Physiol. Res. 44: 261–274, 1995

EDITORIAL

Na,K-Adenosinetriphosphatase: The Paradigm of a Membrane Transport Protein

A. KOTYK, E. AMLER

Institute of Physiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic

Received March 1, 1995
Accepted May 10, 1995

I. History

It was recognized more than half a century ago that specific movements of Na^+ take place across some animal cell layers as well as across the membranes of other cells. Some of these movements are thermodynamically passive, i.e. they proceed along the electrochemical gradient, e.g. when sodium channels open during the passage of an action potential peak along the nerve axon – this is now textbook knowledge, since it was the basis of two Nobel prizes for physiology and medicine (H.H. Dale and O. Loewi 1936, A.L. Hodgkin, A.F. Huxley and J.C. Eccles 1963).

However, another class of these movements are active, proceeding against a gradient of the electrochemical potential and are to be found in a variety of cells and tissues, such as the frog skin (Krogh 1937, Ussing 1950), toad bladder (Leaf *et al.* 1959), bird, turtle and shark rectal gland (Fänge *et al.* 1958, Burger and Hess 1960). At present, this type of active sodium transport has been established for the entire animal kingdom, ranging from primitive *Hydra* polyps, insects and worms to all classes of vertebrates.

Hand in hand with the physiological observations of active sodium transport, often dubbed "the sodium pump", went the biochemical search for the entity responsible for this activity. The two protagonists of this search were J.C. Skou (1957, 1960) and R.L. Post (1959, Post *et al.* 1960).

These authors isolated the microsome-bound ATP-hydrolyzing activity from crab nerves and from human erythrocytes, respectively, and compared it with the concurrent sodium extrusion from cells. It was found in the erythrocytes that (1) both systems are located in the cell membrane, (2) both systems use ATP in contrast to ITP, (3) both require Na^+ and K^+ together, (4) the concentration for half-maximum activation by K^+ in the presence of Na^+ is 3 mM for the enzyme and 2.1 mM for the transport, (5) the concentration for half-maximum activation by Na^+ in the presence of K^+ is 24 mM for the enzyme and 20 mM for the transport, (6) half-maximum inhibition by ouabain in the presence of Na^+ and K^+ was 0.1 μM for the enzyme and 0.07 μM for the transport, (7) NH_4^+ substituted for K^+ but not for Na^+.

The stage was set for attempts to isolate the enzyme. It then took over a decade before reproducible procedures were worked out. In the 1970s, the enzyme was isolated from dog kidney (Kyte 1972, Jørgensen 1974), shark rectal gland and eel electric organ (Perrone *et al.* 1975), duck nasal gland (Hopkins *et al.* 1976) and brine shrimp (Peterson and Hokin 1980). In all these cases, and many others that have been described up to the present, the ATPase was shown to be the enzyme responsible for maintaining an electrochemical disequilibrium of Na^+ (lower concentrations intra- than extracellularly) and of K^+ (higher concentrations intra- than extracellularly) and, simultaneously, for generating the membrane potential of all the cells involved, with the negative side facing inward. Both these functions are essential for nutrient uptake, regulation of cell volume, membrane excitability, electric discharge and others.

II. Biochemistry and Genetics of the Enzyme Molecule

The Na,K-ATPase belongs to the P-type (formerly E_1E_2-type) adenosinetriphosphatases, a family which is composed of perhaps a dozen different enzymes with appreciable homology of amino acid sequences. The number of members of the family is certainly not final, particularly as there is no unanimity in assigning an identical Enzyme Commission number to enzymes with the same function but from different

sources and with a sequence homology of little more than 40 % (Kotyk 1991).

This family differs substantially from other types of ATPases involved in the translocation of cations (H^+ in a vast majority of cases, Na^+ in some alkaliphilic bacteria), viz. the V-type and F-type ATPases. Both these types contain several different subunits, some in multiple copies.

The Na,K-ATPase contains one principal catalytic subunit, designated α and one sugar-rich auxiliary subunit, designated β. There may be an associated subunit γ, the three occurring in a 1:1:1 ratio (Jørgensen 1982, Glynn 1985, Forbush *et al.* 1978).

A. The α subunit

The α subunit has been fully sequenced for enzymes ranging from shrimp to mammals (Kawakami *et al.* 1985, Shull *et al.* 1985, Ovchinnikov *et al.* 1986, Baxter-Lowe *et al.* 1989, Lebovitz *et al.* 1989, Takeyasu *et al.* 1990). Its molar mass is 110–115 kDa and it shows great similarity among species, the most distant enzymes (shrimp vs. man) still showing a 60 % identity. Moreover, it shows a substantial similarity with other P-type ATPases, thus 53 % with the sarcoplasmic reticulum Ca-ATPases, 81 % with gastric mucosa H,K-ATPase, 48 % with the protozoan *Leishmania donovani* H-ATPase, 50 % with the plant *Arabidopsis thaliana* H-ATPase, 47 % with the yeast *Saccharomyces cerevisiae* H-ATPase and 46 % with the bacterium *Streptococcus faecalis* H-ATPase (Serrano 1989, Rao *et al.* 1992, Sachs *et al.* 1992). The evolutionary ancestor of all metazoa was found to possess a Na,K-ATPase α subunit that is highly conserved with respect to its vertebrate counterparts (Canfield *et al.* 1992).

Although the α subunit contains some 20 cysteine residues, there has been hesitation about their assignment to disulfide bridges (Kirley 1989). Perhaps one such bridge is present (Gevondyan *et al.* 1989).

The α subunit occurs in several isoforms, designated $\alpha 1$, $\alpha 2$ and $\alpha 3$, which differ by about 15 % between isoforms from the same source but only by some 7 % between different sources, for instance, isoforms $\alpha 1$ from the rat and from the chicken (Mercer 1993). They are each coded by a different gene, some of them located on different chromosomes (Sweadner 1989). The various isoforms differ primarily in their tissue distribution, $\alpha 1$ predominating in the kidney and lung, $\alpha 2$ in skeletal muscle and $\alpha 3$ in the brain. It is possible that each of these isoforms confers different properties on the Na,K-ATPase which allows effective coupling to the physiological process for which it provides energy in the form of an ion gradient. It is also possible that the multiple isoforms are the result of gene triplication and that they exhibit similar enzymic properties. In this case, the expression of the triplicated genes would be individually regulated to provide the appropriate amount of Na,K-ATPase to the particular tissue and at specific periods of development. While differences are observed in such parameters as Na^+ affinity and sensitivity to cardiac glycosides, it is not known if these properties play a functional role within the cell (Lingrel 1992).

The disposition of the α subunit in the membrane is still not fully clarified. There are three distinct regions of the polypeptide chain: that at the N-terminus with four membrane-spanning segments (H1 to H4), a large cytosolic loop (between H4 and H5) which represents almost one-half of the enzyme's mass (440 amino acid residues within a volume of about 160 nm^3), and a part at the C-terminus, with several, most probably 4 or 6, transmembrane spans (H5 to H10). Fig. 1 compares the two most probable dispositions of the 8-segment and the 10-segment model.

Although a number of authors take the 8-span model as sufficient (Jørgensen and Anderson 1988), evidence from labelling with tritiated spiro (adamantane-4,3-diazirine) and analogy with the closely related plasma membrane Ca-ATPase (Shull and Greeb 1988) speak strongly in favor of the 10-span model. Perhaps an "intermediate" model will provide the correct answer, viz. the one where the polypeptide segment between amino acid residues approximately 975 and 995 is merely embedded in the membrane rather than spanning it fully, without showing an extracellularly exposed section between H9 and H10 (Dzhandzhugazyan and Modyanov 1994).

The α subunit carries the entire catalytic function of the enzyme and this is reflected in its possession of several binding and functional domains. First of all, it is the ATP-binding region which forms a hydrophobic pocket in the H4H5 cytoplasmic loop, represented by Leu, Ile, Val and His between residues 546 and 555, apparently a β-pleated sheet that lies adjacent to the triphosphate part of the ATP molecule. Binding of ATP apparently also involves amino acids located at rather distant positions of the loop. The results that were obtained using the ATP analog 5-(*p*-fluorosulfonyl)-benzoyladenosine (FSBA) suggest amino acids 655-667 and 704-722 and probably Cys-656 and Lys-719, while those yielded by another analog, γ[-(4-*N*-2-chloroethyl-N-methylamino)] benzolamide adenosine triphosphate (Cl-ATP), indicate a role of Asp-710 (Fig. 1).

