

# Fe-catalyzed cleavage of the $\alpha$ subunit of Na/K-ATPase: Evidence for conformation-sensitive interactions between cytoplasmic domains

(Fe ions/energy transduction mechanism/Na/K-pump)

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**ABSTRACT** Incubation of Na/K-ATPase with ascorbate plus H<sub>2</sub>O<sub>2</sub> produces specific cleavage of the  $\alpha$  subunit. Five fragments with intact C termini and complementary fragments with intact N termini were observed. The  $\beta$  subunit is not cleaved. Cleavages depend on the presence of contaminant or added Fe<sup>2+</sup> ions, as inferred by suppression of cleavages with nonspecific metal complexants (histidine, EDTA, phenanthroline) or the Fe<sup>3+</sup>-specific complexant desferrioxamine, or acceleration of cleavages by addition of low concentrations of Fe<sup>2+</sup> but not of other heavy metal ions. Na/K-ATPase is inactivated in addition to cleavage, and both effects are insensitive to OH<sup>•</sup> radical scavengers. Cleavages are sensitive to conformation. In low ionic strength media (E<sub>2</sub>) or media containing Rb ions [E<sub>2</sub>(Rb)], cleavage is much faster than in high ionic strength media (E<sub>1</sub>) or media containing Na ions (E<sub>1</sub>Na). N-terminal fragments and two C-terminal fragments (N-terminals E<sup>214</sup> and V<sup>712</sup>) have been identified by amino acid sequencing. Approximate positions of other cleavages were determined with specific antibodies. The results suggest that Fe<sup>2+</sup> (or Fe<sup>3+</sup>) ions bind with high affinity at the cytoplasmic surface and catalyze cleavages of peptide bonds close to the Fe<sup>2+</sup> (or Fe<sup>3+</sup>) ion. Thus, cleavage patterns can provide information on spatial organization of the polypeptide chain. We propose that highly conserved regions of the  $\alpha$  subunit, within the minor and major cytoplasmic loops, interact in the E<sub>2</sub> or E<sub>2</sub>(Rb) conformations but move apart in the E<sub>1</sub> or E<sub>1</sub>Na conformations. We discuss implications of domain interactions for the energy transduction mechanism. Fe-catalyzed cleavages may be applicable to other P-type pumps or membrane proteins.

Recent studies of structure-function relations of P-type cation pumps, such as Na/K-, H/K-, and Ca-ATPases, have focused on identification of residues of the catalytic subunits involved in cation or ATP binding or on their topological organization, comprising 10 transmembrane segments (M1-M10) (1–3). Cation occlusion sites are located within trans-membrane segments, and site-directed mutagenesis results suggest that side chains within M4, M5, M6, and M8 ligate cations (2, 4–6). The ATP binding site is located within the major cytoplasmic loop, and probable ligating residues are being identified by chemical modification or mutagenesis (1). By comparison with progress in these areas, our understanding of the energy transduction mechanism lags behind. Earlier studies provided a wealth of information on kinetics of cation transport, phosphorylation-dephosphorylation, transport-linked E<sub>1</sub>-E<sub>2</sub> conformational transitions, and cation occlusion (7, 8). Transmission of force between ATP and cation binding sites and their

structural reorganization in E<sub>1</sub> and E<sub>2</sub> conformations must underlie the energy transduction process. However, the structural basis of these events is poorly understood. Some evidence has been obtained using proteolysis or site-directed mutations. Specific proteolytic cleavages of Na/K- or Ca-ATPase in the first cytoplasmic loop between trans-membrane segments M2 and M3, characteristically inhibit ATPase activity and stabilize E<sub>1</sub> conformations (9–11). In addition, there are several highly conserved sequences in the cytoplasmic domains of all P-type pumps, including TGES in the loop between M2 and M3 and MVTGD and TGDGVNDSPALKK in the major cytoplasmic loop before M5. Mutations in these sequences, in Ca- or H-ATPase, inhibit activity and stabilize E<sub>1</sub> forms (and in some cases also inhibit phosphorylation) (2, 12–14). Thus, the conserved sequences serve an important function, perhaps mediating conformational interactions between ATP and cation sites. However, their specific roles are unknown.

This paper describes metal-catalyzed cleavage for study of structure-function relations and conformational transitions of Na/K-ATPase. The use of complexed transition metals, such as EDTA-Fe or phenanthroline-Cu, for selective oxidative cleavage of nucleic acids is well established (15). Although specific metal-catalyzed cleavage of proteins is less characterized, there are several examples of applications to soluble enzymes using complexed or free Fe<sup>2+</sup> and Cu<sup>2+</sup> ions, directed or naturally bound to specific sites (16–20). The term “affinity cleavage reagent” describes substrates or other molecules that complex the heavy metals and direct them to sites where cleavage occurs (16). In principle, analysis of the cleavage pattern should provide spatial information on peptide bonds in proximity to the metal. Applications to membrane proteins include identification of proximal trans-membrane segments of lac permease detected by cleavages dependent on Cu-phenanthroline targeted chemically to engineered cysteines (21) and proximal residues on neighboring subunits of cytochrome *bd* quinol oxidase using Fe-chelate-dependent cleavages (22).

We describe here a chance discovery that incubation of renal Na/K-ATPase with ascorbate and H<sub>2</sub>O<sub>2</sub> causes selective cleavages of the  $\alpha$  subunit. We demonstrate that cleavages depend on the presence of contaminating Fe<sup>2+</sup> ions in the media and describe properties of the Fe-catalyzed cleavages.

