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The Oligomeric Nature of Na/K-Transport ATPase[1]

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Since the discovery of Na/K-ATPase, evidence has accumulated to suggest that 1 mol of ATP hydrolysis occurs *via* the Na^+-occluded ADP-sensitive phosphoenzyme, the K^+-sensitive phosphoenzyme and the K^+-occluded enzyme accompanying active transport of $3Na^+$ and $2K^+$ according the Post-Albers scheme. However, some controversial issues have arisen concerning whether the functional unit of the enzyme is an αβ-protomer or a much higher oligomer, which would be related to the mechanism of transport, either sequential or simultaneous. Detailed studies of oligomer interaction and the reactivity of the enzyme and a comparison of the extent of phosphorylation with ligand-binding capacities in the presence or absence of ATP hydrolysis and others strongly suggest that the functional unit of the enzyme in the membrane is a tetraprotomer, $(\alpha\beta)_4$. They also suggest that each reaction intermediate of the Post-Albers scheme, respectively, reflects half of the site property of the intermediate and that another half binds ATP. These data may be useful not only to answer the long-standing question of whether the mechanism functions in the presence of both Na^+ and K^+ but also contribute to a better understanding of the mechanism of P-type pump ATPase in general.

Key words: Na/K-ATPase, oligomer, P-type ATPase, Post-Albers scheme, tetraprotomer.

Na/K-transport ATPase (*1*) in the plasma membrane was purified to homogeneity (*2*). Its cDNA sequence was determined (*3*) and the enzyme was shown to be composed of an α (catalytic)-chain, a β-chain (a sialoglycoprotein) that plays a role in membrane insertion (*4*, *5*), and a γ-subunit which influences the affinities for Na^+, K^+, and ATP (*6*, *7*). The α chain appears to have 10 transmembrane segments, named M1–M10 (*8*), as in the case of sarcoplasmic Ca/H-ATPase (*8*, *9*), with both the amino- and carboxyl-terminal of the chain on the cytosolic side. Both β and γ subunits have 1 transmembrane segment with the amino-terminal of the chain inside and outside the membrane, respectively. The primary sequence of the catalytic chain has been shown to be homologous to that of gastric H^+/K^+-ATPase (*10*) and sarcoplasmic Ca/H-ATPase (*8*, *9*). The a coupling mechanism of ATP hydrolysis and Na^+ and K^+ transport (*11–14*) is explained by the Post-Albers mechanism (Scheme 1). The atomic structure of the sarcoplasmic reticulum Ca/H-ATPase (*19*) and the similarity of the cDNA sequence between the three ATPases (*4*, *8–10*) suggest that the cation-occluded domain of Na/K- and H/K-ATPase is also localized in the transmembrane segments, M4, M5, M6, and M8. They also suggest that the distance between the ATP binding pocket and the phosphorylation domain, which are both present in a same cytosolic loop beginning at M4 and ending at M5, becomes very close accompanying phosphorylation.

This review describes structural and functional aspects of the oligomericity of Na/K-ATPase. The oligomeric nature of pig stomach H/K-ATPase and sarcoplasmic reticulum Ca/H-ATPase are also described briefly in comparison with Na/K-ATPase. Readers should refer to excellent review articles (*4*, *5*, *8*, *13*, *14*, *20–25*) and papers describing protomeric (*26–30*) or higher oligomeric (*16*, *31–47*) properties of Na/K-ATPase. The proceedings of international conferences on Na/K-ATPase are published every 3 years and are also good sources of an overview of Na/K-ATPase [see also other papers published in these proceedings (*23*, *25*, *29*, *40*)].

Ligand-binding stoichiometry

The maximum amounts of enzyme-bound Na^+ and K^+ (or Rb^+) are, respectively, ~3 and ~2 mol/mol of α-chain. Those of ATP, βγ-bidentate chromium(III)ATP [$Cr(H_2O)_4$-ATP or CrATP], ouabain and vanadate are each ~1 mol/mol of α-chain (molecular mass of αβ complex is 147 kDa) under non-turnover conditions (*27*, *28*). These results indicate that the αβ protomer is the functional unit of the enzyme for ligand binding. However, the functional unit for the Na/K-ATPase reaction or for Na^+ and K^+ transport

