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Molecular Mechanism of the P-Type ATPases

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The recent determination of the structure of the Ca^{2+}-ATPase of sarcoplasmic reticulum to atomic resolution in the Ca^{2+}-bound state and to near atomic resolution in the Ca^{2+}-free, decavanadate-bound state has paved the way for an ultimate complete understanding of the molecular mechanism of the P-type ATPases. Analysis of this new structure information together with the large amount of biochemical information about these enzymes that preceded it has produced important new revelations about how the P-type ATPases work. Most models propose that these transporters operate by a strictly conformational energy coupling mechanism in which conformational changes in the large cytoplasmic head region mechanically drive the ions to be transported from their binding sites in the transmembrane helix region 50 Å away. However, while these enzymes do indeed undergo profound conformational changes, the available evidence suggests that they do not mechanically transduce the chemical energy of ATP hydrolysis into transmembrane ion gradients via these conformational changes. As an alternative, it is proposed that the effects of the chemical events that occur at the phosphorylation/dephosphorylation site in the cytoplasmic region are exerted on the ion-binding sites via two well-defined charge transfer pathways that electronically connect the chemical reaction site with the site of ion binding. The recognition of these charge transfer pathways provides rational explanations of all of the key biochemical features of the P-type ATPase catalytic cycle. Thus, although a few details await elucidation, a nearly complete understanding of the P-type ATPase reaction mechanism may be at hand.

KEY WORDS: P-type ATPase; molecular mechanism; energy coupling; conformational changes; charge transfer pathways; signal transmission.

INTRODUCTION

P-type ATPases are members of a large family of biological energy transducers that utilize the chemical energy of ATP hydrolysis to generate transmembrane electrochemical ion gradients. A central feature of the catalytic cycle of these enzymes is the formation of a phosphoryl-enzyme intermediate at an aspartic acid residue that is invariant in all members of this family. It is certain that the phosphorylation and dephosphorylation of this aspartate during each turn of the catalytic cycle is intimately involved in the molecular mechanism of ion transport catalyzed by these enzymes. Since the discovery of the first member of the P-type ATPase family (Skou, 1957), a huge amount of experimental work has been carried out toward elucidation of the molecular mechanism by which these enzymes catalyze concentrative ion transport (Andersen and Vilsen, 1990; Lutsenko and Kaplan, 1995; Moller *et al.*, 1996). The large amount of information accumulated from these studies laid a firm foundation essential for the formulation of a tenable model for the molecular mechanism of these enzymes. But, as has always been the case with enzymes, the final steps could not be made without the availability of atomic resolution structural information for at least one P-type ATPase. Recently, this long-awaited structural information has been obtained (Toyoshima *et al.*, 2000; Xu *et al.*, 2002), and we are now close to a complete understanding of the P-type ATPase reaction mechanism. Interpretation of the available structural and biochemical information within the framework of established principles of enzyme catalysis and known modes of enzyme conformational dynamics allows the formulation of a straightforward scheme describing the sequence of events that take place as the P-type ATPases proceed through their catalytic

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cycles. The forces that drive the reaction sequence can also be clearly stipulated. Even more importantly, a careful analysis of the available atomic structure has revealed a previously unrecognized energy-transducing element, the charge transfer pathway, two of which run between the phosphorylation/dephosphorylation site and the ion-binding sites. These charge transfer pathways provide the missing link that explains how the chemical events occurring at the phosphorylated aspartate in the cytoplasmic region communicate with the transported ions in their binding sites in the transmembrane region 50 Å away. The results of these considerations indicate that we may be very close to the long-sought goal of understanding the molecular mechanism of the P-type ATPases. They also raise the distinct possibility of the involvement of charge transfer pathways in other transporters, and in numerous cellular signaling systems in which events occurring at one site must be communicated to another site over a considerable distance.

ENZYME CONFORMATIONAL CHANGES AND THE TRANSITION STATE THEORY OF ENZYME CATALYSIS

The P-type ATPases are, of course, enzymes, and so at the outset it is important to briefly mention enzyme conformational changes, the transition state theory of enzyme catalysis, and the manner in which the two are inextricably interrelated. As pointed out by Pauling (1946) and elaborated upon by others (Fersht *et al.*, 1986; Jencks, 1966; Lienhard, 1973; Wolfenden, 1969), enzymes generally are designed to bind with highest affinity to the transition state of the reaction that they catalyze. In doing so, they increase the probability of formation of the transition state, thereby increasing the rate of catalysis (Frost and Pearson, 1961). Implicit in this concept is the existence of numerous favorable bonding interactions between the enzyme and the chemical species undergoing the reaction, which would be maximized in a transition state complex completely surrounded by enzyme functional groups. The problem with this, however, is that substrates could not enter and products could not leave such an enzyme. But nature has solved this problem by evolving enzymes capable of undergoing conformational changes that enclose the reactants after binding to a more open form. In this clever gambit, the conflicting requirements of substrate access and high affinity transition state binding are resolved (Wolfenden, 1974). As atomic structures for enzymes have accumulated over the years, it has become clear how they usually do this. As diagrammed in Fig. 1, enzymes generally comprise two or more relatively rigid domains separated by a deep cleft and held together via a flexible hinge region (Anderson *et al.*, 1979; Eklund *et al.*, 1976; Eklund *et al.*, 1981; Janin and Wodak, 1983; Karplus and McCammon, 1983; Remington *et al.*, 1982; Schulz and Schirmer, 1979).

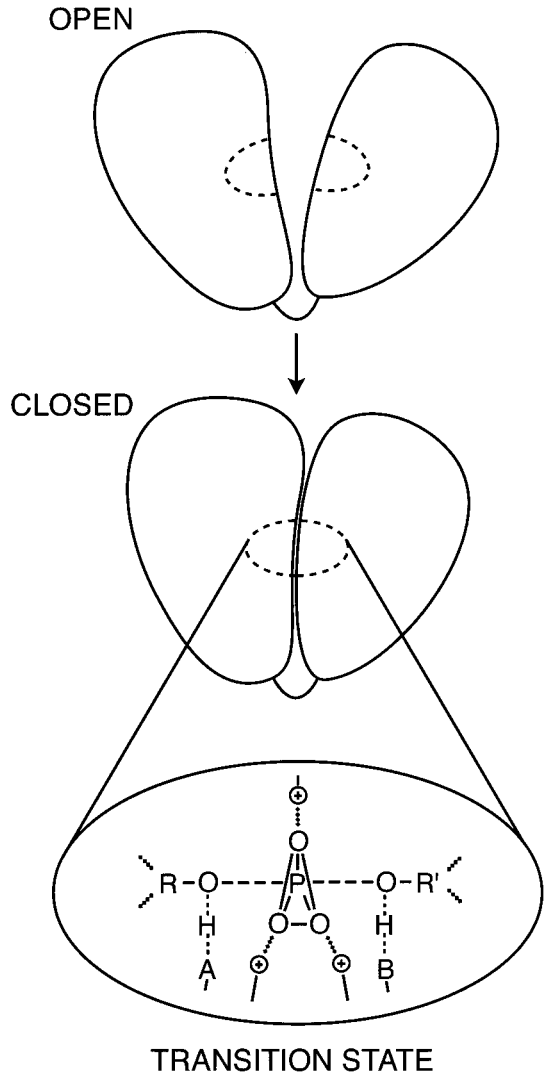

Fig. 1. General features of enzyme catalysis. The key steps that occur in most enzyme-catalyzed reactions are shown. Enzymes usually comprise two or more separate domains separated by a cleft that closes upon the binding of substrates to residues in the active site (dashed semicircles). They are designed to have their maximum affinity for the transition state configuration of the chemical reaction, which is midway between reactants and products. In this example, a typical phosphoryl transfer reaction is shown. General acid (A) and general base (B) assistance of such reactions is common. The circled "+" symbols represent positive centers that assist the reaction by withdrawing electrons that would otherwise hinder the nucleophilic attack by the entering nucleophile. The reacting phosphorus atom is in the pentacovalent trigonal bipyramidal arrangement that exists for most phosphoryl transfer reactions. Adapted from Knowles (1980).

The substrate-binding enzyme functional groups are located in the cleft (dashed semicircles) and upon binding the substrate(s), the domains move together by rigid body motions, closing the cleft and surrounding the reacting species with the appropriate enzyme residues in the proper positions, thereby facilitating the formation of the enzyme-transition state complex. A generalized transition state for phosphoryl transfer reactions (Knowles, 1980) is shown at the bottom of the diagram, with numerous favorable bonding interactions between enzyme functional groups and the reacting chemical species stabilizing the pentacovalent trigonal bipyramidal intermediate. When the transition state decomposes, which it normally does spontaneously at a high rate (Frost and Pearson, 1961), the enzyme reopens to release product(s) and rebind substrate(s) and begin another round of catalysis. Thus, transition state binding affinity is the driving force for enzyme conformational changes. This is an essential point that cannot be overemphasized. It also clearly explains why stable transition state analogues are among the most potent inhibitors of enzymes that are known (Lienhard, 1973; Wolfenden, 1969). Enzymes seize upon transition state analogues, are driven closed, and are then locked there in a nearly irreversible fashion. In sum, the established facts of transition state binding affinity and the hinge-bending conformational change together simply, yet elegantly, explain how enzymes work.

