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REVIEW

Mechanism of allosteric effects of ATP on the kinetics of P-type ATPases

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Abstract The roles of allosteric effects of ATP and protein oligomerisation in the mechanisms of P-type ATPases belong to the most controversial and least well understood topics in the field. Recent crystal structural and kinetic data, however, now allow certain hypotheses to be definitely excluded and consistent hypotheses to be developed. The aim of this review is to critically discuss recent results and, in the light of them, to present a set of conclusions which could form the basis of future research. The major conclusions are: (1) at saturating ATP concentrations P-type ATPases function as monomeric enzymes, (2) the catalytic units of P-type ATPases only possess a single ATP binding site, (3) at non-saturating ATP concentrations P-type ATPases exist as diprotomeric (or higher oligomeric) complexes, (4) protein–protein interactions within a diprotomeric complex enhances the enzymes' ATP binding affinity, (5) ATP binding to both protomers within a diprotomeric complex causes it to dissociate into two separate monomers. The physiological role of protein–protein interactions within a diprotomer may be to enhance ATP binding affinity so as to scavenge ATP and maximize the ion pumping rate under hypoxic or anoxic conditions. For the first time a structural basis for the well-known ATP allosteric acceleration of the E2 → E1 transition is presented. This is considered to be due to a minimization of steric hindrance between neighbouring protomers because of the ability of ATP to induce a compact conformation of the enzymes' cytoplasmic domains.

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Introduction

P-type ATPases are transmembrane ion pumps having a phosphorylated intermediate as part of their reaction cycle. One of their most important members and the first ion pump ever to be discovered (Skou 1957) is the Na^+,K^+-ATPase, which is present in the plasma membrane of all animal cells. This enzyme is responsible for maintaining Na^+ and K^+ electrochemical gradients across the membrane, which are crucial for a variety of physiological functions, e.g. nerve impulse transmission, muscle contraction and nutrient reabsorption. In the plant kingdom a similar role is played by another member of the family, the plasma membrane H^+-ATPase (Palmgren 2001). Other important members include the sarcoplasmic reticulum Ca^{2+}-ATPase (Inesi and Toyoshima 2004), responsible for muscle relaxation, and the H^+,K^+-ATPase (Shin et al. 2004), which causes the acidification of the stomach.

In all of these enzymes the phosphorylated intermediates are formed under physiological conditions by phosphoryl transfer from ATP. The intermediates are of high energy and can only relax to low energy states by conformational changes which simultaneously involve the release of ions to the side of the membrane at which the ions have a higher electrochemical potential. Thus, the hydrolysis of ATP to ADP is coupled to ion transport. However, apart from supplying the energy for ion pumping, it has been known for many years that ATP also plays an allosteric role in the mechanisms of these enzymes, causing an acceleration of some of the partial reactions of their enzymatic cycles without

undergoing hydrolysis. This was first recognized in the case of the Na^+,K^+-ATPase by Post and co-workers (Hegyvary and Post 1971; Post et al. 1972), who found evidence based on measurements of phosphoenzyme levels using radioactively labelled ATP that ATP accelerates the release of K^+ ions from the enzyme. This conclusion was subsequently supported by kinetic measurements of the conformational change associated with K^+ release in the absence of any enzyme phosphorylation using the stopped-flow technique with spectroscopic detection (Karlish et al. 1976; Karlish et al. 1978; Karlish and Yates 1978; Steinberg and Karlish 1989).

Based on the pioneering work of Albers (1967) and Post (Hegyvary and Post 1971; Post et al. 1972) the most widely accepted working hypothesis of the reaction cycle of P-type ATPases is the Albers–Post or E1–E2 model (see Fig. 1). This describes a sequence of ion binding and release reactions coupled to enzyme phosphorylation by ATP and the release of inorganic phosphate. The mechanism shown in Fig. 1 is written specifically for the Na^+,K^+-ATPase, but analogous cycles with different ions and different ion stoichiometries have been proposed for all of the P-type ATPases. The K^+ release reaction of the Na^+,K^+-ATPase, which, as described above, was the first reaction in which an allosteric effect of ATP was discovered, is represented according to the E1–E2 formalism by: $E2(K^+)_2 + 3Na^+ \rightarrow E1(Na^+)_3 + 2K^+$. In this reaction K^+ ions are released to the cytoplasmic medium. In the absence of ATP the reaction is very slow, occurring over a timescale of seconds. However, in the presence of ATP the reaction is dramatically accelerated, reaching an observed rate constant of 65–90 s^{-1} at saturating ATP

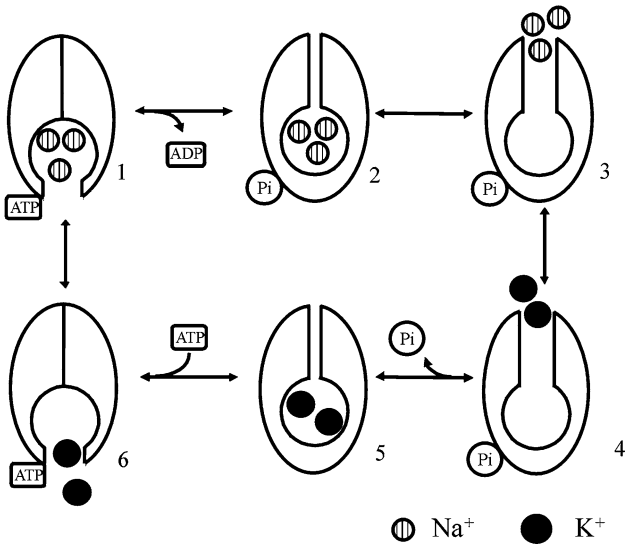

Fig. 1 Albers–Post model of the mechanism of P-type ATPases (shown for the Na^+,K^+-ATPase). The sequence of intermediates from 1 to 6 around the cycle correspond to the following reactions: $E1(Na^+)_3$ ATP $\rightarrow$ $E2P(Na^+)_3 \rightarrow E2P \rightarrow E2P(K^+)_2 \rightarrow E2(K^+)_2 \rightarrow E1(K^+)_2$. The diagram has been modified from Scheiner-Bobis (2002)

concentrations (Lüpfert et al. 2001). The acceleration occurs even in the absence of Mg^{2+} ions (Forbush 1987; Steinberg and Karlish 1989; Pratap et al. 1996; Humphrey et al. 2002; González-Lebrero et al. 2002), which are required as a cofactor of ATP for any phosphorylation reactions to occur. The acceleration must, therefore, come about solely by ATP binding to the E2 state of the enzyme. The dissociation constant of ATP to the E2 state has been estimated to be in the range 71–450 μM (Karlish and Yates 1978; Forbush 1987; Steinberg and Karlish 1989; Kane et al. 1997; Clarke et al. 1998; González-Lebrero et al. 2002). This is much higher than the dissociation constant of ATP to the E1 state, which has been determined to be in the range 0.12–0.63 μM (Nørby and Jensen 1971a; Hegyvary and Post 1971; Fedosova et al. 2003; Grell et al. 2004; Esmann et al. 2008; Pilotelle-Bunner et al. 2008). The site to which ATP binds on E2 and exerts its allosteric effect on K^+ release is, thus, universally referred to as the low affinity site, whereas the site on the E1 state is termed the high affinity site. Whether or not these two sites are in fact two distinct spatially separated sites on the enzyme has been a topic of debate for many years, and it will be one of the issues addressed in this review.