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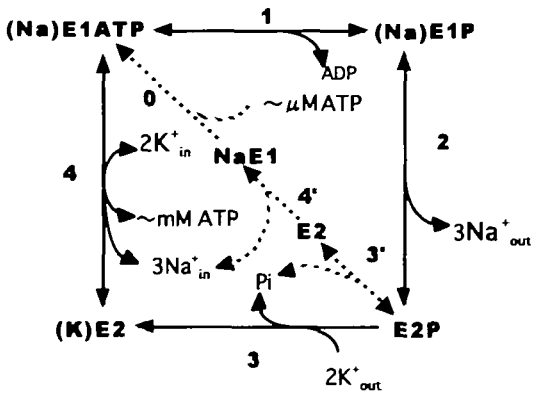
Abbreviations: (Na)E1ATP, Na^+-occluded ATP-bound enzyme; (Na)-E1P, Na^+-occluded ADP-sensitive phosphoenzyme; E2P, K^+-sensitive phosphoenzyme; (K)E2, K^+-occluded enzyme; CrATP, βγ-bidentate chromium (III)ATPor $Cr(H_2O)_4ATP$; EP, phosphoenzyme; NaE1, Na^+-bound enzyme; BIPM, *N*-(*p*-(2-benzimidazolyl)phenyl)-maleimide; (Rb)E2, Rb^+-occluded enzyme; SDS, sodium dodecyl sulfate; DOC, deoxychoric acid; AP_2PL , pyridoxal 5′-diphospho-5′-adenosine; *p*NPP, *p*-nitrophenyl phosphate; (pNPP); CoATP, tetraamine-Co-ATP; CoPi, $Co(NH_3)_4PO_4$; CDTA, 1,2-cyclohexylenedinitrilotetraaceticacid ; C12E8, octaetylener glycol dodecyl ether; FITC, fluorescein 5′-isothiocyanate.



Scheme 1. **According to the Post-Albers mechanism (*11–14*), in step 0 the enzyme accepts an internal $3Na^+$ and MgATP with high affinity (K_d = ~μM ATP) to form the Na^+-occluded ATP-bound enzyme, (Na)E1ATP.** In step 1, it is transformed to the Na^+-occluded ADP-sensitive phosphoenzyme, (Na)E1P, accompanied by the liberation of ADP to the internal medium; and in step 2, it forms the K^+-sensitive phosphoenzyme, E2P, accompanied by the liberation of $3Na^+$ to the extracellular solution. In step 3, $2K^+$ present outside the cell attacks the E2P with a high affinity to form the occluded K^+ form of the enzyme, (K)E2, accompanied by sequential liberation of P_i and Mg^{2+} to the internal medium (*15*). In step 4, the binding of $3Na^+$ and MgATP with a low affinity (K_d = sub-mM), both from inside, to the (K)E2 then induces the liberation of $2K^+$ to the internal medium to form the (Na)E1ATP complex again. Thus, in this model, a low-affinity ATP-binding site appears to be in a steady state in the presence of Mg^{2+}, Na^+, K^+, and ~mM ATP accompanying the active transport of $3Na^+$ and $2K^+$ sequentially by steps 1-2-3-4. Without K^+, a high-affinity ATP-binding site appears along with Na^+-dependent hydrolysis of ATP accompanying the sequential formation of (Na)E1P and E2P *via* the dotted line in the scheme (*14, 16, 17*) by steps 0-1-2-3'-4'. The Na^+-dependent hydrolysis of phosphatase substrates such as *p*-nitrophenylphosphate (*p*NPP) (*16*) and acetyl phosphate (*17, 18*) also occurs by this above route. Without Na^+, no ATP hydrolysis occurs but the K^+-occluded enzyme, (K)E2, functions as a phosphatase to hydrolyze pNPP and acetyl phosphate (*12, 14*). Ouabain binds to E2P, thus reducing the turnover of the enzyme and resulting in strong inhibition of ATP hydrolysis. Vanadate also binds in place of P_i to form a vanadate-enzyme complex and resulting in the strong inhibition. For the sake of simplicity, Mg^{2+} is not included in this scheme.

across the membrane, which consists of partial reactions (Scheme 1), would be much larger. In fact, the simultaneous binding of $3Na^+$ and $2K^+$/phosphoenzyme (EP) had been also reported (*48*). The radiation-inactivation size of the maximum Na/K-ATPase activity is twice that of the ligand-binding capacity such as ATP, ADP, ouabain, and vanadate and the partial reactions (*35*).

Steady-state and transient kinetics are consistent with the Na^+-dependent hydrolysis of ATP proceeding through (Na)E1P and E2P (by steps 0-1-2-3' of Scheme 1) (*11–14*). The presence of two different ATP affinity sites, a high affinity site (step 0) in NaE1 showing a low V_{max}, and a low affinity site (step 4) in KE2 showing a high V_{max}, is generally accepted. Two different types of ATP and ouabain binding occur in Na/K-ATPase (*14, 24, 34, 41*). Sub-μM and ~10 μM ATP concentrations induced, respectively, two different fluorescence changes in an *N*-(*p*-(2-benzimidazolyl)phenyl)-maleimide (BIPM) probe at Cys-964 in the M9 of Na/K-ATPase, accompanying half site phosphorylation (*42, 49*).

