The Role of the M6-M7 Loop (L67) in Stabilization of the Phosphorylation and Ca^{2+} Binding Domains of the Sarcoplasmic Reticulum Ca^{2+}-ATPase (SERCA)*

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The amino acid sequence (L67) intervening between the M6 and M7 transmembrane segments of the Ca^{2+} transport ATPase was subjected to mutational analysis. Mutation of Pro^{826} to Ala interferes with protein expression even though transcription occurs at normal levels. Single mutations of Lys^{819} or Arg^{822} to Ala, Phe, or Glu allow good expression, but produce strong inhibition of ATPase activity. The main defect produced by these mutations is strong interference with enzyme phosphorylation by ATP in the presence of Ca^{2+}, and also by P_i in the absence of Ca^{2+}. The Lys^{819} and Arg^{822} mutants undergo slight and moderate reduction of Ca^{2+} binding affinity, respectively. Reduction of overall steady state ATPase velocity is then due to inhibition of phosphorylated intermediate formation. On the other hand, a cluster of conservative mutations of Asp^{813}, Asp^{815}, and Asp^{818} to Asn interferes strongly with enzyme activation by Ca^{2+} binding and formation of phosphorylated enzyme intermediate by utilization of ATP. Enzyme phosphorylation by P_i in the absence of Ca^{2+} undergoes slight or no inhibition by the triple aspartate mutation. Therefore, the triple mutation interferes mainly with the calcium-dependent activation of the ATPase. The effect of the triple mutation can be to a large extent reproduced by single mutation of Asp^{815} (but not of Asp^{813} or Asp^{818}) to Asn. Functional and structural analysis of the experimental data demonstrates that the L67 loop plays an important role in protein folding and function. This role is sustained by linking the cytosolic catalytic domain and the transmembrane Ca^{2+} binding domain through a network of hydrogen bonds.

The sarcoplasmic reticulum (SR) ATPase is a membrane-bound enzyme that plays a crucial role in sequestration of cytosolic Ca^{2+} in muscle fibers (1–3). The catalytic and trans-
of mutant ATPase (as indicated by Western blot analysis) at 3 °C. The washed three times with 1.0 ml of cold 0.125MPCA and once with cold clinical centrifuge at 5000 rpm for 5 min, and the sediments were protein, and placed in ice. The samples were then spun in a refrigerated Ca2+

ice. The reaction was started by the addition of ATP, run for 10 s at 37 °C following the initial addition of radioactive ATP. Several samples measuring Pi production (18). The reaction mixture contained 20 mM MesTris, pH 6.2, 10 mM MgCl2, 20.0% Me2SO, and 2.0 mM EGTA.

Reverse enzyme phosphorylation by Pi was obtained by adding 30 µg of microsomal protein to 0.2 mM reaction mixture containing 50.0 mM MesTris, pH 6.2, 10 mM MgCl2, 20.0% Me2SO, and 2.0 mM EGTA. Alternatively, the EGTA was omitted and 10, 50, or 100 µM CaCl2 was added. Following a 10-min incubation at 25 °C, 1.0 ml of 1.0 mM ice-cold PCA was added, the samples were transferred into a 1.7-ml Eppendorf tube containing 100 µg of bovine serum albumin as a carrier protein, and placed in ice. Centrifugation, washing, electrophoresis, and detection of phosphoenzyme were then performed as described above for enzyme phosphorylation with ATP.

Measurements of Ca2+ binding to recombinant ATPase in the absence of ATP were performed in microsomes obtained from COS-1 cells infected with adenovirus vectors (as opposed to simple transfections), taking advantage of the higher concentration of recombinant ATPase in virus-transduced samples. The measurements were performed exactly as described by Zhang et al. (12), in the presence of 3 µM free Ca2+, as determined by an EGTA-Ca buffer. The measured Ca2+ binding levels were adjusted to compensate for variations of recombinant ATPase expression in various preparations, with reference to a wild type preparation as indicated by Western blots. The difference between samples incubated in the absence and in the presence of thapsigargin was considered to be specific Ca2+ binding.

Computations—Calculation of free Ca2+ in various reaction mixtures were based on the concentrations of total calcium and EGTA as originally described by Fabiato and Fabiato (19). Simulations of steady state kinetics, yielding overall ATPase velocity and levels of intermediate species, were based on user entered rate constants and concentrations of substrate, ligands, and products, as described by Inesi et al. (20). Computations were performed on a PC microcomputer, using a 14-digit precision MEGABASIC with BCD coding (American Planning Corp, Alexandria, VA).

Copies of the computational programs can be obtained from M. Kurzmacz (La Porte, CO).

