Journal of Bioenergetics and Biomembranes, Vol. 33, No. 5, October 2001 (© 2001)

Structural Organization and Energy Transduction Mechanism of Na^+,K^+-ATPase Studied with Transition Metal-Catalyzed Oxidative Cleavage

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This chapter describes contributions of transition metal-catalyzed oxidative cleavage of Na^+,K^+-ATPase to our understanding of structure–function relations. In the presence of ascorbate/H_2O_2, specific cleavages are catalyzed by the bound metal and because more than one peptide bond close to the metal can be cleaved, this technique reveals proximity of the different cleavage positions within the native structure. Specific cleavages are catalyzed by Fe^{2+} bound at the cytoplasmic surface or by complexes of ATP–Fe^{2+}, which directs the Fe^{2+} to the normal ATP–Mg^{2+} site. Fe^{2+}- and ATP–Fe^{2+}-catalyzed cleavages reveal large conformation-dependent changes in interactions between cytoplasmic domains, involving conserved cytoplasmic sequences, and a change of ligation of Mg^{2+} ions between E_1P and E_2P, which may be crucial in facilitating hydrolysis of E_2P. The pattern of domain interactions in E_1 and E_2 conformations, and role of Mg^{2+} ions, may be common to all P-type pumps. Specific cleavages can also be catalyzed by Cu^{2+} ions, bound at the extracellular surfaces, or a hydrophobic Cu^{2+}-diphenyl phenanthroline (DPP) complex, which directs the Cu^{2+} to the membrane–water interface. Cu^{2+}- or Cu^{2+}-DPP-catalyzed cleavages are providing information on α/β subunit interactions and spatial organization of transmembrane segments. Transition metal-catalyzed cleavage could be widely used to investigate other P-type pumps and membrane proteins and, especially, ATP binding proteins.

KEY WORDS: Na^+,K^+-ATPase; structure–function relations; transition metals; oxidative cleavage.

INTRODUCTION

The Na^+,K^+-ATPase pumps $3Na^+$ and $2K^+$ ions for each ATP molecule hydrolyzed. The process involves a coupling of the scalar process of free energy transfer from ATP to the protein, with vectorial ion transport. The explanation of this mechanism in terms of molecular structure is, of course, the purpose of structure–function studies. The objective of this essay is to point out some of the central mechanistic questions and, in particular, to describe the insights obtained by specific oxidative cleavage catalyzed by bound transition metals ions.

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ENERGY TRANSDUCTION MECHANISM OF P-TYPE CATION PUMPS

Central Functional Issues

The kinetic mechanism of Na^+,K^+-ATPase, as of all other P-type pumps, is now largely understood. We have a wealth of knowledge on transport reactions, covalent phosphorylation, E_1/E_2 conformational transitions, and cation occlusion (see Glynn and Karlish, 1990; Glynn, 1993). The generally accepted Post–Albers mechanism for active Na^+ and K^+ transport involves (1) Na_{cyt}-dependent phosphorylation from ATP and Na^+ occlusion,

0145-479X/01/1000-0387$19.50/0

$E_1 \rightarrow E_1P(Na)$, (2) Na^+ transport outward across the membrane coupled to E_1P–E_2P, (3) K_{exc}-activated dephosphorylation and occlusion, E_2P–$E_2(K)$, and (4) K^+ transport inward across the membrane coupled to $E_2(K) \rightarrow E_1$, accelerated by ATP acting with low affinity. For other pumps, steps (1) or (3) are activated by the appropriate cations, which are transported in steps (2) or (4). It was pointed out many years ago that central requirements for an effective ion pump are strict cation specificity of the phosphorylation and dephosphorylation reactions (1) and (3) and tight coupling of the E_1/E_2 conformational changes to cation movements, in steps (2) and (4), as well as features which minimize energy losses via nonproductive pathways (Jencks, 1983). For example, whereas E_1P, like ATP, has a high free energy of hydrolysis, and can transfer its phosphate to ADP, but is not readily hydrolyzed, E_2P, has a low free energy of hydrolysis and cannot transfer its phosphate to ADP, but it is more readily hydrolyzed, rapidly so when K^+ ions are bound. If, for example, E_1P was readily hydrolyzed, the result would be hydrolysis of ATP without performance of work. Similarly, the property of cation occlusion in the $E_1P(Na)$ and $E_2(K)$ is thought to minimize passive cation slippage fluxes through the protein and so maintain tight coupling of active cation movements to ATP hydrolysis (Karlish *et al.*, 1982). How do the cations move? Studies of charge movements have provided evidence for two negatively charged sites occupied either by $2Na^+$ or $2K^+$ ions and one neutral site for a third Na^+ ion. At the extracellular surface, the $3Na^+$ ions dissociate in sequence from a deep "ion well" or access pathway. At the cytoplasmic surface, the neutral site appears to be located in a shallow "ion-well." Cation transport can be envisaged as the result of movement of "barriers" to free diffusion through the protein. In the E_1 form, barriers are open at the cytoplasmic and closed at the extracellular surface. In $E_1P(3Na)$, with three occluded Na^+ ions, barriers are closed at both surfaces. In E_2P, the extracellular barriers are open and the cytoplasmic barriers remain closed. In $E_2(K)$, with two occluded K^+ ions, barriers at both surfaces are closed (see Rakowski *et al.*, 1997; Apell and Karlish, 2001 for reviews on the transport mechanism).

