The Energy Transduction Mechanism of Na,K-ATPase Studied with Iron-catalyzed Oxidative Cleavage*

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This paper extends our recent report on specific iron-catalyzed oxidative cleavages of renal Na,K-ATPase and effects of $E_1 \leftrightarrow E_2$ conformational transitions (Goldshleger, R., and Karlish, S. J. D. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 9596–9601). The experiments indicate that only peptide bonds close to a bound Fe$^{2+}$ ion are cleaved, and provide evidence on proximity of the different cleavage positions in the native enzyme. A sequence HFIH near trans-membrane segment M3 appears to be involved in Fe$^{2+}$ binding. Previously we hypothesized that $E_2$ and $E_1$ conformations are characterized by formation or relaxation of interactions within the $\alpha$ subunit at or near highly conserved sequences, TGES in the minor cytoplasmic loop and CSDK, MVTGD, and VNDSPALKK in the major cytoplasmic loop. This concept has been tested by examining iron-catalyzed cleavage in both non-phosphorylated and phosphorylated conformations and effects of phosphate, vanadate, and ouabain. The results imply that both $E_1 \leftrightarrow E_2$ and $E_1^P \leftrightarrow E_2^P$ transitions are indeed associated with formation and relaxation of interactions between cytoplasmic domains, comprising the minor loop plus N-terminal tail leading into M1 and major loop, respectively. Furthermore, it appears that either non-covalently or covalently bound phosphate bind near CSDK and MVTGD, and Mg$^{2+}$ ions may bind to residues within TGES and VNDSPALKK and to bound phosphate. Thus cytoplasmic domain interactions seem to occur within or near the active site. We discuss the relationship between structural changes in the cytoplasmic domain and movements of trans-membrane segments that lead to cation transport. Presumably conformation-dependent formation and relaxation of domain interactions underlie energy transduction in all P-type pumps.

The molecular mechanism whereby Na,K-ATPase transduces the free energy of hydrolysis of ATP into active transport of Na$^+$ and K$^+$ ions is unknown. We have a wealth of knowledge on transport reactions, covalent phosphorylation, $E_1/E_2$ conformational transitions, and cation occlusion, embodied in the Post-Albers kinetic mechanism (see Ref. 1). Active cation transport involves Na$^{+}$-dependent phosphorylation from ATP, Na$^+$ movement coupled to $E_1^P \rightarrow E_2^P$, K$^{+}$-activated dephosphorylation, K$^+$ movement coupled to $E_2(K) \rightarrow E_1$. Knowledge of the structural basis for energy transduction is meager due to lack of information on molecular structure. The best structure of Na,K-ATPase at 20–25-Å resolution reveals only the overall shape of the protein and distribution of mass of $\alpha$ and $\beta$ subunits (2). Recent cryoelectron microscopy studies of Ca-ATPase and H-ATPase demonstrate the overall shape at 8-Å resolution, including "head", "neck," and membrane segments, including 10 trans-membrane $\alpha$-helical rods, most of which are tilted at an angle to the membrane (3, 4). The topological organization with 10 trans-membrane segments confirms that shown by a variety of techniques (5). Biochemical and molecular techniques are providing much information on residues involved in cation occlusion within trans-membrane segments (6), primarily M4, M5, and M6 (7–9), or ATP binding within the large cytoplasmic loop (see Ref. 5). These studies indicate the necessity for interactions between the ATP sites and cation occlusion sites. These interactions are mediated by $E_1 \leftrightarrow E_2$ conformational transitions, which have been studied extensively using proteolytic digestion, fluorescent probes, ligand binding, etc. (see Ref. 10 for a review and references). The fact that probes bound at different sites report the $E_1 \leftrightarrow E_2$ transition implies that substantial structural changes must occur. However, the nature of those changes has been largely obscure. All P-type pumps contain the conserved cytoplasmic sequences TGES in the minor loop between M2 and M3, MVTGD in the major loop, and TGDGVNDSPALKK in the so-called "hinge" region before M5 (5). Proteolytic cleavage and site-directed mutagenesis in these sequences usually stabilize $E_1$ conformations, implying an involvement in the conformational transitions (see Refs. 5 and 8 for full references). Based on functional effects of mutations and proteolytic cleavages in the $\beta$-strand minor cytoplasmic loop of sarcoplasmic reticulum Ca-ATPase, an interaction between the minor and major cytoplasmic loop near the phosphorylation site was proposed earlier (11). For yeast H-ATPase, mutations within the minor loop suggested a similar conclusion (12).

Recently, we have described specific iron-catalyzed or copper-catalyzed oxidative cleavage of renal Na,K-ATPase (13–15). The process seems to involve a site-specific mechanism in which peptide bonds close to the bound metals are cleaved, presumably by OH radicals generated locally by the Fenton reaction or a reactive metal-peroxyl derivative (16–19). Because more than one peptide bond can be cleaved from the same metal site, this technique provides information on interacting segments of individual subunits or neighboring subunits (unlike proteolytic cleavage). Incubation of Na,K-ATPase with Fe$^{3+}$/ascorbate/H$_2$O$_2$ induces specific cleavage of the $\alpha$ subunit at the cytoplasmic surface without cleaving the $\beta$ subunit (13). Copper-catalyzed oxidative cleavage occurs at the extracellular surface and both $\alpha$ and $\beta$ subunits are cleaved (15).

