Glycine 105 as Pivot for a Critical Knee-like Joint between Cytoplasmic and Transmembrane Segments of the Second Transmembrane Helix in Ca$^{2+}$-ATPase*

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The cytoplasmic actuator domain of the sarco(endo)plasmic reticulum Ca$^{2+}$-ATPase undergoes large rotational movements that influence the distant transmembrane transport sites, and a long second transmembrane helix (M2) connected with this domain plays critical roles in transmitting motions between the cytoplasmic catalytic domains and transport sites. Here we explore possible structural roles of Gly105 between the cytoplasmic (M2c) and transmembrane (M2m) segments of M2 by introducing mutations that limit/increase conformational freedom. Alanine substitution G105A markedly retards isomerization of the transport sites uncoupling ATP hydrolysis and Ca$^{2+}$ transport. In contrast, this substitution accelerates the ATPase activation (E2 → E1Ca$^{2+}$). Introducing a glycine by substituting another residue on M2 in the G105A mutant (i.e. “G-shift substitution”) identifies the glycine positions required for proper Ca$^{2+}$ handling and kinetics in each step. All wild-type kinetic properties, including coupled transport, are fully restored in the G-shift substitution at position 112 (G105A/A112G) located on the same side of the M2c helix as Gly105 facing M4/phosphorylation domain. Results demonstrate that Gly105 functions as a flexible knee-like joint during the Ca$^{2+}$ transport cycle, so that cytoplasmic domain motions can bend and strain M2 in the correct direction or straighten the helix for proper gating and coupling of Ca$^{2+}$ transport and ATP hydrolysis.

Sarco(endo)plasmic reticulum Ca$^{2+}$-ATPase (SERCA1a), a representative member of P-type ion-transporting ATPases, catalyzes Ca$^{2+}$ transport coupled with ATP hydrolysis (Fig. 1A) (for recent reviews, see Refs. 1–3). The enzyme is activated by the binding of two Ca$^{2+}$ ion to high affinity transport sites facing the cytoplasmic side (E2 to E1Ca$^{2+}$ in Fig. 1) and autophosphorylated at Asp351 with MgATP to form an ADP-sensitive phosphoenzyme (E1P), which reacts with ADP to regenerate ATP in the reverse reaction. Upon E1P formation, the two bound Ca$^{2+}$ are occluded in the transport sites (E1PCa$^{2+}$). The subsequent isomeric transition to the ADP-insensitive E2P form results in rearrangements of the Ca$^{2+}$ binding sites to deocclude Ca$^{2+}$, open the release path, and reduce the affinity, thus releasing Ca$^{2+}$ into the lumen. Finally, the Asp351-acyl-phosphate in E2P is hydrolyzed to form a Ca$^{2+}$-free inactive E2 state.

In the transport cycle, the three cytoplasmic domains N, P, and A undergo large movements and change their organizational state, a repositioning that is coupled to rearrangements in transmembrane helices and thereby changes in the transport sites (1–15). Most remarkable is the motion of the A domain, which functions in cytoplasmic and luminal gating to regulate Ca$^{2+}$ binding and release, as well as E2P hydrolysis. The long helix M2 connects directly with the A domain at their junction (A/M2-junction) and moves largely together with the A domain and also changes its secondary structure, unwinding/rewinding with consequent length changes, during the Ca$^{2+}$ transport cycle (Fig. 1A). The long helix structure of M2 and the A domain motions are common features in P-type ion-transporting ATPases (16–20).

We have demonstrated by extensive mutations throughout M2, disrupting and elongating the helix with glycine insertions (21), that its transmembrane part (M2m), cytoplasmic part (M2c), and junctional region with the A domain (M2top) (Fig. 1B) play different roles in the various catalytic steps, a separation of function apparently needed to control gating at the transport sites and thereby coupling Ca$^{2+}$ transport with ATP hydrolysis. M2 has a role distinct from that of the A/M1’-linker (22–24), although both are needed to coordinate coupling.

There is a glycine residue, Gly105, in the region connecting M2c and M2m. A glycine residue in an α-helix is known to break the helical structure and provide conformational freedom (25), and bending at Gly105 may possibly translate M2c and M2m motions between membrane helices and the cytoplasmic domains, expediting the structural functions of M2. Actually, according to crystal structures, M2 moves, bends, unwinds, and possibly registers tension during the A domain motions in the transport cycle (Fig. 1). In this study, we explore possible structural effects and conformational freedom provided by the glycine residue at the M2c and M2m junction by introducing...
mutations, which are intended to 1) reduce conformational freedom (G105A) and distort helix structure (G105P, V104P, and V106P), 2) increase flexibility (single G-substitution of each residue and GG-substitution of successive residue pairs), and 3) evaluate the positional importance of the glycine (single G-shift substitutions in the G105A mutant). The results demonstrate that Gly105 is crucial for Ca\(^{2+}\)/H\(_{11001}\) occlusion in E\(_P\) and rapid E\(_P\) isomerization and, therefore, for rapid coupled ATP hydrolysis.

Surprisingly, a G-shift substitution at the 7th residue from Gly105 located on the same face of the helix (G105A/A112G) fully restores wild-type kinetic properties and function. The glycine evidently functions as a flexible joint or hinge for M2-mediated coupling between catalytic events and gating during Ca\(^{2+}\) transport, allowing directionally oriented bending and absorbing strain when needed. Its important role in pumping is underscored by the fact that a glycine is conserved in this region in P-type ATPases.

**Results**

**EP Formation at Steady State**—In Fig. 2, we first determined the total amount of EP (E\(_P\)\(_{\text{total}}\), sum of E1P and E2P) and fraction of E2P at steady state for wild type and all mutants in 0.1M K\(^{+}\), which strongly accelerates E2P hydrolysis and therefore suppresses its accumulation at steady state in the wild type (26). The E\(_P\)\(_{\text{total}}\) is >50 pmol/mg of microsomal protein in the wild type and all of the mutants and thus is sufficient to perform
functional analyses. In wild type and all mutants, E1P accumulates almost exclusively.