Structural Features and Mechanistic Questions

The Na^+,K^+-ATPase like the gastric H^+,K^+-ATPase consists of α and β subunits. Renal Na^+,K^+-ATPase also contains a small γ subunit, which is a mainly kidney-specific regulator (Therien and Blostein, 2000). The genes for the Na^+,K^+-ATPase have been cloned, including isoforms, expressed in different tissues (Shull *et al.*, 1985; Lingrel *et al.*, 1990; Ovchinnikov *et al.*, 1986). For the α subunit, consisting of about 1000 residues, various experimental techniques have demonstrated the existence of ten transmembrane segments, both with cytoplasmic N- and C-termini (Møller *et al.*, 1996). The β subunit, consisting of about 300 residues, has a single transmembrane segment, with a cytoplasmic N-terminus, three conserved S–S bridges, and three glycosylation sites in the extracellular domain. The β subunit does not contain ligand binding sites, but is required for stabilization of the α subunit and its passage from the endoplasmic reticulum to the cell membrane (Geering, 1991). Functional sites for cations and ATP are located on the α subunit and are being intensively studied by site-directed mutagenesis, with chimeric proteins, etc., and biochemical techniques, including proteolysis and covalent labeling. It is now clear that cation occlusion sites reside within transmembrane segments (Karlish *et al.*, 1990), primarily M4, M5, M6, and M8 (Jewell-Motz and Lingrel, 1993; Andersen and Vilsen, 1995; Nielsen *et al.*, 1998; see Clarke *et al.*, 1989; Rice and MacLennan, 1996; and MacLennan *et al.*, 1997 for Ca^{2+}-ATPase references). For Na^+,K^+-ATPase, which is the main topic of this essay, there is evidence that E327 in M4, S775, T774, N776, and E779 in M5, D804 and D808 in M6 are involved in monovalent cation binding and occlusion (reviewed in Lingrel *et al.*, 1997; Jorgensen *et al.*, 1998; numbering throughout is as in the pig α1 sequence). ATP binding sites are located within the large cytoplasmic loop between M4 and M5 (see Møller *et al.*, 1996). Since ATP sites are located in the major cytoplasmic loop and cation occlusion sites are located within transmembrane segments, the coupling process must involve communication between them. The communication involves E_1 and E_2 conformational transitions, which have been studied extensively, for example, with fluorescent probes and by selective proteolytic digestion (see Jorgensen and Andersen, 1988; Robinson and Pratap, 1993; for reviews). Probes bound at different sites report the E_1–E_2 transition, implying that substantial structural changes occur. All P-type pumps contain conserved cytoplasmic sequences: ^{212}TGESE in the minor loop between M2 and M3, ^{367}CSDK with the phosphorylated aspartate after M4, and ^{608}MVTGD and ^{708}TGDGVNDSPALKK in the major cytoplasmic loop before M5 (Møller *et al.*, 1996). Proteolytic cleavage and site-directed mutagenesis in these sequences usually stabilize E_1 forms, implying an involvement in conformational transitions (Jorgensen and Andersen, 1988; Møller *et al.*, 1996; MacLennan *et al.*, 1997 for full references). However, their role and, indeed, the structural events which underlie E_1/E_2 transitions have been obscure.

One outstanding event has been the determination of the 2.6 Å crystal structure of sarcoplasmic reticulum

Ca^{2+}-ATPase (Toyoshima *et al.*, 2000). Since the Ca^{2+}-ATPase and the Na^+,K^+-ATPase, are close members of the same family of P-type pumps (Axelsen and Palmgren, 1998) presumably the structure of Na^+,K^+-ATPase resembles that of Ca^{2+}-ATPase, particularly within the cytoplasmic domains with greatest sequence similarity, but may show detailed differences related to the cation specificities and the presence of its β subunit. The structure confirms the topological organization of ten transmembrane segments deduced for Ca^{2+}, Na^+, K^+-, H^+, K^+- and H^+-pumps (Møller *et al.*, 1996), and reveals several unexpected features. For example, the two Ca^{2+} ions are located side by side at a distance of 5.7 Å apart, approximately in the center of the transmembrane segments M4–M6 and M8. M4 and M6 are partly unwound for efficient coordination of the two Ca^{2+} ions. The details of Ca^{2+} occlusion sites fit well with those deduced in extensive mutagenesis studies (Andersen and Vilsen, 1995; MacLennan *et al.*, 1997). It is suggested that an opening between M2, M4, and M6 serves as an access structure on the cytoplasmic side and the outlet of Ca^{2+} at the opposite surface could lie between M3–M5. The cytoplasmic sector is divided into three domains, two domains N (nucleotide) and P (phosphorylation) within the loop between M4 and M5, well separated from a third A (actuator or anchor) domain containing the loop between M2 and M3 and the strand leading into M1. The fold of the P domain is like that of L-2-haloacid dehalogenase (HAD) and related proteins, with homologies to P-type pumps in conserved cytoplasmic sequences (Aravind *et al.*, 1998; Ridder and Dijkstra, 1999). Comparison of the crystal structure (an $E_1$2Ca conformation) with cryoelectron microscope images of Ca^{2+}-ATPase in both E_1 or E_2 conformations (Ogawa *et al.*, 1998), suggested that in E_2 a large movement of domain A brings it into contact with the P/N domain (see below). Electron microscopy of two-dinensional crystals of sarcoplasmic reticulum Ca^{2+}-ATPase (Zhang *et al.*, 1998) and fungal H^+-ATPase (Auer *et al.*, 1998) has led to a model at 8 Å resolution, showing head, neck, and membrane sectors with ten transmembrane segments, and also provides evidence for large conformation-dependent structural rearrangements in the cytoplasmic domain (Stokes *et al.*, 1998). The structure of Na^+,K^+-ATPase has been analyzed by electron microsocopy to about 14 Å resolution (Hebert *et al.*, 2000). Although this provides evidence for similarities to Ca^{2+}-ATPase in the E_2 state, at this resolution significant insight into the functional questions is not obtained.

Essential as it is for predictive and explanatory purposes, the high-resolution structure of Ca^{2+}-ATPase in one conformation ($E_1 \cdot Ca$) does not answer the central questions on the mechanism of cation movement through the transmembrane segments, the structural basis for cation selectivity, and the communication between cation and ATP sites. We need crystal structures in more than one conformation, and information on functional interactions. Thus, lower resolution structural techniques, which provide insight into the functional questions, are also required.

E_1/E_2 CONFORMATIONAL TRANSITIONS AND THE REACTION MECHANISM

Fe^{2+}-Catalyzed Oxidative Cleavage at the Cytoplasmic Surface

Selective Fe^{2+}-catalyzed oxidative cleavage of the α subunit was found as a chance discovery following incubation of renal Na^+,K^+-ATPase with ascorbate/H_2O_2 (Goldshleger and Karlish, 1997). Cleavages are quite specific and there is a characteristic conformation-dependent cleavage pattern. In E_2 conformations, six fragments are observed, whereas in E_1 conformations only two of these fragments appear [see Table I, rows 1 and 2]. The β subunit is not cleaved. Specific cleavage patterns must reflect the native protein structure since, by comparison, denatured Na^+,K^+-ATPase is cleaved to multiple small fragments. Cleavages depend on the presence of contaminant or added Fe^{2+}, as shown by suppression of cleavages by a specific Fe^{3+}-complexant, desferal, or their amplification by addition of micromolar concentrations of Fe^{2+}. Appearance of all the fragments has approximately the same Fe^{2+} concentration dependencies and time-course, indicating that smaller fragments are not products of secondary cleavages. Na^+,K^+-ATPase is inactivated by incubation with submicromolar concentrations of Fe^{2+}/ascorbate/H_2O_2 and inactivation is a little more sensitive to Fe^{2+} concentrations than cleavage of the α subunit, perhaps due to oxidative reactions that do not cleave the chain. Cleavage of the α subunit and inactivation of Na^+,K^+-ATPase are five- to sixfold more sensitive to Fe^{2+} concentrations in Rb^+- compared to Na^+-containing media. Neither cleavage nor inactivation of Na^+,K^+-ATPase is suppressed by the presence of OH radical scavengers (mannitol, formate or *t*-butanol) in the medium.