Iron-catalyzed cleavages are very sensitive to the conformational state (13). In $(E_2 \alpha)$ or $(E_2 \beta)$ conformations, we observed four major and two minor fragments of the $\alpha$ subunit. In $E_1$ or $E_1\alpha Na$ conformations, cleavage was much slower and only one major cleavage was observed. Positions of cleavages were ei-

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ther identified exactly by N-terminal sequencing or, for fragments with blocked N termini, approximately using sequence-specific antibodies. In $E_2$ conformations only, cleavages (identified by short sequences at or near the N termini) were found at $^{214}\text{ESE}$ in the minor loop between $M2$ and $M3$, near $^{387}\text{CSDK}$ after $M4$ which includes the phosphorylated $\text{Asp}^{386}$, near $^{488}\text{MVTGD}$ in the major loop, and at $^{718}\text{VNDS}$ in the “hinge” region before $M5$. In either $E_2$ or $E_1$ conformations, a cleavage was seen near $^{234}\text{IATL}$, before $M3$. The observations suggested strongly that peptide bonds are cleaved with a probability depending on their proximity to a bound Fe$^{2+}$ or Fe$^{3+}$ ion, and thus imply that the different cleavage points are also in proximity to each other. Several cleavages lie in or near the highly conserved cytoplasmic sequences, suggesting that these sequences mediate mutual interactions. We proposed that, in $E_2$ conformations, the minor and major loops interact near the conserved sequences while, in $E_1$ conformations, the loops separate (13).

This paper extends our observations on iron-catalyzed cleavages in two ways. First, we have obtained further evidence for the site-specific mechanism. Second, we have looked at iron-catalyzed cleavages in both phosphorylated and non-phosphorylated conformations and effects of inhibitors. The results provide novel information on the energy transduction mechanism.

**EXPERIMENTAL PROCEDURES**

Na,K-ATPase, with specific activities of 12–17 units/mg protein, was prepared from pig kidney (20) and was stored at $-70^\circ\text{C}$ in a solution of 250 mM sucrose, 25 mM histidine, pH 7.2, and 1 mM EDTA (Tris). Rat azolemma microsomes (2–3 units/mg enzyme) prepared as described in Ref. 21 or rat kidney microsomes (3–4 units/mg protein) were prepared as in Ref. 20. Prior to incubation with Fe$^{3+}$/ascorbate/H$_2$O$_2$, the microsomal membrane preparations (1.5 mg/ml) were preincubated with sodium deoxycholate 1.2 mg/ml for 15 min at 20 °C, washed twice, and resuspended in a solution containing 10 mM Tris-HCl, pH 7.2.

**Cleavage Reactions**—Suspensions of pig kidney Na,K-ATPase (0.1–1 mg/ml) or rat microsomal preparations (1 mg/ml) were incubated at 20 °C with freshly prepared solutions of 5 mM ascorbate (Tris) plus 5 mM H$_2$O$_2$ or with added FeSO$_4$. To arrest the reaction, 5–10 mM EDTA or 5-fold concentrated gel sample buffer with 15 mM EDTA was added. Samples were assayed for Na,K-ATPase activity or applied to PVDF paper, immunoblots, and microsequencing of fragments have been described in detail (22, 23). Anti-$K_{1012}$–$Y_{1016}$, referred to as “anti-KETYY,” was used to detect fragments of the $\alpha$ subunit. Immunoblots were stained with diaminobenzidine with metal ion enhancement (ECL; 3–5 μg of protein/lane) using anti-rabbit IgG horseradish peroxidase conjugate and the protocol supplied with ECL reagents from the 1998 Amersham Pharmacia Biotech catalogue. For quantification of the bands, the stained PVDF paper or developed x-ray films were scanned with a Bio-Rad GS-690 imaging densitometer and analyzed with Bio-Rad Multi-Analyzer software (version 1.01). For quantification of Coomassie-stained $\alpha$ subunit the band was cut out of the gel, and optical density of Coomassie stain extracted into 1% SDS solution was measured at 595 nm (13).

**Calculations**—Non-linear curve-fitting was performed using Enzfitter (Elsevier-Biosoft).

**Materials**—For SDS-PAGE, all reagents were electrophoresis-grade from Bio-Rad. Tris (ultra pure) was from Bio-Lab, Jerusalem, (±)- ascorbic acid catalogue no. 100127), 30% H$_2$O$_2$ (catalogue no. 82287), and α-chymotrypsin (catalogue no. 2307) were from Merck. Phosphocreatine, P6502, creatine phosphokinase P3755, and oligomycin O4876 were from Sigma. All other reagents were of analytical grade.

**RESULTS**

**Properties of Fe$^{2+}$ Activation of Cleavage; Dependence on $E_1/E_2$ Conformations**—Fig. 1 presents an immunoblot using anti-KETYY to detect fragments produced in media containing different proportions of K$^+$ and Na$^+$ ions (sum 150 mM). In 150 mM K$^+$ ($E_2$), we observed five major fragments referred to as: a, near $M1$; b, near $^{214}\text{ESE}$; c, near $^{285}\text{IATL}$ (previously referred to as near IATL, see below); d, near $^{488}\text{MVTGD}$; and e, near $^{718}\text{VNDS}$. Apparent $M_1$ values are 91.3, 80.6, 73.4, 38.2, and 26.3 kDa, respectively. By comparison with previous experiments done in low ionic strength media (13), the cleavage near $^{387}\text{CSDK}$ was largely suppressed, while that near $M1$ was more prominent. About half the $\alpha$ subunit was cleaved in these conditions. As K$^+$ was replaced by Na$^+$ ions, cleavage at ESE, near MVTGD, and at VNDS was progressively suppressed, essentially completely at 150 mM Na$^+$ ($E_2$), while in parallel, the cleavages near $M1$ and HFIH were amplified. A similar change of pattern was observed upon transfer of the enzyme from low ionic strength (10 mM Tris, pH 7, $E_2$) to high ionic strength media (10 mM Tris, pH 7, $E_1$) to high ionic strength media (10 mM Tris, pH 7, 300 mM choline chloride, $E_1$).