CONFORMATIONAL CHANGES IN THE P-TYPE ATPases

Conformational changes in the P-type ATPases have been demonstrated by numerous investigators with a variety of different individual enzymes using a variety of different techniques. It is not the purpose here to discuss the details of those experiments. However, certain experiments carried out with the proton-translocating P-type ATPase (H^+-ATPase) are mentioned because they directly established transition state binding affinity as the driving force for the major P-type ATPase conformational changes, and established the nature of these conformational changes to be in conformity with the general enzyme catalytic principles described above.

Numerous early experiments carried out with the H^+-ATPase had indicated the strong likelihood that it undergoes conformational changes upon binding its substrate, MgATP, and certain other ligands as well (Addison and Scarborough, 1981; Dame and Scarborough, 1980; Scarborough, 1977). With obvious pertinence to the catalytic and transport mechanism, these conformational changes were investigated in more detail in a study of the effects of a variety of ATPase ligands and ligand combinations on the sensitivity of the ATPase to degradation by trypsin (Addison and Scarborough, 1982). To summarize the results of these experiments, with no ligand present, the ca. 100 kDa H^+-ATPase is rapidly degraded by trypsin to small fragments. In the presence of the nonhydrolyzable competitive inhibitor, MgADP, a small piece is rapidly removed from the N-terminus, but further degradation of the resulting ca. 92 kDa enzyme occurs much more slowly, indicating that the H^+-ATPase changes its conformation upon binding its substrate. Similar results have been seen with all of the well-studied P-type ATPases. Importantly, however, in the presence of Mg^{2+} and the potent transition state analogue, orthovanadate (Cantley *et al.*, 1978; see also Macara, 1980; Pope and Dale, 1968), the H^+-ATPase is also markedly protected against tryptic degradation, even when no nucleotide is present at all. This single observation clearly established transition state binding affinity as an important driving force for the H^+-ATPase conformational changes.

The profound effects of the various ATPase ligands on the sensitivity of the H^+-ATPase molecule to trypsinolysis seemed to indicate that the ligand-induced conformational changes are quite extensive. However, ligand protection of only a few key amino acid residues could conceivably have explained the results of these experiments, particularly in the absence of any structural information. The ligand-induced ATPase conformational changes were thus further explored using attenuated total reflection Fourier transform infrared spectroscopy (Goormaghtigh *et al.*, 1994). The results of these experiments showed that the secondary structure components of the H^+-ATPase, i.e., α-helix, β-sheet, turns, and random coil, do not change when the H^+-ATPase undergoes these ligand-induced conformational changes, in agreement with our earlier circular dichroism studies (Hennessey and Scarborough, 1988). But importantly, the hydrogen/deuterium exchange rates of about 175 surface amide linkages in the ATPase polypeptide chain out of a total of about 350 are drastically reduced as the ATPase proceeds from its unliganded conformation to its substrate-binding conformation (i.e. bound to MgADP), and nearly as many surface residues are occluded in the presence of Mg^{2+} and orthovanadate. These results indicated that the ligand-induced H^+-ATPase conformational changes are quite substantial, and strongly suggested hinge-bending rigid body interdomain motions as the underlying mode of the H^+-ATPase conformational transitions. And when the structure of the H^+-ATPase was solved to a resolution of ca. 8 Å (Auer *et al.*, 1998), this interpretation was essentially confirmed. Figure 2 shows a view of the structure of the H^+-ATPase that was obtained. The

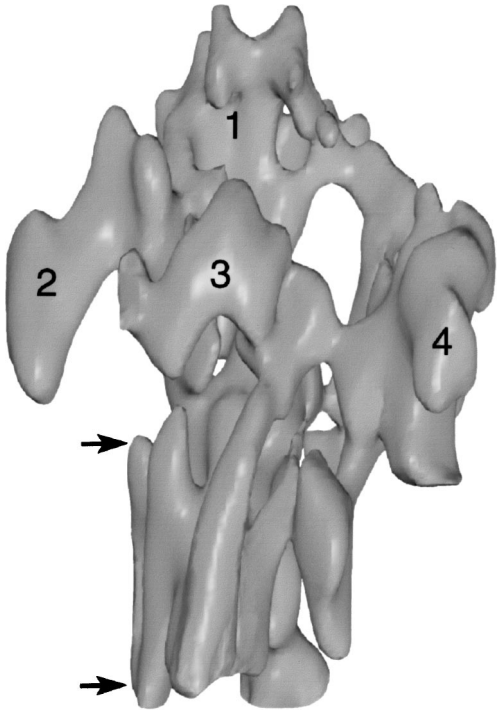

Fig. 2. The 8 Å structure of the H^+-ATPase in its open form. The H^+-ATPase comprises a cytoplasmic region (above) with multiple domains connected to a membrane region (below) with 10 transmembrane helices. The cytoplasmic domains are numbered as described (Auer *et al.*, 1998) and the approximate position of the lipid bilayer is indicated by the arrows.

molecule comprises several cytoplasmic domains (above) connected at several points with a membrane-embedded region (below) consisting of 10 transmembrane α-helices. In view of this multilobed cytoplasmic region, which presumably contained the phosphorylated active site aspartate, it was virtually certain that the H^+-ATPase conforms to the generalized enzyme behavior diagrammed in Fig. 1, with the various lobes closing up like the petals of a flower to engulf the substrate and facilitate the formation of the transition states of the enzyme phosphorylation and dephosphorylation reactions.

Figure 3 shows a similar view of the atomic resolution structure of the closely related Ca^{2+}-ATPase that was recently obtained (Toyoshima *et al.*, 2000). The structure bears a marked similarity to the open H^+-ATPase structure in Fig. 2, with multiple domains named the N, P, and A domains, connected to a transmembrane region with 10 helices. The N domain contains the nucleotide binding site, the P-domain contains the phosphorylation site (asterisk), and the A domain is referred to as the actuator or anchor domain. In the paper describing this elegant structure, Toyoshima *et al*. presented a model for the domain movements that occur when the Ca^{2+}-ATPase changes from its open conformation to its more condensed oligovanadate-bound conformation, the structure of which was solved earlier by Zhang *et al.* (1998) at a resolution of 8 Å using electron crystallography. And this convincing analysis strongly suggests that the domains undergo the rigid body motions indicated by the arrows in Fig. 3. Thus, the A domain rotates nearly 90°, the N domain bends down approaching the P domain, and the cytoplasmic region as a whole tilts to the right by about 20° with respect to the transmembrane helix region. In view of all of the above considerations, it is clear that the generalized model shown in Fig. 1 accurately describes the conformational dynamics of the P-type ATPases in the cytoplasmic region. It also specifically stipulates the forces that drive these profound conformational changes, arising from transition state binding affinity.

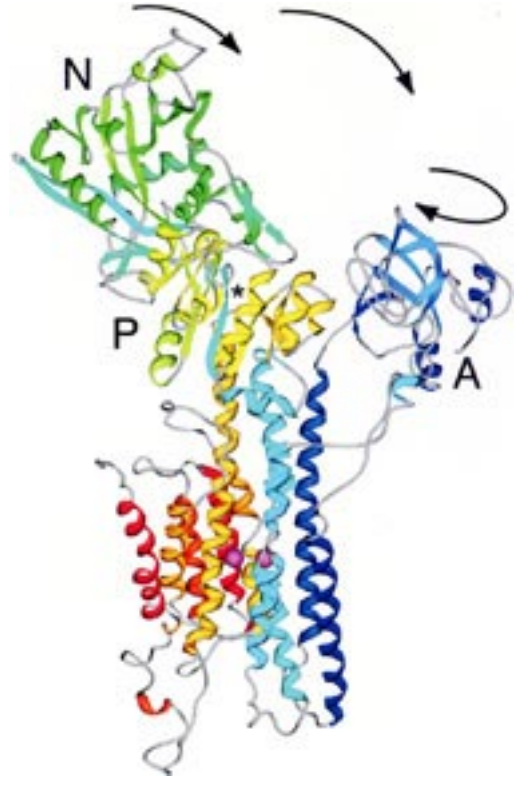

Fig. 3. Interdomain motions of the Ca^{2+}-ATPase. The Ca^{2+}-ATPase with its Ca^{2+} ions bound also possesses an open multidomain structure with a nucleotide-binding (N) domain, and an actuator (A) domain and a phosphorylation (P) domain connected to a ten transmembrane helix region below. During the catalytic cycle, the domains undergo the rigid body movements indicated by the arrows and described in the text. The site of phosphorylation is indicated by the asterisk. The Ca^{2+} ions are shown in purple. The structure was visualized from the PDB file using the program Deep View Swiss-Pdb Viewer (Guex and Peitsch, 1997) and drawn using the program POV-Ray.