Clearly, there is a region with which ATP interacts through phosphorylation of the β-carboxyl group of a particular aspartic acid residue, in usual numbering Asp-372. These regions are remarkably well conserved not only in all P-type ATPases but also in other nucleotide-binding enzymes (Table 1). In addition to the sequences shown there is almost full identity of 17 amino acid residues starting at position 707 where FSBA and Cl-ATP are bound to the ATPase, among all the P-type ATPases examined (Ohta *et al.* 1986).

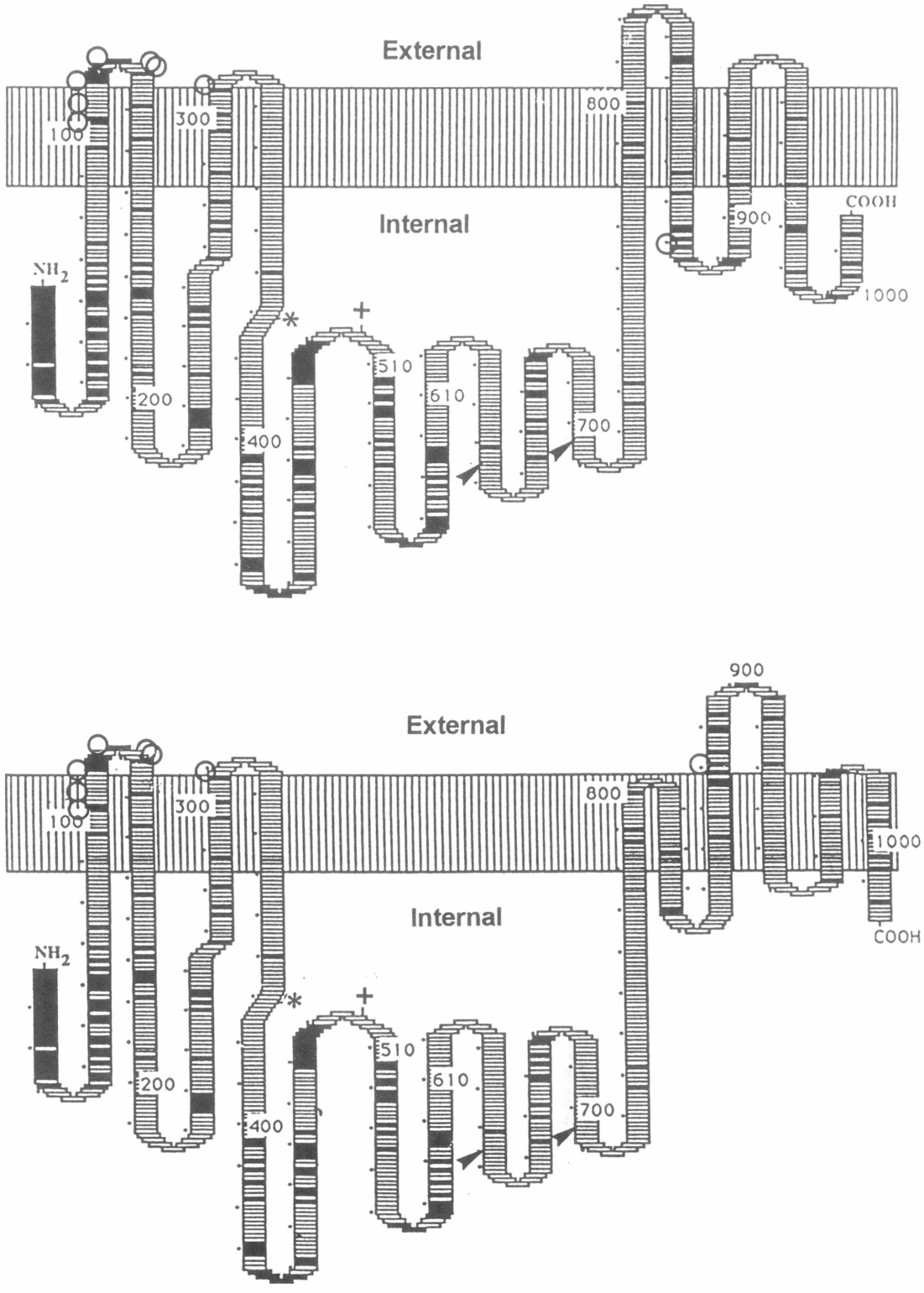

Fig. 1
Topography of the 8-span (A) and 10-span (B) model of Na,K-ATPase subunit in the plasma membrane. The asterisk shows the phosphorylatable Asp residue, the cross shows the binding site for fluorescein isothiocyanate, the arrows point to the parts where the ATP analog FSBA is bound, open circles describe amino acid residues probably involved in the binding of ouabain. (Adapted from Takeyasu *et al.* 1990.)

Table 1
Amino acid sequences in the nucleotide-binding and phosphorylation regions of ATPases and some related enzymes (adapted from Jørgensen and Anderson 1988)

Nucleotide binding	
543 LGERV-LGFCHLFLPDEQFP 561	Na,K-ATPase
613 LKCRT-AGIRVIMVTGDHPI 631	H,K-ATPase
611 QLCRD-AGIRVIMITGDNKG 639	Ca-ATPase of sarcoplasmic reticulum
545 SEARH-LGLRVKMLTGDAVG 563	H-ATPase from yeast
243 EYFRDQEGQDVLLFIDNIFR 262	β subunit of bovine F-ATPase
277 VL-RGNGGAFVLVLYDEIKK 295	ADP/ATP translocator
104 EFERK-IGQPTLLLYVDAGP 122	adenylate kinase
87 EQLKK-HGIQGLVVIGGDGS 105	phosphofructokinase
Fluorescein isothiocyanate binding	
496 PQHLLVMKGAPERILDRCSS 515	Na,K-ATPase
510 PRHLLVMKGAPERVLERCSS 519	H,K-ATPase
508 VGNKMFVKGAPEGVIDRCNY 517	Ca-ATPase of sarcoplasmic reticulum
467 GERIVCVKGAPLSALKTVEE 486	H-ATPase from yeast
388 IDNRMIRKGSVDAIRRHVEA 407	K-ATPase from *Escherichia coli*
Phosphorylation site	
366 TSTICSDKTGTLTQNRM 382	Na,K-ATPase
379 TSVICSDKTKTLTQNRM 395	H,K-ATPase
345 TSVICSDKTGTLTTNQM 361	Ca-ATPase of sarcoplasmic reticulum
372 VEILCSDKTGTLTKNKL 388	H-ATPase from yeast
301 VDVLLLDKTGTITLGNR 317	K-ATPase from *Escherichia coli*
293 QERITTTKKGSITSVQA 309	β subunit of bovine F-ATPase*

* No phosphorylation takes place in the F-ATPase and hence the D residue is missing in the sequence.

At the C-terminus of the α subunit there may be a potential phosphorylation site for the action of cAMP-dependent protein kinase or protein kinase C, represented by the RRNSV sequence of amino acids (Bertorello *et al.* 1991).

The α subunit is also the target of a highly specific inhibitor of the Na^+ and K^+ transport, viz. ouabain and the related digitalis glycosides. The amino acids involved in the binding were identified following the observation that the rat α1 isoform, which is virtually insensitive to the drug, contains several alterations in its amino acid sequence, compared, for instance, with the sheep α subunit (Kent *et al.* 1987, Price *et al.* 1990). The residues involved are apparently Gln-111, Pro-118, Asp-121 and Asn-122 on the outward-facing extramembrane segment between H1 and H2. However, other residues, such as Tyr-308 on the H3H4 loop and Arg-880 on the H7H8 loop (Schultheis *et al.* 1993), as well as some intramembrane residues, such as Cys-104 and Tyr-108, may be implicated. The fact that amino acid residues within the transmembrane region affect ouabain sensitivity suggests that the drug is partially internalized in the lipid bilayer. Since ouabain and ouabagenin (the aglycone) behave identically, it appears that the amino acids identified as determinants of ouabain sensitivity are not likely to interact with the sugar.

