Interactions between Na,K-ATPase α-Subunit ATP-binding Domains*

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The reaction mechanism of the Na,K-ATPase is thought to involve a number of ligand-induced conformational changes. The specific amino acid residues responsible for binding many of the important ligands have been identified; however, details of the specific conformational changes produced by ligand binding are largely underscribed. The experiments described in this paper begin to identify interactions between domains of the Na,K-ATPase α-subunit that depend on the presence of particular ligands. The major cytoplasmic loop (between TM4 and TM5), which we have previously shown contains the ATP-binding domain, was overexpressed in bacteria either with a His6 tag or as a fusion protein with glutathione S-transferase. We have observed that these polypeptides associate in the presence of MgATP. Incubation with [γ-32P]ATP under conditions that result in phosphorylation of the full-length Na,K-ATPase did not result in 32P incorporation into either the His6 tag or glutathione S-transferase fusion proteins. The MgATP-induced association was strongly inhibited by prior modification of the fusion proteins with fluorescein isothiocyanate or by simultaneous incubation with 10 μM eosin, indicating that the effect of MgATP is due to interactions within the nucleotide-binding domain. These data are consistent with Na,K-ATPase associating within cells via interactions in the nucleotide-binding domains. Although any functional significance of these associations for ion transport remains unresolved, they may play a role in cell function and in modulating interactions between the Na,K-ATPase and other proteins.

The Na,K-ATPase is an integral membrane protein that plays a central role in ionic homeostasis in animals by mediating the translocation of Na+ and K+ ions against their electrochemical gradients across the plasma membrane (for a review, see Ref. 1). The Na,K-ATPase functions as a heterodimer consisting of the α-subunit that spans the plasma membrane 10 times (2) and a ~55-kDa glycosylated β-subunit that has a short cytoplasmic N-terminal domain, a single transmembrane domain, and a large extracellular domain. At present, whether the β-subunit plays a part in the transport process remains unclear, but evidence is accumulating that indicates the importance of β in targeting the enzyme complex to the plasma membrane (3, 4). The Na,K-ATPase belongs to a large family of enzymes known as the P2-type ATPases (5). Members of this important protein class couple the hydrolysis of ATP to the transmembrane translocation of cations and have been identified in every taxonomic phylum.

Recently, the three-dimensional structures of several P2-type ATPases including the H-ATPase (6), the Na,K-ATPase (7), and the sarcoplasmic reticulum Ca-ATPase (8, 9) at 8-, 11-, and 2.6-Å resolution, respectively, have been solved. As one might predict, the high resolution sarcoplasmic reticulum Ca-ATPase (SERCA)1 pump structure shows that the 10-transmembrane segments are largely α-helical (8). Indeed, the SERCA crystal structure confirmed several previously proposed structural suggestions based on functional studies, including the suggestion that the fifth and sixth transmembrane domains were surrounded by the other helices as opposed to directly contacting the membrane lipid (10, 11). In addition, the structure of the cytoplasmic loops results in the formation of three distinct domains: 1) the N domain, containing the nucleotide-binding site; 2) the P domain, containing the phosphorylation site; and 3) the A domain, referred to as the “activator” domain by Toyoshima et al. (8, 9), consisting of the N terminus and the M2M3 loop.

The Na,K-ATPase and the gastric H,K-ATPase are the only members of the P2-type ATPase family that possess two obligatory subunits, α and β. Consequently, these enzymes have a quaternary structure; whether this structure is simply an αβ protomer or contains higher oligomers remains a central issue of scientific investigation (see Ref. 1). Identification of the subunit domains involved in assembly and trafficking of Na,K-ATPase has been approached by immune precipitation experiments with truncated β-subunits (12, 13) and chimeras between the Na,K-ATPase and gastric H,K-ATPase α-subunits (14, 15).

The oligomeric state of the Na,K-ATPase remains a controversial issue. Indeed, it seems hard to dispute the findings that monomeric (i.e. detergent-solubilized) αβ protomers of the sodium pump are sufficient to perform Na,K-ATPase activity (16, 17). However, it has been convincingly demonstrated that sodium pump protomer-protomer interactions do indeed take place and that adjacent pumps can be tethered together via chemical cross-linking (18). This apparent paradox has been explained by suggesting that close packing of sodium pumps within high density preparations leads to incidental contact,

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1 The abbreviations used are: SERCA, sarcoplasmic reticulum Ca-ATPase; ADB, ATP-binding domain; FITC, fluorescein 5'-isothiocyanate; PDV, polyvinylidene difluoride; SOD, superoxide dismutase; TBS, Tris-buffered saline; GST, glutathione S-transferase; CAPS, 3-(cyclohexylamino)propanesulfonic acid.
which can be captured via cross-linking (19). Although some functional measurements appear more easily resolved by a functional dipotramer (20–22), more complex models for a single functioning protomer can accommodate many of these findings (23). Nevertheless, it is clear that sodium pump molecules are in close proximity to each other within some cell membrane preparations. Although this association may not be necessary for sodium pump action, it may play a role in cell function by bringing other proteins that interact with the sodium pump into close proximity. For example, the Na,K-ATPase has been shown to be a membrane anchor for phosphoinositide-3 kinase in opossum kidney cells (24).