Acceleration of the E2 $\rightarrow$ E1 transition by ATP and the associated release of K^+ in the mechanism of the Na^+,K^+-ATPase is of great physiological importance, because it has been shown to be the slowest reaction, i.e., the major rate-determining reaction, of the entire cycle (Steinberg and Karlish 1989; Lüpfert et al. 2001; Humphrey et al. 2002; Kong and Clarke 2004). Therefore, ATP plays a crucial role in optimizing the rate of ion pumping as well as providing the necessary energy.

Since the pioneering work of the groups of Post and Karlish (Hegyvary and Post 1971; Post et al. 1972; Karlish et al. 1976; Karlish et al. 1978; Karlish and Yates 1978; Steinberg and Karlish 1989), similar allosteric roles of ATP in the mechanisms of virtually all of the known P-type ATPases have been discovered. In the case of the sarcoplasmic reticulum Ca^{2+}-ATPase from *skeletal* muscle the release of Ca^{2+} into the lumen of the sarcoplasmic reticulum (the equivalent step to Na^+ release to the extracellular medium in the mechanism of the Na^+,K^+-ATPase) is accelerated by ATP binding to a phosphorylated form of the enzyme (Cable et al. 1985; Champeil and Guillain 1986; Cable and Briggs 1988). Champeil et al. (1988) also reported an acceleration of the subsequent dephosphorylation reaction by ATP. In the case of sarcoplasmic reticulum Ca^{2+}-ATPase from *cardiac* muscle, Cable and Briggs (1988) reported that ATP stimulates ion pumping by binding to a non-phosphorylated form of the enzyme, i.e., similar to the behaviour observed in the Na^+,K^+-ATPase. An allosteric effect of ATP has also been found in the mechanism of the gastric H^+,K^+-ATPase (Reenstra et al. 1988). Here the behaviour is analogous to that observed in skeletal

muscle sarcoplasmic reticulum Ca^{2+}-ATPase, i.e., ATP binds to the phosphorylated form of the enzyme and stimulates the release of inorganic phosphate, P_i. The kinetic results obtained with the H^+,K^+-ATPase and the skeletal muscle sarcoplasmic reticulum Ca^{2+}-ATPase, thus, indicate that the binding of ATP and P_i to P-type ATPases can occur simultaneously (Reenstra et al. 1988; Cable et al. 1985; Champeil and Guillain 1986; Champeil et al. 1988; Cable and Briggs 1988). In fact, kinetic data supporting ATP binding to the phosphorylated form of the Na^+,K^+-ATPase has also recently been reported (Clarke and Kane 2007; Pilotelle-Bunner et al. 2008).

Complex ATP activation of the kinetics of both plant (Roberts and Beaugé 1997) and yeast H^+-ATPase (Berberián et al. 1993) has also been reported. Roberts and Beaugé (1997) suggested two ways in which this might come about: (1) the presence of two separate ATP sites on the enzyme, an allosteric (or regulatory) site and a catalytic site, or (2) the presence of a single ATP site that consecutively changes its property from catalytic to allosteric as the enzyme proceeds around its pump cycle. For skeletal muscle sarcoplasmic reticulum Ca^{2+}-ATPase a number of authors have supported the second of these hypotheses (Cable et al. 1985; Bishop et al. 1987; Cable and Briggs 1988). In the light of more recent results it will be shown in this review that a definitive decision in support of a single ATP site with both regulatory and catalytic properties is now possible.

However, in spite of all of these observations of allosteric effects of ATP, no general molecular mechanism has ever been discovered which could account for them. Fortunately, over the last few years X-ray crystal structures of three members of the P-type ATPase family have been solved, the Na^+,K^+-ATPase (Morth et al. 2007), the skeletal muscle sarcoplasmic reticulum Ca^{2+}-ATPase (Olesen et al. 2007; Toyoshima 2008) and the plant H^+-ATPase (Pedersen et al. 2007). In the particular case of the skeletal muscle sarcoplasmic reticulum Ca^{2+}-ATPase, structures have been solved in a number of different conformations around the pumping cycle, thus giving snapshots of the entire ion pumping process. Since these structures appeared, a great deal of information has been gained concerning the pathways followed by the transported ions and the conformational changes associated with enzyme phosphorylation by ATP. However, the aim of this review is to concentrate on the allosteric effects of ATP. Do the crystal structures provide any clue to the origin of these effects?

In any biochemical context whenever one mentions the word "allosteric" the first thing which comes to mind are allosteric globular proteins, the classical example of which is haemoglobin. There is no positive cooperativity in the binding of oxygen by the single polypeptide enzyme myoglobin. Haemoglobin, however, with four subunits, shows pronounced cooperativity, evidenced by a sigmoid oxygen binding curve in contrast to a hyperbolic binding curve for myoglobin. The allosteric binding of oxygen by haemoglobin is attributed to protein–protein interactions between neighbouring subunits, with the interactions increasing the oxygen affinity as the subunits become occupied. By analogy, one could easily imagine that, if P-type ATPases display allosteric effects due to ATP, these could perhaps be related to protein–protein interactions. In fact, the debate as to whether P-type ATPases function as monomers or as oligomers has been going on for approximately the last 30 years, and it is still a very controversial topic. Because the monomer/oligomer question is essential to a discussion of allosteric effects of ATP, some of the evidence for or against either a monomer or a dimer will also be reviewed here.

It must be pointed out at the beginning that the aim here is not to provide an exhaustive review of the topic of allosteric effects and oligomerisation of P-type ATPases, but instead to provide an update on what can be concluded based on recent structural and kinetics findings. It is also hoped that the hypotheses provided will stimulate further discussion leading to advances in the field.

Functional unit

Whether the functional unit of P-type ATPases is a monomer (i.e., an $\alpha\beta$ protomer in the case of the Na^+,K^+-ATPase and the H^+,K^+-ATPase) or a dimer or higher oligomer has been a subject of intense debate for over 30 years. The idea that the Na^+,K^+-ATPase might function as an $(\alpha\beta)_2$ diprotomer was first proposed in 1973 (Stein et al. 1973; Repke and Schön 1973). Since then a number of authors have suggested the involvement of diprotomers or higher oligomers in the mechanism of the Na^+,K^+-ATPase (Blostein 1975; Froehlich et al. 1997; Tsuda et al. 1998; Yokoyama et al. 1999; Tanoue et al. 2006; Clarke et al. 2007; Clarke and Kane 2007; Pilotelle-Bunner et al. 2008). Mechanisms involving dimers or higher oligomers have also been proposed for the H^+,K^+-ATPase (Abe et al. 2002; Shin et al. 2005) and the sarcoplasmic reticulum Ca^{2+}-ATPase (Froehlich et al. 1997; Mahaney et al. 2004; Mahaney et al. 2008). The role of subunit interactions in the mechanism of the Na^+,K^+-ATPase have previously been reviewed by Askari (1987) and again by Taniguchi et al. (2001). A review on the significance of the monomer–oligomer equilibrium for the mechanism of the sarcoplasmic reticulum Ca^{2+}-ATPase was published by Andersen (1989).

