Deletions of Any Single Residues in Glu\textsuperscript{40}–Ser\textsuperscript{48} Loop Connecting A Domain and the First Transmembrane Helix of Sarcoplasmic Reticulum Ca\textsuperscript{2+}-ATPase Result in Almost Complete Inhibition of Conformational Transition and Hydrolysis of Phosphoenzyme Intermediate*

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Possible roles of the Glu\textsuperscript{40}–Ser\textsuperscript{48} loop connecting A domain and the first transmembrane helix (M1) in sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA1a) were explored by mutagenesis. Deletions of any single residues in this loop caused almost complete loss of Ca\textsuperscript{2+}-ATPase activity, while their substitutions had no or only slight effects. Single deletions or substitutions in the adjacent N- and C-terminal regions of the loop (His\textsuperscript{45}–Asn\textsuperscript{39} and Leu\textsuperscript{49}–Ile\textsuperscript{54}) had no or only slight effects except two specific substitutions of Asn\textsuperscript{39} found in SERCA2b in Darius’ disease pedigrees. All the single deletion mutants for the Glu\textsuperscript{40}–Ser\textsuperscript{48} loop and the specific Asn\textsuperscript{39} mutants formed phosphoenzyme intermediate (EP) from ATP, but their isomeric transition from ADP-sensitive EP (E1P) to ADP-insensitive EP (E2P) was almost completely or strongly inhibited. Hydrolysis of E2P formed from P\textsubscript{i} was also dramatically slowed in these deletion mutants. On the other hand, the rates of the Ca\textsuperscript{2+}-induced enzyme activation and subsequent E1P formation from ATP were not altered by the deletions and substitutions. The results indicate that the Glu\textsuperscript{40}–Ser\textsuperscript{48} loop, with its appropriate length (but not with specific residues) and with its appropriate junction to A domain, is a critical element for the E1P to E2P transition and formation of the proper structure of E2P, therefore, most likely for the large rotational movement of A domain and resulting in its association with P and N domains. Results further suggest that the loop functions to coordinate this movement of A domain and the unique motion of M1 during the E1P to E2P transition.

Sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA1a)\textsuperscript{1} is a representative member of P-type ion transporting ATPases and catalyzes Ca\textsuperscript{2+} transport coupled with ATP hydrolysis (Fig. 1) (Refs. 1 and 2, and for recent reviews, see Refs. 3 and 4). In the catalytic cycle, the enzyme is activated by binding of two Ca\textsuperscript{2+} ions (E2 to E1Ca\textsubscript{2}, steps 1 and 2) and then autophosphorylated by MgATP to form ADP-sensitive phosphoenzyme (E1P, step 3). Upon formation of this EP, the bound Ca\textsuperscript{2+} ions are occluded in the transport sites. The subsequent isomeric transition to ADP-insensitive form (E2P, step 4) will result in a reduction in affinity and a change in orientation of the Ca\textsuperscript{2+} binding sites, and thus a Ca\textsuperscript{2+} release into lumen (step 5). Finally, hydrolysis takes place and returns the enzyme into an unphosphorylated and Ca\textsuperscript{2+}-bound form (E2, step 6). E2P can also be formed from P\textsubscript{i} in the presence of Mg\textsuperscript{2+} and absence of Ca\textsuperscript{2+} by reversal of its hydrolysis.

The enzyme has three cytoplasmic domains (N, P, and A), which are widely separated in the Ca\textsuperscript{2+}-bound form (E1Ca\textsubscript{2}) and associated in the Ca\textsuperscript{2+}-unbound and thapsigargin-bound form (E2(TG)) (5, 6) (Fig. 2). The modeling of tubular crystals formed with decavanadate (E2V) revealed (5) that three cytoplasmic domains gather to form a most compactly organized single headpiece in E2V. With the limited proteolysis experiments, we previously showed (7, 8) that E2V is very similar to E2P in domain organization and that E2P is the intermediate having the most compactly organized headpiece in the catalytic cycle. The results further indicated that a large rotation of A domain (by −90° (5)) and its strong association with P and N domains most likely occur during the E1P to E2P transition and suggested that stabilization energy provided by intimate contacts between all three cytoplasmic domains in E2P will provide energy for moving transmembrane helices and release the bound Ca\textsuperscript{2+} ions.