In fact, there have been a great many reports on the presence of a digitalis-like substance produced endogenously by the mammalian body. Such a substance was recently purified from human plasma and identified as a cis-trans-cis steroid of plant origin and similar to the humoral factor produced in the hypothalamus (Hamlyn and Manunta 1992). The factor inhibits the Na,K-ATPase and cross-reacts with a digoxin antibody (Yamada *et al.* 1992). An increased level of the endogenous digitalis-like substance may be involved in the mechanism of hypertension associated with insulin-dependent diabetes (Chen *et al.* 1993) and also in pregnancy-induced hypertension (Amler *et al.* 1993a, 1994). For recent reviews of the physiological role of the digitalis-like factors see, e.g., Schoner (1993) and Doris (1994).

It is somewhat ironical that amino acid residues involved in the actual movement of Na^+ and K^+ ions across the membrane are not known, in spite of considerable effort toward that objective (Jørgensen and Anderson 1988).

When expressed in HeLa cells, the three isoforms of the α subunit exhibit a two- to three-fold difference in their $K_{0.5}$ for Na^+ ($\alpha 1 = \alpha 2 < \alpha 3$) (Jewell and Lingrel 1992). Unfortunately, these experiments do not reveal a region in the $\alpha 1$ or $\alpha 3$ isoform that is clearly responsible for the apparent affinity for Na^+. It is possible that molecular interactions involving amino acids that span virtually the entire Na,K-ATPase molecule contribute to the determination of this parameter.

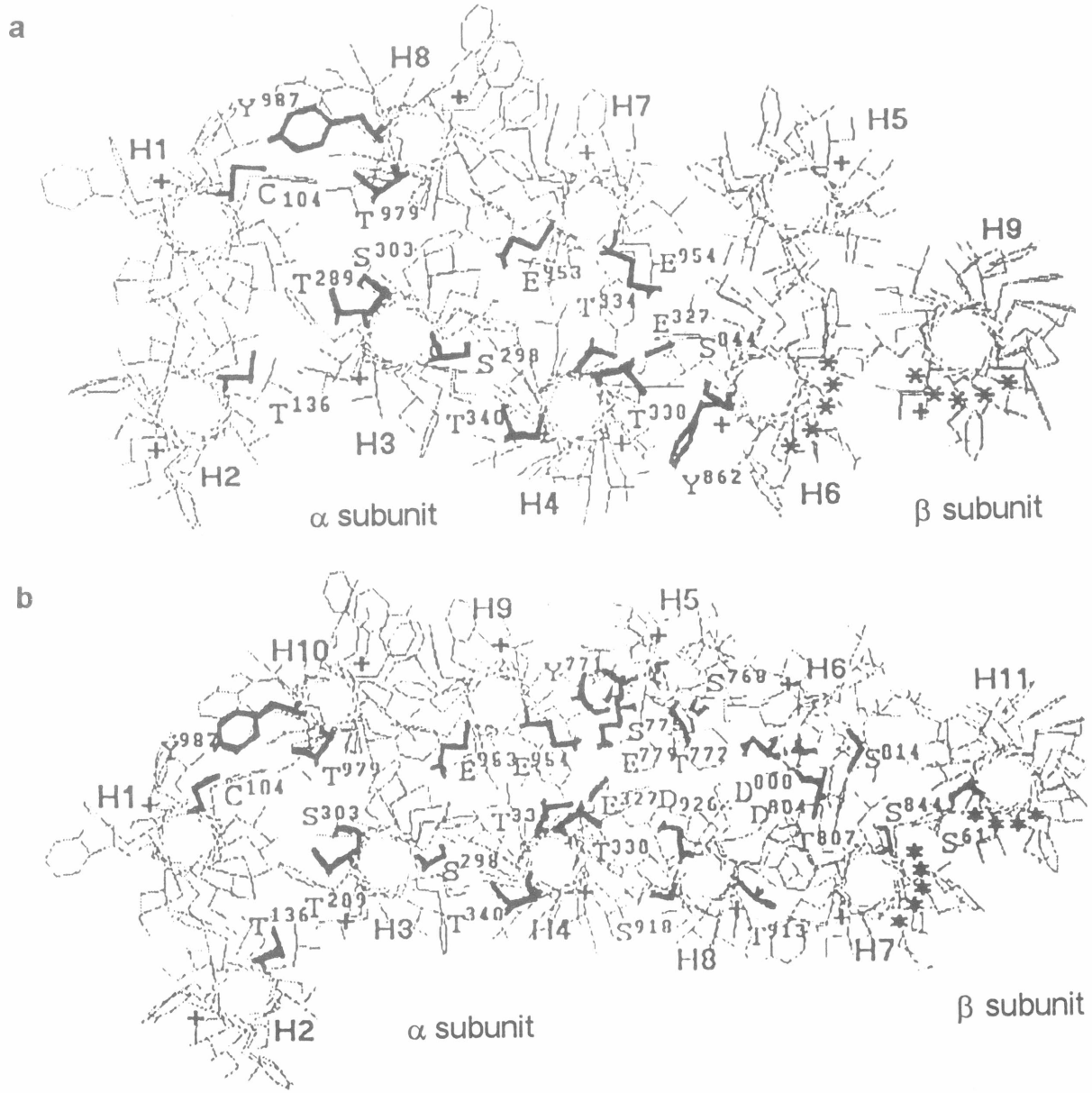

Fig. 2
Extracellular view of the Na,K-ATPase disposition in the membrane, assuming 8 transmembrane segments (a) and 10 transmembrane segments (b). Residues that could form ion-conducting pathways are shown in heavy lines. The most variable sites are marked with crosses (+). (According to Modyanov *et al.* 1992.)

Some acidic amino acid residues, however, have been identified as being involved in sodium or potassium transport across the membrane, especially dicarboxylic amino acids. Incubation of purified renal Na,K-ATPase with the fluorescent carboxyl-selective reagent, 4-(diazomethyl)-7-(diethylamino)-coumarin, results in enzyme inactivation *via* disruption of the univalent cation binding sites and loss of K^+ and Na^+ binding capacity. Modification of one or two carboxyl residues in the α subunit in a K^+- or Na^+-preventable

manner, however, leaves the ATP binding unaltered, and the enzyme is still able to undergo the major conformational transitions (Arguello and Kaplan 1991). The latest work along this path applied site-directed mutagenesis of negatively charged residues within the membrane. There are several of them: Glu-327, Glu-779, Asp-804, Asp-808, Asp-926, Glu-953 and Glu-954 (Fig. 2). Now Glu-779 appears to be essential for the binding of both Na^+ and K^+ (Arguello and Kaplan 1994) while modification of the last two had virtually no effect on the binding (Lingrel *et al.* 1994).

The location of Glu-779 in the fifth transmembrane segment provides a way in which information about ATP binding and phosphorylation in the major cytoplasmic loop of the enzyme is transmitted to intramembrane cation sites during the reaction cycle.

The only thing that appears to be almost certain in this context is that the Na^+-binding sites and the K^+-binding sites are not simultaneously present on the α-subunit molecule (see section on reaction mechanisms).

B. The β subunit

This subunit contains between 302 and 305 amino acid residues, with an approximate molar mass of 35 kDa, depending on the species. The full sequence is now known from several mammals, chicken, frogs and fishes. The homology is high among mammals (about 95 %) but less so in comparison with the amphibian and fish enzymes. There are two highly conserved regions in all the species examined between residues 35 and 63, and between 233 and 250. The first of these is now established as the single membrane-spanning segment of the β subunit while the second is probably in close contact with the membrane and/or with a hydrophobic part of the α subunit.

There are two interesting structural aspects that distinguish the β subunit from the α subunit: (1) the presence of three disulfide bonds at Cys-125–Cys-148, Cys-158–Cys-174 and Cys-212–Cys-275; (2) the presence of sugar-binding sites, three in the mammalian enzyme, four in the fish enzyme, characterized by the Asn-X-Ser or Asn-X-Thr sequence, at Asn-157, Asn-164 and Asn-192 (Ovchinnikov *et al.* 1986). This contrasts with the absence of such situation in the α subunit. The N-linked sugars are oligosaccharides with terminal sialic acid, penultimate galactose and a high mannose content, adding up to a molar mass of as much as 2328 kDa (Miller and Farley 1988).

Similarly as the α subunit, the β subunit occurs in several isoforms. Thus $\beta 2$ with seven potential glycosylation sites was found in the rat brain and in human liver (Martin-Vasallo *et al.* 1989) and a $\beta 3$ isoform was detected in *Xenopus* (Good *et al.* 1990) which differs quite substantially from both $\beta 1$ and $\beta 2$.

