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Why We Must Move On From the E1E2 Model for the Reaction Cycle of the P-Type ATPases

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Recent progress regarding the structure of the Ca^{2+}-translocating ATPase of sarcoplasmic reticulum in several conformational states, and a substantial accumulation of biochemical information about this and other P-type ATPases, have put everything in place for the final convergence of biochemistry and structure that will lead to a complete understanding of the molecular mechanism of these membrane transport enzymes. But the common paradigm used to describe the reaction cycle of the P-type ATPases, the E1E2 model, is seriously flawed, and this is hindering our progress toward this goal. In this paper, it is first shown why the E1E2 model must be discarded. This is followed by a description of the P-type ATPase catalytic cycle that is much more consistent with the structural and biochemical information now available for these enzymes, and also brings to light the origin of the forces that drive the key reaction in the active transport cycle where high-affinity ion-binding sites are converted to low-affinity binding sites capable of releasing the transported ions against a considerable concentration gradient. This new model will therefore serve us better as we seek to unravel the final details of the molecular mechanism of active ion transport catalyzed by these enzymes. It is thus time to move on from the traditional E1E2 model.

KEY WORDS: E1E2 model; P-type ATPases; active ion transport; reaction cycle; energy transduction.

INTRODUCTION

The P-type ATPases are a family of biological energy transducers that catalyze the thermodynamically uphill movements of ions at the expense of the chemical free energy of ATP hydrolysis. The family is widely represented throughout the evolutionary tree (Stangeland *et al.*, 1997) and is so-named for the participation of a uniquely conserved phosphorylated aspartate intermediate in the chemical reaction sequence (Pedersen and Carafoli, 1987). An understanding of the molecular mechanism by which these enzymes catalyze ATP-hydrolysis-driven ion translocation is an important objective of biology. Accordingly, a large amount of biochemical information about these enzymes has been accumulated over the past half-century since the first P-type ATPase was discovered (Skou, 1957). But while the biochemical information regarding the P-type ATPases burgeoned, the equally essential information as to the structure of these enzymes was not forthcoming, and progress toward mechanistic insight was thus agonizingly slow. Fortunately, the wait has ended, and atomic or near-atomic resolution models of the Ca^{2+}-ATPase in conformational states representing three key stages of the reaction cycle have become available over the past 3 years (Toyoshima *et al.*, 2000; Toyoshima and Nomura, 2002; Xu *et al.*, 2001). Unfortunately, this new structural information is likely to yield only minimal progress toward mechanistic understanding under the current state of affairs. This is because the E1E2 model normally used to describe the P-type ATPase reaction cycle is inadequate, and the perceptible reluctance to recognize this fact and discontinue its use is leading to difficulty in interpreting the meaning of experimental results.

The purpose of this paper is to describe the numerous reasons that lead to the conclusion that the E1E2 model is no longer useful, and to then proceed with a description of the main steps of the P-type ATPase reaction cycle based only on model-independent structural and biochemical facts, with attention to established principles of

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enzyme catalysis. The model that emerges from these considerations is completely consistent with all of the major biochemical and structural information that has been accumulated for these enzymes, with none of the obvious pitfalls of the traditional E1E2 model. It also emphasizes the pivotal roles of ligand binding as the driving force for the enzyme conformational changes, and transition state binding as the driving force for catalysis. And considering the reaction cycle in these terms brings new and meaningful insight as to how these forces interplay in the energy-coupling step to bring about the active transport of the ions against a concentration gradient. On the basis of all of these considerations, it is recommended that the E1E2 model be replaced with the more useful description of the P-type ATPase reaction cycle presented here. This will in turn allow us to focus on the few remaining issues that must be resolved before we can say that we truly understand how the P-type ATPases work.

THE E1E2 MODEL AND ITS PROBLEMS

Figure 1 shows the essential features of the E1E2 model for the reaction cycle of the P-type ATPases, adapted from the paper by DeMeis and Vianna (1979), which is most often cited in reference to this model. Because the Ca^{2+}-ATPase is by far the most well-understood P-type ATPase, the model is portrayed for this enzyme. It purports the existence of two, and only two, distinct conformational states of the enzyme, which we shall call E1 and E2 in this paper. The E1 state has high-affinity (micromolar) binding sites for two Ca^{2+} ions that are accessible only from the cytoplasm. The E2 state has two Ca^{2+}-binding sites that are of low affinity (millimolar) and are accessible only from the opposite side of the membrane, or lumen of the sarcoplasmic reticulum in this specific case. In the presence of micromolar concentrations of Ca^{2+}, the