Bound Fe$^{2+}$ ion is predicted to be in contact with more residues in $E_2$ than in $E_1$ forms (see Ref. 13 and model in Fig. 1), and thus the Fe$^{2+}$ should bind more tightly in $E_2$ forms. Fig. 2 depicts the relationship between Fe$^{2+}$ ion concentration and cleavage of the $\alpha$ subunit and inactivation of Na,K-ATPase activity, in Rb$^+$ - and Na$^+$-containing media ($E_2$/Rb) or $E_1$/Na, respectively). The curves are fitted well by simple hyperbolas with $K_{0.5}$ values of 0.57 and 3.49 μM for cleavage of the $\alpha$ subunit and 0.20 and 1.16 μM for inactivation of Na,K-ATPase, respectively. As discussed previously (13, 15), the 2–3-fold lower $K_{0.5}$ value for inactivation of Na,K-ATPase compared with cleavage could imply that oxidative reactions occur in addition to chain cleavage and inactivate the enzyme. In any event, the 5–6-fold lower values of $K_{0.5}$ in the $E_2$/Rb compared with the $E_1$/Na conformation, by either measure, are compatible
Fe2\(^{2+}\) ions can replace Mg2\(^{2+}\) ions in catalysis of Na-dependent phosphorylation (25) and thus the question arose whether Fe2\(^{2+}\) ions bind to the Mg2\(^{2+}\) site and catalyze cleavage from this site. Fig. 3 depicts the cleavages at three concentrations of Fe2\(^{2+}\) (0, 1.5, and 15 \(\mu\)M added in addition to the 0.05 \(\mu\)M contaminant in the solutions), and Mg2\(^{2+}\) from 0 to 7 \(\mu\)M. As the Mg2\(^{2+}\) ion concentration was raised, the cleavages at ESE, near CSDK, and HFIH were amplified (Fig. 3A). Thus, Mg2\(^{2+}\) ions stabilized an E1 conformation, noticeable especially at 7 \(\mu\)M. However, quantification of amounts of the fragments, by scanning the immunoblots, do not reveal any systematic influence of Fe2\(^{2+}\) concentration on effects of Mg2\(^{2+}\) ions, as seen in the examples in Fig. 3B. Hence, Fe2\(^{2+}\) and Mg2\(^{2+}\) ions do not compete and must bind at different sites.

In Ref. 13, we speculated that histidine residues in the sequence HFIH near M3 are involved in Fe2\(^{2+}\) binding. To test this hypothesis, and also the site-specific mechanism, we compared cleavage of rat axolemma and rat kidney enzymes. Rat axolemma enzyme consists of about 65% \(\alpha3\), 25% \(\alpha2\), and only 10% \(\alpha1\) isoforms, while kidney enzyme is essentially all \(\alpha1\) isoform (26–28). In \(\alpha3\) and \(\alpha2\) isoforms, the second histidine is replaced by a glutamine, i.e., HFIQ. The experiment in Fig. 4A compared the time course of cleavage of axolemma and kidney enzymes. For axolemma enzyme, the cleavage near HFIH was largely absent and that at VNDS was less prominent, while the cleavage at ESE and near MVTGD were similar to those of the kidney enzyme. For axolemma, a small amount of the fragment with N terminus near CSDK also appeared, although this is less certain because the control itself contains a minor fragment with the same mobility. (The axolemma fragments cleaved near MVTGD and at VNDS have slightly lower mobility than equivalent fragments of kidney (41.8 versus 39.1 and 29 versus 27 kDa, respectively). However, this cannot be taken to indicate that the cleavage sites are different, since intact \(\alpha3\) and \(\alpha2\) also have a slightly lower mobility than the \(\alpha1\) isoform, even though the \(M_r\) value of rat \(\alpha1\) (112, 566) is slightly higher than \(\alpha2\) (111, 580) or \(\alpha3\) (111, 735) (27, 29)). Fig. 4B depicts cleavage of axolemma enzyme in Rb\(^{-}\)- or Na\(^{-}\)-containing media at different concentrations of added Fe2\(^{2+}\) ions. The two major fragments, with N termini ESE and near MVTGD, increased in amount as Fe2\(^{2+}\) was raised progressively to 10 \(\mu\)M, as did the minor fragments. In parallel experiments with axolemma and kidney enzyme (data not shown), it was found that cleavage of axolemma enzyme required significantly higher concentrations of added Fe2\(^{2+}\) ions. For example, the \(K_{0.5}\) values for appearance of the fragment with N terminus near MVTGD were 1 \(\pm\) 0.2 \(\mu\)M for kidney and 1.95 \(\pm\) 0.2 \(\mu\)M for axolemma, respectively. In the sodium-containing medium, cleavage of both the axolemma and kidney enzymes was suppressed and the fragment with N terminus ESE and the fragment near MVTGD were not observed. In summary, axolemma and kidney enzymes differ in specificity of the cleavages and affinity for Fe2\(^{2+}\) ions, whereas the \(E_2(Rb) \rightarrow E_3\) transition affects cleavage essentially similarly.