## MATERIALS AND METHODS

**Materials.** For SDS/PAGE, all reagents were electrophoresis-grade from Bio-Rad. Tris (ultra pure) was from Bio-Lab, Jerusalem. L(+)-ascorbic acid and 30% H<sub>2</sub>O<sub>2</sub> were from Merck. All other reagents were of analytical grade.

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**Enzyme Preparation.** Na/K-ATPase (13–18 units/mg protein) was prepared from pig kidneys, assayed, and stored at  $-20^{\circ}\text{C}$  in a solution of 250 mM sucrose, 25 mM histidine (pH 7.4), and 1 mM EDTA [as described by Jørgensen (23)]. Before use, membranes were washed twice and suspended in a solution containing 10 mM Tris-HCl, pH 7.4.

**Cleavage Reaction.** Membrane suspensions (0.1–1 mg/ml), with or without added 30 mM RbCl or NaCl, were incubated at  $20^{\circ}\text{C}$  with freshly prepared solutions of 4 mM ascorbate (Tris) plus 4 mM  $\text{H}_2\text{O}_2$ , without or with added  $\text{FeSO}_4$  or other metals, in a total volume of 30–40  $\mu\text{l}$ . To arrest the reaction, 10  $\mu\text{l}$  of 5 mM EDTA or 5-fold concentrated gel sample buffer with 5 mM EDTA was added, and samples were assayed for Na/K-ATPase activity or applied to gels, respectively.

**Gel Electrophoresis, Blotting to Polyvinylidene Difluoride, Immunoblots, Sequencing.** Procedures for running of 10% Tricine (*N*-[tris(hydroxymethyl)glycine] SDS/PAGE, including precautions before sequencing, electroblotting to polyvinylidene difluoride paper, immunoblots, and microsequencing of fragments, have been described in detail (3, 24). In some cases (see Fig. 5), the enzyme (1 mg/ml) was first mixed with 3 mg/ml of the nonionic detergent  $\text{C}_{12}\text{E}_{10}$  (polyoxyethylene 10 lauryl ether), the undissolved protein was removed (50,000 rpm in a Beckman TLX-100 centrifuge), and soluble protein was precipitated with 4 vol of methanol/ether 2:1 and overnight storage at  $-20^{\circ}\text{C}$ . This procedure selectively extracts the pump subunits and their fragments (unpublished work). For quantification of the  $\alpha$  subunit, the band was cut out of the gel, the Coomassie stain was extracted by homogenization in 1% SDS solution, acrylamide was removed by centrifugation, and OD was measured at 595 nm.

**Antibodies.** Rabbit antisera, prepared as described by Grossman *et al.* (25), were raised against fragments of trypsinized Na/K-ATPase, “19-kDa membranes” (5, 24). Antisera included (i) “anti-M1/M2,” prepared from a 11.7-kDa fragment D<sup>68</sup>-R<sup>168</sup>, containing transmembrane segments M1 and M2 and (ii) “anti- $\beta$ ,” prepared from a 16-kDa fragment A<sup>5</sup>-R<sup>142</sup> of the  $\beta$  subunit. Bands were cut out of stained gels, extracted, and precipitated (26), and aliquots of  $\approx 200$   $\mu\text{g}$  were used per injection. Anti-peptide antibodies also were raised against the synthetic peptides L<sup>337</sup>-N<sup>348</sup> and I<sup>263</sup>-P<sup>276</sup>, coupled to keyhole limpet hemocyanin. The epitope of the latter antibody is known to be I<sup>263</sup>-L<sup>266</sup> (A. Shainskaya and S.J.D.K., unpublished work). In addition, the following anti-peptide antibodies, supplied by colleagues, were used to characterize  $\alpha$  subunit fragments: (i) anti-K<sup>1012</sup>-Y<sup>1016</sup>, referred to as “anti-KETYY,” (ii) anti-K<sup>347</sup>-E<sup>358</sup>, (iii) anti-I<sup>366</sup>-N<sup>377</sup>, (iv) anti-D<sup>636</sup>-R<sup>651</sup>, (v) anti-L<sup>815</sup>-Q<sup>828</sup>, and (vi) anti-N<sup>889</sup>-Q<sup>903</sup>.

**Heavy Metals.** Induced coupled plasma atomic emission spectroscopy was performed to determine heavy metal composition of the media (Spectrolabs, Rehovot, Israel). Concentrated solutions of ascorbic acid, Tris-HCl (pH 7.4), and  $\text{H}_2\text{O}_2$  were analyzed, and the contents of the reaction media were then calculated.

**Calculations.** Nonlinear curve-fitting was performed using Enzfitter (Elsevier-Biosoft, Cambridge, U.K.).

## RESULTS

Fig. 1 presents the basic finding that incubation of renal Na/K-ATPase with ascorbate plus  $\text{H}_2\text{O}_2$  produces selective cleavage of the  $\alpha$  subunit but does not cleave the  $\beta$  subunit. Intact  $\alpha$  subunit and its fragments were visualized in immunoblots to detect intact C-terminal residues (anti-KETYY) or the N-terminal region (anti-M1/M2) (see *Materials and Methods*). Five principal fragments with intact C-terminals and five fragments with intact N-terminal fragments were observed. Fragments are numbered as in Table 1, which summarizes apparent molecular mass values (range 22.6–81.8 kDa), antibody binding, and amino-acid sequences. Based on their