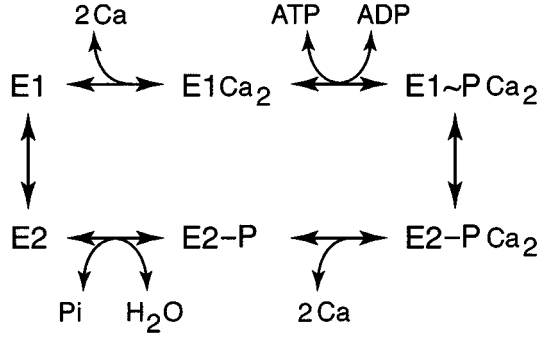

Fig. 1. The E1E2 Model. A traditional E1E2 model for the P-type ATPase reaction cycle adapted from the review by DeMeis and Vianna (1979) is shown (see text for details).

E1 form can be phosphorylated by ATP to form a so-called high-energy phospho-enzyme intermediate, $E1 \sim PCa_2$. It cannot be phosphorylated by inorganic phosphate, Pi. On the other hand, the E2 state can be reversibly phosphorylated by Pi to form a so-called low-energy phospho-enzyme intermediate, E2-P. The mechanism of ion transport catalyzed by this arrangement thus involves binding of two cytoplasmic Ca^{2+} ions to the high-affinity sites followed by phosphorylation by ATP to form the high-energy phospho-enzyme, which then undergoes the essential conformational change to the E2-P state, which releases the two Ca^{2+} ions on the other side. This then hydrolyzes to produce Pi and the unliganded E2 state. The unliganded E2 state is in equilibrium with the unliganded E1 state via a slow conformational isomerization reaction, with the E2 state as the predominant species.

In order for an experimental model to be useful, it must make accurate predictions about the system it is meant to describe. And this is perhaps the most glaring failure of the E1E2 model. It predicts that in the absence of the ions to be transported, the unliganded enzyme rests primarily in the E2 state. This means that the ion-binding sites are of low affinity and are facing the ion-release side of the membrane. Presumably for this reason Xu *et al.* (2002) described their structure of the Ca^{2+}-free, decavanadate-bound form of the Ca^{2+}-ATPase as that of the E2 state. But somewhat surprisingly, in the same communication, it was noted that the tubular crystals of the Ca^{2+}-ATPase that gave rise to their structure are destroyed by micromolar concentrations of Ca^{2+}, suggesting that the Ca^{2+}-binding sites in this form are of high affinity. Moreover, direct Ca^{2+}-binding studies with the same Ca^{2+}-free, decavanadate-bound form of the Ca^{2+}-ATPase independently confirm that the Ca^{2+}-binding sites in this form of the enzyme are of high affinity (Coan *et al.*, 1986). And equally importantly, these studies also clearly demonstrate that the Ca^{2+}-binding sites in this conformational state are accessible from the cytoplasmic side of the membrane, not the lumenal side as predicted by the E1E2 model. These findings thus constitute serious reasons to question the validity of the E1E2 model.

Similarly, Toyoshima and Nomura ascribed their recent new structure of the Ca^{2+}-free form of the Ca^{2+}-ATPase as that of the E2 state (Toyoshima and Nomura, 2002). Curiously, these authors point out that the Ca^{2+}-binding sites in this form of the enzyme appear to be accessible from the cytoplasmic side of the membrane and not the lumenal side, leading them to conclude that their structure "deviates" from the E1E2 model. Perhaps a more constructive conclusion would have been to seriously consider the possibility that the E1E2 model is wrong, since that is what their structure indicates. Parenthetically, it

might be added that since the Ca^{2+}-free structures of Xu *et al.* (2002) and Toyoshima and Nomura (2002) are significantly different, there would need to be two E2 states if there are indeed any.

It is equally perplexing that long ago, straightforward Ca^{2+}-binding studies to the unliganded form of the Ca^{2+}-ATPase by two different groups (Champeil *et al.*, 1983; Dupont, 1982) revealed the presence of at least one rapidly reacting, relatively high-affinity Ca^{2+}-binding site accessible from the cytoplasmic side of the membrane, which is completely inconsistent with the tenets of the E1E2 model. A few years later, Tanford recognized these findings as a fatal flaw in the E1E2 model that justified revision of the model (Tanford, 1985). And yet, he continued to embrace the E1E2 model for reasons that are unclear, but perhaps born of necessity in support of his theoretical studies of the E1 $\sim$ PCa_2 to E2-PCa_2 transition, which as we shall see later, are still of extreme importance today, in spite of a need for a new nomenclature. Then, in a trenchant experimental analysis of the Ca^{2+}-ATPase reaction cycle, Jencks and his colleagues provided overwhelming additional evidence that both of the Ca^{2+}-binding sites of the Ca^{2+}-free, unliganded form of the enzyme are of relatively high affinity and accessible from the cytoplasmic side of the membrane (Jencks, 1989). In response and to his credit, Jencks, who began his studies of the Ca^{2+}-ATPase employing the E1E2 model (Jencks, 1980), rejected the model as false and replaced it with a model more consistent with the experimental facts (Jencks, 1989). He then continued to argue forcefully against the use of the E1E2 model, but as his influence waned, the model returned. The reasons for the extreme resilience of a model that is wrong constitute an interesting socioscientific question, but such issues are outside the scope of this paper.

