Critical Interaction of Actuator Domain Residues Arginine 174, Isoleucine 188, and Lysine 205 with Modulatory Nucleotide in Sarcoplasmic Reticulum Ca$^{2+}$-ATPase*†

Received for publication, September 2, 2008, and in revised form, September 29, 2008 Published, JBC Papers in Press, October 17, 2008, DOI 10.1074/jbc.M806795200

Johannes D. Clausen‡, David B. McIntosh, David G. Woolley, and Jens Peter Andersen

From the 1Department of Physiology and Biophysics, Aarhus University, DK-8000 Aarhus C, Denmark, the 2Institute of Infectious Diseases and Molecular Medicine, Division of Chemical Pathology, Faculty of Health Sciences, University of Cape Town, Observatory, Cape Town 7925, South Africa, and the 3Centre for Membrane Pumps in Cells and Disease—PUMPKIN, Danish National Research Foundation, Denmark

ATP plays dual roles in the reaction cycle of the sarcoplasmic reticulum Ca$^{2+}$-ATPase by acting as the phosphorylating substrate as well as in nonphosphorylating (modulatory) modes accelerating conformational transitions of the enzyme cycle. Here we have examined the involvement of actuator domain residues Arg174, Ile188, Lys205, and Lys205 by mutagenesis. Alanine mutations to these residues had little effect on the interaction of the Ca$^{2+}$E1 state with nucleotide or on the H$^{+}$E2 to Ca$^{2+}$E1 transition of the dephosphoenzyme. The phosphoenzyme processing steps, Ca$^{2+}$E1P to E2P and E2P dephosphorylation, and their stimulation by MgATP/ATP were markedly affected by mutations to Arg174, Ile188, and Lys205. Replacement of Ile188 with alanine abolished nucleotide modulation of dephosphorylation but not the modulation of the Ca$^{2+}$E1P to E2P transition. Mutation to Arg174 interfered with nucleotide modulation of either of the phosphoenzyme processing steps, indicating a significant overlap between the modulatory nucleotide-binding sites involved. Mutation to Lys205 enhanced the rates of the phosphoenzyme processing steps in the absence of nucleotide and disrupted the nucleotide modulation of the Ca$^{2+}$E1P to E2P transition. Remarkably, the mutants with alterations to Lys205 showed an anomalous inhibition by ATP of the dephosphorylation, and in the alanine mutant the affinity for the inhibition by ATP was indistinguishable from that for stimulation by ATP of the wild type. Hence, the actuator domain is an important player in the function of ATP as modulator of phosphoenzyme processing, with Arg174, Ile188, and Lys205 all being critically involved, although in different ways. The data support a variable site model for the modulatory effects with the nucleotide binding somewhat differently in each of the conformational states occurring during the transport cycle.

The sarcoplasmic reticulum Ca$^{2+}$-ATPase (1) is a membrane-bound ion pump that belongs to the family of P-type ATPases (2), named so because their reaction cycles involve the transient phosphorylation of the aspartic acid residue in the universally conserved DKTGT(L/I)T motif. Insight into the structural organization and domain movements that take place during pump activity in P-type ATPases has come from the elucidation over the past 8 years of several high resolution crystal structures of Ca$^{2+}$-ATPase (3–11), each thought to represent a specific intermediate state in the pump cycle (12), as well as the recent crystal structures of pig renal Na$^{+}$,K$^{+}$-ATPase (13) and Arabidopsis thaliana H$^{+}$-ATPase (14). In these P-type pumps the membrane-buried region is made up of 10 membrane-spanning α-helices. The cytoplasmic headpiece is separated into three distinct domains, named A (“actuator”), P (“phosphorylation”), and N (“nucleotide binding”). The ion translocation is achieved by sequential conformational transitions between phosphorylated and dephosphorylated intermediate states (cf. Scheme 1 for Ca$^{2+}$-ATPase) (15, 16). The key to understanding the long distance coupling between formation/decomposition of the acyl phosphate in the cytoplasmic P-domain and the changes in the ion-binding sites buried deep in the membranous region seems to be the relatively large movements of the A-domain, which are transmitted to the transmembrane helices (8, 11).

ATP plays dual roles in the reaction cycle of the Ca$^{2+}$-ATPase by acting both as the phosphorylating substrate as well as in nonphosphorylating (modulatory) modes, the latter regulating the rates of various partial reaction steps (boxed ATP in Scheme 1). The modulatory action of ATP seems to be a general feature in the family of P-type pumps and is a vital means of overcoming rate-limiting reaction steps during pump activity. Thus, the K$^{+}$ deoccluding K$^{+}$E2 $\rightarrow$ E1 transition in Na$^{+}$,K$^{+}$-ATPase and H$^{+}$,K$^{+}$-ATPase as well as the corresponding H$^{+}$E2 $\rightarrow$ E1 transition in Ca$^{2+}$-ATPase are accelerated by ATP with affinity constants in the 10–100 μM range (17–21). In the Ca$^{2+}$-ATPase, ATP furthermore binds to the phosphoenzyme intermediates and stimulates phosphoenzyme turnover (i.e. Ca$^{2+}$E1P $\rightarrow$ E2P and E2P $\rightarrow$ E2 partial reactions (22–30), whereas in Na$^{+}$,K$^{+}$-ATPase the rate of E2P dephosphorylation seems instead to be inhibited by nucleotide (31, 32). Such observations have raised the question whether the modulatory ATP molecule binds at the same site as the ATP molecule undergo-
ing hydrolysis (28, 33, 34) or at a distinct or only partially overlapping site (35, 36). In a recent mutagenesis study (30), we pinpointed Glu439, Phe487, and Arg560 in the N-domain and Arg678 in the P-domain of Ca\(^{2+}\)-ATPase as critical amino acid residues for the stimulation by ATP of E2P dephosphorylation. Phe487, Arg560, and Arg678 are also known as key residues in ATP binding in the phosphorylating mode (5, 7, 30, 37). None of these residues, however, were critical for the modulation by ATP binding in the phosphorylating mode (5, 7, 30, 37). None of these residues, however, were critical for the modulation by additional binding of ATP or MgATP that is not hydrolyzed (‘modulatory ATP’).

**EXPERIMENTAL PROCEDURES**

Site-directed mutagenesis of cDNA encoding the rabbit fast twitch muscle Ca\(^{2+}\)-ATPase (SERCA1a isofrom) inserted into the pMT2 vector (39) was carried out using the QuikChange site-directed mutagenesis kit (Stratagene), and the mutant cDNA was sequenced throughout. To express wild type or mutant cDNA, COS-1 cells were transfected using the calcium phosphate precipitation method. Microsomal vesicles containing either expressed wild type or mutant Ca\(^{2+}\)-ATPase were isolated by differential centrifugation (40). The concentration of expressed Ca\(^{2+}\)-ATPase was determined by an enzyme-linked immunosorbent assay (41). Transport of \(^{45}\)Ca\(^{2+}\) into the microsomal vesicles was measured by filtration, and the ATPase activity was determined by following the liberation of P\(_1\) in the presence of 4 \(\mu\)M ionophore A23187 to prevent inhibition caused by rebinding of Ca\(^{2+}\) to the lumenally facing Ca\(^{2+}\) sites (42, 43). Measurements of phosphorylation and dephosphorylation at 0 °C were carried out by manual mixing (30, 43, 44). Transient state kinetics at 25 °C was analyzed using the Bio-Logic quench-flow module QFM-5 (Bio-Logic Science Instruments, Claix, France) with mixing protocols as described previously (45). The determination of the phosphorylation level by acid quenching followed by acid SDS-PAGE and quantification of the radioactivity associated with the Ca\(^{2+}\)-ATPase band, as well as the photolabeling with [\(\gamma\)-\(^{32}\)P]TNP-8N\(_3\)-ATP,\(^2\) the inhibition of photolabeling by ATP, and the quantification of the label bound specifically to the Ca\(^{2+}\)-ATPase were carried out using the previously established procedures (30, 37, 46–50).