Effects of Phosphorylation, Inorganic Phosphate, Vanadate, and Ouabain—Fig. 5 depicts iron-catalyzed cleavage of the pig kidney Na,K-ATPase in conditions of Na-dependent phosphorylation from ATP. A low concentration of ATP (5 \(\mu\)M) was used together with a regenerating system. In the presence of 140 mM Na\(^{+}\) ions, 0.5 mM Mg2\(^{2+}\) ions, and the regenerating system, we observed cleavages typical for the \(E_1\) form (N termini near M1 and HFIH). Upon addition of ATP and oligomycin, the pump should be phosphorylated and, due to block of the \(E_1\) conformational transition (30), the predominant form should be \(E_2\). This condition indeed produced cleavages typical of an \(E_1\) form. In the absence of oligomycin, the combination of ATP/Na\(^{+}\)/Mg2\(^{2+}\) phosphorylates the pump, but the predominant form should now be \(E_2\). In this condition, we observed two additional cleavages typical of an \(E_2\) form (N termini ESE and VNDS), but, strikingly, the prominent cleavage near MVTGD normally seen for unphosphorylated \(E_2\) or \(E_4(K)\) forms did not appear. The cleavage near CSDK was not seen, but this was expected in the high ionic strength medium.

Addition of a low concentration of Rb\(^{+}\) (2 mM) to the medium containing ATP/Na\(^{+}\)/Mg2\(^{2+}\) accelerated dephosphorylation from high affinity extracellular sites, leading to \(E_2(Rb)\) as the predominant form. In this condition, the fragments with N termini ESE and VNDS were somewhat amplified, but again the cleavage near MVTGD did not appear. The latter result was surprising because the cleavage near MVTGD is very prominent in the \(E_2(Rb)\) form generated directly by adding Rb\(^{+}\) (K\(^{+}\)) ions to the enzyme (see Ref. 13 and this paper). The cleavage pattern in the medium containing 2 mM Rb\(^{+}\), 150 mM Na\(^{+}\), and 0.5 mM Mg2\(^{2+}\), without ATP, shows that the enzyme remained in the \(E_1\) form. Thus, suppression of the cleavage near MVTGD in the presence of Rb\(^{+}\) ions and ATP/Na\(^{+}\)/Mg2\(^{2+}\) was associated with formation of the \(E_2(Rb)\) form via dephosphorylation of \(E_2\). It seemed possible that ATP itself or the product of its hydrolysis, ADP and P\(_i\), were responsible for suppressing the cleavage near MVTGD, and thus a number of control experiments were performed. In low ionic strength media (E9), the cleavage near MVTGD was prominent, but neither ATP nor ADP at 5 \(\mu\)M had any effect (data not shown).

We calculated that, during incubation with ATP/Mg2\(^{2+}\)/Na\(^{+}\)/Rb\(^{+}\) in Fig. 5, 200–250 \(\mu\)M P\(_i\) could accumulate. Therefore, we looked for effects on cleavages of P(Tris), 0.1–1 mM, without or with Mg2\(^{2+}\) and Rb\(^{+}\) ions (Fig. 6). Addition of P\(_i\) to a medium of 39.1 and 29 versus 27 kDa, respectively). However, this cannot be taken to indicate that the cleavage sites are different, since intact \(\alpha3\) and \(\alpha2\) also have a slightly lower mobility than the \(\alpha1\) isoform, even though the \(M_r\) value of rat \(\alpha1\) (112, 566) is slightly higher than \(\alpha2\) (111, 580) or \(\alpha3\) (111, 735) (27, 29).
low ionic strength indeed suppressed the cleavages near CSDK and MVTGD, without significantly affecting other cleavages (Fig. 6A). In this condition, one could expect the enzyme to be in an $E_2$ form, with Pi bound non-covalently. In combination with 1 mM Mg$^{2+}$ ions, addition of 2 mM Pi also partially suppressed the cleavages at ESE and VNDS (Fig. 6B, see legend for quantification based on scans). When added alone, Mg$^{2+}$ ions at 1 mM or lower concentrations had little or no effect (see also Fig. 3). The presence of 1 mM Rb$^+$ ions did not alter the effect of Pi but prevented partial suppression of cleavages by the combination of Pi and Mg$^{2+}$ ions (Fig. 6C, see legend). Another effect of the combination of Pi and Mg$^{2+}$ ions was observed in experiments that examined Pi concentration dependence of suppression of the cleavage near MVTGD at 0–1 mM Mg$^{2+}$. Data from scans of gels (Fig. 7) show that lower concentrations of Pi were required in the presence of Mg$^{2+}$ ions. In the presence of 1 mM Mg$^{2+}$ and 2 mM Pi, a substantial fraction of the enzyme should be phosphorylated as a form referred to as $E_2$-P, which is insensitive to Rb$^+$ ions (31). In the presence of Mg$^{2+}$, Pi, and Rb$^+$, a major fraction should not be phosphorylated ($E_2$(Pi)-P) and a minor fraction could be phosphorylated in a Rb$^+$-bound form ($E_2$-P-Rb) (31). An economical explanation of the results in Figs. 5–7 is that either non-covalently bound phosphate or covalently bound phosphate, derived from ATP or Pi, directly interfere with the cleavages near CSDK and MVTGD. In addition, the cleavages at ESE and VNDS are somewhat suppressed...
iron-catalyzed cleavage of the α subunit. Na,K-ATPase (1 mg/ml) was suspended in a medium containing 5 mM Tris-HCl, pH 7.2, and 0, 0.1, or 1 mM Pi(Tris) (A) or 1 mM MgCl₂ and 2 mM Pi as indicated in B or 1 mM MgCl₂, 2 mM Pi, and 1 mM RbCl as indicated in C, and incubated with 5 μM FeSO₄ and 5 mM ascorbate/H₂O₂ for 2 min at 20 °C. Amounts of fragments in arbitrary units based on scans of immunoblots: ESE: control, 7.2; Mg, 7.3; Pi, 7.1; Pi/Mg, 5.9; Rb, 6.3; Pi/Mg, 6.2.