In this paper, we demonstrate that the isolated nucleotide binding domain can directly associate with full-length Na,K-ATPase purified from dog kidney. In addition, this interaction was significantly enhanced by the binding of magnesium and ATP. To further investigate the sites of interaction, we determined whether the isolated ATP-binding domain (ABD) could interact with itself in the absence of other pump domains. In the presence of MgATP, we found that a GST-tagged ABD associated with a His6-tagged ABD. Consistent with the facilitation of interaction by nucleotide binding was the observation that both FITC and eosin significantly decreased the degree of association by nucleotide binding. Taken together, these data imply that the Na,K-ATPase is capable of self-association and that this quaternary structure is stabilized upon MgATP binding. Preliminary accounts of this work have been previously reported (25).

**EXPERIMENTAL PROCEDURES**

**Reagents and Media**

Glutathione-Sepharose 4B and Rainbow protein molecular weight markers were from Amersham Biosciences. NaCl, KCl, MgCl₂, Na₂HPO₄, NaH₂PO₄, glutathione, Tris-base, Coomassie Brilliant Blue R-250, phenylmethylsulfonyl fluoride, antipain, leupeptin, pepstatin A, FITC, eosin, and imidazole were purchased from Sigma. Recombinant His6-tagged M4M5 loop from pGEM-rat Lys354, Lys₇⁷⁴ with an N-terminal His₆-tagged ABD. Consistent with the facilitation of interaction by nucleotide binding was the observation that both FITC and eosin significantly decreased the degree of association. Taken together, these data imply that the Na,K-ATPase is capable of self-association and that this quaternary structure is stabilized upon MgATP binding. Preliminary accounts of this work have been previously reported (25).

**GST-M4M5 Loop Purification**

The GST-ABD domain was purified by the method of Jorgensen (29) with the modifications described previously (30). In order to assay the interaction between the immobilized GST-ABD and dog kidney ATPase, 50 μg of dog kidney enzyme in 200 μl of 50 mM Tris (pH 7.4) and 0.1% C₅₋F₆ was mixed with a 200-μl slurry of GST-ABD conjugated to glutathione-Sepharose in the presence of either enzyme substrates (as shown in the figure legends). Control assays were performed using slurries of glutathione-Sepharose 4B that was not bound to GST-ABD. Microcentrifuge tubes containing the 400-μl reaction slurries were rotated at room temperature for 60 min, and the unbound enzyme was removed by centrifugation (1000 rpm in a tabletop microcentrifuge for 5 min). The supernatant was discarded, and the Sepharose pellet was resuspended in 1 ml of 50 mM Tris (pH 7.4) and 0.1% C₅₋F₆, and 50-μl aliquots were assayed for ATPase activity as described previously (31). The PVDF membrane was blocked with 10% dry milk protein solution in phosphate-buffered saline for 1 h. The membrane was then incubated with an antibody against the Na,K-ATPase α- or β-subunit for 1 h at room temperature. The primary antibody was removed, and the membrane was washed three times with phosphate-buffered saline and then incubated for 1 h with the appropriate horseradish peroxidase-conjugated secondary anti-IgG at room temperature. The membrane was then washed five times with phosphate-buffered saline plus 0.1% Tween 20, and the proteins were visualized by chemiluminescent detection.
The GST-ABD fusion protein was bound to the glutathione-Sepharose and subsequently washed with TBS (50 mM Tris, 120 mM NaCl, pH 7.4) to remove unbound fusion protein. Domain-domain interactions were initiated by adding 50 µg of the His6-ABD to a slurry of the conjugated GST-ABD in a final volume of 200–400 µl of TBS alone or containing various ligands (see figure legends for details). The fusion proteins were rotated at 4 °C for 1–3 h. After the interaction period, the glutathione-Sepharose was pelleted via centrifugation (1000 rpm in a tabletop microcentrifuge for 5 min) and washed three times with a 20-fold quantity of 50 mM TBS, containing any additives that were present during the interaction period. Finally, the GST loop and any His6 loop bound to it were eluted from the Sepharose with 100 µl of 10 mM reduced glutathione or 100 µl of Laemmli sample buffer. Proteins were separated via SDS-PAGE and electrotransferred to PVDF as described above. Evidence for His6-ABD interaction with GST-ABD was demonstrated via immunostaining with mouse anti-penta-His antibody (Qiagen) and horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (Sigma).