The experiments were generally conducted at least twice, and average values are shown in the figures. The data were analyzed by nonlinear regression using the SigmaPlot program (SPSS, Inc.) or by computation using the SimZyme program (45). Monoexponential functions were fitted to the dephosphorylation time courses. The data obtained in rapid kinetic experiments using quench flow instrumentation were analyzed by the SimZyme program using a three-intermediate reaction scheme, taking into account the contribution by forward phosphoenzyme- and dephosphoenzyme-processing steps to the time course of phosphoenzyme formation, as detailed below and in previous publications (45, 51). The analysis of Ca\(^{2+}\) and MgATP concentration dependences of phosphorylation was based on the Hill equation, \(E_P = E_{P_{\text{max}}} [L]/([K_{0.5} + [L]^n])\). A hyperbolic function plus a linear component was fitted to the [\(\gamma\)-\(^{32}\)P]TNP-8N\(_3\)-ATP labeling data, and the “true” dissociation constant, \(K_{dP}\), for ATP binding was calculated from the measured \(K_{0.5}\) values using the validated equation for competitive inhibition (46). For analysis of the modulatory effect of nucleotide or Pi (abbreviated “S” for substrate) on the rates of the partial reaction steps, the substrate concentration dependence of the rate constant was analyzed according to Equation 1,

\[
 k_{\text{obs}} = k_0 + (k_{\text{max}} - k_0)[S]/([K_{0.5} + [S]]) \tag{1}
\]

Here, \(k_{\text{obs}}\) is the rate constant observed at the indicated substrate concentration; \(k_0\) is the rate constant in the absence of substrate (“basic rate”), and \(k_{\text{max}}\) is the extrapolated value of the rate constant corresponding to infinite substrate concentration. The enhancement factor \(k_{\text{max}}/k_0\) describes the extent of the modulatory effect, and the \(K_{0.5}\) value describes the affinity for the modulatory substrate.

**RESULTS**

Expression and Assays of Overall Function—Eight single mutations were introduced to four amino acid residues in the A-domain of sarcoplasmic reticulum Ca\(^{2+}\)-ATPase. Arg174, Lys204, and Lys205 were each substituted with alanine and glutamate, to examine the consequences of either removing the bulky, charged side chain or reversing the charge, and Ile288 was replaced by alanine and phenylalanine, to examine the importance of the size of the hydrophobic side chain at position 188. The expression levels in COS-1 cells of the six mutant pumps with alterations to Arg174, Ile288, and Lys205 were similar to that obtained with wild type, as evaluated by their immunoreactivity in the specific enzyme-linked immunosorbent assay (data not shown). The expression levels of mutants K204A and K204E were generally around 60 and 10–20%, respectively, of that

\(^2\) The abbreviations used are: TNP-8N\(_3\)-ATP, 2-azido-8-(2,4,6-trinitrophenyl)-azidoadenosine 5'-triphosphate; AMPPCP, adenosine 5′-(β,γ-methylene)-triphosphate; AMPPNP, adenosine 5′-(β,γ-imido)-triphosphate; EPPS, N-2-hydroxyethylpiperazine-N'-3-propanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; PPi, pyrophosphate; SERCA, sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase.
obtained with wild type. Reliable and reproducible biochemical experiments could be performed with K204A, whereas the low expression level of K204E precluded a functional characterization of this mutant.

For R174A, I188F, K205A, and K205E, the molecular rate of Ca\(^{2+}\)-activated ATP hydrolysis ("ATP turnover rate") at 37 °C with 5 mM MgATP was similar to, or slightly lower than (by less than 15%), that of wild type (Fig. 1). I188A and K204A displayed ~30% reduced ATP turnover rates relative to wild type, whereas the ATP turnover rate of R174E was reduced by as much as ~80%. The effects of the mutations to Arg\(^{174}\) on overall ATP turnover are similar to those presented previously (52). The overall activity at 37 °C and saturating substrate concentrations was further evaluated by measuring the ATP-driven accumulation of \(^{45}\)Ca\(^{2+}\) in the microsomal vesicles (Fig. 1). For the mutants with alterations to Arg\(^{174}\), Ile\(^{188}\), and Lys\(^{205}\), the rates of Ca\(^{2+}\) transport and ATP hydrolysis correlated well, implying that the coupling between ATP consumption and Ca\(^{2+}\) translocation is fully retained in the mutants. Mutant K204A, however, displayed only ~40% Ca\(^{2+}\) transport relative to wild type, compared with its ~70% rate of ATP turnover.

The Ca\(^{2+}\) dependence of the steady-state phosphoenzyme level obtained by phosphorylation from 5 \(\mu\)M \([\gamma-\text{P}]\)MgATP and the \([\gamma-\text{P}]\)MgATP dependence of steady-state phosphorylation at 100 \(\mu\)M free Ca\(^{2+}\) were determined at 0 °C and pH 7 (supplemental Fig. S1, left and right panels, respectively). At 10–100 \(\mu\)M free Ca\(^{2+}\) and 5–50 \(\mu\)M MgATP, the concentration ranges of Ca\(^{2+}\) and MgATP applied in the phosphorylation experiments described below, both the wild type and the mutants displayed high levels of phosphoenzyme (>80% of the \(E_{\text{P, max}}\) derived from the fits of the Hill equation to the data).

**Transient State Kinetics of Phosphorylation of Ca\(_{\text{E1}}\) from MgATP—**

The time course of phosphorylation from 10 \(\mu\)M \([\gamma-\text{P}]\)MgATP of wild type or mutant Ca\(^{2+}\)-ATPase pre-equilibrated with Ca\(^{2+}\) was studied at pH 6 and 25 °C using rapid kinetics instrumentation (Fig. 2 and Table 1). Under these conditions, the wild type displays an initial overshoot of phosphorylation because of the contribution to rate limitation by the Ca\(^{2+}\) binding transition (i.e., \(H_{\text{E}}E2 \rightarrow Ca_{\text{E1}}\)), causing a significant amount of dephosphoenzyme to accumulate at steady state. This overshoot can be reproduced by computer simulation based on the simplified three-intermediate reaction cycle shown in the bottom right corner of Fig. 2, with rate constants \(k_{a} = 65 \text{ s}^{-1}\) for phosphorylation of Ca\(_{\text{E1}}\), \(k_{b} = 3 \text{ s}^{-1}\) for phosphoenzyme processing (i.e., Ca\(_{\text{E1P}}\) → E2P → E2, the latter step
A-Domain Interaction of SERCA with Modulatory Nucleotide

being much faster than the former under the conditions applied here, thus allowing them to be represented by a single rate constant, and $k_b = 5 \text{s}^{-1}$ for the $H_3E_2 \rightarrow CaE_1$ reaction sequence. With these rate constants the steady state is reached within $\sim 0.5 \text{s}$ in the wild type, and after this point $36\%$ of the enzyme is in the $E_2$ state, $3\%$ in the $CaE_1$ state, and $61\%$ is phosphorylated.