The paradoxical results just presented led us to inquire whether the expected conformations were indeed being stabilized in the different conditions. Well characterized chymotryptic cleavages (32) showed that this was the case (Fig. 10). Thus, chymotryptic digestion in the Na⁺-containing medium (E₃Na) or in a medium containing 7 mM Mg²⁺ ions (E₃Mg) produced the prominent fragment “a,” 74.6 kDa, while in the Rb⁻-containing medium (E₃Rb)” “a” was suppressed and “b” and “c” were more prominent. Again, as expected in the medium containing ouabain/Mg/Pi (E₃P-ouabain), “b” and “c” were major and “a” was minor. An incidental benefit was that defined chymotryptic fragments allowed better determination of the positions of two iron-catalyzed cleavage fragments, which could not be sequenced due to blocked N termini. The N terminus of fragment “a” is Ala²⁶⁷ (33), and sequencing of fragments “b” and “c” showed that the N termini are Val⁴⁰¹ and Ala⁵⁰¹, respectively. The apparent Mr values of “a” and “c” 74.6 and 39.3 kDa are 1.2 and 1.1 kDa greater than those of the closest iron-catalyzed cleavage fragments, 73.4 and 38.2 kDa respectively. Therefore, the N termini of the latter are approximately 10 residues downstream, i.e., they are near 2⁶⁷HFII and 6⁶⁸MVTGD.

### DISCUSSION

Fig. 11 depicts schematically the proposed arrangement of the peptide sequences around the bound Fe³⁺ ion in different states. Table I summarizes the specificity of the different cleavages and also gives a rough measure of their prominence, which reflects presumably proximity to bound Fe³⁺ ions.

**The Fe³⁺ Binding Site**—Previously, we proposed the site-specific mechanism in which each α subunit is cleaved at only one of the different points of contact between the polypeptide chain and a bound Fe³⁺ ion, as depicted in Fig. 11 (13, 14). The additional observations discussed here support this notion and essentially exclude the possibility that cleavages are catalyzed...
by Fe$^{2+}$ bound at several sites. One might consider a hypothesis that cleavages occur at two Fe$^{2+}$ sites, one of which includes the points near M1 and HFIH and does not change in $E_1$ and $E_2$ forms, while the other includes the points at ES, near CSDK, near MVTGD and at VNDS and exists only in $E_2$ forms.

Although it is difficult to rigorously exclude this idea, the following arguments favor one Fe$^{2+}$ site.

1) Parallel suppression of cleavages at ES, near MVTGD and at VNDS and amplification of cleavages near M1 and HFIH, upon transition from $E_2(K)$ to $E_1Na$ (Fig. 1) or other $E_1$ forms (Fig. 3), is explained most simply by assuming that the contacts at ES, near MVTGD and at VNDS move away while those near M1 and HFIH remain near the bound Fe$^{2+}$ ion. Accordingly, the probability of cleaving either decreases or increases respectively.

2) In Fig. 2, the curves fit well to simple hyperbolae with higher apparent affinities for inactivation or cleavage in a Rb$^+$-containing as opposed to a Na$^+$-containing medium ($K_{0.5}$ of 0.2 or 0.57 versus 1.16 or 3.49 $\mu$M, respectively), consistent with Fe$^{2+}$ binding to a single site. Apparent free energies of Fe$^{2+}$ binding, calculated from the $K_{0.5}$ for inactivation or cleavage, in Rb$^+$- compared with Na$^+$-containing media, are $-9.02$ or $-8.43$ versus $-8.01$ or $-7.36$ kcal/mol, respectively, giving a difference of about $-1$ kcal/mol by either measure. Assuming that the different cleavages between the $E_2(Rb)$ and $E_1Na$ conformations are catalyzed at a separate Fe$^{2+}$ site, the $K_{0.5}$ for Fe$^{2+}$ at this site would correspond to the $-1$ kcal/mol or 0.18 M, i.e. an unrealistic value that argues strongly against the idea of two sites. In contrast, the data imply that most of the binding energy for Fe$^{2+}$ comes from residues that bind in either $E_2$ or $E_1$ forms (e.g. HFIH), while the extra contacts in $E_2$ forms are energetically weak. 2

3) Lack of competition between Mg$^{2+}$ and Fe$^{2+}$ ions (Fig. 3) shows that Mg$^{2+}$ and Fe$^{2+}$ bind at different sites. Other data discussed below (see also Fig. 11) suggest that, in the presence of phosphate, Mg$^{2+}$ ions can interact with the protein near the cleavage sites at ES and VNDS. These observations also favor the notion of one Fe$^{2+}$ site rather than two separate sites, neither of which recognize Mg$^{2+}$ ions.