RESULTS

Expression of Mutants—L67 includes the Gly 808–Gly832 segment of the ATPase sequence, in which we produced several site directed mutations. (Table I). As a comparison, we also mutated residues in the small M8/M9 loop (Ser917–Glu920). Expression of all mutants in COS-1 cells was similar to, or somewhat (10—30%) lower than, that of WT ATPase. An exception was the Pro820 → Ala mutant, whose protein recovery was

| Sample | ATPase (µmoles/mg/min) | E-P (µmoles/mg/min) | E-P (µmoles/mg/min) |
|--------|------------------------|---------------------|---------------------|
| WT     | 100                    | 100                 | 100                 |
| Pro811 → Ala | 98 ± 5.1              | 96 ± 6.3            | 94 ± 15             |
| Pro812 → Ala | 100 ± 1.2             | 76 ± 2.9            | 76 ± 16             |
| Lys819 → Ala | 97 ± 7.0             | 95 ± 2.2            | 72 ± 14             |
| Asp815 → Asn   | 95 ± 3.2             | 95 ± 1.0            | 100 ± 13            |
| Asp816 → Ala   | 95 ± 5.8             | 86 ± 6.9            | 96 ± 15             |
| Asn/Asp816 → Asn| 37 ± 5.7             | 23 ± 3.9            | 96 ± 18             |
| Lys819 → Ala   | 39 ± 0.7             | 16 ± 3.8            | 35 ± 6              |
| Lys819 → Glu   | 44 ± 2.1             | 26 ± 3.7            | 33 ± 4              |
| Pro820 → Ala   | No expression         |                     |                     |
negligible, even though its mRNA level was comparable to that produced by WT cDNA (Fig. 1). It is of interest that Menguy et al. (11) obtained expression of a totally inactive Pro^{820} → Ala mutant. This suggests that mutation of Pro^{820} interferes profoundly with protein folding, resulting in degradation of product by the COS-1 cells used for expression in our experiments, and production of inactive protein by the yeast cells used by Menguy et al. (11).

Functional Characterization of Aspartate Mutants—Falson et al. (10) and Menguy et al. (11) reported that cluster mutations of Asp^{813}, Asp^{815}, and Asp^{818} to Ala reduce the affinity of the ATPase for Ca^{2+}, an effect that was confirmed by direct measurements of Ca^{2+} binding by equilibrium experimentation (12). We now find that even conservative mutation of the Asp^{813}, Asp^{815}, and Asp^{818} cluster to Asn produces strong inhibition of ATPase steady state velocity, and maximal activity cannot be reached even by raising the Ca^{2+} concentration to the 0.1 mM level (Fig. 2A). The effect of the triple mutant can be to a large extent reproduced (data not shown) by single mutation of Asp^{813} (but not Asp^{815} or Asp^{818}) to Asn.

The triple aspartate mutation strongly reduces the steady state levels of phosphorylated intermediate formed with ATP in the presence of Ca^{2+} (Fig. 3 and Table I). However, it affects much less prominently enzyme phosphorylation by Pi in the absence of Ca^{2+} (Fig. 4). In agreement with previous findings (12), direct measurements with radioactive tracer show that the cluster mutation of Asp^{813}, Asp^{815}, and Asp^{818} to Asn displays very low levels of high affinity Ca^{2+} binding in the presence of 3 μM Ca^{2+} (Table II). Direct measurements of Ca^{2+} binding cannot be performed at higher Ca^{2+} concentrations due to unfavorable signal to noise ratio. Nevertheless, we found that phosphorylation of the cluster mutant with Pi is only moderately inhibited by 10–100 μM Ca^{2+} (Fig. 5), indicating that Ca^{2+} binding occurs at lower levels than in the WT enzyme.

Time resolution of enzyme phosphorylation with ATP at low temperature show again significant inhibition, especially when the reaction is started by addition of ATP and Ca^{2+} to enzyme deprived of Ca^{2+}, as compared with addition of ATP to enzyme already activated by Ca^{2+} (Fig. 6). On the other hand, pulse-chase experiments indicate that decay of (already formed) phosphoenzyme is not significant delayed by the triple mutation (Fig. 7).

Single mutations of Asp^{815} to Asn or Ala do not produce significant ATPase inhibition, although slight displacements of the Ca^{2+} concentration dependence of ATPase activation (Fig. 2) and of the inhibitory effect of Ca^{2+} on the P_i reaction are apparent in Figs. 2 and 5, respectively. Ca^{2+} binding in the absence of ATP (3 μM free Ca^{2+}) is not significantly reduced by single Asp^{815} mutations (Table II). Mutation of Leu^{814} to Ala does not produce significantly effects.