The crystal structures which have been published over the last few years of P-type ATPases in different conformational states (Toyoshima et al. 2000; Toyoshima and Nomura 2002; Toyoshima and Mizutani 2004; Toyoshima

et al. 2004; Sørensen et al. 2004; Olesen et al. 2004; Jensen et al. 2006; Morth et al. 2007; Olesen et al. 2007; Pedersen et al. 2007; Toyoshima 2008) provide undisputable evidence for an ion translocation pathway through the centre of a single catalytic unit (or α-subunit in the case of the Na^+,K^+-ATPase). Therefore, there is no structural reason for P-type ion pumps to necessarily aggregate into dimers or higher oligomers in order to translocate ions.

Functional data indicating whether the enzyme is acting as a monomer, dimer or higher oligomer can be obtained by measurements of the phosphorylation capacity. If one takes a molecular mass of an $\alpha\beta$ unit of the Na^+,K^+-ATPase of 147,000 g mol^{-1} (Jørgensen and Andersen 1988), one can theoretically calculate that if every $\alpha\beta$ unit were functionally active the number of moles of phosphorylation sites per milligram of protein should correspond to 6.8 nmol of sites (mg of protein)$^{-1}$. Values close to this theoretical limit have been achieved using highly purified enzyme from rabbit kidney, pig kidney and duck nasal gland (Peters et al. 1981; Vilsen et al. 1987; Martin and Sachs 1999) and on reconstitution of Na^+,K^+-ATPase into phospholipid vesicles (Cornelius 1995). This is clear evidence that the Na^+,K^+-ATPase is acting as an $\alpha\beta$ unit under these conditions, i.e., P-type ATPases can function with only a single catalytic unit. Reconstitution of sarcoplasmic reticulum Ca^{2+}-ATPase from skeletal muscle with excess lipid into vesicles such that only monomeric Ca^{2+}-ATPase is present has also shown that the enzyme retains a high activity (Heegaard et al. 1990). Therefore, both structurally and functionally there is no need for P-type ATPases to aggregate in order to translocate ions. The minimal functional unit is definitely a monomer.

However, in most preparations of the Na^+,K^+-ATPase, phosphorylation capacities much lower than 6.8 nmol of sites (mg of protein)$^{-1}$ are found. Therefore, could it be that too extensive purification breaks apart protein aggregates? If this was true, then larger protein aggregates might still have different kinetics from protein monomers. In order to test this possibility Martin et al. (2000) compared the molecular activity or turnover number of duck salt gland Na^+,K^+-ATPase in purified membranes and in microsomes with different distances of separation between the $\alpha\beta$-protomers. They found no significant difference in the turnover. They, therefore, concluded that: "because monomeric protomers have the same turnover rate as closely packed $\alpha\beta$ protomers, dimerization, if it occurs, makes no notable change in the overall reaction mechanism." However, although the results of Martin et al. (2000) are certainly very valuable, this conclusion is too generalized. In fact what they measured was the maximal turnover under saturating conditions of all of the enzyme's substrates. Therefore, the only justifiable conclusion from their measurements is that the *maximal* turnover is not affected by protein dimerization. This does not exclude effects of protein–protein interactions on the kinetics of P-type ATPases under non-saturating conditions, which are the conditions under which these enzymes function in their physiological environment. In contrast to the results of Martin et al. (2000), Mahaney et al. (2004) showed that complex kinetics of the sarcoplasmic Ca^{2+}-ATPase, which they attributed to protein–protein interactions within an oligomer, disappeared on solubilisation of the enzyme with the detergent $C_{12}E_8$. This detergent is known to break apart protein oligomers and shift the equilibrium within the membrane towards the monomeric state (Esmann 1984).

There is overwhelming evidence in the literature suggesting that P-type ATPases aggregate in their native membrane environment and form either dimers or higher oligomers. In the case of the Na^+,K^+-ATPase this has been shown, for example, by co-immunoprecipitation studies after expression in insect cells (Blanco et al. 1994), radioactive inactivation experiments (Nørby and Jensen 1971b) and thermal denaturation (Donnet et al. 2001). Electronmicroscopic investigations have shown that the Na^+,K^+-ATPase is able to form two-dimensional crystalline arrays containing side-by-side diprotomers within the unit cell (Skriver et al. 1989). It was also found that genetic data could be more easily explained if one assumed the presence of an oligomeric complex (Palladino et al. 2003). Recently, stable $(\alpha\beta)_2$ diprotomers and $(\alpha\beta)_4$ tetraprotomers in addition to $\alpha\beta$ protomers of dog kidney Na^+,K^+-ATPase were isolated chromatographically by Hayashi and his colleagues (Kobayashi et al. 2007; Mimura et al. 2008). In the case of both skeletal and cardiac muscle sarcoplasmic reticulum Ca^{2+}-ATPase the presence of oligomeric protein complexes has been indicated by the determination of the rotational correlation time of phosphorescently labelled protein via measurements of time-resolved phosphorescence anisotropy (Kutchai et al. 1994, 1998). Analysis of the Ca^{2+} concentration dependence of the activity of the sarcoplasmic reticulum Ca^{2+}-ATPase in vivo at non-saturating Ca^{2+} concentrations is also consistent with the enzyme functioning as a dimer (Klein et al. 1991).

In summary, based on the experimental data now available the minimal functional unit of P-type ATPases is a single catalytic unit (or $\alpha\beta$ protomer). Under saturating conditions of all substrates it seems that protein–protein interactions between neighbouring catalytic units either do not exist or they have no effect on the enzyme kinetics. Protein oligomerisation definitely does occur in the native membrane environment, but it probably only affects enzyme kinetics under non-saturating substrate conditions (possibly physiological conditions). The only substrate that all P-type ATPases have in common is ATP. Therefore, in the following sections I shall concentrate on ATP binding and its effects on ATPase conformation and kinetics.

Number of ATP binding sites

It is now universally accepted that ATP plays both a catalytic role and an allosteric role in the mechanism of P-type ATPases. As stated in the "Introduction", there are two possible explanations for this; either the enzymes have two separate ATP binding sites, a catalytic site and an allosteric site, or they have a single ATP binding site which can be either catalytic or allosteric depending on which reaction one is considering.

The hypothesis of two separate ATP binding sites per catalytic subunit has been supported by Ward, Cavieres and their co-workers (Ward and Cavieres 1996; Ward and Cavieres 2003; Ward et al. 2006) based on affinity labelling studies. However, based on activity measurements on highly purified duck nasal gland Na^+,K^+-ATPase in the presence of ligands presumed to label the high and low affinity ATP sites, Martin and Sachs (2000) came to the conclusion that each α-subunit of the enzyme only has a single ATP binding site. One suggestion they offered for these conflicting results was that some ligands, such as FITC, may only partially block the ATP binding site. Two ATP sites on a single α-subunit of the Na^+,K^+-ATPase was suggested by Askari and Huang (1982) on the basis of kinetic evidence indicating that ATP can bind to the enzyme prior to dephosphorylation, i.e., ATP and P_i can be bound to the enzyme simultaneously. This would certainly exclude the possibility of a single ATP site, if the ATP binding site and the phosphorylation site were not sufficiently spatially separated. However, recent crystal structural data indicate that this is not the case. Sørensen et al. (2004) have published a crystal structure of the sarcoplasmic reticulum Ca^{2+}-ATPase with bound ADP and the phosphate analogue AlF_4^-, which represents an E1 ~ PADP transition state. This indicates that nucleotide binding and phosphate binding in P-type ATPases are not mutually exclusive. The results of Askari and Huang (1982) are, therefore, perfectly consistent with a single ATP binding site per α-subunit.