The maximum amount of EP reported was 1.5–6 nmol/mg protein of membrane Na/K-ATPase preparations (*14, 16, 26, 30*): ~6 nmol of EP/mg protein is equal to ~1 mol/mol of α-chain. The variations appear to depend on differences in the purity of the enzyme, its origin, the method used for enzyme purification by detergents as well as the estimation of the membrane protein concentration and the amount of EP. However, the ratio of the stoichiometry of the maximum amount of EP from ATP to that of ouabain-binding capacity under phosphorylation conditions was around 0.5 (*16*). Treatment of the enzyme with trypsin in the presence of 160 mM Na^+ or K^+ showed that the conformational state of the enzyme was completely NaE1 or all (K)E2 (*50*). However, trypsin treatment in the presence of P_i with Mg^{2+} (*43*) or MgATP analogues (*23, 34, 44*) showed that the conformational state of one half of the α-chain is NaE1 and that of the other half is (K)E2 (Scheme 1) according to the definition to the cleavage site (*50*). The amount of Rb^+ (K^+ congener) occlusion/α-chain decreased from 2 under non-turnover conditions to 1 and 0.5 depending on the concentrations of ATP during turnover (*45*). The simultaneous presence of 0.5 mol of enzyme-bound ATP not only with 0.5 mol of EP but also 0.5 mol of (Rb)E2 was unequivocally detected (*45*). Thus, some modification of the Post-Albers scheme (*37, 38, 40–42, 46, 51*) is called for. The difficulty in accepting the half-of-the-site-reactivity (*52*) and the flip-flop (*53*) mechanism of Na/K-ATPase (*11–14*) was, at any instant, half of the site of the intermediate to be phosphorylated (*14*).

The maximum amount of EP/mol of α-chain

Recently, the maximum amount of EP formed from the ATP/α-chain was determined using highly purified sodium dodecyl sulfate (SDS)–treated dog kidney enzymes (*2, 33*) and sodium deoxychoric acid (DOC) + NaI–treated pig kidney enzymes. It was 0.5 mol of EP/mol of α-chain, independent of the purity of the enzyme estimated from the phosphorylation capacity, 1–3.6 nmol EP/mg protein (*42*). A similar half-site phosphorylation by ATP (0.5 mol of EP/mol of α-chain) was observed recently for H/K-ATPase (*54*), which was accompanied by an increase in Trp fluorescence, but full-site phosphorylation by acetyl phosphate decreased the fluorescence intensity by half (*55*). Half-site phosphorylation from P_i was also reported in sarcoplasmic reticulum Ca/H-ATPase (*56*).

Chemical modifications of Na/K-ATPase with pyridoxal 5'-diphospho-5'-adenosine (AP_2PL) showed a half-site reactivity for Lys480 with respect to AP_2PL (*42*). The modification of H/K-ATPase with AP_2PL at Lys497 (unpublished) and the modification of Ca/H-ATPase with fluorescein 5'-isothiocyanate (FITC) at Lys515 (*56*) also suggested, respectively, half site reactivity, with respect to AP_2PL and FITC. In the difference Fourier map of Ca/H-ATPase, the phosphate group of ATP analogue, 2'-(or 3')-*O*-(trinitrophenyl)adenosine-5'-monophosphate is localized near Lys492 in the mouse of ATP binding pocket and the adenine moiety is localized near at Lys515 in the depth of the same pocket (*19*). The corresponding Lys residues (*8*) are also present in Na/K-ATPase, Lys480, and Lys501 (*3*) and in H/K-ATPase, Lys497, and Lys518 (*10*). These data also suggest that presence of a half site reactivity for Na/K-, H/K-, and Ca/H-ATPase in both the phosphorylation domain and ATP binding pocket (*19*).

Phosphorylation of quarter, half, and full site

The second column of Table I shows the phosphorylation capacity of the enzyme/α-chain, a half site phosphorylation, under various conditions. The maximum amount of EP in the presence of Mg^{2+}, Na^+, and ATP was shown to be 0.5 mol/mol of α-chain, (*42*). The addition of ouabain decreased the amount of EP from ATP (second column) to a quarter site (top column) of the α-chain (*11, 31*). Phosphorylation from P_i in the presence of ouabain was also reduced to a quarter when Mg^{2+} was replaced with Ca^{2+} (*57*). The maximum amount of EP in the presence of Ca^{2+} and ATP was a half and a quarter in the presence of 1–2 M and ~0.1 M Na^+, respectively (*58*). Ouabain increased the phosphorylation from P_i in the presence of Na^+ and ADP from zero to a quarter (*59*). The maximum amount of EP in the presence of Mg^{2+} and Na^+ with *p*-nitrophenyl phosphate (*p*NPP) (*16*) appeared to be twice the amount of that with ATP, 1 mol of EP/mol of α-chain (third column). Such full-site phosphorylation seemed to occur transiently in the presence of ~mM ATP (*60*). The amount of EP under steady-state conditions increased significantly with an increase in the time of chymotryptic digestion of the enzyme (*61*). Na/K-ATPase from the nasal salt glands of salt-adapted ducks showed the full-site phosphorylation from P_i in the presence of Mg^{2+} + ouabain (*30*).