ATP Hydrolysis, Ca\(^{2+}\) Transport, and Coupling—In Fig. 3A, the Ca\(^{2+}\) -ATPase activity and oxalate-dependent Ca\(^{2+}\) transport activity were determined at a saturating 10 \(\mu\)M Ca\(^{2+}\) during the initial linear part of P\(_i\) liberation and Ca\(^{2+}\) transport (inset) with the inclusion of 5 mM oxalate (to trap transported Ca\(^{2+}\) in the lumen), and mutants were compared with wild-type activities. The activities were markedly reduced by alanine or proline substitutions of Gly\(^{105}\) (G105A or G105P) and also proline substitutions of Val\(^{104}\) and Val\(^{106}\) (V104P and V106P) at the M2c-M2m connecting region. The activities were also largely reduced by glycine (G) or two-glycine (GG) substitution at the Ile97-Leu98 region on M2m and the Gln108–Ala115 region (Fig. 3B), the ratio of Ca\(^{2+}\) transport activity to ATPase activity (Ca\(^{2+}\)/ATP) of each mutant relative to the wild type is shown. The substitutions G105A, G105P, V104P (but not V104G), V106G, V106P, and W107G around Gly\(^{105}\) markedly reduced Ca\(^{2+}\)/ATP, indicating severe uncoupling (i.e. almost no Ca\(^{2+}\) transport despite fair ATP hydrolysis). The G- or GG-substitutions at the Leu96–Val\(^{104}\) and Gln\(^{108}\)–Ala\(^{115}\) regions did not cause such uncoupling. Interestingly, the single G-substitutions V106G and W107G significantly reduced the transport activity and thus the Ca\(^{2+}\)/ATP ratio, but the GG-substitution of these residues (V106G/W107G, resulting in three successive glycines GGG\(^{107}\) and accompanying conformational freedom to the helical wheel at the M2c-M2m connecting region (Fig. 3D)) somehow restored the wild-type activities and coupled Ca\(^{2+}\) transport.

We then made “G-shift substitutions,” in which the G105A substitution was combined with a glycine substitution of another residue on the M2 helix, to examine whether shifting the position of the glycine residue can restore function. Surprisingly, only one G-shift substitution, G105A/A112G, within the Leu96–Ala115 wide region restored wild-type ATPase activity and coupled Ca\(^{2+}\) transport, both of which were disrupted by the G105A substitution. Residues 105 and 112 are positioned on the same side of the M2 helical wheel in E1Ca\(_2\)−P−ADP\(^{3-}\), E2P ground state, and E2−P\(^{\prime}\), facing M4C, the cytoplasmic part of M4 (Figs. 3C (C and D), 5, and 8). Results demonstrate the critical importance of the glycine position on the M2 helix, both in terms of sidedness and distance from Gly\(^{105}\), for structural communication between the cytoplasmic catalytic domains and transmembrane transport sites.

E1P → E2P Isomerization—We then analyzed each of the steps in the Ca\(^{2+}\) transport cycle. In Fig. 4, the rate of EP isomerization E1P → E2P (i.e. the loss of the ADP sensitivity at the catalytic site) was determined in the presence of K\(^{+}\) under conditions essentially the same as those for E\(_P\) formation in Fig. 2. The isomerization rate is strongly reduced in the G105A and G105P substitutions, but it is comparable with or even higher than that of the wild type in the G- or GG-substitution mutants of other residues, including Ala\(^{112}\), and in the V104P and V106P mutants. Results show the critical importance of Gly\(^{105}\) for rapid EP isomerization, which involves a large A-domain rotation and association with the P domain. Interestingly, the GG-substitution at Ile\(^{103}\)–Val\(^{104}\) (I103G/I104G) accelerates EP isomerization 10-fold; thus, the introduction of three successive glycines and the increase in flexibility of the helix here...
strongly facilitate M2 flexing linked to the large cytoplasmic domain motions.

G-shift substitutions to mutant G105A restored (G105A/I97G, G105A/N101G, G105A/Q108G, G105A/A112G, and G105A/A115G) or even accelerated (G105A/V104G) the sup-
pressed E2P isomerization rate (Fig. 4A). It is notable that all of
the glycines introduced in these restoring G-shift substitutions
are situated on the same side of the M2 helical wheel as Gly105
(Fig. 4, B and C, green), suggesting a directional structural
change in the helix, such as in a knee-bending motion.

E2P Hydrolysis—In Fig. 5, we determined the E2P hydrolysis
rate by first phosphorylating the enzyme with $^{32}\text{P}$, in
the absence of Ca$^{2+}$ and K$^+$ and the presence of 30% (v/v) Me$_3$SO,
which strongly favors E2P formation in the reverse reaction
(27), followed by diluting the phosphorylated protein with a
large volume of nonradioactive P$_i$ and K$^+$ without Ca$^{2+}$. The
hydrolysis rate was not significantly inhibited by the alanine or
proline substitutions of Gly$^{105}$, Val$^{104}$, or Val$^{106}$, nor was it
inhibited by G- or GG-substitutions of all of the residues in the
entire Leu$^{96}$–Ala$^{115}$ region. Rather, the hydrolysis was acceler-
ated with substitutions L98G on M2m and Q108G, A112G,
E113G, and A115G on M2c. Consistently, in the structural
change that takes place during $E_2$:BeF$_3^{-}$ to $E_2$:AlF$_4^{-}$ ($E_2P +$
H$_2$O→$E_2$–$P^\prime$), a part of M2c (Asn$^{111}$–Ala$^{115}$) unwinds (Figs. 1
and 5), due to tilting of the A domain, and presumably glycine
substitutions here expedite helix breaking and unwinding.

