Three Differentially Expressed Na,K-ATPase α Subunit Isoforms: Structural and Functional Implications

Victoria L. M. Herrera,* Janet R. Emanuel,† Nelson Ruiz-Opazo,* Robert Levenson,† Bernardo Nadal-Ginard*

*Laboratory of Molecular and Cellular Cardiology, Howard Hughes Medical Institute, Department of Cardiology, Children's Hospital, and Departments of Pediatrics, Physiology, and Biophysics, Harvard Medical School, Boston, Massachusetts 02115; and †Department of Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06510

Abstract. We have characterized cDNAs coding for three Na,K-ATPase α subunit isoforms from the rat, a species resistant to ouabain. Northern blot and S1-nuclease mapping analyses revealed that these α subunit mRNAs are expressed in a tissue-specific and developmentally regulated fashion. The mRNA for the α1 isoform, ≈4.5 kb long, is expressed in all fetal and adult rat tissues examined. The α2 mRNA, also ≈4.5 kb long, is expressed predominantly in brain and fetal heart. The α3 cDNA detected two mRNA species: a ≈4.5 kb mRNA present in most tissues and a ≈6 kb mRNA, found only in fetal brain, adult brain, heart, and skeletal muscle. The deduced amino acid sequences of these isoforms are highly conserved. However, significant differences in codon usage and patterns of genomic DNA hybridization indicate that the α subunits are encoded by a multigene family. Structural analysis of the α subunits from rat and other species predicts a polytopic protein with seven membrane-spanning regions. Isoform diversity of the α subunit may provide a biochemical basis for Na,K-ATPase functional diversity.

The plasma membrane protein that directly couples the hydrolysis of ATP to the active transport of Na⁺ and K⁺ across the plasma membrane in most animal cells is Na,K-ATPase. This transport produces a Na⁺/K⁺ electrochemical gradient to which is coupled net extrusion or accumulation of many other substances against their concentration gradients (Kyte, 1981). The Na,K-ATPase, therefore, plays a central role in a variety of physiological processes: regulation of cell volume (MacKnight and Leaf, 1977), differentiation (Smith et al., 1982), proliferation (Rozengurt and Heppel, 1975), ion/solute uptake in the stomach, intestine (Allen and Navran, 1984), liver (Blitzer and Boyer, 1978), kidney (Kyte, 1965a, b), and bone (Baron et al., 1986), propagation of the action potential of muscle and nerve (Thomas, 1972), and modulation of synaptic action (Phillis, 1977). In all tissues from which Na,K-ATPase has been identified it has been shown to consist of two subunits, α and β. The α subunit is a polypeptide of Mr~100,000 that contains the ATP- and ouabain-binding sites. The α subunit is phosphorylated and undergoes conformational changes during its reaction cycle (Jorgensen, 1983). The β subunit is a glycosylated polypeptide of Mr~55,000 whose biochemical function is unknown but appears indispensable for enzymatic function (Sweadner and Goldin, 1980). Two α subunit isoforms have been identified in the rat (α and α*) (Sweadner and Gilkeson, 1985; Lytton, 1985) and in brine shrimp (α1 and α2) (Morohashi and Kawamura, 1984). These protein isoforms differ in SDS polyacrylamide gel mobility and amino-terminus amino acid (aa) sequences. Two antigenically different isoforms with different tissue distribution have also been described in the chicken (Fambrough and Bayne, 1983). The primary structure of the α subunit from three ouabain-sensitive species, sheep kidney (β1 and β2) (Morohashi and Kawamura, 1984), electric ray electroplax (Kawakami et al., 1985) and pig kidney (Ovchinnikov et al., 1986), has been determined from cDNA clones.

The reported sequence conservation of the Na,K-ATPase among species is in marked contrast with the wide variety of functions carried out by this enzyme in different cell types, at various developmental stages, and physiological conditions. The present study was undertaken with two main objectives in mind: first, to determine the extent and nature of tissue-specific isoform diversity of the α subunit; and second, to determine the primary structure of the α subunit(s) from an ouabain-resistant species to better understand structure–function relationships, the molecular mechanism(s) of ion transport, and the basis for differential ouabain sensitivity among species.