These data suggest that the phosphorylated subunit or nonphosphorylated subunit might sense the molecular event of either adjacent subunit, thus permitting a quarter, half and full-site phosphorylation. Some desensitization such as induced by chymotrypsin treatment (*61*) may also occur during purification of the enzyme with detergents.

Enzyme bound acid labile ATP

Numerous data indicate the possible presence of a low affinity ATP binding to Na/K-ATPase: the binding of ATP and P_i to the enzyme during Rb^+:Rb^+ exchange (*14*), the binding of P_i and TNP-ADP to the FITC-treated enzyme (*62*), the ATP induced inhibition of the spontaneous breakdown of E2P formed from P_i + Mg^{2+} (*63*) and E2P formed from ATP + Mg^{2+} + Na^+ (*64*). The acceleration of Na/K-ATPase activity by higher concentrations of ATP (*12–14*) and by ADP or adenylyl imidodiphosphate in the presence of low concentrations of ATP (*65*) also suggest the presence of two different sites for ATP, one for high affinity phosphorylation, requiring ATP absolutely (steps 0-1-2 of the Scheme 1) and the other for the low affinity deocclusion of (K)E2 (step 4) (*12, 14*).

Recently, enzyme-bound ^{32}P in the presence of up to 0.2 mM [γ-^{32}P]ATP and [α-^{32}P]ATP, respectively, was detected (*45*) in the steady-state condition for the Na-ATPase reaction (0-1-2-3'). The apparent affinity for ATP is higher for the formation of [^{32}P]EP than that for ^{32}P binding. The maximum amount of ^{32}P binding under conditions of accumulation of (Na)E1P or E2P in the presence of [γ-^{32}P]ATP was around twice the amount of each EP respectively, while the amount of ^{32}P bound to the enzyme under these conditions in the presence of [α-^{32}P]ATP was almost equal to the amount of EP. These data suggest that 0.5 mol of EP and 0.5 mol of EATP or EADP/P_i simultaneously accumulate (*45*).

The Rb^+ (K^+ congener)-occluded enzyme, (Rb)E2, was accumulated *via* E2P by the addition of [α-^{32}P]ATP or [γ-^{32}P]ATP with 320 μM Rb^+ to NaE1 by steps 0-1-2-3 of the Scheme 1. A half mol of ^{32}P was detected with 1 mol of occluded Rb/α-chain in the steady state with no measurable accumulation of phosphoenzyme. The time course for ATP hydrolysis just after the addition of ATP without or with Rb^+ to NaE1 showed an initial P_i burst of ~0.25 or ~0.5 mol, respectively (*45*), which disappeared when the enzyme was preincubated with Rb^+. The data show that the enzyme-bound ^{32}P was due to ATP binding to the phospho- and dephosphoenzyme and exclude the presence of both EADP/P_i (*45*) and an acid-labile EP_i complex (*38*) during the Na/K(or Rb)-ATPase reaction.

The role of 0.5 mol of ATP bound to the enzyme may be related to the high concentrations of ATP required to accelerate Na/K-ATPase (K_m = ~0.3 mM ATP) in order to deocclude K^+ from (K)E2 by steps 3–4 of Scheme 1, where nonhydrolysable ATP analogues including ADP (*14, 63*) are able to mimic the role of ATP. The issue of enzyme-bound ATP, which appeared transiently only in the initial stage of phosphorylation to become (Na)E1P (*58, 66*), however, is different. In the case of pig stomach H/K-ATPase, the maximum amount of EP formation from ATP and the amount of acid-labile enzyme-bound ATP (EATP) have been shown, respectively, to be around 0.5 mol/mol of α-chain, and EATP appears to be broken down *via* EP (*54*).

These data and others (*45*) clearly show the stoichiometry of ligand binding such as ATP, Na^+, and Rb^+ and the extent of phosphorylation change during turnover. The maximum amount of ligand binding under non-turn over conditions serves as a measure of the capability of the enzyme but does not indicate the functional unit of the enzyme required for Mg^{2+}, Na^+, and K^+ dependent ATP hydrolysis or the active transport of Na^+ and K^+.