The chemistry of metal-catalyzed cleavage of proteins is thought to involve OH radicals or metal-peroxyl intermediates generated by Fenton reactions, catalyzed by the bound metal (Berlett and Stadtman, 1997). For the case of Na,K-ATPase, the specificity, Fe^{2+} concentration dependence, similar time-course, lack of effect of

Table I. Fragments Observed in Different Conformational States

Source of bound Fe^{2+} catalyst	Conformation	Cleavage position N-termini of fragments[a]		Domain
Free Fe^{2+}	E_1 or E_1P	nr M1	nr ^{283}HFIH	Membrane [b,c]
		*	***	
Free Fe^{2+}	E_2(X)occ or E_2	nr M1	nr ^{283}HFIH	Membrane [b,c]
		*	***	
		^{214}ESE		A
		***		
		nr ^{367}CSDK	nr ^{608}MVTGD	P
		*	***	
		^{712}VNDS		P
		***		
Free Fe^{2+}	$E_2 \cdot P_i$ or E_2P	nrM1	nr ^{283}HFIH	Membrane[c]
		*	***	
		^{214}ESE		A
		***		
		^{712}VNDS		P
		**		
Free Fe^{2+}	$E_2P \cdot Mg$/ouabain or $E_2V \cdot Mg$	nr M1	nr ^{283}HFIH	Membrane[c]
		*	***	
ATP–Fe^{2+}	E_1 or E_1Na or $E_1P \cdot Fe^{2+}$	nr^{440}VAGDA		N[d]
		*		
		^{712}VNDS (doublet)		P
		***		
ATP–Fe^{2+}	$E_2P \cdot Fe^{2+}$	^{214}ESE		A[d]
		***		
		^{712}VNDS		P
		**		

[a] Numbering is as in the pig α1 sequence. ***, Major; **, Intermediate; *, Minor.
[b] From Goldshleger and Karlish, 1997.
[c] From Goldshleger and Karlish, 1999.
[d] From Patchornik *et al.*, 2000.

OH radical scavengers, as well as the similar effects of conformation on different cleavages, strongly suggest a mechanism of cleavage dependent on specifically bound Fe^{2+} ions (Goldshleger and Karlish, 1997; Goldshleger *et al.*, 1998). In our initial analysis, we inferred two important features. First, Fe^{2+} is bound at a site at the cytoplasmic surface and catalyzes local generation of OH radicals, which themselves or via a Fe-peroxyl intermediate, cleave peptide bonds in proximity to the Fe^{2+} ion. Second, upon chain cleavage, further cleavages do not occur, presumably because the Fe^{2+} no longer binds (Goldshleger and Karlish, 1997). Note that if more than one cleavage is mediated by the bound Fe^{2+}, the cleavage points are of necessity in proximity to each other, although each α subunit is subject to only one cleavage. Thus, this technique has the important advantage of revealing proximity of the different cleavage positions in the native structure, that is, the spatial organization.

Different metal-catalyzed cleavage mechanisms lead either to polypeptides with free N-termini, which mechanism allows N-terminal sequencing (diamide path) or to blocked N-termini, which preclude sequencing (α-amidation path) (Platis *et al.*, 1993; Berlett and Stadtman, 1997). In our case, the exact position of the cleavages could be determined for two fragments, with N-termini ^{214}ESE and ^{712}VNDS. In other cases, cleavage positions were located quite closely, for fragments with blocked N-termini, using sequence-specific antibodies or known proteolytic fragments as standards. A summary of fragments in the different conformational states and also the location of the cleavages in P, N, or A cytoplasmic domains as defined by homology with Ca^{2+}-ATPase, or at the membrane-water surface (membrane), is given in Table I. Note in Table I (row 1) that of the six fragments observed in E_2 or E_2(K) forms, four cleavages are located at or near highly conserved cytoplasmic sequences, at ^{214}ESE in the A domain,

near ^{367}CSDK, near ^{608}MVTGD, and at ^{712}VNDS in the P domain. Two cleavages are located near the entrance of M3 (near ^{283}HFIH) and M1, and these do not differ in E_1 and E_2 conformations (compare Table I, rows 1 and 2). An indication that the second histidine in ^{283}HFIH is involved in Fe^{2+} binding came from an observation that axolemma enzyme, which consists mainly of $\alpha 2$ and $\alpha 3$ isoforms, in which the second histidine is replaced by glutamine, shows no cleavage near ^{283}HFIH. All the other specific cleavages were observed, as were the effects of E_1/E_2 transitions.

Although the assumption of a single Fe^{2+} site explains the kinetics of the cleavages simply, as stated by Goldshleger and Karlish (1999), we could not exclude an alternative hypothesis, which is that there are two Fe^{2+} sites, each site being defined by its own pattern of fragments. We now believe that a two-Fe^{2+} site mechanism is likely, since the crystal structure of Ca^{2+}-ATPase shows that the residues analogous to those near the membrane are far from those in the P domain, which are close to each other (and see also next section and section on Cleavage of Transmembrane Segments Catalyzed by Hydrophobic Cu^{2+} Chelator for new experimental support). This concept is depicted in Fig. 1, for E_2(K) and E_1Na conformations, with the cytoplasmic loops in N, P, and A domains organized as for Ca^{2+}-ATPase (Toyoshima *et al.*, 2000). Site 1 is within the P and A domains and, in E_2 conformations, the Fe^{2+} is bound and mediates cleavage at ^{214}ESE, near ^{367}CSDK and ^{608}MVTGD, and at ^{712}VNDS. In E_1 conformations, Fe^{2+} is not bound at site 1 and cleavages are precluded. We proposed that in the E_2 conformations, residues within a domain formed by the cytoplasmic loop between M2 and M3 and the segment leading into M1 (the A domain), interact with residues within the cytoplasmic loop between M4 and M5 (the P domain), whereas in E_1 conformations, the residues and two domains are well separated (Goldshleger and Karlish, 1997, 1999). This concept of conformation-dependent domain interactions is strongly supported by inferences drawn from the Ca^{2+}-ATPase crystal structure (Toyoshima *et al.*, 2000). (The hypothesis is not affected by the question of one or two Fe^{2+} sites). Site 2 is located near the membrane–water interface and Fe^{2+} catalyzes cleavage near the transmembrane segments M3 (^{283}HFIH) and M1 (near ^{81}EWVK?), which must be in proximity (see also section on Cleavage of Transmembrane Segments Catalyzed by Hsydrophobic Cu^{2+} Chelator and Fig. 4). This site is unaffected by E_1 and E_2 conformations.

