Oxidative Inactivation of Purified Plasma Membrane Ca^{2+}-ATPase by Hydrogen Peroxide and Protection by Calmodulin†

Asma Zaidi,‡,§ Lorena Barŕon,|| Victor S. Sharov,||,§ Christian Schöneich,||,§ Elias K. Michaelis,‡,§ and Mary L. Michaelis*,‡,§

Department of Pharmacology and Toxicology, Higuchi Biosciences Center, and Department of Pharmaceutical Chemistry, University of Kansas, Lawrence, Kansas 66045

Received April 9, 2003; Revised Manuscript Received July 22, 2003

ABSTRACT: Calmodulin (CaM)-regulated plasma membrane Ca^{2+}-ATPase (PMCA) is critical for the regulation of free intracellular Ca^{2+} levels. PMCA activity and levels in neuronal membranes are decreased with aging, possibly due to oxidation-induced inactivation. In the present studies, inhibition of PMCA by H_2O_2 was characterized in enzyme purified from human erythrocyte membranes. Basal and CaM-stimulated PMCA activities were inhibited by exposure to H_2O_2 (25−100 μM). However, neither the concentration-dependent enhancement of PMCA activity by CaM nor the binding of CaM to H_2O_2-exposed PMCA was disrupted by treatment with H_2O_2. Rates of inactivation by H_2O_2 of basal and CaM-stimulated PMCA were nearly identical. The addition of CaM after exposure to H_2O_2 did not protect enzyme activity, although the binding of CaM to PMCA before exposure to H_2O_2 protected the enzyme completely, indicating a CaM-induced conformational state resistant to oxidation. H_2O_2 quenched Trp fluorescence in PMCA, an index of conformational changes, with a rate similar to that observed for enzyme inactivation. H_2O_2 enhanced the solvent accessibility of Trp residues in PMCA, whereas accessibility of the only Trp residue in the CaM-binding domain peptide was unaltered. Exposure of PMCA to H_2O_2 led to aggregate formation partially reversible by dithiothreitol (DTT) but not to recovery of activity. Amino acid analysis indicated Cys modification following H_2O_2 exposure but no Cys oxyacids. Because DTT did not reverse inactivation by H_2O_2, it appears that the disulfide bond formation led to conformational changes that were not fully reversed when the bonds were reduced. Preincubation of PMCA with CaM protected the enzyme from undergoing this conformational change.

Production of a reactive oxygen species (ROS)[1] and a reactive nitrogen species such as a superoxide anion, hydrogen peroxide (H_2O_2), and a peroxynitrite is a normal consequence of oxidative cellular metabolism (*1*). However, the level of oxidants becomes significantly elevated in a variety of pathophysiological disorders as diverse as atherosclerosis, post-ischemic tissue injury, Alzheimer's disease, Parkinson's disease, and also in normal biological aging (*2*−*4*). Increases in oxidative stress lead to the posttranslational modification of proteins resulting in their conformational instability, misfolding, and enhanced propensity for aggregation (*5*). Like other posttranslational modifications, the oxidation of amino acids alters the properties of a number of cellular proteins. Identification of such modifications, and characterization of the underlying structural and functional changes in crucial proteins, would be helpful in elucidating mechanisms of protein modification and the cellular pathways disrupted in disease processes involving oxidative stress.

One of the most common perturbations found in cells subjected to oxidative stress is a significant alteration in cellular Ca^{2+} homeostasis (*6*−*8*). The maintenance of tightly regulated free intracellular Ca^{2+} levels is accomplished by the extrusion of cytosolic Ca^{2+} by transporters such as the plasma membrane Ca^{2+}-ATPase (PMCA) and the sodium calcium exchanger (NCX), sequestration by the sarcoplasmic-endoplasmic reticulum Ca^{2+}-ATPase (SERCA), and buffering by high affinity Ca^{2+}-binding proteins such as calmodulin (CaM). Currently, there are limited examples of functionally relevant Ca^{2+}-regulatory proteins in which oxidative modification of specific amino acids and associated functional consequences have been identified. For example, studies on CaM from senescent rat brain showed that methionine residues were oxidized to the corresponding sulfoxides, resulting in reduced stimulation of the CaM-activated Ca^{2+}-ATPase (*9*, *10*). Also, SERCA obtained from the skeletal muscle of aged rats was found to have multiple posttranslational modifications, including oxidized cysteines (Cys) and nitrated tyrosines (Tyr) (*11*−*13*). Structural alterations in

† Supported by NIH Grant PO1AG12993, American Federation for Aging Research, and the American Heart Association.

* Correspondence should be addressed to the following author. Fax: (785) 864-5219. Phone: (785) 864-3905. E-mail: mlm@ku.edu.

‡ Department of Pharmacology and Toxicology.

§ Higuchi Biosciences Center.

|| Department *o*f Pharmaceutical Chemistry.

[1] Abbreviations: BCIP, 5-bromo-4-chloro-3-indolyl phosphate; BSA, bovine serum albumin; CaM, calmodulin; DNPH, dinitrophenylhydrazine; DTT, dithiothreitol; Gdn-HCl, guanidine hydrochloride; H_2O_2, hydrogen peroxide; MSA, methane sulfonic acid; NBT, nitrotetrazolium blue; NCX, sodium−calcium exchanger; PMCA, plasma membrane Ca^{2+}-ATPase; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; SERCA, sarco-endoplasmic reticulum Ca^{2+}-ATPase; SPMs, synaptic plasma membranes.

10.1021/bi034565u CCC: $25.00
Published on Web 09/23/2003

SERCA were accompanied by a marked decrease in its conformational stability, particularly at the nucleotide-binding site. The age-related loss of function of these two critical Ca^{2+}-regulatory proteins is consistent with the observed increase in intracellular Ca^{2+} levels within senescent cells (*5*).

Age-related posttranslational modifications in other Ca^{2+}-regulating proteins, such as PMCA, have not been reported, despite an age-dependent loss of activity in synaptic plasma membranes (SPMs) isolated from brains of aging rats (*14*, *15*). We found that brain membrane PMCA is highly susceptible to in vitro exposure to a variety of ROS (*16*), suggesting that the reduction in PMCA might be due to age-dependent oxidative stress in the brain. Treatment of SPMs with H_2O_2 under relatively mild conditions led to a marked loss of PMCA activity, apparently due to aggregation of PMCA molecules as a result of Cys oxidation to disulfides and increased hydrophobic interactions (*16*). In view of the fact that these studies were conducted in SPMs, it was not clear whether inactivation of PMCA was due to direct effects of the ROS on the protein or to secondary effects resulting from peroxidation of the polyunsaturated fatty acids abundant in SPMs. Thus, in the present study we have purified PMCA from erythrocyte membranes and investigated functional and structural alterations in the isolated protein as a result of exposure to H_2O_2, an oxidant with physiological relevance (*17*, *18*). Our data shed some light on the molecular mechanisms involved in H_2O_2 -induced PMCA inactivation and also demonstrate that CaM protects PMCA against both functional and structural effects of H_2O_2. Biochemical and biophysical evidence indicated that CaM binding to the enzyme induces global conformational changes in PMCA that stabilize the protein in a conformation that makes critical sites inaccessible to H_2O_2. This observation may be of significance in understanding the altered regulation of intracellular Ca^{2+} in an aging brain in which both an oxidation of CaM (*19*) and a decrease in the levels of SPM-associated CaM occurred with increasing age (*15*).