Functional Characterization of Lysine and Arginine Mutants—Single mutations of Lys^{819} to Ala or Glu, and of Arg^{822} to Phe, Glu, or Ala produce very strong inhibition of the steady state ATPase velocity even at relatively high Ca^{2+} concentrations (Fig. 2B). WT enzyme and mutants reach their own maximal activity within the same ATP concentration range (data not shown), indicating that the steady state ATPase depend-
ence on the ATP concentration is not significantly changed by the Lys and Arg mutations. Direct measurements of Ca\(^{2+}\) binding in the absence of ATP show slight and moderate reduction in the Lys\(^{810}\) and Arg\(^{822}\) mutants, respectively (Table II). This finding is in agreement with the interference with the inhibitory effect of Ca\(^{2+}\) on the P\(_i\) reaction (Fig. 5).

A marked effect of the Lys\(^{819}\) and Arg\(^{822}\) mutations is strong reduction of the steady state levels of phosphorylated intermediate formed by utilization of ATP. This reduction is only slightly compensated for by raising the Ca\(^{2+}\) concentration in the medium (Fig. 3). Low levels of phosphoenzyme are already apparent in the initial phase of its formation upon simultaneous addition of ATP and Ca\(^{2+}\) to enzyme pre-incubated with EGTA, or even when ATP alone is added to enzyme preincubated with Ca\(^{8+}\). In the former case the phosphoenzyme level rises slowly due to slow activation by Ca\(^{2+}\) (Fig. 6A), while in the latter case the steady state level of phosphoenzyme is reached faster since the enzyme is already activated by Ca\(^{2+}\) (Fig. 6B). It is clear that, in either case, net formation of phosphoenzyme is much lower in the mutants than in the WT samples.

We also conducted pulse-chase experiments to test whether hydrolytic cleavage of the phosphorylated intermediate (once formed) is influenced by the Lys\(^{819}\) and Arg\(^{822}\) mutations (Fig. 7). We found that the time course of phosphoenzyme cleavage is not significantly affected by the Lys and Arg mutations (Fig. 7).

It should be pointed out that the experiments on phosphoenzyme formation and cleavage were conducted at low temperature and non-saturating ATP concentrations in order to obtain kinetic resolution. The relevance of these findings to the steady state behavior of the enzyme at 25°C is considered under “Discussion.”

It is of interest that the equilibrium levels of phosphoenzyme formed with P\(_i\) in the absence of Ca\(^{2+}\) and ATP, are also very much reduced (Fig. 4). These experiments were conducted at 25°C and, consistent with the ATP experiments conducted at low temperature, indicate that Lys\(^{819}\) and Arg\(^{822}\) play an important role in determining the functional integrity of the phosphorylation domain. It is then noteworthy that the phosphorylation defect resulting from the Lys\(^{819}\) and Arg\(^{822}\) mutations can be observed in the presence and in the absence of Ca\(^{2+}\), and at low and high temperature.

**Functional Characterization of Proline Mutants**—We found no inhibition of ATPase activity in the Pro\(^{811}\) → Ala and Pro\(^{812}\) → Ala mutants, and moderate inhibition in the Pro\(^{821}\) → Ala, Pro\(^{824}\) → Ala, and Pro\(^{827}\) → Ala mutants (Fig. 2). As noted above, however, the Pro\(^{820}\) mutation resulted in negligible protein recovery, despite normal mRNA levels. All tested proline mutants exhibited a Ca\(^{2+}\) concentration dependence nearly identical to that of the WT ATPase, independent of whether the ATPase velocity was reduced or not (Fig. 2). The proline mutants yielded phosphoenzyme levels similar to those obtained with WT ATPase, by utilization of either ATP in the presence of Ca\(^{2+}\) (Fig.
3) or P, in the absence of Ca\(^{2+}\) (Fig. 4). In agreement with previous reports (11), we found that the proline mutations did not interfere significantly with Ca\(^{2+}\) inhibition of enzyme phosphorylation with P\(_i\) (Fig. 5).

**Functional Characterization of Mutants in the L89—** A set of single mutations were produced in the loop intervening between the M8 and M9 transmembrane segments (L89), to obtain a comparative evaluation with the effects obtained...
with mutations in L67. We found that the Ser$^{917}$ → Ala and Gln$^{920}$ → Ala mutants sustained ATPase activity at rates equal to the WT enzyme, while the Glu$^{918}$ → Ala and Asn$^{919}$ → Ala exhibited slightly lower rates. The Ca$^{2+}$ concentration dependence of the M8/M9 loop mutants was nearly identical to that of the WT ATPase (Fig. 2).