As seen in Fig. 2 and Table 1, all seven mutants displayed phosphorylation rates in the $65$–$80 \text{s}^{-1}$ range (column labeled “$CaE_1 \rightarrow CaE_{1P}$” in Table 1), i.e. at least as fast as that of wild type, implying that Arg$^{174}$, Ile$^{188}$, Lys$^{204}$, and Lys$^{205}$ are not critical for $E_1$ form. Because the phosphorylation was carried out at a subsaturating concentration of MgATP (the $K_D$ for MgATP at pH 6 and 20 °C is $\sim 10 \text{mM}$ (53, 54)), the wild type-like phosphorylation rates of the mutants further suggest that Arg$^{174}$, Ile$^{188}$, Lys$^{204}$, and Lys$^{205}$ are not critical for binding of MgATP to Ca$^{2+}$-saturated $E_1$, as any change in nucleotide affinity induced by the mutations should also affect the phosphorylation rate (see below for direct measurements of MgATP binding to Ca$^{2+}$-free $E_1$).

K205A and K205E both displayed more pronounced phosphorylation overshoots relative to wild type, with substantial levels of dephosphoenzyme accumulated at steady state (Fig. 2), implying either rapid phosphoenzyme processing or inhibition of the Ca$^{2+}$ binding transition of the dephosphoenzyme. In such cases where the phosphorylation overshoot is substantial, fairly accurate values of $k_b$ and $k_c$ can be derived from the computer simulations, because the steepness of the descending phase following the overshoot directly reflects the rate of phosphoenzyme processing (51). The computational analysis showed that phosphoenzyme processing was $2.7\times$ and $6\times$ enhanced for K205A and K205E, respectively, relative to wild type, whereas the Ca$^{2+}$ binding $H_3E_2 \rightarrow CaE_1$ transition was rather unaffected by the mutations. For the remaining mutants, the size of the phosphorylation overshoot was either similar to or smaller than that of wild type (Fig. 2), and in such cases more latitude exists with respect to the determination of $k_b$ and $k_c$ (51). A reduced overshoot can result either from an inhibition of phosphoenzyme processing or from an enhanced Ca$^{2+}$ binding transition, or a combination of both. Direct measurements of the rates of the partial reactions involved in phosphoenzyme and dephosphoenzyme processing are presented below.

Affinity of E1 for MgATP—The nucleotide binding properties of wild type and mutants were examined in the absence of Ca$^{2+}$ (to avoid ATP hydrolysis) and at pH 8.5 (to accumulate $E_1$ even in the absence of Ca$^{2+}$ (55)), by using the previously described and validated assay in which the ATPase is photolabeled with $[\gamma\text{-}^32\text{P}]\text{TNP-8N3-MgATP}$ (30, 46, 47, 49). The $[\gamma\text{-}^32\text{P}]\text{TNP-8N3-MgATP}$ dependence of the photolabeling and the competitive inhibition of the photolabeling with MgATP were studied, and results are shown in Fig. 3 and supplemental Fig. S2, with the derived affinity constants listed in Table 2. In accordance with the wild type-like phosphorylation rates obtained with the mutants at a subsaturating concentration of MgATP (Fig. 2), the effects of the mutations on nucleotide binding were moderate. Thus, the $K_{f,0.5}$ values for the binding of the photolabel and the $K_{a}$ values for MgATP inhibition of the photolabeling generally deviated less than 2-fold from the values obtained with wild type (Table 2). Exceptions to this pattern, however, were R174E and I188A, the former displaying a significant and reproducible 4-fold reduced MgATP affinity relative to wild type, and the latter displaying 2–3-fold increased TNP-8N3-MgATP and MgATP affinities, suggesting that under the conditions of the binding assay Arg$^{174}$ and Ile$^{188}$ may interact with the nucle-
otide to a slightly more significant extent than seen in the phosphorylation assay described above. The flexibility of the A-domain may be higher in the absence of Ca^{2+} than in its presence, thereby allowing the A-domain to “sample” E2 conformations, in which Arg^{74} comes close to and possibly interacts with the nucleotide (see under “Discussion”). Studies of the ADP dependence of ADP-induced dephosphorylation of phosphoenzyme formed from 5 μM [γ-32P]MgATP also showed only moderate deviations of the mutants from wild type with respect to the K_{0.5} for ADP, suggesting that the mutations interfere little with ADP binding to Ca_{2}E1P (supplemental Fig. S3) and therefore an obvious target for regulation of activity. Indeed, acting in a nonphosphorylating modulatory mode, MgATP has a prominent activating effect on the rate of the conformational change of the phosphoenzyme (22–25, 30).

The time course of the forward processing of phosphoenzyme formed by phosphorylation of Ca^{2+}-saturated enzyme from 5 μM [γ-32P]MgATP was measured by a chase of the accumulated phosphoenzyme with excess EGTA (to remove Ca^{2+} and thus terminate phosphorylation) and varying concentrations of nonradioactive MgATP. Under the phosphorylation conditions applied here (0°C at pH 7 and high [K^{+}]), the steady-state phosphoenzyme accumulated prior to the initiation of the chase was almost exclusively ADP-sensitive Ca_{2}E1P for wild type as well as mutants (supplemental Fig. S3), demonstrating that the conformational transition of the phosphoenzyme (i.e. Ca_{2}E1P → E2P) was indeed the rate-limiting step in phosphoenzyme processing. The dephosphorylation time courses obtained at selected MgATP concentrations are shown in supplemental Fig. S4, and the basic rate constants (i.e. those corresponding to absence of MgATP in excess of the amount used for phosphorylation) obtained by fitting of a monoeponential decay function to the data are listed in Table 1 (column labeled “Ca_{2}E1P → E2P”). R174E and I188A displayed significant 8- and 3-fold reduced basic rates of Ca_{2}E1P → E2P relative to wild type, whereas the rate of R174A was only mildly reduced, and the rates of I188F and K204A were wild type-like. K205A and K205E displayed 2- and 4-fold enhanced basic rates of Ca_{2}E1P → E2P, respectively, in accordance with the large, steep phosphor-
duced activation of phosphoenzyme processing throughout the MgATP concentration range studied, and accordingly, no MgATP affinity constant could be obtained for these two mutants. The ability of K205A and K205E to undergo MgATP-induced stimulation of phosphoenzyme processing was also notably impaired. Thus, K205A displayed only a 1.7-fold enhancement of phosphoenzyme processing over the MgATP concentration range applied, suggesting very low MgATP affinity (although a \( K_{0.5} \) value could not be derived), and in K205E the affinity appeared even lower with almost no MgATP modulation throughout the nucleotide concentration range.