There have been various reports on the function of the β subunit or its lack. It appears to be essential for the transport of the whole enzyme complex and its insertion into the plasma membrane and for its activity (McDonough *et al.* 1990). It was shown to influence the stability of the cation-binding site on the α subunit, particularly with respect to K^+ (Lutsenko and Kaplan 1993).

C. The γ subunit

This is a hydrophobic proteolipid of molar mass roughly 10 kDa. Although very little is known about its function, it does appear to be obligatorily associated with the $\alpha\beta$ complex. It occurs in a 1:1 ratio with the other subunits, is labelled with ouabain derivatives in much the same manner as the α subunit is and its amino acid sequence is well conserved from shark to lamb (Hardwicke and Freytag 1981), suggesting some important function for it.

It spans the membrane once, the external C-terminus being highly charged (Mercer *et al.* 1991, Mercer 1993). Its amino acid composition (58 residues) corresponds to a peptide of molar mass of 6.5 kDa.

Its importance is emphasized by the fact that other P-type ATPases are accompanied by analogous proteolipids. The best explored of these is the one associated with the yeast H^+-transporting plasma membrane ATPase (Navarre *et al.* 1992); it contains 38 amino acids and may occur as a dimer.

D. Quaternary structure and assembly of the enzyme

It is a commonly accepted fact that the α and β subunits exist in a complex. The assembly of the α and β subunits is necessary for the cell-surface delivery of the active enzyme. In view of the similarity between the Na,K-ATPase and the gastric H,K-ATPase, results obtained with the latter's incorporation into the membrane may be relevant (Gottardi and Caplan 1993). The α subunit requires its β subunit for efficient cell-surface expression. The H,K-ATPase β protein was able to reach the cell surface unaccompanied by any α subunit but not *vice versa*.

A recent study proved the direct extracellular interaction of the α and β subunits. Förster energy transfer studies localized the oligosaccharides of the α subunits to the vicinity of the ouabain-binding site. This site should be at an average distance of 1.8 nm from Lucifer Yellow (LY) attached to the β subunit (Amler *et al.* 1991).

The expression of the Na,K-ATPase α and β subunit genes is influenced by a complex series of regulatory pathways. For example, unequal amounts of subunit mRNAs are detected in several tissues, both at rest and upon mRNA induction, even though equal quantities of subunit proteins exist. Differential translational efficiency may contribute to the equal

biosynthesis of subunit proteins in those tissues in which subunit mRNAs either exist in unequal amounts or are differentially induced (Devarajan *et al.* 1992). However, in the membrane the molar ratio between the α and β subunits is one.

The minimum functional unit associated with ATP hydrolysis, ion transport and regulation *in vivo* is certainly the $\alpha\beta$ heterodimer (frequently designated as the protomer). However, over the years evidence has kept accumulating that in fact it is an $(\alpha\beta)_2$ heterotetramer (sometimes called the diprotomer). Radiation studies have in general reported a target size for the Na,K-ATPase activity greater than the sum of the α and β subunit pair (Ottolenghi and Ellory 1983). For example, studies of Norby and Jensen (1989), using the frozen enzyme, suggest that the inactivation size of the enzyme for nucleotide, vanadate and ouabain binding, as well as K^+-*p*-nitrophenylphosphatase activity, is close to the mass of only 112 000 while the Na,K-ATPase activity and Tl^+ occlusion reflect an apparent molecular weight of 195 000.

Some laboratories showed that with dodecyl octaethylene monoether both protomers and diprotomers are present (Brotherus *et al.* 1983, Hayashi *et al.* 1989). Also negative staining and computer-enhanced electron microscopy imaging of two-dimensional crystalline arrays of the membrane-embedded enzyme show both protomers and diprotomers, depending on the conditions (Skriver *et al.* 1981, Beall *et al.* 1989)

The existence of the diprotomers was supported recently by the observation that coexpression of different subunits in cultured cells resulted in a stable association into oligomeric complexes (Blanco *et al.* 1994).

Förster energy transfer measurements provide an additional means of investigating the quaternary structure of Na,K-ATPase. The method has the great advantage of yielding information on the enzyme in its native state. By using this technique, it was concluded (Amler *et al.* 1992, 1993b) that the diprotomer $(\alpha\beta)_2$ is the organizational unit of Na,K-ATPase in its native state. Fig. 3 represents the possible disposition of the diprotomer of the enzyme in the native membrane.

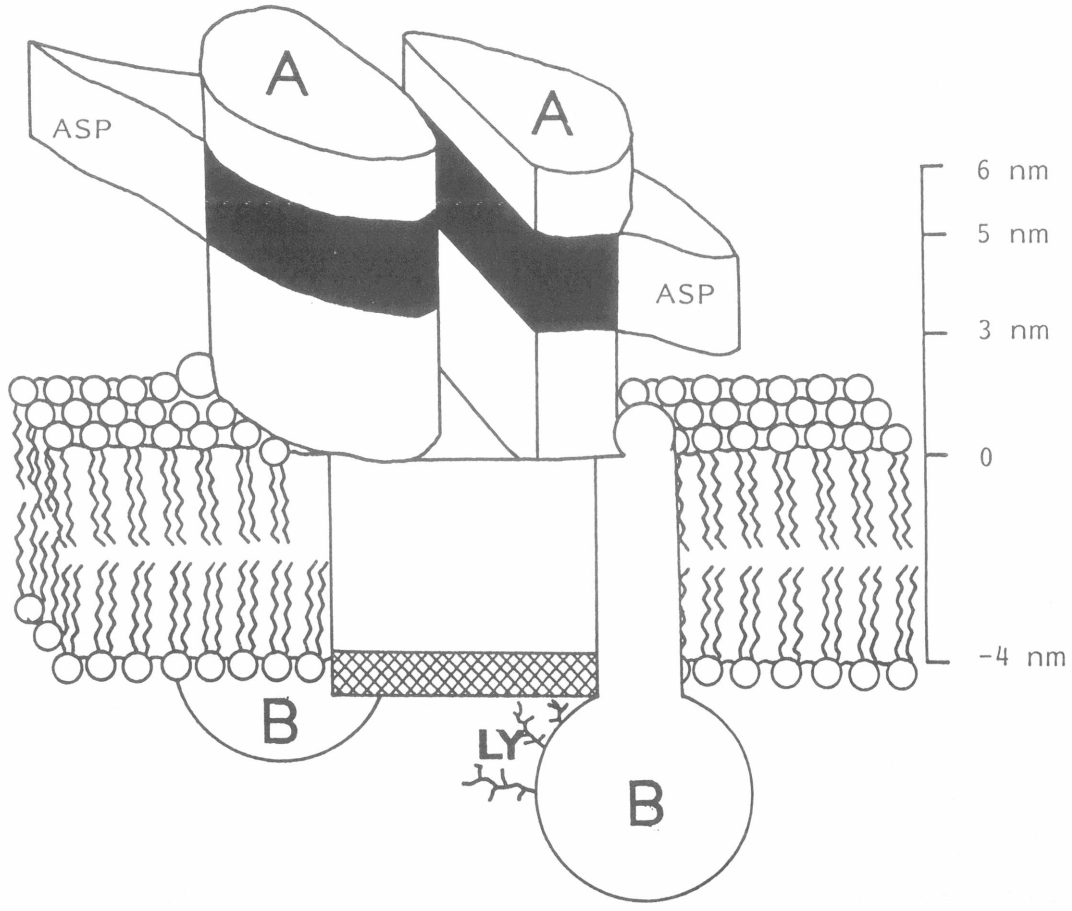

Fig. 3
Disposition of the $(\alpha\beta)_2$ Na,K-ATPase in the lipid bilayer. A, α subunit; B, β subunit. The black region stands for the ATP-binding site (defined by FITC binding), ASP shows the phosphorylatable aspartate residue, the hatched region shows the ouabain binding area. The binding sites for Lucifer Yellow (LY), for FITC and for ouabain form a triangle such that the distance between LY and FITC is 8.5 nm, that between LY and ouabain 1.8 nm and that between FITC and ouabain 7.0 nm. The FITC site is about 2.8 nm from the centre between the two $\alpha\beta$ protomers.

The membrane-bound enzyme appears to be closely associated with the lipid milieu, some 60 lipid molecules lying within the protein-associated boundary layer (Brotherus *et al.* 1980). Even the purified enzyme carries with it some 200 phospholipid molecules, the limiting number for its activity being about 100. Phosphatidylserine and phosphatidylinositol are probably more important for the activity than other phospholipids (Wheeler and Whittam 1970).

