Tryptophan 1093 is Largely Responsible for the Slow Off Rate of Calmodulin from Plasma Membrane Ca\(^{2+}\) Pump 4b*

Tryptophan 1093 resides in the 28-residue calmodulin-binding/autoinhibitory domain of the plasma membrane Ca\(^{2+}\) pump (PMCA). Previous studies with the isolated calmodulin-binding/autoinhibitory peptide from PMCA have shown that mutations of the tryptophan residue decrease the affinity of the peptide for calmodulin and its affinity as a result of a proteolytically activated pump. In this study, the PMCA mutation in which tryptophan 1093 is converted to alanine (W1093A) was constructed in the full-length PMCA isoform 4b. The mutant pump was expressed in COS cells, and its steady state and pre-steady state kinetic properties were examined. The W1093A pump exhibited an increased basal activity in the absence of calmodulin, so the activation was 2-fold (it is 10-fold in the wild type). The W1093A mutation also lowered the steady state affinity for calmodulin from $K_{on}$ of 9 nM for wild type to 144 nM (assayed at 700 nM free Ca\(^{2+}\)). Pre-steady state measurements of the rate of activation by Ca\(^{2+}\)-calmodulin revealed that the W1093A mutant responded 2.5-fold faster to calmodulin. In contrast to these relatively modest effects, the halftime of inactivation of the mutant was reduced by more than 2 orders of magnitude from 41 min to 7 s. We conclude that tryptophan 1093 does not play a substantial role in Ca\(^{2+}\)-calmodulin recognition; rather it functions primarily to slow the inactivation of the calmodulin-activated pump.

The plasma membrane Ca\(^{2+}\) ATPase or pump (ATP2B1–4, referred to here as PMCA) and the Na\(^+\)/Ca\(^{2+}\) exchanger are the main mechanisms for removing Ca\(^{2+}\) from the cell. Ca\(^{2+}\) removal is required to maintain a steady Ca\(^{2+}\) disequilibrium of $10^{5}$ between the cytoplasm and the extracellular milieu and to create the Ca\(^{2+}\) spikes, waves, and oscillations that are the most ubiquitous signaling systems in the cell. Regulation of PMCA occurs via allosteric activation by Ca\(^{2+}\)-calmodulin. Before activation, PMCA is inhibited by an intramolecular interaction between the inhibitory domain at the C terminus and the catalytic core of the molecule. The inhibitory domain contains the calmodulin-binding region of the molecule. A rise in intracellular Ca\(^{2+}\) results in 4 Ca\(^{2+}\) binding to calmodulin; the Ca\(^{2+}\)-calmodulin complex then binds to the inhibitory domain, activating the pump. Activation results in an increase in the affinity of the pump for Ca\(^{2+}\) at its transport site as well as an increase in its maximal turnover.

PMCA in mammals is encoded by four genes with additional diversity provided by alternate splicing at two or more sites (for a recent review of PMCA genes, splicing variants, and nomenclature, see Strehler and Zacharias (1)). PMCA isoforms differ among themselves in three ways: 1) basal activities in the absence of calmodulin, 2) rates of activation by Ca\(^{2+}\)-calmodulin, and 3) rates of inactivation by calmodulin dissociation. These differences are caused by differences in the interactions between the inhibitory domain, calmodulin, and the catalytic core. The present study investigates the effect of changing one residue in the inhibitory domain upon these three properties.

These differences in PMCA responses are integrated into cellular physiology. A cell that utilizes PMCA to respond rapidly to a Ca\(^{2+}\) spike requires an isoform that has a high basal activity, such as isoform 3f in heart muscle (2), and/or one that is activated rapidly by calmodulin, such as isoform 2a in inner ear hair cells. In contrast, a cell that requires a sustained Ca\(^{2+}\) signal may utilize isoforms with low basal activity and slow activation by Ca\(^{2+}\)-calmodulin, such as isoform 4b in Jurkat T lymphocytes (3). In addition to their response to a single Ca\(^{2+}\) spike, PMCA isoforms also have a potentially wide range of responses to a sequence of spikes. An isoform such as 4b, with low basal activity, relatively slow calmodulin activation, but much slower inactivation will allow the first spike to develop for several seconds before becoming activated but may remain activated for several minutes after the spike is dissipated, thus responding much faster to the next Ca\(^{2+}\) spike. We have termed this the "memory effect" of the pump (4), and it is thought to be a major driving force for the large number of PMCA isoforms since it is a response that cannot be regulated simply by differences in activity or expression levels.