TREATMENT OF FUSION PROTEIN ABDs WITH ATP SITE PROBES

Eosin—Eosin, tetrabromofluorescein, has been shown to be a potent inhibitor of the Na,K-ATPase by competing with ATP (32). Thus, we performed the domain-domain interaction experiments described above in the presence and absence of 10 µM eosin.

FITC—FITC is a potent irreversible inhibitor of the Na,K-ATPase and has been shown to specifically label Lys454, which resides in the nucleotide binding site (33, 34). The GST loop was modified by incubating the protein, bound to glutathione-Sepharose 4B, with 20 µM FITC in 50 mM Tris buffer (pH 9.0, 30 min, 25 °C). The unbound FITC was removed by washing the Sepharose twice with a 10-volume quantity of TBS. FITC labeling was confirmed by UV illumination of the labeled protein on a gel (see Fig. 7B, middle panel). The His6 loop was labeled similarly, except the protein was not bound to the Ni2+-nitrilotriacetic acid; thus, excess FITC was removed via dialysis (12-kDa cut-off) overnight against a 1000-volume quantity of 50 mM Tris (pH 8.0). In order to determine whether FITC modification was necessary at either or both ABD partners, both FITC-labeled and unlabeled GST-ABD were incubated in the presence of 5 mM MgATP and 50 µg of either FITC-labeled or unlabeled His6-ABD.

Na+K+ATPase Activity Measurements—Na+,K+-ATPase activity was determined in a standard assay medium containing 1 mM EGTA, 130 mM NaCl, 20 mM KCl, 3 mM MgCl2, 3 mM NaATP, 50 mM imidazole, pH 7.2, and 0.5 µM of purified dog kidney enzyme (or enzyme solubilized with C12E8). The mixture was incubated at 37 °C for 15 min, and the amount of inorganic phosphate released through osmohem-sensitive ATP hydrolysis was measured as described previously (35).

Phosphorylation with [32P]ATP—The phosphorylation measurements were carried out essentially as described previously (36) in 50 µl of medium containing 100 mM NaCl (or 100 mM KCl), 5 mM MgCl2, 50 mM Tris-HCl, pH 7.2, and 50 µg of protein. The reaction was initiated by the addition of ATP ([γ-32P]ATP (PerkinElmer Life Sciences) and 7.3 µM Tris-ATP) and incubated in an ice bath for 60 s. The phosphorylation was stopped with 750 µl of “ice-cold” 5% (v/v) perchloric acid containing 0.5 mM Tris-ATP and 1.5 mM Tris-phosphate. The samples were filtered through Millipore filters (pore size 0.45 µm), washed three times with 3 ml of stopping buffer, and counted in a scintillation counter. Specific phosphorylation was calculated from the difference between 32P incorporation in native protein preparations and those where perchloric acid was added before ATP to denature the proteins.

RESULTS

Interaction between Purified Dog Kidney Na+,K+-ATPase with a Recombinant ATP-binding Domain—For these studies, a GST fusion protein of the large cytoplasmic loop between the fourth and fifth transmembrane segments was constructed. We reported previously that this GST-M4M5 loop was able to bind ATP as determined via protection against FITC labeling (37). More recently, the corresponding domain in the full-length rabbit fast twitch SERCA has been shown to coordinate TNP-AMP binding by x-ray crystallographic analysis (8).

To determine whether the GST-M4M5 loop (GST-ABD) associated with purified full-length Na+,K+-ATPase, C12E8-solubilized dog kidney Na+,K+-ATPase (50 µg) was incubated at 25 °C with a slurry of the GST loop bound to a glutathione-Sepharose affinity resin. The incubation was performed both in the absence and presence of pump substrates (Fig. 1A). There was a clear interaction between the GST-ABD and the intact Na+,K+-ATPase in the presence of MgATP (Fig. 1A, lane 6). Occasion-
properties of the His6-ABD were described previously (26); in the same polypeptide as the GST-ABD. The nucleotide-binding domain comprises nearly 40% of the mass of the catalytic subunit. Furthermore, it has been shown that this domain undergoes dramatic conformational changes in response to substrate binding (39, 40). Consequently, we decided to test whether the interactions observed between the intact Na,K-ATPase and the His6-ABD were mediated via contacts within the ATP-binding domain. These experiments were performed using either the GST-ABD or a His8-tagged fusion protein with the same polypeptide as the GST-ABD. The nucleotide-binding properties of the His8-ABD were described previously (26); in addition, we found that the His8-ABD, like the GST-ABD, associates with the intact Na,K-ATPase in a MgATP-dependent manner (Fig. 3). Therefore, the observed interactions between native Na,K-ATPase and the fusion proteins were mediated by enzyme contacts with the ATP-binding domain and not by either of the fusion protein affinity tags.