EXPERIMENTAL PROCEDURES

Reagents. $C_{12}E_8$ and bovine brain CaM were obtained from Calbiochem (La Jolla, CA). ThioGlo-1 was from Covalent Associates (Woburn, MA) and CaM-Sepharose from Pharmacia Biotech (Piscataway, NJ). ATP, 5-bromo-4-chloro-3-indolyl phosphate (BCIP), nitrotetrazolium blue (NBT), phosphatidylcholine, H_2O_2, anti-dinitrophenyl (DNP) antibodies, and alkaline phosphatase-conjugated anti-mouse secondary antibody were purchased from Sigma Chemical Company (St. Louis, MO). All other reagents were of the highest purity grade available.

Purification of PMCA. Human erythrocyte ghosts, depleted of CaM, were used to purify PMCA by affinity chromatography using CaM-sepharose as described (*20*). Briefly, the supernatant obtained after solubilization of the membranes with Triton X-100 (1 mg/mg of protein) was stabilized with phosphatidylcholine and Ca^{2+} (final concentrations of 0.5 mg/mL and 100 μM, respectively). The supernatant was applied to a CaM-sepharose column equilibrated with a buffer containing 10 mM Hepes, pH 7.2, 120 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.5 mg/mL phosphatidylcholine, and 0.32% $C_{12}E_8$. Following application of the supernatant, the column was washed with several bed volumes of equilibration buffer lacking Ca^{2+} to remove the nonspecifically bound proteins. The retained PMCA was eluted in a buffer containing 10 mM Hepes, pH 7.2, 120 mM NaCl, 1 mM $MgCl_2$, 0.5 mg/mL phosphatidylcholine, 0.05% $C_{12}E_8$, 2 mM EDTA, and 5% glycerol (buffer A), and protein was monitored by absorbance at 280 nm. Each of the fractions was checked for PMCA activity using the malachite green method to monitor inorganic phosphate (P_i) formation (*21*). Active fractions were pooled, and $MgCl_2$ and $CaCl_2$ were added to neutralize the EDTA and yield a final free concentration of 1.5 mM $MgCl_2$ and 4.8 μM $CaCl_2$. The enzyme was stored at −80 °C with no loss of activity. Protein concentrations were estimated by the Coomassie brilliant blue dye-binding method according to the manufacturer's instructions (Pierce, Rockford, IL).

Measurement of PMCA Activity. The activity of PMCA was determined in 96-well microplates. Each well contained the following in a final volume of 100 μL: 25 mM Tris-HCl, pH 7.4, 50 mM KCl, 1 mM $MgCl_2$, 0.1 mM ouabain, 4 μg/mL oligomycin, 200 μM EGTA, and $CaCl_2$ added to yield a final concentration of 10 μM Ca^{2+}. The final concentration of PMCA in the reaction mixture was 0.022 nmol/mL (0.022 μM). The final free Ca^{2+} concentration was calculated using a modified version of a computer program that calculates the multiple equilibria between all ligands in solution (*22*). PMCA activity measured in the presence of 10 μM Ca^{2+} but in the absence of added CaM is referred to as basal activity and that in the presence of 10 μM Ca^{2+} plus 340 nM CaM as CaM-stimulated activity. After a 5 min preincubation of the control or H_2O_2-treated PMCA with other components of the assay, the reaction was started by the addition of 1 mM ATP, continued for 20 min at 37 °C, and stopped by addition of the malachite green solution (*21*). The contents were acidified by adding 19.5% H_2SO_4, incubated for 45 min, and the color read at 650 nm in a microwell plate reader. The PMCA activity was defined as the Ca^{2+}-activated ATP hydrolysis and expressed as nmol of P_i liberated per mg of protein per min, based on values from a standard curve of the absorbance using various concentrations of free Pi.

Immunoblot Analysis. Isolated PMCA preparations were checked for purity by SDS−PAGE on 7.5% polyacrylamide gels, followed by silver staining (*23*). For immunoblot analysis, the protein was transferred to PVDF membranes and probed with a pan antibody (Affinity Bioreagents, Golden, CO) or isoform-specific antibodies to PMCA 4 (1:1000) (a gift from Dr. Penniston, Mayo Clinic). The formation of carbonyls on PMCA was assessed by reacting the protein with dinitrophenylhydrazine and detecting immunoreactivity with anti-DNP antibodies (1:500). Alkaline phosphatase-conjugated secondary antibodies (1:1000) were used, and color was developed by the BCIP/NBT method (*15*). Immunoblots were scanned, and the densitometric analysis was performed using Adobe Photoshop 5.0.

Exposure of PMCA to H_2O_2. Unless indicated otherwise, aliquots of the purified PMCA (0.22 nmol/mL) were exposed to various concentrations of H_2O_2 for 10 min at 37 °C. The reaction with H_2O_2 was terminated by the addition of catalase (2 units), and the tubes were kept at 23 °C for 5 min to allow the remaining H_2O_2 to decompose. In some experiments, a strong metal chelator, either desferrioxamine (100 μM) or

diethylenetriaminepentaacetic acid (100 μM), was present during exposure to H_2O_2 to ensure chelation of trace metals in the medium and to minimize free radical formation. The H_2O_2-reacted PMCA was added to microwells containing the assay mixture described previously. Data analyses for H_2O_2 inhibition included nonlinear least-squares fitting to either hyperbolic decay or single and biexponential decay functions for concentration- and time-dependent inhibition of enzyme activity, respectively. The goodness of fit of the calculated functions to the experimental data was assessed by statistical comparisons of the sum of squares of the residuals, normality, and constant variance tests (SigmaPlot 2000).

CaM Overlay. Aliquots of control and H_2O_2-exposed PMCA (0.5 μg of protein) were subjected to SDS−PAGE (7.5% acrylamide gels) and transferred to PVDF membranes. The membranes were incubated overnight at 4 °C with biotinylated CaM (0.5 μg/mL in PBS-0.05% Tween20) in the presence of either 0.5 mM $CaCl_2$ or 5 mM EDTA. Following extensive washing with PBS-Tween20 to remove unbound CaM, the membranes were incubated with avidin-conjugated alkaline phosphatase (1:1000), and the color was developed using the BCIP/NBT system.

Assessment of Conformational Changes by Fluorescence Spectroscopy. The intrinsic fluorescence of Trp residues in PMCA was used to detect conformational changes induced in the protein by H_2O_2. Fluorescence intensities were measured in a Fluoro Max-2 fluorometer with the excitation wavelength set at 290 nm and the emission recorded between 300 and 400 nm. Control experiments were carried out to correct for changes in fluorescence emission due to the introduction of buffer A, the buffer in which PMCA was present. The extent to which the Trp residues were accessible to solvents was assessed by monitoring the quenching of Trp fluorescence in the presence of increasing concentrations of acrylamide. In these experiments, a freshly prepared acrylamide stock solution was added in small volumes to a cuvette containing either the control or the H_2O_2-treated PMCA (0.22 nmol/mL). Fluorescence intensities were recorded at 335 nm (λ_{max} of the PMCA emission spectrum) both in the presence and in the absence of acrylamide, and the data were analyzed according to the Stern−Volmer equation: $F_o/F = 1 + \mathrm{Ksv}Q$ (*24*). F_o and F are the fluorescence intensities in the absence and presence of the quencher, acrylamide; Ksv is the Stern−Volmer constant; and Q is the molar concentration of acrylamide. In some experiments, the quenching of the single Trp residue in C28W, a peptide homologous to the CaM-binding domain in PMCA, was measured to explore the effects of H_2O_2 on peptide conformation.