Since much of the information about the role of the inhibitory region of PMCA has been obtained through the use of synthetic peptides (5, 6), a discussion of the data is necessary as background for the present study. The synthetic peptides have been referred to by names such as C28 with C representing the calmodulin-binding domain and the numerals indicating the length of the peptide beginning with leucine 1086 (Fig. 1). C28 is the full-length peptide containing all of the residues necessary for high affinity calmodulin binding (7). Peptide C28 inhibited the calmodulin-activated form of the pump by competing with the enzyme for calmodulin. The calculated dissociation constant for calmodulin was 0.1 nM (5), which was comparable to the constant obtained by direct binding experiments with danyal-calmodulin.

In other sets of experiments the C28 peptide was added to a...
Trp in PMCA Gives Slow Dissociation of Calmodulin

Construction of W1093A—Overlapping PCR was used to create a cassette with a silent SacII site at the nucleotide positions partially encoding phenylalanine 1094 to glycine 1096 in the center of the 28-residue calmodulin binding motif of hPMCA4b. The front PCR product was amplified with the primer pair 5′-TGACAACATAACACACGCC-3′/GGGAGAACCGCGGATGTCGGCTCCGGCGG-G. The back PCR product was amplified with the primer pair 5′-CCCGGGCCCTGGAAC-3′/CTCAGAAGGGTACCTAACTGATGCCTTAG3′. The silent SacII sites are underlined, and the residues to change the trypthofan codon are alanine shown with bold letters. The template for the PCRs was full-length h4b in pMM2 (pMM2 was originally called pMT2-m (13)). The front PCR product contained a unique endogenous XbaI site near the 5′-end, while a unique KpnI site was added to the 3′-end of the back PCR product immediately after the stop codon. Two PCR products were separately cloned into the pCR Blunt II TOPO vector (Invitrogen) and ligated at the SacII site. The W1093A-encoding insert was excised from pCR Blunt II TOPO with XbaI and KpnI, and ligated to the vector fragment of an XbaI/KpnI-digested full-length h4b in pMM2. This produced a construct ending immediately after the 28-residue calmodulin binding motif. This construct was named ct92 W1093A (C-terminal mutant truncated of residue 92).

Heterologous Expression of PMCA in COS Cells—COS cells were transfected with the constructs using LipofectAMINE as described previously (14).

Isolation of Microsomes—Crude microsomal membranes from COS cells were prepared as described previously (15).

Ca2+ Transport Activity—Ca2+ transport was measured by the incorporation of 45Ca2+ into inside-out vesicles prepared from COS cells that transiently expressed PMCA. The conditions of the assay were 25 mM tris(hydroxymethyl)aminomethane, pH 7.2, 7 mM MgCl2, 40 mM KH2PO4/K2HPO4, 10 mM EGTA, 2.5 mM Na2ATP, 160 mM KCl, 5 mM MgCl2, 160 mM CaCl2, 0.2 mM EGTA, and 1 mg/ml aprotinin, 1 mg/ml leupeptin, and 0.5% Triton X-100. Where V0 is the activity in the absence of calmodulin, Vmax in the activity in the presence of saturating calmodulin, and v is the activity at each plotted calmodulin concentration.