The recently published crystal structures of the sarcoplasmic reticulum Ca^{2+}-ATPase from skeletal muscle (Sørensen et al. 2004; Toyoshima and Mizutani 2004; Jensen et al. 2006; Olesen et al. 2007) and the plasma membrane H^+-ATPase of plants (Pedersen et al. 2007) with the non-hydrolysable ATP derivative AMPPCP also show no evidence for more than one nucleotide binding site on the catalytic unit of these enzymes. From crystal structures alone, however, it is extremely difficult to discern whether a ligand is playing purely a catalytic role or a regulatory role. For this kinetic measurements are required. As mentioned earlier, it is well established that the E2 → E1 transition of the Na^+,K^+-ATPase is accelerated by ATP binding at a low affinity site (K_d = 71–450 μM) on the E2 state of the enzyme. However, recently it was shown that the reverse reaction, i.e., E1 → E2, is accelerated by ATP binding in the sub-micromolar range, with a K_d of approximately 0.25 μM (Clarke et al. 2007). A K_d of this magnitude is typical for high affinity binding of ATP to the E1 state, which is generally referred to as the catalytic site, because its occupation is required, together with Mg^{2+}, for phosphorylation. Therefore, the results of Clarke et al. (2007) indicate that the high affinity ATP site can be either catalytic or regulatory depending on which reaction one is studying, i.e., phosphorylation or the E1 → E2 transition. This is strong evidence in favour of a single ATP binding site on the catalytic sub-unit of P-type ATPases which can fulfil both catalytic and regulatory functions. There is no functional need for separate sites and there is no structural evidence for separate sites.

The acceleration of the E1 → E2 transition by high affinity binding of ATP to E1 may seem strange, because it would seem to imply that high affinity ATP binding would drive the Na^+,K^+-ATPase in reverse. However, the observation of this effect requires the absence of Mg^{2+} ions, which is not a physiological condition. In the presence of physiological concentrations of Mg^{2+} the phosphorylation reaction with its much faster rate constant (~200 s^{-1}) would far outweigh the reverse reaction E1 → E2 with a rate constant of ~1 s^{-1}, so that no inhibition due to ATP would be apparent.

Before proceeding, it should be mentioned that, although the recent results which have been discussed here strongly favour the hypothesis of a single ATP site on each catalytic subunit of P-type ATPases, this does not preclude the possibility of the simultaneous presence of both high and low affinity bound ATP, if the ATP molecules are bound to different catalytic subunits within an oligomer and if two or more protomers of the oligomer are present in different states of the pump cycle, i.e., the protomers are out-of-phase. This corresponds to a hypothesis put forward by Scheiner-Bobis et al. (1987) based on measurements in which they could still measure ATP binding and enzyme phosphorylation of the Na^+,K^+-ATPase even after blockage of the low affinity ATP binding site with $Co(NH_3)_4ATP$. Later measurements from the same group (Buxbaum and Schoner 1990, 1991) showed that inactivation of the enzyme in the E2 state by complexation with $Co(NH_3)_4PO_4$ still allowed the measurement of all activities, such as Na^+–Na^+ exchange and Na^+-ATPase activity, associated with the E1 state. Conversely, inactivation of the ATP site on the E1 state by reaction with the chromium complex of adenosine 5′-[β,γ-methylene]triphosphate (Hamer and Schoner 1993) or fluorescein 5′-isothiocyanate (FITC) (Schoner et al. 1998) was found to still allow the measurement of K^+-activated *p*-nitrophenylphosphatase, a reaction associated with the E2 state. Via the measurement of the efficiency of

fluorescence energy transfer between erythrosin 5′-isothiocyanate bound to the low affinity ATP site and FITC bound to the high affinity ATP site, Linnertz et al. (1998) determined a distance between the two sites of 6.5 nm, i.e., greater than the diameter of a single α-subunit. These results give further support to the hypothesis of Scheiner-Bobis et al. (1987) of two coexisting low and high affinity ATP binding sites on different α-subunits within an $(\alpha\beta)_2$ diprotomer. The asynchronicity of pump protomers, which the hypothesis of Scheiner-Bobis et al. (1987) requires, is the subject of the following section.

Synchronicity of conformational changes

Although it is well known that the E2 → E1 transition of the Na^+,K^+-ATPase is stimulated, i.e., accelerated, by the binding of ATP to the E2 state with low affinity, what is probably less well recognized is that ATP also changes the mechanism of the E2 → E1 transition and its associated K^+ release to the cytoplasm. At zero or very low concentrations of ATP the release of K^+ or its congener Rb^+ from the enzyme follows biexponential kinetics, whereas as the ATP concentration is increased the kinetics become increasingly monoexponential (Forbush 1987; González-Lebrero et al. 2002; Montes et al. 2004). Similarly, measurements of the kinetics of the E2 → E1 transition via the voltage-sensitive fluorescent probe RH421 using a stopped-flow apparatus after mixing the enzyme with NaCl show a biexponential fluorescence change, but only at low ATP concentrations (i.e., ≤25 μM) (Clarke et al. 1998; Humphrey et al. 2002; Clarke et al. 2007). RH421 responds to the kinetics of the E2 → E1 transition with a drop in fluorescence, which has been attributed to the rapid binding of Na^+ to the E1 state following the transition (Schneeberger and Apell 2001; Apell and Diller 2002; Clarke et al. 2007), i.e., the formation of the $E1(Na^+)_3$ state.

What is the origin of biphasic kinetics at low ATP concentrations? Could this mean that the enzyme cycle contains an additional previously undiscovered intermediate state? Could it be due to sequential release of two K^+ or Rb^+ ions? In order to answer these questions it is useful to analyse more deeply the RH421 fluorescence results. RH421 has probably been one of the most useful probes of Na^+,K^+-ATPase kinetics for a number of reasons. Firstly it does not require covalent modification of the enzyme, because it inserts itself into the membrane adjacent to the protein. Therefore, if it is used at a sufficiently low concentration it has no inhibitory effect on protein function (Frank et al. 1996; Kane et al. 1997). Another great advantage of RH421 is that it responds to a number of different reactions around the enzyme's pump cycle and can, thus, be used to gain kinetic information on several different reaction steps, i.e., phosphorylation by ATP, phosphorylation by P_i, dephosphorylation and E1P → E2P in addition to E2 → E1 (Forbush and Klodos 1991; Stürmer et al. 1991; Klodos 1994; Apell et al. 1996; Kane et al. 1998; Cornelius et al. 1998; Cornelius 1999; Clarke et al. 2007; Clarke and Kane 2007). Based on the agreement between RH421 fluorescence transients and kinetic measurements of pump activity by electrical methods, the probe is believed to respond to changes in electric field strength within the membrane associated with electrogenic partial reactions of the enzyme, in particular ion binding and release (Bühler et al. 1991; Domaszewicz and Apell 1999). From careful equilibrium titrations Apell and co-workers (Domaszewicz and Apell 1999; Schneeberger and Apell 2001; Apell and Diller 2002) were able to show that, in the case of ion binding to the cytoplasmic side of the Na^+,K^+-ATPase, RH421 responds only to the binding of the third Na^+ ion, not to the first or the second. This finding has important consequences for the interpretation of the biphasic kinetics observed using RH421 to study the E2 → E1 transition. It means that each kinetic phase observed must be due to the formation of enzyme in the $E1(Na^+)_3$ state, i.e., the formation of $E1Na^+$ or $E1(Na^+)_2$ are insufficient to cause a fluorescence change. If either the binding of the first or the second Na^+ ion could produce a fluorescence change, then a biphasic fluorescence change could be consistent with a two step Na^+ binding process to an enzyme monomer. However, if only binding of the third Na^+ ion is detected by RH421, this is no longer possible. Therefore, because it is well established that no more than three Na^+ ions can bind per α-subunit, the only possible explanation for two phases is that the enzyme exists as a diprotomer.

