Inactivation and Self-association of Ca\textsuperscript{2+}/Calmodulin-dependent Protein Kinase II during Autophosphorylation*

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The time-dependent loss in enzyme activity associated with the autophosphorylation of Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaM-kinase) was altered by both pH and ATP concentration. These parameters also influenced the extent to which soluble CaM-kinase undergos self-association to form large aggregates of sedimentable enzyme. Specifically, autophosphorylation of CaM-kinase in 0.01 mM ATP at pH 6.5 resulted in the formation of sedimentable enzyme and a 70% loss of enzyme activity. Under similar conditions at pH 7.5, the enzyme lost only 30% of its activity, and no sedimentable enzyme was detected. In contrast to 0.01 mM ATP, autophosphorylation of CaM-kinase at pH 6.5 in 1 mM ATP did not result in a loss of activity or the production of sedimentable enzyme, even though the stoichiometry of autophosphorylation was comparable. Electron microscopy studies of CaM-kinase autophosphorylated at pH 6.5 in 1 mM ATP revealed particles 100–300 nm in diameter that clustered into branched complexes. Inactivation and self-association of CaM-kinase were influenced by the conditions of autophosphorylation in vitro, suggesting that both the catalytic and physical properties of the enzyme may be sensitive to fluctuations in ATP concentration and pH in vivo.

Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaM-kinase) is highly enriched in brain where it is thought to perform a multifunctional role in the transduction of Ca\textsuperscript{2+} signals. Substrates phosphorylated by CaM-kinase are involved in neurotransmitter synthesis and release, cytoskeletal function, intracellular Ca\textsuperscript{2+} homeostasis, carbohydrate metabolism, ion channel function, and synaptic plasticity (for reviews, see Refs. 1 and 2). Purified forebrain CaM-kinase is isolated as a holoenzyme of approximately 460–650 kDa (3, 4) and estimated to contain 10–12 subunits consisting primarily of 50 kDa (a) and 60 kDa (b) isoforms in a 3:1 ratio, respectively (3). Although substrate phosphorylation is stringently coupled to Ca\textsuperscript{2+}/CaM binding, the activity and regulatory properties of CaM-kinase are altered by autophosphorylation (for reviews, see Refs. 1, 2, and 5). Previously reported autophosphorylation-associated changes in the functional properties of CaM-kinase include generation of the Ca\textsuperscript{2+}/CaM-independent (autonomous) form (6–10), Ca\textsuperscript{2+}/CaM insensitivity (11–15), CaM trapping (16, 17), and inactivation (18–24).

We have demonstrated a loss of CaM-kinase activity in both in vivo and in situ models of ischemia (25, 26). Coincident with inactivation in these models was a redistribution of soluble CaM-kinase to particulate fractions. One possible explanation for the loss of activity and redistribution of soluble CaM-kinase is that ischemic-induced alterations in the cellular environment alter the properties of the enzyme, potentially through an autophosphorylation-mediated process. Biochemical alterations observed during ischemia include ionic imbalances with marked reductions in intracellular pH and ATP concentrations (see "Discussion"). Although several investigators have observed a time-dependent decrease in the activity of autophosphorylated CaM-kinase (i.e. inactivation) in vitro (18–24), the physiological significance and role of autophosphorylation in this loss of activity is unknown. Lou et al. (22) reported that ATP concentration influences autophosphorylation-associated losses in the activity of CaM-kinase, with low ATP maximizing and high ATP preventing enzyme inactivation. The generation of autonomous activity and the sites of autophosphorylation also were influenced by ATP concentration (22). Other parameters reported to influence the loss of enzyme activity and sites of autophosphorylation include reaction temperature (27) and the type and concentration of divalent ions (24, 27). These findings indicate that the conditions of autophosphorylation are important determinants for alterations in the activity of CaM-kinase. The redistribution and loss of soluble CaM-kinase activity associated with ischemic conditions led us to further examine the influence of conditions of autophosphorylation, specifically pH and ATP concentration on the activity and physical characteristics of CaM-kinase in vitro. In this study, we observed that pH and ATP concentration influenced the inactivation of CaM-kinase associated with autophosphorylation. These parameters also influenced the self-association of soluble CaM-kinase into sedimentable enzyme complexes, suggesting that cells undergoing fluctuations in pH and ATP concentration could produce autophosphorylation-associated changes in both the physical and catalytic properties of the enzyme in vivo.