We have isolated and characterized cDNAs coding for

1. Abbreviations used in this paper: aa, amino acid; GES, Goldman-Engleman-Steitz scale; H, hydrophobic region.

V. L. M. Herrera's and N. Ruiz-Opazo's present address is Section of Molecular Genetics, Cardiovascular Institute, Boston University Medical School, Boston, MA 02118.
three Na,K-ATPase α subunit isoforms from rat, an ouabain-resistant species. Our results suggest that in the rat Na,K-ATPase α subunit is encoded by a multigene family that is expressed in a tissue-specific and developmentally regulated manner. Comparative analysis of the primary and deduced expressed in a tissue-specific and developmentally regulated ATPase α subunit is encoded by a multigene family that is resistant species. Our results suggest that in the rat Na,K-ATPase α subunit isoforms from rat and other species predicts a polytopic protein containing seven putative membrane-spanning domains and two putative regions involved in ouabain binding. While the overall structure of the α subunit from rat and other species appears to be conserved, structural differences between rat isoforms were detected. Such differences are consistent with the hypothesis that Na,K-ATPase α subunit isoforms play specific functional roles.

Materials and Methods

Isolation and Characterization of cDNA Clones

Rat brain and liver AgtI cDNA libraries were plated (50,000–100,000 recombinant plaque-forming units per plate) and transferred to nitrocellulose filters (Schleicher & Schuell, Inc., Keene, NH). Filters were prepared for hybridization by the method of Benton and Davis (1977). Random hexanucleotide-primed 32P-dCTP (Amersham Corp., Arlington Heights, IL)-labeled cDNA (RB5) probe was prepared by the method of Feinberg and Vogelstein (1983) to a specific activity of 108 cpn/μg DNA, and hybridized to the filters overnight. Filters were washed at varying stringencies, followed by autoradiography. Positive clones were plaque purified and their cDNA inserts characterized by standard restriction endonuclease-mapping procedures.

Isolation of mRNA and Northern Blot Analysis

Fetal (18-d gestation) and adult rat tissue mRNAs were isolated from Sprague-Dawley rats by the method of Chirgwin et al. (1979). A panel of fetal and adult total cellular RNA (20 μg of RNA per sample) was separated electrophoretically on 1% agarose gels containing formaldehyde as previously described (Lehrach et al., 1977). The RNA was transferred to nitrocellulose filters (Schleicher & Schuell) and hybridized with 0.5–1 × 106 cpn/ml random hexanucleotide-primed 32P-dCTP-labeled probes, at 42°C in 5× SSC (1× SSC = 150 mM NaCl, 50 mM sodium citrate), 50% formamide (Thomas, 1980). Washes were performed at 65°C in 0.1× SSC, 0.1% SDS for 1 h.

SI-Nuclease Mapping Analysis

End-labeled, double-strand cDNA restriction fragment probes (Fig. 3 C) were prepared either by kinase (New England Biolabs, Beverly, MA) labeling with [γ-32P]ATP (5′-end labeled probes) or with 32P-dATP (Amersham Corp.) using terminal transferase (New England Biolabs) (3′-end-labeled probes). Double-strand end-labeled probes were hybridized to 20 ng of total cellular RNA under RNA-looping conditions as described previously (Casey and Davidson, 1977; Berk and Sharp, 1977). The reaction mixture was incubated with 200 μM of SI-nuclease (Boehringer Mannheim Biochemicals, Indianapolis, IN) at 25°C for 1 h. SI-nuclease-resistant products were ethanol precipitated and size separated on 8% polyacrylamide gels. Negative controls using tRNA were run to assess for any reannealing of the end-labeled probes.

Southern Blot Analysis

Rat liver genomic DNA was isolated by a modification of the method described by Billa and Stafford (1976). Genomic DNA was digested with a panel of restriction endonucleases and the DNA fragments separated electrophoretically on 1% agarose gels. The DNA was transferred to Zeta-bind filters (AMF Cuno Precision Control Products, Meriden, CT) by the method of Southern (1975). Hybridization was carried out with cDNA probes specific for each α subunit isoform.