**DISCUSSION**

Functional Analysis—Our experiments demonstrate that single or cluster mutations within L67 interfere profoundly with the ATPase function. It is remarkable that L67 can affect the molecular events occurring at both phosphorylation site (Asp$^{351}$) and Ca$^{2+}$ binding sites, which are separated by a more than 50-Å distance. L67 plays a crucial role even in protein folding. In fact, mutation of Pro$^{820}$ (unique among six L67 prolines) interferes with the appearance of significant levels of expressed protein. By comparison, mutations within L89 produce little or no interference. We want to clarify here how the twenty five residues of the L67 loop can interfere with such various aspects of the enzyme structure and function.

Particularly interesting are single mutations of Arg$^{822}$ and Lys$^{819}$. Mutations of these residues inhibit strongly ATPase activity, due to interference with formation of phosphorylated enzyme intermediate both by utilization of ATP in the presence of Ca$^{2+}$, and of P$_i$ in the absence of Ca$^{2+}$. It is remarkable that L67 can affect Ca$^{2+}$ dependent enzyme phosphorylation by ATP. Inhibitors of steady state velocity is observed even at saturating ATP. Yet another type of functional defect is produced by mutations of L67 aspartate residues, as originally pointed out by Falson et al. (10) and Menguy et al. (11) who mutated these residues to Ala. We find that removal of negative charge by conservative mutations of aspartate residues interferes with high affinity Ca$^{2+}$ binding and Ca$^{2+}$ dependent enzyme phosphorylation by ATP. Inhibition of steady state velocity is observed even at saturating Ca$^{2+}$. No significant inhibition of enzyme phosphorylation with P$_i$ in the absence of Ca$^{2+}$ is produced by the aspartate mutations.

Considering the heterogeneity of effects produced by mutations within the L67 loop, we sought to verify by kinetic analysis whether the observed functional behavior of various mutants could be accounted quantitatively for by perturbations of the phosphorylation reaction, or rather by interference with Ca$^{2+}$ binding and enzyme activation. For this purpose we utilized a complete reaction sequence (Fig. 8) to simulate the steady state ATPase behavior. The rate constants listed in Fig. 8, obtained at 25 °C as explained previously (20), generate a simulated pattern of ATPase Ca$^{2+}$ activation that is identical to that obtained experimentally with the WT enzyme (Figs. 2 and 9). Any of the rate constants can then be changed to explore the effects of specific perturbations of partial reactions on the overall steady state behavior of the ATPase. A change of the forward rate constant for any particular reaction must be accompanied by a proportional change of the reverse rate constant of the same or of another appropriate reaction so that the overall equilibrium constant (corresponding of that ATP hydrolysis to ADP and P$_i$) remains unchanged.

To simulate the triple mutant (Asp cluster) behavior we considered that the mechanism of Ca$^{2+}$ binding and ATPase activation includes three steps: fast binding of the first Ca$^{2+}$, followed by a relatively slow isomeric transition, and cooperative binding of the second Ca$^{2+}$ (21). Enzyme activation occurs only after binding of the second Ca$^{2+}$, when involvement of
transmembrane helices M4, M5, M6 and M8 in Ca\(^{2+}\) complexation is complete (12). We then found in our simulation that a simple inhibition of the enzyme affinity for Ca\(^{2+}\) through reduction of fast steps (reactions 1 forward and 8 reverse in Fig. 8) yields a displacement of the Ca\(^{2+}\) curve, with full activation at higher Ca\(^{2+}\) (Fig. 9A, squares). This type of curve was not observed experimentally (Fig. 2A). Combined inhibition of the slow Ca\(^{2+}\) induced transition and of enzyme phosphorylation with ATP (reactions 2 and 5 in Fig. 8) is required to generate a pattern (Fig. 9A, diamonds) matching precisely the pattern observed experimentally with the cluster Asp\(^{813}\), Asp\(^{815}\), and Asp\(^{818}\) to Asn mutant (Fig. 2A, diamonds). Thus the simulation suggests that the aspartate negative charges are not involved in direct coordination of Ca\(^{2+}\), but play an important role in maintaining the structural integrity of the L67 loop, thereby permitting Ca\(^{2+}\) binding and transmission of the activation signal to the phosphorylation domain.

Simulating the behavior of the Lys and Arg mutant is more straightforward, and is obtained just by lowering the rate constants of ATP phosphorylation by ATP and Pi (reactions 5 and 6 in Fig. 8). As in the case of the aspartate cluster, any manipulation of rate constants was balanced in the forward and reverse directions so as to maintain an identical overall equilibrium constant for the entire reaction cycle.

