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Molecular cloning of the β-subunit of the Na,K-ATPase in the brine shrimp, *Artemia*

The cDNA-derived amino acid sequence shows low homology with the β-subunits of vertebrates except in the single transmembrane and the carboxy-terminal domains

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A cDNA encoding the β-subunit of the Na,K-ATPase of brine shrimp (*Artemia*) has been cloned. Its nucleotide sequence and predicted amino acid sequence have been determined. The amino acid sequence shows considerable divergence from that of chicken, dog, human, pig, rat, sheep, *Torpedo*, and *Xenopus*. This is not entirely unexpected since brine shrimp is a 'fast clock' organism which diverged from the precursor of the vertebrates 0.5–1.0 billion years ago. However, a highly hydrophobic putative transmembrane domain and the carboxy-terminal domain show considerable conservation. The relatively small degree of conservation in the β-subunit of *Artemia* should provide information about the functional significance of this protein.

Na,K-ATPase; β-Subunit; cDNA cloning; Nucleotide sequence; Amino acid sequence; *Artemia*

1. INTRODUCTION

The Na,K-ATPase is an integral membrane protein which utilizes ATP as the energy source and maintains a low intracellular Na^+ concentration and a high intracellular K^+ concentration in all animal cells (see reviews [1–4]). These concentration gradients are necessary for a variety of physiological functions, including regulation of cell volume, maintenance of membrane excitability, and net transport of Na^+, K^+, sugars, and amino acids. The enzyme molecule consists of equimolar amounts of the catalytic α-subunit and a glycosylated β-subunit of unknown function. The cDNAs coding for the β-subunit of the Na,K-ATPase have been cloned and sequenced in chicken [5], dog [6], human [7], pig [8], rat [9], sheep [10], *Torpedo* [11], and *Xenopus* [12].

We have utilized the brine shrimp, *Artemia*, as a useful model system for studying developmental regulation of the Na,K-ATPase at the molecular level [13–20]. The Na,K-ATPase of *Artemia* is the best characterized invertebrate system. *Artemia* is an ancient organism which diverged from a precursor of the vertebrates 0.5–1 billion years ago. It is a 'fast clock' organism [21], thus accumulating mutations at a relatively high rate, presumably because of its rapid generation time of a few days. One might, therefore, anticipate low homology in amino acid sequences in nonessential domains.

We previously sequenced an α-isoform of the brine shrimp [22] and elicited its membrane topology through analysis of the primary structure of the protein, as deduced from the nucleotide sequence. The α-subunit cDNA sequence shows less homology with the higher forms, but the hydropathy plot of the predicted amino acid sequence is essentially identical to that in the other organisms.

We report here the molecular cloning, nucleotide sequence, and predicted amino acid sequence of the β-subunit of Na,K-ATPase from brine shrimp in the hope of understanding further the molecular architecture of this enzyme and its conservation through evolution.

2. MATERIALS AND METHODS

2.1. *Construction of cDNA libraries*

The cDNA libraries from the size fractionated mRNA of brine shrimp (San Francisco Bay Brand, Lot 1521) grown for 18 h was constructed in λgt10 and λgt11 vectors, as described by Baxter-Lowe et al. [22].

2.2. *Screening of cDNA libraries*

The λgt11 expression library was screened using polyclonal antisera raised against the purified β-subunit of brine shrimp Na,K-ATPase

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[14,23]. Antibody screening was carried out using the ProtoblotTM Immunoscreening system from Promega Biotec.

The λgt10 library was screened using standard hybridization procedures [24] with the largest cDNA probes (obtained in the antibody screening), which were radiolabelled with ^{32}P, either by nick translation [24] or by using an oligolabelling kit by Boehringer Mannheim Biochemicals.

2.3. *Analysis and screening of cDNA*

The positive clones were plaque purified and then analyzed by restriction mapping using standard procedures [24]. The inserts were subcloned into M13mp18 and M13mp19 vectors and sequenced by the dideoxy chain termination method of Sanger [25] using a sequencing kit (Sequenase, United States Biochemical). The entire sequence was determined by sequencing restriction fragments in both strands of cDNA and analyzed with the UWGCG sequence analysis program [26].