DNA Sequence Analysis

DNA sequence analysis was performed by the dideoxy chain-termination method of Sanger et al. (1977) using 35S-dATP (Amersham Corp) as radioactive label. Appropriate restriction fragments were isolated from low melt agarose gels and subcloned directly into the corresponding M13 vectors and the nucleotide sequence obtained. (●) Origin and direction of nucleotide sequencing; (●) synthetic oligonucleotide-primed nucleotide sequence. The cDNA clones are aligned according to their deduced amino acid sequences.

Results

Isolation of Three Classes of Na,K-ATPase α Subunit cDNA Clones

To search for Na,K-ATPase α subunit isoforms from an ouabain-resistant species, several rat cDNA libraries were screened using the previously characterized RB5 cDNA clone (Schneider et al., 1985) as probe. Several clones were isolated. Rα1-a, Rα2-a, Rα2-d, Rα3-a, and Rα3-b were isolated from a random-primed adult rat brain AgtI cDNA library. Another clone, Rα1-b, was isolated from an adult rat liver AgtI cDNA library. Restriction map analysis identified three classes of cDNA clones (Fig. 1): class 1 (α1) consisted of Rα1-a and Rα1-b; class 2 (α2) consisted of Rα2-a and d; while class 3 (α3) consisted of Rα3-a and Rα3-b. Clones representing 3′ extensions of α2, Rα2-b and c were subse-
quent isolation from an oligo dT-primed adult rat brain cDNA library using the 3'-most 400-bp Pst I/Eco RI restriction fragment of Ra2-a as probe (Fig. 1). Many other a2 clones were isolated from several libraries but did not extend further toward the 3' UT presumably because of the presence of internal Eco RI sites which were not methylated during the cDNA cloning procedure.

**Three a Subunit mRNAs, Each Encoded by a Different Gene, Are Expressed in a Tissue-specific and Developmentally Regulated Fashion**

The cDNA clones shown in Fig. 1 were used to investigate the tissue distribution and size of each a subunit mRNA isoform. Probes were prepared from the cDNA clones spanning the phosphorylation and ATP-binding sites from each class: a1 (Ral-b), a2 (Ra2-a), and a3 (Ra3-a) (Fig. 1). Each probe was hybridized to separate but identical Northern blots containing equivalent amounts (20 μg) of fetal (18-d gestation) and adult rat tissue total cellular RNA. At the same stringent conditions, strikingly different patterns of mRNA distribution were detected (Fig. 2). As shown in Fig. 2 B, the a1 probe hybridized to a ~4.5-kb mRNA present in varying amounts in all tissues examined. Different patterns of expression are also detected during development. The a1 mRNA is more abundant in fetal than adult kidney, whereas it is more abundant in adult than fetal brain. An almost equal level of a1 mRNA is detected in fetal and adult heart, as well as in fetal and adult skeletal muscle. As shown in Fig. 2 C, the a2 probe also detects a ~4.5-kb mRNA but this species is present only in brain and fetal heart. Like the a1 mRNA, the a2 isoform is also more abundant in adult than fetal brain. As shown in Fig. 2 D, the a3 cDNA probe detects two mRNAs of different sizes, ~6 and ~4.5 kb. The ~6-kb mRNA is detected only in fetal brain and adult brain, heart,

**Figure 2. Northern blot analyses of Na,K-ATPase a subunit isoforms.** Three separate but identical Northern blots of equivalent amounts (20 μg) of rat adult (a) and fetal (f) tissues were hybridized to a cDNA probe for each Na,K-ATPase a subunit isoform spanning comparable regions. (A) Ethidium bromide-stained picture of the gels before transfer. (B, C, and D) The pattern of hybridization to a1 (codons 402-938), a2 (5'UT to codon 940), and a3 (codon 44 to the 3' end) cDNA clones, respectively. (Arrow) The hybridizing mRNA species. Size of ribosomal RNA subunits (28S, 18S, 5S) are noted on the left. Sk. M., skeletal muscle. Longer exposure of the Northern blot in D (not shown) reveals a ~4.5-kb mRNA hybridizing band in fetal liver and skeletal muscle.