Dephosphorylation of E2P and Its Modulation by ATP—Like the \( \text{Ca}_2\text{E}1P \rightarrow \text{E}2P \) conformational transition of the phosphoenzyme, the dephosphorylation of \( \text{E}2P \) is also a target for stimulatory modulation by nucleotide in a nonphosphorylating mode (22, 24, 26–28, 30). Contrary, however, to the nucleotide modulation of \( \text{Ca}_2\text{E}1P \rightarrow \text{E}2P \), which is rather independent of whether \( \text{Mg}^{2+} \) is present or absent (30), the modulation of \( \text{E}2P \rightarrow \text{E}2 \) depends on metal-free ATP. High concentrations of \( \text{Mg}^{2+} \) eliminate the accelerating effect, because only metal-free ATP binds to \( \text{E}2P \) with reasonable affinity (in the 10–100 \( \mu \text{M} \) range) (24, 28–30).

To study the rate of \( \text{E}2P \) dephosphorylation, wild type and mutants were phosphorylated by \( ^{32}\text{P} \) in the reverse direction relative to the normal turnover cycle, under favorable conditions for \( \text{E}2P \) accumulation (saturating concentration of \( ^{32}\text{P} \), and \( \text{Mg}^{2+} \), absence of \( \text{Ca}^{2+} \), acidic pH, presence of dimethyl sulfoxide, and absence of \( \text{K}^+ \)). Next, the phosphoenzyme decay was followed upon a chase of the accumulated phosphoenzyme with excess EDTA (to chelate \( \text{Mg}^{2+} \) and thus terminate phosphorylation) and varying concentrations of ATP. The time courses obtained with wild type and mutants at selected ATP concentrations are shown in supplemental Fig. S5, and the basic rate constants (i.e. those obtained in the absence of ATP) are listed in Table 1 (column labeled “\( \text{E}2P \rightarrow \text{E}2 \)”). In the absence of ATP, R174E, K205A, and K205E displayed significantly reduced \( \text{E}2P \) stability relative to wild type (4.1-, 3.6-, and 7.9-fold increased basic rate constants, respectively), whereas the rate constants obtained with R174A, I188A, I188F, and K204A deviated less than 2-fold from that of wild type.

The ATP dependence of the rate of \( \text{E}2P \) dephosphorylation is shown in Fig. 5, and the ATP affinities and enhancement factors derived from fits of a hyperbolic function to the data are listed in Table 3 (columns labeled “\( \text{E}2P \rightarrow \text{E}2, \text{ATP} \)”). For wild type, the affinity of \( \text{E}2P \) for ATP was found to be 34 \( \mu \text{M} \) with an enhancement factor of 2.4, comparable with our previously published values of \( K_{0.5} = 19 \mu \text{M} \) and \( k_{\text{max}}/k_0 = 2.9 \) (30). The parameters derived from the analysis of K204A deviated only marginally from those of wild type (1.8-fold reduced ATP affinity and wild type-like enhancement factor). In contrast, R174A and R174E displayed 7.6- and, at least, 15-fold reduced ATP affinities, respectively (note the different scales on the abscissa for R174A and R174E in Fig. 5), but with significant (more than 3-fold) activation of \( \text{E}2P \) dephosphorylation over the range of ATP concentrations applied, suggesting involvement of the Arg174 side chain in binding of the modulatory ATP to \( \text{E}2P \). The dephosphorylation of I188A was not modulated by ATP throughout the concentration range studied. On the contrary, the \( \text{E}2P \) dephosphorylation of I188F was modulated quite efficiently (\( k_{\text{max}}/k_0 = 4.6 \)) by ATP with an affinity only 2.2-fold lower than that of wild type, implying that a bulky, hydrophobic side chain at position 188 is a prerequisite for a functional modulatory ATP-binding site in \( \text{E}2P \).
Modulation of E2P Dephosphorylation by PPi—Millimolar concentrations of PPi (pyrophosphate) exert a stimulatory effect on the overall ATP turnover rate, resembling the low affinity effect of ATP/MgATP on ATPase activity (58). Furthermore, PPi has been shown to competitively inhibit the binding of both ATP and the phosphate transition-state analogue vanadate to Ca\(^{2+}\)-deprived E2, in either case with an affinity around 1–2 mM (58–60), suggesting overlapping binding sites for PPi, vanadate, and the modulatory nucleotide.

To study the ability of PPi to modulate E2P → E2, we measured the PPi dependence of the rate of E2P dephosphorylation. The method was essentially the same as that described above for the ATP dependence of E2P dephosphorylation, except that ATP was replaced by PPi. The results are shown in Fig. 6, with the time courses at selected PPi concentrations depicted in supplemental Fig. S6, and the PPi affinities and enhancement factors derived from fits of a hyperbolic function to the data listed in Table 3 (columns labeled “E2P → E2, PPi”). It is seen in Fig. 6 that the rate of E2P dephosphorylation of the wild type is accelerated by PPi, with an affinity constant of 3 mM and an enhancement factor of 4.7. K204A and R174A deviated only marginally from wild type with respect to affinities and enhancement factors. R174E displayed 2.3-fold higher rate at 5 mM PPi than in its absence and a significantly reduced PPi affinity (an affinity constant could not, however, be derived for R174E, because the dephosphorylation rate did not reach a steady level within the concentration range of PPi applied). I188A and I188F both displayed affinities for PPi at least as high as that of the wild type, although the enhancement factor of I188A was only 2.4 compared with 6.0 for I188F. The ability of I188A to undergo PPi-induced stimulation of E2P dephosphorylation sharply contrasts its insensitivity to ATP modulation, suggesting that Ile\(^{188}\) contributes to ligation of the adenosine moiety of the modulatory ATP in E2P. Neither K205A nor K205E were able to undergo PPi-induced stimulation of E2P → E2, both mutants displaying a rather constant high E2P dephosphorylation rate throughout the PPi concentration range studied.
Ca$^{2+}$ binding) was first examined in the absence of nucleotide. The wild type or mutant enzyme was pre-equilibrated in the absence of Ca$^{2+}$ at pH 6 to accumulate the E2 state, followed by incubation with excess Ca$^{2+}$ for varying time intervals and subsequent incubation with [$\gamma$-32P]MgATP for 34 ms. This assay takes advantage of the fact that only the Ca$^{2+}$E1 state can be phosphorylated by MgATP (61) and that the phosphorylation step is much faster than the preceding deprotonation and Ca$^{2+}$ binding conformational changes for mutants as well as wild type (cf. Table 1). The amount of phosphoenzyme obtained for each time interval of Ca$^{2+}$ incubation thus reflects the amount of Ca$^{2+}$E1 present prior to the addition of [$\gamma$-32P]MgATP. The time courses of H$_{p}$E2 → Ca$^{2+}$E1 obtained from such experiments are shown in supplemental Fig. S7A, and the rate constants derived from fits of a monoexponential function to the data are listed in Table 1 (column labeled “H$^{\text{const}}$”). Only R174E displayed a basic rate of H$_{p}$E2 → Ca$^{2+}$E1 deviating more than 2-fold from that of the wild type (2.7-fold enhanced rate).