Cytoplasmic domain interactions depicted in Fig. 1 have some striking implication (see also section on Consequences of Cytoplasmic Domain Movements). For example, since the sequences inferred to be in mutual proximity are highly conserved, one could propose that they mediate their mutual interactions. Thus, interference with the interactions could be predicted to stabilize E_1 forms in which they are already relaxed. In fact there is much evidence that proteolytic cleavage or mutations near the ^{212}TGESE, ^{608}MVTGD, and ^{712}VNDS sequences of Na^+, K^+-, Ca^{2+} and H^+ pumps do stabilize E_1 states (see Jorgensen and Andersen, 1988; Moller *et al.*, 1996). The involvement of conserved sequences and appropriateness of these predictions suggests that the concept in Fig. 1 may be a general feature in P-type pumps. Indeed, recent work shows that gastric H^+,K^+-ATPase is cleaved very similarly to Na^+,K^+-ATPase, with similar differences between E_1 and E_2K conformations (J. M. Shin, R. Goldshleger, G. Sachs, and S. Karlish, 2001, submitted for publication).

Effects of P_i and Mg^{2+} Ions

Conformation-dependent domain movements and cleavage patterns should accompany both E_1P/E_2P and E_1/E_2 transitions. Fe^{2+}-catalyzed cleavages of phosphorylated Na^+,K^+, ATPase and enzyme incubated with P_i, Mg^{2+}, ouabain, and vanadate, Mg^{2+} did indeed suggest that the domain interactions occur also in the $E_1P \leftrightarrow E_2P$ transition (Goldsheger and Karlish, 1999). In addition, some surprising observations suggested that the interactions occur within the phosphorylation site. Thus, noncovalently bound P_i or covalently bound phosphate were found to selectively suppress cleavages at ^{367}CSDK and near ^{608}MVTGD, whereas Mg/P_i/ouabain or vanadate/Mg completely suppressed all cleavages in the P domain, producing an E_1-like, rather than the expected E_2-like, pattern of cleavages. These paradoxical findings were explained by assuming that the phosphate group interacts with residues near the ^{367}CSDK and ^{608}MVTGD sequences and Mg^{2+} ions interact within the ^{212}TGESE and ^{708}TGDGVNDSPALKK sequences, as well as with the bound phosphate. Thus both ligands directly interfere with Fe^{2+} binding . This explanation supports the two Fe^{2+} site model in Fig. 1, assuming that P_i and Mg^{2+} ions interfere with Fe^{2+} binding only in site 1 (P and A domains). The inferred sites for P_i and Mg^{2+} binding in the E_2 conformations are consistent with the crystal structures of the active sites of HAD and Ca^{2+}-ATPase (Ridder and Dijkstra, 1999; Toyoshima *et al.*, 2000), except that the involvement of the ^{212}TGESE sequence in Mg^{2+} binding is not predicted. Note, however, that HAD has no ^{212}TGESE sequence and does not undergo E_1/E_2 transitions, whereas the structure of Ca^{2+}-ATPase is for a E_1Ca conformation only.

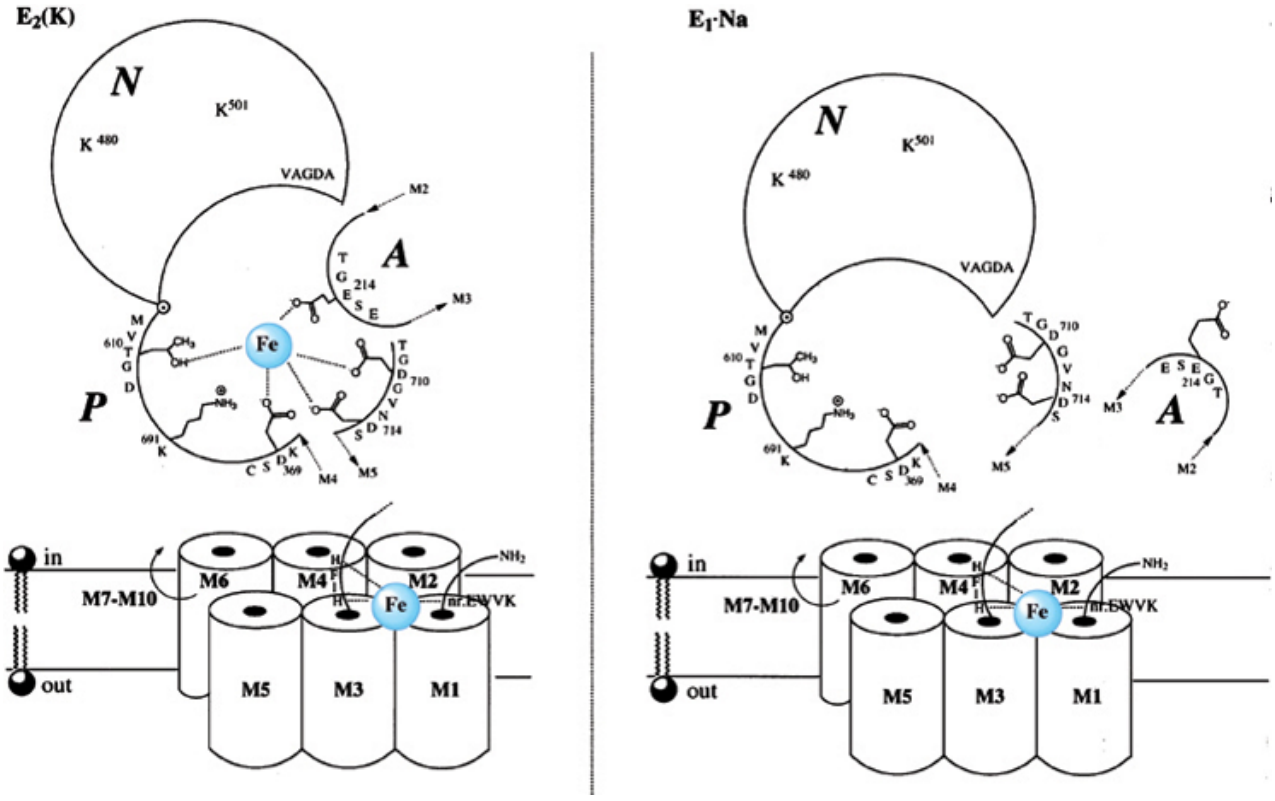

Fig. 1. Models of cytoplasmic domains and transmembrane segments M1–M6 s howing two Fe^{2+} sites. Organization of the cytoplasmic domain in $E_2(K)$ and E_1Na conformations. Dotted lines connect the bound Fe^{2+} with residues on the protein, at or near which cleavage occurs. Division of the cytoplasmic section into N, P, and A domains follows that of Ca-ATPase crystal structure (Toyoshima *et al.*, 2000). For simplicity, transmembrane segments M7–M10 have been omitted. Site 1 is in the P and A domains; site 2 is at the membrane–water interface.