TABLE I. **Quarter, half, and full-site phosphorylation of Na/K-ATPase.**

EP formation	Condition	Ref. No.
Quarter	Mg^{2+} + 16 mM Na^+ + ATP^{32} + ouabain	*11, 31*
	Ca^{2+} + $^{32}P_i$ + ouabain	*57*
	Ca^{2+} + 126 mM Na^+ + ATP^{32}	*58*
	Mg^{2+} + 16 mM Na^+ + ADP + $^{32}P_i$ + ouabain	*59*
Half	Mg^{2+} + 16 mM – 2 M Na^+ + ATP^{32}	*42, 59*
	Mg^{2+} + $^{32}P_i$ + ouabain	*57*
	Ca^{2+} + 1 or 2 M Na^+ + ATP^{32}	*58*
	Mg^{2+} + 16 mM Na^+ + (^{32}P)acetyl phosphate	*17, 49*
Full	Mg^{2+} + 16 mM Na^+ + $pNPP^{32}$	*16*
	Mg^{2+} + 150 mM Na^+ + mM ATP^{32} (transient)	*60*
	Mg^{2+} + 100 mM Na^+ + μM ATP^{32} (chymotrypsin-treated enzyme)	*61*
	Mg^{2+} + $^{32}P_i$ + ouabain (salt-adapted duck enzyme)	*30*

Quarter, half, and full-site reactivity

The top column of Table II shows the quarter-site reactivity of Na/K-ATPase. The addition of acetate to (Na)E1P formed in the presence of Mg^{2+} + 2 M Na^+ + acetyl phosphate induced a half-rapid and half-slow breakdown in spite of nearly complete breakdown as the result of added ADP (*17*). The addition of *p*-nitrophenol to (Na)E1P, which is formed in the presence of Mg^{2+} + 2M Na^+ + ATP, induced only half the amount of *p*NPP formation (*16*). When the enzyme was incubated with Rb^+ in the absence of any ligand, 2 mol of Rb^+ occlusion/mol of α-chain (*22, 27, 28, 45*) occurred (third column). On addition of 160 mM Na^+ with

TABLE II. **Quarter, half, and full-site reactivity of Na/K-ATPase.**

Reactivities	Condition	Ref. No.
Quarter	acetate sensitivity to (Na) E1P	*17*
	PNP sensitivity to (Na) E1P to form *p*NPP	*16*
	Rb^+ occlusion in the presence of Mg^{2+} (or Ca^{2+}) + Na^+ + ~mM ATP	*45*
Half	CoPi binding to E2, Co ATP binding to E2, Cr ATP binding to E1	*24, 34*
	Bound AP_2PL at Lys480	*68*
	ADP sensitivity to (Na) E1P, K^+ sensitivity to E2P	*59*
	Na^+ occlusion in the presence of Mg^{2+} + P_i + ouabain	*28*
	Rb^+ occlusion in the presence of Mg^{2+} + Na^+ + sub-mM ATP	*45*
Full	ATP binding, Cr ATP binding in the presence of Mg^{2+} + Na^+, ouabain binding in the presence of Mg^{2+} + P_i	*27, 28*
	[^{32}P] binding in the presence of Mg^{2+} + Na^+ + (γ-^{32}P)ATP	*45*
	$2Rb^+$ occlusion	*27, 28, 45*
	$3Na^+$ occlusion	*27, 28*

0.43 or 4 mM Mg^{2+} or 16 mM Na^+ with 1 mM Ca^{2+}, complete deocclusion occurred. However, the amount of Rb^+ occlusion increased to nearly 1 mol (second column) and then decreased to a nearly constant level of ~0.5 mol of Rb^+/mol of α-chain (top column) with increasing concentrations of ATP. ATP has the dual effect of increasing the amount of Rb^+ occlusion (*45*). The occluded Rb^+ was shown to turn over. A similar half-occluded Rb^+ release has been reported (*67*). Such dynamic changes would only be possible as the result of subunit interactions. The presence of oligomeric interaction in Ca/H-ATPase was clearly shown by cooperative and noncooperative Ca^{2+} binding, Trp fluorescence change (*76*), the half of the site reactivity (*56*) and fluorescence energy transfer (*78*). The crystal structure of the calcium pump of the sarcoplasmic reticulum at 2.6 Å resolution showed (*19*) that two Ca^{2+}s occluded sites are liganded by the same seven corresponding amino acid residues as are found in both Na/K- and H/K-ATPase (*3, 8–10*) and three different residues. A direct cation-binding assay and Na^+-dependent phosphorylation in Na/K-ATPase showed that four carboxyl residues are essential for Na^+ and K^+ binding (*73*), in which and the corresponding 4 residues in Ca/H-ATPase are all liganded to Ca^{2+}. These data are consistent with overlapping binding sites for cations in these three ATPases (*74*). Three different cation-liganded residues seem to participate not only in the recognition of specific occlusion site structures for each P-type ATPase, but also affect the affinities for ATP and specific cations to induce phosphorylation and dephosphorylation (*75*), respectively.