Addition of Na,K-ATPase to phospholipid vesicles changes their structure, they become larger and a multilamellar population appears (Volet *et al.* 1994). The presence of a protein in the membrane phase or of a compound in the water phase can influence and direct vesicle formation *in vitro*.

The interface between the lipids and the intramembranous domains was conserved on removal of the extramembranous parts of the protein (Esmann *et al.* 1994). The ability to occlude Rb^+ was also retained by the trypsinized membranes, as previously observed with pig kidney Na,K-ATPase. Interestingly, the rotational mobility of the tryptic fragments in the Na^+-trypsinized membranes was lower than for the Rb^+-trypsinized membranes, indicating rearrangement of the peptides. In addition, the occlusion capacity was lost when trypsinization was carried out in the presence of Na^+, suggesting a correlation between structure and function in the trypsinized membranes.

III. Kinetics and the Reaction Cycle

The overall kinetic and energetic performance of the Na,K-ATPase is characterized by the transport of Na ions (ideally three) out of cells and of K ions (ideally two) into cells, at the expense of the free energy of hydrolysis of the terminal phosphate of ATP. The partial reactions responsible for this process are many and, in spite of 35 years of systematic effort starting with Skou's work in the late 1950s and proceeding to latest schemes (Goldschleger *et al.* 1994), still not unanimously accepted.

Several properties of the reaction cycle are now firmly established.

1. The enzyme exists in two distinct conformations. E_1 and E_2, the first of these displaying a high affinity for sodium and being amenable to ATP binding and phosphorylation, the second showing a high affinity for potassium and being amenable to dephosphorylation.

2. ATP binds to the E_1 form (or possibly, by binding to the E_2 form, it changes it to the E_1 form).

3. At a certain stage of the reaction cycle, ATP phosphorylates an Asp residue and ADP leaves the molecule.

4. At another stage of the cycle, the enzyme is dephosphorylated.

5. The Na and K ions bind alternatively to the enzyme. Three Na^+ are accepted by the ATP-binding E_1 inside the cell and, after several reaction steps, released on the external face of the membrane. This may occur in two steps, first one Na ion, then two Na ions being ejected. Two K^+ are accepted on the outside, immediately as the three Na^+ are released and, after several steps, are ejected inside the cell.

6. There are stages in the process where the ions are bound to the enzyme in an "occluded" form when they cannot be released or exchanged. This occurs with Na^+ in the E_1 form and with K^+ in the E_2 form. In fact, the number of binding sites for sodium and potassium is still widely discussed (Jørgensen 1992). In the E_1 form stabilized as an oligomycin complex, three sites for Na^+ occlusion were found per α subunit. In E_2 forms stabilized as the phosphorylated ouabain complex, two Na^+ or two Rb^+ were occluded per α subunit. Selective chymotryptic cleavage (Leu-266) in the second cytoplasmic loop completely blocked the E_1-E_2 transition, and ion and charge translocation without affecting ligand binding to the protein. Structural transitions of this segment reflect changes in capacity and orientation of cation sites that are part of the ion-translocation process in the α subunit of the Na,K-pump.

7. The sodium release is an electrogenic process, being mainly responsible for the generation of the membrane potential across the ATPase-containing membranes.

Cation occlusion from the cytoplasmic surface is suggested to occur in two steps (Or *et al.* 1993). In an initial recognition, either transported cations or sodium antagonists interact with carboxyl groups. The second step is selective for transported cations and involves occlusion of cations (either potassium or sodium) and a conformational change to a compact structure which is resistant to proteolysis and thermal inactivation.

The latest model of the Na,K-ATPase operation is shown in Fig. 4. It should be made clear that in the classical model, first formulated by Albers *et al.* (1963) and by Sen *et al.* (1969), and generally accepted by most of the leading authorities in the field (Kaplan 1985, Glynn 1988, Jørgensen and Anderson 1988, Skou 1990), the transport steps (i.e. release of Na^+ and K^+ at the two opposite sides of the membrane) were concurrent with the switch from E_1 to E_2 and back, respectively. At present the view gains acceptance (Repke and Schön 1992) that, in fact, both forms are able to bind transiently either Na^+ or K^+.

Kinetically, the basic feature of the models is that the binding sites are open to either one or the other side of the membrane but not to both at the same time – fundamental characteristic of a carrier as opposed to a channel (Kotyk *et al.* 1988). Of the two mechanistic possibilities involved, viz. a moving site or a moving barrier, the latter appears to be correct here.

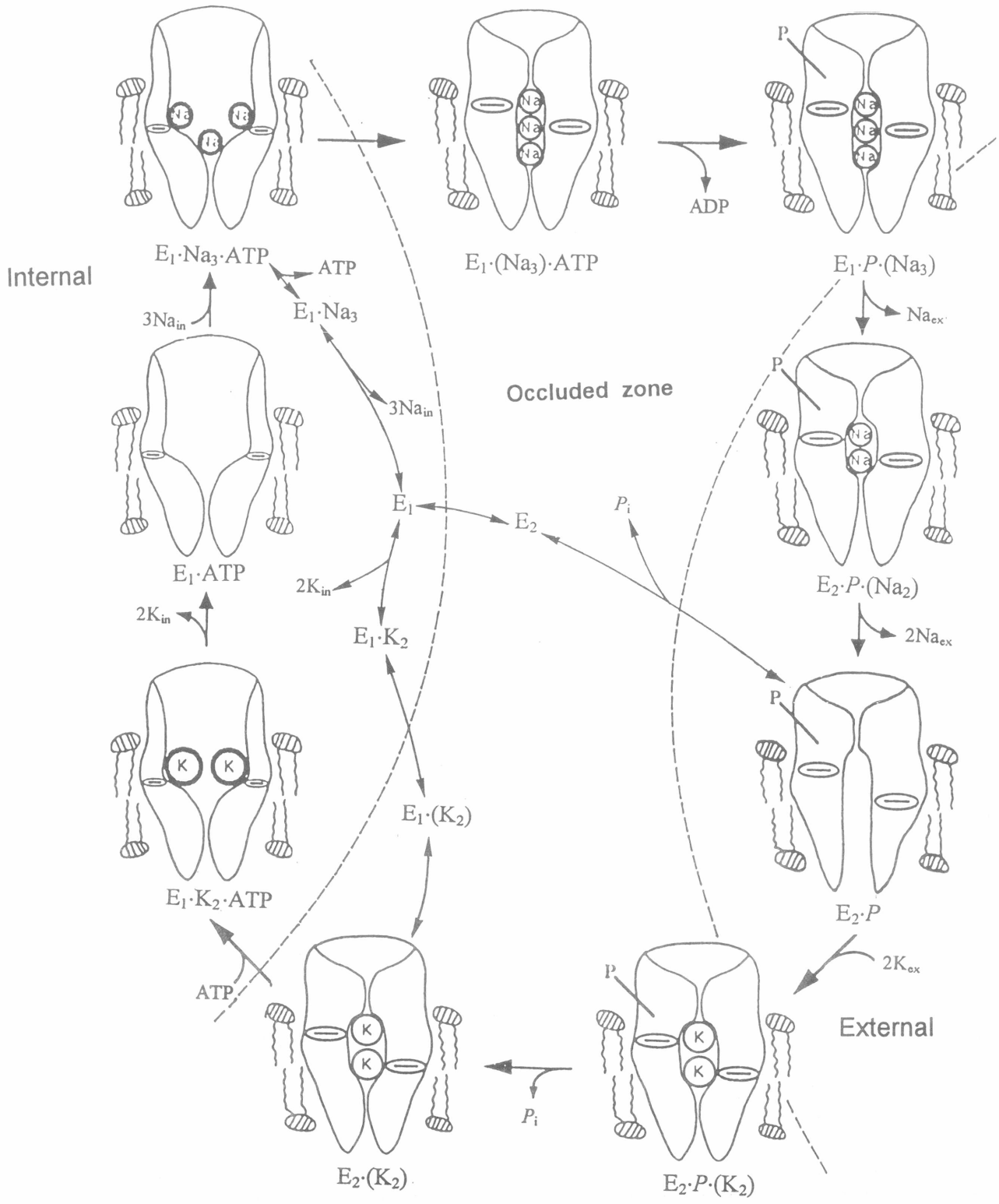

Fig. 4
A contemporary model for the reaction cycle of Na,K-ATPase. The occluded zone may be considered as intracellular because both ADP and P_i release occur within the cells. (Adapted from Goldschleger *et al.* 1994.)

