Critical Role of Val-304 in Conformational Transitions That Allow Ca\(^{2+}\) Occlusion and Phosphoenzyme Turnover in the Ca\(^{2+}\) Transport ATPase*

Received for publication, July 31, 2007, and in revised form, November 26, 2007 Published, JBC Papers in Press, December 5, 2007, DOI 10.1074/jbc.M706315200

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Site-directed mutations were produced in the distal segments of the Ca\(^{2+}\)-ATPase (SERCA) transmembrane region. Mutations of Arg-290 (M3–M4 loop), Lys-958, and Thr-960 (M9–M10 loop) had minor effects on ATPase activity and Ca\(^{2+}\) transport. On the other hand, Val-304 (M4) mutations to Ile, Thr, Lys, or Ala inhibited transport by 90–95% while reducing ATP hydrolysis by 83% (Ile, Thr, and Lys), 56% (Ala), or 45% (Glu). Val-304 participates in Ca\(^{2+}\) coordination with its main-chain carbonyl oxygen, and this function is not expected to be altered by mutations of its side chain. In fact, despite turnover inhibition, the Ca\(^{2+}\) concentration dependence of residual ATPase activity remained unchanged in Val-304 mutants. However, the rates (but not the final levels) of phosphoenzyme formation, as well as the rates of its hydrolytic cleavage, were reduced in proportion to the ATPase activity. Furthermore, while the Val-304 → Glu mutant, which retained the highest residual ATPase activity, it was possible to show that occlusion of bound Ca\(^{2+}\) was also impaired, thereby explaining the stronger inhibition of Ca\(^{2+}\) transport relative to ATPase activity. The effects of Val-304 mutations on phosphoenzyme turnover are attributed to interference with mechanical links that couple movements of transmembrane segments and headpiece domains. The effects of thermal activation energy on reaction rates are thereby reduced. Furthermore, inadequate occlusion of bound Ca\(^{2+}\) following utilization of ATP in Val-304 side-chain mutations is attributed to inadequate stabilization of the Glu-309 side chain and consequent defect of its gating function.

The Ca\(^{2+}\)-ATPase (SERCA) is required for active transport of Ca\(^{2+}\) into intracellular stores, whereby Ca\(^{2+}\) is available for subsequent release and signaling functions. SERCA is a membrane-bound protein that includes ten transmembrane helical segments (M1 to M10), and three headpiece domains (“N,” “P,” and “A”) protruding from the cytosolic side of the membrane (1, 2). The catalytic and transport cycle begins with high affinity binding of two Ca\(^{2+}\) derived from the cytoplasmic medium (“outside”), followed by ATP utilization to form a phosphorylated enzyme intermediate. The bound Ca\(^{2+}\) then undergoes dissociation onto the luminal side (“inside”) of the membrane, against a concentration gradient. Finally, hydrolytic cleavage of the phosphoenzyme completes the cycle (3, 4). Due to a relatively large distance between Ca\(^{2+}\) binding in the transmembrane region and the ATP site in the headpiece domains, coupling of ATP utilization and Ca\(^{2+}\) transport requires a “long range intramolecular linkage,” which is operated by protein conformational changes (5).