In sum, a substantial amount of solid experimental evidence indicates that the Ca^{2+}-free form of the Ca^{2+}-ATPase rests in a state in which the Ca^{2+}-binding sites are of relatively high affinity and accessible from the cytoplasmic side of the membrane. This alone constitutes a compelling case for rejecting the E1E2 model, which wrongly predicts otherwise, and replacing it with a model that more closely approximates reality.

Another problem with the E1E2 model that has apparently never been mentioned is that it is a Maxwell's Demon as formulated. Again, using the Ca^{2+}-ATPase as an example, if the E1 form of the enzyme has high-affinity Ca^{2+}-binding sites facing the cytoplasm, and this is in equilibrium with the E2 form which has low-affinity Ca^{2+}-binding sites facing the opposite side of the membrane, then this device alone would catalyze the active transport of Ca^{2+} without any input of energy. This is of course impossible and calls for restrictions on the model to prevent this from being predicted. Such restrictions could readily be incorporated, but in view of the foregoing discussion, it seems counterproductive to attempt to fix a model that is wrong on entirely different grounds.

In addition to the above errors of commission of the E1E2 model, there are errors of omission as well. Whereas these might be considered somewhat less serious, it is important to point them out, because proper attention to them will aid in the construction of a new model that more accurately describes the events that transpire as the P-type ATPases proceed through their catalytic cycles, and the forces that drive these events, both of which are required for an adequate model.

First, the E1E2 model never stipulates any relationship between the enzyme conformational changes it proposes and the various ligands that also intimately participate in the catalytic mechanism. It changes its conformation to move the ion-binding sites to the release side of the membrane and expel the ions, and it changes its conformation to return the low-affinity ion-binding sites to the uptake side of the membrane and change them back to high-affinity sites in the process. And it does this with no interaction with the various ligands involved. But the fact of the matter is that the overwhelming majority of conformational changes that are known to occur for all kinds of proteins are driven by ligand-binding reactions. Few, if any, of significance occur without ligand-binding. Thus, as we shall see below, a major conformational change in the Ca^{2+}-ATPase occurs in response to the binding of Ca^{2+} ions, and another must occur in response to nucleotide binding during the enzyme phosphorylation reaction. And the most crucial conformational change of all must occur when the chemical potential of the phosphoryl-enzyme intermediate changes in concert with the Ca^{2+}-binding affinity, leading to the expulsion of the ions on the release side of the membrane. Although Tanford's elegant treatment of the thermodynamics of this latter reaction recognizes the importance of the participating enzyme ligands (Tanford, 1981, 1985), most discussions of the E1E2 mechanism pay little regard to the importance of ligand binding in any stage of the P-type ATPase reaction cycle, which is clearly a mistake.

Another significant error of omission of the E1E2 model is the failure to recognize transition state binding as the driving force that propels the P-type ATPases, and all enzymes, through their catalytic cycles. We know from a large body of accumulated biochemical and structural information that enzymes perform their catalytic magic by binding most avidly to the transition state(s) of the reaction(s) that they catalyze (Fersht, 1986; Jencks, 1966; Lienhard, 1973; Pauling, 1946; Wolfenden, 1969). In so doing, they raise the concentration of the transition

state(s), which drives the reaction. And because they can do this most efficiently by binding to the transition state(s) from all angles, transition state binding is usually, if not always, accompanied by conformational changes in the enzyme that allow for facile substrate access and high-affinity transition state binding (Wolfenden, 1974). Thus, because this behavior of enzymes is universal, any complete description of the P-type ATPase reaction cycle should include considerations of transition state binding and the associated protein conformational changes driven by transition state binding for both the enzyme phosphorylation and dephosphorylation reactions.

AN IMPROVED MODEL FOR THE P-TYPE ATPASE REACTION CYCLE

Having rejected the E1E2 model for a variety of reasons, we can now consider a revised model for the reaction cycle of the P-type ATPases based solely on model-independent biochemical and structural information and taking into account all of the objections to the E1E2 model discussed in the preceding paragraphs. Such a model is presented in Fig. 2, again using the Ca^{2+}-ATPase as a specific example. We can begin with the unliganded enzyme, or E form, in its resting state, which is shown at the lower left of the figure. In a normal catalytic cycle, the E form may already have an essential Mg^{2+} ion bound from the last cycle, but this is not included for the sake of simplicity. The structure of the Ca^{2+}-free form of the enzyme at this stage of the reaction cycle is known, and is the structure recently determined by Toyoshima and Nomura (Toyoshima and Nomura, 2002; PDB ID IWO).