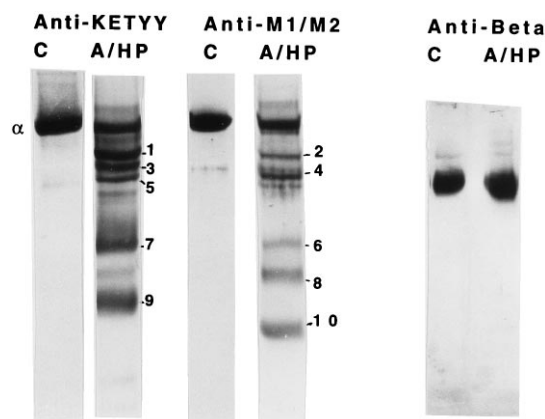


FIG. 1. Selective cleavage of the  $\alpha$  subunit induced by incubation with ascorbate and  $\text{H}_2\text{O}_2$ . Enzyme, 1 mg/ml, was incubated for 60 min in the standard conditions (without added Rb or  $\text{FeSO}_4$ , see *Materials and Methods*). C, control; A, ascorbate; and HP,  $\text{H}_2\text{O}_2$ . The figure presents immunoblots using anti-KETYY, anti-M1/M2, or anti- $\beta$  (see *Materials and Methods*).

apparent lengths, the following pairs of fragments appear to be complementary: 10 and 1, 8 and 3, 6 and 5, 4 and 7, and 2 and 9 (for supporting evidence see Table 1).

Both ascorbate and  $\text{H}_2\text{O}_2$  were required for efficient specific cleavage. Ascorbate alone was slightly effective whereas  $\text{H}_2\text{O}_2$  alone was ineffective. This finding hinted at possible involvement of transition metal ions as catalysts of oxidation/reduction reactions although no metal ions were added. The experiments in Fig. 2 tested this hypothesis. In Fig. 2A various heavy metal chelators were added to the medium; 25 mM histidine (Hist) partially protected the  $\alpha$  subunit whereas 2 mM EDTA or 1 mM phenanthroline (Phen) completely prevented cleavage. The result indicated the requirement for a heavy metal, which is either tightly bound to the enzyme or is present as a contaminant in the medium. The following experiment showed that the first possibility is unlikely. We incubated the enzyme in a medium containing 4 mM ascorbate/ $\text{H}_2\text{O}_2$  and 30 mM EDTA(Tris) so as to release and chelate any bound ions, washed the enzyme twice in 10 mM Tris-HCl to remove EDTA, and then reincubated with ascorbate/ $\text{H}_2\text{O}_2$ . The enzyme was cleaved to the same extent as control enzyme (not shown). Analysis of heavy metal content of other components showed that a medium consisting of 4 mM ascorbic acid, 4 mM  $\text{H}_2\text{O}_2$  and 10 mM Tris-HCl contains: Fe, 47.5 nM; Ni, 14.4 nM; Cu, 8 nM; Mn, 3.3 nM; Cr, 1.12 nM; Mo, 1.1 nM; Zn, 0.9 nM; and Sn, 0.8 nM. One approach to identify the relevant contaminant metal ion has been to use metal-specific complexants. The  $\text{Fe}^{3+}$ -selective complexant desferrioxamine suppresses cleavage at 10–100  $\mu\text{M}$  (Fig. 2B) whereas neither a Cu-selective complexant, bicinchoninic acid, nor a Ni-specific complexant, dimethyl glyoxime, prevented cleavage at 100–1000  $\mu\text{M}$  (not shown). The result points to  $\text{Fe}^{2+}$  or  $\text{Fe}^{3+}$  ions, but it might be misleading because the complexant is in large molar excess over all contaminants and, because selectivity is not absolute, other metal ions might also be complexed. More conclusive evidence was obtained by looking at effects of added  $\text{Fe}^{2+}$  and other metals. The experiments used short incubation times (2 minutes) to minimize cleavage due to the contaminant  $\text{Fe}^{2+}$  ions. Fig. 2C shows that addition of 0.25–10  $\mu\text{M}$   $\text{Fe}^{2+}$  ions greatly amplifies the extent of cleavage. Note that the same five cleavages appear as in the absence of added  $\text{Fe}^{2+}$  ions and that the cleavages display a similar  $\text{Fe}^{2+}$  concentration dependence. Fig. 2D shows that, of the heavy metals ions  $\text{Fe}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Ni}^{2+}$ , or  $\text{Mn}^{2+}$  added at 5  $\mu\text{M}$ , only  $\text{Fe}^{2+}$  ions were effective.  $\text{Cr}^{3+}$ ,  $\text{Ru}^{3+}$ ,  $\text{Sn}^{2+}$ ,  $\text{Ag}^+$ ,  $\text{Pb}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Cd}^{2+}$ , and  $\text{Ti}^{3+}$  ions also were ineffective (not shown). Fig. 3 presents the  $\text{Fe}^{2+}$ -concentration dependence of cleavage of