It is thus crucial to find out structural elements essential for the A domain movement and resulting domain organization and for transmitting these changes to transmembrane helices. We have recently identified the Lys\textsuperscript{189}–Lys\textsuperscript{205} outermost loop of A domain as to make intimate contact with P domain for formation of the proper structure of Ca\textsuperscript{2+}-released form of E2P in step 5 (9). P domain was actually documented with the crystal structures E1Ca\textsubscript{2} and E2(TG) to function as a coordinator for transmitting the movements of the transmembrane helices to the cytoplasmic domains (6). In addition, it is also possible that the loops connecting A domain with the transmembrane helices (M1-M3) may play roles in the movement of A domain and in transmitting the movement to transmembrane helices. The likely importance of interactions of M2- and M3-connecting loops with P domain was previously pointed out (6, 10), and in fact the proteolytic cut or mutation of the M3-
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connecting loop at or near the interaction sites was shown to cause almost complete loss of the Ca\textsuperscript{2+}-ATPase activity due to blockage of the E1P to E2P transition (10–14). On the other hand, the Glu\textsuperscript{40}–Ser\textsuperscript{48} loop with an extended structure, the major part of the M1-connecting loop, is well separated from and not having significant interactions with other parts of the molecule in E2V as well as in E1Ca\textsubscript{a} and E2(TG) (Fig. 2), and possible roles of this loop remain unknown. Interestingly, not only A domain, but also M1 connected to this loop, seems to undergo very large and unique structural changes during Ca\textsuperscript{2+} transport cycle, i.e. up-and-down and horizontal movements and bending near the membrane surface (6).

In the present study, we focused on and explored possible roles of the Glu\textsuperscript{40}–Ser\textsuperscript{48} loop by site-specific mutagenesis and found that deletions of any single residues in this loop result in almost complete loss of the Ca\textsuperscript{2+}-ATPase activity, while their substitutions have no or only slight effects. Results further showed that both the E1P to E2P transition and the E2P hydrolysis are almost completely inhibited in all these single deletion mutants. The results indicate that the Glu\textsuperscript{40}–Ser\textsuperscript{48} loop is critical for formation of the proper structure of E2P and further suggest that the loop may coordinate the motions of A domain and M1 during the E1P to E2P transition and thus possibly contribute to the rearrangement of the transmembrane helices.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis and Expression**—Mutations were created by PCR using the QuikChange\textsuperscript{TM} site-directed mutagenesis kit (Stratagene) and plasmid pGEM7-Zf(+) (Promega) containing the ApaI-KpnI fragment of the rabbit SERCA1a cDNA as a template. The ApaI-KpnI fragments were then excised from the PCR products and used to replace the corresponding region in the full-length SERCA1a cDNA in the pMT2 expression vector (15). The pMT2 DNA was transfected into COS-1 cells by the liposome-mediated transfection method. Microsomes were prepared from the cells as described previously (16). The “control microsomes” were prepared from COS-1 cells transfected with the pMT2 vector containing no SERCA1a cDNA. The amount of expressed SERCA1a was quantified by a sandwich enzyme-linked immunosorbent assay as described previously (17). The expression levels of all the mutants in the microsomes were comparable with that of the wild type.