**Figure 3. S1-nuclease mapping analysis of Na,K-ATPase a1 mRNA isoform.** (A) S1-nuclease protection of the 190-bp-long end-labeled Kpn/Eco RI fragment of clone Ral-b shown as probe A in C by RNA isolated from different rat fetal (f) and adult (a) tissues. The full-length protected fragment is indicated by a solid arrow. The undigested probe is shown in the first lane. End-labeled Hae III-digested φX174 markers are shown in the second lane in base pairs. (B) S1-nuclease protection pattern of the 3' portion of clone Ral-b extending from the Kpn site into the plasmid vector and shown as probe B in C. The fully protected fragment is indicated by a solid arrow. Sm. Intest. and Sk. M., small intestine and skeletal muscle, respectively. The first lane shows the double-stranded labeled probe. End-labeled Hind III-digested λ DNA markers in the second lane are in base pairs. (C) Map of the different end-labeled restriction fragments used for S1-nuclease mapping experiments with ← indicating the labeling site and its 5' or 3' position. These probes span the Na,K-ATPase a subunit phosphorylation site (P); FITC-binding site; stop codon (COOH); and poly-A tail (AAAA). (→) Vector sequences.
and skeletal muscle. The lower hybridizing band (~4.5 kb) is similar in size to the α1 and α2 mRNAs. However, it is unlikely that this band represents hybridization to α1 and/or α2 mRNAs since the relative abundance of the mRNAs that are detected by the α3 probe in individual tissues is strikingly different. Whether the two mRNAs that are detected by the α3 probe are the products of the same or different and highly homologous genes remains to be determined. The smaller mRNA species in adult heart, skeletal muscle, and fetal liver have a slightly faster mobility than in other tissues. It has not yet been determined whether the tissues that exhibit this mobility difference express yet another isoform, although considering the stringency of hybridization, this is unlikely.

To confirm that the α1 cDNA probe truly detected α1 mRNA and not other cross-hybridizing isoforms mRNAs, S1-nuclease mapping analysis was performed using different regions of the entire RB5 and Ratb cDNA clones (Fig. 3 C). The results of two representative experiments are shown in Fig. 3, A and B. Full protection of each end-labeled probe used, spanning different regions of the mRNA, was detected with the mRNA from all fetal and adult tissues studied. Partially protected fragments were also observed that varied in intensity in proportion to the fully protected fragments. These bands were not reproducible in other S1-nuclease mapping experiments using end-labeled probes of different sizes in the 5' and 3' direction and probably represent artifacts of S1-nuclease digestion. These results confirm that the α1 mRNA is expressed in every tissue examined and that the α1 mRNA detected by the α1 cDNA probe is not the result of cross-hybridization with other α subunit mRNAs.

To investigate the genomic complexity of Na,K-ATPase α subunit, rat genomic DNA fragments were hybridized with cDNA probes for each of the α subunits spanning comparable regions of each isoform: α1 (5'-3'UT), α2 (5'UT-codon 940 [α1 numbering]), and α3 (codon 44-3'UT [α1 numbering]). As shown in Fig. 4, each α subunit probe hybridized to a unique pattern of DNA restriction fragments. This result suggests that each α subunit isoform is encoded by a different gene. Low stringency hybridization of the genomic blot shown in Fig. 4 detects additional hybridizing bands for each of the probes, suggesting the existence of additional genomic sequences that are homologous but not identical to any of the three cDNAs reported here (data not shown).