According to the above argument the mechanism of the E2 → E1 transition in the absence and presence of ATP can be described by the scheme shown in Fig. 2 (Clarke et al. 2007). Based on this scheme and experimentally determined observed rate constants of the RH421 fluorescence transients one can estimate rate constants for the $E2(K^+)_2$:$E2(K^+)_2$ → $E2(K^+)_2$:$E1(Na^+)_3$ and the $E2(K^+)_2$:$E1(Na^+)_3$ → $E1(Na^+)_3$:$E1(Na^+)_3$ transitions of 6.9 and 0.33 s^{-1}, respectively. It appears, therefore, that, in the absence of ATP, after one protomer has undergone the transition and bound three Na^+ ions, a change in protein–protein interactions between the two protomers must occur so that the second protomer undergoes the transition to E1 with a lower rate constant. However, in the presence of ATP both protomers appear to undergo the transition synchronously, i.e., the whole reaction occurs in a single step $E2(K^+)_2ATP$:$E2(K^+)_2$ → $E1(Na^+)_3ATP$:$E1(Na^+)_3$. The rate constant estimated for this reaction was 34 s^{-1} (Clarke et al. 2007). Therefore, ATP not only accelerates the E2 → E1 transition (which has been known for many years); it also synchronizes the transition of two protomers within a dimer.

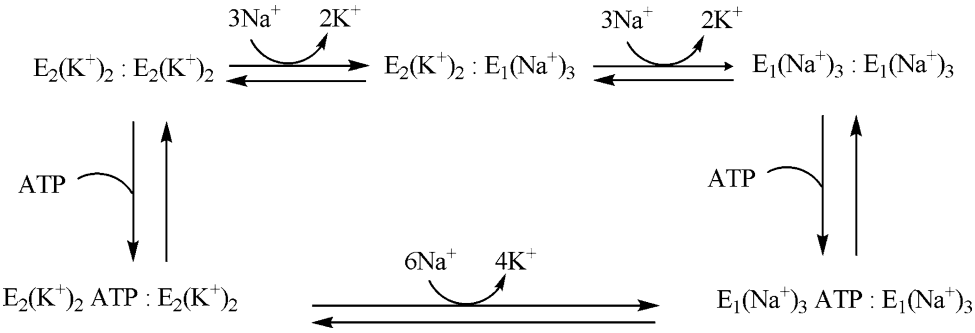

Fig. 2 Dimer model of the E2 → E1 conformational change of the Na^+,K^+-ATPase

A further interesting allosteric effect of ATP on the mechanism of the Na^+,K^+-ATPase is that increasing the ATP concentration causes an increase in the *rate constant* of the phosphorylation reaction (Clarke and Kane 2007). Of course the normal physiological phosphorylation of the enzyme by ATP cannot occur in the absence of ATP and the *rate* of phosphorylation must, therefore, increase with increasing ATP concentration. However, recent results show that the concentration dependence of the amplitudes of RH421 fluorescence transients associated with the reaction $E1(Na^+)_3 \rightarrow E2P + 3Na^+$ (Clarke and Kane 2007; Pilotelle-Bunner et al. 2008) can only be explained if one assumes that the enzyme is capable of binding two molecules of ATP, with each ATP binding step leading to an acceleration of the phosphorylation reaction. As discussed earlier, it now seems certain that each α-subunit of the Na^+,K^+-ATPase only has a single ATP binding site. Therefore, if two ATP binding equilibria occur, the only logical explanation for this is again that the enzyme exists in the membrane as a diprotomer, with two separate ATP binding equilibria coming about because of protein–protein interactions causing a cooperativity in ATP binding. The kinetic model describing this is shown in Fig. 3. Based on this model it was possible to reproduce the RH421 fluorescence amplitudes observed in stopped-flow experiments over five orders of magnitude of the ATP concentration (Clarke and Kane 2007). The K_d of the initial ATP binding used was 0.25 μM, which agrees with equilibrium binding studies of ATP to the E1 conformation of the enzyme (Nørby and Jensen 1971a; Hegyvary and Post 1971; Fedosova et al. 2003; Grell et al. 2004; Esmann et al. 2008; Pilotelle-Bunner et al. 2008). In order to best reproduce the data, the K_d of the second ATP binding step was taken as 7.0 μM. This implies a negative cooperativity between the two protomers, i.e., the binding of the first ATP molecule weakens the binding of ATP to the second protomer. A second ATP binding step with a K_d of around 7 μM has so far never been observed in equilibrium binding studies, although values of this order of magnitude have frequently been determined from pre-steady-state kinetic studies (Froehlich et al. 1983; Borlinghaus and Apell 1988; Fendler et al. 1993; Friedrich et al. 1996; Kane et al. 1997; Clarke et al. 1998). A possible explanation for this is that the second ATP binding site only becomes accessible when all of the physiological substrates are present, i.e., ATP, Na^+ and Mg^{2+}. In equilibrium binding studies one of these must always be omitted in order to achieve the equilibrium condition.