Assessment of Cys Oxidation. Aliquots of purified PMCA were incubated for up to 60 min with 10 μM ThioGlo-1 in 25 mM sodium phosphate buffer, pH 7.4, with either 2% SDS or 2% SDS plus 6 M guanidine hydrochloride (GdnHCl). Fluorescence in the samples was measured either in a Shimadzu RF5000U spectrofluorometer or in a BIO-TEK FL600 microplate fluorescence reader. A calibration curve with increasing concentrations of Cys was used for quantification.

Amino Acid Analysis. Gas-phase acid hydrolysis was performed in 6 N HCl and 1% (w/v) phenol under vacuum at 115 °C for 22 h (*25*). Protein hydrolysates were analyzed by reverse phase HPLC (Varian 9012) on an Inertsil C18 ODS3 column (250 × 4.6 mm), using precolumn derivatization with phenylisothiocyanate (absorbance detection at 254 nm, Varian 9050). Samples were dissolved in mobile phase A (10 mM potassium phosphate buffer, pH 6.5), injected onto a column preequilibrated with mobile phase A at 40 °C, and eluted at 1 mL/min with mobile phase B (75% acetonitrile/25% phase A) using a binary gradient linearly increasing to 11% (v/v) by 15 min, to 14% by 17 min, to 36% by 39 min, and to 45% by 48 min. Amino acids were quantified on the basis of the area under the chromatographic peaks, using amino acid standards. BSA hydrolyzed under the same conditions was analyzed and served as a standard.

Methane sulfonic acid (MSA) hydrolysis of protein samples was used for the determination of labile amino acids, Trp and Met sulfoxide, and for the oxidized forms of Cys (sulfinic and sulfonic acid) that are completely lost under conditions of acid hydrolysis. Samples were hydrolyzed with 4 M MSA at 115 °C for 22 h in an air-evacuated chamber (∼40 mTorr). After hydrolysis, samples were neutralized by the addition of an equal volume of 3.5 M NaOH and analyzed by reverse phase HPLC on a Supelcosil LC-18T column (150 × 4.6 mm), using precolumn *o*-phthaldialdehyde derivatization and fluorescence detection (330 nm excitation and 450 nm emission) as described (*26*). The column was equilibrated at 35 °C with mobile phase A (95% v/v 25 mM sodium acetate buffer, pH 5.8, 5% tetrahydrofuran). The samples were injected onto the column and eluted at 0.7 mL/min using the following gradient program: mobile phase B (95% methanol and 5% tetrahydrofuran) increasing linearly from 0 to 15% (v/v) between 0 and 0.5 min, isocratic elution between 0.5 and 19 min, mobile phase B linearly increasing to 45% between 10 and 20 min, isocratic elution between 20 and 30 min, and mobile phase B increasing linearly to 100% between 30 and 40 min. Quantification of the amino acids was based on the coelution of the respective commercially available standards. BSA hydrolyzed under the same conditions was analyzed to determine the efficiency of MSA hydrolysis.

RESULTS

H_2O_2-Induced Inactivation of PMCA Activity. Exposure of purified erythrocyte membrane PMCA to increasing concentrations of H_2O_2 (25−500 μM) for a limited period of time, 10 min at 37 °C, led to a concentration-dependent inhibition of enzyme activity (Figure 1A). Analysis of the data using a hyperbolic function revealed no significant differences between the fractional inhibition produced by a given concentration of H_2O_2 on the basal PMCA activity and that observed following stimulation of the enzyme by CaM (the latter measured in the presence of saturating concentrations of CaM). The very similar sensitivity of basal and CaM-stimulated PMCA to increasing concentrations of H_2O_2 is shown in the inset of Figure 1A, depicting the data as a percent of control PMCA activity. Addition of either desferrioxamine (100 μM) or diethylenetriaminepentaacetic acid (100 μM) to the incubation buffer at the time of exposure of PMCA to H_2O_2 had no effect on the peroxide-mediated PMCA inactivation (data not shown), thus ruling out the participation of trace metals in the reaction and formation of a free radical species from metal-catalyzed reactions.

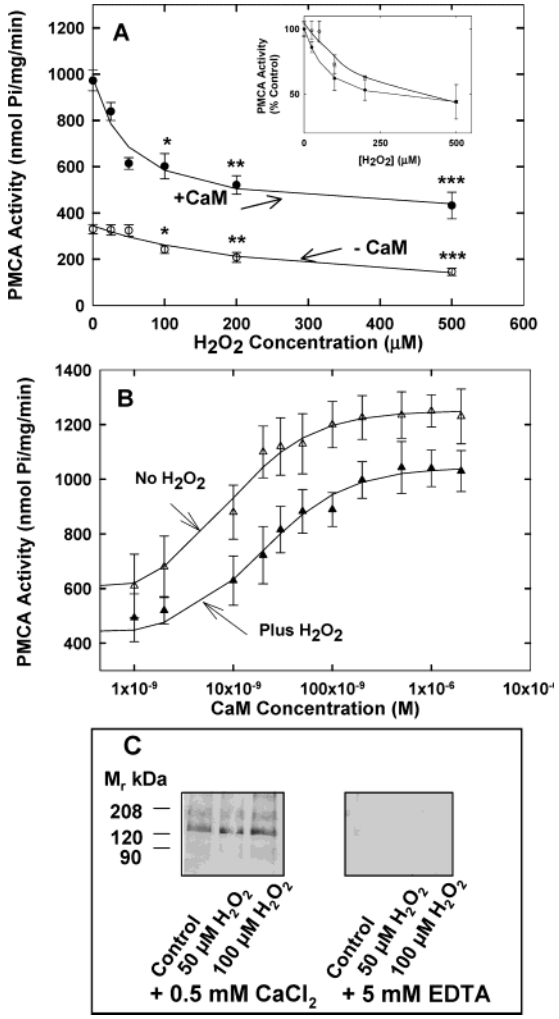

FIGURE 1: Effects of H_2O_2 on PMCA activity and CaM binding to PMCA. Purified PMCA (20 pmol) was incubated at 37 °C with increasing concentrations of H_2O_2 for 10 min, and H_2O_2 was inactivated by addition of catalase (2 units). After 3 min, aliquots were withdrawn and assayed for PMCA activity or run on SDS−PAGE for CaM overlay analysis. (A) PMCA activity was measured in the presence of 10 μM Ca^{2+} without (○) or with (●) 340 nM bovine brain CaM added to the medium. Data are expressed as nmol of P_i generated per mg of PMCA per min and represent means ± SEM from five experiments with different PMCA preparations and quadruplicate determinations in each. Statistical significance between untreated and H_2O_2-treated samples was determined by Student's *t* test (*$p < 0.05$, **$p < 0.04$, and ***$p < 0.02$). The inset to the figure shows the effects of H_2O_2 on PMCA activity expressed as a percent of control. The curves shown represent fitting of the data to a hyperbolic function. (B) PMCA activity was measured in controls (△) and samples exposed to 100 μM H_2O_2 for 10 min at 37 °C (▲) in the presence of increasing concentrations of CaM. Data represent means ± SEM from four experiments with different PMCA preparations using quadruplicate samples per experiment. Statistical significance of differences between H_2O_2-treated and untreated enzyme was determined by ANOVA (described in the text). The curves drawn represent fitting of the data to a hyperbolic function with three parameters. (C) Binding of CaM to control and H_2O_2-treated PMCA was assessed in a CaM overlay assay. Following exposure to 50 or 100 μM H_2O_2 at 37 °C for 10 min, PMCA (0.5 μg of protein) was subjected to SDS−PAGE, transferred to PVDF membranes, and probed for CaM binding as described under Experimental Procedures. Blots were scanned in Adobe Photoshop 5.0, and densitometric analyses were carried out by determination of pixel density of the ~140 kDa band indicative of the CaM-PMCA complex. Intensity of control PMCA was taken as 100%. The data are representative of results obtained in three experiments with different PMCA preparations.