An improved electron crystallographic structure of the Ca^{2+}-free, decavanadate-bound form of the Ca^{2+}-ATPase at a resolution of 6 Å has recently been presented by Xu *et al.* (2002). As part of this work, these authors have presented a pseudo-atomic model for the structure based on fitting various parts of the atomic structure presented by Toyoshima *et al.* (2000) into their 6 Å density. This model essentially confirms the cytoplasmic domain motions proposed by Toyoshima *et al.* (2000), with a 53° oblique rotation of the P domain and its associated N domain toward the A domain and a 90° rotation of the A domain about a vertical axis. But even more importantly, this model provides some detail as to the differences in the structure of the transmembrane helix region, including the Ca^{2+}-binding site region, that exist between the two conformational states. The model indicates that most of the transmembrane helices (TM) do not change substantially, but the structures of TM2, TM4, and TM5 change significantly. TM2 becomes inclined by ca. 14° and TM5 becomes bent. In addition, the top part of TM4 is displaced laterally and downward by about 4–5 Å, and this movement is associated with folding of the unwound region of TM4 to a shorter, somewhat more helical conformation. The changes in TM4 and TM5 appear to be directly related to the 53° rotation of the P domain. As a result of these conformational changes, primarily those in TM4 and TM5, the arrangement of the atoms that constitute the Ca^{2+}-binding sites changes substantially, and these movements are almost certainly intimately involved in the mechanism of active ion transport. However, as we shall see below, the sweeping interdomain movements in the cytoplasmic region and the associated conformational changes in the transmembrane helix region do not directly bring about the change in ion-binding affinity that is needed for release of the ions at millimolar concentrations on the release side of the membrane, as recent models (Stokes *et al.*, 1999; Toyoshima *et al.*, 2000; Xu *et al.*, 2002; Zhang *et al.*, 1998) propose, because the form of the enzyme that results from these conformational changes retains high affinity Ca^{2+} binding. It is thus more likely that the differences in the conformations of the P domain, TM4 and TM5, and the Ca^{2+}-binding ligands between the two structures simply reflect the conformations that these elements adopt in the presence and absence of Ca^{2+}. We shall return to this key conformational change in the Ca^{2+}-binding site region and its relation to the cytoplasmic domain motions in more detail below.

In summary, the P-type ATPases do indeed undergo profound conformational changes, particularly in the large cytoplasmic region, which are primarily sweeping, rigid body, hinge-bending interdomain movements of the N, P, and A domains. These conformational changes are intimately involved in the molecular mechanism of active ion transport catalyzed by these enzymes, but are not directly and mechanically related to the changes in the ion-binding sites that alter the ion affinities. Rather, they are primarily conformational changes common to most enzymes, the function of which is to optimize the binding affinities of the enzyme for the transition states of the aspartate phosphorylation and dephosphorylation reactions, and thereby maximize catalytic throughput for the ATP hydrolysis reaction, in accord with the established tenets of the transition state theory of enzyme catalysis.

CATALYTIC CYCLE OF THE P-TYPE ATPases

With this essential foundation formed, it is now possible to discuss the events that transpire as the P-type ATPases proceed through their catalytic cycles. It is useful to begin the discussion with a consideration of the recent model of Xu *et al.* (2002), which is reasonably explicit and takes into account all of the structural information currently available for the P-type ATPases. It is a purely conformational coupling model whereby interdomain movements in the cytoplasmic region mechanically drive conformational changes in the Ca^{2+}-binding sites via several of the TM helices, leading to a decreased affinity for Ca^{2+} and release of the Ca^{2+} on the far side of the membrane. It is also a strict E1-E2 type model (DeMeis and Vianna, 1979), and exemplifies the thoughts of virtually everyone past and present in the P-type ATPase field. It is thus a worthy object with which to begin this discussion. Figure 4 shows this model, adapted from Xu *et al.* (2002). It begins with MgATP bound to the Ca^{2+}-liganded form of the enzyme in the E1 conformation ($E1Ca_2$), the structure of which was solved by Toyoshima *et al.* (2000). Having bound ATP, the N domain, which is proposed to move freely about a hinge region that connects it with the P domain, approaches the P domain because of thermal motion, and when the domains are in the proper position, the γ-phosphoryl group of ATP is transferred to the active site aspartate in the P domain. This results in the formation of the E1 $\sim$ P conformational state with the ions bound ($E1 \sim PCa_2$). The A domain, termed the β domain by Xu *et al.* (2002), is not involved in these initial motions. The β domain then undergoes its 90° rotation, again driven by thermal motion, about the flexible hinge region connecting it with TM1, TM2, and TM3 in the transmembrane helix region. Next, when the highly conserved TGES sequence in the β domain collides with the aspartyl phosphate in the phosphorylation/dephosphorylation site, it latches on to it, fixing the β domain in its rotated orientation. This in turn is proposed to put a strain on the loops connecting

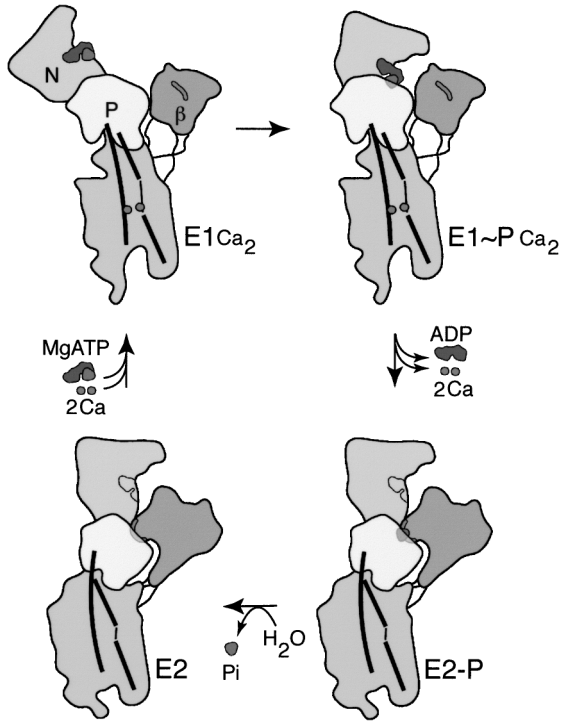

Fig. 4. Recent conformational coupling model for the Ca^{2+}-ATPase reaction cycle. The model shown is adapted from the recent model of Xu *et al.* (2002). N and P refer respectively to the nucleotide-binding and phosphorylation domains defined by Toyoshima *et al.* (2000), and β refers to the A domain defined by the same authors. The transmembrane helix region lies below these domains. The thick lines in the transmembrane region represent TM5 (left) and TM4 (right). The icons for MgATP, ADP, Ca^{2+} ions, and Pi are identified in the figure. The elongated feature in the β domain is the highly conserved TGES sequence of residues 181–184. See text for details.

the β domain to TM1, TM2, and TM3, and this, in an unspecified manner, causes Ca^{2+} release on the far side of the enzyme via TM1 and TM2 and drives the P domain rotation and concomitant conformational changes in TM4 and TM5. The sum of these changes after E1 $\sim$ P is the traditional E1 $\sim$ P to E2—P conformational change. The aspartyl phosphate linkage in E2—P then hydrolyzes to form the E2 state, in which the A and P domain rotations and the changes in the transmembrane helix region are maintained. Then, upon binding of a new pair of Ca^{2+} ions from the cytoplasmic side of the membrane, the A and P domain rotations and the changes in the transmembrane helix region are reversed, regenerating the E1 form of the enzyme with the Ca^{2+} ions bound.

Several features of this model merit further discussion. On the positive side, it recognizes for the first time since the landmark studies of Jencks *et al.* (1989), and a few efforts of my own (e.g. Addison and Scarborough, 1982; Scarborough, 1985, 1992, 1996, 2000a,b), that major conformational changes do indeed occur in the catalytic cycles of the P-type ATPases that are not related to the putative E1/E2 conformational change that changes the reactivity of the enzymes to ADP or water and changes the sidedness and the affinities of the ion-binding sites at the same time. Thus, the major conformational change proposed to occur in the enzyme phosphorylation reaction is a significant step in the right direction. On the other hand, a weakness of the model is that it fails to recognize the essential role of transition state binding affinity as the driving force for this reaction and the other major conformational changes undergone by the P-type ATPases during their catalytic cycles. While this may be considered tolerable, it is not good from an heuristic point of view, since an ultimate complete description of the molecular mechanism of these enzymes will most certainly include this critical aspect of the reaction cycle.

Another problem with the model is the stipulation that the A or β domain does not undergo a conformational change in the enzyme phosphorylation reaction, which is almost certainly incorrect. In the case of the H^+-ATPase, the enzyme is strongly protected against tryptic hydrolysis in the presence of MgADP, which is a substrate analogue (Addison and Scarborough, 1982) for the enzyme phosphorylation reaction, and by Mg β,γ-methylene ATP, which is a transition state analogue of this reaction (Addison and Scarborough, 1982). Because the A domain contains numerous residues that are capable of being cleaved by trypsin in the absence of ligands (Addison and Scarborough, 1982; Hennessey and Scarborough, 1990; Scarborough and Hennessey, 1990), this profound protection against tryptic degradation can only be explained by a conformational change in the A domain induced by MgADP and β,γ-methylene ATP, presumably a rotation analogous to that proposed by Toyoshima *et al.* (2000) and by Xu *et al.* (2002) later in the reaction cycle (Fig. 4). This premise is further supported by the fact that the greatest extent of surface residue occlusion in the H^+-ATPase is induced by MgADP, as evidenced by the hydrogen/deuterium exchange rates in the presence and absence of this ligand (Goormaghtigh *et al.*, 1994). And finally, recent ca. 17 Å resolution structures of the H^+-ATPase obtained by single particle analyses of the enzyme in the presence and absence of MgADP (Rhee *et al.*, 2002), indicate that the outermost density of the H^+-ATPase hexamers moves toward the sixfold axis upon binding of MgADP. Since the A domain analogue in the H^+-ATPase (domain 4 of Fig. 2) is the outermost domain in the H^+-ATPase hexamers, and the N and P domain analogues are more centrally located (Auer *et al.*, 1998), this provides direct evidence of substantial movement of domain 4 of the H^+-ATPase toward the N and P domains

upon the binding of MgADP. Importantly, the domain 4 movement is accompanied by detectable changes in the transmembrane helix region (Rhee *et al.*, 2002). In view of all of the above considerations, it is clear that the A domain undergoes a substantial conformational change during the enzyme phosphorylation reaction, and this change leads to movements in the transmembrane helix region. This makes it less likely that the A domain rotation and associated movements of TM1–3 can be the driving force for the proposed E1 ∼ P to E2—P conformational change that expels the bound Ca^{2+} ions from their binding sites, because the majority of these movements probably occur before this step.