**ATPase Activity**—The rate of ATP hydrolysis was determined at 25 °C in a mixture containing 20 μg/ml microsomal protein, 0.1 mm [γ-\textsuperscript{32}P]ATP, 1 μM A23187, 7 mm MgCl\textsubscript{2}, 0.1 m KCl, 50 mm MOPS/Tris (pH 7.0), 0.55 mm CaCl\textsubscript{2}, and 0.5 mm EGTA. The Ca\textsuperscript{2+}-ATPase activity was obtained by subtracting the Ca\textsuperscript{2+}-independent ATPase activity, which was determined in the presence of 5 mm EGTA without added CaCl\textsubscript{2}, otherwise as above. The specific ATPase activity/mg of expressed SERCA1a protein was calculated from the amount of expressed SERCA1a and the Ca\textsuperscript{2+}-ATPase activity of expressed SERCA1a, which was obtained by subtracting the Ca\textsuperscript{2+}-ATPase activity of the control microsomes from that of the microsomes expressing SERCA1a. This background level with the control microsomes was as low as 3% of the activity of microsomes expressing the wild-type SERCA1a.

**Formation and Hydrolysis of EP**—Phosphorylation of SERCA1a in microsomes with [γ-\textsuperscript{32}P]ATP or \textsuperscript{32}P, and dephosphorylation of \textsuperscript{32}P-labeled SERCA1a, were performed under conditions described in the legends to figures. The reactions were quenched with ice-cold trichloroacetic acid containing P\textsubscript{i}. Rapid kinetics measurements of phosphorylation and dephosphorylation were performed with a homemade rapid mixing apparatus (18) or otherwise as above. The precipitated proteins were separated at pH 6.0 by 5% SDS-polyacrylamide gel electrophoresis according to Weber and Osborn (19). The radioactivity associated with the separated Ca\textsuperscript{45}–ATPase was quantitated by digital autoradiography as described previously (20). The amount of EP formed with the expressed SERCA1as was obtained by subtracting the background radioactivity with the control microsomes. This background was less than 4% of the radioactivity of EP formed with the expressed wild-type SERCA1a. The amount of EP/mg of SERCA1a protein was calculated from the amount of EP thus obtained and the amount of expressed SERCA1a.

**Miscellaneous**—Protein concentrations were determined by the method of Lowry et al. (21) with bovine serum albumin as a standard. Data were analyzed by nonlinear regression using the program Origin (Microcal Software, Inc., Northampton, MA). Three-dimensional models of the enzyme were reproduced by the program VMD (Theoretical Biophysics Group, University of Illinois at Urbana-Champaign).

**RESULTS**

**Effects of Deletions and Substitutions on ATP Hydrolysis**—The specific Ca\textsuperscript{2+}-ATPase activities of the expressed mutant and wild-type SERCA1a were determined at 25 °C (Fig. 3). Deletions of any single residues in the Glu\textsuperscript{40}–Ser\textsuperscript{48} loop resulted in almost complete loss of the activity, while their non-conservative substitutions caused only partial decrease or rather slight increase in the activity. Single deletions or substitutions of the residues in the adjacent N-terminal (His\textsuperscript{2}-Asn\textsuperscript{39} and C-terminal (Leu\textsuperscript{49}–Ile\textsuperscript{54}) regions of the Glu\textsuperscript{40}–Ser\textsuperscript{48} loop had only slight or moderate effect on the activity, except that the specific substitutions of Asn\textsuperscript{39} (N39D and N39T, but not N39A) had a significantly reduced activity. Quadruple alanine substitutions of Asn\textsuperscript{39}, Glu\textsuperscript{40}, Glu\textsuperscript{44}, and Glu\textsuperscript{45} were previously shown to cause no loss of function (22), and the present results are in essential agreement.