3. RESULTS AND DISCUSSION

3.1. *Nucleotide sequence of the brine shrimp Na,K-ATPase β-subunit cDNA*

When the λgt11 expression library was screened with a polyclonal antibody raised against the β-subunit of Na,K-ATPase, 17 positive clones were obtained on duplicate filters from 3.5×10^5 clones of the amplified library. Seven phage clones remained immunoreactive through five subsequent rounds of screening and plaque purification. The largest of these clones contained an insert (B11) of approximately 1100 nucleotides long, as determined by agarose gel electrophoresis.

DNA sequence analysis revealed that B11 was missing approximately 60 nucleotides of coding region from the 5′-end relative to known sequences of β-subunits from other species. B11 was used as a hybridization probe to screen a λgt10 library containing inserts 1300–4000 nucleotides long. 126 positive clones were isolated in the initial screening, 12 of which were brought through four rounds of screening and plaque purification. Restriction analysis and subsequent sequence determination revealed that one of these cDNA clones [29, 1794 nucleotides long) contained sequence information for the entire coding region of the β-subunit and 5′- and 3′-untranslated regions. Sequence analysis of this cDNA clone (29) showed an open reading frame of 945 nucleotides, which codes for a peptide containing 315 amino acids (Fig. 1). The molecular weight of the peptide was calculated to be 35954 Da, which agrees well with the reported molecular weight of β-subunits from other species [5–12].

The nucleotide triplet, at position 72 of the cDNA sequence, is an ATG codon, which we assign as the start methionine for the initiation of translation for the following reasons: (i) This ATG triplet is the first one that appears downstream of a nonsense codon (TGA at position 54 through 56; (see also [6–11]). (ii) The amino-terminal residue, just after the methionine, is alanine, which is true for all the mammalian, avian, amphibian and elasmobranch β-subunits sequenced so far [5–12]. (iii) The position of ATG agrees well with the Kozak survey, which indicates a high frequency of certain short defined base sequences around the start ATG sequence [27]. Like all other β-sequences from different species, *Artemia* β also does not have a leader sequence.

The translation termination codon, which begins at position 1017, is followed by 778 nucleotides of 3′-untranslated sequence containing two potential polyadenylation signals (AATAAA) starting at residues 1371 and 1707.

3.2. *The predicted amino acid sequence of the β-subunit of the brine shrimp Na,K-ATPase and comparison with higher organisms*

The predicted amino acid sequence of the brine shrimp β-subunit is shown in Fig. 1, and a comparison of this sequence with that of other species is shown in Fig. 2. The *Artemia* β-peptide (315 amino acids long) is 10 to 12 amino acids longer than that of other species. Within mammalian species, the β-subunit shows >90% identity, and *Torpedo* shows >60% identity with mammals. The brine shrimp β-subunit amino acid sequence shows approximately 60% similarity with previously reported mammalian, *Torpedo* and *Xenopus* sequences and only 35% identity. If *Torpedo* and *Xenopus* are excluded from the amino acid sequence comparisons there is somewhat higher identity between the brine shrimp and the mammals. The polypeptide sequence contains large insertions and deletions relative to reported mammalian and *Torpedo* sequences (Fig. 2).

In a previous paper [22], we reported that the protein sequence of one of the α-subunits of the brine shrimp shows about 83% similarity and 69% identity with higher organisms. The brine shrimp α-sequence shows the largest reported deviation from the α-subunit consensus sequence. It appears that the primary structure of the β-subunit of brine shrimp diverges even more from the higher species. The limited number of conserved sequences should provide information about the functional significance of the various domains of the β-subunit.

The N- and C-terminal domains of the *Artemia* β-subunit show high similarity with those of other species, suggesting that these regions may be functionally significant. The N-terminal region of high similarity is a stretch of 35 uncharged amino acids, including many non-polar residues, which represents the transmembrane segment. This is evident from the hydropathy plot (Fig. 3) made according to Kyte and Doolittle [28].