Another problem with the model is that it misleadingly overemphasizes the role of the enzyme conformational changes in the overall transport process. The conformational changes that occur as the enzyme proceeds from the E1 ∼ P form with Ca^{2+} tightly bound to the E2—P state represented by the structure of Xu *et al.* (2002) are proposed to convert high affinity Ca^{2+}-binding sites facing the cytoplasm to low affinity sites facing the other side of the membrane, from which the Ca^{2+} ions are released. Then, after dephosphorylation to the E2 state, the same conformation is capable of high affinity Ca^{2+}-binding from the cytoplasm that reverses all of these conformational changes to regenerate the El form. This appears to be a paradox from a purely conformational point of view. That is, if the conformational changes per se drive the Ca^{2+}-binding sites to a low affinity state facing the exocytoplasmic side of the membrane, then how can high affinity binding of cytoplasmic Ca^{2+} to these sites reverse the conformational changes? This suggests that one or the other of these premises is incorrect. Importantly Coan, *et al* have clearly shown by direct Ca^{2+}-binding studies that the Ca^{2+}-free, decavanadate-bound form of the enzyme representing the E2—P state in the model of Fig. 4 has high affinity Ca^{2+}-binding sites that are accessible from the cytoplasmic side of the membrane (Coan *et al.*, 1986). Moreover, the tubular crystals of the Ca^{2+}-ATPase that have yielded the 6 Å structure of this conformational state are destroyed by micromolar concentrations of Ca^{2+} (Xu *et al.*, 2002), indicating that the ATPase molecules in these crystals have a high affinity for Ca^{2+}. Thus, the premise that the purely conformational machinations that give rise to the structure of Xu *et al.* (2002) also convert the Ca^{2+}-binding sites to a low affinity form appears to be erroneous. The answer almost certainly lies in important differences in the ATPase molecule before and after the enzyme dephosphorylation reaction, but this is not appropriately recognized in the model.

Finally, again on the positive side, the model specifically stipulates that the latching of the TGES sequence in the β or A domain to the aspartyl phosphate in the phosphorylation/dephosphorylation site initiates the subsequent events that lead to expulsion of the Ca^{2+} ions from their binding sites on the far side of the membrane. This is likely to be true, and represents for the first time, a specific suggestion as to what might actually transpire when the traditional E1 ∼ P form of the enzyme "decides" to undergo a conformational change to the E2—P form and debind the transported ions. It is additionally attractive because latching of the TGES sequence to the aspartyl phosphate is tantamount to formation of the transition state of the enzyme dephosphorylation reaction, and high affinity binding of the enzyme to this transition state is almost certainly the driving force that makes the reaction proceed, in keeping with the generalized scheme shown in Fig. 1.

Beyond that, however, the model is too vague with respect to the events that happen next. After the TGES sequence latches to the aspartyl phosphate, a strain is put on the loops connecting the β domain with TM1–3, and this drives the Ca^{2+} ions from their binding sites. But it is difficult to see how this might actually be, particularly in view of the apparent flexibility of these connecting loops. Related to this, if the strain is strong enough to wrench the tightly bound Ca^{2+} ions from their binding sites and bring about rotation of the P domain, it is not clear why these conformational changes would not immediately reverse after the aspartyl phosphate linkage has been hydrolyzed. It is also proposed that the release of the Ca^{2+} ions from their binding sites then somehow relaxes the transmembrane helices, which conformationally signals back to the aspartyl phosphate via TM4 and TM5 to undergo hydrolysis. Again, while this may happen, it is difficult to imagine how it might actually occur. These vague aspects of the model are therefore of questionable heuristic value.

We are thus in need of a better model for the catalytic cycle of the P-type ATPases. Figure 5 shows a model that addresses most of the above concerns of the model of Xu *et al.* (2002), again using the Ca^{2+}-ATPase as the example. It begins (top left) at the same point of the catalytic cycle, with MgATP bound to the Ca^{2+}-liganded, open form of the Ca^{2+}-ATPase determined by Toyoshima *et al.* (2002). Except for the bound MgATP, this state is also represented by the ca. 8 Å structure of the H^+-ATPase determined by Auer *et al.* (1998). After binding MgATP, and the ions to be transported, the P-type ATPases then begin the enzyme phosphorylation reaction, which proceeds via the first transition state of the cycle, indicated as TSI. The structure of the P-type ATPases in this state is unknown, but the active site has to look something like that shown in Fig. 6, with numerous binding interactions stabilizing the transition state configuration, and with the aspartate to

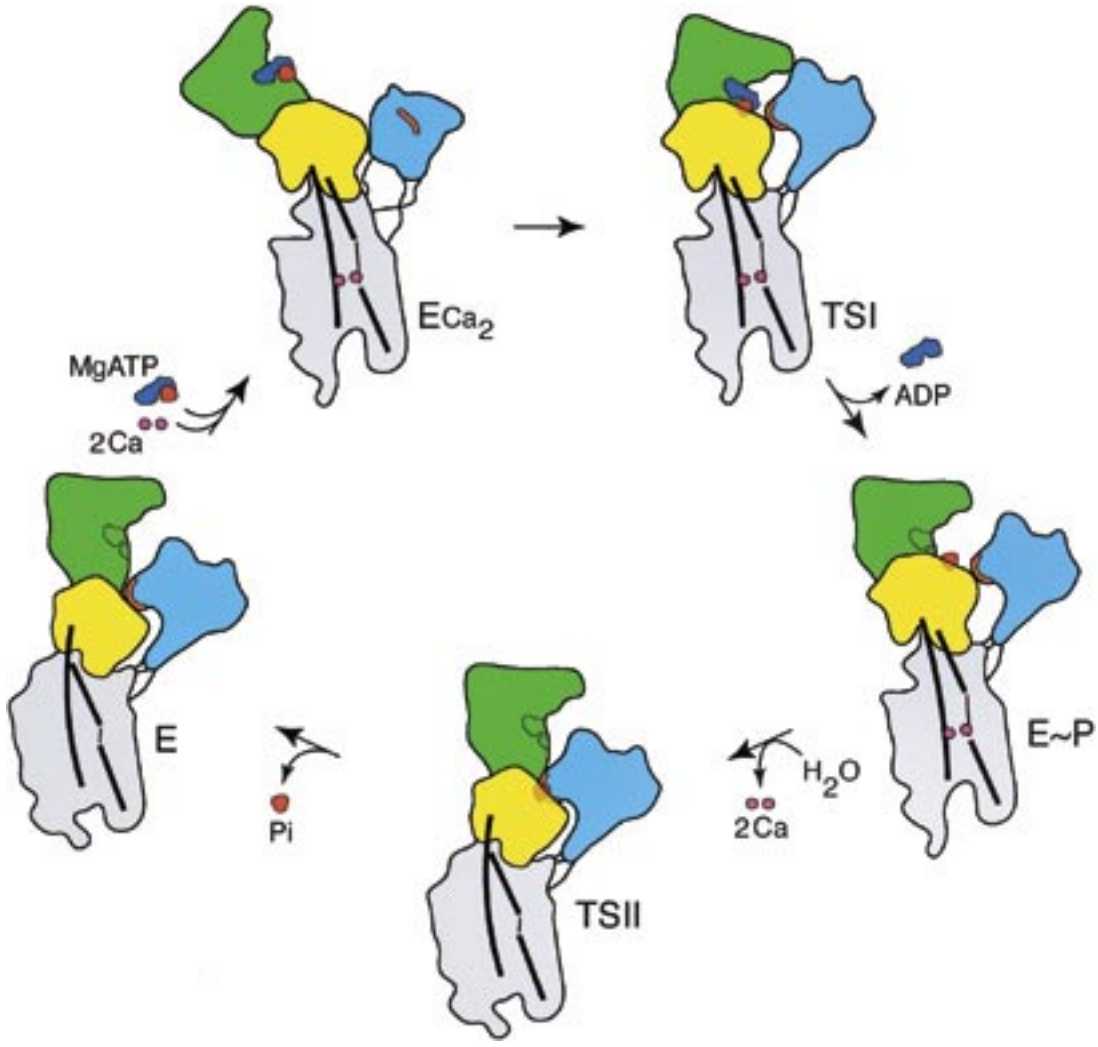

Fig. 5. Improved model for the catalytic cycle of the P-type ATPases. The key steps that occur during the catalytic cycle of the P-type ATPases are shown. The N, P, and A domains defined by Toyoshima *et al.* (2000) are colored green, yellow, and blue respectively. The transmembrane helix region below is colored gray. Other features are the same as those used in Fig. 4. The TGES sequence is colored orange. See text for details.

be phosphorylated as the incoming nucleophile and ADP as the leaving group. This form also must involve tipping down of the N domain to bring the MgATP close to the active site aspartate, and as mentioned above, it probably also involves rotation of the A domain. In keeping with Fig. 1, these interdomain motions and cleft closures are driven by the favorable bonding interactions approximated in Fig. 6 (Transition State I). For the H^+-ATPase, this is manifest as profound resistance to tryptic degradation in the presence of the nonhydrolyzable ATP analogue, Mg-β,γ-methylene ATP (Addison and Scarborough, 1982). For the Ca^{2+}-ATPase, this is near the form of the enzyme locked closed by CrATP, and by this point, the Ca^{2+} ions are occluded inside the molecule, as has been shown by several groups, including Serpersu *et al.* (1982) and Vilsen and Andersen (1992). But even though they are occluded, the ion-binding sites retain high affinity, as shown by Vilsen and Andersen (1992).