4) Although there are significant differences in sequences of the rat a3, a2, and a1 isoforms (85–86% identity; Ref. 29), it is likely that the different cleavage patterns of axolemma (mainly a3 and a2) and kidney (a1) enzyme (Fig. 3) are attributable to the His$^{286}$Gln substitution. First, the cleavage near HFIH is essentially absent in axolemma enzyme. Second, the fact that cleavages, at ES and near MVTGD, occur in both axolemma and kidney enzymes and undergo a similar response to the $E_2(Rb) \rightarrow E_1$ transition (Fig. 4B) indicates that spatial organi-

If a fraction of added Fe$^{2+}$ is bound to the membranes, ascorbate, etc., the true binding affinity of Fe$^{2+}$ will be higher than the calculated affinity, but the relative difference in Rb$^+$ versus Na$^+$-containing media will remain. This possibility can only strengthen the argument against two Fe$^{2+}$ sites.

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**Fig. 10.** Chymotryptic digestion of Na,K-ATPase. Na,K-ATPase (1 mg/ml) was suspended in a medium containing 5 mM Tris-HCl, pH 7.2, 20 mM NaCl, or 20 mM RbCl or 7 mM MgCl$_2$ (A) or 1 mM P, 1 mM MgCl$_2$, and 1 mM ouabain (B). α-Chymotrypsin was added at a ratio of 1:20 (w/w) with respect to Na,K-ATPase and incubated together at 37 °C for 10 min. 150 mM RbCl was added and then 1 mM PMSF, and the mixture was incubated for 15 min at room temperature before centrifugation to remove the chymotrypsin, and solubilization of the pellet in the gel buffer. In A, 25 µg of protein were applied per lane (diaminobenzidine stain), and, in B, 5 µg were applied per lane (ECL).

**Fig. 11.** Schematic drawings of interactions between bound Fe$^{2+}$ ions and the Na,K-ATPase in different conformational states.
zation of the α3 and α1 isoforms is similar. Thus, reduced cleavage at VNDS in addition to that at HFIH suggests that the Gln for His substitution alters the disposition of both VNDS and HFIH next to the bound Fe2⁺ ions and weakens Fe2⁺ binding. Conversely, reduced cleavage at VNDS catalyzed at a separate Fe2⁺ site is a less likely possibility since the sequences of α1, α2, and α3 isoforms are identical over a long stretch either side of VNDS. Unequivocal evidence for involvement of histidine residues will require site-directed mutagenesis.

5) Treatment of renal Na,K-ATPase with the histidine-specific reagent diethylpyrocarbonate prevents all iron-catalyzed cleavages, clearly implicating histidine residues.³ Histidines are found only at the cleavage site near HFIH and MVTGD.

Effects of Phosphorylation, Inorganic Phosphate, Vanadate, and Ouabain—Two salient features in Fig. 5 are as follows: 1) in E₂P (ATP/Na⁺/Mg²⁺/oligomycin), the cleavage pattern is typical for E₂; 2) in E₂P (ATP/Na⁺/Mg²⁺/Pi), two cleavages typical of E₂ or E₂(Rb) appear (at ESE and VNDS), if less prominently than in the non-phosphorylated form (ATP/Na⁺/Mg²⁺/Rb⁺), but the major cleavage near MVTGD is not observed. Fig. 6 clarifies the difference between the phosphorylated and unphosphorylated E₂ forms by showing the following: 1) Non-covalent binding of Pi in E₂P selectively suppresses the cleavages near CSDK and MVTGD (Fig. 6A). An equivalent form is achieved in the presence of P/Mg²⁺/Rb⁺ (Fig. 6C), or ATP/Na⁺/Mg²⁺/Rb⁺ due to ATP hydrolysis (Fig. 5). 2) In E₂⁻P (Pi, Mg²⁺), the pattern is the same as in E₂⁻P (Fig. 6B). E₂⁻P and E₂⁻P represent different subconformations, as indicated by different responses to alkali cations, methyl hydroxylamine, and vanadate (31, 34, 35). Since their iron-cleavage pattern is the same, presumably the difference between E₂⁻P and E₂⁻P is restricted to the microenvironment of the bound phosphate. On the basis of Figs. 5 and 6, we propose that either non-covalently bound (E₂⁻P shown in Fig. 11) or covalently bound phosphate (E₂⁻P, E₂⁻P) interacts directly with residues near CSDK and MVTGD, hindering access of the bound Fe²⁺ and cleavage.

Mg²⁺ ions are tightly bound to the phosphoenzyme formed from either ATP (E₂⁻P) or P₄ (E₂⁻P) (31, 35). In order to explain the less prominent cleavages at ESE and VNDS in the E₂⁻P complex compared with non-phosphorylated forms (seen in both Figs. 5 and 6), and the synergism between P₄ and Mg²⁺ ions in suppressing cleavage near MVTGD (Fig. 7), we propose that Mg²⁺ ions are bound near ESE and VNDS and also to the bound phosphate. In this way, access of the Fe²⁺ to the sites at ESE and VNDS may be reduced. In the presence of P₄, Mg²⁺ ions, and ouabain (Fig. 8), ouabain may induce the Mg²⁺ ions in the E₂⁻P⁻Mg complex to bind to its contact residues more tightly or in such a way as to completely prevent access of the bound Fe²⁺ ions and cleavage at ESE, near CSDK and MVTGD, and at VNDS (Fig. 11). Similarly vanadate may bind near CSDK and MVTGD with Mg²⁺ ions bound near ESE and VNDS and to the vanadate itself, suppressing cleavages at ESE, near CSDK and MVTGD, and at VNDS (Fig. 11). Thus, the paradox that Pi/Mg²⁺/ouabain or vanadate/Mg²⁺ give E₂⁻like cleavage patterns, although these ligands stabilize E₂⁻ forms, is explained by assuming that the bound ligands directly hinder access of the bound Fe²⁺ ions to the cleavage sites. Finally, the paradox (Fig. 9) that preincubation of P₄ with Fe²⁺ leads to a pattern similar to that with P₄/Mg²⁺/ouabain or vanadate/Mg²⁺ may imply that, Fe²⁺ ions slowly gain access to

³ D. Tal, J. Capasso, and S. J. D. Karlsh, manuscript in preparation.