G-shift substitutions at Val$^{103}$ and Val$^{106}$ next to Gly$^{105}$ (i.e.
G105A/V104G and G105A/V106G but not the G-substitutions
V104G and V106G possessing Gly$^{105}$) markedly retarded the
hydrolysis, suggesting that the required flexing is strictly in
a particular direction.

The E2P ground state possesses luminally open low affinity
Ca$^{2+}$ transport sites with $K_a$ of ~1 M (23, 28–34), and in $E_2$→
$E_2$–$P^\prime$ during E2P hydrolysis, the luminal gate is tightly
closed, which prevents luminal Ca$^{2+}$ access and possible Ca$^{2+}$
leakage (7). Actually, E2P hydrolysis is inhibited by a high con-
centration of luminal Ca$^{2+}$ due to Ca$^{2+}$ binding at the
luminally open transport sites in E2P. We determined the E2P hydrolysis
rate of the G105A mutant in the presence of 3 and 20 mM lumi-
cal Ca$^{2+}$ otherwise, as in Fig. 5A, and found that the hydrolysis
is markedly retarded by the luminal Ca$^{2+}$ in the G105A mutant
as in the wild type. Actually, the hydrolysis rates (s$^{-1}$) in
the presence of 0, 3, and 20 mM Ca$^{2+}$ are 0.587 ± 0.078 ($n = 6$),
0.006 ± 0.005 ($n = 3$), and 0.003 ± 0.003 ($n = 3$), respectively
in the wild type and 0.501 ± 0.112 ($n = 3$), 0.018 ± 0.004 ($n = 3$),
and 0.007 ± 0.001 ($n = 3$), respectively in the G105A mutant.
Thus, luminal Ca$^{2+}$ access to the transport sites in the E2P
ground state in G105A is as in the wild type, and evidently
gating here is not impaired.

$E_2$→$E_1$ Transition—For ATPase activation $E_2$→$E_1$→
$E_1$Ca$_2$, the enzyme is isomerized first to a transient $E_1$ state to
gain an open cytoplasmic gate and high Ca$^{2+}$ affinity at the

FIGURE 3. Ca$^{2+}$-ATPase activity and oxalate-dependent Ca$^{2+}$ transport activity. A, the activities of the expressed SERCA1a mutants were determined as
described under “Experimental Procedures” and shown as the values relative to the respective wild-type activities (ATP hydrolysis, 47.8 ± 1.8 n mole P/min/mg
microsomal protein ($n = 5$); oxalate-dependent Ca$^{2+}$ accumulation in the wild type and mutant G105 are shown in the inset. B, the ratio, Ca$^{2+}$ transport activity per Ca$^{2+}$-ATPase activity (Ca$^{2+}$/ATP) is
shown as the percentage of the wild-type ratio. The wild type (light gray), G105A and G-substitution mutant (open), G-shift substitution mutant (closed), and
GG-substitution mutant (lattice) are shown as indicated. C and D, M2 is viewed from a direction parallel to the membrane plane (C) or from the position
indicated by the red arrow in C (D) in the crystal structure as indicated (E1Ca$_2$:AlF$_4^{-}$:ADP). The effect of G-shift substitution of residues on Ca$^{2+}$/ATP in B
is visualized with $\alpha$-carbon coloring as follows. Green, wild type (Gly$^{105}$)-like coupled transport; red, ~36% of wild type (severely uncoupled). Error bars, S.D.
transport sites and then binds two Ca\(^{2+}\) ions. The E2 \(\rightarrow\) E1 transition rate can be assessed by measuring the rate of E1PCa\(_2\) formation from the Ca\(^{2+}\)-deprived E2 state following the addition of Ca\(^{2+}\) plus ATP. The assay takes advantage of the fact that subsequent steps (Ca\(^{2+}\) binding, ATP binding, and phosphorylation) are relatively fast (21). In Fig. 6A, the rates of E1PCa\(_2\) formation from the Ca\(^{2+}\)-deprived E2 state and from the Ca\(^{2+}\)-bound activated E1Ca\(_2\) state were determined, and the ratio of the two rates is shown in Fig. 6B.

The rate of E1PCa\(_2\) formation from the E1Ca\(_2\) state was hardly affected by the mutations, but that from the E2 state was mostly increased or reduced, depending on the mutations, as compared with the wild type. In the alanine substitution G105A, the E2 \(\rightarrow\) E1 transition was faster, and the ratio increased 3-fold (Fig. 6B). In contrast, in the proline substitution G105P as well as V104P and V106P, the transition was increased 3-fold (Fig. 6).

The G-substitution of Ile97 and Leu98 on M2m and of Gln108, Ala112, and Ala115 on M2c markedly reduced the E2 \(\rightarrow\) E1 transition rate and ratio (Fig. 6, C and D, asterisks). The G-shift substitutions of these residues, G105A/L98G, G105A/Q108G, G105A/A112G, and G105A/A115G, restored the wild-type rate and ratio by reducing the increased rate and ratio of the G105A mutant (except for G105A/I97G) (Fig. 6, C and D, green). These residues are again on one side of the M2 helical wheel facing M4C/M6, suggesting the importance of a directional motion of M2 and consequent formation of a more rigid integral helix structure in the E2 \(\rightarrow\) E1 structural transition.