The relatively similar interference of H_2O_2 with the function of PMCA under basal and CaM-activated states suggested that H_2O_2 did not interfere with CaM-induced stimulation of the PMCA activity. This possibility was assessed by measuring the CaM-dependent stimulation of enzyme activity in untreated samples of PMCA and in samples pretreated for 10 min with 100 μM H_2O_2 (Figure 1B). The addition of increasing amounts of CaM (1 nM to 5 μM) to samples of PMCA that were pretreated with H_2O_2 produced activation of the enzyme that closely paralleled that observed for PMCA that was not preexposed to H_2O_2, although the enzyme activity was significantly lower at each CaM concentration (ANOVA $F = 5.2$, d$f = 1{:}22$, $p = 0.03$). Fitting of the data to hyperbolic functions indicated that, despite a lower estimated basal activity of the H_2O_2-treated protein at zero CaM concentration (416.9 nmol mg^{-1} of protein vs 542.9 nmol mg^{-1} of protein for H_2O_2-treated and untreated PMCA, respectively), the K_{act} by CaM of the treated enzyme differed from that of the untreated PMCA by only a factor of 2 ($K_{act} = 1.9 \times 10^{-8}$ M for the H_2O_2-treated vs 0.8×10^{-8} M for the untreated). The maximal net CaM activation values in the treated and untreated PMCA differed by only 13% (626.9 nmol mg^{-1} of protein for the H_2O_2-treated and 707.9 nmol mg^{-1} of protein for the untreated PMCA). These results indicated that inhibition of the enzyme by H_2O_2 was likely due to a chemical modification of the protein that led to an overall loss of enzyme activity, without altering the sensitivity of the enzyme to CaM activation.

Further evidence that the oxidative modification by H_2O_2 responsible for enzyme inhibition did not directly affect CaM binding to PMCA was obtained by using a CaM overlay technique to examine the effect of H_2O_2 treatment on CaM binding to the enzyme. Biotinylated CaM coupled to avidin-conjugated alkaline phosphatase was used to detect CaM binding to PMCA, in the presence or absence of 0.5 mM $CaCl_2$. The PVDF membranes onto which control and H_2O_2-treated PMCA had been transferred showed a prominent protein band at ~140 kDa to which the biotinylated CaM bound (Figure 1C, left panel). Samples that contained 5 mM EDTA during incubation with CaM did not show any labeled bands (Figure 1C, right panel), supporting the Ca^{2+} dependence of the interaction of CaM with PMCA in the overlay assay. Densitometric analyses of the labeled bands at ~140 kDa showed a 13% increase in CaM binding to the H_2O_2-treated PMCA sample as compared to untreated controls. Thus, H_2O_2 treatment did not interfere with either CaM binding or CaM activation of the enzyme.

Assuming that H_2O_2 caused oxidation of certain amino acid residues in PMCA, an irreversible type of inhibition would be expected to result from such treatment. The residual activity of the enzyme detected at 10 min of incubation, even at high H_2O_2 concentrations (Figure 1A), was likely an indication of relatively slow rates of irreversible inhibition of the enzyme by H_2O_2. This prediction was born out by the results of the kinetic characterization of PMCA inhibition by H_2O_2 (Figure 2A). The time-dependent decay in basal and CaM-stimulated PMCA activity following exposure to 100 μM H_2O_2 was best fit by a biexponential function. The estimated pseudo-first-order kinetic constants for inhibition by 100 μM H_2O_2 were $k'_1 = 0.303$ and $k'_2 = 0.028$ min^{-1} for basal PMCA activity and $k'_1 = 0.339$ and $k'_2 = 0.029$

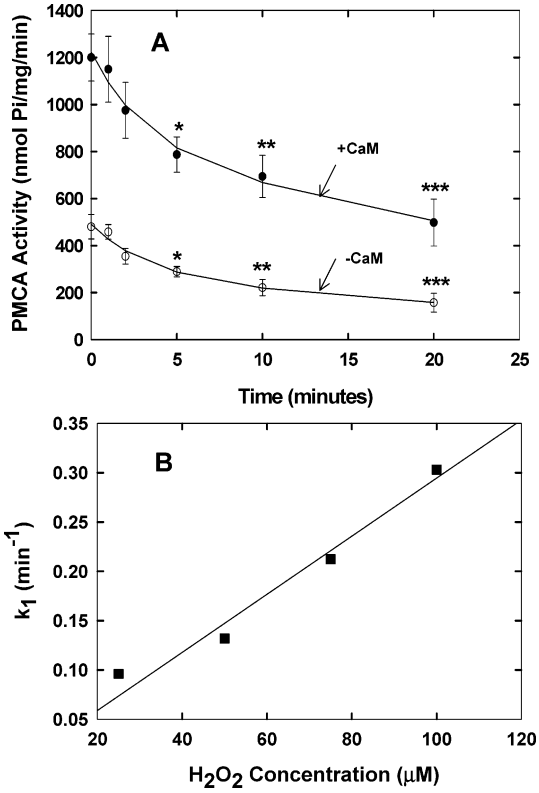

FIGURE 2: Kinetics of inactivation of PMCA by H_2O_2. (A) Time-dependent inactivation of purified PMCA. The enzyme was incubated with 100 μM H_2O_2 for the indicated times, followed by addition of catalase (2 units) and determination of activity in the absence (○) or presence (●) of 340 nM CaM as described under Experimental Procedures. Curves shown represent the fit of the data to a double-exponential decay function. Statistical significance of differences between untreated and H_2O_2-treated samples was determined by Student's t test (*p < 0.05, **p < 0.04, and ***p < 0.02). (B) Concentration dependence of rates of inactivation of PMCA by H_2O_2. The rates of inactivation of basal PMCA activity were determined as described in panel A following incubation of the enzyme with 25, 50, 75, or 100 μM H_2O_2. The data were fit to a double-exponential decay function to determine the k_1' values for enzyme inactivation at each concentration of H_2O_2.

min^{-1} for CaM-stimulated PMCA activity (Figure 2A). Thus, both basal and CaM-stimulated PMCA activities were inhibited by H_2O_2 with approximately equal rates of inhibition for both the rapid and the slow phases of such inhibition. Similar kinetic analyses applied to the inhibition of PMCA activity by increasing concentrations of H_2O_2 indicated that the rate constants for the inhibition of basal PMCA increased linearly with an increasing concentration of H_2O_2 (Figure 2B).