Table 1. Fragments produced by Fe-catalyzed cleavage of the  $\alpha$  subunit

Fragment approximate molecular mass, kDa	Antibody binding	Sequence found	Position of cleavages
1. $81.8 \pm 2.5$ ( $n = 5$ )	Anti-KETYY	ESEPQTR	E <sup>214</sup> -Y <sup>1016</sup>
2. $79.8 \pm 2$ ( $n = 4$ )	Anti-M1/M2	GRDKYEP	G <sup>1</sup> -G <sup>711</sup>
3. $76 \pm 2.2$ ( $n = 4$ )	Anti-KETYY	Blocked N terminus	$\geq$ (I <sup>262</sup> -L <sup>266</sup> )-Y <sup>1016</sup>
4. $69.1$ ( $n = 1$ )	Anti-M1/M2	Not sequenced	G <sup>1</sup> - $\leq$ (D <sup>636</sup> -R <sup>651</sup> )
5. $68.8$ ( $n = 2$ )	Anti-KETYY	Not sequenced	$\geq$ (I <sup>366</sup> -N <sup>377</sup> )-Y <sup>1016</sup>
6. $41.5 \pm 0.3$ ( $n = 3$ )	Anti-M1/M2	GRDKYEP	G <sup>1</sup> - $\geq$ (I <sup>366</sup> -N <sup>377</sup> )
7. $39.1 \pm 1$ ( $n = 5$ )	Anti-KETYY	Blocked N terminus	$\leq$ (D <sup>636</sup> -R <sup>651</sup> )-Y <sup>1016</sup>
8. $33.9 \pm 1.5$ ( $n = 3$ )	Anti-M1/M2	GRDKY	G <sup>1</sup> - $\geq$ (I <sup>262</sup> -L <sup>266</sup> )
9. $27.4 \pm 0.7$ ( $n = 5$ )	Anti-KETYY	VNDSPALKK	V <sup>712</sup> -Y <sup>1016</sup>
10. $22.6 \pm 0.4$ ( $n = 4$ )	Anti-M1/M2	GRDKYEPAAV	G <sup>1</sup> -G <sup>213</sup>

the  $\alpha$  subunit and, for comparison, inactivation of Na/K-ATPase. Inactivation of the enzyme is attributable to the combined action of Fe<sup>2+</sup>/ascorbate/H<sub>2</sub>O<sub>2</sub> because elimination of any of these components prevented effective inactivation. Both inactivation of Na/K-ATPase and cleavage show hyperbolic dependence on added Fe<sup>2+</sup> ions with fitted  $K_{0.5}$  values of  $180 \pm 12$  and  $560 \pm 23$  nM, respectively. The presence of OH<sup>-</sup> radical scavengers such as mannitol, formate, or tert-butanol (20 mM) did not affect inactivation of Na/K-ATPase or cleavage (not shown).

In the course of optimizing conditions, the rate of cleavage was found to be greatly slowed down in media containing 150 mM choline chloride or at pH 8. These conditions are known to favor E<sub>1</sub> conformations (27), so it was of interest to compare

cleavages in Na- or K(Rb)-containing media, conditions that stabilize the E<sub>2</sub>(Rb) or E<sub>1</sub>Na conformations, respectively. Fig. 4 shows the time course of appearance of fragments visualized with either anti-KETYY (A and B) or the anti-M1/M2 antibody (C and D) and numbered as in Table 1. In the presence of Rb ions (Fig. 4A and C), we observed four major fragments. The intensity of staining of all fragments rises in parallel, i.e., smaller fragments are not products of secondary cleavages. Fragments 1, 3, 7, and 9 recognize anti-KETYY, and complementary fragments 2, 4, 8, and 10 recognize anti-M1/M2. By comparison with the low ionic strength medium (Figs. 1 and 2), which also stabilizes primarily an E<sub>2</sub> form, fragments 5 and 6 were less pronounced in the presence of Rb ions. A minor fragment of  $\approx 100$  kDa that binds anti-KETYY also was seen in these experiments. In the Na-medium (Fig. 4B and D), the rate of cleavage was slow, and only one major cleavage was observed, producing fragment 3, which recognizes anti-KETYY, and fragment 8, which recognizes anti-M1/M2. Fig. 5 shows Coomassie-stained gels of fragments produced in Rb- or Na-containing media and digested for 20 or 60 min, without added Fe<sup>2+</sup> ions. To clearly distinguish pump fragments from contaminant proteins, intact and cleaved Na/K-ATPase were selectively solubilized with C<sub>12</sub>E<sub>10</sub> (see *Materials and Methods*). In the Rb medium, six fragments marked 1/2, 3, 7, 8, 9, and 10 were observed. Fragment 4 is concealed by the  $\beta$  subunit. In the Na-medium, the  $\alpha$  subunit is much less cleaved, and again, only two fragments, 3 and 8, were observed.

Fragments from gels, as in Fig. 5, were transferred to polyvinylidene difluoride paper and sequenced (Table 1).