The number and location of potential N-linked glycosylation sites with the sequence Asn-X-Ser/Thr, where X can be any amino acid, have been conserved among the β-subunits of all mammalian species studied to date [6–10]. *Torpedo californica* has one additional site which is not present within the mammalian system. In *Artemia*, there are three potential glycosylation sites at positions 198, 222, and 296, which, with the excep-

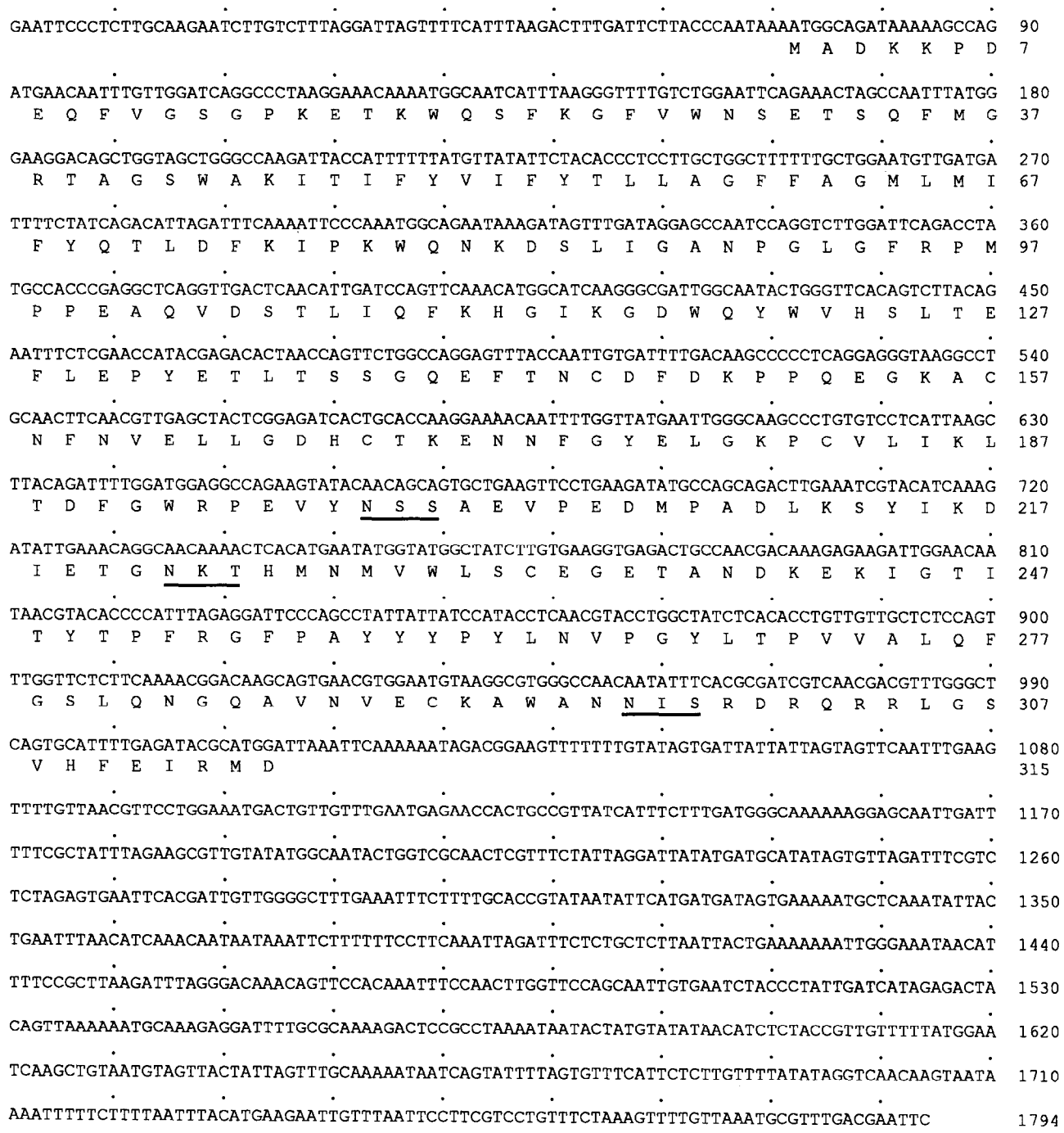

Fig. 1. The nucleotide sequence of brine shrimp Na,K-ATPase β-subunit cDNA and the deduced amino acid sequence of the encoded protein. Nucleotide residues are numbered in the 5′ to 3′ direction beginning with the first base of the cDNA insert, and the deduced amino acid residues are numbered beginning with the amino-terminal start methionine. Potential glycosylation sites are underlined.