The second column shows that the saturated binding of CrATP or tetraamine-Co-ATP, $Co(NH_3)_4ATP$ (CoATP), or tetraamine-Co phosphate, $Co(NH_3)_4PO_4$ (CoPi), is 0.5 mol/mol of α-chain. However, after 0.5 mol of CrATP binding, an additional 0.5 mol of Co-ATP or CoPi binding was detected in the absence of Na^+ and K^+ (*23, 34*). The amount of Na^+ occlusion (*28*) in the presence of Mg^{2+}, P_i, and ouabain was around half of that induced by oligomycin. The modification of Na/K-ATPase with pyridoxal was saturated at around 0.5 mol pyridoxal probe binding at Lys-480 at the entrance of the ATP-binding pocket (second column). The probe-bound enzyme showed that ATP induced two different reversible pyridoxal fluorescence changes in the presence of Mg^{2+} and Na^+, a decrease by sub-μM ATP and an increase by ~μM ATP (*49, 68*). The data suggest the presence of two different ATP-binding sites, namely, two quarter sites. The enzyme preparations showed half-site phosphorylation capacity with respect to acetyl phosphate with Mg^{2+} and Na^+ as a non-treated enzyme, independent of the incorporation of 1 mol of the FITC probe to Lys501 in the depth of the pocket. Further, ~mM ATP accelerated the conformational change in the transition of (K)E2 to NaE1ATP (Scheme 1, step 4) as detected by fluorescence changes of both pyridoxal and FITC probes of the doubly labeled enzyme. The enzyme showed neither EP formation by steps 0-1-2-3, nor high affinity ATP binding in the presence of CDTA, nor Na/K-ATPase activity. These data suggest that the preparation is able to bind ATP with both high and low affinity. However, these modifications had little influence on Mg^{2+} and Na^+-dependent EP formation by substrates which are less bulky than ATP, such as acetyl phosphate (*17, 18*) or pNPP (*16*), or on MgATP binding to the low affinity site or K^+-*p*NPPase activity. It is clear that the modification of these Lys residues does not disrupt the ATP binding site, which was confirmed by direct ATP-binding in the presence of CDTA (*68*).

Site-directed mutagenesis (*69, 70*) or chemical modification (*42, 49, 68, 71*) of Lys480 and Lys501, which are present, respectively, at the entrance and in the depth of the same ATP-binding pocket (*3, 19*), induced different effects on the high and the low ATP affinity sites. These data suggest that the number of ATP-binding sites is 1 mol/mol of α-chain rather than 2 (*72*). Thus 2 or 4 different ATP affinity sites, as detected by dynamic fluorescence changes, reflected ATP-induced conformational events in each quarter site of the enzyme molecules. In the case of H/K-ATPase, the maximum amount of ATP binding under non-turnover conditions was around 1 mol/mol of α-chain, which is equal to the maximum amount of EP formed from acetyl phosphate or P_i but twice the amount of EP from ATP (*55*).

The bottom column shows that the maximum amount of CrATP that binds in the presence of Mg^{2+} + Na^+ (*28*), and the amount of ^{32}P binding under fully phosphorylated conditions (~0.5 mol of EP/mol of α-chain) in the presence of Mg^{2+} and Na^+ with [γ-^{32}P]ATP (*45*) was, respectively 1. The maximum amount of ouabain binding has been shown to be 1 mol/mol of α-chain (*27, 28*). In the case of Ca/H-ATPase, CrATP binds to neither the adenine-binding domain nor the nucleotide-binding domain but cross-links between the two domain (*24*).

The data described above suggest a quarter-site reactivity of the enzyme and a higher oligomerization, such as $(\alpha\beta)_4$, as the functional unit of the enzyme during turnover. The full-site phosphorylation in the case of nasal salt glands of salt-adapted ducks (*30*) was taken as evidence for rejecting models including a half of the sites reactivity (*52, 53*). However, this simply shows one of the capacities of the full-site reactivity of the enzyme as not requiring ATP hydrolysis, such as CrATP binding (*28*) and ouabain binding (Table I).