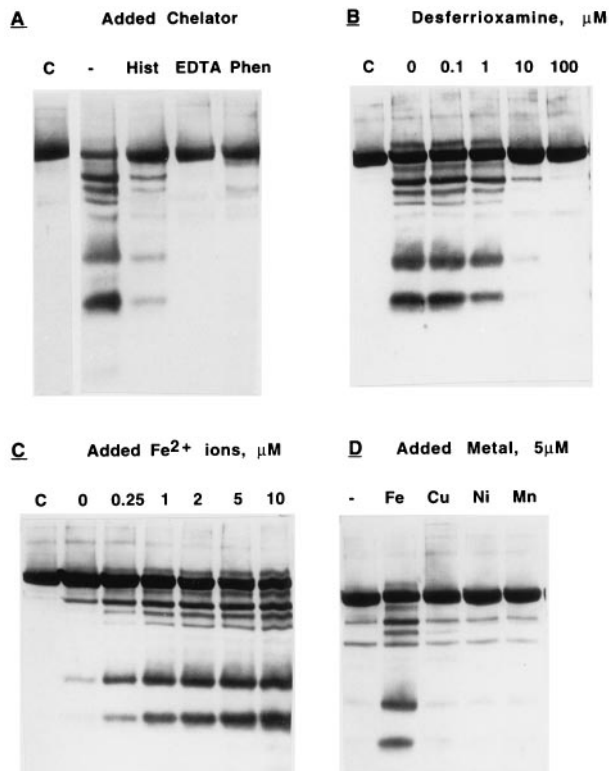


FIG. 2. Demonstration that selective cleavages are dependent on presence of contaminant or added Fe<sup>2+</sup> (or Fe<sup>3+</sup>) ions. (A) Enzyme, 1 mg/ml, was incubated for 30 min in the standard conditions (without added Rb or FeSO<sub>4</sub>) without or with 25 mM histidine or 2 mM EDTA or 1 mM phenanthroline. (B) As in A but incubation with indicated concentrations of desferrioxamine. (C) Enzyme, 0.1 mg/ml, was incubated for 2 min in standard condition with indicated concentrations of added FeSO<sub>4</sub>. (D) Incubation as in C without or with 5  $\mu$ M added FeSO<sub>4</sub>, CuSO<sub>4</sub>, NiCl<sub>2</sub>, or MnCl<sub>2</sub>. The figure presents immunoblots using anti-KETYY.

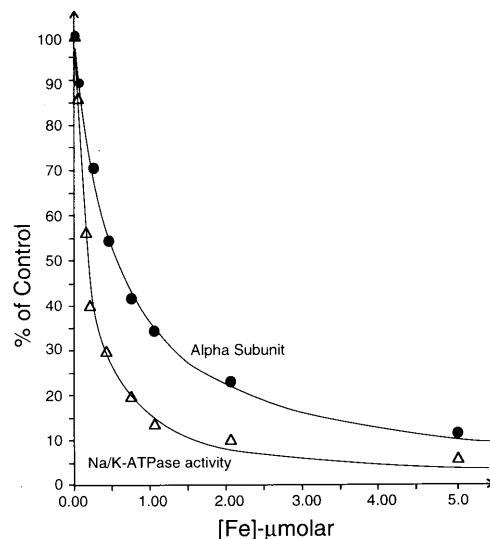


FIG. 3. Fe<sup>2+</sup> concentration dependence of cleavage and inactivation of Na/K-ATPase activity. Incubation was as in Fig. 2C. The curves represent best-fits to experimental points assuming a single site inhibition model.

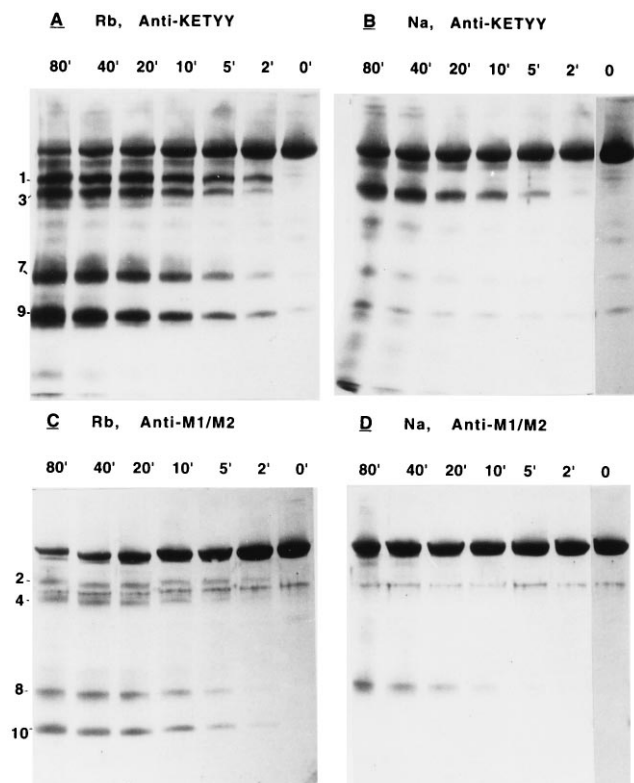


FIG. 4. Time course of cleavage in  $E_2(\text{Rb})$  or  $E_1\text{Na}$  conformational states. The enzyme, 1 mg/ml, with added 30 mM RbCl or NaCl, was incubated for the indicated times in the standard conditions without added  $\text{Fe}^{2+}$  ions. The figure presents immunoblots using anti-KETYY or anti-M1/M2. Anti-M1/M2 stains much less intensely than anti-KETYY, thus precluding quantitative comparisons of amounts of fragments binding the two antibodies.