tion of residue 198, do not correspond closely to the potential glycosylation sites of the higher species (Fig. 2). There is almost complete conservation of cysteine residues in the β-subunits of *Artemia*, mammals, *Xenopus*, and *Torpedo*.

The brine shrimp is a 'fast clock' organism; i.e., it demonstrates a high rate of mutation [21]. Many of the mutations which produce homologous proteins in different species will be neutral; i.e., they will show no effect on the function of the protein. The possibility for neutral mutations is dependent upon the amount of variation permissible in the amino acid sequences. If the precise amino acid sequence is not crucial for function, a large fraction of the total mutations will be neutral, and the sequence will be more subject to rapid change. Since only those mutations which are not deleterious

```
          1                                                  50
ARTEMIA   MA.DKKPDEQ FVGSGPKETK WQSFKGFVWN SETSQFMGRT AGSWAKITIF
CHICKEN   MARGKAND.. ..GDG..... ..NWKKFIWN SEKKELLGRT GGSWFKILLF
   DOG    MARGKAKEE. ..G....... ..SWKKFIWN SEKKEFLGRT GGSWFKILLF
 HUMAN    MARGKAKEE. ..G....... ..SWKKFIWN SEKKEFLGRT GGSWFKILLF
   PIG    MARGKAKEE. ..G....... ..SWKKFIWN SEKKEFLGRT GGSWFKILLF
   RAT    MARGKAKEE. ..G....... ..SWKKFIWN SEKKEFLGRT GGSWFKILLF
 SHEEP    MARGKAKEE. ..G....... ..SWKKFIWN SEKKEFLGRT GGSWFKILLF
TORPEDO   MAREKSTDD. ..GGG..... ...WKKFLWD SEKKQVLGRT GTSWFKIFVF
XENOPUS   MARDKAKET. .DG....... ..GWRKFIWN ADKKEFLGRT GGSWFKILLF
          **  *        *           0 0* *    00  0*** 0 **0**  *

          51                                                 100
ARTEMIA   YVIFYTLLAG FFAGMLMIFY QTL.DFKIPK WQNKDSLIGA NPGLGFRPMP
CHICKEN   YVIFYGCLAG IFIGTIQVML LTVSEFE.PK YQDR....VA PPGLT..QVP
   DOG    YVIFYGCLAG IFIGTIQVML LTISEFK.PT YQDR....VA PPGLT..QIP
 HUMAN    YVIFYGCLAG IFIGTIQVML LTISEFK.PT YQDR....VA PPGLT..QIP
   PIG    YVIFYGCLAG IFIGTIQVML LTISEFK.PT YQDR....VA PPGLT..QIP
   RAT    YVIFYGCLAG IFIGTIQVML LTISELK.PT YQDR....VA PPGLT..QIP
 SHEEP    YVIFYGCLAG IFIGTIQVML LTISEFK.PT YQDR....VA PPGLT..QIP
TORPEDO   YLIFYGCLAG IFIGTIQVML LTISDFE.PK YQDR....VA PPGLS.HS.P
XENOPUS   YLIFYGCLAG IFIGTIQVLL LTISEFE.PK YQDR....VA PPGLT..QLP
          * ***00*** 0*0*0000 0 0*      *  0*00    0* 0***     *

          101                                                150
ARTEMIA   PEAQVDSTLI QFK.HGIKGD WQYWVHSLTE FLEPYETLTS SGQE.....F
CHICKEN   ...QVQKTEI SFTVNDPKS. YDPYVKNLEG FLNKY....S AGEQTDNIVF
   DOG    ...QIQKTEI SFRPNDPKS. YEEYVRNIVR FLEKYK...D SAQK.DEMIF
 HUMAN    ...QIQKTEI SFRPNDPKS. YEAYAMNIVR FLEKYK...D SAQ.RDDMIF
   PIG    ...QSQKTEI SFRPNDPQS. YESYVVSIVR FLEKYKDLAQ KDD....MIF
   RAT    ...QIQKTEI SFRPNDPKS. YEAYVLNIIR FLEKYK...D SAQK.DDMIF
 SHEEP    ...QIQKTEI AFRPNDPKS. YMTYVDNIDN FLKKYR...D SAQK.DDMIF
TORPEDO   ..YAV.KTEI SFSVSNPNS. YENHVNGLKE LLKNYN...E SKQD.GNTPF
XENOPUS   ..RAV.KTEI SFSPSDSNS. YQEYVKSMDN FLSKYNNEKQ GS....NM.F
               0*0*  *      0  0           *  *               *

          151                                                200
ARTEMIA   TNC.....DF .DKPP...QE G..KACNFNV ELLGDHCT.K ENNFGYELGK
CHICKEN   QDCGDIPTDY KERGPYNDAQ GQKKVCKFKR EWLENCSGLQ DNTFGYKDGK
   DOG    EDCGNMPSEI KERGEFNNER GERKVCRFKL EWLGNCSGIN DETYGYRDGK
 HUMAN    EDCGDVPSEP KERGDFNHER GERKVCRFKL EWLGNCSGLN DETYGYKEGK
   PIG    EDCGNVPSEL KERGEYNNER GERKVCRSRL EWLGNCSGLN DETYGYKDGK
   RAT    EDCGSMPSEP KERGEFNHER GERKVCRFKL DWLGNCSGLN DESYGYKEGK
 SHEEP    EDCGNVPSEL KDRGEFNNEQ GERKVCRFKL EWLGNCSGIN DETYGYKEGK
TORPEDO   EDCGVIPADY ISRGPIEESQ GQKRVCRFLL QWLKNCSGID DPSYGYSEGK
XENOPUS   EDCGTIPGPY HERGALNKDE GMKKSCVFRR EWLQNCSGLN DPSYGFADGK
           0*          00        *    *       0* 0000   0   *   **
```