Direct evidence for oligomeric interactions

Twenty years ago, the cross-linking between the phos-

phorylated α-chain and the non-phosphorylated α-chain was reported to suggest a minimum of four catalytic subunits, $(\alpha\beta)_4$ (*32*). Further studies demonstrated the contact points on the C-terminal side of Ala439 (*39*) as in the case of the C-terminal side of Ca/H-ATPase (*78*). Cross-linking between the transmembrane helices M1–M2 and M7–M10 occurred both in the solubilized and the membrane-bound states in extensively trypsin-digested Na/K-ATPase (*74*), which retains the ability to bind Na^+, K^+, and ouabain (*77*). The similarity of P-type ATPase transmembrane sequences (*8*), in which M1–M2 is far from M7–M10 in the crystal structure of Ca/H-ATPase (*19*), also supports the cross-linking between different α-chains (*32*). Cross-linking in H/K-ATPase between Cys565 and/or Cys615 has been shown (*79*). The corresponding residues are also present in Na/K-ATPase (*3*), Cys556 and Cys606, and in Ca/H-ATPase (*9*), Cys561 and Cys614, in the ATP-binding domain. These data suggest that ligand-dependent contacts between subunits occur during ATP hydrolysis not only in the case of trans-membrane segments near the cation-occluded domain but in the adenine-binding domain as well. This may be related to the reversible change in the light scattering of Na/K-ATPase (*80*) and H/K-ATPase (*81*) accompanying phosphorylation.

More quantitative studies using low-angle laser light scattering photometry coupled with a high-performance gel chromatography clearly show that an oligomeric interaction is required for the hydrolysis of ATP in the case of C12E8 solubilized enzyme (*33*, *36*, *47*, *82*). These data constitute the strongest direct evidence in favor of oligomeric interactions in Na/K-ATPase. They also show that the C12E8 solubilized enzyme is not a protomer but a mixture of oligomers, such as protomers (150 kDa), diprotomers (300 kDa), and tetraprotomers (*45*, *82*). When the concentration of Na^+ was decreased in the Mg^{2+} and ATP-dependent phosphorylation reaction, an increase in the fraction of diprotomer and higher oligomers with a corresponding decrease in protomers was directly measured (*82*). The data show the fractions of E2P and (Na)E1P, respectively, increased and decreased with a decrease in Na^+ concentration and *vice versa* (*59*). The molecular structure of the membrane-associated enzyme appeared to be in dynamic equilibrium between loosely associated diprotomers in NaE1 and (Na)E1P and a tightly associated diprotomer in E2P and KE2 (*82*). Thus the rather widely accepted conclusions based on C12E8 solubilized enzyme being composed of an αβ-protomer (*28–30*, *83*) need to be abandoned.

Fluorescence energy transfer

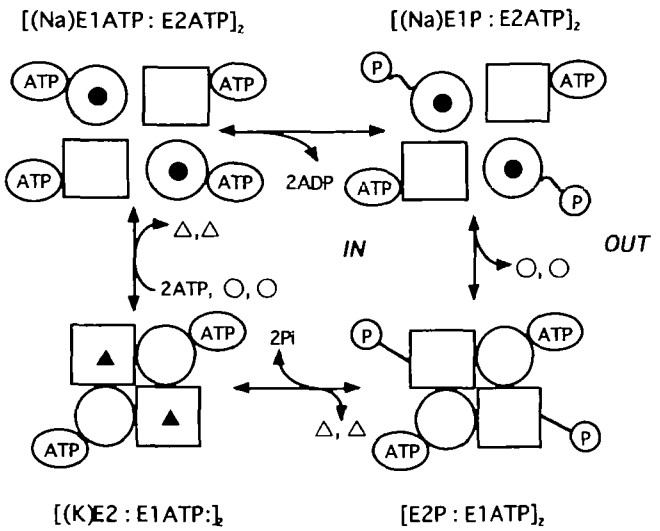
The fluorescence energy transfer from the BIPM probe at Cys964 in the M9 of Na/K-ATPase to the FITC probe at Lys501 in the depth of the ATP binding pocket increased from NaE1 to E2P via acetate-sensitive (Na)E1P and decreased from E2P to NaE1 *via* (K)E2 (*18*). These data suggest that NaE1 and (Na)E1P are rather relaxed states compared with E2P and (K)E2 (*14*, *59*), as has also been suggested by the relative fluorescence intensity of the Trp residue and the BIPM probe at Cys964 in these states (*14*, *59*). An Na/K-ATPase preparation was divided into two portions, and one was labeled with a fluorescence donor (BIPM) at Cys-964 in M9, and the other with the acceptor (*N*-(7-dimethylamino-4-methylcoumarynyl) maleimide (DACM) probe at a Cys residue in the adjacent α-chain. A significant increase in fluorescence energy transfer from the BIPM to the DACM probe occurred when solubilized with C12E8 (unpublished), which is consistent with oligomeric interactions for Na/K-ATPase in the C12E8-solubilized enzyme (*33*, *47*, *82*).