After the first transition state, the phosphoenzyme (E $\sim$ P) is formed. Little is known about the structure of this form. It may remain predominantly closed to aid the efficiency of the subsequent reaction, but at least the N domain must tip up to some extent with significant frequency, to allow the ADP/ATP exchange reactions that have been measured for most of the well-studied P-type ATPases. From the experiments of Andersen *et al.* (1985) with the Ca^{2+}-ATPase, this ADP-sensitive form of the enzyme also retains the bound Ca^{2+} ions at high affinity

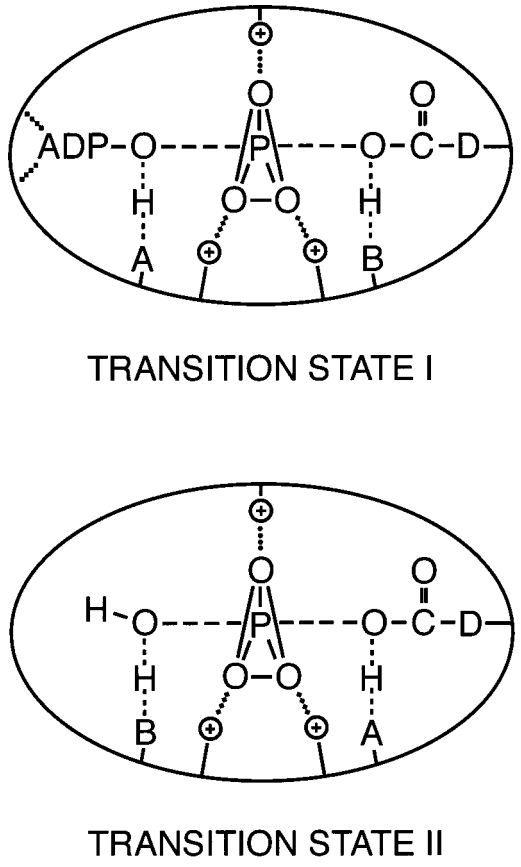

Fig. 6. Approximate transition states of the P-type ATPase phosphorylation and dephosphorylation reactions. The approximate arrangements of the reacting atoms at the transition state of the enzyme phosphorylation reaction (Transition State I) and the enzyme dephosphorylation reaction (Transition State II) for the P-type ATPases are shown. The D represents the phosphorylated aspartate. In the enzyme phosphorylation reaction above, ADP is the leaving group and the aspartate carboxylate is the entering group. In the enzyme dephosphorylation reaction below, the aspartate carboxylate is the leaving group and water is the entering group. Other symbols are as for Fig. 1.

sites. Thus, this form of the enzyme has all of the key characteristics of the classical E1 $\sim$ P state.

The next step in the reaction cycle is a nucleophilic attack by the hydrolytic water molecule on the phosphoryl-aspartate linkage, with the resultant formation of the second transition state (TSII in Fig. 5). The active site in this reaction must look something like that shown in Fig. 6 (Transition State II), with water attacking and the phosphorylated aspartate leaving the phosphoryl phosphorus atom. This state must also involve extensive cleft closure, driven by the favorable bonding interactions indicated in Fig. 6 and in accord with the general scheme of Fig. 1. This is the form that these enzymes assume in the presence of orthovanadate, a transition state analogue of the enzyme dephosphorylation reaction, as mentioned above. For the H^+-ATPase, this is manifest again as extreme resistance to tryptic cleavage (Addison and Scarborough, 1982) and extensive occlusion of the surface residues of the H^+-ATPase in this form, as indicated by our hydrogen/deuterium exchange experiments (Goormaghtigh *et al.*, 1994). Interestingly, the tryptic cleavage pattern is indistinguishable from that of the enzyme locked in transition state I (Addison and Scarborough, 1982), which seems to indicate that the nucleotide binding domain remains involved at this stage of the cycle, and that the two structures are quite similar. Importantly, site-directed mutagenesis studies of the Ca^{2+}-ATPase strongly suggest that the highly conserved TGES sequence is involved at this stage of the reaction (Andersen and Sorensen, 1996), but not before, and this sequence is very close to the phosphorylated aspartate in the structure of Xu *et al.* (2002) of the Ca^{2+}-free, decavanadate-bound form of the enzyme. It thus stands to reason that the structure of Xu *et al.* represents the conformational state of the enzyme very near transition state II of the catalytic cycle. Thus, the structure termed TSII in the model of Fig. 5 represents the structure obtained by Xu *et al.*, named the E2—P state in the model of Fig. 4, at least with respect to the cytoplasmic domain and transmembrane helix arrangements. As indicated in the model, this form of the enzyme has indeed lost its affinity for its bound Ca^{2+} ions and has released them, but as mentioned above, this is not due to the domain movements per se. The actual underlying reasons for this constitute the essence of the energy coupling problem for the P-type ATPases and are discussed in some detail below.

Upon the breakdown of the second transition state, the enzyme is dephosphorylated, resulting in the unliganded E state shown in the model of Fig. 5. Again, the structure of this form is unknown, but on the basis of Fe-catalyzed cleavage studies of the TGES region of the Na^+/K^+-ATPase (Patchornik *et al.*, 2000), it is likely that the active site aspartate and the TGES sequence remain close, as shown. This is the form of the enzyme that can be phosphorylated by Pi in the absence of Ca^{2+} ions, so it is close to the state traditionally referred to as E2. But importantly, there is ample evidence that by this point of the cycle, i.e., the dephospho-enzyme, the ion-binding sites are again high affinity and accessible from the cytoplasmic side of the membrane. This was established long ago by Dupont (1982), and in separate studies by Champeil *et al.* (1983). The failure of the unliganded enzyme to rapidly return at least one of the ion-binding sites rapidly to the cytoplasmic side of the membrane was cited by Tanford as a fatal flaw in the traditional E1–E2 model (Tanford, 1985). And this important premise was proved beyond reasonable doubt in the thorough experimental analysis of

Petithory and Jencks (1988). Nevertheless, the proposed conformation of the dephosphorylated E state of the enzyme in the model of Fig. 5 indicates that the atoms that constitute the Ca^{2+}-binding sites, TM4 and TM5, and the rotated orientation of the P domain remain similar to that in TSII, in which the Ca^{2+} ions are released at low affinity. This is not a contradiction because, as discussed above, this arrangement of the binding site atoms, TM4 and TM5, and the P domain does not, per se, bring about Ca^{2+} ion release. They remain in this conformation in the E state simply because this is the most stable arrangement that they can assume in the absence of Ca^{2+} ions.

In the final step of the reaction cycle, the enzyme binds a new pair of Ca^{2+} ions from the cytoplasmic side of the membrane and reverts to a state near the Ca^{2+}-bound structure solved by Toyoshima *et al.* (2000). This step of the model is same as that in the model of Xu *et al.* (2002). Because the two Ca^{2+}-binding sites are not well formed in the E form of the enzyme, this process must involve a concerted reaction in which the binding of the Ca^{2+} ions to a few of the binding site atoms brings about a rearrangement of the other Ca^{2+} ligands, which regenerates the two complete Ca^{2+}-binding sites with the ions bound, and drives the remaining conformational changes in the transmembrane helices and the cytoplasmic domains. It is pertinent in this regard that simple side chain rotations of the Ca^{2+} site I ligands (Toyoshima *et al.*, 2000) of the Ca^{2+}-free structure generate an essentially complete Ca^{2+}-binding site. This concerted rearrangement occurs spontaneously in the presence of Ca^{2+} ions because this is the most stable arrangement of the various elements when Ca^{2+} ions are bound. This sequence of events in the process of Ca^{2+} binding to the E form of the enzyme is completely consistent with the cooperative Ca^{2+}-binding process characterized in the direct Ca^{2+}-binding studies of Petithory and Jencks (1988).

As an additional note regarding this step, the analysis of Stahl and Jencks (1987) indicates that during a normal catalytic cycle, the reaction does not proceed via the wide open Ca^{2+}-bound structure of the enzyme solved by Toyoshima *et al.* (2000) as depicted in Fig. 3. Rather, it normally proceeds by rapid binding of ATP and the Ca^{2+} ions to the E form followed by phosphoenzyme formation at a high rate. Thus, in normal cycling, the more vertical orientation of the N domain depicted for the E form is probably maintained after the binding of MgATP and the two Ca^{2+} ions. In fact, although beyond the scope of this paper, a much more detailed reaction cycle model for the Ca^{2+}-ATPase could readily be constructed with energies and rates and approximate structures using the values carefully measured and compiled by Pickart and Jencks (1984) together with the structural information currently available.