Mutational (6–8) and structural (9) studies have shown that, within the membrane-bound region of the ATPase, the Asn-768, Glu-771, Thr-799, Asp-800, and Glu-908 side chains, and two water molecules, contribute oxygen atoms for Ca\(^{2+}\) coordination on site I. Ca\(^{2+}\) binding on neighboring site II is stabilized by Glu-309 (M4), Asn-796, and Asp-800 (M6) side chain, as well as by Val-304, Ala-305, and Ile-307 (M4) main-chain carbonyl oxygen atoms. Binding of the two Ca\(^{2+}\) is cooperative (10), and single mutations in site I prevent binding at either site. On the other hand, following single mutations on site II, the enzyme retains non-cooperative binding of one Ca\(^{2+}\) on site I. Most importantly, occupancy of site II by Ca\(^{2+}\) is required to trigger the long range conformational change permitting activation of the catalytic site and utilization of ATP (11, 12). The complex formed by binding of ATP substrate to the activated enzyme (ATP:E1P2Ca\(^{2+}\)) presents a specific conformation (13, 14), and, as the phosphoenzyme intermediate is formed, changes in the space relationship of polypeptide chains occur (15) while the bound Ca\(^{2+}\) undergoes a process of occlusion, which prevents isotopic exchange with medium Ca\(^{2+}\) (12, 16). The phosphoenzyme intermediate (ADP:E1P2Ca\(^{2+}\)) then undergoes a further conformational transition (17–19), coupled with dissociation of ADP into the cytosolic medium and Ca\(^{2+}\) dissociation into the luminal medium. Finally, the so derived phosphoenzyme conformer (E2P) undergoes hydrolytic cleavage. The entire cycle is reversible, starting with enzyme phosphorylation by P, in the absence of Ca\(^{2+}\) (20) and ending with synthesis of ATP upon addition of Ca\(^{2+}\) and ADP (21).

With the experiments described here we mutated several amino acids near and distal to the Ca\(^{2+}\) binding sites, with the initial intention of exploring a possible interference with the
exit of transported Ca\(^{2+}\) through the luminal side of the membrane. However, as we studied step by step the sequential reactions involved in utilization of ATP and Ca\(^{2+}\), the most interesting finding was that the well conserved Val-304 residue sustains an important role in allowing conformational transitions coupled with utilization of ATP by the Ca\(^{2+}\)-activated enzyme.

MATERIALS AND METHODS

PCR Mutagenesis and Protein Expression—The chicken fast muscle SERCA-1 cDNA (22) was subcloned into the SV40-pAdlox vector for site-directed mutagenesis. Primers of 20–30 bp in length were synthesized for each individual mutation. These primers were utilized to anneal the DNA sequences internal to the flanking primers and were used for PCR mutagenesis by overlap extension (23). The mutant DNA fragment was then exchanged with the corresponding fragment of WT cDNA in SV40-pAdlox. After DNA sequencing, the cDNA was transfected into COS-1 cells for overexpression of protein under the control of SV40 promoter (24). Alternatively, WT SERCA and selected mutants (Val-304 → Glu, Asn-796 → Ala, and Glu-309 → Gin) were obtained by exogenous gene expression in COS-1 cells infected with adenovirus vectors carrying the corresponding cDNA (25).

Microsomal Preparation and Immunodetection of Expressed Protein—The microsomal fraction of transfected or infected COS-1 cells was obtained by differential centrifugation of homogenized cells (25). Immunodetection of expressed ATPase in the microsomal fraction was obtained by Western blotting using 9E10 monoclonal antibody against the c-Myc tag of SERCA-1.

Functional Studies—For all functional measurements the protein concentration of microsomes containing mutant SERCA-1 was corrected by the expression level revealed by Western blots with reference to WT enzyme, so that the final functional level refer to the same concentration of recombinant enzyme.

SERCA ATPase activity was assayed in 1 ml of reaction mixture containing 20 mM MOPS, pH 7.0, 80 mM KCl, 2 mM MgCl\(_2\), 5 mM sodium azide, 30 μg of total microsomal protein, 3 μM ionophore A23187, 3 μM ATP, in the absence (with 2 mM EGTA and without any addition of Ca\(^{2+}\)) or presence of 10 μM free Ca\(^{2+}\). The reaction was started at 37 °C by adding ATP, and samples were taken at serial times for P\(_i\) determination (26). The Ca\(^{2+}\)-dependent activity was calculated by subtracting the Ca\(^{2+}\)-independent ATPase activity from the total ATPase activity and then corrected by the expression level ratio between the wild-type and mutant microsomes revealed by Western blot assay. The Ca\(^{2+}\) dependence of SERCA ATPase activity was measured under the same condition except that serial concentrations of free Ca\(^{2+}\) were established by adding various amount of EGTA to reaction mixtures containing 0.1 mM CaCl\(_2\).