Stimulation of PMCA for ATPase Assays—COS cell membrane preparations were solubilized and reconstituted as described previously (16). Briefly, COS cell membrane preparations containing 100–200 μg of protein were pelleted by centrifugation in a microcentrifuge tube and resuspended in 80 μl of solubilization buffer containing 60 mM TES triethanolamine, pH 7.2, 240 mM KCl, 10 mM MgCl2, 400 μM EGTA, 10 mM Na2ATP, 2.5 mM Na2ATP, 1 wash diethiothreitol, 1 mM ouabain, 8 μg/ml oligomycin, 400 nM thapsigargin, 4 μg/ml aprotinin, 1 μg/ml leupeptin, and 0.5% Triton X-100 at 4 °C. After 4 min, 320 μl of dilution buffer (same as solubilization buffer except 0.5 mg/ml phosphatidylcholine in place of Triton X-100) was added to the tube, and then 200 μg of Bio-Beads SM-2 were added. The tube was placed on a gel rocker for 1 h at 4 °C, the bulk of the Bio-Beads was removed by centrifugation, and the few residual beads were removed by filtration with a 0.45-μm spin filter.
nucleoside phosphorylase at 1 unit/ml. In the presence of purine nucleoside phosphorylase, MESG is phosphorylated by inorganic phosphate to produce ribose-1-phosphate and 2-amino-6-mercapto-7-methylpurine. The time course of phosphorylation of MESG was monitored on a Beckman DU 70 spectrophotometer as an increase in absorbance at 360 nm at 37 °C.

Inactivation by Calmodulin Removal—Experiments to measure the rate of inactivation upon calmodulin removal were conducted in the same assay medium as for calmodulin activation. The enzyme was allowed to reach steady state at 0.5 μM free Ca^{2+} and 235 μM calmodulin, and then 10 μM calmodulin-binding peptide RRKWQKTGHAVRAIGRLSS from chicken smooth muscle myosin light chain kinase (17) was added to the reaction.

RESULTS

Steady State Transport Assays—W1093A was constructed in PMCA isoform h4b and transiently expressed in COS cells. COS cell membrane preparations (inside-out vesicles) were assayed for ATP-dependent incorporation of ^{45}Ca^{2+}. Fig. 2 shows that the full-length W1093A mutant has about 50% basal activity (in the absence of calmodulin), while the wild type has 15%. The W1093A mutant was also constructed in a truncated isoform 4b lacking the C-terminal 92 residues (ct92). ct92 ends with the last residue of the 28-residue calmodulin-binding domain and therefore lacks some downstream inhibitory determinants (18). Because of this, wild-type ct92 is also shown (7) (Fig. 2). The combination of truncation and W1093A showed a significant decrease in Ca^{2+} transport at 21 μM free Ca^{2+} plus or minus 544 μM calmodulin. The grey bars indicate the activity without calmodulin, and the black bars indicate the activity with calmodulin. Data are plotted relative to the mean activity with added calmodulin. Triplicate assays were done, and the S.E. is shown by the error bars.

Steady State Dependence of Ca^{2+} Transport on Calmodulin—Since it was demonstrated that the W1093A mutant was indeed activated by calmodulin, kinetic experiments were performed to examine the steady state affinity for Ca^{2+}-calmodulin. These curves were performed at 0.7 μM free Ca^{2+}. Fig. 3 shows that the calmodulin affinity of W1093A was much lower than the wild type. The K_{d,5} for calmodulin was 144 nM for W1093A compared with 9 nM for the wild type shown in Fig. 2 exist at all Ca^{2+} levels.

Steady State Ca^{2+} Transport Curves—The curves in Fig. 4 were performed with either 0 or 2 μM added calmodulin. Increasing Ca^{2+} results in more Ca^{2+} at the transport site and more of the activator Ca^{2+}-calmodulin complex. W1093A showed a significant decrease in Ca^{2+} affinity relative to wild type in the presence of 2 μM total calmodulin. The K_{d,5} for Ca^{2+} was 0.35 ± 0.03 μM for the W1093A mutant compared with 0.19 ± 0.01 μM for the wild-type h4b. Since the activating species of PMCA is the Ca^{2+}-calmodulin complex, the change in calmodulin affinity of the W1093A mutant was also reflected in a change in K_{d,5} for Ca^{2+} at constant calmodulin. These curves show that the differences in basal rates between the mutant and the wild type shown in Fig. 2 exist at all Ca^{2+} levels.