Ca\(^{2+}\) Oclusion in E1PCa\(_2\)—For coupling Ca\(^{2+}\) transport with ATP hydrolysis, the Ca\(^{2+}\) occlusion in E1PCa\(_2\) and subsequent deocclusion with luminal gate opening during E1PCa\(_2\) \(\rightarrow\) E2PCa\(_2\) \(\rightarrow\) E2P \(+\) 2Ca\(^{2+}\) are key processes. To examine a possible cause of the uncoupling of mutants, V104P, G105A, V106G, V106P, W107G, and the G-shift mutants having the G105A substitution (except for the coupled G105A/E112G) (cf. Fig. 3), the Ca\(^{2+}\) occlusion in E1PCa\(_2\) was assessed in Fig. 7 under the conditions in Fig. 2, in which all of the mutants as well as the wild type accumulate mostly E1P at steady state. In the experiments, E1P was formed by ATP with the Ca\(^{2+}\)-activated enzyme in the presence of \(^{45}\)Ca\(^{2+}\), and then unbound and unoccluded \(^{45}\)Ca\(^{2+}\) were washed out with membrane filtration. By this method, two Ca\(^{2+}\) ions were determined to be occluded in E1PCa\(_2\) in wild type.

In the mutants G105A, G105P, V104P, and V106P, the Ca\(^{2+}\) occlusion in E1P was largely reduced and thus defective, showing that the Ca\(^{2+}\) in E1PCa\(_2\) was not occluded. Because Ca\(^{2+}\) that escaped or transported into the lumen would be trapped by oxalate present in the transport assay system, the observed uncoupling in these mutants (Fig. 2) indicates that Ca\(^{2+}\) escape is not toward the lumen but to the cytoplasmic side. All of the G- or GG-substitution mutants occluded two Ca\(^{2+}\) ions in E1PCa\(_2\) as in the wild type, in agreement with their coupled
Ca\(^{2+}\) transport, but an exception was found with the mutants V106G and W107G, which showed uncoupling despite Ca\(^{2+}\) occlusion in E1P\(\text{Ca}^{2+}\) (for more analysis, see below).

The G-shift substitutions in the Leu\(^{96}\)-Ile\(^{103}\) M2m region (Fig. 7A, closed bars) did not restore the E1P Ca\(^{2+}\) occlusion that was disrupted by the G105A substitution, in agreement with their uncoupling. Surprisingly, G-shift substitutions in the Leu96-Ile103 M2m region (i.e. G105A/Q108G, G105A/R110G, G105A/A112G, and G105A/A115G) fully restored Ca\(^{2+}\) occlusion in E1P, despite their uncoupling (except for the coupled G105A/A112G mutant). In these uncoupled G-shift mutants and the above noted uncoupled mutants V106G and W107G, the occluded Ca\(^{2+}\) in E1P\(\text{Ca}^{2+}\) may possibly escape to the cytoplasmic side during the E\(\text{P}\) isomerization (i.e. E1P\(\text{Ca}^{2+}\) → E2P\(\text{Ca}^{2+}\) → E2P).

Therefore, in Fig. 8, we examined Ca\(^{2+}\) occlusion in E2P\(\text{Ca}^{2+}\) for these mutants. Ca\(^{2+}\) Occlusion in E2P\(\text{Ca}^{2+}\)—The transient E2P\(\text{Ca}^{2+}\) state with occluded Ca\(^{2+}\) before Ca\(^{2+}\) release can be trapped by elongation of the A-domain/M1’ linker (A/M1’-linker, Glu\(^{50}\)→Ser\(^{49}\)) by a four-glycine insertion between Gly\(^{46}\) and Lys\(^{47}\) (4Gi-46/47 in Fig. 8) (23, 24). We introduced additional mutations on M2 to this A/M1’-linker elongation mutant (+4Gi-46/47). All of these +4Gi-46/47 mutants as well as the control 4Gi-46/47 mutant accumulate almost exclusively E2P in the steady state, in contrast to the exclusive E1P accumulation in the wild type (Fig. 8A). Actually, this E2P species is very stable, and its decay was nearly completely blocked in all of the +4Gi-46/47 mutants as in the control 4Gi-46/47 mutant (Fig. 8B).

Approximately two Ca\(^{2+}\) ions were occluded in the wild type and control 4Gi-46/47 mutant as E1P\(\text{Ca}^{2+}\) and E2P\(\text{Ca}^{2+}\), respectively, as found previously (23, 24). In the A/M1’-linker elongated V106G and W107G mutants and all of the G-shift mutants except for G105A/A112G + 4Gi-46/47, Ca\(^{2+}\) occlusion in E2P\(\text{Ca}^{2+}\) was markedly reduced and thus defective, in contrast to their Ca\(^{2+}\) occlusion in E1P\(\text{Ca}^{2+}\). This finding agrees with the view that in these mutants, the occluded Ca\(^{2+}\) in E1P\(\text{Ca}^{2+}\) escapes to the cytoplasmic side during \(\text{E}\)\(\text{P}\) isomerization E1P\(\text{Ca}^{2+}\) → E2P\(\text{Ca}^{2+}\), causing uncoupling.

Notably, the A/M1’-linker elongated G105A mutant (G105A + 4Gi-46/47) was not able to occlude Ca\(^{2+}\) in E2P\(\text{Ca}^{2+}\), but the G-shift mutant G105A/A112G + 4Gi-46/47 occluded two Ca\(^{2+}\) ions. Thus, the single G105A mutation prevents Ca\(^{2+}\) occlusion both in E1P\(\text{Ca}^{2+}\) and trapped E2P\(\text{Ca}^{2+}\) states, and the G-shift mutation G105A/A112G restores occlusion in both of these states, in agreement with the finding that the G-shift mutation G105A/A112G restored coupled Ca\(^{2+}\) transport (Figs. 3, 7, and 8).