Ca\(^{2+}\) transport by SERCA was measured at 25 °C in a reaction mixture containing 50 mM MOPS, pH 7.0, 80 mM KCl, 2 mM MgCl\(_2\), 50 μg of microsomal protein/ml, 5 mM potassium oxalate, 10 μM free Ca\(^{2+}\) with \(^{45}\)Ca tracer. ATP (3 mM) was added to start the reaction, and at various times 1 ml of reaction mixture was loaded onto a 0.45-μm Millipore Filter by vacuum suction and washed with 15 ml of 2 mM LaCl\(_3\) and 10 mM MOPS, pH 7.0. The filter was then processed for determination of radioactivity by scintillation counting.

Enzyme phosphorylation by ATP was measured in an ice-cold reaction mixture containing 50 mM MOPS, pH 7.0, 80 mM KCl, 2 mM MgCl\(_2\), 10 mM free Ca\(^{2+}\), 5 mM A23187, 100 μg of microsomal protein from COS-1 cells transfected with wild-type SERCA-1 or mutated cDNA. The reaction was started by the addition of 10 μM [γ-\(^{32}\)P]ATP and quenched at various times with 1 mM perchloric acid. Enzyme phosphorylation by P\(_i\) was obtained at 35 °C for 10 min in a reaction mixture containing 50 mM MES-Tris, pH 6.0, 10 mM MgCl\(_2\), 2 mM EGTA, 20% (CH\(_3\))\(_2\)SO, 0.5 mM [\(^{32}\)P]P\(_i\), and 100 μg of microsomal protein from COS-1 cells transfected with wild-type SERCA-1 or mutated cDNA. The reaction was quenched with 1 mM perchloric acid.

Decay experiments with phosphoenzyme obtained by utilization of ATP were performed in an ice-cold reaction mixture containing 50 mM MOPS, pH 7.0, 80 mM KCl, 2 mM MgCl\(_2\), 10 μM free Ca\(^{2+}\), 5 mM A23187, and 50 μg of microsomal protein. The reaction was started by the addition of 10 μM [γ-\(^{32}\)P]ATP, and after 30 s the [γ-\(^{32}\)P]phosphoenzyme was chased by the addition of 0.5 mM non-radioactive ATP. Then the reaction was quenched at various times by the addition of 1 mM perchloric acid. In all cases, the quenched reaction mixture was loaded onto a 0.45-μm Millipore Filter and washed with 20 ml of 0.125 M perchloric acid and 10 ml of water. The filter was then processed for determination of radioactivity by scintillation counting. Decay of phosphoenzyme obtained by utilization of P\(_i\) was started by a 5-fold dilution of the phosphorylation mixture with a medium containing 50 mM MES-Tris, pH 6.0, 10 mM MgCl\(_2\), 2 mM EGTA, and 5 mM P\(_i\) at 20 °C. The decay reaction was quenched at various times by the addition of 1 mM perchloric acid, filtered, and processed as described above.

Calcium binding to the ATPase was determined by incubating COS-1 microsomal vesicles (100 μg/ml) in a medium containing 50 mM MOPS, pH 7.0, 80 mM KCl, 5 mM MgCl\(_2\), 5 mM A23187 calcium ionophore, and 10 μM [\(^{45}\)Ca]Ca\(^{2+}\) (including contaminant Ca\(^{2+}\)). Determination of the time course of Ca\(^{2+}\) dissociation from the fluoroaluminate-bound SERCA form was performed in the same reaction mixture but with the addition of 1 mM ADP, 50 μM AlCl\(_3\), and 2 mM potassium fluoride. Thapsigargin (TG) was added to half of the samples (1 μM) to provide controls exhibiting no specific Ca\(^{2+}\) binding in both conditions. Following 30-min incubation at 25 °C, 1-ml samples were placed on a 0.45-μm Millipore filter, and the medium was removed by suction. The vesicles were then perfused with 10 mM EGTA for dissociation of bound [\(^{45}\)Ca]Ca\(^{2+}\). The experiments were performed in a BioLogic rapid filtration apparatus, and the filters were then blotted and processed for determination of radioactivity by scintillation counting.