Although exposure of purified PMCA to H_2O_2 did not alter CaM binding or activation of the enzyme, these observations did not address the effect of reacting the PMCA with CaM before exposure of the enzyme to H_2O_2. Because CaM binding to PMCA relieves the autoinhibition of the activity of the enzyme, it seemed possible that CaM-induced conformational changes in PMCA might protect the enzyme from H_2O_2-induced inactivation. When PMCA was preincubated with a 3-fold molar excess of CaM in the presence of 100 μM $CaCl_2$ (10 min at 23 °C), prior to exposure to H_2O_2, PMCA activity was completely resistant to the inhibitory effect of H_2O_2 (Figure 3). The enzymatic activity in control samples preincubated with CaM was 2-fold greater than that of samples with no CaM (Figure 3), and further

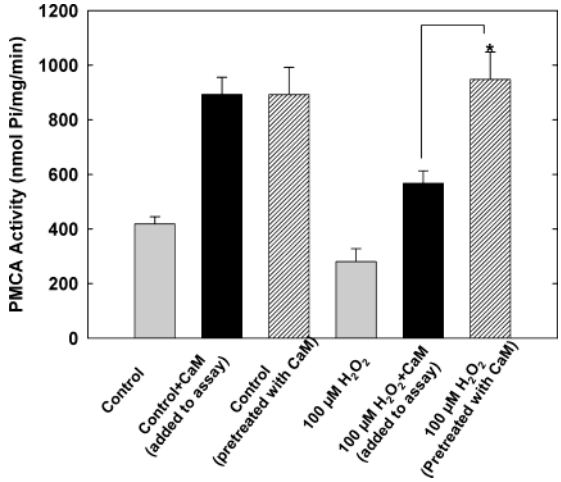

FIGURE 3: Effects of preincubation of PMCA with CaM on H_2O_2-induced inactivation. Purified PMCA (18 pmol) was exposed to 100 μM H_2O_2 for 10 min at 37 °C either before or after a 5 min preexposure to 54 pmol CaM. The PMCA activity was determined as described under Experimental Procedures in the presence or absence of 340 nM CaM added to the assay as indicated under each bar. Data represent means ± SEM from four experiments with different PMCA preparations and quadruplicate determinations in each. Statistical significance of differences between samples was determined by Student's t test (*p < 0.05).

addition of CaM to the PMCA assay did not increase activation in these prereacted samples. As shown also in the studies described previously, the PMCA that had not been pretreated with CaM but was exposed to CaM only at the time of the assay remained sensitive to H_2O_2-induced inhibition, whereas the enzyme prereacted with CaM before the H_2O_2 was fully active (Figure 3). This marked protection of PMCA by prereaction with CaM suggests that the CaM binding to PMCA induced conformational changes in the protein that masked sites that undergo oxidative modification by H_2O_2 in the absence of this regulatory protein.

Conformational Changes in PMCA Induced by H_2O_2. The results described previously were suggestive of H_2O_2-induced modifications of amino acids in PMCA that were accessible to the oxidant only when the enzyme was in the basal, non-CaM bound state. In subsequent studies, the accessibility of solvent in the medium surrounding the enzyme to specific amino acids of the PMCA protein was determined under basal conditions and following H_2O_2 treatment to assess changes in protein conformation under these treatment conditions. The assessment of solvent access to certain amino acids in a protein is based on the well-known changes in the fluorescence emission spectrum and shifts in the λ_{max} of Trp residues in peptides and proteins following changes in the polarity of the microenvironment of such residues (*27*). Therefore, the fluorescence of Trp residues in PMCA was measured prior to and following exposure to H_2O_2, to determine the effects of such exposure on the conformation of the enzyme. Fluorescence emission spectra of PMCA had a λ_{max} of 335 nm, indicating an overall hydrophobic environment of its 12 Trp residues (*28*). Exposure of PMCA for 10 min to increasing concentrations of H_2O_2 (100−500 μM) led to the quenching of Trp fluorescence that was dependent on the H_2O_2 concentration (Figure 4A,B). Exposure of PMCA to 100 μM H_2O_2 for increasing time intervals (1−10 min) resulted in a progressive, statistically significant quenching of fluorescence intensity (Figure 4C). The de-

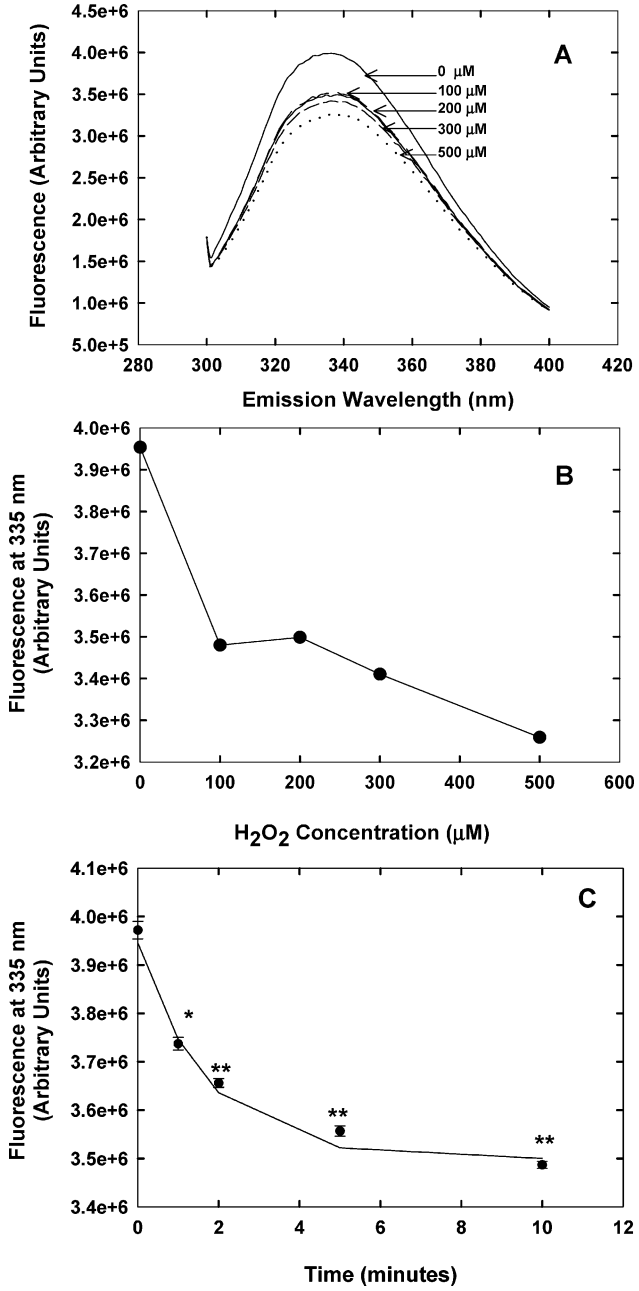

FIGURE 4: Concentration and time-dependent quenching of the PMCA fluorescence by H_2O_2. (A and B) Concentration-dependent quenching of PMCA fluorescence. In panel A, PMCA was exposed to increasing concentrations of H_2O_2 (100−500 μM) for 10 min at 37 °C, and fluorescence spectra (excitation 290 nm, emission 300−400 nm) were recorded. In panel B, fluorescence intensity of PMCA emission at 335 nm was plotted as a function of increasing concentrations of H_2O_2. Data shown are representative of four experiments. (C) Time-dependent quenching of PMCA fluorescence by 100 μM H_2O_2. Fluorescence of PMCA at 335 nm was measured following exposure to H_2O_2 for the indicated time periods. Data represent means ± SEM from three experiments. The curve shown was fit to the data using a single-exponential decay function. Statistical significance of differences between untreated and H_2O_2-treated samples was determined by Student's *t* test (*$p < 0.01$ and **$p < 0.005$).