Fluorescence life time measurements showed that the distance between the FITC probe at Lys501 and an erythrosine isothiocyanate probe at Cys549 and bound CoATP was respectively, 6.9 and 6.5 nm (*84*). Corresponding Lys and Cys residues are also present in the nucleotide-binding domain in both Ca/H-ATPase (*9*) and H/K-ATPase (*10*). These long distances reflect the distance between ATP-binding pockets (*19*) in different α-chains (*77*, *84*). In contrast, the distance between the FITC probe at Lys501 and the bound CoADP was reported to be 1.8 nm as the distance between two different nucleotide sites in the same α-chain. (*72*). However, it is reasonable to suggest that the distance is a reflection of the depth and the mouth of the same ATP-binding pocket (*19*). Ca/H-ATPase has a typical Rossman fold in the P-domain, where Asp351 is situated in the C-terminal end of the central β-strand and critical residues for ATP hydrolysis are clustered (*19*). ATP bound in the ATP-binding pocket at a distance of up to 25 Å from the P-domain appears to be close to the P-domain (*19*), a distance of a few Å (*24*), in order to be stabilized by the Rossman fold and to donate a γ-phosphoryl group to Asp351 to form E1P.

Electron microscopic observations

Freeze-fractured and rotary shadowed purified membrane-bound Na/K-ATPase from rat kidney and intact baso-lateral plasma membranes of the Henle's loop showed four membrane-spanning partite structures with a diameter about 10 nm, which were sensitive to negative staining giving particles with a diameter 4.3 nm (*85*). Electron microscopy of rotary shadowed C12E8 solubilized and gel-filtered dog kidney Na/K-ATPase fractions confirmed the tetraprotomeric structure, $(\alpha\beta)_4$. Each fraction showed tetrameric, dimeric, and monomeric structures (*45*), which have a unitary structure of pear-like shape of ~21 nm in length and ~11 nm in width at the widest portion. The presence of these oligomers in each fraction suggests the time–dependent equilibrium between soluble oligomers. The membrane-bound pig enzyme also showed morphologically similar structures to those described above. When Na/K-ATPase molecules modified with FITC at Lys501 were complexed with anti-FITC antibodies, the presence of a tetramer with 1–4 molecules of antibodies attached to the wider end of the pear-like structure, the cytosolic side, was detectable (*45*).

A protomer of Na/K-ATPase in the p1 crystal and a diprotomer in the p21 crystal both induced by vanadate/Mg^{2+} and a diprotomer in tetrameric crystal induced by CoATP have been obtained (*86*). Quite recently, single particle analysis was applied to Na/K-ATPase two-dimensional crystals (*87*), of which a quarter part appeared to show slightly different crystal structure. Strong protein–protein interactions and the formation of a tetraprotomer have been reported from the vanadate/Mg^{2+}-induced two-dimensional crystals of membrane bound gastric H/K-ATPase (*88*). Ligand-induced subunit interactions appear to affect not only reactivity, as shown in the tables, but also the formation of oligomeric crystal structures.



Scheme 2. **Tetraprotomer model of Na/K-ATPase.** (Na)E1ATP, (Na)E1P, E2P, and (K)E2 in the Post-Albers model (Scheme 1), respectively, are shown as [(Na)E1ATP:E2ATP]$_2$, [(Na)E1P:E2ATP]$_2$, [E2P:E1ATP]$_2$, and [(K)E2:E1ATP]$_2$. Large open circles represent the conformational state of the enzyme accepting Na^+ and ATP with a high affinity (*11–14*). This conformation is susceptible to trypsin at Arg262 (*29*) has a lower fluorescence intensities of Trp and the BIPM probe at Cys964 (*16*, *59*). Large open squares represent the conformational state of the enzyme accepting P_i, K^+ and ATP with a lower affinity. This conformation is susceptible to trypsin at Arg438 (*29*) and has higher fluorescence intensities of Trp and BIPM probe (*59*). Small circles and triangles, respectively, designate 3 sodium and 2 potassium ions and corresponding closed symbols designate occluded cations. Large open squares and circles with ATP-bound forms might contain cations, which remain to be determined with the step of hydrolysis of bound ATP.

Tetraprotomeric hypothesis of Na/K-ATPase

The reaction intermediates of the Post-Albers scheme have been shown to contain stoichiometric amounts of acid-labile enzyme-bound ATP accompanying ATP hydrolysis in the presence of Mg^{2+} and Na^+ with or without Rb^+ (K^+). One may ask if the simultaneous presence of EP and EATP in oligomeric structure could be explained as an equilibrium between EATP in an αβ-protomer form and EP in another αβ-protomer form. The fact that a high concentration of ATP inhibited the breakdown of isolated EP, and additional data presented above, clearly exclude such a possibility. Here, we propose the tetraprotomer hypothesis of ATP hydrolysis shown in Scheme 2. Further studies will be required to understand the mechanism of transport, sequential or simultaneous, and energy transduction in not only Na/K-ATPase but also other P-type ATPases, which show half of the site phosphorylation.

Addendum—After this review was submitted for publication, the data that the Na/K-ATPase associated as a tetramer *via* its cytoplasmic domain (*89*) and ligand regulated β,β-interactions (*90*) were reported.

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