As a final point regarding the catalytic cycle of the P-type ATPases, examination of the fundamental reaction scheme deduced by Jencks *et al.* (1989) on the basis of their analysis of the Ca^{2+}-ATPase reaction cycle reveals that it contains the same essential features as the catalytic cycle described in Fig. 5. Thus, in the scheme of Jencks, the ion-binding site access changes are driven by phosphorylation and dephosphorylation of these enzymes. The ion-binding sites face the uptake side in the unphosphorylated form and are capable of debinding the ions to the release side after phosphorylation. Upon dephosphorylation, the ion-binding sites rapidly become accessible again on the uptake side of the membrane. This is a much more realistic view of the catalytic cycle of the P-type ATPases than the traditional E1–E2 scheme, and there is a great deal of solid evidence in support of it.

THE ENERGY COUPLING MECHANISM

The results of the foregoing analysis obviously beg the following question: If the sweeping conformational changes that convert the structure of the Ca^{2+}-bound form of the Ca^{2+}-ATPase elucidated by Toyoshima *et al.* (2000) to the Ca^{2+}-free form elucidated by Xu *et al.* (2002) per se do not expel the Ca^{2+} ions from their binding sites as posited above, then what does? Somehow, the chemical events occurring during the enzyme dephosphorylation reaction in the cytoplasmic region exert effects on the ion-binding sites 50 Å away, leading to a decreased affinity for the ions, and the basis for this is the key remaining question in the P-type ATPase field. A related question is the basis for Pickart and Jencks' inescapable conclusion that there is mutual repulsion between the phosphorylated aspartate and the bound ions in the Ca^{2+}-ATPase (Pickart and Jencks, 1984). And another similar question is the basis for the observation that the binding of Ca^{2+} ions to the Ca^{2+}-free orthovanadate-bound form of the Ca^{2+}-ATPase can drive the orthovanadate out of its tightly bound transition state II complex with D351 and other residues at the dephosphorylation site (Coan *et al.*, 1986). These are all probably manifestations of the same phenomenon of communication between the phosphorylation/dephosphorylation site and the ion-binding sites through a considerable distance. And the means by which this communication is achieved constitutes the solution to the energy coupling problem for the P-type ATPases.

The traditional answer to this problem has been the proposal that the communication is achieved by long-range conformational changes in the molecule. However, we have already ruled out the direct involvement of all of the conformational changes in the P-type ATPases known

to exist, and so this answer would require the oft-used gambit of "further conformational changes" (MacLennan *et al.*, 1997) to a new state. But this would only delay a solution as we wait for the structure of that new state, which will never be obtained if it does not exist.

There is, however, an attractive alternative solution to the problem, and it can be almost completely developed and described using structural information that is already available. Since both the dephosphorylation reaction and ion debinding are really only electronic rearrangements among the atoms involved, all that is needed is one or more paths in the ATPase molecule through which electronic signals can be transmitted. An ideal building block for such a path in proteins is the peptide bond itself. As explained by Pauling *et al.* (1951) and diagrammed in Fig. 7, the peptide bond is a hybrid structure of two limiting electronic forms linked by resonating π electrons smeared between the C′—O and C′—N bonds. It is this

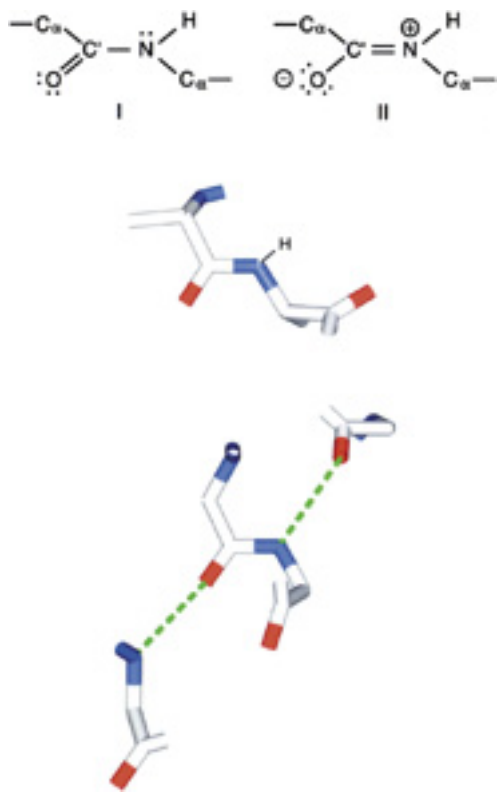

Fig. 7. The peptide bond as a charge transfer unit. The upper part of the figure shows the two limiting electronic structures of the peptide bond, labeled I and II. Below these is a sticks representation of the peptide bond viewed from a similar perspective. The bottom part of the figure shows a sticks representation of a small charge transfer complex comprising three peptide linkages and two hydrogen bonds. See text for details.

electronic arrangement of the peptide linkage that gives rise to its characteristic bond lengths and fixed planar nature, and importantly in the present context, its large dipole moment (Schulz and Schirmer, 1979). Thus, a positive charge placed near the carbonyl oxygen of one residue of a dipeptide linkage will attract the π electrons, thereby transmitting the positive charge to the amide —NH of the second residue. Moreover, any carbonyl oxygen that is hydrogen-bonded with that amide —NH will receive the positive charge pulse and transmit it to its amide —NH partner by the same mechanism. A small charge transfer complex of this sort involving three peptide linkages and two hydrogen bonds is shown at the bottom of Fig. 7. It follows that any hydrogen-bonded chain involving C′—O and C′—NH linkages will transfer charge along its entire length. This is similar to the ionic defect transfer part of the hydrogen-bonded chain mechanism for proton conduction in proteins suggested by Onsager (1969) and reviewed by Nagle and Tristram-Nagle (1983), but with no second rotation steps and hence no proton conduction.

Figure 8 shows that two such paths do indeed exist in the Ca^{2+}-ATPase molecule. The first of these, path I, is shown in Fig. 8a. It begins at the water molecule bound to the Ca^{2+} ion in Ca^{2+}-binding site I and extends via 764 O (lower arrow) all of the way up to 353 N (upper arrow) within a few angstroms of D351 (asterisk). It runs primarily along TM5, but crosses at the top of TM5 via 740 N into the β sheet section of the P domain and makes the final connections via the strand that contains D351. The second path, path II, is shown in Fig. 8b. It begins at the Ca^{2+} ion in Ca^{2+}-binding site II directly liganded to the carbonyl oxygen, 307 O (lower arrow), and extends all the way up to amide 723 N (upper arrow) near the probable active site residue, D703 (filled circle). It travels up the TM4 α-helix and then crosses over to the β-sheet structure of the P domain, where the final connections are made between several of the adjacent strands. Interestingly, this path passes right through the unwound helix region of TM4, which contains the strictly conserved PEGLP sequence beginning at P308. The strict conservation of this sequence in virtually all P-type ATPases would appear to be necessary in order to maintain the integrity of the charge transfer pathway in this region. This also probably explains why mutation of P312 interferes with the E1P to E2P transition (Andersen and Sorensen, 1996). Amazingly, both charge transfer pathways consist entirely of main chain atoms. This may serve as a very effective means of protecting the paths against loss of function due to random mutational events, since most side chain mutations would only indirectly affect the function of the charge transfer pathways.

Figure 8c shows both of the charge transfer pathways together. It also shows that each pathway actually