Structural State of Trapped E2P\(\text{Ca}^{2+}\) State Revealed by Proteolytic Analysis—In the EP isomerization and Ca\(^{2+}\) release E1P\(\text{Ca}^{2+}\) → E2P\(\text{Ca}^{2+}\) → E\(\text{P}\) + 2Ca\(^{2+}\), the A domain largely rotates and docks on the P domain (E1P\(\text{Ca}^{2+}\) → E2P\(\text{Ca}^{2+}\)) causing loss of ADP sensitivity, and then the associated A and P domains are pulled and inclined by the A/M1’-linker, thereby
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causing deocclusion and release of bound Ca\(^{2+}\) into the lumen (E2PCa\(_2\) → E2P + 2Ca\(^{2+}\)) (23, 24). These structural changes can be monitored by changes in the availability of specific cleavage sites for trypsin and proteinase K (prtK) (5, 7, 23, 35).

In the two top panels (trypsin) of Fig. 9, trypsin proteolysis was performed, and the ATPase chain and its fragments were probed with a monoclonal antibody that recognizes Ala\(^{199}\)–Arg\(^{505}\) (the tryptic fragment A1) of SERCA1a. In the wild type and all of the mutants, the T1 site (Arg\(^{505}\)) on the outermost loop of the N domain is very rapidly cleaved to produce the fragment A (Met\(^{1}\)–Arg\(^{505}\) probed by the antibody) and the fragment B (Ala\(^{506}\) to the C terminus Gly\(^{994}\)). T2 site Arg\(^{198}\) on the outermost loop (Asp\(^{196}\)–Val\(^{200}\)) is included in the fragment A1 (probed) and A2 (Met\(^{1}\)–Arg\(^{198}\), not probed). By contrast, in E2PCa\(_2\), exclusively accumulated with the elongated A/M1\(^{-}\)-linker control mutant (4Gi-46/47) and all of the +4Gi-46/47 mutants on M2, the A1 fragment band is very faint and extremely slow to make its appearance. Thus, the control mutant 4Gi-46/47 and all of the +4Gi-46/47 mutants possess the characteristic property of the ADP-insensitive E1P (E2PCa\(_2\) as well as E2P); namely, the A domain has largely rotated from its position in E1PCa\(_2\) and associated with the P domain at the Val\(^{200}\) loop, including Arg\(^{198}\) thereby causing the loss of the ADP sensitivity and blocking sterically the tryptic attack (5, 7, 23, 35).

In the two bottom panels (prtK) of Fig. 9, the same set of experiments was performed with prtK. As demonstrated previously (23, 24), in the E2PCa\(_2\) state trapped by the A/M1\(^{-}\)-linker elongation, prtK cleavage occurs at Leu\(^{119}\) on M2, producing the fragment p95 (see the control mutant 4Gi-46/47), in contrast to its nearly complete resistance in E1PCa\(_2\) (see the wild type E1PCa\(_2\)) as well as in the Ca\(^{2+}\)-released E2P, as demonstrated previously (23).

In all of the +4Gi-46/47 mutants on M2 with the elongated A/M1\(^{-}\)-linker, the accumulated E2P species is cleaved at Leu\(^{119}\), producing the p95 fragment, as in the control elongation mutant 4Gi-46/47 (i.e. there is no indication of a large change by these substitutions on M2 in the overall structure of the
trapped $E_{2PCa_2}$ species). Thus, all of the $+4Gi-46/47$ mutants accumulate $E_{2PCa_2}$ with its characteristic structure found in the control elongation mutant ($4Gi-46/47$). The defect of $Ca^{2+}$ occlusion in $E_{2PCa_2}$ caused by the mutations on M2 is not due to a large structural effect on the $E_{2P}$ species but can be ascribed to some specific effects on the cytoplasmic $Ca^{2+}$ gate, Glu$^{309}$.

**Discussion**

Gly$^{105}$ on M2 Functions as a Flexible Joint—Glycine is a typical helix breaker and as part of an $\alpha$-helix gives conformational freedom to the structure, such that bending and loosening, with or without unwinding and elongation, become possible. A typical example exists in the $Ca^{2+}$-ATPase at Gly$^{770}$ on M5 at the transport sites, where there is a pivoting point for a tilting transition, whereas forcing helix disruption by Gly$^{105}$P, V$^{104}$P, and V$^{106}$P and increased flexibility by G-substitutions of residues on M1 (particularly Leu$^{65}$) has been loosened benefiting for straightening of M2 to gain the $Ca^{2+}$-coordinating residue Glu$^{309}$ and normally fixed by residues on M1 (particularly Leu$^{65}$) has been loosened to allow $Ca^{2+}$ to escape to the cytoplasmic side. Interestingly, the defect is corrected by a second mutation two turns up on the M2 helix at Ala$^{112}$. Thus, pump G105A/A112G has wild type-like activity and coupling.

A second significant finding is that the $E_2 \rightarrow E_1$ structural transition is accelerated by mutation G105A, in contrast to the marked retardation of the $E_P$ isomerization, and both wild-type rates are recovered by the G-shift mutation at Ala$^{112}$. The inhibition of ATPase activity can be ascribed to the retardation of either $E_P$ isomerization (e.g. G105A and G105P) or $E_2 \rightarrow E_1$ transition (e.g. G105P, but not G105A) or both. We will argue through analysis of our mutations in this region and the crystal structures that Gly$^{105}$ is actually a hinge point, where conformational flexibility in a particular direction (a knee-like bending, possibly with elongation) is paramount for occlusion but where excessive flexing is largely inhibitory and less freedom beneficial for straightening of M2 to gain the $E_1$ state.

$E_2 \rightarrow E_1$ Transition—Examining the $E_2 \rightarrow E_1$ transition first, where mutation G105A facilitates a rapid $E_2 \rightarrow E_1$ structural transition, whereas forcing helix disruption by G105P, V104P, and V106P and increased flexibility by G-substitutions of resi-
dues located on the same side of M2 as Gly\textsubscript{105} facing M4C/M6 are inhibitory, and where these same G-shift substitutions restore the wild type rate from the accelerated one in G105A (Fig. 6), it is apparent that the intermediate properties of the single wild-type glycine control the structure here such that the helix is neither too bent and flexible nor too rigid.