Next, we studied the ability of MgATP to accelerate H$_{p}$E2 → Ca$^{2+}$E1. The enzyme was again pre-equilibrated in the absence of Ca$^{2+}$ at pH 6 to accumulate H$_{p}$E2, and the H$_{p}$E2 → Ca$^{2+}$E1 → Ca$^{2+}$E1P reaction sequence was then followed by simultaneous addition of excess Ca$^{2+}$ and varying concentrations of [$\gamma$-32P]MgATP, with subsequent acid quenching at varying time intervals. The resulting time courses obtained at selected MgATP concentrations are shown in supplemental Fig. S7B. As noted above, the Ca$^{2+}$ binding transition is considerably slower than the subsequent phosphorylation step at pH 6 for wild type as well as mutants (cf. Table 1), and the observed time course of phosphoenzyme formation thus reflects the rate of H$_{p}$E2 → Ca$^{2+}$E1. In Fig. 7, the measured rate constants are shown as a function of the MgATP concentration, and Table 3 lists the MgATP affinity constants ($K_{\text{0.5}}$, and enhancement factors ($k_{\text{max}}/k_0$) derived from fits of a hyperbolic function to the data (columns labeled “H$_{p}$E2 → Ca$^{2+}$E1, MgATP”). For the wild type, the MgATP affinity determined was 37 $\mu$M with an enhancement factor of 34, in good agreement with our previously published values (30). The parameters derived from the analysis of I188A, K204A, and K205A were almost identical to those obtained with the wild type. R174A and R174E displayed wild type-like MgATP affinities, but 2.0- and 3.8-fold reduced enhancement factors, respectively, possibly related to their respective 2- and 3-fold enhanced basic rates of H$_{p}$E2 → Ca$^{2+}$E1 (Table 1). I188F and K205E both displayed reduced MgATP affinity. Thus, in both mutants the basic rate of H$_{p}$E2 → Ca$^{2+}$E1 was almost identical to that of the wild type, whereas the rates measured at MgATP concentrations in the 5–50 $\mu$M range were significantly reduced. The low MgATP affinities of I188F and K205E excluded an accurate determination of the affinity constants for these two mutants. However, assuming that the $k_{\text{max}}$ value of I188F and K205E is wild type-like (i.e. corresponding to wild type-like enhancement factors, because of the fact that the $k_0$ is wild type-like for both mutants), their $K_{\text{0.5}}$ for MgATP would be ~100 $\mu$M (Table 3), i.e. 3-fold reduced MgATP affinity relative to wild type.

**FIGURE 7.** MgATP dependence of the rate of the Ca$^{2+}$ binding transition. The H$_{p}$E2 → Ca$^{2+}$E1 rate constants derived from the data in supplemental Fig. S7 and from data obtained in a similar way at other MgATP concentrations in the phosphorylation medium are shown here as a function of the MgATP concentration. The lines represent the best fits to the data of the hyperbolic function described under “Experimental Procedures” (see Equation 1), the derived parameters being listed in Table 3 (column H$_{p}$E2 → Ca$^{2+}$E1, MgATP). The broken lines correspond to the wild type curve from the top left panel.

**DISCUSSION**

Here we have investigated the functional consequences of mutations to Arg$^{174}$, Ile$^{188}$, Lys$^{204}$, and Lys$^{205}$ in the A-domain of the Ca$^{2+}$-ATPase, and the results demonstrate that the A-domain is an important player in the function of ATP as nonphosphorylating modulator of the partial reaction steps, with Arg$^{174}$, Ile$^{188}$, and Lys$^{205}$ all being critically involved in the stimulation of phosphoenzyme processing by nucleotide, although in different ways.

The crystal structures of the Ca$^{2+}$-ATPase provide useful (although not necessarily perfect) models of the various conformational states of the enzyme cycle, and for the present purpose of discussing the mutational effects on nucleotide interaction, it is helpful to consider the arrangement of the nucleotide binding region in the crystal structures in some detail, in particular the contribution of the A-domain (Fig. 8). During the transport cycle, the A-domain undergoes quite dramatic movements relative to the N- and P-domains. The intermediates Ca$^{2+}$E1-ATP and Ca$^{2+}$E1P-ADP are represented by the crystal structures of Ca$^{2+}$E1-AMPPCP (5, 7) and Ca$^{2+}$E1P-AMPPNP (11), respectively, the $\gamma$-phosphate of AMPPNP being transferred to Asp$^{351}$ in the latter. In these structures, residues in the N- and P-domains...
A-Domain Interaction of SERCA with Modulatory Nucleotide

form a tight complex with the nucleotide at the catalytic site, whereas the A-domain does not contribute to nucleotide binding (Fig. 8, upper left panel), in good agreement with our data showing that for all the mutants analyzed functionally here the rate of phosphorylation of Ca$_{2+}$E1 from a subsaturating concentration of MgATP was indistinguishable from that of the wild type. Following Ca$_{2+}$E1P formation, an ~90° clockwise rotation (viewed from the cytoplasm) of the A-domain in a plane roughly parallel to the membrane allows for the insertion of the conserved 181TGES phosphatase motif (38) of the A-domain into a cleft formed between the P- and N-domains, leading to the E2P ground state (represented by the E2-BeF$_3^-$ crystal structure (10, 11) shown in Fig. 8, upper right panel). The E2-BeF$_3^-$ structure was obtained in the absence of nucleotide; however, docking of ADP or ATP into the structure on the basis of a structural alignment of the N-domain provided a “snug fit” with Arg$_{174}^{2+}$ binding the nucleotide (11). During the Ca$_{2+}$E1P $\rightarrow$ E2P transition ADP dissociates from the catalytic site (whether ADP actually dissociates before (10) or after (11, 62) the A-domain rotation is not clear). Moreover, the A-domain rotation leads to opening of the Ca$_{2+}$ sites toward the lumen and release of the Ca$_{2+}$ ions (56). In E2P, a subsequent further ~25° rotation of the A-domain brings Glu$_{183}^{2+}$ in the 181TGES motif in position for binding the water molecule attacking the aspartyl phosphate (38), thus resulting in initiation of the hydrolysis by formation of the E2-P transition state (represented by the E2-AlF$_4^-$-AMP-NPP crystal structure (11) shown in Fig. 8, lower right panels). In the latter structure, the side chains of Arg$_{174}^{2+}$, Ile$_{188}^{2+}$, and Lys$_{205}^{2+}$ are closely associated with the bound nucleotide in good agreement with our findings of mutational effects on ATP modulation of E2P dephosphorylation. The liberation of the phosphoryl group from the catalytic site seems to be accompanied by the N-domain swinging back down upon the P-domain, the 181TGES motif in the A-domain leaving the catalytic site and Arg$_{174}^{2+}$, Ile$_{188}^{2+}$ and Lys$_{205}^{2+}$ retreating from their association with the nucleotide (cf. Fig. 8, lower left panel, showing the E2-AMP-NPP crystal structure (9), representing E2-ATP). Completion of the cycle is associated with a reversal of the A-domain rotation during the Ca$_{2+}$-binding