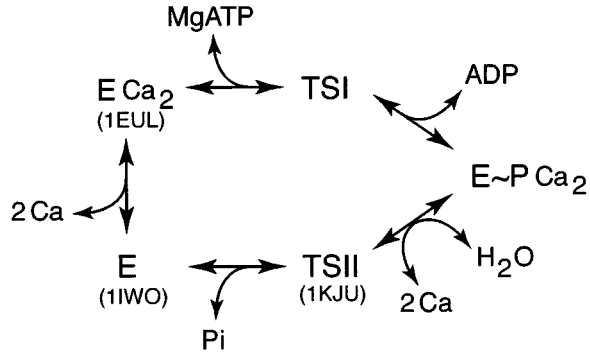

Fig. 2. Improved Model for the Reaction Cycle of the P-type ATPases. The model depicts the key steps that occur in the catalytic cycle of the P-type ATPases, using the Ca^{2+}-ATPase as a specific example. The structures of this enzyme at three stages of the cycle are known. The E state represents the Ca^{2+}-free structure determined by Toyoshima and Nomura (2002; PDB ID 1IWO). The ECa_2 state represents the Ca^{2+}-liganded structure obtained by Toyoshima *et al.* (2000; PDB ID 1EUL). The structure of the enzyme near the transition state of the enzyme dephosphorylation reaction, TSII, was determined by Xu *et al.* (2002) to a resolution of 6 Å. The PDB ID of the model generated from this structure is 1KJU. The structures of the enzyme in the transition state of the enzyme phosphorylation reaction (TSI) and the $E \sim PCa_2$ state are not known, but their structures can be approximated as desribed in the text (see text also for other details).

The E form of the Ca^{2+}-ATPase can undergo two different reactions, depending on the ligands it meets. The primary reaction, at least in a normal catalytic cycle, is the Ca^{2+}-binding reaction, which proceeds upward from the E state indicated in Fig. 2. Binding of the substrate, MgATP, probably occurs simultaneously, but the reaction also occurs in the absence of MgATP. The structure of the product of this reaction is also known. This is the structure of the Ca^{2+}-bound form of the enzyme determined by Toyoshima *et al.* (2000; PDB ID 1EUL), and designated ECa_2 in the figure. There is thus little left to the imagination regarding the Ca^{2+}-binding step of the reaction cycle. In the E state of the enzyme, the Ca^{2+}-binding sites are not well-formed, but the access pathway from the cytoplasm to one of the Ca^{2+}-binding residues, E309, is open (Toyoshima and Nomura, 2002). Thus, as suggested by Toyoshima and Nomura (2002), the first event in the Ca^{2+}-binding reaction is probably a collision between a cytoplasmic Ca^{2+} ion and E309. This then leads to a massive, concerted conformational change that generates the Ca^{2+}-bound form, ECa_2, in which the two Ca^{2+}-binding sites are completely formed with their Ca^{2+} ions in place (Toyoshima *et al.*, 2000). This involves major movements of the cytoplasmic domains and substantial rearrangements of several of the transmembrane helices (TM) (Toyoshima *et al.*, 2000; Toyoshima and Nomura, 2002).

It is very satisfying to consider how well this concerted Ca^{2+}-binding reaction, which we now understand in structural detail, fits with numerous biochemical studies of this reaction reported earlier. It explains the independent results of Dupont (1982) and Champeil *et al.* (1983), which noted a fast-reacting, relatively high-affinity Ca^{2+}-binding reaction at one Ca^{2+}-binding site followed by the slower generation of a second Ca^{2+}-binding site. From the structures now known for this reaction, it is clear what they were observing. The initial encounter complex between E309 and the first Ca^{2+} ion initiates a conformational change that allows access to the other Ca^{2+}-binding site II ligands (Toyoshima *et al.*, 2000) and in concerted fashion, all of the other conformational changes occur that change the various domain and transmembrane helix arrangements, and generate both complete Ca^{2+}-binding sites as well, leading to the E Ca_2 form of the enzyme (Toyoshima *et al.*, 2000). The Ca^{2+}-binding reaction structures also

nicely rationalize the results of Jencks and his colleagues regarding this reaction (Jencks, 1989). Both of the Ca^{2+}-binding sites are potentially accessible from the cytoplasm but a substantial conformational change is needed to unmask and generate the second site. This is precisely what was concluded by Petithory and Jencks on the basis of their direct Ca^{2+}-binding studies (Petithory and Jencks, 1988). This sequence also explains how binding of the first Ca^{2+} ion can be somewhat of lower affinity than binding of the second (Petithory and Jencks, 1988). And it also indicates that the mechanism of cooperativity in the Ca^{2+}-binding reaction proposed by Petithory and Jencks (1988) is exactly right. Thus, the cooperativity of the Ca^{2+}-binding reaction (Jencks, 1989), often cited as evidence for the E1E2 model, is in fact the result of an entirely different mechanism, as pointed out by Petithory and Jencks (1988).