![Fig. 2. GST-ABD interaction with C12E8-solubilized dog kidney Na,K-ATPase at varying [MgATP].](image)

The GST-ABD bound to glutathione-Sepharose was incubated with 50 μg of purified Na,K-ATPase solubilized with 0.1% C12E8. The incubation medium contained 50 mM Tris (pH 7.4) with the indicated concentrations of magnesium and ATP. After the incubation period, the Sepharose was washed with the corresponding media, and bound proteins were removed by adding Laemmli sample buffer. Aliquots were run on a 7.5% Laemmli gel, and proteins were electrotransferred to a PVDF membrane. The membranes were probed with anti-α antibody (Affinity Bioreagents). The lack of protein-protein interactions at low MgATP suggests that the GST-ABD must be in the nucleotide-bound state to interact with the α-subunit.

Evidence Suggesting That Interactions Take Place within the Nucleotide-binding Domain—The Na,K-ATPase nucleotide-binding domain comprises nearly 40% of the mass of the catalytic α-subunit. Therefore, we incubated a bacterially produced sodium pump that interacted with the bacterially produced ATP-binding domains of the full-length Na,K-ATPase that interacted with the bacterially produced ATP-binding domains remained to be determined. Thus, we initially designed experiments to determine whether the interactions observed between the two purified cytoplasmic loops (i.e. the M4M5 loop of the intact enzyme with the equivalent heterologously expressed isolated domain). We tested this hypothesis by measuring interactions between the two purified cytoplasmic loops themselves. The soluble His8-ABD was incubated with GST-ABD bound to glutathione-Sepharose resin in the presence of varying substrates. We observed that the two constructs did associate and that this association depended upon the simultaneous presence of both magnesium and ATP (Fig. 4A). In other words, neither ATP alone nor magnesium alone was an effective promoter of the interaction. MgADP and MgAMP were unable to facilitate interaction between the two nucleotide-binding domains (Fig. 4B). The inability of MgADP to promote interactions between the GST-ABD and the His8-ABD was somewhat surprising, considering that it did facilitate interactions between the GST-ABD and intact Na,K-ATPase (Fig. 1A). Thus, this finding may suggest that MgADP binding to the full-length α-subunit elicits a conformation slightly different than when it is bound to the isolated M4M5 loop alone. Indeed, the nucleotide-bound crystal structure of SERCA indicates that both the N terminus and the M2M3 cytoplasmic loop (i.e. the “A” domain) are in close proximity to the M4M5 loop (8, 9); the A domain is obviously absent from our bacterial constructs.

It was important to ensure that MgATP does not facilitate nonspecific protein interactions with GST-ABD or glutathione-Sepharose. Therefore, we incubated a bacterially produced superoxide dismutase (SOD) fusion protein with the GST-ABD under the same conditions that promote interaction with the His8-ABD. Fig. 5 shows that there was no interaction between the GST-ABD and SOD in the presence or absence of MgATP. The Western blot was probed with an anti-SOD antibody, and a positive control lane shows that the immunostaining was successful (Fig. 5) and that SOD would have been detectable had it interacted with the GST-ABD.

Specificity of the Interactions between ATP-binding Domains—To more directly examine the MgATP-dependent asso-
In the absence of ATP, as a positive control for immunodetection, we ran 1 μg of purified SOD in an adjacent lane (Fig. 6). Lane 1, interactions between the ABDs in the presence of 1 mM MgATP and 10 μM eosin. Lane 2, interactions between the ABDs in the presence of 1 mM MgATP alone. Both the Coomassie-stained gel (left panel) and the immunoblot (right panel) indicate much smaller amounts of the His6-tagged loop associated with the GST fusion protein in the presence of eosin. His6-ABD protein was detected with anti-penta-His antibody (Qiagen). Experiments were performed essentially as in Fig. 5 with the addition of eosin.

FITC is a fluorescent amine-reactive molecule that labels Lys residues in the purified Na,K-ATPase; this reaction is prevented by the simultaneous presence of ATP (33, 34). Similarly, ATP has been shown to protect both the His6-ABD (26) and the GST-ABD (37) against FITC labeling. When both the GST-ABD and His6-ABD were labeled with FITC, domain-domain interactions were substantially reduced (Fig. 7B, right panel). Fig. 7B (middle panel) shows FITC incorporation into the GST-ABD, whereas FITC-labeled His6-ABD is not observed, since it did not associate with the GST-ABD and thus was lost during the washing steps. (The FITC labeling protocol used for His6-ABD modification was identical to those published previously (26).) Interestingly, FITC labeling of only one of the interacting fusion proteins did not inhibit the interaction; clearly, FITC modification of the His6-ABD (Fig. 7A, middle panel) did not significantly reduce its ability to associate with the nonmodified GST-ABD (Fig. 7A, right panel).