RESULTS

We produced several site-directed, single mutations of amino acids residing within or distal to the Ca\(^{2+}\) binding domain, including the M1, M2, and M4 transmembrane helices as well as on the M3/M4 and M9/M10 loops (Table 1). Muta-
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TABLE 1
Expression, ATPase activity, and Ca$^{2+}$ transport activities of recombinant WT and mutant ATPase
The data in bold refer to expression from cells infected with adenovirus vectors whereby a greater efficiency of expression was obtained.

| Expression | ATPase activity | Ca$^{2+}$ transport |
|------------|-----------------|---------------------|
| %          | mmol P$_i$/mg protein/min | mmol Ca$^{2+}$/mg protein/min |
| WT         | 100             | 156 ± 4             | 40 ± 2 |
| R290A      | 40              | 132 ± 6             | 38 ± 2 |
| R290E      | 62              | 140 ± 2             | 38 ± 2 |
| V304A      | 89              | 70 ± 4              | 4 ± 1  |
| V304E      | 94              | 86 ± 8              | 2 ± 1  |
| V304I      | 83              | 26 ± 6              | 6 ± 1  |
| V304K      | 90              | 25 ± 2              | 1 ± 2  |
| V304T      | 80              | 27 ± 5              | 5 ± 2  |
| K958A      | 38              | 123 ± 7             | 25 ± 2 |
| K958E      | 54              | 120 ± 6             | 24 ± 3 |
| T960A      | 67              | 125 ± 5             | 22 ± 3 |
| WT         | 100             | 1,092 ± 14          | 302 ± 18 |
| V304E      | 102             | 592 ± 26            | 4 ± 1  |
| E309Q      | 96              | 0                  | 0      |
| N796A      | 92              | 0                  | 0      |

FIGURE 1. ATPase activity of WT ATPase and various mutants (A and B). Recombinant proteins were recovered in the microsomal fractions of transfected COS-1 cells (see "Materials and Methods"). SERCA ATPase activity was measured at 37°C in the presence of 20 mM MOPS, pH 7.0, 80 mM KCl, 5 mM MgCl$_2$, 5 mM sodium azide, 30 μg of total microsomal protein/ml, 3 μM ionophore A23187, 3 mM ATP, in the presence or absence (with 2 mM EGTA and without any addition of Ca$^{2+}$) of 10 μM free Ca$^{2+}$. The difference in ATPase activity in the presence and the absence of Ca$^{2+}$ was considered as specific SERCA ATPase.

E2P ↔ E2 + P, sequence. It is shown in Fig. 5 that the rate of decay is clearly reduced by Val-304 mutations to Glu or Ala, in proportion to reduction of overall ATPase activity. In fact, the rate of decay was reduced even further (not shown) for Val-304 → Lys, Val-304 → Thr, and Val-304 → Ile in accordance with the more drastic reduction of ATPase activity (Table 1).