of H$_{P}E2 \rightarrow$ Ca$_{2+}$E1 transition (4). The role of the modulatory nucleotide in connection with H$_{P}E2 \rightarrow$ Ca$_{2+}$E1 is probably to help release the A-domain from the P-domain by interfering with inter-domain bonds (30). Our results indicating that alanine mutations to Arg$_{174}^{2+}$, Ile$_{188}^{2+}$, Lys$_{205}^{2+}$, and Lys$_{205}^{2+}$ do not disturb the modulation of the H$_{P}E2 \rightarrow$ Ca$_{2+}$E1 transition by nucleotide are in good accordance with the E2-AMP-NPP structure, where these A-domain residues neither bind to the nucleotide nor to the P-domain (Fig. 8, lower left panel).

Role of Arg$_{174}^{2+}$—The quite moderate effects of the R174A mutation on the basic rates of Ca$_{2+}$E1P $\rightarrow$ E2P, E2P $\rightarrow$ E2, and H$_{P}E2 \rightarrow$ Ca$_{2+}$E1 suggest that the Arg$_{174}^{2+}$ side chain is of minor mechanistic importance for the progression of the conformational transitions of the enzyme cycle in the absence of modulation by nonphosphorylating nucleotide, although the

FIGURE 8. Structural arrangement of Arg$_{174}^{2+}$, Ile$_{188}^{2+}$, Lys$_{205}^{2+}$, and Lys$_{205}^{2+}$ in relation to the nucleotide-binding site in Ca$_{2+}$-ATPase crystallized in the Ca$_{2+}$E1P-ADP state (Ca$_{2+}$E1P-AMPPN), the E2P state (E2-BeF$_3^-$), the E2-P-ATP transition state (E2-AlF$_4^-$-AMP-NPP), and the E2-P state (E2-AMPPCP). The respective Protein Data Bank accession codes corresponding to the structures shown are 3BA6 (11), 3B98 (11), 3B9R (11), and 2C88 (9). Amino acid side chains are shown for residues discussed in the text. Carbon and aluminum atoms are shown in gray, beryllium in green, nitrogen in blue, oxygen in red, fluoride in cyan, and phosphorus and nucleotide in orange (except in the boxed close-up view of the nucleotide site in E2-AlF$_4^-$-AMP-NPP, in which the nucleotide is shown with carbon atoms in gray, oxygen in red, nitrogen in blue, and phosphorus in orange). Arrows indicate the direction of the enzyme cycle. The phosphorylation, nucleotide binding, and actuator domains are indicated by “P,” “N,” and “A,” respectively.
significant slowing of phosphoenzyme processing in R174E indicates that Arg174 does approach other parts of the enzyme during the A-domain transitions. In the E2-BeF4− structure (E2P ground state analog) the side chain of Arg174 is indeed only 4.2 Å from that of Glu439 of the N-domain (upper right panel in Fig. 8).

Both R174A and R174E were severely affected with respect to modulation by MgATP of the Ca2+1P → E2P transition (Fig. 4). Moreover, both R174A and R174E displayed markedly reduced affinity for ATP modulation of E2P dephosphorylation (Fig. 5). In contrast, the ability to undergo PPi-induced stimulation of E2P dephosphorylation was rather unaffected by the R174A mutation and even occurred to a significant extent in R174E, although the affinity for PPi was significantly reduced in R174E (Fig. 6), showing that the PPi induces the negative charge of the glutamate side chain. These observations support the hypothesis that the role of Arg174 in nucleotide modulation of Ca2+1P → E2P and E2P dephosphorylation relates to direct interactions with the nucleotide. Because PPi may be considered representative of the phosphate moiety of ATP, it appears that, at least in connection with the modulation of E2P dephosphorylation, it is the adenosine part of the nucleotide that interacts with Arg174, thereby providing a functional correlate of the close proximity of Arg174 to the adenine ring seen in the E2-BeF4−·AMPPCP structure (E2-P transition state analog, Fig. 8, lower right panels).

Role of Ile188—The reduced basic rate of Ca2+1P → E2P in I188A suggests that Ile188, like Arg174, may interact with other parts of the enzyme during the Ca2+1P → E2P transition. MgATP modulation of Ca2+1P → E2P in I188A and I188F occurred with affinities and enhancement factors at least as high as that of wild type, indicating that Ile188 is not crucial to the interaction with the nucleotide modulating Ca2+1P → E2P. In contrast, the stimulation by ATP of E2P dephosphorylation was completely abolished in I188A. I188F, on the other hand, displayed only a moderate 2-fold reduction of affinity for the modulation by ATP of E2P dephosphorylation, showing that a bulky hydrophobic side chain at position 188 is a prerequisite for ATP modulation of this reaction step. Contrasting the conspicuous effect of the I188A mutation on the modulation by ATP, the affinity of I188A for the PPi-induced modulation of E2P dephosphorylation was at least as high as that of the wild type. Hence, Ile188 may contribute directly to the binding of the adenosine moiety of the modulatory nucleotide in the E2-P transition state. In the E2-BeF4−·AMPPCP crystal structure Ile188 is indeed closer to the adenine ring than to other parts of the nucleotide (lower right panels in Fig. 8).

Role of Lys205—Contrary to Lys204, the Lys205 side chain is unmistakably critical for the phosphoenzyme processing steps, as evidenced by the enhanced basic rates of Ca2+1P → E2P and E2P dephosphorylation in K205A and K205E (Table 1). Even more intriguing are the effects of the mutations to Lys205 on the modulation by MgATP/ATP of phosphoenzyme processing. Thus, K205A displayed rather low affinity for modulation by MgATP of Ca2+1P → E2P, and in K205E there was almost no MgATP modulation of Ca2+1P → E2P (Fig. 4). Remarkably, rather than stimulating the dephosphorylation of E2P, as seen for wild type, ATP inhibited this partial reaction step in K205A and K205E (Fig. 5). The affinity of K205A for the inhibition by ATP of E2P dephosphorylation was indistinguishable from the affinity of the wild type for the stimulation by ATP of E2P dephosphorylation (Table 3), and the affinity of K205E was only 3-fold lower, showing that Lys205 is not very critical for the binding of the nucleotide modulating E2P dephosphorylation, even though it is only 3.8 Å from the terminal phosphate of AMPPCP in the E2-BeF4−·AMPPCP structure and may interact through salt bridge formation (lower right panels in Fig. 8). Such interaction could, of course, require breakage of other bond(s) to the nucleotide, thereby explaining the unaltered affinity upon alanine substitution of Lys205. Hence, the importance of Lys205 in connection with E2P dephosphorylation may lie mostly in the mechanistic aspects, like transmittance of the effect of binding of ATP to the A-domain (see below), and not so much in contributing to the affinity for the nucleotide. The finding that in K205A and K205E the E2P dephosphorylation was not stimulated by PPi, either is consistent with a mechanistic role of the interaction of Lys205 with the phosphate group, and it could mean that Lys205 is absolutely essential to PPi binding.