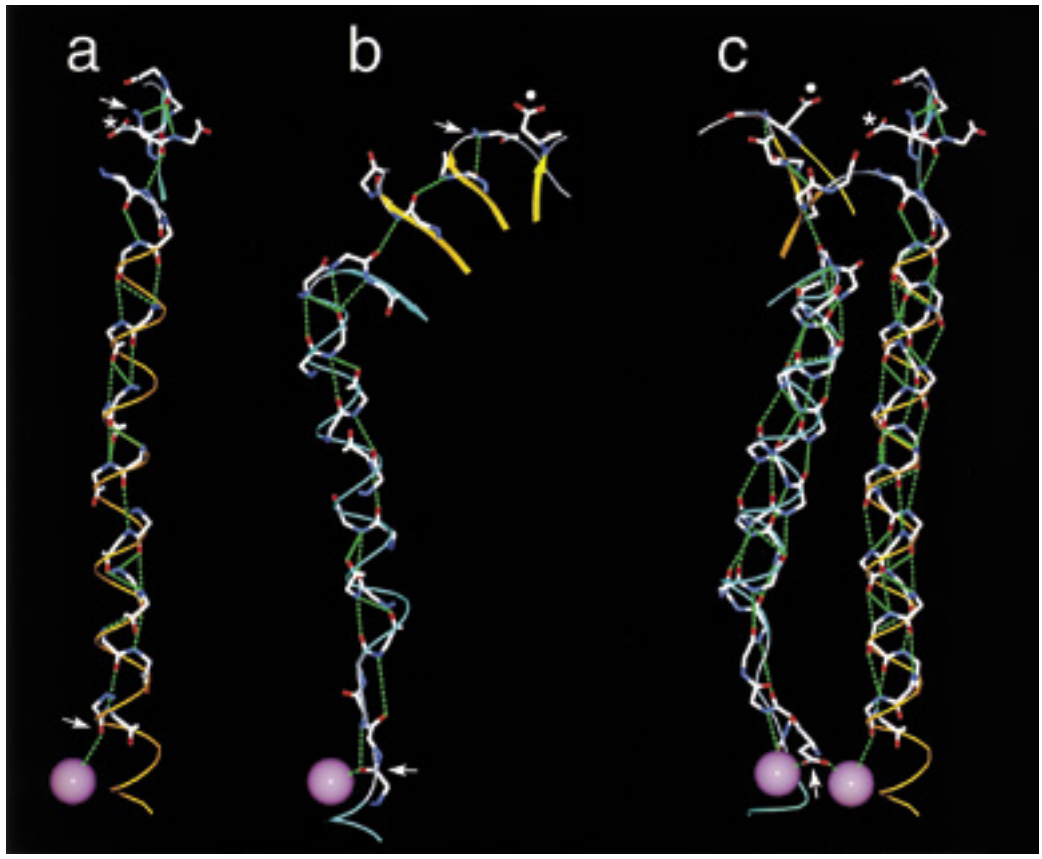

Fig. 8. Charge transfer pathways in the Ca^{2+}-ATPase. Two resonating H-bond pathways capable of charge transfer connect the Ca^{2+} ions in their binding sites with residues very near the phosphorylation/dephosphorylation site. The H-bonds or direct Ca^{2+} linkages are shown as green dashed lines and the Ca^{2+} ions are shown in purple. Panel a shows one of the charge transfer pathways. The lower arrow points to 764 O and the upper arrow points to 353 N. The asterisk marks the side chain carboxyl group of D351. Panel b shows the other charge transfer pathway. The lower arrow points to 307 O and the upper arrow points to 723 N. The filled circle marks the side chain carboxyl group of D703, another key residue. Panel c shows the two charge transfer pathways together. The filled circle and the asterisk mark the side chain carboxyl groups of D703 and D351 as in Panels a and b. The arrow in Panel c points to the side chain carboxyl group of D800. Only selected main chain residues and H-bonds are shown in Panels a and b. Panel c shows a more complete representation of the main chain residues and H-bonds that constitute the charge transfer pathways. The presence of H-bonds was judged by the programs RasMol and Deep View Swiss-Pdb Viewer using the default settings. The structures shown were generated as for Fig. 3.

splits into three electrically parallel paths for a substantial distance. Path I splits just above 764 O and then converges again at 741 O at the top of TM5. Path II splits at 314 N, just after the unwound helix region of TM4 and then converges again at the top of TM4. Since the two pathways are linked via D800 (arrow in Fig. 8c), they too are linked in an electrically parallel fashion. This design of the charge transfer pathway complex may be significant. First, it should substantially decrease the electrical resistance of the signalling pathway. But possibly more importantly, this feature would also help prevent loss of function of the paths due to indirect structural perturbations caused by mutation, by providing alternate charge transfer routes for each of the paths.

In addition to the points of connection between the bound Ca^{2+} ions and the charge transfer pathways described above, there are probably other connections as well. But at a structure resolution of 2.6 Å, and without the coordinates of probable bound water molecules or the actual density for inspection, it is difficult to propose others with certainty. However, there appears to be a substantial amount of extra density in the region of the bound Ca^{2+} ions (Toyoshima *et al.*, 2000) that may be due to numerous other bound water molecules, which could serve

as alternative or additional links between the bound Ca^{2+} ions and the charge transfer pathways. As a good example of this, D771 Oε1 is a Ca^{2+} ligand and D771 Oε2 in resonance with it is 4.3 Å away from 768 O, which is connected with path I. A water molecule in this space could make another connection between the Ca^{2+} ion in site I and path I. Additional possibilities can be seen, but they are not discussed here.

In sum, it is very likely that residues at the phosphorylation/dephosphorylation site and both bound Ca^{2+} ions are electronically linked via two charge transfer pathways consisting of resonating main chain C′, O, N, and H atoms, and possibly water molecules as well. It is proposed that one or both of these pathways transmit electronic signals generated during the enzyme dephosphorylation reaction to the bound Ca^{2+} ions, promoting their debinding and diffusion away on the lumenal side of the enzyme. Likewise, the bound Ca^{2+} ions influence the chemical reactions at the phosphorylation/dephosphorylation site via the same pathways. These charge transfer pathways thus constitute the atomic and molecular basis of the energy coupling mechanism.

MOLECULAR MECHANISM OF THE P-TYPE ATPases

The final step to an understanding of the molecular mechanism of the P-type ATPases will be to precisely define the side chain movements and atomic rearrangements occurring during the enzyme dephosphorylation reaction that give rise to the charge pulse that drives the Ca^{2+} ions from their binding sites via the charge transfer pathways described above. Unfortunately, this will be possible only when an atomic resolution structure of a P-type ATPase in transition state II, complete with the chemical reactants, becomes available. Fortunately, because most, if not all, P-type ATPases tightly bind the transition state II analogue orthovanadate, it can be anticipated that even this final step may become possible quite soon because it should be possible to crystallize at least one of these in the presence of Mg plus orthovanadate. But even as we wait, on the basis of the structural and biochemical information we have now, it is possible to visualize an outline of the complete molecular mechanism.

The enzyme dephosphorylation reaction must proceed via a transition state II arrangement something like that shown in Fig. 6. The presence of bound Ca^{2+} ions will generate positivity via the charge transfer pathways at certain active site residues, which may include one or more of the positive centers indicated by the circled "+" symbols. This will delocalize the electron shield protecting the phosphorus atom and thereby assist the nucleophilic attack by the hydrolytic water molecule as shown. As indicated by the A, positivity at another residue may also provide general acid assistance to the leaving group, which is probably D351 Oδ1. Binding of the hydrolytic water molecule via a general base B as shown will also promote the formation of transition state II, as would binding of the other water hydrogen atom to a second general base not shown. Together, these forces will stabilize transition state II as shown and thereby promote the enzyme dephosphorylation reaction. This is presumbly at least part of the basis for the mutual repulsion between the bound Ca^{2+} ions and the phosphoryl aspartate, concluded to exist by Pickart and Jencks (1984). And the positive push on the leaving group readily explains the observation of Coan *et al.* (1986) that Ca^{2+}-binding drives orthovanadate off of the enzyme. Moreover, if the general base or bases are linked to either or both of the charge transfer pathways, then binding of the positive end of the strongly dipolar hydrolytic water molecule would push back on the bound Ca^{2+} ions, promoting their release from the Ca^{2+}-binding sites. And, since the charge transfer pathways actually extend somewhat below the ion-binding site region, the positive push should assist the diffusion of the ions toward the release side of the membrane. This positive push would also contribute to the mutual repulsion between the phosphoryl aspartate and the bound Ca^{2+} noted by Pickart and Jencks (1984). It is pertinent that all of the Ca^{2+}-binding atoms in both binding sites are electronically interconnected with each other and both charge transfer pathways. Thus, a positive push via either charge transfer pathway would act on all of the Ca^{2+}-binding ligands. These proposed interactions are also reasonably consistent with the data of Andersen *et al.* (1985), which suggest that the Ca^{2+} ions debind before the E2P hydrolysis reaction has occurred. Within the context here, they would leave at transition state II, which would still probably be measured as phosphoenzyme under the acid quench conditions of these experiments. Pickart and Jencks also raised the possibility that the Ca^{2+} actually dissociates from the transition state of the phosphoenzyme hydrolysis reaction (Pickart and Jencks, 1984).

The above forces alone may be adequate to explain how the formation of transition state II could drive the Ca^{2+} ions from their binding sites, promoting their release to the far side of the Ca^{2+}-ATPase. However, the measurements of Coan *et al.* (1986) indicate that Ca^{2+} drives the transition state II analogue, orthovanadate, off of the Ca^{2+}-ATPase with an affinity of about 50 μM. This may mean that the Ca^{2+} sites have not reached their lowest affinity at transition state II. This could possibly reflect an imperfectness of orthovanadate as a transition state

analogue of the enzyme dephosphorylation reaction, or it might indicate that a complete breakdown of transition state II and the resultant full release of the hydrolytic water molecule protons are needed to fully drive the Ca^{2+} ions from their binding sites via the charge transfer pathways. If the latter possibility is correct, it would presumably indicate that the liberated Pi, properly positioned in the binding site, would still revert to phosphoenzyme under the acid quench conditions of the experiments of Andersen *et al.* (1985). It is also conceivable that the hydrolytic water proton(s) exert a more direct effect on the ion-debinding reaction by diffusion or hopping from the dephosphorylation site to the ion-binding site via a conspicuous channel that runs between these sites.

Although not essential to the energy coupling mechanism, it should be mentioned for the sake of completeness that the bound Ca^{2+} ions probably also electronically promote the formation of the transition state of the enzyme phosphorylation reaction (transition state I) via the charge transfer pathways in ways similar to those proposed here for the enzyme dephosphorylation reaction but with different attacking and leaving groups.