**Formation of EP from ATP**—We then performed detailed kinetic analysis with the mutants. EP was formed from ATP at 0 °C under conditions otherwise similar to those for the ATPase assay. All the mutants possessed the ability to form EP, and the amount formed was comparable with that of wild type (Fig. 4). In Fig. 5, the fraction of E2P accumulated was determined at steady state (15 s after addition of ATP). In the presence of K\textsuperscript{+}, which strongly accelerates decay of E2P and thus suppresses its accumulation in the wild type (23), the amount of E2P accumulated in all the mutants was very low as in the wild type. In the absence of K\textsuperscript{+}, the fraction of E2P largely increased in the wild type (to 73% of the total amount of EP (E1P plus E2P), but it remained very low in the single deletion mutants for the Glu\textsuperscript{40}–Ser\textsuperscript{48} loop and in the mutants N39D and N39T, indicating that almost all EP accumulated was E1P in these mutants. On the other hand, as in the wild type, the fraction of E2P largely increased in the other mutants, in which the residues in the loop were substituted or those in the adjacent N- and C-terminal regions of the loop were deleted or substituted (with the exception of N39D and N39T). The results strongly suggest that the E1P to E2P transition in step 4 is inhibited in the single deletion mutants for the Glu\textsuperscript{40}–Ser\textsuperscript{48} loop and in the substitution mutants N39D and N39T. We, therefore, examined the decay of E1P accumulated in the presence of K\textsuperscript{+}.

**Decay of EP Formed from ATP**—Decay of EP formed from ATP in the presence of K\textsuperscript{+} was determined at 0 °C by first phosphorylating with [γ-\textsuperscript{32}P]ATP in the presence of K\textsuperscript{+} and Ca\textsuperscript{2+} for 15 s under the conditions in which almost all EP
formed was E1P (see Fig. 5) and then terminating phosphorylation by adding excess EGTA to prevent further phosphorylation and thus allow decay of $^{32}$P-labeled EP. The EP decay was well fitted with a single exponential as shown in Fig. 6 with the representative mutants and wild type. The decay rates were thus obtained with all the mutants and summarized in Table I. The decay was almost completely blocked by any single deletions in the Glu$^{40}$–Ser$^{48}$ loop, and the rates were only 1–3% of that in the wild type. On the other hand, the rates in the substitution mutants for the Glu$^{40}$–Ser$^{48}$ loop and in all the deletion and substitution mutants for the adjacent regions of the Glu$^{40}$–Ser$^{48}$ loop were comparable with or only slightly lower than that in the wild type, except the mutants N39D and N39T (but not N39A) that had the significantly reduced rate (dashed lines in Fig. 6).

**Hydrolysis of E2P Formed from P$_7$—E2P was formed by P$_i$ in the absence of Ca$^{2+}$ and K$^+$ and presence of 35% (v/v) Me$_2$SO, which extremely favors E2P formation (24). In some of the single deletion mutants, including those for the Glu$^{40}$–Ser$^{48}$ loop, the amount of EP formed was somewhat reduced (Fig. 7A). Nevertheless the hydrolysis of $^{32}$P-labeled E2P was examined with all the mutants at 0 °C by diluting the above phosphorylated samples with a large volume of a solution containing K$^+$ and non-radioactive P$_i$. The conditions were thus made otherwise identical to those used for the decay of EP formed from ATP in Fig. 6. The hydrolysis of E2P proceeded with first-order kinetics as shown in Fig. 7. B and C, with the representative mutants and wild type. The hydrolysis rates were thus obtained with all the mutants and summarized in Table I. The hydrolysis was markedly slowed or blocked by any single deletions in the Glu$^{40}$–Ser$^{48}$ loop, and the rates were only 0.3–3% of that in the wild type. In contrast, the hydrolysis in the substitution mutants for the Glu$^{40}$–Ser$^{48}$ loop and in the deletion and substitution mutants for the adjacent regions of the loop including N39D and N39T (dashed lines in Fig. 7C) was as rapid as, or only slightly slower than, that in the wild type.