In the crystal structure E\textsubscript{2}(TG), the M2 helix is unwound at Asn\textsubscript{111}–Ala\textsubscript{115} and in the transition to E\textsubscript{1}Mg\textsubscript{2}/H\textsubscript{11001} moves toward M4C/M6 and straightens, a rigidity stabilized by interactions of M2c/M2m with M4C/M6 (cf. Fig. 1). The marked retardation of E\textsubscript{2} transition by the GG-substitutions at the Gln\textsubscript{108}–Ala\textsubscript{115} region on M2c is consistent with these changes of M2.

How then is Gly\textsubscript{105} influencing this step; does the residue bear on the rewinding higher up or perhaps on the interactions with M4 and M6? We speculate that M2 is actually bent at Gly\textsubscript{105} in the E\textsubscript{2} structure of wild type (not seen in the crystal structure of E2(TG) but possible in the physiological “flexible” E\textsubscript{2} assisted by unoccupied Ca\textsuperscript{2+} transport sites and no interaction with M4/M6), which would fit with the unwinding higher up being due to a pulling strain imposed by A domain movements, and that mutation G105A helps reverse this tug and facilitate helix straightening, thus favoring the rapid E\textsubscript{2} \rightarrow E\textsubscript{1} transition.

The marked reduction of the E\textsubscript{2} \rightarrow E\textsubscript{1} transition rate by G-substitutions L98G, Q108G, A112G, and A115G facing M4C/M6 (Fig. 6, B and C) is in contrast to the acceleration of E\textsubscript{2}P hydrolysis by these substitutions (Fig. 5; see below) but fits very well with the reverse structural change that occurs during the hydrolysis; namely, M2 unwinds at M2c, bends, and moves away from M4C/M6, the opposite, as we have discussed, of what occurs during E\textsubscript{2} \rightarrow E\textsubscript{1}.

**EP Isomerization E\textsubscript{1}Ca\textsubscript{2} \rightarrow E\textsubscript{2}Ca\textsubscript{2}—Phosphorylation of E1Ca\textsubscript{2} is hardly affected by any of the mutations. In contrast, the next step, the EP isomerization, is markedly retarded by substitutions G105A and G105P, an inhibition reversed by G-shift substitutions of residues facing M4C (Fig. 5). The G- or GG-substitutions of all other residues as well as V104P and V106P, all possessing Gly\textsubscript{105}, actually accelerate the isomerization. Thus, flexibility of M2 in one direction (and possibly a
loosening and elongation; see schematic model in Fig. 10) is crucial for rapid EP isomerization, which involves a large A-domain rotation swinging away from the P domain and docking on the P domain that inclines toward the A domain, and consequent rearrangement of the M2top/M2c interaction with the M4C/P domains and strain imposed on M2 in E2PCa2 state (as indicated by the prtK cleavage at Leu119 on M2top) (23, 24).

Ca2+ occlusion in E1PCa2—We determined that the uncoupling of Ca2+ transport from ATP hydrolysis in mutants G105A, G105P, V104P, and V106P is due to defective Ca2+ occlusion in E1PCa2, and evidently flexibility without distortion in M2 around the glycine is needed to prevent this in E1PCa2. The Ca2+ occlusion is restored in G-shift substitution of the residues facing M4C and some facing other sides (Fig. 7), indicating the importance of bending and loosening (elongation). To occlude Ca2+ at the transport sites, the side-chain configuration of the Ca2+ ligand and cytoplasmic gate Glu309 needs to be fixed by residues on M1 (particularly Leu65 (36)), which forms the rigid V-shaped structure with M2m upon the kinking of M1 during the E1PCa2 formation (see