While the above events of the phosphoenzyme hydrolysis reaction and the Ca^{2+}-debinding reaction coupled via the charge transfer pathways are proceeding, forces are also at work on the protein structure all along the charge transfer pathways. Figure 9 shows some of the major interactions. When the positivity from the hydrolytic water

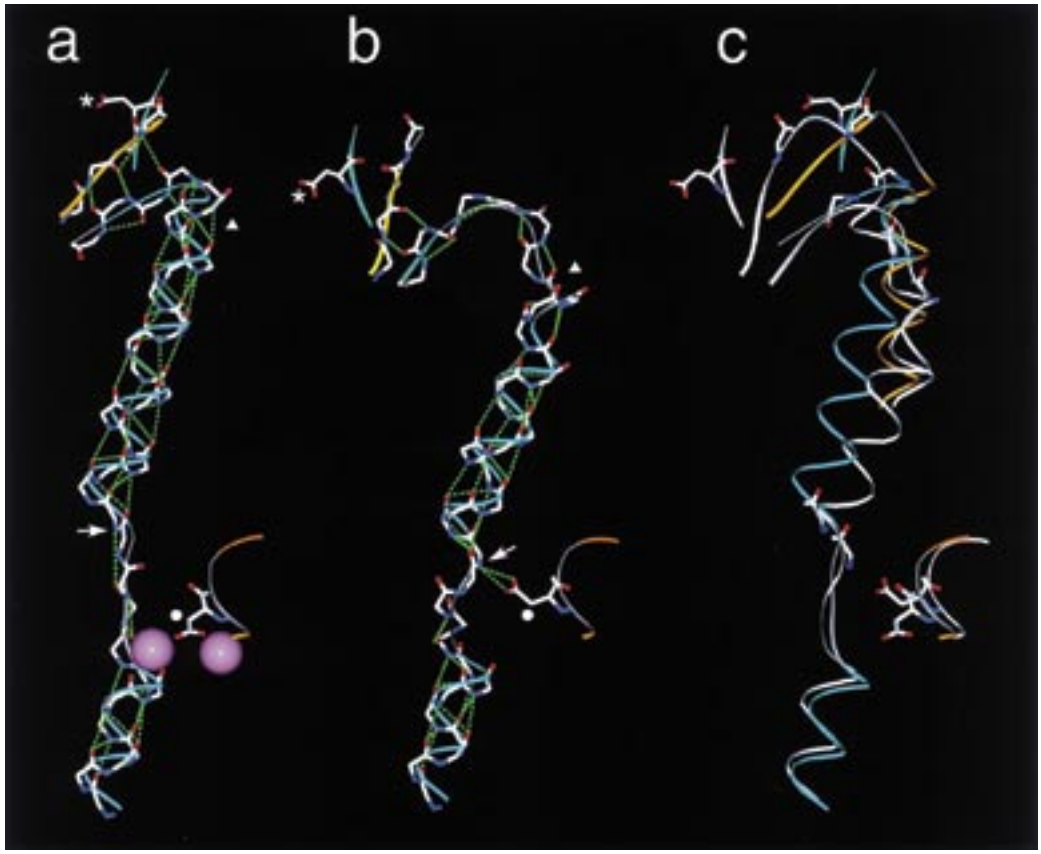

Fig. 9. Key structural changes along the charge transfer pathways. Important structural changes that occur along the charge transfer pathways as the Ca^{2+}-liganded form of the Ca^{2+}-ATPase determined by Toyoshima *et al.* (2000) is converted to the Ca^{2+}-free structure of Xu *et al.* (2002) are shown. Asterisk: D351 side chain carboxyl group; filled triangle: region of M326; arrow: 311 N; filled circle: D800 side chain. Panel a shows the Ca^{2+}-liganded structure. TM4 is shown in cyan; the β-strand at the top of TM5 is light orange; the part of TM6 where D800 is located is in darker orange; H-bonds are in green; the bound Ca^{2+} ions are in purple. Panel b shows the Ca^{2+}-free structure. The color scheme is the same as for Panel a. Panel c shows the same region of the two structures together for comparison, with the top part of TM5 included. In this case, colored ribbons indicate the Ca^{2+}-liganded structure, and white ribbons indicate the Ca^{2+}-free form. The alignment of the two structures was done by the program Deep View Swiss-Pdb Viewer selecting only TM5 residues 742–778 for the fitting function. The presence of H-bonds was judged as for Fig. 8 and the structures shown were generated as for Fig. 3.

molecule in the dephosphorylation site begins to exert its presence on the bound Ca^{2+} ions via the charge transfer pathways, the positivity at 311 N (arrow) increases. Since the hydrogen bond is a continuous electrostatic energy function of the charge on the bonding partners and the distance and angle between them (Kabsch and Sander, 1983), the increased positivity at 311 N will strengthen and shorten the long H-bond between 311 N and 308 O, resulting in a downward movement of the upper part of TM4 and assisting in Ca^{2+} expulsion by distorting Ca^{2+}-binding site II. At the same time, the positivity on 311 N pulls on the D800 side chain (filled circle) carboxyl group, or favors the closer rotamer, causing it to swing up toward 311 N and hydrogen-bond with it. This further distorts both Ca^{2+}-binding sites since the D800 carboxyl group is a ligand for both. The strengthening connection between the D800 side chain carboxyl group and 311 N further pulls the upper part of TM4 down, leading to further distortion of Ca^{2+}-binding site II. This downward movement of the upper part of TM4 pulls the P domain down with it, causing the P-domain rotation, and causing TM5 to bend. But with resistance from the P domain and TM5 and torsional constraints, this downward movement also opens the tight turn at the top of TM4, breaking several H-bonds in the region of M326 (filled triangle) in the process, and partially separates the β-sheet connecting the tops of TM4 and TM5. To the extent that the model of this 6 Å resolution structure is accurate, this process also breaks both charge transfer pathways by a few angstroms. And finally, although not shown in Fig. 9, by this time, all of the former Ca^{2+}-binding atoms except the main chain carbonyls 304 O, 305 O, and 307 O, have found new partners via H-bonds. These three potential Ca^{2+} ligands presumably remain free to initiate the concerted conformational change that will occur when the next cytoplasmic Ca^{2+} ions bind.

The occurrence of all of the above events in concert results in the completion of the phosphoryl aspartate hydrolysis reaction and release of the Ca^{2+} ions, and the enzyme structure is near that determined by Xu *et al.* (2002). It is thus nearly ready to bind a new pair of cytoplasmic Ca^{2+} ions and begin another round of the cycle. But a significant problem remains at this point, as is the case for any model for the P-type ATPases. If the driving forces for the release of the Ca^{2+} ions arise from the events occurring during the enzyme dephosphorylation reaction as proposed above, then it must be explained why the released ions do not simply rebind when the enzyme dephosphorylation reaction has been completed. And the answer with respect to this model is that if this were to begin to occur, the charge transfer pathways would reform (if they are indeed broken in the real structure), and the bound hydrolytic water protons would prevent any further rebinding via their positive push back down the charge transfer pathway(s). This resistance to rebinding would persist until the enzyme has reopened enough to release the hydrolytic water protons and the Pi as well. At that point, preferential ion binding from the cytoplasmic side may be aided by a more efficient arrangement of ligands for removing the hydration shells of the Ca^{2+} ions in the cytoplasmic entry path than in the exocytoplasmic exit path.

CONCLUSION

In light of the discussion presented, it is likely that the P-type ATPases do not work by the traditional mechanochemical coupling mechanism in which events occurring at the site of phosphorylation and dephosphorylation mechanically drive the ion-binding site residues from one side of the membrane to the other and change their affinity in the process. While sweeping conformational changes do occur in these enzymes, their primary function is to facilitate the course of the chemical reaction sequence by optimizing transition state binding affinity. But the effects of the chemical events occurring at the phosphorylation/dephosphorylation site must somehow be transmitted to the ion-binding sites in the membrane so as to drive the release of the ions to the other side of the membrane. And the means by which this is accomplished is the essence of the molecular mechanism of the P-type ATPases. Rather than purely conformational coupling, it is proposed that these enzymes use an energy coupling mechanism of a different sort. Electronic events occurring at the phosphorylation/dephosphorylation site are transmitted to the ion-binding sites via two well-defined charge transfer pathways that run between the sites. These electronic events directly contribute to expulsion of the transported ions and additionally drive conformational changes in the binding site region that further assist ion debinding. The recognition of these charge pathways allows the formulation of a first-generation model for the complete molecular mechanism of the P-type ATPases. All that remains before the complete solution are atomic details of the structure of a P-type ATPase in the transition state of the enzyme dephosphorylation reaction.

Finally, with the likelihood that charge transfer pathways are essential in the function of the P-type ATPases, it is worth considering the possibility that they may operate in other enzymes as well. One group of enzymes that comes to mind in this regard is the F_1F_0 ATP synthases of mitochondria, chloroplasts, and bacteria with their long helical γ subunit (Bianchet *et al.*, 1998; Gibbons *et al.*, 2000) that transmits a long-range signal from the F_0 sector in the membrane to the sites of ATP synthesis

in the $\alpha_3\beta_3$ complex. Another is the animal cell tyrosine-specific protein kinase coupled receptors with only a single transmembrane helix through which the signal transmission must occur. Similar signalling between intracellular enzymes and effectors is equally feasible.

ACKNOWLEDGMENT

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