Fragments 4 and 5 could not be isolated because they are overlapped by the  $\beta$  subunit. Note that fragments that bind anti-M1/M2 begin at the N-terminal  $G^1$  of the  $\alpha$  subunit. Of the fragments that bind anti-KETYY, sequence was obtained from fragments 1 and 9 whereas fragments 3 and 7 appear to have blocked N termini. The band marked 1/2 in Fig. 5 consists of a mixture of two fragments with sequences ESEPQTR (N-terminal  $E^{214}$ ) and GRDKEY (N-terminal  $G^1$ ). Fragment 9 gave a single sequence VNDSPALKK (N-terminal  $V^{712}$ ). The C termini of the complementary fragments 2 and 10, beginning at  $G^1$ , can be assigned as  $G^{71}$  and  $G^{213}$ . For fragments that could not be sequenced, limits on the positions of cleavages were determined using anti-peptide antibodies. Salient findings are: (i) Fragment 8 binds anti- $I^{263}\text{-P}^{276}$  whereas its complementary fragment 3 does not, (ii) fragment 6 binds anti- $K^{347}\text{-E}^{358}$  and anti- $I^{366}\text{-N}^{377}$  whereas its complementary fragment 5 does not, and (iii) fragment 7 binds  $D^{636}\text{-R}^{651}$  whereas its complementary fragment 4 does not (see Table 1). Finally, use of anti- $L^{815}\text{-Q}^{828}$ , between M6 and M7, or  $N^{889}\text{-Q}^{903}$ , between M7 and M8, produced the same cleavage pattern as observed with anti-KETYY. The latter finding demonstrates that only fragments binding either anti-KETYY or anti-M1/M2 are produced. The data in Table 1 indicate that the splits are cytoplasmic (see also Fig. 6). Using right side-out renal microsomal vesicles to define sidedness, the  $\text{Fe}^{2+}$ -dependent cleavages also have been shown directly to occur at the cytoplasmic surface, i.e., vesicles must first be opened by addition of a detergent (M. Bar Shimon and S.J.D.K., unpublished).

## DISCUSSION

**Mechanism of Cleavage of the  $\alpha$  Subunit.** The following properties of Fe-dependent cleavages suggest that  $\text{Fe}^{2+}$  (or

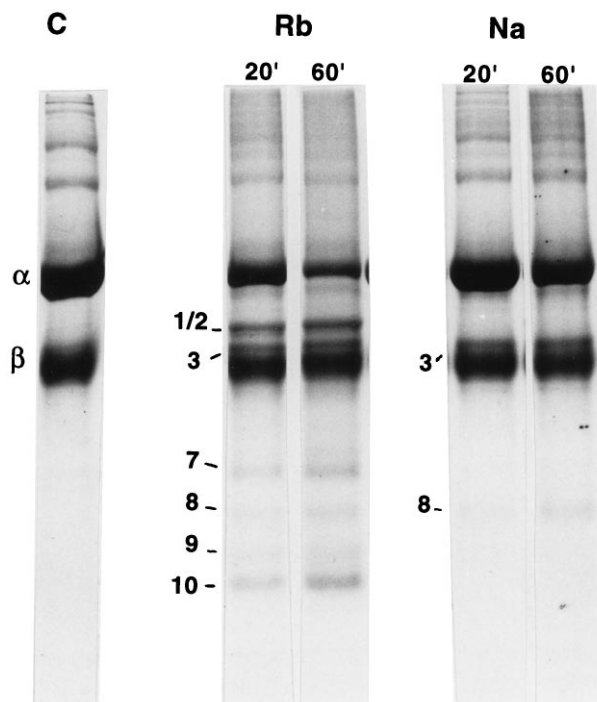


FIG. 5. Fragments of the  $\alpha$  subunit produced in  $E_2(\text{Rb})$  and  $E_1\text{Na}$  conformations. The enzyme, 1 mg/ml, with added 30 mM RbCl or NaCl, was incubated at  $20^\circ\text{C}$  for 20 or 60 min in the standard conditions, without added  $\text{Fe}^{2+}$  ions. The figure presents a Coomassie-stained gel.

$\text{Fe}^{3+}$ ) ions bind with high affinity at the cytoplasmic surface of the  $\alpha$  subunit and catalyze cleavages of peptide bonds close to or in contact with the bound  $\text{Fe}^{2+}$  ion. (i) Cleavages were specific. We detected five cleavages and only fragments containing either intact C or N termini. (ii) Cleavage and inactivation of Na/K-ATPase displayed hyperbolic dependence on low  $\text{Fe}^{2+}$  concentrations ( $K_{0.5}$ , 560 and 180 nM, respectively) (Fig. 3). (iii) All cleavages showed a similar  $\text{Fe}^{2+}$  concentration dependence (Fig. 2C). (iv) Cleavages showed a similar time course of appearance. (Fig. 4A). (v) Four cleavages were virtually absent in Na compared with the Rb medium (see Figs. 4 and 5). One cleavage producing fragments 3 and 8 occurred slowly. This is discussed below. (vi) Cleavages were insensitive to  $\text{OH}\cdot$  scavenging reagents in the medium. These properties suggest further that (a) cleavages at the different positions occur with a probability depending on the spatial arrangement of the polypeptide chain and proximity of the different peptide bonds to the bound  $\text{Fe}^{2+}$  ion and that (b) once the chain is cleaved at a particular position further cleavages do not occur to a significant extent. Selective Fe-catalyzed cleavages may be applicable to other pumps or membrane proteins (28).