ATP–Fe^{2+} Complexes Used as Specific Affinity Cleavage Reagents

The story of Fe^{2+}-catalyzed cleavage has taken a new turn recently with the demonstration that in the presence of ascorbate/H_2O_2, ATP–Fe^{2+}, or AMPPNP–Fe^{2+} complexes act as affinity cleavage reagents, mediating selective cleavage of the α subunit of Na^+,K^+-ATPase at high-affinity ATP-Mg^{2+} sites (Patchornik *et al.*, 2000). It is well known that ATP or other adenine nucleotides complex Fe^{2+} ions and still allow efficient generation of OH radicals by Fenton reactions (Floyd and Lewis, 1983; Rush *et al.*, 1990). Our evidence indicates that selective cleavages of Na^+,K^+-ATPase occur only in conditions permitting ATP binding and that cleavages are prevented by high concentrations of ATP, which compete with the ATP–Fe^{2+} complex for the site, or by Mg^{2+} ions, which compete with Fe^{2+} ions for the ATP. Cleavages are also prevented by the presence of K^+ ions or by FITC labeling of Na^+,K^+-ATPase (Karlish, 1980), both of which preclude ATP binding. The cleavages reveal contact points of Fe^{2+} or Mg^{2+} ions. In E_1 and E_1Na conformations, two major cleavages are detected within the conserved ^{708}TGDGVNDSPALKK sequence (at V712 and nearby) in the P domain, and one (E_1Na) or two (E_1) minor cleavages near the sequence ^{440}VAGDA in the N domain (Table I).

A particular advantage of this method of cleavage is that in media containing sodium and ATP, Fe^{2+} substitutes for Mg^{2+} in activating phosphorylation and ATP hydrolysis (Rendi and Uhr, 1964; Fukushima and Post, 1978). This property permits detection of changes in the cleavages in different conformations of the catalytic cycle that reflect changes in ligation of the Fe^{2+} (Mg^{2+}) ions. In the E_1P conformation, cleavages are the same as in E_1. Fe^{2+} is not bound tightly. By contrast, in the E_2P conformation, the pattern is different. A major cleavage occurs near the conserved sequence ^{212}TGESE, whereas

those in ^{708}TGDGVNDSPALKK are less prominent. Fe^{2+} is bound very tightly. Upon E_2P hydrolysis, the Fe^{2+} dissociates.

Figure 2 presents schematic models depicting residues ligating the Fe^{2+} ion and the γ-phosphate of ATP or covalently bound phosphate, in the different conformations. The models are based on the cleavage experiments using both free Fe^{2+} and ATP–Fe^{2+} complexes. Cleavages using ATP–Fe^{2+} complexes strongly support the concept of large conformation-dependent domain movements and sites for P_i and Mg^{2+} ions and add a number of new insights. The models fit well with the crystal structures of Ca^{2+}-ATPase and the HAD and CheY response regulator proteins with a homologous fold of the phosphorylation domain (Toyoshima *et al.*, 2000; Aravind *et al.*, 1998; Ridder and Dijkstra, 1999) . There are two major features. First, the E_1/E_2 conformational transitions are characterized by a pattern of reciprocal interactions between N, P, and A domains. In E_1 or E_1P conformations, N docks onto P with A displaced to one side. In E_2(K) and E_2P conformations, A docks onto P and N is displaced from the P domain. Second, the E_1P–E_2P conformational transition is associated with a change in Mg^{2+} binding from the P domain in E_1P to the A domain in E_2P. Presumably these features apply to other P-type pumps. We now discuss each conformation in detail.

E_1Na

ATP–Fe^{2+} or AMPPNP–Fe^{2+} complexes bind in the high-affinity ATP–Mg^{2+} site in E_1 or $E_1 \cdot Na$ conformations. Bound Fe^{2+} is ligated to the β and γ phosphates and the purine ring of ATP, to D710 and D714 of the TGDGVNDS sequence in the P domain and, farther away, to a sequence near ^{440}VAGDA in the N domain. In Ca^{2+}-ATPase, the residues equivalent to D710 and D714 are located on one side of a triad with the phosphorylated D369 at the apex (Toyoshima *et al.*, 2000) and in CheY Mg^{2+} binds to the residues equivalent to D710 and D714 (Ridder and Dijkstra, 1999). For bound Fe^{2+}, this arrangement explains well the two major cleavages at and just beyond V712. Mutations of D710 and N713 in Na^+,K^+-ATPase α subunit have recently been shown to affect Mg^{2+} interactions (Pedersen *et al.*, 2000). The sequence ^{440}VAGDA of Na^+,K^+-ATPase aligns with ^{438}EATET of Ca^{2+}-ATPase, which contains a residue T441, shown to lie within the ATP binding pocket in the N domain (Toyoshima *et al.*, 2000). Thus, ^{440}VAGDA is close to D710 and D714 when ATP is bound, indicating that the N domain comes into proximity with the P domain. Proximity of N (nucleotide) and P (phosphorylation) domains in the E_1 conformation explains simply the well-known fact of high-affinity ATP binding in this state ($K_{0.5}$ submicromolar) (Hegevary and Post, 1971; Norby and Jensen, 1971). The Fe^{2+} is shown as making contact with the purine ring since it is this part of the ATP molecule, which interacts with the N domain. Various transition metals, including Mn^{2+}, which substitutes for Mg^{2+}, interact with the purine ring as well as with β- and γ-phosphates of ATP (Grisham, 1988). The γ-phosphate of ATP interacts with D369 as well as with the conserved K691 and T610 of the ^{608}MVTGD sequence, as proposed for Ca^{2+}-ATPase and CheY. This arrangement is consistent with lack of cleavages by the ATP–Fe^{2+}complex at these positions, indicating that bound Fe^{2+} (Mg^{2+}) is not in direct contact with D369, T610 or K691. A suggestion that Mg^{2+} ions bind to D586 of ^{586}DPPR and in the ^{608}MVTGD sequence (Kasho *et al.*, 1997) is not supported by the cleavage data or the crystal structure of Ca^{2+}-ATPase. The A domain is separated and oriented away from the N and P domains, precluding cleavage by ATP–Fe^{2+} at ^{212}TGESE.

E_1P

Fe^{2+} is still bound to D710 and D714, near ^{440}VAGDA, and to covalently bound phosphate. The other features are the same as in E_1Na, thus explaining the same cleavages in E_1P. Fe^{2+} is not very tightly bound, whereas Mg^{2+} is tightly bound.