Several features of the process are worth noting.

1. ATP is the substrate of choice, the second best CTP providing roughly 15 % activity (Skou 1974).

2. Mg ions are required for full function of the enzyme; they can be replaced at 10 % efficiency with Mn^{2+} and Co^{2+}. In the presence of Mg^{2+}, a number of divalent cations are inhibitory: Fe^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+}, Ba^{2+}, Sr^{2+} and Be^{2+}.

3. The dissociation constant of Na^+ binding in the native enzyme is 0.19–0.26 mM, that of K^+ binding about 9 mM, that of ATP binding 0.1–0.2 μM. There is distinct cooperativity in Na^+ binding (Hegyvary and Post 1971). The specificity for Na^+ is nearly absolute (Post *et al.* 1965) but K^+ can be replaced with other cations in the following sequence of efficiency: $Tl^+ > Rb^+ = K^+ > Cs^+ = NH_4^+ > Li^+$ (Robinson 1977).

4. The Na,K-ATPase is able to hydrolyze various phosphorylated compounds, e.g. *p*-nitrophenylphosphate (Robinson 1977).

5. The rate constants for interconversion of the four cardinal forms of the enzyme, based on data of Karlish *et al.* (1978) working the with pig kidney enzymes, are as follows:

$E_1.Na_3.ATP$	$\longrightarrow$	$E_1\sim P.(Na_3)$	180 s^{-1}
$E_1\sim P.(Na_3)$	$\longrightarrow$	$E_2\sim P.(K_2)$	75 s^{-1}
$E_2\sim P.(K_2)$	$\longrightarrow$	$E_2.(K_2)$	230 s^{-1}
$E_2.(K_2)$ + ATP	$\longleftrightarrow$	$E_2.K_2.ATP$	$K_D = 0.45$ mM
$E_2.K_2.ATP$	$\longrightarrow$	$E_1.Na_3.ATP$	54 s^{-1}

IV. Note in conclusion

In spite of its having been investigated for well over 30 years, the Na,K-ATPase remains a subject of study, attracting scientists to specialized meetings and extracting from them perhaps a hundred primary publications every year. Many of the aspects addressed in these publications are of clinical nature and many of them reflect attempts to utilize the knowledge of ATPase function for therapeutical purposes. However, there remains one basic feature that has defied analysis – and it is one shared by other ion-transporting ATPases, viz. the path taken by the transported ions and the individual amino acid residues taking part in this process. Will this intriguing problem be solved before the end of the century ?

References

ALBERS R.W., FAHN S., KOVAL G.J.: The role of sodium ions in the activation of *Electrophorus* electric organ adenosine triphosphatase. *Proc. Natl. Acad. Sci. USA* **50:** 474–481 1963.

AMLER E., MALAK H., ABBOTT A., LAKOWICZ J., BALL W.J.: Investigation of the lateral and rotational motions of the carbohydrate chains of the subunit of Na,K-ATPase. *Biophys. J.* **59:** 342, 1991.

AMLER E., ABBOTT A., BALL W.J.: Structural dynamics and oligomeric interactions of Na^+,K^+-ATPase as monitored using fluorescence energy transfer. *Biophys. J.* **61:** 553–568, 1992.

AMLER E., CESTER N, MAGNANELLI R., MAZZANTI R., KOTYK A., ROMANINI C.: Na,K-ATPase from placenta of women with pregnancy- induced hypertension exhibits an increased affinity for cardiac glycosides. *Physiol. Res.* **43:** 33–36, 1993a.

AMLER E., STAFFOLANI R., KOTYK A.: The frequency-domain method reveals the dimeric structure of Na,K-ATPase. *J. Fluoresc.* **3:** 243–244, 1993b.

AMLER E., CESTER N., SALVOLINI E., STAFFOLANI R., BURKHARD M., MAZZANTI L., KOTYK A., ROMANINI C.: Human hypertensive placenta contains an increased amount of Na,K-ATPase with higher affinity for cardiac glycosides. *Cell Biol. Int.* **18:** 723–727, 1994.

ARGUELLO J.M., KAPLAN J.H.: Evidence for essential carboxyls in the cation-binding domain of the Na,K-ATPase. *J. Biol. Chem.* **266:** 14627–14635, 1991.

ARGUELLO J.M., KAPLAN J.H.: Glutamate 779, an intramembrane carboxyl, is essential for monovalent cation binding by the Na,K-ATPase. *J. Biol. Chem.* **269:** 6892–6899, 1994.

BAXTER-LOWE L.A., GUO J.Z., BERGSTROM E.E., HOKIN L.E.: Molecular cloning of the Na,K-ATPase α-subunit in developing brine shrimp and sequence comparison with higher organisms. *FEBS Lett.* **257:** 181–187, 1989.

BEALL H.C., HASTINGS D.F., TING-BEALL H.P.: Digital image analysis of two-dimensional Na,K-ATPase crystals: dissimilarity between pump units. *J. Microsc.* **154:** 71–82, 1989.

BERTORELLO A.M., APERIA A., WALAAS S.I., NAIRN A.C., GREENGARD P.: Phosphorylation of the catalytic subunit of Na^+,K^+-ATPase inhibits the activity of the enzyme. *Proc Natl. Acad. Sci. USA* **88:** 11359–11362, 1991.

BLANCO G., KOSTER J.C., MERCER R.W.: The alpha subunit of the Na,K-ATPase specifically and stably associates into oligomers. *Proc. Natl. Acad. Sci. USA* **91:** 8542–8546 1994.

BROTHERUS J.R., JOST P.C., GRIFFITH O.H., KEANA J.F.W., HOKIN L.E.: Charge selectivity at the lipid-protein interface of membranous Na,K-ATPase. *Proc. Natl. Acad. Sci. USA* **77:** 272–276, 1980.

BROTHERUS J.R., JACOBSEN L., JØRGENSEN P.L.: Soluble and enzymatically stable (Na^+,K^+)-ATPase from mammalian kidney consisting predominantly of protomer $\alpha\beta$ units. Preparation, assay and reconstitution of active Na^+ and K^+ transport. *Biochim. Biophys. Acta* **731:** 290–303, 1983.

BURGER J.W., HESS W.N.: Function of the rectal gland in the spiny dogfish. *Science* **131:** 670–671, 1960.

CANFIELD V.A., XU K.Y., D'AQUILA T., SHYAN A.W., LEWENSON R.: Molecular cloning and characterization of Na,K-ATPase from *Hydra vulgaris:* implications for enzyme evolution and ouabain sensitivity. *Nature New Biol.* **4:** 339–348. 1992.

CHEN S., YUAN C., CLOUGH D., SCHOOLEY J., HADDY F.J., PAMNANI M.B.: Role of digitalis-like substance in the hypertension of streptozotocin-induced diabetes in reduced renal mass rats. *Am. J. Hypertens.* **6:** 397–406, 1993.

DEVARAJAN P., GILMORE-HEBERT M., BENZ E.J.Jr.: Differential translation of the Na,K-ATPase subunit mRNAs. *J. Biol. Chem.* **267:** 22435–22439, 1992.

DORIS P.A.: Regulation of Na,K-ATPase by endogenous ouabain-like materials. *Proc. Soc. Exp. Biol. Med.* **205:** 202–212, 1994.

DZHANDZHUGAZYAN K.N., MODYANOV N.N.: Probing the α-subunit folding by the dot-sandwich immunochemical analysis. In: *The Sodium Pump.* E. BAMBERG, W. SCHONER (eds), Steinkopff, Darmstadt, 1994, pp. 366–369.

ESMANN M., KARLISH S.J., SOTTRUP-JENSEN L., MARSH D.: Structural integrity of the membrane domains in extensively trypsinized Na,K-ATPase from shark rectal glands. *Biochemistry* **33:** 8044–8050, 1994.

FÄNGE R., SCHMIDT-NIELSEN K., ROBINSON M.: Control of secretion from the avian salt gland. *Am. J. Physiol.* **195:** 321–326, 1958.

FORBUSH B., KAPLAN J.H., HOFFMAN J.F.: Characterization of a new photoaffinity derivative of ouabain labeling of the large polypeptide and of a proteolipid component of the Na,K-ATPase. *Biochemistry* **17:** 3667–3676, 1978.