crease in Trp fluorescence with time was best fit by a single-exponential function with an estimated rate constant $k' = 0.59$ min^{-1} (Figure 4C). This rate constant was approximately double that describing the rapid phase of enzyme inactivation by 100 μM H_2O_2 ($k_1 = 0.30$ min^{-1}) and might indicate a slightly more rapid change in the PMCA conformation preceding enzyme inactivation in the presence of H_2O_2. Unlike the H_2O_2-induced inactivation of PMCA, Trp fluorescence quenching in the presence of H_2O_2 was best fit by a single-exponential function with a single rate constant. The quenching of PMCA Trp fluorescence by H_2O_2 was accompanied by a 2 nm red shift, an additional indication of a slight overall increase in the polarity of H_2O_2-exposed PMCA molecules. When 500 μM concentrations of H_2O_2 were used, all fluorescence quenching took place within the first minute (data not shown), suggesting that the rate of Trp fluorescence quenching was dependent on the concentration of H_2O_2 added. This dependence was a linear function of the concentration of H_2O_2 (data not shown).

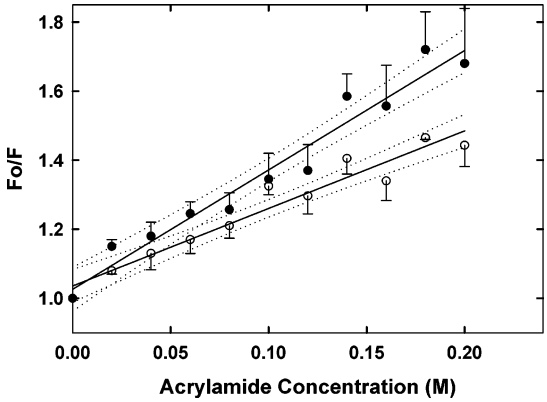

FIGURE 5: Effects of H_2O_2 on acrylamide-induced quenching of Trp in PMCA fluorescence (solvent accessibility). Following exposure of PMCA to H_2O_2 (100 μM for 10 min at 37 °C), emission spectra were acquired as described under Experimental Procedures. The ratio of the initial fluorescence intensity of PMCA (F_o) to that in the presence of increasing concentrations of acrylamide (F) was plotted against the acrylamide concentration. Stern−Volmer plots for both control and H_2O_2-treated PMCA are shown. The solid lines represent fitted regression lines of data for control (○) and H_2O_2-treated PMCA (●), and the dotted lines represent the 95% confidence interval for each set of data. The data shown are the means ± SEM of fluorescence ratios from three experiments with different PMCA preparations.

An alternative approach used to probe for possible changes in the solvent accessibility of Trp residues in H_2O_2-treated PMCA was the measurement of Trp quenching by the collisional quencher acrylamide. The addition of increasing quantities of acrylamide to the protein resulted in concentration-dependent decreases in fluorescence intensity in both control samples of PMCA and those pretreated with H_2O_2 (100 μM, 10 min). This effect of acrylamide indicated solvent exposure of at least some of the Trp residues in PMCA. Plotting the ratio F_o/F as a function of acrylamide concentration resulted in linear plots (Figure 5). The Stern−Volmer constant (Ksv) calculated from the slope of the lines indicated that Trp residues in PMCA were relatively exposed to solvent (Ksv = 2.25 ± 0.3 M^{-1}, mean ± SEM, $n = 3$ PMCA preparations). Following exposure to 100 μM H_2O_2 for 10 min, the solvent had greater access to the Trp residues, Ksv = 3.46 ± 0.6 M^{-1} (Figure 5). The increase in accessibility of the Trp residues to the solvent following exposure to H_2O_2 was 54%, but this increase was not significant (ANOVA, $F = 2.41$, df = 1:18, $p = 0.13$). Although the results were consistent with H_2O_2-induced alterations in the conformation of the enzyme, the changes might have been localized to specific protein domains and did not affect all Trp residues equally.

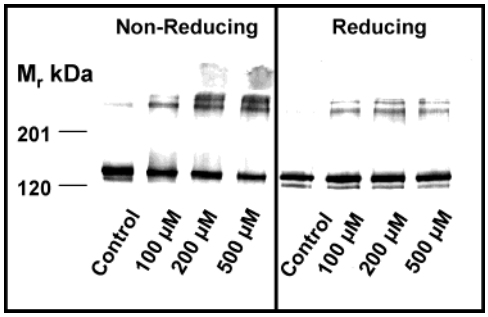

FIGURE 6: Effect of H_2O_2 on PMCA migration on SDS−PAGE. Following exposure of the protein to H_2O_2, 0.1 μg protein aliquots were subjected to SDS−PAGE in the presence or absence of 50 mM DTT, transferred to PVDF membranes, and probed with a PMCA 4 antibody (1:1000 dilution). Blots were scanned, and densitometric analyses of the ~140 kDa PMCA monomer and the higher molecular weight aggregates were performed as described under Experimental Procedures. The density of the PMCA monomer band in the control sample was taken as 100%. This immunoblot is representative of data obtained in five experiments with different PMCA preparations.

To assess local changes in the conformation of PMCA as a result of H_2O_2 exposure, studies were conducted with the synthetic peptide C28W, a 28 amino acid peptide that represents the sequence of the CaM-binding domain in PMCA (*29*−*31*). Changes in fluorescence of the single Trp in the peptide were measured. Fluorescence emission spectra of the Trp in C28W yielded a λ_{max} of 350 nm, characteristic of a fully exposed Trp. The addition of equimolar amounts of CaM caused a significant spectral blue shift (data not shown), indicating increased hydrophobicity of the Trp in accordance with previous observations (*31*−*35*). Exposure of the peptide to peroxide did not significantly alter the estimated Ksv of the peptide (Ksv = 11.5 ± 0.9 M^{-1} for untreated peptide and 14.6 ± 0.8 for the H_2O_2-treated peptide, data not shown), indicating that the CaM-binding domain of PMCA was not directly modified by exposure to H_2O_2.

H_2O_2-Induced PMCA Aggregation. The analysis of Trp fluorescence in intact PMCA suggested that an H_2O_2-induced loss of activity might be correlated with some structural alterations in the protein. To determine whether H_2O_2-induced enzyme inactivation correlated with large-scale changes in protein structure, such as protein aggregation, control and H_2O_2-treated samples were subjected to SDS−PAGE under nonreducing and reducing conditions, and immunoblot analyses were performed using monoclonal antibodies against PMCA 4, the major isoform in erythrocyte membranes. The anti-PMCA 4 antibodies reacted strongly with a band of ~140 kDa as expected (*36*), and exposure of the purified PMCA to increasing concentrations of H_2O_2 for 10 min at 37 °C altered protein migration (Figure 6). The intensity of the ~140 kDa PMCA band under nonreducing conditions decreased progressively with increasing H_2O_2 concentration. Densitometric analyses revealed a 40% decrease in immunoreactivity of the 140 kDa band at 500 μM H_2O_2 and a concomitant increase in high molecular weight species that, under nonreducing conditions, never entered the separating gel (Figure 6, left panel). When electrophoresis was performed in the presence of a reducing agent, 50 mM DTT, the large aggregates nearly disappeared (Figure 6, right panel), suggesting that much of the aggregation was due to intermolecular disulfide bridges. However, the fact that reducing conditions did not completely reverse aggregate formation suggested that cross-linking through Cys disulfide bond formation was not the only mechanism underlying the altered migration pattern.

