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

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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, 1976a, 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-terminal 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 (Shull et al., 1985), 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.
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 play specific functional roles.

Materials and Methods

Isolation and Characterization of cDNA Clones

Rat brain and liver Agt11 cDNA libraries were plated (50,000-100,000 recombinant plaque-forming units per plate) and transferred to nitrocellulose filters (Schleicher & Schuell, 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 109 cpm/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 tissue total cellular RNA (20 µg of RNA per sample) was separated electrophoretically on 1% agarose gels containing formaldehyde as previously described (Lerach et al., 1977). The RNA was transferred to nitrocellulose filters (Schleicher & Schuell) and hybridized with 0.5-1 × 106 cpm/ml random hexanucleotide-primed 32P-DCTP-labeled probes, at 42°C in 5x SSC (1x SSC = 150 mM NaCl, 50 mM sodium citrate), 50% formamide (Thomas, 1980). Washes were performed at 65°C in 0.1x 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 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 µg 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 Blin and Stafford (1976). Genomic DNA was digested with a panel of restriction endonucleases and the DNA fragments separated electrophoretically on 1% agarose gels containing formaldehyde as previously described (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.

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 radio-active label. Appropriate restriction fragments were isolated from low melt agarose gels and subcloned directly into the corresponding M13 vectors (mpl8 and/or mpl9) (Messing, 1983). Single-strand DNA templates were isolated and subjected to nucleotide sequence analysis (Bethesda Research Laboratories, Gaithersburg, MD). An average of 300-350 nucleotide sequences was obtained. A significant amount of the sequence was obtained using template-specific 17-mer primers synthesized on a DNA synthesizer (Biosearch, San Rafael, CA) based on the known DNA sequence. Each region was sequenced at least twice to reduce ambiguities and confirm overlapping regions. Overlapping regions of respective cDNA clones were sequenced multiple times in both orientations.

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. Ral-1, Ra2-2, Ra2-2, Ra3-3, Ra3-3, and Ra3-3 were isolated from a random-primed adult rat brain Agt11 cDNA library. Another clone, Ral-b, was isolated from an adult rat liver Agt11 cDNA library. Restriction map analysis identified three classes of cDNA clones (Fig. 1): class 1 (α1) consisted of Ra1-1 and Ra1-1-b; class 2 (α2) consisted of Ra2-2 and d; while class 3 (α3) consisted of Ra3-3 and Ra3-3-b. Clones representing 3' extensions of α2, Ra2-2 and c were subse-
sequently isolated 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 α2 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 α 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 α subunit mRNA isoform. Probes were prepared from the cDNA clones spanning the phosphorylation and ATP-binding sites from each class: α1 (Ra1-b), α2 (Ra2-a), and α3 (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 α1 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 α1 mRNA is more abundant in fetal than adult kidney, whereas it is more abundant in adult than fetal brain. An almost equal level of α1 mRNA is detected in fetal and adult heart, as well as in fetal and adult skeletal muscle. As shown in Fig. 2 C, the α2 probe also detects a ≈4.5-kb mRNA but this species is present only in brain and fetal heart. Like the α1 mRNA, the α2 isoform is also more abundant in adult than fetal brain. As shown in Fig. 2 D, the α3 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 4. Southern blot analysis of rat liver DNA. (a1) Southern blot of rat liver DNA digested with different restriction endonucleases (Ava I, Bam HI, Eco RI, Nco I, Pst I), hybridized to an α1 isoform-specific DNA probe spanning the 5'UT to the 3'UT. (a2) Subsequent hybridization of the same blot with an α2 isoform-specific DNA probe spanning from 5'UT to aa 940 (α1 numbering.) (a3) Subsequent hybridization of the same Southern blot with an α3 isoform-specific DNA probe spanning aa 44 to 3'UT (α1 numbering). Hae III- and Hind III-digested phage DNA markers are on the left in kilobase pairs.

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, SI-nuclease mapping analysis was performed using different regions of the entire RB5 and Rat-β cDNA clones (Fig. 3C). The results of two representative experiments are shown in Fig. 3A 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 SI-nuclease mapping experiments using end-labeled probes of different sizes in the 5' and 3' direction and probably represent artifacts of SI-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'-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 (Ovchinikov et al., 1986). Surprisingly, the rat α2 isomorph is only 82–85% homologous with rat α1, and the previously characterized α-subunits from sheep kidney (Shull et al., 1985), pig kidney (Ovchinikov 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 αa sequences (fine print, unnumbered) are aligned below. Spaces mark shifts in either sequence to allow optimal alignment for αa insertions/deletions. □ marks every 10 αa in α1. (. and -) Nucleotide and αa homology between α1 and α2 isoforms, respectively. Hydrophobic putative membrane-spanning regions H1-H4 are indicated; I- and 2-OUA-R, putative ouabain-binding regions; P*, phosphorylation site; FSB4, 5-(p-fluorosulfonyl) benzoyladenine-binding site; FITC, fluorescein 5-isothiocyanate–binding site; I, the I0-aa-long region of nonhomology.