FIGURE 9. Structural analysis of E2PCa2 state by limited proteolysis. Microsomes expressing wild type or the mutants shown in Fig. 8 were phosphorylated at 25 °C for 10 s in 6 μl of a mixture containing 0.12 mg/ml microsomal protein, 0.5 mM ATP, 0.1 M KCl, 7 mM MgCl2, 5 mM CaCl2, 1 μM A23187, and 50 mM MOPS/Tris (pH 7.0), and then 0.72 mg/ml trypsin (top panels) or prtK (bottom panels) was added in a small volume and incubated for the indicated time periods. In the wild type, EP accumulated was exclusively E1PCa2, and its decay was extremely slowed during the proteolysis periods due to the feedback inhibition by the high concentration of Ca2+. In the mutant, EP accumulated was exclusively E2P, and its decay was extremely slow (see Fig. 8, A and B). The proteolysis was terminated by 2.5% (v/v) trichloroacetic acid, and the digests were subjected to Laemmli SDS-PAGE. The ATPase chain and its fragments separated on the gel were blotted onto a polyvinylidene difluoride membrane and visualized by immunodetection with a monoclonal antibody that recognizes the Ala199–Arg505 peptide (tryptic fragment A1) of SERCA1a, as described under “Experimental Procedures.” The tryptic fragments were as follows; A, Met1–Arg505, A1, Ala199–Arg505. The fragments formed by prtK were p95 (Lys120–Gly994), p81 (Met1–Met733), and p83 (Glu243–Gly994) (48, 49). The positions of the Ca2+–ATPase chain and its fragments and those of the molecular mass markers are indicated on the left and right, respectively.
Figure 10. Schematic for structural change in EP processing (A) with M2-M4C contact in an E2P model (B) and sequence alignment around M2 for P-type ATPases (C). A, proposed model of Gly105 functions as a flexible joint of M2c-M2m segments for tilting, bending, and loosening of M2 helix during EP formation E1Ca2 → E1P Ca2−, EP isomerization E1P Ca2− → E2P Ca2−, and subsequent Ca2+ release E2P Ca2− → E2P + 2Ca2+. The A and P domain and selected transmembrane helices are shown; the N domain, M3, and M7–M10 are not depicted for simplicity. The positions of Gly105, Ala112, and Leu119 are indicated. Open red arrows, movements of M2c and M2m. The yellow arrow on E1Ca2 and E1P Ca2− and the red arrow on E2P Ca2− indicate the motions (tilting and rotation) of the A domain and the tilting of associated A-P domains, respectively, for the subsequent step. In E1Ca2 → E1P Ca2−, the A domain slightly tilts due to the P domain’s conformational change upon the Mg2+ ligand and phosphorylation, thereby pulling up M1 and M2 (broken purple arrows), and the M1/M2m is produced and fixes the Glu309 cytoplasmic gate; thus, the Ca2+ occlusion is accomplished. In E1P Ca2− → E2P Ca2−, the A domain largely rotates and docks on the P domain; thereby, M2 connected with the A domain is pulled and moved, M2c is detached from the cytoplasmic part of M4 (M4C) and strained, and the top part of M2c (M2top) is unwound (as found with Leu119 exposed to prtK in E2P Ca2− (see Fig. 9i)). In the latter, E2P Ca2− → E2P + 2Ca2+, the associated A-P domains are inclined due to the strain imposed on the A/M1 ′-linker in E2P Ca2− (23); thereby, the M2m/M1 rigid V-shaped body pushes M4L to open the lumenal path (gate) to release Ca2+ (9). B, as a consequence, in E2P (depicted with its model E2P-BeF3− (PDB 22BE (11)), M2 straightens a steric collision of the Gly105 region with M4C that inclines toward Gly105-M2c by the P domain inclination, and the structure is stabilized by interactions in the Tyr112–hydrophobic cluster (Leu119–Tyr122 on M2c/M2top with the A and P domains), the M1′–M2c (Val116–Arg119) interaction, and the M2m/M1 V-shaped body (M2m). Gly105 (or Gly112 of G-shift G105A/A112G mutant) with its conformational freedom is critical for rapid processing of these large motions while keeping the cytoplasmic gate closed.

C, the protein sequence of rabbit SERCA1a Ca2+–ATPase (UniProt P04191) is aligned using ClustalW version 2.1 with pig H11001, pig H11002, human flippase ATP8A2 (UniProt Q9NTI2). Letter colors denote fully conserved (green) or highly conserved residues (blue). The color bars above the sequence alignment indicate the M2m, M2c, and M2top regions of Ca2+–ATPase. The glycine residue at the M2m-M2c connecting region is shown in red.
Glycine 105 on Second Transmembrane Helix of Ca\textsuperscript{2+}-ATPase

(23, 24), and the M1/M2m rigid V-shaped body leans and pushes M4L to open the luminal gate while keeping the cytoplasmic gate closed, as seen in the E2-BeF\textsubscript{3} crystal structure (Fig. 8, D and E). M2 straightens significantly from the structure seen in E1Ca\textsubscript{2}·AlF\textsubscript{3}·ADP (E1PCa\textsubscript{4}), and flexibility at the glycine would obviously be needed for this (see Figs. 8 (D and E) and 10). The straightening may be due to a steric collision of the Gly\textsuperscript{105} region with M4C that inclines toward Gly\textsuperscript{105}·M2c by the P domain inclination (Fig. 10). The E2p ground state structure with luminally open gate, under the influence of M2, is stabilized by the interaction networks at the Tyr\textsuperscript{122}-hydrophobic cluster (formed with Leu\textsuperscript{119}/Tyr\textsuperscript{122} on M2top and hydrophobic residues on the A and P domains (28, 37, 38)), at M1’-M2c (Val\textsuperscript{106}–Arg\textsuperscript{110}) interaction, and at the M1/M2m V-shaped body.

**E2p Hydrolysis**—Finally, during the subsequent E2p hydrolysis process from the ground state to the transient state E2p → E2→P\textsuperscript{3}, the luminal gate becomes tightly closed (7), due to the unwinding at M2top and overall downward movement of M2 upon the slight (25°) rotation of the A domain by the water attack (according to the crystal structural model (11)). The Gly\textsuperscript{105}A substitution does not affect the E2p hydrolysis kinetics with coupled luminal gate closure; thus, it appears that there is no bending movement, at least around the glycine, during the hydrolysis reaction. It fits with the need for most of the helix to remain straight for the downward movement to close the luminal gate (see Fig. 5b as a model for E2→P\textsuperscript{3}).

An interesting question is why the unwinding of M2 helix (due to distortion imposed on M2 upon the A domain motion) occurs in the Asn\textsuperscript{111}–Ala\textsuperscript{115} region and not at Gly\textsuperscript{105}, as seen in the crystal structural change E2·BeF\textsubscript{3} → E2·AlF\textsubscript{3} (TG) (Figs. 5 and 8). It needs to be borne in mind that unwinding and elongation at any position in the Ile\textsuperscript{103}–Ala\textsuperscript{115} region forced by a five-successive glycine insertion exhibits very rapid E2p hydrolysis with coupled tight luminal gate closure (21). The unwinding in the wild type seen in the E2→P\textsuperscript{3} model probably occurs because 1) the Gly\textsuperscript{105} region is fixed by the interaction with M1’, and 2) the M2top region (Leu\textsuperscript{119}/Tyr\textsuperscript{122}) is fixed by the Tyr\textsuperscript{122}-hydrophobic cluster with the A and P domain, whereas 3) the Asn\textsuperscript{111}–Ala\textsuperscript{115} region is detached from the P domain upon the A domain’s tilting motion in E2p → E2→P\textsuperscript{3} (E2·BeF\textsubscript{3} → E2·AlF\textsubscript{3}) and therefore unsupported and susceptible to unwinding. Retardation of hydrolysis by the G-shift substitution at Val\textsuperscript{104} and Val\textsuperscript{106} (Fig. 5) may possibly be due to a disruption of the interaction with M1’, resulting in M2 bending in a direction unfavorable to proper A domain motion relative to the P domain on water attack of the phosphoryl group.