We also characterized the decay of phosphoenzyme obtained by incubation with P$_i$ in the absence of Ca$^{2+}$ (i.e. E2P). In agreement with the phosphoenzyme levels obtained with ATP, we found that following 5-min incubation at 25°C, similar levels of phosphoenzyme were obtained with WT protein and Val-304 mutants. It is reassuring that decay of E2P obtained with recombinant WT protein occurs at a nearly identical rate as that of the E2P obtained with the native ATPase of muscle SR translocations of Leu-75 or Phe-88 (M1 and M2) to Ala, Glu, or Lys strongly reduced the SERCA protein expression, likely due to interference with transcription or, possibly, enhancement of mRNA turnover. In fact, an 80-85% reduction of mRNA levels was demonstrated by Northern blots and reverse transcription-PCR (not shown). On the other hand, mutations of Arg-290, Val-304, Lys-958, or Thr-960 resulted in protein expression at levels nearly as high, or moderately lower than the WT ATPase (Table 1). Expression of WT, Val-304 → Glu, Asn-796 → Ala, and Glu-309 → Gln in COS-1 cells infected with adenovirus vectors (8) yielded microsomes with an ~7-fold higher SERCA content than obtained following simple transfections (Table 1).

From the functional point of view, we found that the Arg-290 → Ala, Arg-290 → Glu (M3/M4 luminal loop), Lys-958 → Ala, Lys-958 → Glu, or Thr-960 → Ala mutations (M9–M10 luminal loop) did not produce major inhibition of ATPase activity or Ca$^{2+}$ transport (Figs. 1A and 2A and Table 1). On the other hand, interesting functional effects were obtained with Val-304 mutations (M4 transmembrane helix). These mutations did not interfere with protein expression, but inhibited almost completely Ca$^{2+}$ transport in all cases, while reducing to various extents the ATPase activity (Figs. 1B and 2B). An overall account of mutations, levels of expressed protein, and residual functional activities is given in Table 1. It is of interest that the Ca$^{2+}$ concentration dependence of the residual ATPase activity was not altered by Val-304 mutations (Fig. 3), even though the steady-state velocity (i.e. enzyme turnover) was reduced. This indicates that high affinity Ca$^{2+}$ binding is not affected by Val-304 mutations. On the other hand, we found that the rates of phosphoenzyme formation by utilization of [γ-$^{32}$P]ATP in the presence of Ca$^{2+}$ were reduced, although steady-state levels of phosphoenzyme equal to those obtained with WT enzyme were finally reached with all mutants, as demonstrated in Fig. 4 for the Val-304 → Glu and Val-304 → Ala mutants. We then proceeded to determine the time dependence of phosphoenzyme decay by first obtaining maximal levels of [γ-$^{32}$P]phosphoenzyme with [γ-$^{32}$P]ATP in the presence of Ca$^{2+}$, and then diluting the medium with excess non-radioactive ATP. Under these conditions, the decay of [γ-$^{32}$P]phosphoenzyme represents the overall time dependence of the ADP-E1·P·2Ca$^{2+}$ ↔ ADP + Ca$^{2+}$ in + E2P ↔ E2 + P, sequence. It is shown in Fig. 5 that the rate of decay is clearly reduced by Val-304 mutations to Glu or Ala, in proportion to reduction of overall ATPase activity. In fact, the rate of decay was reduced even further (not shown) for Val-304 → Lys, Val-304 → Thr, and Val-304 → Ile in accordance with the more drastic reduction of ATPase activity (Table 1).
vesicles (Fig. 6). However, even in this case, hydrolytic cleavage of $E_2P$ obtained with mutant protein proceeded at slower rates (Fig. 6), in proportion to the overall reduction of ATPase activity.