GEVONDYAN N.M., GEVONDYAN V.S., GAVRILYEVA E.E., MODYANOV N.N.: Analysis of free sulfhydryl group and disulfide bonds in Na^+,K^+-ATPase. *FEBS Lett.* **255:** 265–268, 1989.

GLYNN I.M.: The Na^+,K^+-transporting adenosine triphosphatase. In: *The Enzymes of Biological Membranes.* A.N. MARTONOSI (ed.), Plenum Press, New York, 1985, pp. 35–114.

GLYNN I.M.: The coupling of enzymatic steps to the translocation of sodium and potassium. *Progr. Clin. Biol. Res.* **268A:** 435–460, 1988.

GOLDSCHLEGER R., TAL D.M., SHAINSKAYA A., OR E., HOVING S., KARLISH S.J.D.: Organization of the cation binding domain of the Na^+/K^+-pump. In: *The Sodium Pump.* E. BAMBERG, W. SCHONER (eds), Steinkopff, Darmstadt, 1994, pp. 309–320.

GOOD P.J., RICHTER K., DAWID I.B.: A nervous system-specific isotype of the β subunit of Na^+,K^+-ATPase expressed during early development of *Xenopus laevis. Proc. Natl. Acad. Sci. USA* **87:** 9088–9092, 1990.

GOTTARDI C.J., CAPLAN M.J.: Molecular requirements for the cell-surface expression of multisubunit ion-transporting ATPases. Identification of protein domains that participate in Na,K-ATPase and H,K-ATPase subunit assembly. *J. Biol. Chem.* **268:** 14342–14347, 1993.

HAMLYN J.M., MANUNTA P.: Ouabain, digitalis-like factors and hypertesion. *J. Hypertens.* **10** (Suppl. 7): S99–S111, 1992.

HARDWICKE P.M.D., FREYTAG J.W.: A proteolipid associated with Na,K-ATPase is not essential for ATPase activity. *Biochem. Biophys. Res. Commun.* **102:** 250–257 1981.

HAYASHI Y., MIMURA K., MATSUI H., TAKAGI T.: Minimum enzyme unit for Na,K-ATPase is the $\alpha\beta$ protomer. *Biochim. Biophys. Acta* **983:** 217–229, 1989.

HEGYVARY C., POST R.L.: Binding of adenosine triphosphate to sodium and potassium stimulated adenosine triphosphatase. *J. Biol. Chem.* **246:** 5234–5240, 1971.

HOPKINS B.L., WAGNER H.W.Jr., SMITH T.W.: Sodium- and potassium-activated adenosine-triphosphatase of the nasal salt gland of the duck (*Anas platyrhynchos*). *J. Biol. Chem.* **251:** 4365–4371, 1976.

JEWELL E.A., LINGREL J.B.: Chimeric rat Na,K-ATPase $\alpha 1/\alpha 3$ isoforms. Analysis of the structural basis for the differences in Na^+ requirements in the $\alpha 1\,\alpha\xi\delta\,\alpha 3$ isoforms. *Ann. N.Y. Acad. Sci.* **671:** 120–132 1992.

JØRGENSEN P.L.: Purification and characterization of ($Na^+ + K^+$)-ATPase. III. Purification from the outer medulla of mammalian kidney after selective removal of membrane components by sodium dodecylsulphate. *Biochim. Biophys. Acta* **356:** 36–52, 1974.

JØRGENSEN P.L.: Mechanism of the Na^+,K^+ pump. Protein structure and conformations of the pure (Na^+K^+)-ATPase. *Biochim. Biophys. Acta* **694:** 27–68, 1982.

JØRGENSEN P.L.: Functional domains of Na,K-ATPase: conformational transitions in the α subunit and ion occlusion. *Acta Physiol. Scand.* Suppl. 607: 89–95, 1992.

JØRGENSEN P.L., ANDERSON J.P.: Structural basis for E1-E2 conformational transitions in Na,K-pump and Ca-pump proteins. *J. Membr. Biol.* **103:** 95–120, 1988.

KAPLAN J.H.: Ion movements through the sodium pump. *Annu. Rev. Physiol.* **47:** 535–544, 1985.

KARLISH S.J.D., YATES D.W., GLYNN I.M.: Conformational transitions between Na^+-bound and K^+-bound forms of $(Na^+ + K^+)$-ATPase, studied with formycin nucleotides. *Biochim. Biophys. Acta* **525:** 252–264, 1978.

KAWAKAMI K., NOGUCHI S., NODA M., TAKAHASHI H., OHTA T., KAWAMURA M., NOJIMA H., NAGANO K., HIROSE T., INAYAMA S., HAYASHIDA H., MIYATA T., NUMA S.: Primary structure of the α-subunit of *Torpedo californica* $(Na^+ + K^+)$ATPase deduced from cDNA sequence. *Nature* **316:** 733–736, 1985.

KENT R.B., EMANUEL J.R., BEN-NERIAH Y., LEVENSON R., HOUSMAN D.E.: Ouabain resistance conferred by expression of the cDNA for a murine Na^+,K^+-ATPase α subunit. *Science* **237:** 901–903, 1987.

KIRLEY T.L.: Determination of three disulfide bonds and one free sulfhydryl in the β subunit of (Na,K)-ATPase. *J. Biol. Chem.* **264:** 7185–7192, 1989.

KOTYK A.: Is the plasma membrane H^+-ATPase of fungal and plant origin the same enzyme? *Acta Pharmacol. Yugosl.* **41:** 361–368, 1991.

KOTYK A., JANÁČEK K., KORYTA J.: *Biophysical Chemistry of Membrane Functions.* John Wiley, Chichester, 1988, pp. 185–213.

KROGH A.: Sympathetic innervation of cold spots, brought about in reflex from pharynx to skin in man. *Scand. Arch. Physiol.* **71:** 1–6, 1934.

KYTE J.: Properties of the two polypeptides of sodium- and potassium-dependent adenosine triphosphatase. *J. Biol. Chem.* **247:** 7642–7649, 1972.

LEAF A., PAGE L.B., ANDERSON J.: Respiration and transport in isolated toad bladder. *J. Biol. Chem.* **234:** 1625–1629, 1959.

LEBOVITZ R.M., TAKEYASU K., FAMBROUGH D.M.: Molecular characterization and expression of the $(Na^+ + K^+)$-ATPase alpha-subunit in *Drosophila melanogaster. EMBO J.* **8:** 193–202, 1989.

LINGREL J.B.: Na,K-ATPase: isoform structure, function and expression. *J. Bioenerg. Biomembr.* **24:** 263–270, 1992.

LINGREL J.B., VAN HUYSSE J., JEWELL-MOTZ E., SCHULTHEIS P., WALLICK E.T., O'BRIEN W., ASKEW G.R.: Na,K-ATPase: cardiac glycoside binding and functional importance of negatively charged amino acids of transmembrane regions. In: *The Sodium Pump.* E. BAMBERG, W. SCHONER (eds), Steinkopff, Darmstadt, 1994, pp. 276–285.

LUTSENKO S., KAPLAN J.H.: An essential role for the extracellular domain of the Na,K-ATPase β-subunit in cation occlusion. *Biochemistry* **32:** 6737–6743, 1993.

MARTIN-VASALLO P., DACKOWSKI W., EMANUEL J.R., LEVENSON R.: Identification of a putative isoform of the Na,K-ATPase β subunit. Primary structure and tissue-specific expression. *J. Biol. Chem.* **264:** 4613–4618, 1989.

McDONOUGH A.A., GEERING K., FARLEY R.A.: The sodium pump needs its β subunit. *FASEB J.* **4:** 1598–1605, 1990.

MERCER R.W.: Structure of the Na,K-ATPase. *Int. Rev. Cytol.* **137C:** 139–168, 1993.

MERCER R.W., BIEMSEDERFER D., BLISS D.P.Jr., COLLINS J.H., FORBUSH B. III: In: *The Sodium Pump: Structure, Mechanism and Regulation.* J. H. KAPLAN, P. DE WEER (eds), Rockefeller University Press, New York, 1991, pp. 37–41.

MILLER R.P., FARLEY R.A.: All three potential N-glycosylation sites of the dog kidney $(Na^+ + K^+)$-ATPase beta subunit contain oligosaccharides. *Biochim. Biophys. Acta* **954:** 50–57, 1988.