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(Shull et al., 1985; Kawakami et al., 1985; Ovchinnikov et al., 1986; this paper) reveals the presence of a highly conserved five amino acid–long amino-terminal peptide that has not been detected in the mature protein. This peptide does not have the characteristics of a signal sequence (Kriel, 1981) and suggests the existence of a novel and so far undescribed posttranslational modification. Furthermore, the α2 isoform is also 10 aa longer than the brine shrimp α1 and α2 subunits, raising the possibility of a similar amino-terminal processing event for this isoform. The nature and significance of these posttranslational modifications remain to be elucidated.

The peptides identified for certain functional domains and all the putative membrane-spanning regions are all highly conserved at the aa level in the different α subunit cDNAs characterized to date, including the rat isoforms (Fig. 5). These regions include the phosphorylation site, CSDK (Bastide et al., 1973); putative ouabain–binding site, YTWLE (Shull et al., 1985); and two ATP analogue–binding sites, FITC (HLLUMKGAPER [Farley et al., 1984]) and 5′-(p-fluorosulfonyl) benzoyladenosine (FSBA)–binding sites ([α] MTAHMWFDOHEADT, and [β] DISHEOHILH-YHTEIVF (Ohta et al., 1985)).

A region of significant nonhomology between α1 and α2 isoforms was found in proximity to the ATP analogue (FITC)–binding site. This region (I) is located 5 aa upstream to the lysine residue identified as the FITC-binding site (Fig. 7). It spans 10 consecutive aa and differences in net charge, predicted secondary structure (Chou and Fasman, 1978), and hydrophilicity (Kyte and Doolittle, 1982) are noted between the Ca-ATPase isoforms (Brandl et al., 1986), also in proximity to the FITC-binding site (Fig. 7).

**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. α1 and α2, 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.

**Figure 7.** Regions of nonhomology in the Na,K-ATPase α subunit isoforms α1 and α2. Comparison with Ca-ATPase isoforms. A 10-aa-long region of nonhomology (I) is noted between α1 and α2 located in proximity to the FITC-binding site. This region differences in charged aa composition (+/−) and in predicted secondary structure, α-helix (†), β-sheet (−), and turn (−−−) by the Chou and Fasman (1978) algorithm are noted. A similar region of nonhomology also exists in the slow and fast twitch rabbit muscle isoforms of the Ca-ATPase (Brandl et al., 1986).

**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. Hydrophathy plots of α1 and α2 were obtained using the Kyte-Doolittle (Kyte and Doolittle, 1982) and the Goldman-Engelmann-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 hydrophathy plots identify seven putative membrane-spanning regions. Five regions, H₁ and H₃-6 (Fig. 8), fulfill the GES 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-
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; FSBA, 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 (H1-H7) 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 aa 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 ≈6.0- 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...
Figure 9. Structural diagram of the Na,K-ATPase α subunit topography. The putative topography of the Na,K-ATPase α subunit is deduced from a detailed analysis of α1 isoform's hydropathy plot, predicted secondary structure, and helical wheel analysis of the putative membrane-spanning regions. (A) Putative membrane spanning regions H₁-H₇ delineate the extracellular and intracellular domains. An alternative intracellular location of the carboxyl terminus (COOH) is designated should the 7th hydrophobic region not span the membrane (stippled area). Unit aa residues, (O), are notated in α-helix ( ), β-sheet ( ), or turn ( ), with the corresponding charge (+ or −), if any. Predicted alternative secondary structures with equal propensities based on the Chou and Fasman (1978) algorithm are marked alongside the primary unit aa structure; ( ), alternative α-helix; ( ), alternative β-sheet, ( ), alternative turn. (B) Helical wheel analysis of the seven putative membrane-spanning regions (H₁-H₇) suggests a putative relatively hydrophilic pore. The number of hydrophilic residues lining the aqueous pore and those situated in the outer region are noted. The location of charged residues (+ or −) is designated alongside the membrane-spanning regions.

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 α 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 α 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 α 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 α subunit raises the question as to the existence of isoform diversity for the β subunit. The possibility of specific α₁-β₃ subunit quaternary associations could generate more complex assembly and functional interactions. Furthermore, the existence of α 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 α 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 α subunit are most likely involved in the ion translocation
process. However, the topology of Na,K-ATPase α subunit remains controversial. Kyte-Doolittle hydrophathy plot analyses of three previously characterized α subunits (Shull et al., 1985; Kawakami et al., 1985; Ovchinikov 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., 1985), using the GES hydrophobicity scale. This scale has been proposed as appropriate for identifying nonpolar transbilayer helices in a sequence 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 hydrophathy 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 H2, 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 iris effect of the transmembrane helices of the gap junction in response to Ca2+ (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 (Ovchinikov 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 H2 and H6 (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 cryptic peptides, amino terminus (41 kD) and carboxyl terminus (58 kD), are both covalently labeled with H-N-(ouabain)-N (2-nitro-4-azidophenyl) ethylenediamine (Jorgensen et al., 1982), and with the location proposed by H-anthroyl-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 steric hindrance of conformational changes involving the respective flanking transmembrane regions by the glycoside. 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 aa sequences are highly homologous among the three species. On the other hand, there are differences between the α1 type and the α2 isoform. The α2 isoform like the electric ray electroplax 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.

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