Comparative Analysis of the Primary Structure of the Na,K-ATPase α Subunit Isoforms

Nucleotide sequences were obtained for all α1 and α2 cDNA clones, confirming the alignment shown in Fig. 1 by the existence of overlapping regions with identical sequences obtained in both orientations. Partial nucleotide sequences have been obtained for α3. Comparison of nucleotide sequences (Fig. 5) show α1 and α2 to be 74% homologous with 66% of the differences being at the wobble position. Partial nucleotide sequence confirmed that α3 represents a third α subunit isoform differing from α1 and α2 in codon usage and deduced amino acid sequence (data not shown).

Analysis of the deduced aa sequences of the rat α subunit isoforms (Fig. 5) and previously characterized α subunits shows that α1 from the rat is almost identical (97% homology) with the α subunit polypeptides from sheep (Shull et al., 1985) and pig kidney (Ovchinnikov et al., 1986). Surprisingly, the rat α2 isoform is only 82-85% homologous with rat α1, and the previously characterized α-subunits from sheep kidney (Shull et al., 1985), pig kidney (Ovchinnikov et al., 1986), and electric ray electroplax (Kawakami et al., 1985). The amino terminus of α2 differs markedly from other identified sequences (Fig. 6) including the previously described rat brain isoform, α+ (Lytton, 1985). It is shorter than α1 by 10 aa and lacks histidine 13, like the brine shrimp α subunit amino terminus (Morohashi and Kawamura, 1984). The lysine rich regions are highly conserved, however, among all the amino termini characterized to date. Interestingly, comparison of the protein- (Hopkins et al., 1976; Cantley, 1981; Collins et al., 1983; Morohashi and Kawamura, 1984) and cDNA-derived α1-type aa sequences...
Figure 5. Nucleotide and deduced amino acid sequence homology between Na,K-ATPase α subunit isoforms α1 and α2. The α1 nt sequences (fine print, numbered) and aa sequences expressed in one letter code (bold print, numbered) are presented. The α2 aa (bold print, unnumbered) and amino acid sequence homology between Na,K-ATPase α subunit isoforms α1 and α2.

Herrera et al. Na,K-ATPase α Subunit Isoforms
Figure 6. Comparison of amino-terminal sequences of different Na,K-ATPase α subunits. Amino-terminal sequences of different α subunit isoforms are aligned. The different α subunits presented are: RAT α+ (Lytton, 1985); RAT α1 and α2 (this paper); S. K. cDNA, sheep kidney α subunit (Shull et al., 1985); P. K. cDNA, pig kidney α subunit (Ovchinnikov et al., 1986); E. R. E. cDNA, electric ray electroplax α subunit (Kawakami et al., 1985); B. S. a1 and a2, brine shrimp α1 and α2 isoforms, respectively (Morohashi et al., 1984). The homologous regions among the different sequences have been boxed. Sequences have been aligned and gaps (-) introduced to maximize homology. The five cDNA-derived aa that are absent from the respective characterized mature protein are segregated from the remainder of the sequences.

Na,K-ATPase α Subunit Topography

To gain some insight into the rat α1 and α2 subunit topography, we analyzed the aa sequences to determine the putative hydrophobic membrane-spanning regions. Hydrophyto plots of α1 and α2 were obtained using the Kyte-Doolittle (Kyte and Doolittle, 1982) and the Goldman-Engleman-Steitz (GES) (Engelman et al., 1986) scales. The GES hydrophathy plots were obtained using a window averaging of 20 aa since this is the average length required for an α-helix to cross the plasma membrane lipid bilayer (Engelman et al., 1986). A 14-aa window averaging was also applied because the minimum number of aa to span the plasma membrane has been experimentally shown to be 12–14 aa (Adams and Rose, 1985). Comparative analysis of the different hydrophyto plots identify seven putative membrane-spanning regions. Five regions, H1 and H3-6 (Fig. 8), fulfill the Goldman-Engleman-Steitz criteria for membrane-spanning regions (Engelman et al., 1986). The minimum total free energy of transfer to water for a 12-aa membrane-spanning region was calculated to be 20 kcal/mol from the GES hydrophathy plot of an experimentally proven transmembrane region of 12 aa (Adams and Rose, 1985; En-
Hydropathy plots of the rat α1 and α2 polypeptides are presented using the GES scale. The vertical axis marks the free energy of transfer to water per amino acid averaged over 14 aa, and the horizontal axis, the unit amino acid. Putative hydrophobic membrane-spanning domains are noted 1-7. Previously identified functional domains are noted for reference: 1- and 2-OUA-R, putative ouabain-binding regions; W, tryptophan; C, cysteine; P*, phosphorylation site; D, aspartic acid; K, lysine; FITC, fluorescein 5'-isothiocyanate-binding site; FSB4, 5'-(p-fluorosulfonyl) benzoyladenosine-binding sites. The 10-aa-long region of nonhomology (I) is also noted.