Apart from causing a weakening of the ATP affinity, it was found that ATP binding to the diprotomer affected the rate constant for the formation of E2P from E1, increasing from only 15 s^{-1} when only a single ATP was bound per diprotomer to 173 s^{-1} when two ATP molecules are bound

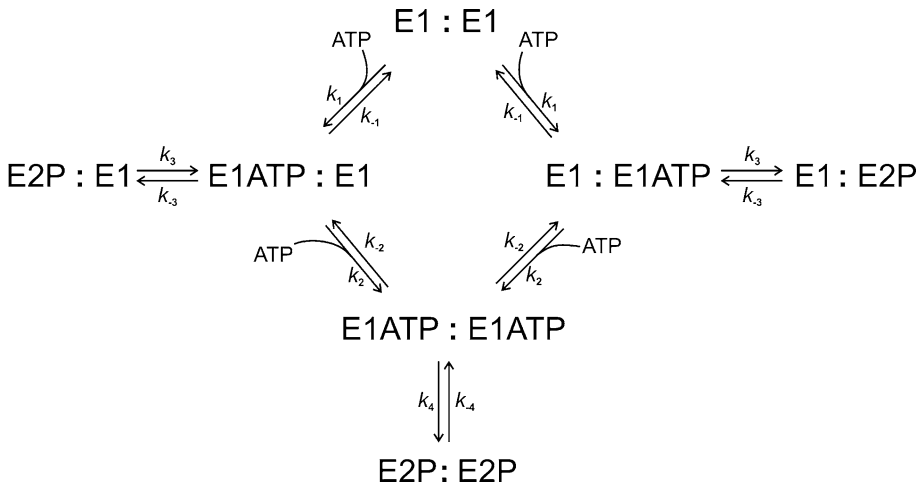

Fig. 3 Dimer model of Na^+,K^+-ATPase phosphorylation by ATP

per diprotomer (Clarke and Kane 2007). It is, thus, proposed that the enzyme has two gears of ion pumping. This leads to the modification to the Albers–Post cycle shown in Fig. 4. This scheme actually represents two classical Albers–Post cycles combined. In the lower faster cycle both protomers proceed around the Albers–Post cycle in unison. In the upper slower cycle one protomer stays locked in the $E1(Na^+)_3$ state while the other protomer proceeds around the Albers–Post cycle. Therefore, the scheme shown in Fig. 4 does not involve the introduction of any new intermediate states. The enzyme simply exists as a diprotomer in which either a single protomer pumps ions alone or both protomers pump ions synchronously.

The performance of quenched-flow measurements analogous to the fluorescent-based stopped-flow measurements of Clarke and Kane (2007) is difficult because the experiments require high concentrations of radioactively labelled ATP. However, experiments have in fact been reported by Peluffo et al. (1992). They found that the amount of phosphoenzyme formed continued to increase at ATP concentrations above 10 μM, where one would have expected based on a single high affinity ATP site with a K_d of around 0.2 μM that all of the enzyme's ATP binding sites should be saturated. They termed this observation "superphosphorylation". The results of Peluffo et al. (1992) are, therefore, consistent with two ATP binding equilibria with different affinities, both leading to phosphorylation, as shown in Fig. 4. Thoenges and Schoner (1997) reported a 30-fold increase in the rate of ATP hydrolysis when two ATP binding sites are occupied relative to when only one is occupied. At the time they performed their work, however, in the absence of any crystal structural information it was not possible for either Peluffo et al. (1992) or Thoenges and Schoner (1997) to distinguish between the possibilities of phosphorylation on two separate sites on a single $\alpha\beta$ protomer or phosphorylation on separate α-subunits within an $(\alpha\beta)_2$ diprotomer. Based on the information discussed in the previous section, the first of these possibilities can now be excluded.

In the kinetic scheme shown in Fig. 4 the inactive protomer in the lower gear of pumping is shown in the $E1(Na^+)_3$ state. The reason for this is that in the experiments carried out by Clarke and Kane (2007), the enzyme was equilibrated in a solution containing 130 mM NaCl, which would be expected to stabilize the E1 state. However, this does not preclude the possibility that this protomer could exist in other conformations. For example, if one of the protomers was locked into the E1 state via the

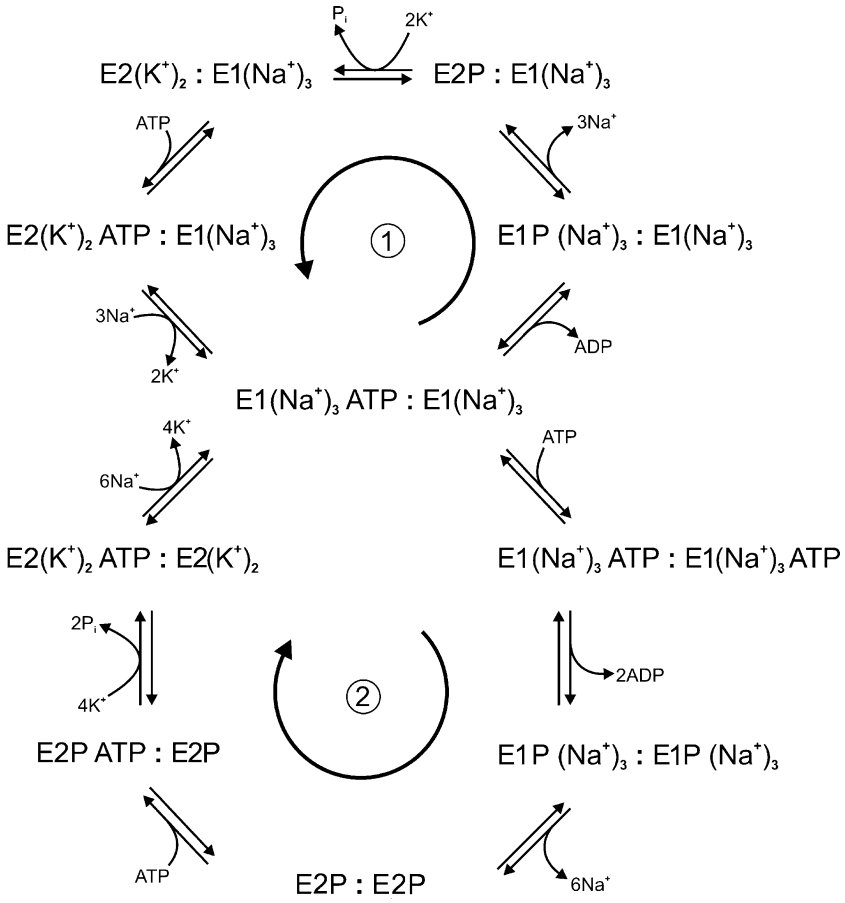

Fig. 4 Two-gear dimeric model of Na^+,K^+-ATPase function. The upper cycle (*1*) represents the pathway followed at low concentrations of ATP, when only one of the ATP binding sites of an α-subunit is occupied. The lower cycle (*2*) represents the pathway followed at high concentrations of ATP, when both of the ATP sites of the $(\alpha\beta)_2$ dimer are occupied. The *arrows* indicate the physiological direction of cycling by the enzyme

binding of a non-phosphorylating ATP derivative or FITC and sufficient K^+ ions were present in solution, its likely that the other protomer would convert to the $E2(K^+)_2$ state. It is, furthermore, possible that this protomer could be capable of undergoing phosphorylation by inorganic phosphate, even while the other protomer says in the E1 state. This would be consistent with the results of Schoner and co-workers (Buxbaum and Schoner 1990, 1991; Hamer and Schoner 1993; Schoner et al. 1998) showing that blocking of the enzyme in either the E1 or the E2 state does not abolish enzyme activity associated with the other state.

It should be pointed out that a bicyclic model of the Na^+,K^+-ATPase mechanism involving subunit interactions has previously been proposed by Plesner and co-workers (Plesner et al. 1981; Plesner 1987). However, the model which they presented consisted of two separate cycles depending on the ionic conditions. They proposed an "Na^+–enzyme cycle" for conditions with no K^+ ions in the medium and an "($Na^+ + K^+$)-enzyme cycle" for conditions when both Na^+ and K^+ ions are present in millimolar concentrations. In contrast, in the bicyclic model shown in Fig. 4 the cycle followed by the enzyme does not depend on the Na^+ or K^+ concentrations but on the ATP concentration.