Phosphorylation Cannot Explain Domain-Domain Interactions—It is clear that MgATP, and not ATP alone, promotes the observed interactions. An obvious possibility might be that the proteins are undergoing magnesium-dependent phosphorylation. However, there was no difference between the K+–containing control with the intact Na,K-ATPase and either the His6-ABD or the GST-ABD in the presence of Na+ or K+ (Fig. 8). This may not be surprising, since the isolated ABDs are devoid of cation-binding sites. Nonetheless, the phosphorylation levels observed for the fusion proteins (Fig. 8), compared with the level of 32P, captured on the filter with perchloric acid-denatured protein (i.e. background), was minimal. Moreover, considering that the fusion proteins are less than half the molecular mass of the Na,K-ATPase and constitute a purer protein preparation, the difference in phosphoprotein production between the intact enzyme and the fusion proteins is a very conservative estimate (Fig. 8). Also, considering that
Fig. 7. Effect of FITC modification on MgATP-induced ABD associations. These experiments were designed to determine whether FITC modification of both the GST- and His₆-ABDs was required to prevent the MgATP-dependent association. For FITC modification, the fusion proteins were treated with 20 µM FITC for 30 min at room temperature (50 mM Tris, pH 9.0). Unreacted FITC was removed via dialysis (see “Experimental Procedures”). A. Unlabeled GST-ABD, tethered to glutathione-Sepharose, was incubated with a 50-µg quantity of either unlabeled His₆-ABD or FITC-labeled His₆-ABD. Interactions were measured in the presence of 1 mM MgATP. Equal aliquots from the respective interactions were run in separate lanes on a 12% Laemmli gel. The gel was cut in half, and one section was used for Western analysis with anti-penta-His antibody (right panel), whereas the other half was first photographed under UV illumination (middle panel) and then stained with Coomassie Brilliant Blue (left panel). Lane 1 in each panel shows interactions in the complete absence of FITC labeling. Lane 2 in each panel shows the interaction between unlabeled GST-ABD and FITC-labeled His₆-ABD. Clearly, FITC-modified His₆-ABD was still able to associate with unlabeled GST-ABD in the presence of 1 mM MgATP (right panel, lane 2). B. FITC-labeled or unlabeled GST-ABD, tethered to glutathione-Sepharose, was incubated with a 50-µg quantity of either unlabeled His₆-ABD or FITC-labeled His₆-ABD. Interactions were measured in the presence of 1 mM MgATP. Equal aliquots from the respective interactions were run in separate lanes on a 12% Laemmli gel. The gel was cut in half, and one section was used for Western analysis with anti-penta-His antibody (right panel), whereas the other half was first photographed under UV illumination (middle panel) and then stained with Coomassie Brilliant Blue (left panel). Lane 1 in each panel shows interactions when both fusion proteins were labeled with FITC. Lane 2 is the control, showing normal protein interactions occurring in the complete absence of FITC modification. Clearly, FITC modification of the fusion proteins dramatically reduced their ability to associate with one another (right panel, lane 1).

neither the His₆-ABD nor the GST-ABD can hydrolyze ATP (data not shown), the inability to isolate a phosphorylated ABD (Fig. 8) cannot be due to a more labile acyl-phosphate intermediate. Rather, it seems likely that the ABD constructs cannot “close” sufficiently to bring the N and P domains together, a requirement of phosphoenzyme formation (8, 9). This inability to close is probably due to the lack of both the membrane domains and the A domain in the isolated ABDs. Thus, our data suggest that the protein conformation producing the strongest domain-domain interactions is produced simply by the binding of both magnesium and ATP and not phosphoenzyme formation.

DISCUSSION

The high resolution structures of the Ca-ATPase (8, 9) clearly show that, as predicted previously (41), the cation and nucleotide binding domains of P₇-type ATPases are not only functionally separate but are spatially separate as well. In other words, all of the residues involved in ATP-binding and hydrolysis are located in the cytoplasmic loop between M4 and M5, forming the two separate N and P subdomains. Conversely, all of the residues suggested to be involved with cation coordination in the occluded state are located in the transmembrane-spanning regions of the enzyme (1). Since occupation of the cation-binding site dramatically alters nucleotide affinity, it is obvious that communication exists between the cation-binding membrane domains and the cytoplasmic ATP-binding domain. Indeed, the conformational changes observed between the E₁ and E₂ SERCA structures (see Refs. 8 and 9, respectively) show dramatic movements of transmembrane helices and changes in their structure, which push and pull on large cytoplasmic domains. Instability of helices associated with cation binding has been observed previously in both the Na,K-ATPase (10) and the gastric H,K-ATPase (11).