Sites and Possible Mechanisms of the Modulatory Actions of Nucleotide on the Phosphoenzyme Processing Steps—It has been a controversial issue whether the ATP modulation of the Ca2⁺-ATPase reaction cycle represents binding to a site (or sites) separate from the catalytic site (35, 36), and whether more than one ATP molecule might bind simultaneously on each Ca2⁺-ATPase polypeptide chain (28, 33, 34). Our previous finding that the residues Phe697, Arg560, and Arg567 contributing to the binding of the phosphorylating ATP at the catalytic site are also critical for the stimulation by ATP of E2P dephosphorylation clearly indicated some degree of overlap between the catalytic site and the modulatory nucleotide site on E2P (or the E2-P transition state), but on the other hand these residues did not seem to contribute to binding of the MgATP molecule that modulates Ca2+1P → E2P (30). The present findings with the Ile188 mutants likewise indicate that Ile188 is required for the binding of the ATP molecule modulating E2P dephosphorylation, whereas Ile188 is not important for MgATP modulation of Ca2+1P → E2P, again suggesting that two different modulatory sites could be involved. Our data for Arg174 show, however, that this residue is critical to nucleotide modulation of either of these phosphoenzyme processing steps, thereby indicating a significant overlap between the two nucleotide sites involved with Arg174 contributing to both. Lys205 also seems to be involved in nucleotide modulation of Ca2+1P → E2P as well as E2P dephosphorylation, being a rather critical determinant of
TABLE 4

Conclusions regarding the functional roles of the amino acid residues studied

| Residue | Ca\textsubscript{2}E1 \textarrow{Ca\textsubscript{2}E1P} | Ca\textsubscript{2}E1P \textarrow{E2P} | E2P \textarrow{E2} | H\textsubscript{2}E2 \textarrow{Ca\textsubscript{2}E1} |
|---------|----------------|----------------|----------------|----------------|
| Arg\textsuperscript{174} | No prominent role | Contribution to binding of modulatory MgATP; critical for basic rate (reduced in alanine mutant) but not for modulation by MgATP | Contribution to binding of the adenosine moiety of modulatory ATP | No prominent role |
| Ile\textsuperscript{188} | No prominent role | No prominent role | No prominent role | No prominent role |
| Lys\textsuperscript{204} | No prominent role | Critical for basic rate (enhanced in mutants); contribution to binding of modulatory MgATP | No prominent role | No prominent role |
| Lys\textsuperscript{205} | No prominent role | Critical for the basic rate (enhanced in mutants); critical for the modulatory effect of ATP binding (transmitted through phosphate moiety) with anomalous inhibition seen in mutants | No prominent role | No prominent role |

Conclusions regarding the functional roles of the amino acid residues studied

The affinity for the MgATP modulating Ca\textsubscript{2}E1P \textarrow{E2P} and playing a more mechanistic role in connection with the ATP modulation of E2P dephosphorylation. Both roles may be exerted through direct interaction with the nucleotide. Accordingly, none of the crystal structures published so far contains more than one nucleotide per Ca\textsuperscript{2+}-ATPase polypeptide chain, and in all cases the nucleotide is found in the same region of the protein. Taken together the data thus support a variable site model (37), in which the site is reconfigured from slightly different portions of the P-, N-, and A-domains in each of the conformational states occurring during the transport cycle. The modulatory nucleotide probably binds and dissociates several times in each major intermediate.

Although Arg\textsuperscript{174} and Lys\textsuperscript{205} are outside the possible range of interactions with nucleotide in the Ca\textsubscript{2}E1P state, we nevertheless found that both residues are critical for the nucleotide modulation of Ca\textsubscript{2}E1P \textarrow{E2P}, implying that the modulation does not involve a destabilization of Ca\textsubscript{2}E1P, but rather a stabilization of one or more of the subsequent E2-type intermediates where the A-domain has undertaken its 90° rotation, bringing Arg\textsuperscript{174} and Lys\textsuperscript{205} into contact with the nucleotide. Stabilization of Ca\textsubscript{2}E2P (a proposed intermediate state between Ca\textsubscript{2}E1P and E2P (56)) relative to Ca\textsubscript{2}E1P would indeed cause slowing of the back-reaction Ca\textsubscript{2}E2P \textarrow{Ca\textsubscript{2}E1P} and thus increase the net rate of the forward transition from Ca\textsubscript{2}E1P to E2P. It seems likely that ATP/MgATP bound in exchange for the leaving ADP may assist in stabilizing Ca\textsubscript{2}E2P relative to Ca\textsubscript{2}E1P by bridging the interactions of the A-domain with the P- and N-domains. It emerges from our discussion that Arg\textsuperscript{174} and Lys\textsuperscript{205} would interact with the modulatory nucleotide in Ca\textsubscript{2}E2P as well as in the E2-P transition state in the E2P dephosphorylation reaction, whereas Ile\textsuperscript{188} seems to interact with the nucleotide only in the E2-P transition state.

The inhibition in the mutant Ca\textsuperscript{2+}-ATPases brings to mind the situation in the wild type Na\textsuperscript{+},K\textsuperscript{+}-ATPase for which it is well documented (31, 32) that E2P dephosphorylation is inhibited by nucleotide, and it also demonstrates that the opposite behaviors of the wild type Ca\textsuperscript{2+}- and Na\textsuperscript{+},K\textsuperscript{+}-ATPases in response to ATP may be caused by rather subtle differences between these enzymes. A survey of the available P-type ATPase amino acid sequences and crystal structures (see supplemental Fig. S8 and associated table) clearly indicates a high conservation of positively charged residues corresponding to Arg\textsuperscript{174} and Lys\textsuperscript{205} among both the type IIA (SERCA) Ca\textsuperscript{2+}-ATPases and the type IIC (Na\textsuperscript{+},K\textsuperscript{+}- and H\textsuperscript{+},K\textsuperscript{+}-) ATPases, thus illustrating that their different responses to ATP is because of differences elsewhere; perhaps the presence of a polar serine or threonine in the Na\textsuperscript{+},K\textsuperscript{+}-ATPase at the position corresponding to Ile\textsuperscript{188} in the Ca\textsuperscript{2+}-ATPase slightly changes the position of the modulatory ATP molecule and thereby its mode of interaction.

Conclusion—The A-domain is critically involved in the modulatory effects of MgATP/ATP on the rates of Ca\textsubscript{2}E1P \textarrow{E2P} and E2P dephosphorylation. Arg\textsuperscript{174} is an important residue for binding of the modulatory nucleotide in both of these phosphoenzyme processing steps, whereas Ile\textsuperscript{188} interacts only with the ATP that modulates E2P dephosphorylation. Lys\textsuperscript{205} seems to be involved differentially in nucleotide modulation of Ca\textsubscript{2}E1P \textarrow{E2P} and E2P dephosphorylation (Table 4).