The first step of the reaction cycle of the P-type ATPases, the cooperative Ca^{2+}-binding reaction to the E state, is therefore understood in substantial structural and biochemical detail. The only question remaining is whether or not the nucleotide-binding (N) domain tips back from its position in the E state (Toyoshima and Namura, 2002) to the extremely supine position it occupies in the ECa_2 structure (Toyoshima *et al.*, 2002) during a normal catalytic cycle. The analysis of Stahl and Jencks (1987) suggests that when the enzyme is actively cycling, ATP binding and phospho-enzyme formation subsequent to Ca^{2+}-binding occur at such high rates that the *N* domain may not have the time to tip as far back as it is in the ECa_2 structure (Toyoshima *et al.*, 2000). But this is only a minor uncertainty, and probably matters little in terms of our understanding of the molecular mechanism.

The other reaction in which the E state can participate is phosphorylation by Pi. If the Ca^{2+}-free, resting enzyme is presented with Pi in the absence of Ca^{2+} ions, it can become phosphorylated in a reaction that proceeds by way of the transition state of the enzyme dephosphorylation reaction, or TSII, as also shown in Fig. 2. The breakdown of TSII to form the Ca^{2+}-free phospho-enzyme is not shown because it probably does not occur in a normal catalytic cycle. Inspection of the structure of the unliganded E form (Toyoshima and Nomura, 2002) shows that the phosphorylation (P) domain containing the phosphorylated aspartate, D351, and the actuator (A) domain containing the highly conserved TGES sequence involved in reactions subsequent to the enzyme phosphorylation reaction (Andersen and Sorensen, 1996), almost certainly the enzyme dephosphorylation reaction, are situated such that the TGES sequence and D351 are only about 9 Å away from each other at the closest point. Thus, in this conformation, the A and P domains are arranged such that Pi binding can readily induce or stabilize the minimal interdomain movements that are probably needed for the formation of TSII, which is required to form the phosphoryl-enzyme intermediate from Pi. The structure of the enzyme at TSII is not known, but as we shall see below, it is probably quite close to the structure of the Ca^{2+}-free, decavanadate-bound form of the enzyme determined by Xu *et al.* (2002). In the model generated from this structure (PDB ID 1KJU), the TGES sequence and D351 are within hydrogen-bonding distance of each other. Thus, the relative ease with which the Ca^{2+}-ATPase is phosphorylated by Pi is not surprising. It only requires Pi binding and miminal movements of the A and P domains to bring the Pi, the TGES sequence, and the atoms around D351 to within reaction distance so that TSII can be formed. This is not the case after the Ca^{2+} ions have bound. In the form of the enzyme that results from Ca^{2+}-binding, the TGES sequence, and D351 are roughly 22 Å apart (Toyoshima *et al.*, 2000). This is presumably at least one of the reasons why phosphorylation of the enzyme by Pi does not occur in the presence of Ca^{2+}.

There is no reason to ascribe any special energetic significance to the formation of a phosphoryl-aspartate linkage from Pi and the side-chain carboxyl group of D351. Whereas this would form only with great difficulty from Pi and aspartate in aqueous solution, it forms with ease on the enzyme. Attainment of the transition state configuration is assisted by uniquely placed enzyme residues in TSII (Fig. 2) and this spontaneously breaks down to form the Ca^{2+}-free aspartyl-phosphoryl-enzyme intermediate and a water molecule (not shown). Moreover, whereas aspartyl phosphate is relatively unstable in water, it will be more stable in a less-aqueous milieu. This is likely the situation in the vicinity of the phosphorylated D351, particularly when the A and P domains are close. As we shall see below, the milieu around the phosphoryl-aspartate linkage is an extremely important issue and is at the heart of the mechanism of energy coupling of the P-type ATPases.