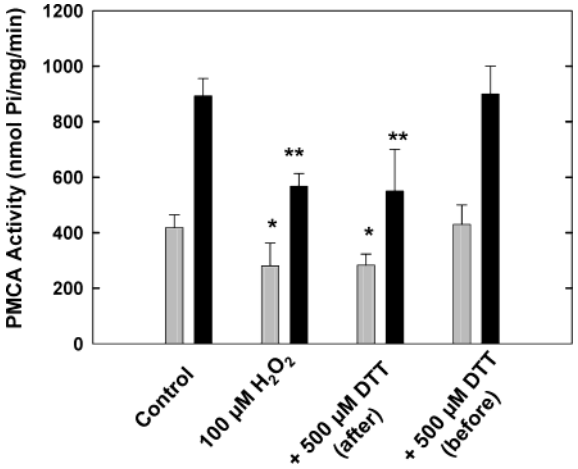

FIGURE 7: Effects of DTT on H_2O_2-induced inhibition of PMCA activity. The PMCA was measured in control samples, in samples preincubated with 100 μM H_2O_2 for 10 min at 37 °C, in samples exposed to 100 μM H_2O_2 followed by DTT (500 μM), and in samples first preincubated with DTT (500 μM), followed by addition of H_2O_2, as indicated. Activity was measured in the absence (gray bars) and presence (black bars) of 340 nM CaM added to the assay. Data represent means ± SEM from four experiments with different PMCA preparations and quadruplicate samples in each. Statistical significance of differences in basal activity between control and H_2O_2-treated samples (*p < 0.05) and in activity between CaM-stimulated control and H_2O_2-treated samples (**p < 0.05) was determined by Student's t test.

One possible alternative mechanism for oxidation-induced protein aggregation is through bityrosine formation, a phenomenon previously observed in SERCA exposed to peroxyl radicals (*37*). To determine if bityrosine formation occurred, the PMCA was exposed to H_2O_2 at 100 and 500 μM for 10 min, and fluorescence spectroscopy was used to determine if the characteristic bityrosine peak at 420 nm appeared. Both control and H_2O_2-treated PMCA samples were excited at 325 nm, and emission was recorded between 375 and 500 nm (*38*), but no peak at 420 nm was detected in either sample.

Effects of DTT on PMCA Activity. The observation that reducing conditions partially reversed the H_2O_2-induced aggregation of PMCA (Figure 6) raised the possibility that reducing disulfide bonds in the protein might lead to a reversal of the inactivation resulting from exposure to H_2O_2. However, when the enzyme was exposed to 500 μM DTT after incubation with H_2O_2 and the activity was measured, no recovery of the activity was observed (Figure 7). As expected, when the DTT was present at the time of the exposure to H_2O_2, the activity was not inhibited due to the rapid inactivation of the H_2O_2 by the large excess of DTT.

Determination of Cys Oxidation in PMCA. The partial reversal of PMCA aggregation by reducing agents suggested that Cys oxidation and disulfide formation were modifications to PMCA that resulted from exposure to H_2O_2. In efforts to evaluate the number of free sulfhydryls in PMCA, we measured the reaction of the enzyme with ThioGlo-1, a fluorescent maleimide probe (*39*), before and after exposure to H_2O_2. Reacting most Cys residues in PMCA with ThioGlo-1 proved to be difficult, and only two out of 21 Cys residues present in PMCA 4 could be labeled in protein

preparations treated with 2% SDS. We reasoned that the phosphatidylcholine used during the purification procedure might have interfered with the reaction of ThioGlo-1 with Cys residues through the formation of tight vesicles containing unreactive PMCA. The PMCA was purified in the absence of any phospholipids, but this manipulation did not enhance the efficiency of Cys labeling. In efforts to unfold the protein more completely, PMCA samples were exposed to 6 M Gdn-HCl, in addition to 2% SDS. This treatment increased the number of labeled Cys residues to 5.8 ± 0.6 mol of Cys/mol of PMCA (n = 4 determinations). The labeling of only six of 21 Cys residues in PMCA was surprising, given that the techniques used in the present studies were the same as those employed to achieve a high labeling efficiency in SERCA (*11*, *39*−*42*). Failure to label more than a third of the potentially available thiol groups might have been due to the presence of some intramolecular disulfide bonds or some mixed protein−glutathione disulfide bonds. To test this possibility, we reduced the protein with the reducing agent Tris[2-carboxyethyl]phosphine prior to Cys labeling. However, neither this manipulation nor the addition of Ca^{2+} plus CaM to move the C-terminal autoinhibitory domain away from the rest of the protein enhanced the labeling (7.0 ± 1.5 mol of Cys/mol of PMCA). Nevertheless, despite the small number of Cys residues that could be labeled, exposure of PMCA to 100 μM H_2O_2 decreased the number of free SH groups to 4.5 ± 0.4 mol of Cys/mol of PMCA. Higher concentrations of H_2O_2 (500 μM) did not lead to a further decrease in available Cys residues (4.3 ± 0.4 mol of Cys/mol of PMCA). Similar results were obtained with another SH-labeling agent, 4-(dimethylamino)phenylazophenyl-4′-maleimide.

Analysis of Other Amino Acid Modifications in PMCA. A global assessment of other possible amino acid modifications in PMCA was made by subjecting the protein to amino acid analyses after acid hydrolysis. No significant change in any amino acid residue was detected after exposure of PMCA to 100 μM H_2O_2. Although such analyses did not provide data on Trp and Met, modifications of His, Val, Leu, Ser, and Tyr could be excluded as the basis for the observed structural and functional changes. A parallel analysis of PMCA subjected to hydrolysis with 4 M MSA was used to quantify Met sulfoxide and possible formation of oxy acids from Cys. After exposure of PMCA to 100 μM H_2O_2, the protein contained only ∼0.06 mol of Met sulfoxide/mol of PMCA, or ∼0.002 Met sulfoxide/mol of Met, as there are 28 Met residues in PMCA 4. Additionally, in protein samples hydrolyzed with MSA, there were no detectable levels of Cys sulfinic or sulfonic acids. Finally, analysis of the carbonyl formation in the presence of 100 μM H_2O_2 did not reveal any significant increase in immunoreactivity of the 140 kDa band labeled by anti-DNP antibodies, indicating the absence of additional carbonyl formation in PMCA treated with H_2O_2.