EXPERIMENTAL PROCEDURES

Materials—ATP was purchased from Pharmacia Biotech Inc., [\textsuperscript{32P}]ATP (3,000 Ci/mmol) from Amersham Corp., and AMP-PCP from Boehringer Mannheim. Tabbed-copper grids (400 mesh) and 90-nm polystyrene sizing beads were obtained from Ted Pella. Ultrafiltration units (regenerated cellulose, M.W.C.O. 100,000) were purchased from Millipore. Low molecular weight standards and Tween-20 were obtained from Bio-Rad. CaM was purified from bovine brain as described by Gopalakrishna and Anderson (28). Soluble CaM-kinase was purified from rat forebrain essentially as described by Kelly and Schenolikar.
with the omission of gel filtration (29). The enzyme was quantified using either Bradford or BCA (Pierce) methods, with bovine serum albumin as the standard. Multiple forebrain preparations of CaM-kinase generated similar results throughout this study. Specific activity of enzyme preparations under the assay conditions described below were between 12 and 30 μmol/min/mg. The peptide substrate syntide (1-7) (Novabiochem) was synthesized on an automated peptide synthesizer (Applied Biosystems) and high performance liquid chromatography purified.

Autophosphorylation and Processing of CaM-kinase— Standard autophosphorylation conditions are defined as 50 mM MES/Hepes, pH 6.5, 0.4 mM DTT, 0.5 mM CaCl2, 10 mM MgCl2, 1 μM CaM, 10 μM ATP, 3 μCi [γ-32P]ATP, 10% (v/v) glycerol and 0.1% Tween-20 (100 μl, final volume) were initiated with the addition of enzyme (1 μg). The enzyme was autophosphorylated (or simply incubated depending upon the reaction conditions) for 0.5 min on ice before an initial time 0 point was collected. The purpose of the preincubation step was to allow the equilibration of the reaction components and to generate a similar phosphorylation state on ice before allowing the enzyme to undergo further autophosphorylation at 30 °C. In the absence of this step, similar alterations in sedimentation and Ca2+/CaM-dependent inactivation were observed. Following preautophosphorylation, the timer was started, and the reaction mixture was placed in a 30 °C shaking water bath for collection of samples at subsequent time points. Aliquots (10 μl, 100 ng of enzyme) were collected over time and diluted 6-fold in 50 μl of ice-cold 50 mM MES/Hepes, pH 7.2 with 10 mM EDTA, terminated by autophosphorylation of the enzyme. Samples were then subjected to centrifugation for 30 min at 15,000 × g at 4 °C. The supernatant and pellet were separated and resuspended in SDS-loading buffer (10% glycerol, 15 mM DTT, 2.3% SDS, and 62.5 mM Tris, pH 6.8) for SDS-polyacrylamide electrophoresis (PAGE).