Figure 8. Hydropathy plots of Na,K-ATPase α subunit isoforms, α1 and α2. Hydropathy plots of the rat α1 and α2 polypeptides are presented using the GES scale. The vertical axis marks the free energy of transfer to water per amino acid averaged over 14 aa, and the horizontal axis, the unit amino acid. Putative hydrophobic membrane-spanning domains are noted 1-7. Previously identified functional domains are noted for reference: 1- and 2-OUA-R, putative ouabain-binding regions; W, tryptophan; C, cysteine; P*, phosphorylation site; D, aspartic acid; K, lysine; FITC, fluorescein 5'-isothiocyanate-binding site; FSB4, 5'-(p-fluorosulfonyl) benzoyladenosine-binding sites. The 10-aa-long region of nonhomology (I) is also noted. (Inset) Number of aa residues (aa) per hydrophobic region and total free energy of transfer to water in kcal/mol (E) for the α1 and α2 subunits. Weakly hydrophobic regions (8 and 9) in α1 are also presented with their corresponding number (aa) and total free energy of transfer to water in kcal/mol (E).

Discussion

Na,K-ATPase Isoform Diversity Generated by a Tissue-specific and Developmentally Regulated Multigene Family

Three isoforms of Na,K-ATPase α subunit, α1, α2, and α3, have been unambiguously identified by the isolation of three classes of cDNA clones. The respective mRNAs have distinct nucleotide and deduced aa sequences, as well as characteristic differential patterns of expression. The two mRNA species ≥4.0- and ≥4.5-kb mRNAs detected by the α3 cDNA at stringent conditions of hybridization most likely represent transcriptional products of the same gene. Differential utilization of polyadenylation signals could account for their size difference as has been observed in other genes (Setzer et al., 1980; Parnes et al., 1983; Capetenaki et al., 1983; Carroll et al., 1986). The significance of the observed tissue-specific variation of mRNA isoforms detected by the α3 cDNA clone remains to be elucidated. The existence of two protein isoforms produced by alternative splicing is unlikely but cannot be formally excluded at present. Other isoforms in addition to the three presented here most likely exist. This conclusion is supported by the preliminary characterization of several rat genomic clones which differ in primary sequence from the isoforms presented here (unpublished data).

Our results suggest that the three rat α subunit isoforms presented here are encoded by three distinct genes. This view...
is supported by the differences in codon usage and genomic DNA blot restriction fragment hybridization pattern. Chromosomal-mapping studies further support this conclusion. We have found that the three \( \alpha \) subunit cDNA probes map to three different mouse chromosomes (Kent et al., 1987b). The high degree of nucleotide and aa sequence homology suggests that the three \( \alpha \) subunit genes probably arose from a common ancestor. The sequence conservation of these three isoforms with other ATPases further supports the idea that different ion transport ATPases derived from a common ancestral gene (Serrano et al., 1986).

**Significance of Na,K-ATPase \( \alpha \) Subunit Isoform Diversity**

The identification of isoform diversity exhibiting tissue-specific and developmental regulation is highly significant as it could provide the basis for Na,K-ATPase functional diversity. This diversity includes markedly different tissue-specific responses to different physiologic conditions (Charlemagne et al., 1986) and hormonal regulation (Lytton et al., 1985), differences in ouabain affinity (Sweadner, 1985; Charlemagne et al., 1986), as well as specific cellular (Sweadner, 1979; Fambrough and Bayne, 1983) and subcellular localization (Caplan et al., 1986). Direct support for this hypothesis awaits the determination of the functional characteristics as well as the cellular and subcellular location of each isoform. The differences in primary and secondary structure among isoforms, most notably in the amino termini and in proximity to the ATP-binding site may be involved in such isoform-specific functional characteristics.