Pre-steady State Activation Measurements—Previous work
were allowed to reach steady state in 500 nM Ca\(^{2+}\)/H\(_{11002}\) than 4 Ca\(^{2+}\)

ative events initiated by the binding of calmodulin with less Ca\(^{2+}\). The activation rate constant (k\(_{\text{act}}\)) was 0.02443 ± 0.00008 s\(^{-1}\) for h4b and 0.057 ± 0.018 s\(^{-1}\) for W1093A. These values for k\(_{\text{act}}\) at 235 nM calmodulin correspond to half-times of 29 and 12 s, respectively. The data shown are representative of at least three experiments, and the constants are given as mean ± S.E. Coefficients of determination (r\(^2\)) for the fitted curves were all greater than 0.9998. The dotted straight lines in the figures are lines fitted to the maximum rate achieved at long times and are shown to allow a visual estimate of the curvature.

has shown that the wild-type h4b has a slow activation rate (half-time of about 1 min) and an extremely slow inactivation rate (half-time of more than 20 min) (16). For comparison, we did similar studies on the W1093A mutant. To perform the pre-steady state activation experiments (Fig. 5), wild-type or W1093A pump was allowed to reach steady state at 500 nM Ca\(^{2+}\) and then 235 nM calmodulin was added, and PMCA was allowed to proceed to a new, activated steady state. The activation rate constant (k\(_{\text{act}}\)) was then obtained from the change in slope that occurred upon addition of calmodulin using the equation in the legend to Fig. 5. Wild-type h4b was activated by 235 nM calmodulin with a k\(_{\text{act}}\) for the fitted curve of 0.0245 s\(^{-1}\), while the k obtained for W1093A was larger at 0.0574 s\(^{-1}\). The values of k yield k\(_{\text{act}}\) of 1.043 × 10\(^5\) M\(^{-1}\) s\(^{-1}\) for h4b and 2.443 × 10\(^5\) M\(^{-1}\) s\(^{-1}\) for W1093A. We used the total concentration of calmodulin (235 nM) for the calculation of k\(_{\text{act}}\). The actual concentration of the activator Ca\(^{2+}\)-calmodulin would be dependent on the macroscopic constants for Ca\(^{2+}\) binding to calmodulin (19) as well as cooperative events initiated by the binding of calmodulin with less than 4 Ca\(^{2+}\).

Pre-steady State Inactivation Measurements—The next experiment to examine the kinetic properties associated with tryptophan 1093 was to measure the rate of enzyme inactiva-

tion by calmodulin removal. Since the steady state affinity of W1093A for calmodulin was lower than that of the wild-type h4b and the rate of activation by calmodulin was faster, it could be assumed that the rate of inactivation would be much faster for W1093A than wild-type h4b. Inactivation experiments were performed by allowing the enzyme to reach steady state at 500 nM Ca\(^{2+}\) and 235 nM calmodulin, and then 10 \(\mu\)M competing calmodulin-binding peptide from myosin light chain kinase was added to the reaction. To compare the time scale of the inactivation of the mutant with that of the wild type, the data for W1093A is included in an inset in the upper panel. Comparison of the inset with the 4b data makes it clear that the inactivation of the mutant is very much faster, too fast to be visible on the collapsed time scale. Data were fitted to Equation 7 from Caride et al. (16): Y(t) = Y\(_0\) - \(v_0\) \(t\)/k\(_{\text{act}}\) + (\(v_0\) + \(v_1\))/k\(_{\text{act}}\) \(\exp(-k_{\text{inact}}t)\), where Y\(_0\) is the absorbance at 360 nm at time 0, \(v_0\) is the steady state slope in the absence of calmodulin, and \(v_1\) is the steady state slope in the presence of 235 nM calmodulin. The apparent rate constant for inactivation (k\(_{\text{inact}}\)) was 0.00028 ± 0.00002 s\(^{-1}\) for h4b and 0.100 ± 0.004 s\(^{-1}\) for W1093A. These values of k\(_{\text{inact}}\) correspond to half-times of 2483 and 7 s, respectively. The data shown are representative of at least three experiments, and the constants are given as mean ± S.E. Coefficients of determination (r\(^2\)) for the fitted curves were all greater than 0.9998. The dotted straight lines in the figures are lines fitted to the minimum rate achieved at long times and are shown to allow a visual estimate of the curvature.
DISCUSSION