Electrophoresis was performed as described elsewhere (30), using a 10% resolving gel. Following SDS-PAGE, the enzyme was visualized using either Coomassie Blue staining or Western blotting. Coomassie staining of proteins was accomplished by incubation of gels in fixative (20% methanol, 7% acetic acid) containing 0.002% of Coomassie Brilliant Blue R-250 for 30 min and destained overnight in fixative. For Western blotting, the enzyme was transferred to nitrocellulose using a Genie transfer apparatus (Idea Scientific). Transfer was performed at 4 °C in 20% methanol, 190 mM glycine, and 26 mM Tris, pH 8.0, for 1–2 h at a constant 15–20 V. After transfer, the nitrocellulose was blocked overnight in 5% dry milk in phosphate-buffered saline. Monodonal antibodies to the 50-(2D5) (31) and 60- (Cb1-1) (32) kDa subunits of CaM-kinase were diluted 1:2000 in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, and 0.05% Tween-20) and incubated at 30 °C for 1 h. Following incubation, the membrane was washed in TBST, followed by incubation in anti-mouse IgG AP-conjugated secondary antibody (Promega) diluted 1:5000 in TBST for 1 h. The membrane was washed again in TBST and the immunoreactive proteins were visualized using the alkaline phosphatase substrates, nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, and developed with Coomassie Blue stained 50- and 60-kDa subunits after SDS-PAGE was performed essentially as described for standard reaction conditions, with modifications as indicated in the figure legends. Aliquots of the reaction mixture (10–20 μl) were collected at the indicated times and diluted with high performance liquid chromatography purified and destained were performed as described above. An aliquot of the reaction mixture was taken before addition of the enzyme and spotted directly onto the filter paper. The counts/min from each reaction mix (filter paper) and the radioactivity incorporated into the subunits of CaM-kinase (dried gel) were determined simultaneously using a BetaScope 603 (Betagen, counting efficiency of 19%). Densitometric analysis of Coomassie Blue stained 50- and 60-kDa subunits after SDS-PAGE was used to determine a ratio of 3:1 (50:60 kDa) giving an average molecular mass per mol of CaM-kinase of 52.5 kDa. Stoichiometry is reported as moles of phosphate incorporated per mol of CaM-kinase.

Transmission Electron Microscopy (TEM)— Autophosphorylation was performed under standard conditions with the indicated changes in ATP concentration. Reactions were terminated by the addition of EDTA (final concentration of 50 mM) to each reaction mix and held on ice for 10 min. The samples were deposited onto carbon-coated Formvar grids in a Millipore 100,000 MW sample concentrator using a refrigerated microcentrifuge at 4,000 × g for 3 min. The grids were incubated in 2% uranyl acetate in 25% methanol for 1–2 min and then washed in distilled water and air dried. Additionally, 90-nm polystyrene sizing beads were applied to the grid by the drop method for a size reference. Transmission electron microscopy was performed using conventional irradiation procedures on a J OEL-100CX operating at 80 kV.

RESULTS

Autophosphorylation of CaM-kinase was associated with a time-dependent decrease in Ca2+/CaM-dependent enzyme activity at pH 7.5 (Fig. 1A). Activity measurements from enzyme autophosphorylated at pH 7.0 and 6.5 indicated that a greater percentage of enzyme activity was lost over time with a decrease in reaction pH (Fig. 1A). Autophosphorylation of CaM-kinase at pH 7.5 resulted in a 15% decrease in enzyme activity at 2 min, whereas pH 7.0 and pH 6.5 were both associated with a 40% loss of enzyme activity at 2 min. After 5 min, the extent of enzyme inactivation at pH 7.5 (30%) was less than both pH 7.0 (50%) and pH 6.5 (70%). Activity measurements also were performed in the presence of EGTA to monitor changes in Ca2+/CaM-independent activity (Fig. 1B). The time course of Ca2+/CaM-independent inactivation at pH 6.5 (65% and 40% at 2 and 5 min, respectively) and pH 7.0 (80 and 58% at 2 and 5 min, respectively) were similar to decreases in Ca2+/CaM-dependent activity over time. However, at pH 7.5 a 40% increase in independent activity was observed at 2 min (120%) and a modest decrease in activity observed at 5 min (90%). These data suggested that maximal Ca2+/CaM-independent activity had not been obtained during the 0.5-min autophosphorylation at pH 7.5. This conclusion was supported by measuring the percent Ca2+/CaM-independent activity generated at time 0 for pH 6.5, 7.0, and 7.5, which were 55 ± 2.1, 54.2 ± 5.4, and 42 ± 0.2, respectively (n = 3, ±S.D.) following the standard 0.5-min preautophosphorylation protocol. Extending the preautophosphorylation to 1, 2, or 3 min on ice increased the percent Ca2+/CaM-independent activity generated at pH 7.5 at time 0 to 52.1 ± 0.09, 55.3 ± 0.06, and 54.6 ± 0.04, respectively (n = 3, ±S.D.). After extending the preautophosphorylation on ice to 2 min, we observed no differences in the time course of autonomous inactivation for pH 6.5 and 7.0 conditions (data not shown). However, at pH 7.5, the percent inactivation observed for Ca2+/CaM-independent activity (90 and 60% at 2 and 5 min, respectively) was similar to the Ca2+/CaM-dependent loss of activity over time (see solid bar in Fig. 1B). These data indicated that the loss of CaM-kinase activity observed during autophosphorylation was influenced by pH and that the extent of inactivation observed is similar for both Ca2+/CaM-dependent and independent activities.
mobility were also observed for the β subunit. The loss of immunostaining is not due to a loss of the 60-kDa subunit (see autoradiogram in Fig. 3) or specific to any reaction pH and may represent changes in the affinity of the monoclonal antibody to the autophosphorylated subunit and/or a decrease in the threshold for immunostaining. It should be noted that an increase in the preautophosphorylation period from the standard 0.5 min to 2 min did not alter the formation of sedimentable enzyme in the different pH conditions, nor did omitting the preautophosphorylation step completely (data not shown).