**Gly\textsuperscript{105} Conservation**—Gly\textsuperscript{105} of SERCA1a on the M2c-M2m connecting region is conserved in the P-type ATPase family; the position of the glycine is shifted three residues earlier in the sequence in H\textsuperscript{+}·K\textsuperscript{+}·ATPase, Na\textsuperscript{+}·K\textsuperscript{+}·ATPase, and flippase (Fig. 10C) and presumably serves the same function. A knee-like bending movement of M2 may be needed in these P-type ATPases.

**Experimental Procedures**

**Mutagenesis and Expression**—The pMT2 expression vector (39) carrying rabbit SERCA1a cDNA with a desired mutation was constructed as described previously (23). Transfection of pMT2 DNA into COS-1 cells and preparation of microsomes from the cells were performed as described (40).

**Ca\textsuperscript{2+}-ATPase Activity and Ca\textsuperscript{2+} Transport Activity**—Activities of expressed SERCA1a were obtained essentially as described previously (41). The rate of ATP hydrolysis was determined at 25 °C in a mixture containing 1–5 μg of microsomal protein, 0.1 mM [γ\textsuperscript{32}P]ATP, 0.1 mM KCl, 7 mM MgCl\textsubscript{2}, 10 μM CaCl\textsubscript{2}, 5 mM potassium oxalate, and 50 mM MOPS/Tris (pH 7.0). The Ca\textsuperscript{2+}-ATPase activity of expressed SERCA1a was obtained by subtracting the ATPase activity determined in the presence of 1 μM thapsigargin (TG), a highly specific and subnanomolar affinity SERCA inhibitor (42) with conditions otherwise as above. The rate of Ca\textsuperscript{2+} transport was determined with [γ\textsuperscript{32}P]ATP and nonradioactive ATP and otherwise as above. The Ca\textsuperscript{2+} transport activity of expressed SERCA1a was obtained by subtracting the activity determined in the presence of 1 μM TG, with conditions otherwise as above.

**Formation and Hydrolysis of EP**—Phosphorylation of SERCA1a in microsomes with [γ\textsuperscript{32}P]ATP or [γ\textsuperscript{32}P]ADP, and dephosphorylation of [γ\textsuperscript{32}P]-labeled SERCA1a were performed under conditions described in the figure legends. The reaction was quenched with ice-cold trichloroacetic acid containing Pi. Precipitated proteins were separated by 5% SDS-PAGE at pH 6.0 according to Weber and Osborn (43). The radioactivity associated with the separated Ca\textsuperscript{2+}-ATPase was quantified by digital autoradiography as described (44). The amount of EP in expressed SERCA1a was obtained by subtracting the background radioactivity determined in the presence of 1 μM TG, with conditions otherwise as above. We confirmed that 1 μM TG reduces the EP value in the wild type and all mutants to a background radioactivity level (i.e., <1% of the maximum EP level, which is actually the same as that obtained in the absence of Ca\textsuperscript{2+} without TG).

**Ca\textsuperscript{2+} Occlusion in EP**—Microsomes were phosphorylated for 1 min at 0 °C in a mixture containing 1–5 μg of microsomal protein, 10 μM ATP, 0.1 mM KCl, 7 mM MgCl\textsubscript{2}, 10 μM 45CaCl\textsubscript{2}, 1 μM A23187, and 50 mM MOPS/Tris (pH 7.0), and immediately filtered through a 0.45-μm nitrocellulose membrane filter (Milipore). The filter was washed extensively with a washing solution (1 mM EGTA, 0.1 mM KCl, 7 mM MgCl\textsubscript{2}, and 20 mM MOPS/Tris (pH 7.0)), and 45Ca\textsuperscript{2+} remaining on the filter was quantified as described (23). The amount of Ca\textsuperscript{2+} occluded at the transport sites of EP in the expressed SERCA1a was obtained by subtracting the amount of nonspecific Ca\textsuperscript{2+} binding determined in the presence of 1 μM TG, with conditions otherwise as above. The amount of EP formed was determined with nonradioactive Ca\textsuperscript{2+} and [γ\textsuperscript{32}P]ATP under otherwise the same conditions as above by membrane filtration, and the radioactivity remaining on the filter was quantified.

**Limited Proteolysis and Western Blotting Analysis**—Ca\textsuperscript{2+}-ATPase was phosphorylated and subjected to structural analysis by limited proteolysis with trypsin and prtK as described in the legend to Fig. 9. The digests were separated by 10.5 or 7.5% SDS-PAGE according to Laemmli (45) and blotted onto a polyvinylidene fluoride membrane and then incubated with IIH11 monoclonal antibody to rabbit SERCA1a (Affinity Bioreagents), which recognizes an epitope between Ala\textsuperscript{199} and...
Arg505. After incubation with secondary antibody (goat anti-mouse IgG, horseradish peroxidase-conjugated), the signal was detected with Pierce Western blotting substrate (Thermo Fisher Scientific).

Miscellaneous—Protein concentration was determined by the method of Lowry et al. (46) 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 (47). The data represent the mean ± S.D. for 3–6 independent experiments (or 10–20 experiments in Figs. 7 and 8C).

Author Contributions—T. D. conceived, designed, performed, and analyzed the experiments. T. D. and H. S. coordinated the study and wrote the paper. K. Y. and S. D. provided critical discussion and technical advice. All authors reviewed the results and approved the final version of the manuscript.

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