**Transition from E2 to E1Ca$_2$—**The mutants and wild type were preincubated in the absence of Ca$^{2+}$ at pH 6 where equilibrium between E1 and E2 is most shifted to E2 (25) and then phosphorylated at 0 °C by simultaneous addition of saturating concentrations of Ca$^{2+}$ and ATP under conditions otherwise similar to those for the ATPase assay. The time course of EP formation was well described by the first-order kinetics as shown in Fig. 8 with the representative mutants and wild type. The rates were thus obtained with all the mutants and summarized in Table I. The rates of all the mutants, including the single deletion mutants for the Glu$^{40}$–Ser$^{48}$ loop, were comparable with that of the wild type and not significantly reduced. When ATP was added to the enzyme preincubated with Ca$^{2+}$ otherwise as above, the EP formation proceeded at much faster rate (5 s$^{-1}$ in the wild type and the comparable rate (4–7 s$^{-1}$).

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**Fig. 2. Structural comparison of E1Ca$_2$, E2V, and E2(TG) for the Glu$^{40}$–Ser$^{48}$ loop.** The coordinates for the structures E1Ca$_2$, E2V, and E2(TG) were obtained from the Protein Data Bank (accession codes 1EUL, 1FQU, and 1IWO, respectively (5, 6). The residues in the Glu$^{40}$–Ser$^{48}$ loop, Arg$^{40}$, and Asp$^{41}$ (phosphorylation site) are shown in white (carbon atom), red (oxygen atom), and blue (nitrogen atom). The Lys$^{392}$–Lys$^{395}$ loop and Thr$^{131}$–Ser$^{134}$ loop are shown in blue and red, respectively. The three structures were manually fitted with M8–M10, which do not move virtually in the three structures. To realize the states in E2V and E2(TG) from E1Ca$_2$: A domain largely rotates by more than ~90° and also P and N domains incline as indicated by arrows. Note that the Glu$^{40}$–Ser$^{48}$ loop is extended and does not have significant interactions with other parts of the molecule in the three structures and that, in E2V and E2(TG), M3–M5 directly associated with P domain and M6 (with the loop connecting M6 and M7) are inclined and bent toward M1 and the top part of M1 is bent to form an amphipathic helix M1' likely at the membrane surface (5, 6). Also note that E2V has the most compactly organized domain structure and E2(TG) has the organized but more relaxed one.
in all the mutants). The results show that the Ca$^{2+}$-induced E2 to E1Ca$_2$ transition, which is rate-limiting for the EP formation from E2, is essentially not inhibited in all the mutants.

**DISCUSSION**

Roles of Glu$^{40}$–Ser$^{48}$ Loop—In the present study, we explored possible roles of the Glu$^{40}$–Ser$^{48}$ loop connecting A domain and M1 helix by mutagenesis and found that deletions of any single residues within this loop (but not their substitutions) almost completely block or strongly inhibit both the E1P to E2P transition and the E2P hydrolysis. Results indicate that the loop with its appropriate length (but not with specific residues) is critical for the rapid isomeric transition and hydrolysis of EP.

During the E1P to E2P transition and Ca$^{2+}$ release into lumen, a large rotation of A domain by $-90^\circ$ and its intimate contact with P and N domains occur to form the most compactly organized cytoplasmic domains in E2P without bound Ca$^{2+}$ (7, 8). In this E2P, the hydrophobic atmosphere (24, 26–28) is thus realized around the phosphorylation site and a specific water molecule can now attack the acylphosphate bond to hydrolyze. It is therefore likely that the formation of the proper structure of E2P, i.e. the movement of A domain and resulting domain organization, was impaired by the deletions of even single residues ($-3.5 \AA$ shortening) in the Glu$^{40}$–Ser$^{48}$ loop. The facts found in the structural model E2V, an E2P analogue (7, 8), that the Glu$^{40}$–Ser$^{48}$ loop is extended and not interacting with other parts of the molecule (Fig. 2), is consistent with the view that no specific residues are involved in the role of this loop but that its length being crucial.