Returning to the forward cycle, after the binding of the Ca^{2+} ions to be transported and MgATP, the next step in the reaction sequence is phosphorylation of the side chain carboxyl group of D351 by the γ-phosphoryl group of ATP to form the aspartyl-phosphoryl-enzyme intermediate, $E \sim PCa_2$. This reaction occurs via transition state I, or TSI, as shown in Fig. 2. Although the structures of neither TSI nor $E \sim PCa_2$ are known, it is clear from the structure of the ECa_2 form of the enzyme (Toyoshima *et al.*, 2000), that this must involve a major movement of the ATP binding site on the N domain toward D351 on the P domain, because these two sites are more than 25 Å apart in the ECa_2 structure (Toyoshima *et al.*, 2000). The nature of this

movement as hinge bending at the region connecting the N and P domains was mentioned in the original description of the ECa_2 structure (Toyoshima *et al.*, 2000) and the details of this movement were made even more clear by Xu *et al.* (2002). These authors modeled the N-domain movement as a hinge-bending rotation of the N domain only, pivoting at N359 and R604 in the two strands connecting the N and P domains, and showed that this motion brings the γ-phosphoryl group of an ATP molecule in the nucleotide binding site into close proximity of the side-chain carboxyl group of D351 in the P domain. Whereas the initial movement of the N domain may be thermally driven (Xu *et al.*, 2002), the final stages of this reaction are surely driven and stabilized by favorable bonding interactions between enzyme functional groups and the transition state configuration of the aspartate phosphorylation reaction (Scarborough, 2002). This is probably all that happens with respect to the N and P domains in the formation of TSI and the subsequent formation of $E \sim PCa_2$. But there is extensive evidence available indicating that the A domain undergoes a substantial conformational change during this reaction as well. Most of this evidence has been recently described (Scarborough, 2002), so it will not be reiterated here. Substantial movement of the A domain and its associated TM helices toward the P domain and the other TM helices at this stage of the reaction cycle is consistent with the facts that the two bound Ca^{2+} ions have become occluded by this time (Serpersu *et al.*, 1982; Vilsen and Andersen, 1992) and the Ca^{2+} access pathway appears to be between these two regions of the molecule (Toyoshima *et al.*, 2000; Toyoshima and Nomura, 2002).

The events of the forward cycle described thus far have produced the aspartyl-phosphoryl-enzyme intermediate with the two Ca^{2+} ions tightly bound and probably occluded, designated as $E \sim PCa_2$ in the model of Fig. 2. This form of the enzyme is capable of phosphorylating ADP and catalyzing ADP/ATP exchange reactions, indicating that the N and P domains can probably separate to some extent and that ADP debinds and rebinds at a reasonable rate. This is thus the ADP-sensitive form of the enzyme traditionally referred to as $E1 \sim PCa_2$. Thus far, nothing of much bioenergetic significance has happened, but at this point, something has to happen to convert the high-affinity ion-binding sites to low-affinity sites so that the ions can be released to the far side of the membrane. The traditional E1E2 model recognized this, but gave no indication of what this might entail, simply stating that the "high-energy" form, $El \sim PCa_2$, changes to a "low-energy" form, $E2\text{-}PCa_2$, with a concomitant change in the accessibility and affinity of the Ca^{2+}-binding sites. This has always been difficult to comprehend in molecular terms, and the nature of the driving force behind this pivotal event is equally obscure. Jencks dismissed this step in his models because he could find no experimental evidence for it (Jencks, 1989), settling on the conclusion that the phosphate moiety of the phospho-enzyme intermediate and the bound Ca^{2+} ions share a "mutual destabilization" (Pickart and Jencks, 1984). This is almost as vague as the traditional $E1 \sim PCa_2$ to $E2 \sim PCa_2$ reaction but is an improvement because it presents the notion that the Ca^{2+} ions in their binding sites somehow repel the phosphate moiety of the phosphoenzyme intermediate and vice versa, thereby introducing the involvement of enzyme ligands into the picture and providing at least a hint of molecular insight as to what is going on in this step. But Tanford gave this step a more molecular and thermodynamic meaning when he explained how the chemical potential of bound ligands at different sites may be exchanged via interactions between the sites (Tanford, 1981). Perhaps the most explicit description of what this means, particularly with respect to the Ca^{2+}-ATPase and the reaction we are discussing, can be found in the drawing on p. 133 of a review by Tanford (1985). It shows how tightly bound Ca^{2+} ions (low chemical potential) in their binding sites can be converted to loosely bound Ca^{2+} ions (higher chemical potential) in much weaker binding sites, if a loosely bound phosphate moiety (high chemical potential, high energy) of the phospho-enzyme intermediate, $E1 \sim PCa_2$, is simultaneously converted to a tightly bound phosphate moiety (low chemical potential, low energy) of $E2\text{-}PCa_2$, and the two sites are linked. Or in other words, a change in the state of the phosphoryl group of $E1 \sim PCa_2$ to a state where it is more tightly bound can provide the energy to convert the Ca^{2+}-binding sites from high to low affinity at the same time, providing that the sites are linked. This is almost certainly what happens in the reaction traditionally known as the $E1 \sim PCa_2$ to $E2\text{-}PCa_2$ transition. As almost everyone does, Tanford chose a mechanical coupling model for this event, but there are other ways to accomplish the same thing (Scarborough, 2002). But regardless of the model used, Tanford's thermodynamic treatment of this reaction is still valid and very important today, because it is model-independent and thoroughly developed. This reaction is the essence of the energy transduction mechanism for the P-type ATPases.