Acknowledgments—We thank Lene Jacobsen and Karin Kracht (Aarhus University, Denmark) for expert technical assistance. Drs. Pou1 Nissen and Claus Olesen (Aarhus University, Denmark) are thanked for providing information about crystal structures prior to publication.
A-Domain Interaction of SERCA with Modulatory Nucleotide

REFERENCES

1. Hasselbach, W., and Makino, M. (1961) Biochim. Biophys. Acta 56, 208–212
2. Axelsson, K. B., and Palmgren, M. G. (1998) J. Mol. Biol. 282, 1149–1157
3. Toyoshima, C., Nakasako, M., Nomura, H., and Ogawa, H. (2000) Biochemistry 39, 14595–14601
4. Toyoshima, C., and Nomura, H. (2002) Biochemistry 41, 6399–6406
5. Olesen, C., and Vilsen, B. (2003) Biochemistry 42, 1118–1123
6. Sørensen, T. L., and Andersen, J. P. (2004) J. Biol. Chem. 279, 14056–14062
7. Clausen, J. D., McIntosh, D. B., Vilsen, B., Woolley, D. G., and Andersen, J. P. (2005) J. Biol. Chem. 280, 16157–16162
8. Baginski, E. S., Foa, P. P., and Zak, B. (1967) Cell. Biol. 23, 132–133
9. Sørensen, T., Vilsen, B., and Andersen, J. P. (1997) J. Biol. Chem. 272, 10491–10496
10. McIntosh, D. B., Woolley, D. G., Vilsen, B., and Andersen, J. P. (1999) J. Biol. Chem. 274, 15227–15232
11. Clausen, J. D., McIntosh, D. B., Woolley, D. G., and Andersen, J. P. (2001) J. Biol. Chem. 276, 35741–35750
12. McIntosh, D. B., and Andersen, J. P. (2003) J. Biol. Chem. 278, 8257–8262
13. Söderhäll, K., and Söderhäll, K. (1983) Biochemistry 22, 2867–2875
14. Kabakayashi, S., Ogura, S., and Shigekawa, M. (1986) J. Biol. Chem. 261, 9762–9769
15. Chappell, P., and Guillain, F. (1986) Biochim. Biophys. Acta 851, 9–15
16. Bogey, A. L., and Jencks, W. P. (1987) J. Biol. Chem. 262, 13997–14004
17. Shigekawa, M., and Dougerty, J. P. (1978) J. Biol. Chem. 253, 1451–1457
18. Arkle, M., and Boyer, P. D. (1980) Biochemistry 19, 2001–2004
19. Chappell, P., Jollet, S., Orlowski, S., Guillain, F., Seebregts, C. J., and McIntosh, D. B. (1988) J. Biol. Chem. 263, 12288–12294
20. Clausen, J. D., McIntosh, D. B., Anthonissen, A. N., Woolley, D. G., Vilsen, B., and Andersen, J. P. (2007) J. Biol. Chem. 282, 20686–20697
21. Askari, A., and Huang, W. (1982) Biochim. Biophys. Acta 704, 1447–1453
22. Mahmoud, Y. A. (2008) Proc. Natl. Acad. Sci. U. S. A. 105, 1757–1761
23. Cable, M. B., Feher, J. J., and Briggs, F. N. (1985) Biochemistry 24, 5612–5619
24. Bishop, J. E., Al Shawi, M. K., and Inesi, G. (1987) J. Biol. Chem. 262, 4658–4663
25. Coll, R. J., and Murphy, A. J. (1991) Biochemistry 30, 1456–1461
26. Suzuki, K., Kubota, T., Kubo, K., and Kanazawa, T. (1990) Biochemistry 29, 7040–7045
27. Clausen, J. D., McIntosh, D. B., Vilsen, B., Woolley, D. G., and Andersen, J. P. (2003) J. Biol. Chem. 278, 20245–20258
28. Clausen, J. D., Vilsen, B., McIntosh, D. B., Einholm, A. P., and Andersen, J. P. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 7776–7781
29. Kaufman, R. J., Davies, M. V., Pathak, V. K., and Hershey, J. W. (1989) Mol. Cell. Biol. 9, 946–958
30. Maruyama, K., and MacLennan, D. H. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 3314–3318
31. Vilsen, B., Andersen, J. P., and MacLennan, D. H. (1991) J. Biol. Chem. 266, 16157–16164
32. Baginski, E. S., Foa, P. P., and Zak, B. (1967) Chem. 3, 132–133
33. Sørensen, T., Vilsen, B., and Andersen, J. P. (2006) Proc. Natl. Acad. Sci. U. S. A. 104, 19831–19836
34. Olesen, C., Picard, M., Winther, A. M., Gyurp, C., Morth, J. P., Oxvig, C., Muller, J. V., and Nissen, P. (2007) Nature 450, 1036–1042
35. Danko, S., Yamasaki, K., Dahi, T., and Suzuki, H. (2004) J. Biol. Chem. 279, 14991–14998
36. Morfill, M., Pedersen, B. P., Tutschek-Jensen, M. S., Sørensen, T. L., Petersen, J., Andersen, J. P., Vilsen, B., and Nissen, P. (2007) Nature 451, 1043–1049
37. Pedersen, B. P., Buch-Pedersen, M. J., Morth, J. P., Palmgren, M. G., and Nissen, P. (2007) Nature 450, 1111–1115
38. Shigekawa, M., and Dougerty, J. P. (1978) J. Biol. Chem. 253, 1458–1464
39. de Meis, L., and Vianna, A. L. (1979) Annu. Rev. Biochem. 48, 275–292
40. Scofano, M. H., Vieyra, A., and de Meis, L. (1979) J. Biol. Chem. 254, 1021–1027
41. Wakabayashi, S., and Shigekawa, M. (1990) Biochemistry 29, 7309–7318
42. Karlish, S. J., and Yates, D. W. (1978) Biochim. Biophys. Acta 527, 115–130
43. Clarke, R. J., Apell, H. J., and Kong, B. Y. (2000) Biochemistry 39, 7034–7044
44. Reenstra, W. W., Coethers, J., and Forte, J. G. (2007) Biochemistry 46, 10145–10152
45. McIntosh, D. B., and Boyer, P. D. (1983) Biochemistry 22, 2867–2875
46. McIntosh, D. B., and Boyer, P. D. (1983) Biochemistry 22, 2867–2875
47. Kabakayashi, S., Ogura, S., and Shigekawa, M. (1986) J. Biol. Chem. 261, 9762–9769
48. Chappell, P., and Guillain, F. (1986) Biochemistry 25, 7623–7633
49. Bodley, A. L., and Jencks, W. P. (1987) J. Biol. Chem. 262, 13997–14004
50. Shigekawa, M., and Dougerty, J. P. (1978) J. Biol. Chem. 253, 1451–1457
51. Askari, A., and Huang, W. (1982) Biochim. Biophys. Acta 704, 1447–1453
52. Mahmoud, Y. A. (2008) Proc. Natl. Acad. Sci. U. S. A. 105, 1757–1761
53. Cable, M. B., Feher, J. J., and Briggs, F. N. (1985) Biochemistry 24, 5612–5619