Additional simulations can be performed by the interested reader by changing the values of the rate constants in Fig. 8, and evaluating the behavior of ATPase with the various mutants.
forward and 11 reverse in Fig. 8). The simulations reproduce satisfactorily the curves observed experimentally, including the low steady state velocity and an apparent saturation by Ca\(^{2+}\) at lower concentrations than observed with the WT enzyme (Compare Figs. 2B and 2B). This indicates that the observed reduction of overall steady state ATPase velocity at 25 °C can be attributed to mutational perturbation of the phosphorylation domain and consequent inhibition of phosphoenzyme formation (Fig. 6).

Structural Considerations—L67 is an extended loop, consisting of ~25 residues. Its pathway seems to be determined by a hydrogen bond network with other protein segments; that is, with the L89 loop near its N-terminal end, with helices in the P domain and M5 in the middle part, and with M3 and M5 near its C-terminal end. Two pairs of Pro may also play an important part in determining the L67 path, by restricting the main chain torsion angles (Fig. 10).

The effect of L67 aspartate mutations on Ca\(^{2+}\) binding are likely to be indirect, since their distance from the transmembrane domain (8) precludes their direct participation in the binding of the two activating calcium ions. As illustrated in Fig. 10, Asp\(^{813}\), Asp\(^{815}\), and Asp\(^{818}\) are involved in a hydrogen bonding network with Arg\(^{755}\) and Asp\(^{755}\) (cytosolic extension of M5), Ser\(^{917}\) and Glu\(^{920}\) (L69), and Ala\(^{617}\) (P2). Our experiments suggest that involvement of Asp\(^{813}\) in these bonds is most important. Since aspartate mutations impair Ca\(^{2+}\) binding, the hydrogen bonding network is likely to maintain M6 (with its three Ca\(^{2+}\) binding residues) and neighboring segments in optimal position for Ca\(^{2+}\) binding and Ca\(^{2+}\) dependent enzyme activation.

It is of interest that the L67 apex sustains a crucial role in protein folding, as the Pro\(^{820}\) → Ala mutant fails to yield significant levels of expressed protein even though transcription occurs normally. Mutation of the neighboring Arg\(^{755}\) (in the cytosolic extension of the long M5 transmembrane helix) also interferes with expression and functional competence of recombinant ATPase (22). It is shown in Fig. 10 that Pro\(^{820}\) is very close to Arg\(^{755}\), and hydrogen-bonded at the carbonyl oxygen atom of the preceding residue (Lys\(^{819}\)). Thus this hydrogen bond appears critical for proper folding of the protein and Pro\(^{820}\) is likely to be important for the correct positioning of this carbonyl group (presumably by restricting the main chain torsion angle).

Mutations of Lys\(^{819}\) and Arg\(^{822}\), also within the L67 apex, produce strong functional inhibition and specific interference with formation of the phosphorylated enzyme intermediate. Lys\(^{819}\) and Arg\(^{822}\) are in close proximity and hydrogen-bonded to residues of the P2 (Asp\(^{16}\)) and P1 (Glu\(^{346}\)) helices of the phosphorylation domain (Fig. 10). Mutation of Ser\(^{346}\), which is located in the loop connecting β1 and P1, and is hydrogen-bonded to Glu\(^{856}\) (Fig. 10), also produces catalytic interference (23). It is likely that, in the process of enzyme activation, M4 and M5 undergo large conformational changes (8), and M6 is repositioned by engagement of three of its residues (Asn\(^{796}\), Thr\(^{799}\) and Asp\(^{800}\)) in Ca\(^{2+}\) complexation (12). Thereby, the L67 loop and the P1 and P2 helices are affected as well. This would in turn affect the neighboring β-strand (strand 1 in Fig. 10) that includes the residue undergoing phosphorylation (Asp\(^{351}\)). This strand is connected to transmembrane segment M4, which is also involved in Ca\(^{2+}\) complexation through the intervention of Glu\(^{859}\). The entire region appears to be stabilized by the M5 helix that extends from the lumenal surface of the membrane up to the end of the P domain (8). Thus, precise conservation of structural interactions in this region is required for competence of the phosphorylation and the Ca\(^{2+}\) binding domains, and their long range functional linkage.

Acknowledgment—Manuscript and figures were edited by Lisa Schuertz.

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J. Biol. Chem. 2001, 276:15232-15239.
doi: 10.1074/jbc.M010813200 originally published online February 5, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M010813200

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