E_2P

In E_2P, the major cleavage occurs near the conserved ^{212}TGESE, whereas those at or near ^{712}VNDS are less prominent. Fe^{2+} is very tightly bound and, upon hydrolysis of E_2P, the bound Fe^{2+} ion dissociates. The model depicts a large movement and reorientation of domain A toward the P domain, as predicted from the cleavage experiments with free Fe^{2+}, and the inferred structure of Ca^{2+}-ATPase in the E_2 conformation. E214 in ^{212}TGESE sequence in the A domain makes contact with tightly bound Fe^{2+}, explaining the major cleavages near ^{212}TGESE, whereas D710 and D714 are somewhat displaced in order to account for the less prominent cleavage at this position. Since the fragment near ^{440}VAGDA is not observed in this state, the N domain has moved away from the P domain. The model fits well with conclusions based on cleavage catalyzed by free Fe^{2+} , namely, that Mg^{2+} is ligated by residues in both the ^{712}VNDS and ^{212}TGESE sequences and phosphate by residues in the ^{367}CSDK and

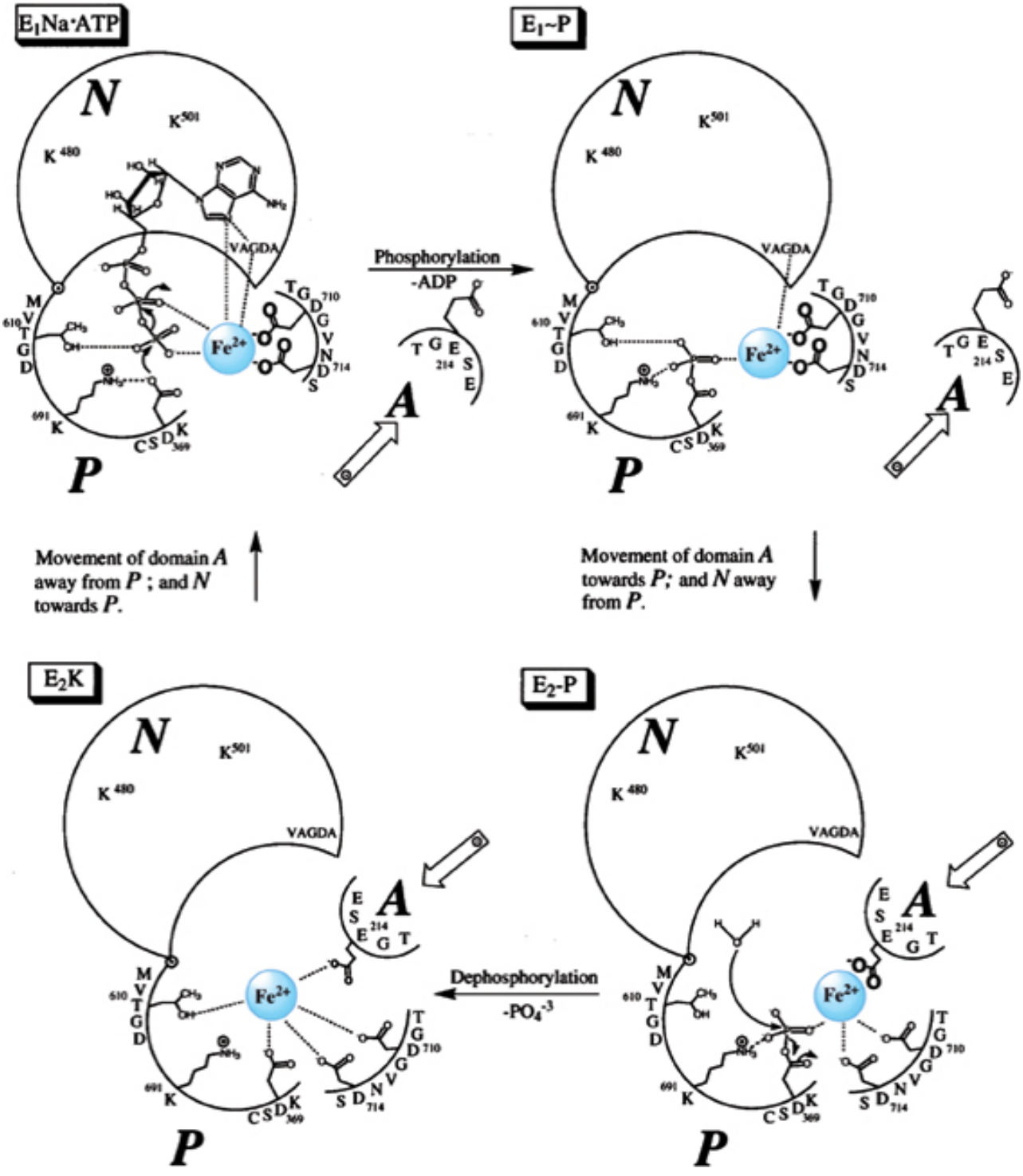

Fig. 2. Models of the active site with bound Fe^{2+} (Mg^{2+}) ions in all conformations of the catalytic cycle. Details are as in Fig. 1. K501 and K480 represent lysine residues in the N domain known to be involved in ATP binding. The Fe^{2+} ion can be replaced by a Mg^{2+} ion.

^{608}MVTGD sequences (Goldshleger and Karlish, 1999). The recent work on mutations of D710 and N713 shows that, by contrast with their role on Mg^{2+} binding in the E_1 conformation, they are not required for Mg^{2+} interactions in the E_2P conformation (Pedersen *et al.*, 2000). The conclusion is compatible with that from the cleavage work although, of course, the identity of residues involved in Mg^{2+} binding in E_2P (i.e., ^{212}TGESE) were not established by these mutations.

E_2(K)

An Fe^{2+} ion is bound in the absence of ATP, as concluded from the earlier work (Fig. 1). At high ATP concentrations free Fe^{2+} is chelated in the ATP–Fe^{2+} complex, which is not bound with high affinity in E_2(K). Presumably the bound Fe^{2+} in site 1, occupies a site which can normally recognize a Mg^{2+} ion, in the absence of bound ATP. Previously, we were unable to demonstrate competition between Mg^{2+} and Fe^{2+}. This was taken to indicate that Mg^{2+} and Fe^{2+} occupy different sites, but the result is complicated by the fact that Mg^{2+} ions stabilize an E_1 state in the absence of Fe^{2+} ions (Goldshleger and Karlish, 1999). The residues within ^{212}TGESE in the A domain and ^{367}CSDK, ^{608}MVTGD, and ^{712}VNDSPALKK in the P domain are in proximity. The N domain is displaced from the P domain and thus it is not cut (at near ^{440}VAGDA). Separation of the N and P domains in the E_2(K) conformation can explain simply the low-affinity ATP binding in this state ($K_{0.5}$ hundreds of micromolar) (Hegevary and Post, 1971; Norby and Jensen, 1971). In the inferred structure of Ca^{2+}-ATPase in an E_2 state, the A domain docks onto the P domain and the ^{212}TGESE and ^{608}MVTGD sequences are close to one another (Toyoshima *et al.*, 2000).