In the detailed and well accepted reaction mechanism for the E1P to E2P transition and Ca$^{2+}$ release, the process consists of two steps (steps 4 and 5, Fig. 1). The single deletions in the Glu$^{40}$–Ser$^{48}$ loop obviously caused blocking of the loss of ADP...
sensitivity in step 4 (i.e., the E1P to E2P transition). We have recently found (9) that the mutations in the Lys189–Lys205 outermost loop on A domain do not inhibit the loss of ADP sensitivity but strongly inhibit the subsequent processing of E2P in step 5 and indicated that the final process of gathering of A and P domains is accomplished in step 5 to release the bound Ca\(^{2+}\) by the intimate contact of the Lys189–Lys205 loop with P domain. Together with this study, we thus could identify two structural elements crucial for the successive, but distinct, two steps; the large rotation of A domain and its association with P and N domains (to some extent) in step 4 and the final process for intimate contact of A and P domains in step 5.

Importantly, it was found in comparison of the structures EcA2 and Ec2(TG) (6) that upon Ca\(^{2+}\) dissociation, M1 connected to the Glu40–Ser48 loop undergoes horizontal and upward movements and a large structural change, i.e., bending at Asp38, forming an amphipathic helix M1 that has hydrophobic residues on one side and charged residues on the other (Fig. 2). M1 is thus likely to be situated at the membrane surface by the intimate contact of the Lys205 loop (hydrolysis of E2P transition). We have previously observed (7, 8) that cytoplasmic domain organization in the more relaxed E2 state is significantly different from that in the most compactly organized E2P state. The view may also be compatible with the notion (6) that the shortening of the Glu40–Ser48 loop by the deletions may also be compatible with the notion (6) that the shortening of the Glu40–Ser48 loop functions in coordinating these motions of A and P domains for the transition (and subsequent E2P transition and thus in the formation of the proper structure of E2P). It is possible that the Glu40–Ser48 loop functions in coordinating these motions of A domain and M1 during the E1P to E2P transition and thus in rendering A domain to accept the inclined P domain at an appropriate position and contributing to cross-talk between the cytoplasmic and transmembrane domains.

The observation showing essentially no effects of the deletions in the Glu40–Ser48 loop on the rate of the E2 to EcA2 transition (and subsequent E1P formation) (steps 1–3, Fig. 8) indicates that the structural importance of the Glu40–Ser48 loop may be already lost in E2. This view is consistent with our previous observation (7, 8) that the cytoplasmic domain organization in the more relaxed E2 state is significantly different from that in the most compactly organized E2P state. The view may also be compatible with the notion (6) that E2 is likely in
Fig. 7. Hydrolysis of E2P formed from P_i. Microsomes were phosphorylated with 32P; at 25 °C for 10 min in 50 μl of a mixture containing 2 μg of microsomal protein, 0.1 mM 32P, 1 mM A23187, 7 mM MgCl₂, 50 mM MOPS/Tris (pH 7.0), and 5 mM EGTA. At zero time, 50 μl of the buffer containing 20 μM (γ-32P)ATP and 1.4 mM CaCl₂, in place of EGTA otherwise as above, was added to the microsome suspension. At various times after this addition, the amount of E2P formed was determined. The time courses of the wild type (WT) and representative mutants are shown and indicated in the figure with different symbols. Solid and dashed lines show the least squares fit to a single exponential. The rate constants were thus obtained with all the deletion and substitution mutants and given in Table I. The values for the amount of E2P obtained at infinite time in the fitting are normalized to 100%.

Fig. 8. Phosphorylation upon simultaneous addition of ATP and Ca²⁺. Microsomes expressing the wild-type or mutant SERCA1a (2 μg of microsomal protein) were preincubated in the absence of Ca²⁺ at 0 °C for 5 min in 50 μl of a buffer containing 1 mM A23187, 7 mM MgCl₂, 0.1 mM KCl, 50 mM MOPS/Tris (pH 6.0), and 1 mM EGTA. At zero time, 50 μl of the buffer containing 20 μM (γ-32P)ATP and 1.4 mM CaCl₂, in place of EGTA otherwise as above, was added to the microsome suspension. At various times after this addition, the amount of E2P formed was determined. The time courses of the wild type (WT) and representative mutants are shown and indicated in the figure with different symbols. Solid and dashed lines show the least squares fit to a single exponential. The rate constants were thus obtained with all the deletion and substitution mutants and given in Table I. The values for the amount of E2P obtained at infinite time in the fitting are normalized to 100%.