In the current work, we provide evidence that tight protein-protein interactions occur between two differentially tagged constructs of the large cytoplasmic loop between M4 and M5 of the Na,K-ATPase. This loop has been shown to contain all of the residues that compose the nucleotide binding domain in the sodium pump (26) as well as other members of the P₇-type ATPase family (8, 42). The ABD-ABD interaction reported here was dependent upon the presence of both magnesium ions and ATP, yet neither fusion protein was phosphorylated (Fig. 8); nor did they possess significant ATPase activity (26).
Ligand-specific Binding between Sodium Pump ABDs

One Versus Two ATP Molecules Bound—Whether the MgATP-dependent interaction between the M4M5 loops required both partners to have MgATP bound remained a question. Thus, we determined the MgATP concentration dependence for the interaction between GST-ABD and intact (C12E8-solubilized) Na,K-ATPase. It was shown previously that the isolated ATP-binding domain has a $K_{d}^{MgATP}$ of ~500 $\mu$M, consistent with the ABD existing in an $E_{2}$-like conformation (26). In contrast, the binding affinity for ATP of intact Na,K-ATPase is less than 1 $\mu$M (43). Thus, we measured interactions between the GST-ABD and intact enzyme at varying MgATP concentrations (Fig. 2). At low MgATP concentrations (e.g., <50 $\mu$M), where intact enzyme is saturated with ATP and GST-ABD is less than 10% occupied by ATP, no protein-protein interactions were detected (Fig. 2). However, at MgATP concentrations greater than 500 $\mu$M, when both the GST-ABD and intact enzyme are largely in the ATP-bound state, the GST-ABD was able to pull down the intact Na,K-ATPase $\alpha$-subunit (Fig. 2). These observations are consistent with the necessity of ATP binding to both interacting proteins to facilitate association, or alternatively, they may reflect a necessary MgATP-dependent stabilization or decrease in flexibility of the soluble GST-ABD to achieve this stable protein-protein interaction.

If ATP-binding domain interactions only occur when each protein is in the nucleotide-bound form, then modification of one of the fusion proteins with FITC should eliminate ABD-ABD interactions, because the FITC-labeled partner would be unable to bind MgATP. However, we found that labeling either the GST-ABD or the His$_{8}$-ABD could not disrupt the MgATP-dependent association between the two (Fig. 7A). Rather, FITC modification of both fusion proteins was required to block the GST-ABD/His$_{8}$-ABD interaction (Fig. 7B, right panel). Consequently, it appears that MgATP binding is only necessary to one of the constructs to facilitate protein-protein interactions, or that a single ATP molecule is binding in part to both fusion proteins and forming a bridge between the two ABDs. In other words, FITC modifies Lys$^{501}$ that forms part of the binding site for the adenosine moiety in the N domain of the Na,K-ATPase $\alpha$-subunit. Since FITC binding to both partners is required to prevent association, either partner can supply this segment. The other partner may provide the terminal phosphate binding segment (close to Asp$^{166}$) in the P domain. In this way, the ATP molecule bridges two ABD polypeptide loops via N and P domains from each member of the dimer (see below).

Intermolecular Interactions between Adjacent Sodium Pumps—Whether P-type ATPases exist as monomers, dimers (diprotomers for the H,K- and Na,K-ATPases), or higher oligomers is a subject of debate. There have been several reports demonstrating oligomeric forms of various P-type ATPases (e.g., the sarcoplasmic reticulum Ca-ATPase (44), the Na,K-ATPase (45), and the H,K-ATPase (46)). In particular, there have been several studies that suggest the Na,K-ATPase exists as an $\alpha/\beta$ multimer (i.e., $\alpha/\beta_{2}$). Initially, $\alpha/\beta_{2}$ models were proposed to explain the biphasic kinetics of ATP on enzyme activity (47) and later expanded to $\alpha/\beta_{n}$ models (48). Structural support for oligomeric models comes from a number of diverse studies, including cross-linking (18), fluorescence resonance energy transfer between the ATP-site probes FITC and ErITC (22), and more directly by co-immunoprecipitation experiments (49).

It has been suggested that the findings of the cross-linking and energy transfer studies could be a result of the high density of Na,K-ATPase in kidney membrane preparations used in these studies (19). Indeed, a recent study reported that thermal denaturation of the Na,K-ATPase resulted in the formation of $\alpha/\alpha$ dimer and tetramer aggregates (50). Interestingly, these oligomers were devoid of $\beta$- and $\gamma$-subunits, demonstrating that the important contacts were within the $\alpha$-subunit (50).