Consequences of Cytoplasmic Domain Movements. Communication Between the Cytoplasmic and Membrane Domains

Changes in ligation of the Mg^{2+} ion from the P domain in E_1 and E_1P to the A domain in E_2P must have an important mechanistic implication. Mg^{2+}ions are required for phosphorylation and, presumably, by shielding the negative charge and raising electrophilicity of the phosphorus atom, they facilitate nucleophilic attack by the carboxylate of D369 on the γ-phosphate of ATP. Tight binding of Mg^{2+} ions in E_2P is necessary for its normal reactivity to water (Fukushima and Post, 1978). An important implication of altered ligation of Mg^{2+} in E_2P, by comparison to that in E_1 or E_1P, is that geometry of ligands surrounding the bound phosphate must change. This factor could be crucial for facilitating nucleophilic attack by water on the phosphorus atom of the O—P bond. ATP hydrolysis occurs with overall retention of stereochemical configuration of released phosphate and the simple explanation is that both phosphorylation and dephosphorylation reactions involve "in-line" nucleophilic reactions, via penta-coordinate transition state intermediates, each with inversion of configuration (Webb and Trentham, 1981). K^+ ions, which greatly accelerate hydrolysis, act at a distance, and a likely mechanism involves induction of an appropriate configuration for "in-line" nucleophilic attack by water on the phosphorus. Effects of organic solvents suggested that the environment of the C—O—P bond is hydrophobic (De Meis *et al.*, 1980). A hydrophobic environment should amplify shielding of negative charge by Mg^{2+} ions on the phosphate oxygens and facilitate E_2P hydrolysis. Upon hydrolysis of E_2P, the Mg^{2+} ion dissociates.

Movement of the A domain toward the P domain and separation of P and N domains accompanying $E_1P \rightarrow E_2P$ must be coupled to movements of transmembrane segments (M4, M5, M6, M8?), which release Na^+ ions at the exterior. Conversely, docking of N and P and separation of A and P domains accompanying the reverse $E_2(K) \rightarrow E_1Na$ transition must be coupled to movements of transmembrane segments that release K^+ ions to the interior. How these changes in cytoplasmic domain interactions are transmitted to the transmembrane segments remains an open question. In Ca^{2+}-ATPase, the cytoplasmic extensions of M4 and M5 make contact with residues in the P domain and also with the cytoplasmic loop L6/7 between M6 and M7 (Toyoshima *et al.*, 2000). Thus changes in cytoplasmic domain interactions could affect these interactions and be transmitted onward to the membrane. Details of relevant interactions remain to be established. There is evidence that negatively charged residues in L6/7 play a role as an entrance port for cation occlusion sites (Menguy *et al.*, 1998; Shainskaya *et al.*, 2000), perhaps indicating a role for the L6/7 in the coupling mechanism.

Three Na^+ or two K^+ ions bound or occluded within transmembrane segments induce conformational information, which is transmitted to the cytoplasmic domains. This process is crucial for triggering selective Na^+-dependent phosphorylation or K^+-dependent dephosphorylation, respectively, but again, little or nothing is known of the molecular details. A small, but significant, difference in cleavage in the Na^+-rich (E_1Na) and choline-rich media (E_1) was detected in the N domain (near ^{440}VAGDA) (one or two fragments, respectively). In both conditions, the enzyme is an E_1 form. Thus the observation indicates that in E_1Na, Na^+ ions induce a long-range structural change

in the N domain. A small difference between E_1 and E_1Na in fluorescence of FITC bound at K501 has also been reported (Schneeberger and Apell, 1999). Rearrangement within the ATP site represents an essential step in which Na^+ ions trigger phosphorylation, possibly by bringing D369 close to the γ-phosphate of ATP ion altering ligation of Mg^{2+} ions.

SPATIAL ORGANIZATION OF TRANSMEMBRANE SEGMENTS AND α/β SUBUNIT INTERACTIONS

Cu^{2+}-Catalyzed Cleavages at the Extracellular Surface

In another application of the cleavage technique, we have shown that Cu^{2+}/ascorbate/H_2O_2 cause specific oxidative cleavage of both α and β subunits of Na^+, K^+-ATPase at the extracellular surface of right-side-out renal microsomal vesicles (Bar Shimon *et al.*, 1998). Again, a site-specific mechanism is involved, with cleavage of peptide bonds close to a bound Cu^{2+} (Fig. 3). This led to several conclusions on subunit interactions and spatial organization. (1) Two major cleavages of the α subunit lie within the extracellular loop L7/8 between M7 and M8, as well as minor cleavages in loop L9/10. In the β subunit, two cleavages were detected, one before the first S–S bridge and the other between the second and third S–S bridges (Fig. 3). The two cleavage sites in L7/8 of the α subunit and two cleavage sites of the β subunit represent points of interaction of the subunits. The two sites in L7/8 lie within a stretch of about ten residues near the sequence SYGQ close to the entrance to M8, which is known to be important for the α/β interaction (Colonna *et al.*, 1997). (2) Incubation with Cu^{2+}/ascorbate/ H_2O_2 inactivates Na^+, K^+-ATPase and Rb^+ occlusion, whereas ouabain binding remains intact. Thus L7/8 may play a role in cation occlusion and transport. (3) Comparison of the locations of Cu^{2+}-catalyzed cleavages with Fe^{2+}-catalyzed cleavages (Goldshleger *et al.*, 1998) suggests that the membrane sector is divided into two domains comprising M1–M6 and M7–M10/Mβ, respectively (see Fig. 3). This notion fits very well with the crystal structure of Ca^{2+}-ATPase in which a division of M1–M6 and M7–M10 is clearly seen (Toyoshima *et al.*, 2000) and also with the fact that Type 1 P-type ATPases lack M7–M10 (Lutsenko and Kaplan, 1995; Moller *et al.*, 1996). Separation of M1–M6 and M7–M10/Mβ provides a strong constraint on the packing arrangement of transmembrane segments of Na^+,K^+-ATPase. Combination of this data with information on covalent cross-linking of fragments of an extensively tryptically digested preparation, 19-kDa membranes (Karlish *et al.*, 1990), led to a tentative model of the helix arrangement of the α/β subunits (Or *et al.*, 1999).