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The extensive hydrogen bond network is actually formed between the TGES* loop, the Asp* loop, Asp* (the residues in Lys* and Arg* on N domain, and stabilizing the associated state of the domains. Asp* and Glu* on the TGES* loop were predicted by the iron-catalyzed cleavage of Na*/H* to participate together in the Mg* transition by the specific substitutions, strongly suggest that the above residues (Asp*, Asp*, Asp* and Asp*) or at least some of them are involved in the A-P domain interaction together with the TGES* loop for the loss of the ADP sensitivity, although information on the three-dimensional structure of E2PCa* cannot reach or access any more the covalently bound phosphate in E2P (38, 40) and thus in the possible change in the immediate vicinity of the phosphate for the loss of ADP sensitivity.

Obviously the above docking of A domain with P domain to situate the TGES* loop at their appropriate interface requires the large rotational movement of A domain during the E1P to E2P transition and also the inclination of P domain toward A domain (see also Fig. 2). The Glu*−Ser* and Gly* loops are both apart from this interacting face. The Glu*−Ser* loop is most likely important for the rotational movement of A domain and coordinated motions of M1 to result in the appropriate domain docking, as discussed above. The Gly*−Pro* loop is interacting with the lower part (helix P6) of bent forward P domain in E2(TG) (and in E2V) but separated in E1Ca* and it was suggested that this loop moves for the E1P to E2P transition from a peripheral to the more central position where it interacts with the bent forward P domain (by the hydrogen bonds between Glu*−Lys, Gln*−Thr*−Met*). The residues of which the contributions of the Gly*−Pro* loop for anchoring A domain on P domain that has likely inclined toward A domain together with the motions of M3−M6, M2, and M1. These coordinated movements and the domain interactions are obviously made possible by the conformational energy gained in steps 2 and 3 with the Ca*− and ATP bindings and accumulated in E1Ca* which has P and N domains with their most closed configuration as realized in the E1Ca* ATP complex, P domain thus being distorted (41, 42) and A domain with the M3- and M2-connecting loops being repositioned from E1Ca* (and thus ready for its subsequent large rotation) as revealed by the complete protection of P domain and these loops against proteinase K and V8 (but not trypsin at Arg* in our systematic proteolysis (7, 8) and also in the very recent mutation and proteolysis study (40). It is also important to note that P and N domains should be opened in step 4 from this most closed configuration to some extent (likely by the reverse movement of N domain and/or further inclination of P domain toward A domain), and hence A domain can rotate in and associate with P and N domains to cause the loss of ADP sensitivity (i.e. the β-phosphate of ADP cannot reach or access any more the covalently bound phosphate at Asp* (38)). (In E2V, the A-N domain association involves the residues near the adenine binding pocket (Glu* and Glu*, the dashed circle in Fig. 9) besides Arg* at the three-domain interface.) In the subsequent step 5, the final process of gathering of A and P domains is accomplished by the formation of strong interaction between the polar residues surrounding Val* (Arg*, Asp*, Gln*, and Asp*) on the

![Diagram](image_url)

**Fig. 9.** Residues previously found to be essential for the E1P to E2P transition on the structural model E2V. The residues of which mutations were previously shown to inhibit the loss of ADP sensitivity (step 4) are represented with Asp* (phosphorylation site) and Arg* as a CPK model in cyan (carbon atom), red (oxygen atom), and dark blue (nitrogen atom) in E2V (Protein Data Bank accession code 1FQU (5)). The dashed circles show the regions of the hydrogen bonding interactions between the Gly*−Pro* loop and helix P6 on P domain and between A and N domains. The Lys*−Lys* loop is also shown. For details, see “Discussion.”