Finally, to explain the stronger reduction of Ca$^{2+}$/H$^{+}$ transport, despite significant residual ATPase activity (Table 1), we tested the ability of the Val-304→Glu mutant to retain occluded Ca$^{2+}$ following enzyme phosphorylation by ATP. Occlusion of bound Ca$^{2+}$ can be demonstrated by trapping radioactively labeled $[^{45}\text{Ca}]\text{Ca}^{2+}$ in the stable ADP-fluoroluminate transition analog of the ADP-E$1P$-2Ca$^{2+}$ intermediate, and measuring the rate of exchange with (non-radioactive) Ca$^{2+}$ added to the medium. To obtain sufficient signal intensity for these measurements, we constructed adenovirus vectors carrying WT and Val-304→Glu SERCA cDNA under control of the cytomegalovirus promoter for efficient gene transfer and protein expression in COS-1 cells. The yield of recombinant protein in cells infected with adenovirus vector turned out to be ~7-fold higher than in cells subjected to simple transfections.
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(DIP-1P-2Ca\(^{2+}\)). Recombinant proteins were recovered in the microsomal fractions of transfected COS-1 cells. Decay of radioactive \([\gamma^{32}P]\)phosphoenzyme was measured following a chase with non-radioactive P, at 20 °C (see "Materials and Methods"). The experimental points were obtained with WT protein (○), Val-304 → Glu (●), and Val-304 → Ala (□).

It is shown in Fig. 7 that the WT enzyme retains the occluded \([{}^{45}\text{Ca}]\text{Ca}^{2+}\) for at least 2 s before significant exchange with medium Ca\(^{2+}\) is noted. On the other hand, exchange occurs with a 0.5-s half time when the Val-304 → Glu mutant is used. This behavior is similar to that observed with the Glu-309 → Gln and Asn-796 → Ala mutants (12), although the release of occluded \([{}^{45}\text{Ca}]\text{Ca}^{2+}\) is affected to a different extent by the three mutations (Fig. 7). None of these three mutants sustains significant Ca\(^{2+}\) transport activity (Table 1). It should be pointed out that the Glu-309 and Asn-796 are involved with their side chains in Ca\(^{2+}\) coordination at site II. For this reason, the Glu-309 → Gln and Asn-796 → Ala mutants retain binding of only one Ca\(^{2+}\) at site I, whereas the lack of Ca\(^{2+}\) on site II completely prevents ATPase activation. On the other hand, Val-304 participates in coordination with its main carbonyl oxygen, and its side-chain mutation to Glu allows retention of significant ATPase activity (Table 1).

**DISCUSSION**

The most important results of the experiments reported here are related to Val-304 mutations, whose inhibition of Ca\(^{2+}\) transport was noted previously (27, 28). Five different mutations of this amino acid did not interfere significantly with protein expression, but strongly inhibited Ca\(^{2+}\) transport, while ATPase activity was inhibited (Table 1) 83% by the Val-304 → Ile, Thr, and Lys mutations, 56% by the Val-304 → Ala mutation, and 45% by the Val-304 → Glu mutation. The Ca\(^{2+}\) concentration dependence of the residual ATPase activity was not affected by Val-304 mutations, indicating that high affinity Ca\(^{2+}\) binding occurred normally. This is not surprising, because Val-304 participates in Ca\(^{2+}\) coordination with its main-chain carbonyl oxygen (Fig. 8), and not with the side chain, which is subjected to mutations. However, characterization of the subsequent partial reactions of the ATPase cycle revealed inhibition of the rates of phosphoenzyme formation...
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Val-304 is a well conserved residue, which, as mentioned above, contributes with its main-chain carbonyl oxygen to coordination of Ca\(^{2+}\) bound at site II within the transmembrane region (Fig. 8). The question is then why mutation of the Val-304 side chain, located in the M4 transmembrane segment (Fig. 9), reduces the rates of phosphoenzyme formation and cleavage occurring at a relatively large distance within the P domain of the ATPase headpiece. It should be noted in this regard that the M4 helix is integrated with the P domain through structural continuity. Perturbations of catalytic and transport activity by mutations on the M4 segment approaching the P domain were previously noted (25).