Isoform diversity of the \( \alpha \) subunit raises the question as to the existence of isoform diversity for the \( \beta \) subunit. The possibility of specific \( \alpha_{\alpha} - \beta_{\beta} \) subunit quaternary associations could generate more complex assembly and functional interactions. Furthermore, the existence of \( \alpha \) subunit isoform heterogeneity described here makes it imperative to correlate enzymatic and pharmacologic parameters with the specific isoforms in order to assess respective physiological roles.

**Structural Implications on the Mechanism of Ion Transport**

Analysis of the topography of Na,K-ATPase \( \alpha \) subunit, as determined by the identification of membrane-spanning regions, is central to the goal of eventually understanding the mechanism of ion transport. The membrane-spanning segments of the \( \alpha \) subunit are most likely involved in the ion translocation...
process. However, the topology of Na,K-ATPase α subunit remains controversial. Kyte-Doolittle hydropathy plot analyses of three previously characterized α subunits (Shull et al., 1985; Kawakami et al., 1985; Ovchinnikov et al., 1986) have suggested different numbers of hydrophobic membrane-spanning regions. Because the Kyte-Doolittle scale does not address conformational and environmental aspects relevant to membrane proteins in the lipid bilayer (Engelman et al., 1986), using the GES hydrophobicity scale. This scale has been proposed as appropriate for identifying nonpolar transbilayer helices in α sequences of membrane proteins (Engelman et al., 1986). These analyses identified a consensus of seven putative membrane-spanning regions that can form a putative hydrophilic pore. Although the accuracy of the different methods of relative amphiphilicity determination remain to be tested experimentally, the striking analogies between the transmembrane structures of Na,K-ATPase α subunit (presented here) and bacteriorhodopsin suggest their validity. The latter has been shown by electron microscopy to have seven transmembrane helices (Henderson and Unwin, 1975), that are also identified by GES hydropathy analysis (Engelman et al., 1986). Furthermore, neutron scattering data suggests the existence of a relatively hydrophilic pore (Engelman and Zacai, 1980), that could provide a pathway for proton translocation (Tanford, 1982). More interestingly, the hydrophilicity of the putative pore defined by the Na,K-ATPase α subunit transmembrane segments is greater than the one in bacteriorhodopsin. Furthermore, hydrophobic regions H1, H3, and H6, all greater than 20 aa (Fig. 9B), are sufficiently long to cross the lipid bilayer in a path other than a perpendicular one. This may allow these transmembrane segments to be involved in conformational changes important in ion transport much like the tilting effector of the transmembrane helices of the gap junction in response to Ca++ (Unwin and Ennis, 1984); or as proposed in the chemical potential change model of ion transport (Tanford, 1982). With the amino terminus previously shown to be in the cytoplasmic side (Jorgensen et al., 1982a, b; Farley et al., 1986), the presence of seven hydrophobic regions places the carboxyl terminus on the extracellular side. This putative topology more closely approximates the observation from previous biochemical data that the molecular mass of the extracellular domain of Na,K-ATPase α subunit is ~20–30% of the intracytoplasmic domain (Ovchinnikov et al., 1985).

Interestingly, Chou and Fasman (Chou and Fasman, 1978) predicted secondary structure propensity of the extramembrane regions of α1 and α2 (Fig. 9A; complete data not shown) shows that several regions have almost equal predicted propensities to adopt an α-helix and/or β-sheet and/or turn conformation. Changes between these two conformations could be the structural basis for the E1-E2 conformational transitions.