Fig. 2. Alignment of amino acid sequences of Na,K-ATPase β-subunits. Complete sequences of brine shrimp, mammals, *Xenopus*, and *Torpedo* are shown. Identity in all species is indicated by an asterisk, and identity in all species except brine shrimp is indicated by a '0'. No assignment is made where gaps are present.

allow survival, sites that mutate most quickly will be those that do not have adverse effects on the protein.

Conservation of the cysteine residues, the transmembrane section, and the carboxyterminal sequences is consonant with the view that the tertiary structure has been highly conserved during evolution. A three-dimensional structure defined at an early stage of evolution in the common ancestor would be the crucial

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          201                                                  250
ARTEMIA   PCVLIKLTD. FGWR...PEV YNSSAEVPED MPADLK.S.Y IKDIETGNKT
CHICKEN   PCILVKLNRI IGFKPKAPE. .NES..LPSD L.AG.KYNPY LIPVHCVAK.
   DOG    PCVLIKLNRV LGFKPKPPK. .NES..L.EA YPV.MKYSPY VLPVQ.....
 HUMAN    PCIIIKLNRV LGFKPKPPK. .NES..L.ET YPV.MK...Y .......NP.
   PIG    PCVIIKLNRV LGFKPKPPK. .NES..L.ET YPV.MKYNPY VLPV......
   RAT    PCIIIKLNRV LGFKPKPPK. .NES..L.ET YPLTMK...Y .......NP.
 SHEEP    PCVIIKLNRV LGFKPKPPK. .NES..L.ET YPV.MKYNPY VLPVQ.....
TORPEDO   PCIIAKLNRI LGFYPKPP.. KN.GTDLPEA WQANYN..QY VLPI......
XENOPUS   PCVIVKLNRI LAFKPVPPQ. .NNS..L... .PPEMT.LNY .......NPY
          **   **00    0    *    *    0             *