It is important to consider in this regard that a global involvement of the ATPase protein conformation takes place, as ATP utilization, isomerization of the resulting intermediate, and phosphoenzyme hydrolytic cleavage are accompanied by large movements of the headpiece domains with mechanical links to movements and bending of transmembrane helices. In fact, following nucleotide substrate binding and in concomitance with A domain tilting, the M1 helix is displaced by two helical turns in the direction of the cytosolic surface of the membrane, and relative to M4 (13). M1 then approximates M4 and limits movements of the Glu-309 side chain, which is also stabilized by interactions with Asn-796 and Leu-65. In this state, the upper M4 segment pulls down the P domain, whereas the lower M4 undergoes angular bending. The N domain then approximates with a swinging movement, and an overall conformation that favors catalytic activation and phosphoenzyme formation is reached.

As for enzyme phosphorylation with P, and its hydrolytic cleavage (E2 \(\rightarrow\) E2P and E2P \(\rightarrow\) E2 reactions), a behavior similar to that produced by Val-304 mutants is observed following a proteinase K cut at Lys-120 (29). In this case the reaction rates, but not the final phosphoenzyme levels, are reduced. In fact, movements of the A domain and of the M4 lower segment occur in these reactions, again coupled through important mechanical links. As a consequence of perturba-

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**FIGURE 8.** Alignment of Ca\(^{2+}\) binding residues in E1-2Ca\(^{2+}\) (atom color) and AMPPCP-E1-2Ca\(^{2+}\) (magenta). Note that Val-304 participates in Ca\(^{2+}\) binding on site II with its main-chain carbonyl oxygen, rather than with the side chain that was subjected to mutations. Note also that the Glu-309 side chain position, depending on its interactions (see Fig. 9), plays an important role in sealing the Ca\(^{2+}\) binding cavity. Derived from PDB accession numbers 1sv4 (E1-2Ca\(^{2+}\)) and 1vfp (AMPPCP-E1-2Ca\(^{2+}\)).

**FIGURE 9.** Structural models around Val-304 in E1-2Ca\(^{2+}\) (a), E1-AlF\(_{4}\)-ADP (b), E2-BeF\(_{3}\)-tTG (c), and E2(TG+BHQ) (d), viewed from the cytoplasm approximately perpendicular to the membrane. In E1-2Ca\(^{2+}\) (a), Glu-309 side chain can take different conformations (depicted with green sticks) so that site II Ca\(^{2+}\) can detach and undergo exchange; here, Val-304 \(\rightarrow\) Glu can be accommodated without steric clashes. On the other hand, in E1-AlF\(_{4}\)-ADP (b), due to the movements of M1 and M2 helices, the space around Glu-309 and Val-304 is much more confined, thereby producing occlusion of bound Ca\(^{2+}\). The Glu-309 side chain is fixed by several van der Waals contacts (shown with gray dotted lines) with Leu-65 and Ile-97; the Val-304 \(\rightarrow\) Glu mutated side chain cannot reside there without interference with either M1 or M2 helix. If Val-304 is mutated to Ala or Lys (not shown), Leu-65 side chain, and, as a consequence, the Ile-97 side chain can take other conformations that will allow the Glu-309 side chain to move. When mutated to Thr (not shown), the Val-304Thr side chain will acquire a different conformation by forming a hydrogen bond with Val-300 or Ala-301, again conferring freedom to the Leu-65 side chain. The red spheres are water molecules, and the blue spheres are calcium ions. In E2-BeF\(_{3}\)-tTG (c) and E2(TG+BHQ) (d), orientation and interactions of the Val-304 side chain are shown in the E2P ground state analog and in the final E2 state, suggesting a role of Val-304 in concerted movements of transmembrane helices, including van der Waals contacts with Ile-94 (M2) and Leu-65 (M1). PDB accession numbers: 1sv4, 2z8g, 2zbe (30), and 2agv.
tions of these mechanical links, such as evidently produced by interference with Val-304 optimal fitting, the movements of M4 lower segment and of the A domain become to some extent independent. The A domain is then less likely to move, with consequent stabilization of the E2 state and resistance to phosphorylation by Pi. Conversely, once E2P is formed, impairment of mechanical links renders the cytosolic domains less sensitive to movements of the transmembrane helices, with consequent stabilization of the phosphoenzyme. A role of Val-304 in concerted movements of transmembrane helices through van der Waals contacts with Ile-94 (M2) and Leu-65 (M1) appears (Fig. 9, c and d) in recently obtained structures of E1-AlF4-ADP, E2-BeF4(--TG), E2-BeF4(TG), and E2-AlF4(TG) forms of the ATPase (PDB ID codes 2ZBD, 2ZBE, 2ZBF, and 2ZBG; ref. 30).