Looking at this crucial reaction in the P-type ATPase catalytic cycle in terms of well-established, general principles of enzyme catalysis brings on an even deeper understanding of what is going on at this step. That is, in enzyme catalytic terms, after the formation of the phosphoryl-enzyme intermediate, the next step is the binding of the hydrolytic water molecule and formation of the transition state of the enzyme dephosphorylation reaction. And

according to the widely accepted transition state theory of enzyme catalysis (Fersht *et al.*, 1986; Jencks, 1966; Lienhard, 1973; Pauling, 1946; Wolfenden, 1969), this involves increased binding interactions between the enzyme functional groups and the chemical reactants that together make up the transition state of this reaction (Scarborough, 2002), which include the covalently bound phosphoryl group of the state called $E \sim PCa_2$ in Fig. 2. Thus, putting Tanford's thermodynamics and general enzyme catalytic principles together, it seems certain that the energy transduction event for the P-type ATPases, where the chemical potentials of the bound Ca^{2+} ions and the phospho-enzyme intermediate are exchanged, occurs at the transition state of the enzyme dephosphorylation reaction or very near it, that is, TSII in the model of Fig. 2.

A substantial amount of information is available as to the protein structural changes that occur during this key step in the reaction cycle. Although the structure of the enzyme in the $E \sim PCa_2$ state is not known, with the Ca^{2+} ions firmly in place, it is safe to assume that the P domain and the TM helices containing the Ca^{2+}-binding ligands are in similar positions and conformations as they are in the ECa_2 state. But the A domain with its essential TGES sequence is probably much closer to the P domain, since it is in TSI as mentioned previously. It therefore has probably rotated a significant part of the 90° rotation originally described (Toyoshima *et al.*, 2000), and may even be hyperrotated as it is in the ligand-free E state, that is, about 110° horizontally from the position it occupies in the ECa_2 state (Toyoshima *et al.*, 2000; Toyoshima and Nomura, 2002). And the N domain is probably tipped back to some degree from the TSI state to allow the approach of the TGES sequence on the A domain to the phosphoryl moiety of the phospho-enzyme linkage at D351. As mentioned previously, the structure of the end product of this reaction, TSII, is also not known, but it must be very close to that of the Ca^{2+}-free, decavanadate-bound structure of Xu *et al.* (2002; 1KJU), because the TGES sequence in this structure is within hydrogen-bonding distance of D351, the bound Ca^{2+} ions are gone, and the Ca^{2+}-binding sites have been disorganized.

Thus, upon the formation of the encounter complex between the TGES region in the A domain and the phosphoryl group of the D351 phospho-enzyme intermediate in the P domain, an extensive conformational rearrangement occurs that includes an approximately 53° oblique rotation of the P domain and its associated N and A domains, bending of TM5, and displacement of the top of TM4 laterally and downward by about 4–5 Å, which leads to a somewhat more helical arrangement of the unwound region of TM4. TM2 probably also inclines to its position in the structure of Xu *et al.* (2002), but since its position in the $E \sim PCa_2$ state is not known, the extent of this change is uncertain. Other more minor changes in the TM region are likely as well. So the protein conformational changes that occur during this key energy-transducing reaction in the catalytic cycle of the P-type ATPases are for the most part reasonably clear. The only remaining question therefore is the nature of the forces that drive this conformational transition after the collision between the TGES region and the phosphorylated aspartate has occurred. For a satisfactory understanding of the molecular mechanism of these enzymes, it is insufficient to merely stipulate that the conformational changes occur and the Ca^{2+} ions debind. This quintessential remaining question will be briefly mentioned again below.