A further interesting point about the schemes shown in Figs. 2 and 4 is that inherent in both schemes is the idea that as the ATP concentration increases the enzyme protomers should become increasingly synchronous. Asynchronicity is only present at low ATP concentrations in both schemes. A number of researchers have postulated kinetic models involving asynchronous pumping by P-type ATPases in the past, i.e., for the Na^+,K^+-ATPase (Froehlich et al. 1997; Taniguchi et al. 2001; Yokoyama et al. 1999; Tanoue et al. 2006), the sarcoplasmic reticulum Ca^{2+}-ATPase (Froehlich et al. 1997; Mahaney et al. 2004; Mahaney et al. 2008) and the H^+,K^+-ATPase (Abe et al. 2002; Shin et al. 2005). Consistent with these models, the lower gear of pumping shown in Fig. 4 (when only one ATP has bound to the diprotomer, i.e., upper cycle) does involve the presence of dimeric species in which the two protomers can be in two different intermediate states of the pump cycle. However, once the enzyme becomes saturated with ATP, the schemes shown in Figs. 2 and 4 indicate that both protomers should be synchronous. How might synchronicity come about? The simplest possible way would be if all protomers were completely dissociated from one another, i.e., if the oligomers broke up into monomers. In this case every monomer would possess the same rate constant and they would appear synchronous in a stopped-flow experiment simply because they were synchronously stimulated, e.g. by mixing with ATP or with NaCl. If this is true then the faster cycle in Fig. 4 (when two ATP molecules have bound per diprotomer) would simply correspond to individual α-subunits proceeding around the pump cycle independently of one another.

In summary, in the light of all of the experimental evidence available it would seem that the highest rate of pumping by the Na^+,K^+-ATPase occurs when the enzyme exists as a monomer. A lower gear of pumping, which comes about due to protein–protein interactions, only occurs at non-saturating ATP concentrations. ATP binding itself is responsible for the dissociation of the diprotomers into monomers. These conclusions are perfectly consistent with the results of Peters et al. (1981), Vilsen et al. (1987), Martin and Sachs (1999), Cornelius (1995) and Martin et al. (2000), discussed earlier, which indicate, based on measurements of phosphorylation capacity and turnover at different pump densities in the membrane, that under saturating substrate conditions the enzyme behaves as a monomer. Nevertheless, the kinetic scheme shown in Fig. 4 also allows the possibility that protein–protein interactions play an important physiological role when the ATP concentration is non-saturating.

What might the physiological significance be of protein–protein interactions, as described by the scheme shown in Fig. 4? Physiologically there would seem to be no advantage for an ion pump to have a slow gear of pumping. A fast pumping rate could more effectively counter passive diffusion of ions across the membrane so that a cell would need to express fewer ion pump molecules in the membrane to establish or maintain the ion concentration gradients necessary for each associated physiological process (e.g. nerve activity, muscle contraction and relaxation, digestion, nutrient reabsorption). However, although the slow gear of pumping has a lower turnover than the faster gear, the slower gear is also associated with a higher affinity for ATP, i.e., $K_d \approx 0.25$ μM (in comparison to 7 μM for the second ATP binding step). The physiological role of protein–protein interactions may not be to produce a slower turnover of ion pumping, but instead to increase the ATP affinity. Even if the turnover is lower when only a single ATP molecule is bound to a diprotomer, at very low ATP concentrations, if the ATP affinity is higher than that of a monomer, then the enzyme could more effectively bind ATP and this could in fact lead to an overall higher rate of ion pumping than if the enzyme existed as a monomer. The results of Clarke and Kane (2007) suggest that this might be the case at sub-micromolar concentrations of ATP. Therefore, the physiological importance of protein–protein interactions in P-type ATPases may be to maximize the rate of ion pumping under extreme hypoxic or anoxic conditions when the ATP level in the cell falls to a very low level.

If the role of protein–protein interactions is to enhance ATP binding affinity, it is probably more appropriate to speak of a *positive* cooperative effect. Experimentally the kinetic results indicate that the affinity for ATP decreases after the binding of the first ATP molecule to a diprotomer, which formally would be described as a *negative* cooperative

effect. However, it now seems that this negative cooperativity is due to the dissociation of the diprotomer, not to protein–protein interactions themselves. The protein–protein interactions are only present at low ATP concentrations where they positively enhance ATP binding. This interpretation is in accord with results obtained many years ago by Møller et al. (1980) on the sarcoplasmic reticulum Ca^{2+}-ATPase. They found that solubilisation of the protein into monomers using the detergent $C_{12}E_8$ decreased the enzyme's ATP affinity. For aggregated enzyme in its native membrane environment they measured a K_d for ATP of 2 μM, whereas for detergent-solubilized enzyme they measured a K_d of 7 μM. Although this difference is much smaller than that suggested here based on experiments on the Na^+,K^+-ATPase, the direction is the same. For both enzymes (and perhaps for all P-type ATPases), protein–protein interactions enhance the ATP affinity. The next important question is what the mechanism of this enhancement might be.

Structural effect of ATP binding

Over the last few years, crystal structures of the sarcoplasmic reticulum Ca^{2+}-ATPase from skeletal muscle have been determined in a number of different conformational states around the enzyme cycle. With respect to the allosteric role played by ATP in the mechanism of P-type ATPases, the most interesting results are those published by Sørensen et al. (2004) and Jensen et al. (2006), who determined structures of both the calcium-free E2 state and the calcium-bound E1 state in the presence and absence of the non-hydrolysable ATP analogue AMPPCP. The cytoplasmic domains of the relevant structures are shown in Fig. 5, kindly reproduced by permission of the authors. There it can be seen that the E2, E2-ATP and Ca_2E1-ATP states are all in a compact closed conformation with tight association between the protein's N- and P-domains. The Ca_2E1 state is, however, in a much more open state with wider separation of the N- and P-domains. Conversion between the E2 and the Ca_2E1 conformations would, therefore, require a much greater structural rearrangement than between the E2-ATP and Ca_2E1-ATP states. It has recently been found that phosphorylated states of the pump cycle, such as Ca_2E1 ~ P-ADP, E2P and E2P-ATP, also have compact closed conformations (Olesen et al. 2007). Therefore, Olesen et al. (2007) have proposed that ATP has an important modulatory role in maintaining the enzyme in a compact state throughout the whole enzymatic cycle. Based on the structures shown in Fig. 5, Jensen et al. (2006) proposed that under normal physiological conditions the cycle of the Ca^{2+}-ATPase would not include the Ca_2E1 state at all. Because of the similarity between the published structures of the Ca^{2+}-ATPase and the Na^+,K^+-ATPase (Morth et al. 2007), the same is very likely to be true for the Na_3E1 state. A typical intracellular ATP concentration is 1–5 mM, depending on the type of cell (Gribble et al. 2000). At such a high ATP concentration, the ATP-free enzyme states E2 and E1 would not be expected to be appreciably occupied. Under such conditions the enzyme could, thus, maintain its closed conformation throughout the process of releasing K^+ ions to the cytoplasm and binding Na^+ ions.