Blanco et al. (49) demonstrated associations between different $\alpha$-subunit isoforms (i.e., $\alpha_{1}$ and $\alpha_{3}$) heterologously expressed in insect cells, via co-immunoprecipitation experiments with isoform-specific antibodies. In these experiments, the expressed Na,K-ATPase is less than 10% of the total membrane protein and most likely closer to 1–2% (3). Clearly, the pump density in insect cell preparations is sufficiently low that $\alpha/\alpha$ interactions are unlikely to have been caused by overcrowding. In the present study, the ability of both GST- and His$_{8}$-ABD to pull down intact $\alpha$-subunit from a C$_{12}E_{8}$-solubilized kidney preparation of Na,K-ATPase (Figs. 1 and 3) in the presence of defined ligands is more consistent with specific interactions than incidental contact. Indeed, the lack of interaction between the ABDs and intact Na,K-ATPase $\alpha$-subunit, in the absence of solubilization, suggests that endogenous pump-pump associations must be broken before the exogenous ABDs can bind. Moreover, the demonstration that the two tagged ABDs associate with the same substrate dependence as with the intact Na,K-ATPase indicates that pump-pump interactions may be solely through interactions between the large cytoplasmic loops. Indeed, Koster et al. (45) reached similar conclusions based upon $\alpha/\alpha$ associations measured between different Na,K- and H,K-ATPase chimeric enzymes expressed in insect cells. Specifically, the sodium pump $\alpha_{i}$ subunit selectively associated with chimeras containing the Na,K-M4M5 loop but not with constructs containing the H,K-ATPase large cytoplasmic loop (45). In fact, these authors observed that wild-type sodium pump $\alpha_{i}$ associated with a chimera containing only the sodium pump M4M5 loop inserted within the H,K-ATPase (45).

Significance of $\alpha/\alpha$ Interactions—It seems clear that the catalytic properties of Na,K-ATPase can be mediated by a functional monomeric form (i.e., $\alpha/\beta$) of the enzyme (16, 19, 23, 38). Consequently, the relevance of the higher oligomeric states sometimes proposed for this enzyme remains a puzzle. There are several reports measuring various kinetic properties of Na,K-ATPase function that can be easily explained by $\alpha/\alpha$ interactions. For example, fluorescence resonance energy transfer between several Na,K-ATPase labeling probes reveals distances great enough to indicate that the probes reside on different protomers (22). These observations are directly in contrast with FRET measurements from another laboratory indicating that FITC and Co(NH$_{3}$)$_{4}$ATP are close enough to reside on a single $\alpha/\beta$ protomer (52). In addition, the ability of TNP-ADP to inhibit the residual $p$-nitrophenyl phosphatase activity of FITC-labeled $\alpha/\beta$ protomers, solubilized with C$_{12}E_{8}$, makes it difficult to justify invoking the involvement of additional protomers (21). Indeed, Martin and Sachs (23) convincingly demonstrate that the appearance of the “low affinity” nucleotide site, which allows TNP-ADP to bind and inhibit $p$-nitrophenyl phosphatase activity, is actually a result of FITC modification and does not appear on native enzyme. Thus, there seems to be no compelling functional reason to suggest that the Na,K-ATPase exists as a diprotomer (or higher oligomer). Nevertheless, the current observations (Figs. 1–3) as well as previous reports from other laboratories (18, 45, 48–50) strongly suggest that under certain conditions the Na,K-ATPase does self-associate.

A Cell Biological Role of Dimerization?—Recently, Kaplan and colleagues (53) initially identified, using the yeast two-hybrid system, several cellular proteins that interacted with the Na,K-ATPase. Following careful characterization, specific candidates were identified: 1) polycystin-1, involved in polycystic kidney disease; 2) SNAPAP, a protein involved in vesicular targeting; and 3) the catalytic subunit of protein phosphatase
Alternative dimerization between Na,K-ATPase ABDs may perform a necessary cellular function as a membrane anchor for other signaling proteins (24, 53). Experiments are under way to confirm whether the associations presented here are dimers or higher oligomers and to further understand the role that these sodium pump interactions may play in cellular physiology.