          251                                                  300
ARTEMIA   HMNMVWLSCE GETANDKEKI GTITYTPFRG FPAY...YYP .Y...LNVPG
CHICKEN   .....R.... DEDA.DK..I GMVEYYGMGG YPGFALQYYP YYGRLLQ.PQ
   DOG    ........CT GKRDEDKDRI GNVEYFGLGG YPGFPLQYYP YYGKLLQ.PK
 HUMAN    ..NVLPVQCT GKRDEDKDKV GNVEYFGLGN SPGFPLQYYP YYGKLLQ.PK
   PIG    H.......CT GKRDEDKEKV GTMEYFGLGG YPGFPLQYYP YYGKLLQ.PK
   RAT    ..NVLPVQCT GKRDEDKDKV GNIEYFGMGG FYGFPLQYYP YYGKLLQ.PK
 SHEEP    ........CT GKRDEDKEKV GSIEYFGLGG YPGFPLQYYP YYGKLLQ.PK
TORPEDO   H.......CQ AKKEEDKVRI GTIEYFGMGG VGGFPLQYYP YYGKRLQ.KN
XENOPUS   ...VIPIHCQ AKKEEDIEKI KEVKYYGMGG FAGFPLTYYP YYGKLLQ.PD
                     *         * 0 0    00   ***  *   *0

          301                                                  350
ARTEMIA   YLTPVVALQF GSLQNGQAVN VECKAWANNI ..S.RDR.QR RLGSVHFEIR
CHICKEN   YLQPLVAVQF TNLTYDVEVR VECKEYGQNI QYSDKDRFQG RFD.IKFDIK
   DOG    YLQPLLAVQF TNLTMDTEIR IECKAYGENI GYSEKDRFQG RFD.VKIEVK
 HUMAN    YLQPLLAVQF TNLTMDTEIR IECKAYGENI GYSEKDRFQG RFD.VKIEVK
   PIG    YLQPLMAVQF TNLTMDTEIR IECKAYGENI GYSEKDRFQG RFD.VKIEVK
   RAT    YLQPLLAVQF TNLTLDTEIR IECKAYGENI GYSEKDRFQG RFD.VKIEVK
 SHEEP    YLQPLLAVQF TNLTMDTEIR IECKAYGENI GYSEKDRFQG RFD.VKIEVK
TORPEDO   YLQPLVGIQF TNLTHNVELR VECKVFGDNI AYSEKDRSLG RF.....EVK
XENOPUS   YLQPLIAVQF TNITFDAEVR IECKAYGENI DYHDKDRFQG RFD.VKFDIK
          **0*0   ** 00 0   0 0  ***  0 **     0**  0 *0       0

          351
ARTEMIA   MD...
CHICKEN   SS...
   DOG    S....
 HUMAN    S....
   PIG    S....
   RAT    S....
 SHEEP    S....
TORPEDO   IEVKS
XENOPUS   SS...
```

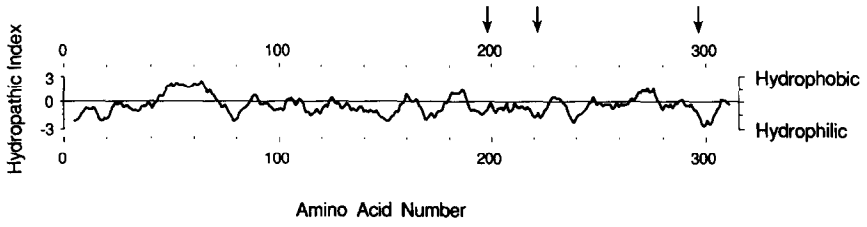

Fig. 3. Hydropathy profile of the Na,K-ATPase β-subunit. Seven-residue averages have been plotted using a computer program based on Kyte and Doolittle [28]. The locations of potential N-linked glycosylation sites (arrows) are indicated.

parameter for the functioning of the protein. The high conservation of the amino acid sequences among the mammalian β-subunits suggests that there may be some species-specific limitations on its structure which are not necessary for the brine shrimp. Alternatively, the relatively recent appearance of mammals as compared to *Artemia*, along with a slower rate of mutation in the former, may account for the high similarity of the β-subunits in various mammalian species.

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