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**Table I**

Fragments observed in different conformational states

| Condition | Conformation | Fragments | Location |
|-----------|--------------|-----------|----------|
| Na (high µ) | E₁Na | nrM1 | nrHFIH | Fig. 1 |
| Mg (~7 mM) | E₁Mg | nrM1 | nrHFIH | Fig. 3 |
| High µ | E₁ | nrM1 | nrHFIH | Not shown |
| Low µ or Mg (<1 mM) | E₂ | nrM1 | ESE | nrHFIH | Fig. 6 |
| K, Rb, Cs, Tl (high µ) | E₂(X)acc | nrM1 | ESE | nrHFIH | Fig. 1 |
| P (low µ) | E₂P₁ | nrM1 | ESE | nrHFIH | Fig. 6 |
| P/Mg (<1 mM) (low µ) | E₂⁻P⁻Mg | nrM1 | ESE | nrHFIH | Fig. 6 |
| Na/Mg/ATP/oligomycin (high µ) | E₂⁻P | nrM1 | ESE | nrHFIH | Fig. 5 |
| Na/Mg/ATP (high µ) | E₂⁻P | nrM1 | ESE | nrHFIH | Fig. 5 |
| P/Mg (<1 mM) ouabain (low µ) | E₂⁻P⁻Mg/ouabain | nrM1 | nrHFIH | Fig. 8 |
| Vanadate/Mg (low µ) | E₂⁻V⁻Mg | nrM1 | nrHFIH | Fig. 8 |
| P/Fe (low µ) | E₂⁻P⁻Fe | nrM1 | nrHFIH | Fig. 9 |
normal Mg\(^{2+}\) binding sites (see Ref. 25) to produce a complex with the P\(_i\). In this complex with P\(_i\), the Fe\(^{2+}\) is itself redox-inactive, but access of the redox active Fe\(^{2+}\) bound near HFIH (Fig. 11) to the cleavage sites is hindered.

Although our findings do not identify precisely co-ordinating residues for Mg\(^{2+}\) ions and phosphate, some reasonable suggestions can be made. Based on a structural analogy to EF hand proteins and cation binding to synthetic peptides, it was proposed that Mg\(^{2+}\) ions are co-ordinated with Asp\(^{707}\) in the sequence EITAMTGDVN\(^{707}\)DSPALKK of Ca-ATPase. (37). Studies of O\(^{18}\) exchange between P\(_i\) and water after mutagenesis of Asp\(^{966}\) of Na,K-ATPase, and sequence and structural homologies with adenylate kinase, suggest that conserved sequences DPPRR and \(^{608}\)MVTGDHPITAK may be involved in co-ordination of Mg\(^{2+}\) ions and the \(\gamma\)-phosphate group of ATP (38, 39). Proximity of the \(\gamma\)-phosphate of ATP to the sequence VNDs can be inferred from covalent labeling at Asp\(^{710}\) (40) and at Lys\(^{719}\) (41). Mg\(^{2+}\) ions could be co-ordinated to several residues, such as Asp\(^{714}\) and His\(^{211}\) at VNDs and ESE as depicted in Fig. 11. Non-covalently bound phosphate could interact with Lys\(^{370}\) in CSDK, and both non-covalently and covalently bound phosphate could also interact with Lys\(^{618}\) near MVTGd (or Arg\(^{659}\) in the DPPR sequence).

The effect of \(\text{P}\) alone (Fig. 6) and synergism between \(\text{P}\) and Mg\(^{2+}\) (Fig. 7) indicate that P\(_i\) and Mg\(^{2+}\) bind in an unordered but positively co-operative fashion (see also Refs. 36 and 42). Our findings do not agree with a proposal that P\(_i\) binds after Mg\(^{2+}\) ions (38).

**Implications for the Energy Transduction Mechanism**—By comparison with our initial study of iron-catalyzed cleavages, several additional conclusions emerge.

1) Characterization of \(E_1\) and \(E_1\) forms by formation or relaxation of interactions between minor and major cytoplasmic loops can be modified slightly to include a role of the N-terminal cytoplasmic segment. Parallel behavior of the cleavages near M1 and M3 (HFIH) implies proximity between M1 and M3 and interactions between the segment leading into M1 and loop between M2 and M3 (probably via salt bridges; Ref. 33). Proteolytic cleavage or truncated mutants in the N-terminal segment shift the conformational equilibrium toward \(E_1\) (33, 43), as do proteolytic cleavages or mutations in the loop between M2 an M3 (Refs. 32 and 44; see Ref. 5 for references to other P-type pumps). Recently, an interaction between the cytoplasmic tail and the loop between M2 and M3 and possibly with the large cytoplasmic loop (45) has been inferred on the basis of strong synergism in the functional effects of a double mutant (with a truncation at residue 32 (a1M32) and also G233K or G233Q mutation, referred to as a1M32G233KQ). Our results fit this interpretation assuming that the N-terminal segment together with the minor loop constitute one domain and the major loop a second domain.