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REFERENCES

1. Kaplan, J. H. (2002) Annu. Rev. Biochem. 71, 511–535
2. Hu, Y.-K., and Kaplan, J. H. (2000) J. Biol. Chem. 275, 19185–19191
3. Catto, G., McLoud, S. M., and Kaplan, J. H. (2001) Am. J. Physiol. 281, C982–C992
4. Geering, K., Begah, A., Good, P., Girardet, S., Roy, S., Schaer, D., and Jaunin, P. (1996) J. Cell Biol. 133, 1193–1204
5. Lutsenko, S., and Kaplan, J. H. (1995) Biochemistry 34, 5607–5613
6. Kuhlbrandt, W., Auer, M., and Scarborough, G. A. (1998) Curr. Opin. Struct. Biol. 8, 510–516
7. Rice, W. J., Young, H. S., Martin, D. W., Sachs, J. R., and Stokes, D. L. (2001) Biophys. J. 80, 2187–2197
8. Toyoshima, C., Nakasako, M., Nomura, H., and Ogawa, H. (2000) Nature 405, 647–655
9. Toyoshima, C., and Nomura, H. (2002) Nature 416, 605–611
10. Lutsenko, S., Andersko, R., and Kaplan, J. H. (1995) Proc. Natl. Acad. Sci. 92, 7936–7940
11. Catto, G., Lutsenko, S., Shin, J.-M., Sachs, G., and Kaplan, J. H. (1999) J. Biol. Chem. 274, 13737–13746
12. Jaunin, P., Horisberger, J.-D., Richter, K., Good, P. J., Rossier, B. C., and Almers, W. (1997) Proc. Natl. Acad. Sci. 94, 1636–1641
13. Lutsenko, S., and Kaplan, J. H. (1998) Biochemistry 37, 569–576
14. Laemmli, U. K. (1970) Nature 227, 680–685
15. Bradford, M. M. (1976) Anal. Biochem. 72, 248–252
16. Jorgensen, P. L. (1974) Biochim. Biophys. Acta 356, 36–52
17. Catto, G., Thornewell, S. J., Holden, P. J., and Kaplan, J. H. (1999) J. Biol. Chem. 274, 24985–25003
18. Matsudaira, P. (1987) J. Biol. Chem. 262, 10035–10038
19. Skou, J. C., and Esmann, M. (1981) Biochim. Biophys. Acta 647, 232–240
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33. Farley, R. A., Tran, M. C., Carilli, C. T., Hawke, D., and Shively, J. E. (1984) J. Biol. Chem. 259, 9532–9535
34. Kirley, T. L., Wallick, E. T., and Lane, L. K. (1984) Biochem. Biophys. Res. Commun. 125, 767–773
35. Gatto, C., S. Lutsenko, S. Lutsenko, and Kaplan, J. H. (1997) Arch. Biochem. Biophys. 340, 90–100
36. Pedemonte, C. H., and Kaplan, J. H. (1988) Biochemistry 27, 7966–7973
37. Kaplan, J. H., Lutsenko, S., Gatto, C., Daoud, S., and Kenna, L. J. (1997) Ann. N. Y. Acad. Sci. 834, 45–55
38. Ward, D. G., and Cavieres, J. D. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5332–5336
39. Karlish, S. J. D. (1980) J. Bioenerg. Biomembr. 12, 111–136
40. Lutsenko, S., and Kaplan, J. H. (1994) J. Biol. Chem. 269, 4555–4564
41. Kaplan, J. H., Gatto, C., Holden, J. P., and Thorne, S. J. (1998) Acta Physiol. Scand. 163, 99–105
42. Moutin, M.-J., Cuilliel, M., Rapin, C., Miras, R., Anger, M., Lopre, A.-M., and Dupont, Y. (1998) J. Biol. Chem. 269, 11147–11154
43. Askari, A. (1987) J. Bioenerg. Biomembr. 19, 359–374
44. Merino, J. M., Gutierrez-Merino, C., and Henao, F. (1999) Arch. Biochem. Biophys. 368, 298–302
45. Koster, J. C., Blanco, G., and Mercer, R. W. (1995) J. Biol. Chem. 270, 14332–14339
46. Shin, J. M., and Sachs, G. (1996) J. Biol. Chem. 271, 1904–1908
47. Askari, A. (1982) Mol. Cell Biochem. 43, 129–143
48. Taniguchi, K, Kaya, S., Abe, K, and Maruh, S. (2001) J. Biochem. (Tokyo) 129, 335–342
49. Blanco, G., Koster, J. C., and Mercer, R. W. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8542–8546
50. Donnet, C., Arystarkhova, E., and Sweadner, K. H. (2001) J. Biol. Chem. 276, 7357–7365
51. Sachs, J. R. (1994) Biochim. Biophys. Acta 1193, 199–211
52. Farley, R. A., Elquza, E., Kane, D. J., Kasho, V., and Faller, L. D. (2000) Biophys. J. 78, 279 (abstr.)
53. Pagel, P., Kimura, T., and Caplan, M. J. (2001) J. Am. Soc. Nephrol. 12, 59 (abstr.)
54. Moody, J. E., Millen, L., Binns, D., Hunt, J. F., and Thomas, P. J. (2002) J. Biol. Chem. 277, 21111–21114
55. Hopfner, K. P., Karcher, A., Shin, D. S., Craig, L., Arthur, L. M., Carney, J. P., and Tainer, J. A. (2000) Cell 101, 789–800
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