MODYANOV N.N., VLADIMIROVA N.M., GULYAEV D.I., EFREMOV R.G.: Architecture of the sodium pump molecule. Vectorial labeling and computer modelling. *Ann. N.Y. Acad. Sci.* **671:** 134–146, 1992.

NAVARRE C., FERROUD C., GHISLAIN M., GOFFEAU A.: A proteolipid associated with the plasma membrane H^+-ATPase of fungi. Ann. *N.Y. Acad. Sci.* **671:** 189–193, 1992.

NORBY J.G., JENSEN J.: A model for the stepwise radiation inactivation of the $\alpha 2$ dimer of Na,K-ATPase. *J. Biol. Chem.* **264:** 19548–19558, 1989.

OHTA T., NAGANO K., YOSHIDA M.: The active site structure of Na^+/K^+-transporting ATPase: location of the 5'-(*p*-fluorosulfonyl) benzoyl adenosine binding site and soluble peptides released by trypsin. *Proc. Natl. Acad. Sci USA* **83:** 2071–2075, 1986.

OR E., DAVID P., SHAINSKAYA A., TAL D.M., KARLISH S.J.: Effects of competitive sodium-like antagonists on Na,K-ATPase suggest that cation occlusion from the cytoplasmic surface occurs in two steps. *J. Biol. Chem.* **268:** 16929–16937, 1993.

OTTOLENGHI P., ELLORY J.C.: Radiation inactivation of (Na,K)-ATPase, an enzyme showing multiple radiation-sensitive domains. *J. Biol. Chem.* **258:** 14895–14907, 1983.

OVCHINNIKOV Y.A., MODYANOV N.N., BROUDE N.E., PETRUKHIN K.E., GRISHIN A.V., ARZAMAZOVA N.M., ALDANOVA N.A., MONASTYRSKAYA G.S., SVERDLOV E.D.: Pig kidney Na^+,K^+-ATPase. Primary structure and spatial organization. *FEBS Lett.* **201:** 237–245, 1986.

PERRONE J.R., HACKNEY J.F., DIXON J.F., HOKIN L.E.: Molecular properties of purified (sodium + potassium)-activated adenosine triphosphatases and their subunits from the rectal gland of *Squalus acanthias* and the electric organ of *Electrophorus electricus*. *J. Biol. Chem.* **250:** 4178–4184, 1975.

PETERSON G.L., HOKIN L.E.: Improved purification of brine shrimp (*Artemia salina*) ($Na^+ + K^+$)-activated adenosinetriphosphatase and amino-acid and carbohydrate analyses of the isolated subunits. *Biochem. J.* **192:** 107–118, 1980.

POST R.L.: Relationship of an ATPase in human erythrocyte membranes to the active transport of sodium and potassium. *Fed. Proc.* **18:** 121, 1959.

POST R.L., SEN A.K., ROSENTHAL A.S.: A phosphorylated intermediate in adenosine triphosphate-dependent sodium and potassium transport across kidney membranes. *J. Biol. Chem.* **240:** 1437–1445, 1965.

POST R.L., MERRITT C.R., KINSOLVING C.R., ALBRIGHT C.D.: Membrane adenosine triphosphatase as a participant in the active transport of sodium and potassium in the human erythrocyte. *J. Biol. Chem.* **235:** 1796–1802, 1960.

PRICE E.M., RICE D.A., LINGREL J.B.: Structure-function studies of Na,K-ATPase. Site-directed mutagenesis of the border residues from the H1-H2 extracellular domain of the α subunit. *J. Biol. Chem.* **265:** 6638–6641, 1990.

RAO R., NAKAMOTO R.K., VERJOVSKI-ALMEIDA S., SLAYMAN C.W.: Structure and function of the yeast plasma membrane H^+-ATPase. *Ann. N.Y. Acad. Sci.* **671:** 195–203, 1992.

REPKE K.R.H., SCHÖN R.: Role of protein conformation changes and transphosphorylations in the function of Na^+/K^+-transporting adenosine triphosphatase. *Biol. Rev.* **67:** 31–78, 1992.

ROBINSON J.D.: K^+ stimulation of ADP/ATP exchange catalyzed by the ($Na^+ + K^+$)-dependent ATPase. *Biochim. Biophys. Acta* **484:** 161–168, 1977.

SACHS G., SHIN J.M., BESANCON M., MUNSON K., HERSEY S.: Topology and sites in the H,K-ATPase. *Ann. N.Y. Acad. Sci.* **671:** 204–216, 1992.

SCHONER W.: Endogenous digitalis-like factors. *Progr. Drug Res.* **41:** 249–291, 1993.

SCHULTHEIS P.J., WALLICK E.T., LINGREL J.B.: Kinetic analysis of ouabain binding to native and mutated forms of Na,K-ATPase and identification of a new region involved in cardiac glycoside interactions. *J. Biol. Chem.* **268:** 22686–22694, 1993.

SEN A.K., TOBIN T., POST R.L.: A cycle for ouabain inhibition of sodium- and potassium-dependent adenosine triphosphatase. *J. Biol. Chem.* **244:** 6596–6604, 1969.

SERRANO R.: Structure and function of plasma membrane ATPase. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **40:** 61–94, 1989.

SERRANO R., PORTILLO F.: Catalytic and regulatory sites of yeast plasma membrane H^+-ATPase studied by directed mutagenesis. *Biochim. Biophys. Acta* **1018:** 195–199, 1990.

SHULL G.E., GREEB J.: Molecular cloning of two isoforms of the plasma membrane Ca^{2+}-transporting ATPase from rat brain. Structural and functional domains exhibit similarity to Na^+,K^+- and other cation transport ATPases. *J. Biol. Chem.* **263:** 8646–8657, 1988.

SHULL G.E., SCHWARTZ A., LINGREL J.B.: Amino-acid sequence of the catalytic subunit of the ($Na^+ + K^+$)ATPase deduced from a complementary DNA. *Nature* **316:** 691–695, 1985.

SKOU J.C.: The influence of some cations on an adenosine triphosphatase from peripheral nerve. *Biochim. Biophys. Acta* **23:** 394–401, 1957.

SKOU J.C.: Further investigations on a $Mg^{++} + Na^+$-activated adenosinetriphosphatase, possibly related to the active, linked transport of Na^+ and K^+ across the nerve membrane. *Biochim. Biophys. Acta* **42:** 6–23, 1960.

SKOU J.C.: Effect of ATP on the intermediary steps of the reaction of the ($Na^+ + K^+$)-dependent enzyme system (Parts I,II,III). *Biochim. Biophys. Acta* **339:** 234–273, 1974.

SKOU J.C.: The energy coupled exchange of Na^+ for K^+ across the cell membrane. The Na^+,K^+-pump. *FEBS Lett.* **268:** 314–324, 1990.

SKRIVER E., MAUNSBACH A.B., JØRGENSEN P.L.: Formation of two-dimensional crystals in pure membrane-bound Na,K-ATPase. *FEBS Lett.* **131:** 219–222, 1981.

SWEADNER K.J.: Isozymes of the Na^+/K^+-ATPase. *Biochim. Biophys. Acta* **998:** 185–220, 1989.

TAKEYASU K., LEMAS V., FAMBROUGH D.M.N.: Stability of Na^+,K^+-ATPase α subunit isoforms in evolution. *Am. J. Physiol.* **259:** C619–C630, 1990.

USSING H.H.: The distinction by means of tracers between active transport and diffusion. *Acta Physiol. Scand.* **19:** 43–56, 1950.

VOLET B., LACOTTE D., MOOSMAYER M., ANNER B.M.: Na,K-ATPase and carboxyfluorescein distinctly alter vesicle formation in vitro. *Biochim. Biophys. Acta* **1191:** 1–6, 1994.

WHEELER K.P., WHITTAM R.: The involvement of phosphatidylserine in adenosine triphosphatase activity of the sodium pump. *J. Physiol. Lond.* **207:** 303–328, 1970.

YAMADA H., IHARA N., TAKAHASHI H., YOSHIMURA M., SANO Y.: Distribution of the endogenous digitalis-like substance (EDLS)-containing neurons labeled by digoxin antibody in hypothalamus and three circumventricular organs of dog and macaque. *Brain Res.* **584:** 237–243, 1992.

Reprint Requests

Dr. A. Kotyk, Institute of Physiology, Academy of Sciences of the Czech Republic, 142 20 Prague 4, Vídeňská 1083, Czech Republic.