From the data for \( k_{\text{act}} \) and \( k_{\text{inact}} \) of ATPase experiments it is possible to calculate dissociation constants for calmodulin and compare them to the apparent \( K_d \) values obtained from steady state \( \text{Ca}^{2+} \) transport. The calculated \( K_d \) for h4b from the pre-steady state experiments (performed at 500 nM \( \text{Ca}^{2+} \)) is ~3 nM. This was somewhat less than the apparent \( K_d \) of 9 nM obtained for the steady state experiments (performed at 700 nM \( \text{Ca}^{2+} \)). The calculated \( K_d \) for W1093A is ~407 nM compared with an apparent \( K_d \) of 144 nM. The agreement of these numbers is quite good considering that phosphatidylycholine membranes were used for the ATPase measurement, and native COS cell membrane vesicles were used for the \( \text{Ca}^{2+} \) transport assays. We consider the \( K_d \) values obtained from the pre-steady state data to be more accurate because PMCA is enriched about 10-fold by the reconstitution, and the \( \text{Ca}^{2+} \) concentration is better controlled with the buffer system used for the pre-steady state assays.

The data presented here show that the W1093A mutant differs from the wild type in three ways. The first is that the mutant has a less effective interaction of the calmodulin-binding domain with the catalytic core so that it retained only half of the normal autoinhibitory properties. The second is that the rate of activation by calmodulin is faster for the mutant, and the third is that the mutant exhibits a much faster dissociation of the \( \text{Ca}^{2+} \)-calmodulin complex.

The basal activity in the absence of calmodulin is controlled by the interaction of the calmodulin-binding domain with the catalytic core. The degree of autoinhibition is also affected by the downstream regulatory region (7). In the absence of calmodulin, an equilibrium exists between closed and open conformations (in the closed conformation, the calmodulin-binding domain interacts with the catalytic core, while in the open conformation it does not). The catalytic core and calmodulin may be viewed as competing for the open conformation of the calmodulin-binding domain. Changing tryptophan 1093 to alanine increased the basal activity of the pump (in the absence of calmodulin); in the wild type the basal activity was 15% of the maximal calmodulin-stimulated activity, while in the mutant it was 50%. This indicates that the mutant pump was shifted more toward the open conformation, and thus the calmodulin-binding domain was more accessible to calmodulin. As was mentioned in the discussion of Fig. 2 (see “Results”), the tryptophan side chain was responsible for ~50% of the autoinhibitory properties of the calmodulin-binding domain. This confirms what had been seen with the peptide studies that show that tryptophan 1093 has a vital role in the autoinhibitory interaction as well as being an important anchor for calmodulin binding.

The displacement of PMCA toward the open state by the mutation should also make the calmodulin-binding domain of the mutant more accessible to \( \text{Ca}^{2+} \)-calmodulin. This accessibility may be reflected in an acceleration in the rate of activation by \( \text{Ca}^{2+} \)-calmodulin. Such an effect was observed for the W1093A mutation, which increased the rate of activation ~2.5-fold. The effect of opening the pump is probably adequate to account entirely for the activation rate increase without needing to invoke any effect of tryptophan 1093 in calmodulin recognition.

In contrast with the small effect of the mutant on activation, the mutant greatly accelerates the inactivation (350-fold). This large effect indicates that tryptophan 1093 has a major role in slowing dissociation of \( \text{Ca}^{2+} \)-calmodulin from the pump.

The small role of tryptophan 1093 in the initial interaction with calmodulin is rather surprising because of the dominant role of this tryptophan in stabilizing the fully formed complex with calmodulin. The NMR solution structure showed the tryptophan interacting with nine residues of calmodulin compared with only three calmodulin residues interacting with the next most important residues in the calmodulin-binding domain of the pump (9). These nine interactions evidently contribute very strongly to the stabilization of the pump-calmodulin complex and hinder its dissociation. However, the initial binding of calmodulin must involve recognition of a number of important features among which the tryptophan is not a major contributor.
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