The chemistry of selective Fe-mediated cleavage of peptide bonds is uncertain. Both oxidative and hydrolytic mechanisms have been proposed (16–18, 29). In the presence of ascorbate,  $\text{H}_2\text{O}_2$ , and  $\text{Fe}^{2+}$  ions,  $\text{OH}\cdot$  radicals are generated by the Fenton reaction, but  $\text{OH}\cdot$  radicals in the medium are not involved in cleavage of Na/K-ATPase. One hypothesis, for Fe-dependent, specific oxidative cleavages, is that  $\text{OH}\cdot$  radicals generated within sites are responsible (16). Resulting C- or N-terminal fragments are the amide and ketoacyl derivatives, respectively (16, 29). The blocked N termini preclude sequencing of C-terminal fragments by Edman degradation. Another oxidative mechanism (18) produces an isocyanate derivative at the N terminus of the cleaved fragment, which can be sequenced because the NCO derivative is rapidly hydrolyzed. In the hydrolytic mechanism (17), a bound Fe-peroxo intermediate acts as a nucleophile to attack the carbonyl carbon of the

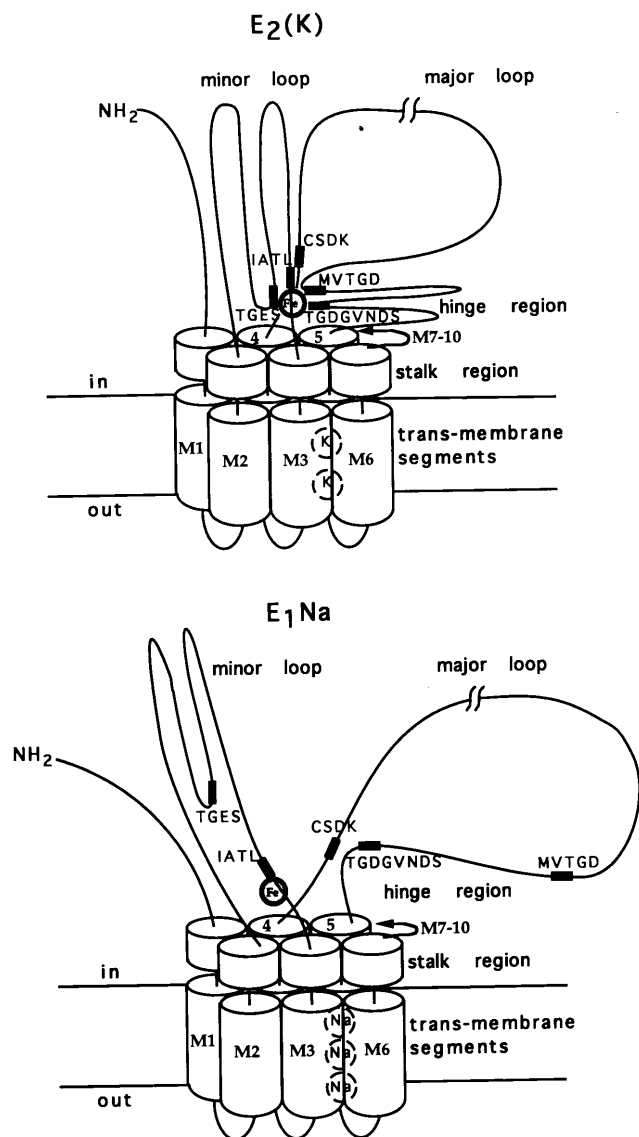


FIG. 6. Schematic models depicting formation [E<sub>2</sub>(K)] or disruption (E<sub>1</sub>Na) of interactions between cytoplasmic domains.

peptide bond, producing an intermediate that rearranges to generate free carboxyl and amino groups. This reaction produces fragments that can be sequenced. Our results are compatible with any of these mechanisms and suggest that different reactions can occur simultaneously because two C-terminal fragments could be sequenced (1 and 9) while two fragments (3 and 7) were blocked. Both E<sup>214</sup> and V<sup>712</sup> in the sequenced fragments lie adjacent to glycine residues, hinting that the same reaction mechanism operates in these cases. Another observation compatible with different types of oxidative reactions is the higher sensitivity to Fe<sup>2+</sup> of inactivation of Na/K-ATPase compared with cleavage (Fig. 3). In time course experiments, the rate of enzyme inactivation is also faster than cleavage. A reasonable explanation is that enzyme inactivation precedes chain cleavage, but not all inactivating events lead to cleavage, e.g., oxidation of side chains (29). Similar phenomena have been described for Fe<sup>2+</sup>- and Cu<sup>2+</sup>-dependent cleavage of enzymes (19, 20).

**Interactions Between Cytoplasmic Domains: Implications for Energy Transduction.** Fig. 6 presents schematic models of peptide segments close to the bound Fe<sup>2+</sup> ion in the K(Rb)-bound, E<sub>2</sub>(K), or Na-bound, E<sub>1</sub>Na state. Sites for 2 K or 3 Na ions are depicted within transmembrane segments in a domain

comprising M1-M6 (2, 4-6). The C-terminal region may also be involved in cation binding, but for simplicity transmembrane segments M7-M10 and the  $\beta$  and  $\gamma$  subunits are omitted. Approximate positions of cleavages that could not be determined exactly are assigned on the basis of antibody binding, apparent molecular mass values, and topological considerations. Cleavages in the minor loop between M2 and M3 include (i) E<sup>214</sup> in TGES (fragments 1 and 10) and (ii) that producing fragments 3 and 8 that lies beyond IATL but before the entrance to M3, i.e., close to IATL. The cleavage producing fragments 5 and 6 is located after M4 at or beyond I<sup>366</sup>-N<sup>377</sup>, near the phosphorylation site CSDK. The two other cleavages in the major loop are (i) that producing fragments 7 and 4 that lies before D<sup>636</sup>-R<sup>651</sup> and may be near the segment MVTGD and (ii) V<sup>712</sup> in TGDGVNDSPALKK (fragments 9 and 2), within the so-called "hinge region" that leads into M5.