The extensive hydrogen bond network described above the covalently bound phosphate in E2P (38, 40) and thus in the possible change in the immediate vicinity of the phosphate for the loss of ADP sensitivity.

Obviously the above docking of A domain with P domain to situate the TGES* loop at their appropriate interface requires the large rotational movement of A domain during the E1P to E2P transition and also the inclination of P domain toward A domain (see also Fig. 2). The Glu*−Ser* and Gly* loops are both apart from this interacting face. The Glu*−Ser* loop is most likely important for the rotational movement of A domain and coordinated motions of M1 to result in the appropriate domain docking, as discussed above. The Gly*−Pro* loop is interacting with the lower part (helix P6) of bent forward P domain in E2(TG) (and in E2V) but separated in E1Ca* and it was suggested that this loop moves for the E1P to E2P transition from a peripheral to the more central position where it interacts with the bent forward P domain (by the hydrogen bonds between Glu*−Lys, Gln*−Thr*−Met*). The residues of which the contributions of the Gly*−Pro* loop for anchoring A domain on P domain that has likely inclined toward A domain together with the motions of M3−M6, M2, and M1. These coordinated movements and the domain interactions are obviously made possible by the conformational energy gained in steps 2 and 3 with the Ca*− and ATP bindings and accumulated in E1Ca* which has P and N domains with their most closed configuration as realized in the E1Ca* ATP complex, P domain thus being distorted (41, 42) and A domain with the M3- and M2-connecting loops being repositioned from E1Ca* (and thus ready for its subsequent large rotation) as revealed by the complete protection of P domain and these loops against proteinase K and V8 (but not trypsin at Arg* in our systematic proteolysis (7, 8) and also in the very recent mutation and proteolysis study (40). It is also important to note that P and N domains should be opened in step 4 from this most closed configuration to some extent (likely by the reverse movement of N domain and/or further inclination of P domain toward A domain), and hence A domain can rotate in and associate with P and N domains to cause the loss of ADP sensitivity (i.e. the β-phosphate of ADP cannot reach or access any more the covalently bound phosphate at Asp* (38)). (In E2V, the A-N domain association involves the residues near the adenine binding pocket (Glu* and Glu*, the dashed circle in Fig. 9) besides Arg* at the three-domain interface.) In the subsequent step 5, the final process of gathering of A and P domains is accomplished by the formation of strong interaction between the polar residues surrounding Val* (Arg*, Asp*, Gln*, and Asp*) on the
Lys189–Lys205 loop (another outermost loop of A domain besides the TGES loop) and those on P domain (Arg147, Glu40 on the basis of E2V) to produce the most compactly organized single headpiece (9). This intimate contact of A and P domains will likely provide the conformational energy enough to further distort P domain and rearrange the transmembrane helices and thus open the luminal gate and release the bound Ca2+. The intimate contact of the domains also produces the hydrophobic atmosphere (and the fine-tuning) around the phosphorylation site, so that the specific water molecule can attack the acylphosphate bond and the essential residues can participate in the hydrolysis. Upon the molecule can attack the acylphosphate bond and the essential ing) around the phosphorylation site, so that the specific water

Mutants in Darier’s Disease—Darier’s disease, a human autosomal dominant skin disorder, was shown to be caused by substitutions (Thr226), and others in G1629 loop) and those on P domain (Arg147, Glu40, Lys205 loop. The intimate contact of the domains also produces the hydrophobic atmosphere (and the fine-tuning) around the phosphorylation site, so that the specific water molecule can attack the acylphosphate bond and the essential residues can participate in the hydrolysis. Upon the molecule can attack the acylphosphate bond and the essential

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