At ATP concentrations below the normal physiological level it is, however, still possible that open states such as Ca_2E1 or Na_3E1 could still influence pump kinetics even if they are not part of the physiological pump cycle. If at low ATP concentrations P-type ATPases exist as dimers, as discussed in the previous section, then open states such as Ca_2E1 and Na_3E1 could influence the pumping rate of the neighbouring protomer within the dimer via protein–protein interactions. In the lower gear of pumping of the kinetic model shown in Fig. 4 (upper cycle) one can see that one protomer is thought to stay fixed in the $E1(Na^+)_3$ state while the other protomer proceeds around the pump cycle. If the two protomers of the dimer are in close proximity to one another, then the open conformation of the $E1(Na^+)_3$ state would be expected to increase the extent of protein–protein interactions between the cytoplasmic domains of the neighbouring protomers, which could reduce the rate of pumping of the active protomer but increase its ATP affinity. At higher ATP concentrations the initially inactive $E1(Na^+)_3$ state would also bind ATP and convert to the closed $E1(Na^+)_3$ATP state. With both protomers in closed states, the extent of protein–protein interactions would be significantly reduced and this would enable both protomers to pump ions individually as monomers at a higher rate. The two gear model shown in Fig. 4 in which P-type ATPases function as diprotomers at low ATP concentrations and monomers at high ATP concentrations is, thus, not only consistent with kinetic data, there is also a basis for it in the published crystal structures.

The acceleration and synchronisation of the E1 → E2 transition by ATP also makes sense in terms of the crystal structures (see Fig. 2). In the $E2(K^+)_2$:$E2(K^+)_2$ state both protomers would be in a closed state based on crystal structural data and, therefore, the interaction between their cytoplasmic domains would be expected to be small. However, the sequence of reactions $E2(K^+)_2$:$E2(K^+)_2$ → $E2(K^+)_2$:$E1(Na^+)_3$ → $E1(Na^+)_3$:$E1(Na^+)_3$ would involve an opening of the cytoplasmic domains of a protomer at each step. Each opening would increase the extent of protein–protein interactions between the two protomers and presumably increase steric hindrance, thus leading to the successive deceleration of the transition which has been experimentally observed (Clarke et al. 2007). However, if both protomers initially have ATP bound, then the conversion of

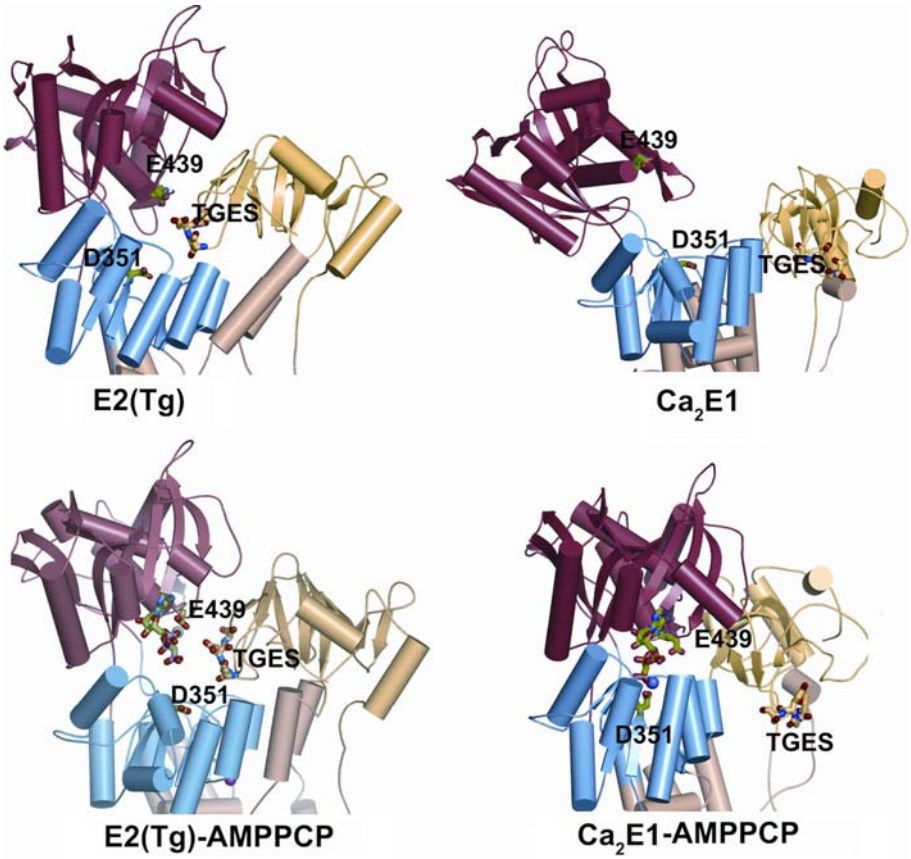

Fig. 5 Crystal structures of the cytoplasmic domains of the skeletal muscle sarcoplasmic reticulum Ca^{2+}-ATPase in the E_2, E_2-ATP, Ca_2E_1-ATP and the Ca_2E_1 states (Sørensen et al. 2004; Jensen et al. 2006), reproduced by kind permission of the authors. Thapsigargin (*Tg*) is an inhibitor which stabilizes the enzyme in the E_2 conformation. Adenosine (*β–γ* methylene)-triphosphate (*AMPPCP*) is a non-hydrolysable ATP analogue. E439 is a glutamate residue involved in ATP binding. D351 is an aspartate residue to which a phosphate group from ATP is transferred. The TGES motif is a threonine–glycine–glutamate–serine amino acid sequence which catalyses dephosphorylation of the enzyme (Olesen et al. 2004)

$E2(K^+)_2ATP$ to $E1(Na^+)_3ATP$ does not involve any opening of the cytoplasmic domains at all, because both are in a compact closed conformation. Therefore, under these conditions all protomers could remain as monomers and, because the steric interaction with a neighbouring monomer within the membrane is minimal, each could undergo the conformational transition from E1 to E2 at an accelerated rate. Finally then, based on the idea of a diprotomeric enzyme complex, a structural basis for the allosteric acceleration of the E2 → E1 transition by ATP has been obtained.

Conclusion

Much has been learnt in the past few years from crystal structural data concerning the atomic detail of ion transport through the catalytic unit of P-type ATPases and the conformational changes associated with phosphorylation by ATP. The crystallization of these proteins has been a magnificent achievement and has led to a renaissance in research into their function. However, it is important to bear in mind that these are *membrane* proteins. In diagrams showing enzyme structures the membrane is generally not shown at all, and in the crystallization process the lipids constituting the membrane are removed and replaced by detergent. Therefore, the consideration of the crystal structure of a single catalytic unit of a P-type ATPase removed from its physiological environment of the membrane is unlikely to give a full picture of the mechanism of these enzymes. Nevertheless, the crystal structures do give very valuable information, and, together with kinetic data obtained in the native membrane environment, as demonstrated here a much clearer description of their mechanism is evolving. This review has been devoted to allosteric effects of ATP in the mechanism of P-type ATPases, for which up to now no explanation has been offered based on crystal structural data. If the views expressed in this paper are accepted, the reason for this is that the basis for these effects lies not in the structure of a single catalytic unit of these enzymes but in the interaction between them in their membrane surroundings.

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