*A priori*, it is likely that segments close to bound Fe<sup>2+</sup> also interact with each other. The sequences CSDK, TGES, and VAVTGDGVNDSPALKK, and MVTGD, are the most highly conserved in P-type pumps. A strong implication is that major and minor loop domains do interact (in E<sub>2</sub> states), and the sequences are highly conserved because the interactions serve an important function. In E<sub>1</sub> states, major and minor loops may move apart and change configuration so that the conserved segments no longer make contact with the Fe<sup>2+</sup> ion (see Fig. 6). The bond near IATL is cleaved (slowly), even in the E<sub>1</sub>Na state. A possible explanation is that Fe<sup>2+</sup> binding residues are near IATL in the primary sequence and IATL cannot move away. The sequence HFIH, near the entrance of M3, might form part of a Fe coordination site, especially if the imidazole nitrogens of the two histidines are oriented in an  $\alpha$ -helical arrangement. In the E<sub>2</sub>(K) state (Fig. 6), of the five cleavage points, CSDK is placed furthest away from the Fe<sup>2+</sup> ion and is perhaps barely in contact with it because the split producing fragments 5 and 6 is a minor one (Fig. 4). In an E<sub>2</sub> conformation (absence of Rb and low ionic strength), this split is more pronounced (Figs. 1 and 2) and CSDK might lie closer to the Fe<sup>2+</sup> ion.

Are the models in Fig. 6 compatible with known characteristics of E<sub>1</sub>/E<sub>2</sub> conformational transitions? The notion that the major and minor loops move apart in the E<sub>2</sub>/E<sub>1</sub> transition fits well with studies on proteolytic digestion of Na/K-ATPase. In the E<sub>1</sub>Na state, the  $\alpha$  subunit is cleaved at R<sup>262</sup> by trypsin (9) or at L<sup>266</sup> by chymotrypsin (10), consistent with exposure of the IATL sequence to proteases in the medium. By contrast, in the E<sub>2</sub>(Rb) state, the IATL sequence is inaccessible to proteases, and the  $\alpha$  subunit is cleaved in the major loop. The model predicts that structural modifications, such as mutations or proteolytic splits, could interfere with the domain interactions and stabilize E<sub>1</sub> states in which such interactions are already suppressed. Stabilization of E<sub>1</sub> states of Ca- Na/K- or H-ATPase is indeed observed when these proteins are mutated at or close to the positions of the Fe-catalyzed cleavage sites described here (2, 12-14, 30, 31). Specific proteolytic cleavages of Na/K- and Ca-ATPase in the minor loop also stabilize E<sub>1</sub> forms (9-11). Conversely, modifications that favor interactions could stabilize E<sub>2</sub> states. As a recent example, mutations of D<sup>369</sup> to neutral residues N and A have been found to stabilize E<sub>2</sub> forms (32).

What are the implications for the energy coupling mechanism? One important issue concerns catalytic effects of bound cations and ATP-cation interactions. Binding of Na ions at cytoplasmic sites stabilizes the E<sub>1</sub>Na state, in which ATP is bound with high affinity ( $K_d \approx 0.2 \mu\text{M}$ ) and catalyzes phosphorylation of D<sup>369</sup>. Conversely, binding of K(Rb) at cytoplasmic sites stabilizes E<sub>2</sub>(K) and antagonizes ATP binding ( $K_d \approx 200-300 \mu\text{M}$ ). A simple hypothesis to explain K-ATP antagonism could assume that interactions of TGDVNDSPALKK or the MVTGD sequence with the minor loop [in the E<sub>2</sub>(K) state] distort the major loop and interfere with high affinity

ATP binding. Conversely, these interactions are suppressed in the  $E_1Na$  state, allowing reorganization of the major loop for high affinity ATP binding and phosphorylation of  $D^{369}$ . Consistent with the hypothesis, chemical modification of  $D^{710}$  and  $K^{719}$ , using an ATP analog, CIRATP [ $\gamma$ -(4-*N*-2-chloroethyl-*N*-methylamino)benzylamide-adenosine triphosphate; ref. 33], with the reactive group attached to the  $\gamma$  phosphate or the adenosine derivative FSBA [*p*-(fluorosulfonyl)benzoyladenine; ref. 34] demonstrates proximity of  $D^{710}$  or  $K^{719}$  with  $D^{369}$  and the  $\gamma$  phosphate of ATP in the  $E_1$  state. The mechanism could also explain the finding (10) that chymotryptic cleavage of Na/K-ATPase at  $L^{266}$  stabilizes  $E_1$  with high affinity ATP binding and phosphorylation but also leaves intact Rb occlusion, a characteristic of  $E_2$ . Cleavage may hinder domain interactions and so uncouple normal K-ATP antagonism. The second major issue concerns coupling of conformational transitions to active cation transport. One can envisage that the interactions between the minor and major cytoplasmic loops might cause movement of trans-membrane segments M4-M6, which are considered to play a major role in cation binding and transport (1). However, formulation of more specific hypotheses will require comprehensive information on cleavages in the different phosphorylated and non-phosphorylated states of the catalytic cycle. Experiments to obtain such information are in progress.

A final question of interest concerns the possibility that binding of  $Fe^{2+}$  ions to the Na/K-pump *in vivo* and subsequent cleavage reactions have physiological significance or a pathological role in oxidative stress (35). Alternatively, the Fe-catalyzed cleavages may have no significance *in vivo* and serve only as a useful tool for *in vitro* experiments.

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