2) Formation or relaxation of the domain interactions in \(E_1\) and \(E_2\) Na forms are paralleled in the phosphorylated conformations \(E_1\) and \(E_2\) and, we suggest, constitute an essential element of energy transduction. These interactions seem to occur within or near the active site, so explaining interference with cleavages of non-covalently or covalently bound phosphate or vanadate (Fig. 11). A change of microenvironment of bound phosphate from hydrophilic in high energy \(E_1\) to hydrophobic in low energy \(E_1\) has been proposed based on effects of organic solvents (46). The aqueous or less aqueous microenvironment of bound phosphate in \(E_1\) or \(E_2\) could be explained by the open or closed domain structure, respectively. A Mg\(^{2+}\) ion is tightly bound in \(E_1\) and it can be proposed that tight co-ordination of the Mg\(^{2+}\) ion near TGES and VNDS and to the covalently bound phosphate induces the interactions between the minor and major loops which underlies the \(E_1 \rightarrow E_2\) transition. In \(E_2\), the bound phosphate (at CSDK and MVTGd) and the interacting sequences of the minor and major loops (TGES and VNDS) must lie fairly close to the membrane in order to explain proximity to HFIH near the entrance to M3. Accordingly, the high affinity ATP site in \(E_1\) should be organized such that the \(\gamma\)-phosphate of ATP interacts near CSDK and MVTGd, while the purine binding residues (such as Lys\(^{501}\) and \(\text{Gly}\)^{502}) (47, 48) and Lys\(^{480}\) (49) are located toward the periphery of the major cytoplasmic loop.

3) Cation transport involves deocclusion of Na\(^{+}\) or K\(^{+}\) ions accompanying \(E_1\) \(\rightarrow E_2\) and \(E_1\) \(\rightarrow E_2\), respectively, and requires opening or closing of “gates,” which act as barriers to dissociation of cations. “Gates” could be formed by interacting residues of adjacent trans-membrane segments. In \(E_1\) and \(E_2\), “gates” are closed at both surfaces. In \(E_1\), an extracellular “gate” is open and a cytoplasmic “gate” is closed, while in \(E_2\) the cytoplasmic “gate” is open and an extracellular “gate” is closed. We propose that formation or relaxation of interactions between cytoplasmic domains alters the twist or tilt or perhaps stretch of the relevant trans-membrane helices and so alters their mutual interactions, i.e. the “gates.” Prime candidates as mobile trans-membrane segments are M4, M5, and M6, which contain residues involved in cation occlusion (7–9). Movement of M5, particularly Asn\(^{776}\), Ser\(^{775}\), Thr\(^{774}\) and M6, which contain residues involved in cation occlusion is itself redox-sensitive, and so alters their mutual interactions, i.e. the “gates.” Prime candidates as mobile trans-membrane segments are M4, M5, and M6, which contain residues involved in cation occlusion (7–9). Movement of M5, particularly Asn\(^{776}\), Ser\(^{775}\), Thr\(^{774}\) and M6, which contain residues involved in cation occlusion is itself redox-sensitive, and so alters their mutual interactions, i.e. the “gates.” Prime candidates as mobile trans-membrane segments are M4, M5, and M6, which contain residues involved in cation occlusion (7–9). Movement of M5, particularly Asn\(^{776}\), Ser\(^{775}\), Thr\(^{774}\) and M6, which contain residues involved in cation occlusion.

**Conclusion**—Because the sequences involved in cytoplasmic domain interactions are highly conserved, the mechanism of energy coupling proposed here should apply to all P-type pumps. In this respect, it is remarkable that recent cryoelectron microscope images of Ca-ATPase indeed reveal a compact cytoplasmic domain in the \(E_1\) form but an open structure in the \(E_2\) form, with distinct lobes separated by a deep gorge (54).

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**REFERENCES**

1. Glynn, I. M., and Karlish, S. J. D. (1990) Annu. Rev. Biochem. 59, 171–205
2. Maunsbach, A. B., Skriver, E., and Hebert, H. (1991) Soc. Gen. Physiol. U. S. A. 87, 4566–4570
3. Zhang, P., Toyoshima, C., Yonekura, K., Green, N. M., and Stokes, D. L. (1998) Nature 392, 835–839
4. Auer, M., Scarbrough, G. A., and Kuhlbrandt W. (1998) Nature 392, 840–843
5. Møller, J. V., Juul, B., and Le Maire, M. (1996) Biochim. Biophys. Acta 1266, 1–51
6. Karlish, S. J. D., Goldshlegger, R., and Stein, W. D. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 101–106
7. Jewell-Motz, E. A., and Lingrel, J. B. (1993) Science 260, 13523–13530
8. Andersen, J. P., and Vilsen, B. (1995) FEBS Lett. 359, 101–106
9. Nielsen, J. M., Pedersen, P. A., Karlish, S. J. D., and Jørgensen, P. L. (1998) Biochemistry 37, 1961–1968
10. Robinson, J. D., and Pratap, P. R. (1993) Biochim. Biophys. Acta 1154, 83–104
11. Green, N. M., and Stokes, D. L. (1992) Acta Physiol. Scand. 152, 59–68
12. Harris, S. L., Perlin, D. S., Seto-Young, D., and Haber, J. E. (1991) J. Biol.
Iron-catalyzed Cleavage of Na,K-ATPase