Referring again to Fig. 2, the next step in the reaction is the breakdown of TSII and regeneration of the unliganded E form of the enzyme, after debinding of the products of the reaction, Pi and one or two protons (not shown). The conformational changes that occur in this reaction are moderate, and are simply the reverse of the conformational changes described previously for the phosphorylation of the Ca^{2+}-free, unliganded E form of the enzyme by Pi. They are thus represented by the differences between the structure of Toyoshima and Nomura (2002; 1IWO) and the structure of Xu *et al.* (2002; 1KJU). The breakdown of TSII occurs spontaneously at a high rate (Frost and Pearson, 1961). This very nicely solves a conundrum inherent in Tanford's thermodynamic treatment of the energy exchange reaction that precedes this one. That is, because very high affinity binding of enzyme functional groups to the phosphoryl moiety of the phospho-enzyme intermediate is necessary to weaken the binding of the two bound Ca^{2+} ions (Tanford, 1985), after the Ca^{2+} ions have debound, this phosphoryl group binding would be even tighter, which would constitute an energy well that would probably slow the overall reaction to an unacceptable rate (Eyring *et al.*, 1949). Tight binding to TSII obviates this problem because this species has such a fleeting existence.

One final particularly appealing aspect of the P-type ATPase reaction cycle as formulated here is that it accomplishes the essential $E1 \sim PCa_2$ to E_2–PCa_2 reaction of the E1E2 model without leaving behind the problematic E1E2 conformational equilibrium reaction (Fig. 1), which has always led to predictions inconsistent with the experimental facts.

THE MOLECULAR MECHANISM OF ENERGY COUPLING

What remains is to briefly discuss the means by which binding of the TGES region in the A domain to

the phosphoryl moiety of the phospho-enzyme intermediate at D351 in the E $\sim$ PCa_2 form of the enzyme elicits a response in the molecule that leads to the observed major conformational changes in the cytoplasmic and transmembrane regions that produce the structure of Xu *et al.* (2002) and expel the Ca^{2+} ions from their binding sites, that is, the E $\sim$ PCa_2 to TSII reaction shown in Fig. 2. A recent effort to explain these events proposes that the binding of the TGES region to the phosphoryl group at D351 induces a strain in the molecule that drives the subsequent conformational changes, which in turn disorganize the Ca^{2+}-binding sites (Xu *et al.*, 2002). This is a purely mechanical explanation of the energy-coupling mechanism and represents the predominant theme of most models past and present addressing this critical question. A weakness of this proposed energy coupling mechanism is that it is too vague to impart a sense of how the strain is generated and how it is transmitted to the Ca^{2+}-binding sites leading to their distortion. It also seems unlikely for a more specific reason. Since more than a dozen hydrogen bonds exist between the TGES region of the A domain and the P and N domains in the structure of Xu *et al.* (2002), and these are unable to prevent high-affinity Ca^{2+}-binding to this structure that induces the reverse conformational changes (Coan *et al.*, 1986; Scarborough, 2002; Xu *et al.*, 2002), it seems unlikely that the binding of the TGES region to the phospho-enzyme intermediate could be so much stronger that it could drive the conformational changes in the other direction and expel the Ca^{2+} ions. These are not necessarily grounds to dismiss the mechanical coupling model, but they are significant reasons for concern.

An alternative, more explicit mechanism has recently been proposed in which electrochemical signals generated at the chemical reaction site are transmitted to the Ca^{2+}-binding sites via two well-defined charge transfer pathways that connect the sites (Scarborough, 2002). The charge pulses generated at the site of the phosphoenzyme hydrolysis reaction are conducted to the Ca^{2+}-binding site region, where they repel the bound Ca^{2+} ions and drive local conformational changes, which in turn are responsible for the more global domain and TM helix movements that occur (Scarborough, 2002). Although this represents a largely new concept in bioenergetics and biological signaling as well, it is nevertheless sound and worthy of serious consideration.

In any case, it is this crucial energy-coupling reaction that occurs during the conversion of the E $\sim$ PCa_2 state of the molecule to the TSII state, where future experimental work and thinking about the molecular mechanism of the P-type ATPases should be directed, because there are few, if any, other questions left to be resolved.

CONCLUSION

It is clear from the foregoing discussion that the E1E2 model for the reaction cycle of the P-type ATPases is fatally flawed and that its use is now acting as a barrier to progress toward understanding the molecular mechanism of these fascinating transport machines. But when the wealth of available structural and biochemical information about these enzymes is interpreted outside the box of the E1E2 model, with attention to known modes of protein conformational dynamics and established principles of enzyme catalysis, a working model emerges that is in harmony with virtually everything that has been learned about these enzymes since they were discovered, and about enzyme reactions in general. It is hoped that the arguments leading to this new model will be found convincing, and accordingly that the use of the E1E2 model is discontinued. With that done, it will be possible for everyone to focus on the last mechanistic question remaining in the P-type ATPase field, the precise nature of the events that transpire during the E $\sim$ PCa_2 to TSII reaction and the forces that drive them. And when that question is answered, we shall truly understand the molecular mechanism of the P-type ATPases.

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