2$ state [24]. Fifth, even the complex between ouabain and Na^+/K^+-ATPase solubilized with $C_{12}E_8$ and isolated by column chromatography remains stable in the $\alpha_2\beta_2$ form [37]. Sixth, digoxin connected at the sugar side chain with a large protein or with Sepharose via a long polyamide or hydrocarbon bridge, does nevertheless inhibit Na/K-ATPase suggesting that the glycoside binding site is located in a cleft accessible from the external surface of the cell membrane (reviewed in [38]).

In conclusion, the simplest, if not the only tenable, explanation for the above observations is that one digitalis molecule intercalates itself in the interprotomeric cleft of a Na^+/K^+-ATPase molecule that thus serves as digitalis receptor-site.

4. Perspective

Knowledge on the location and amino acid composition of the digitalis receptor site is urgently required for the prediction of 3D structure of the peptide sequences enveloping a digitalis molecule, and for composing an operative model of the binding site capable of predicting the binding affinity of designed compounds prior to synthesis (cf. [39,40]).

Acknowledgements: The review has been rendered possible by a grant of the Deutsche Forschungsgemeinschaft (Re 878/1–4).

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