Cleavage of Transmembrane Segments Catalyzed by a Hydrophobic Cu^{2+} Chelator

Very recently, we have been able to obtain additional information on spatial organization of transmembrane segments using hydrophobic Cu^{2+} complexes (Goldshleger *et al.*, 2000; Tal *et al.*, submitted). Renal Na^+, K^+-ATPase is incubated with ascorbate/H_2O_2, in the presence of Cu^{2+} ions complexed with different phenanthroline derivatives. Cu^{2+} complexes of several phenanthrolines catalyze rather nonspecific cleavages, but the Cu^{2+} complex of 4,7-diphenyl-1,10-phenanthroline (DPP) catalyzes three specific cleavages of the α subunit. The β and γ subunits remain intact. Cleavages occur close to the membrane/cytoplasm interface of M1, M3, and M10. The kinetics imply that the M1 and M3 cleavages, and possibly also the cleavage in M10, are mediated by a single Cu^{2+}/DPP complex interacting with the protein. The fragments near M1 and M3 are indistinguishable from those produced by Fe^{2+}-catalyzed cleavage near M1 and M3 (site 2, Fig. 1). Because the Cu^{2+}/DPP complex can be assumed to be located at the membrane–water interface, the results strongly support the notion in Fig. 1 that the Fe^{2+}-catalyzed cleavages near M1 and M3 (site 2) occur at a different site from those in the P domain (site 1). Like Fe^{2+}-catalyzed cleavages, Cu^{2+}/DPP-catalyzed cleavages near M1 and M3 are unaffected by the conformational state, whereas that near M10 is most prominent in the E_2P and $E_2(K)$ states. An implication is that M1 and M3 are static, whereas M10 moves in the E_1–E_2 transition. Overall, the results suggest that M1, M3, and possibly also M10 (particularly in E_2 conformations) are in proximity at the cytoplasmic surface. A homology model, with Na^+, K^+-ATPase residues within transmembrane segments and connecting loops substituted into the crystal structure of Ca^{2+}-ATPase, shows close proximity of M3 and M1 near HFIH (M3) and EVWK (M1), confirming the conclusions based on cleavages mediated by Cu^{2+}/DPP or Fe^{2+} at site 2 (Tal *et al.*, submitted). The helix packing, seen from the cytoplasmic surface, is slightly modified from our previous proposal (Or *et al.*, 1999) in that M1 to M3 are in proximity, rather than M1 and M2. The arrangement of M1–M8 is now similar to that in the crystal structure of Ca^{2+}-ATPase at the cytoplasmic surface (Toyoshima *et al.*, 2000). Nevertheless, differences in helix arrangement of Na^+, K^+-ATPase or H^+,K^+-ATPase in the M7–M10 segments might occur as a result of strong interactions of the β

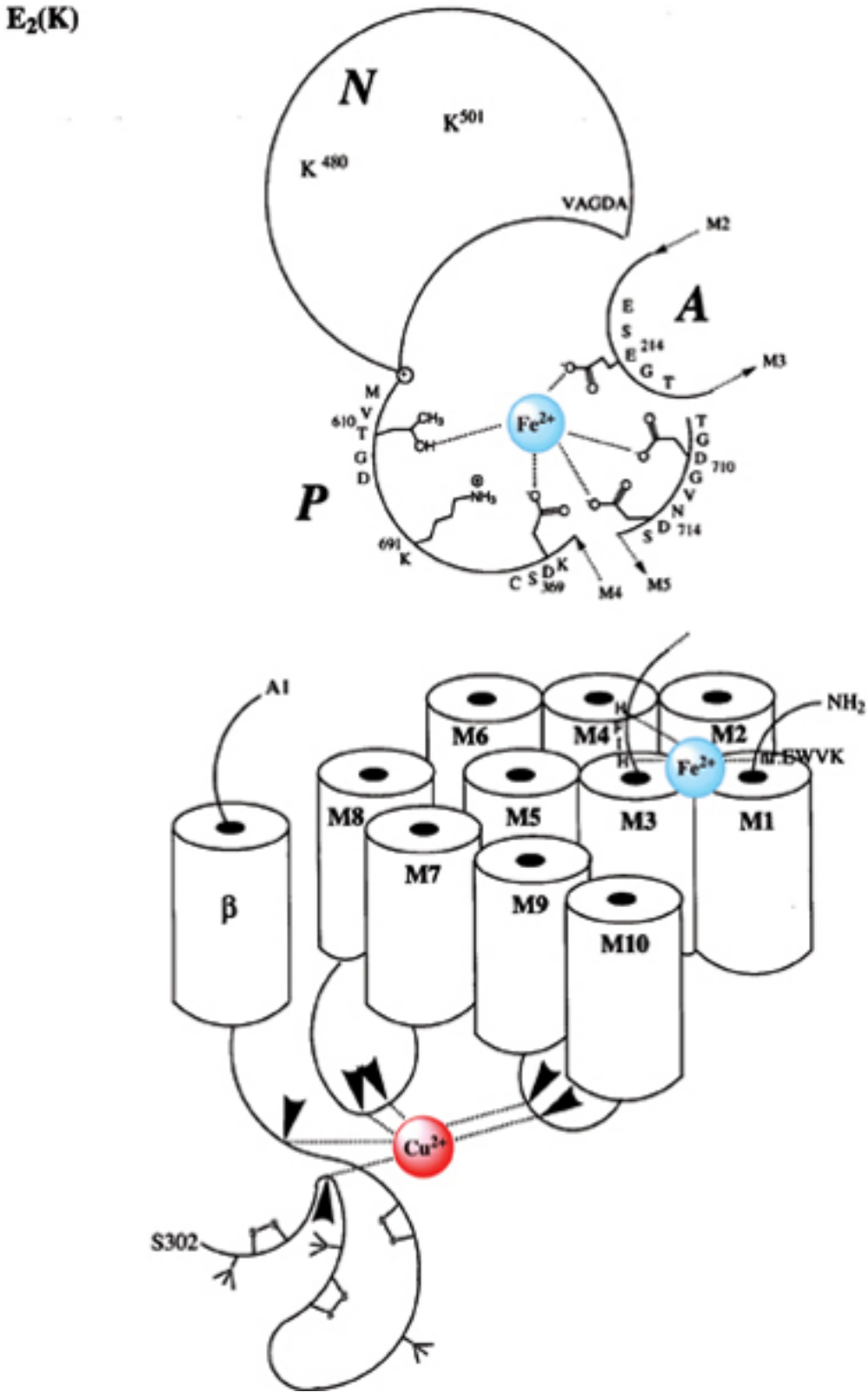

Fig. 3. Sites for bound Fe^{2+} ions at the cytoplasmic surface and a bound Cu^{2+} ion at the extracellular surface.

subunit with the α subunit near M8 (Colunna *et al.*, 1997; Or *et al.*, 1999). The position of M9 and M10 remains uncertain and must be defined by further experimental work.

CONCLUSION AND PERSPECTIVES

The strength of the cleavage technique is that it provides information on proximity of cleavage sites in the native protein. This complements methods such as site-directed mutagenesis, which provide information on individual residues or motifs. As discussed here, when used together with information from the crystal structure of the Ca^{2+}-ATPase, the cleavage approach is much more powerful. On the one hand, the structure has obliged a modification of the model to include two Fe^{2+} sites. On the other hand, the cleavages have provided insights on conformational changes and ligand binding, which are not provided by the crystal structure. The full strength of the technique will be realized when the exact position of all cleavages can be determined by mass spectrometry and especially when it is applied to recombinant Na^+,K^+-ATPase, which has been modified to remove native metal sites or create new sites in positions of choice. The cleavage technique may of course be applied to other P-type pumps, membrane proteins, and especially to ATP binding proteins.

ACKNOWLEDGMENT

This work was supported by a grant 15/00-1 from the Israel Science Foundation.

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