DISCUSSION

Treatment of PMCA with H_2O_2 led to a marked loss of enzymatic activity, even after only a limited exposure to this oxidant. Furthermore, the sensitivity of PMCA to H_2O_2 was greater than that estimated previously for other Ca^{2+} regulatory proteins such as CaM, SERCA, and NCX (*11*, *43*−*45*). An interesting observation made in the present study was that the inhibitory effect of H_2O_2 was apparently not through an effect on either the binding of CaM to PMCA or the activation of the enzyme by CaM. Following pretreatment of the enzyme with H_2O_2, there was a moderate (2-fold) increase in the K_{act} by CaM as compared with the K_{act} of the native protein, and the V_{max} of the CaM-activated state of the preoxidized enzyme was a modest 13% lower than that of the native PMCA.

The possibility that the effects of H_2O_2 treatment on CaM-stimulated enzyme activity might have been due to the direct oxidation of CaM seemed unlikely for three reasons. First, the relatively modest changes in K_{act} and maximal activation of PMCA by CaM were not consistent with the marked loss of stimulation of erythrocyte PMCA by CaM that was preoxidized with H_2O_2 (*46*). Second, in the present study, the exposure of PMCA to H_2O_2 was followed by an amount of catalase sufficient to convert all H_2O_2 to H_2O before any CaM was added. Third, the fact that the activity of purified PMCA in the absence of any CaM was just as sensitive to oxidation as the activity of the enzyme in the presence of CaM suggested that the mechanism of inhibition by the oxidant did not involve direct oxidation of CaM.

It was reported previously that PMCA purified from erythrocyte plasma membranes (ghosts) forms dimers or oligomers that lead to a full activation of the enzyme in the absence of any CaM and that the introduction of varying concentrations of CaM to the oligomerized form of the enzyme produced no further stimulation of activity (*47*−*49*). Thus, the possibility was considered that the inhibition of PMCA activity by H_2O_2 in the absence of any CaM in the assay might be due to the disruption of the enzyme dimerization or oligomerization through the oxidative modification of key amino acids in PMCA. The process of PMCA dimerization or oligomerization is dependent on the concentration of the enzyme in solution and reaches its maximum at a PMCA concentration of approximately 20−40 nM (*48*, *50*). Although the concentration of PMCA at the time of exposure to H_2O_2 was 220 nM, the final concentration of the PMCA protein in the enzyme assay was 22 nM (i.e., at the threshold of forming dimers or oligomers). However, either any oligomerization of the purified PMCA used in the present studies had no effect on the sensitivity of the enzyme to CaM or the purified PMCA preparations used did not undergo oligomerization. The effects of dimerization on the CaM-induced activation of PMCA are still being debated as the PMCA protein in erythrocyte plasma membranes exists in the form of dimers, yet it is fully responsive to CaM (*51*). In the present study, CaM activated PMCA with an estimated K_{act} equal to 8 nM (when the total CaM concentration in solution was considered), an activation constant that was not substantially different from that previously reported for the CaM activation of PMCA in its nonoligomerized state, K_{act} = 6.4 nM (*47*).

In the present study, we observed that the prereaction of CaM with PMCA completely protected the enzyme from inactivation by H_2O_2. This protective effect was interpreted to indicate the presence of at least two conformations of the enzyme, one being sensitive to exposure to H_2O_2 and the other, induced by CaM, being insensitive to H_2O_2. There are other instances in which protein−protein interactions protect against oxidative damage to target proteins, including instances of protection of CaM from the oxidation when it

is complexed with the peptide melittin or with the CaM-dependent enzyme inducible nitric oxide synthetase (*52*). With respect to PMCA, CaM appears to alter the conformation of PMCA beyond merely dissociating the CaM-binding domain from the catalytic site, as demonstrated by the effects that CaM binding has on PMCA oligomerization in solution (*50*). Focusing on the effects of H_2O_2 on PMCA conformations, it was noted that PMCA pretreated with H_2O_2 in the absence of CaM underwent a detectable change in conformation, whereas a similar exposure of the CaM-binding peptide C28W to H_2O_2 did not cause detectable conformational changes in the peptide. Thus, it appeared that the primary effect of H_2O_2 was to alter the conformation of PMCA but not within the CaM-binding domain.

The H_2O_2-induced aggregation of PMCA and the reversal of such aggregation by DTT, as well as the decrease in ThioGlo-1-reactive Cys, suggested a cross-linking of PMCA monomers through disulfide linkages. Since the maleimide reagents usually attack the most accessible thiols first, the ~33% reduction of ThioGlo-reactive Cys upon exposure to 100 μM H_2O_2 was an indication that H_2O_2 itself may target the most accessible Cys residues. Some of the targeted residues might be critical for the functional integrity of PMCA. The oxidation of functionally important Cys residues could cause conformational changes in the active site through the formation of the bulky disulfide bond and interference with events such as ATP binding, phosphorylation, dephosphorylation, and Ca^{2+} transport. The absence of detectable sulfinic or sulfonic acids of Cys was an indication that the primary modification of Cys residues was likely the formation of disulfide bonds. However, the inhibition of enzyme activity was not reversed by DTT, indicating that even after the reduction of the disulfide bond, the PMCA did not refold properly into its native conformation.

Although the loss of two Cys residues was the only amino acid modification of the PMCA protein detected after exposure of the protein to H_2O_2, the possibility that other residues were oxidatively modified by H_2O_2 or by free radicals generated during exposure of the enzyme to H_2O_2 was explored. The global assay of carbonyl groups in PMCA following exposure to H_2O_2 did not reveal a significant increase in DNPH immunoreactivity, nor did the chelation of free metals in the buffer media used during the reaction modify the effects of H_2O_2 on PMCA activity. Therefore, it appeared unlikely that H_2O_2 modified PMCA through the generation of free radicals and the formation of carbonyls.

What was more interesting was that PMCA, when associated with CaM, exhibited a more open conformation that was more solvent exposed. This conformation was completely resistant to structural changes induced by peroxide and appeared to stabilize a form that was less sensitive to H_2O_2. The structural change was consistent with the effectiveness of CaM in protecting PMCA from H_2O_2-induced functional inactivation (Figure 5). The observation that CaM binding protects the PMCA from H_2O_2-induced inactivation may have important physiological implications. Some CaM present in neurons is very tightly bound to the synaptic membranes, with evidence for constitutive binding of CaM to the L-type Ca^{2+} channels (*53*). In view of the minimal stimulation of PMCA in synaptic membranes by exogenous CaM (30−40% as compared to 300% in membranes from other tissues), it is quite likely that a substantial amount of CaM is tightly bound to PMCA in synaptic membranes. If this is the case, then periodic elevations of H_2O_2 or other oxidants in neurons might have only limited effects on neuronal PMCA under normal physiological conditions. However, our previous observation that the amount of CaM bound to brain membranes from aged animals is significantly reduced might suggest that the PMCA activity in brain neurons of aged animals is more susceptible to oxidative inactivation. In aged brain and under other oxidative stresses the PMCA could be significantly inactivated, leading to intracellular Ca^{2+} accumulation. This, in turn, would become a stimulus for the increased release of ROS from mitochondria and the perpetuation of a cycle of Ca^{2+} dysregulation that has been observed in both pathological states and in brain aging.

ACKNOWLEDGMENT

The authors thank Drs. T. S. Squier and D. J. Bigelow (Pacific Northwest National Labs, Richland, WA) for insightful advice and discussions on this project. We also thank Dr. J. Penniston and A. Filoteo (Mayo Clinic, Rochester, MN) for the kind gift of PMCA 4 antibodies. The peptide C28W was a gift from Dr. J. Urbauer (University of Kansas).

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BI034565U