**Na,K-ATPase α Subunit Interaction with Ouabain: Inhibition and Resistance**

The binding of ouabain to the α subunit most likely involves two regions located between transmembrane regions H3 and H5 (1-OUA-R, Figs. 5 and 9A), and between H3 and H6 (2-OUA-R, Figs. 5 and 9A). These two regions are implicated by the location of a unique tryptophan (tryp 318, α1) and cysteine residue (cys 809, α1), respectively, previously shown to be involved in ouabain binding (Goeldner et al., 1983; Kirley et al., 1986). There are no other extracellular tryp/cys residues between membrane-spanning regions. The predicted regions involved in ouabain binding are consistent with previous findings that the two E2 major tryptic peptides, amino terminus (41 kD) and carboxyl terminus (58 kD), are both covalently labeled with 3H-N-(ouabain)-N"-(2-nitro-4-azidophenyl) ethylenediamine (Jorgensen et al., 1982), and with the location proposed by 3H-ouabain fluorescence resonance energy transfer study (Cantley et al., 1982). The proximity of the putative regions involved in ouabain binding to transmembrane domains could result in ionic hindrance of conformational changes involving the respective flanking transmembrane regions by the glucoside. This hypothesis is consistent with the finding that ouabain binding keeps the Na,K-ATPase in the E2(K) form (Jorgensen, 1983).

Na,K-ATPases of rats, mice, and hamsters have been found to be ouabain resistant (Willis and Emory, 1983). Chromosome-mediated gene transfer experiments suggest that only the α1 isoform segregates with the ouabain-resistant phenotype. DNA sequences coding for the α2 and α3 isoforms and the β subunit were not transferred to recipient cells selected for ouabain resistance. Furthermore, direct transfer of mouse or rat α1 subunit cDNA is sufficient to confer ouabain resistance to ouabain-sensitive CV-1 cells (Kent et al., 1987a, b). These results suggest that the α1 isoform is responsible for the differential ouabain sensitivity among species (Fallows et al., 1987). Comparison of the rat α1 primary and secondary structures with the α1 equivalents from sheep and pig kidney, both ouabain-sensitive species, did not, however, reveal any insightful differences that could account for the differences in ouabain sensitivity. The two putative ouabain-binding regions and flanking α sequences are highly homologous among the three species. On the other hand, there are differences between the α2 type and the α2 isoform. The α2 isoform, like the electric ray electropla α subunit, has a noncharged residue (glycine) substituting for a charged one (glutamic acid) in the putative ouabain-binding region, I-OUA-R (Fig. 5). Furthermore, in α2, an aspartic acid substitution for valine in the extracellular-flanking region of the putative ouabain-binding region 2-OUA-R (Fig. 5) induces a six aa-long hydrophilic interruption in a hydrophobic stretch present in rat α1, sheep, pig, and electric ray subunits. This hydrophilic region could affect ouabain binding.

In addition to α1, ouabain resistance may also involve expression of other α subunits isoform(s), as well as different levels of expression of these isoforms. Moreover, the ouabain resistance conferred by intrinsic differences in the α subunit isoforms must be distinguished from other phenomena observed in acquired ouabain resistance, such as gene amplification (Emanuel et al., 1986; Pauw et al., 1986), and the expression of an ouabain resistance gene (Levenson et al., 1984).

The availability of cloned cDNAs for several α subunit isoforms should allow the test of putative structure–function relationships and define the pharmacologic and biochemical properties of the α subunit isoforms through the expression of functional subunits from the isolated cDNAs and their in vitro–generated mutants.

We thank Dr. David Atkinson of the Biophysics Institute, Boston University Medical Center, for advice on the analysis of the protein structure, Ms. Lynne Stone for technical assistance on some of the work, Dr. Charles Sim-
moms for critical reading of the manuscript, and Ms. Sharon Ward for her excellent secretarial assistance.

This work was supported in part by grants (to B. Nadal-Ginard and R. Levinson) from the National Institutes of Health and the American Heart Association; by the Simeon Burt Wolbach Research Fund of the Children's Hospital of Boston (to V. L. Herrera), R. Levenson is an Established Investigator of the American Heart Association.

Received for publication 17 December 1986, and in revised form 2 July 1987.

References