An important point to note is that the rates of reactions, rather than the final levels of intermediate, are affected by the Val-304 mutations. To explain this effect, we consider that the headpiece domains are quite constrained following ATP binding and establishment of hydrogen bonds. For this reason, phosphoenzyme turnover is rate-limiting and requires significant energy of activation. In fact, thermal motion develops mostly in the transmembrane region, and is normally transmitted to the headpiece domain as activation energy. However, proper packing of the helices is required for transmission of thermal motion (i.e. energy of activation) to the headpiece. It is then apparent that optimal mechanical coupling requires proper positioning of Val-304 (see relationship with M1 and M2 helices in Fig. 9), whereas mutational interference with this transmission reduces the reaction rates. Impairment of mechanical links, as evidently produced by mutations of the well conserved Val-304 residue, diminishes significantly the long range effect of thermal perturbation. Consequently the rates of reactions are significantly lower, whereas the final levels of products are not significantly affected.

Although Val-304 mutations produce various levels of ATPase inhibition, depending on the side-chain substitution, all mutations produce nearly total inhibition of Ca\(^{2+}\) transport (Table 1). We were particularly intrigued by the Val-304 \(\rightarrow\) Glu mutant that retains 55% ATPase activity while sustaining no significant Ca\(^{2+}\) transport. We then found that Ca\(^{2+}\) occlusion by the fluoroaluminate analog of ADP-E1P-2Ca\(^{2+}\) (i.e. first intermediate following utilization of ATP) is defective in this mutant (Fig. 7). This defect is similar to that produced by Glu-309 and Asn-796 mutations. In fact, Glu-309 and Asn-796 are involved with their side chains in Ca\(^{2+}\) coordination at site II (9), whereas the lack of Ca\(^{2+}\) at site II prevents ATPase activation. On the other hand, the Val-304 mutants retain significant ATPase activity, with a normal Ca\(^{2+}\) concentration dependence (Fig. 3). It is then apparent that the Val-304 side chain mutation to Glu (rather than affecting directly Ca\(^{2+}\) binding) hinders critical movements of transmembrane helices and engagement of Glu-309. Structural models illustrating the interference of Val-304 mutations with stabilization of the Glu-309 side chain are presented in Fig. 9. Because Glu-309 sustains a very important gating role in locking bound Ca\(^{2+}\) (12), the Val-304 \(\rightarrow\) Glu mutation is sufficient to impair Ca\(^{2+}\) occlusion. Leak of occluded Ca\(^{2+}\) onto the outer medium, and reversal of the occluded state, would then interfere with the net rate of phosphoenzyme formation and with Ca\(^{2+}\) transport.

In conclusion, we find that mutations of the well conserved Val-304 residue interfere with occlusion of bound Ca\(^{2+}\) upon utilization of ATP (31, 32). This effect is attributed to altered constraint of the Glu-309 side chain and consequent defect of its gating function, resulting in reduction of the net rate of phosphoenzyme formation and inhibition of vectorial Ca\(^{2+}\) transport. In addition, phosphoenzyme turnover and overall ATPase activity are reduced by Val-304 mutations through interference with mechanical coupling and transmission of transmembrane segments movements to headpiece domains. Thereby, the kinetic effect of thermal activation energy is also reduced.

Acknowledgment—G. I. is grateful to Dr. Francesco Tadini-Buoninsegni for attentive reading of the manuscript and helpful suggestions.

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