A more comprehensive time course was conducted to observe changes in sedimentability and activity of CaM-kinase during autophosphorylation at pH 6.5. The Western blot in Fig. 3 demonstrates that, under standard reaction conditions, CaM-kinase was detected in the pellet as early as 1 min. Similar to Fig. 2, changes in the electrophoretic mobilities of the subunits of CaM-kinase occurred with autophosphorylation. A 54-kDa form of the 50-kDa subunit was observed as early as 1 min, appearing temporally with the detection of sedimentable enzyme. By 5 min, the 50-kDa form was predominantly shifted to the 54-kDa form. The mobility of the 60-kDa subunit was also altered during autophosphorylation and although not visible by immunostaining in Fig. 3, the formation of a 62-kDa form was evident after 5 min of autophosphorylation on the autoradiogram (Fig. 3). The autoradiogram of the Western blot in Fig. 3 also demonstrates alterations in the distribution of CaM-kinase between supernatant and pellet and altered mobilities of the 50- and 60-kDa subunits of the enzyme. The signal from the 32P-labeled phosphoproteins clearly illustrates both the presence of the 60-kDa subunit throughout the period of autophosphorylation and the generation of the 62-kDa form after 5 min (Fig. 3).

The time course of CaM-kinase inactivation during autophosphorylation as described in Fig. 3, is shown in Fig. 4 (left ordinate axis). At 1.5 min, the enzyme was completely sedimentable (see Fig. 3), yet retained over 60% of its initial activity. After 7 min, the enzyme retained less than 25% of the initial activity. Quantitative analysis of 32P incorporation into CaM-kinase during autophosphorylation indicated an increase in the incorporation of 32P over time, with approximately 2.3 mol of phosphate incorporated per mol of enzyme after 7 min of autophosphorylation (Fig. 4, right ordinate axis). The time course of enzyme inactivation parallels an increase in the stoichiometry of autophosphorylation, suggesting that autophosphorylation contributes to the time-dependent loss in enzyme activity.

The autophosphorylation dependence of the formation of sedimentable CaM-kinase at pH 6.5 was investigated by sub-
were obtained when the reaction mix contained 0.01 mM ADP. Rylation, sedimentable enzyme was not formed. Similar results were obtained under similar reaction conditions, yet in the absence of Ca²⁺/CaM retained 90% of its activity after 5 min (data not shown). This loss of substrate phosphorylation by CaM-kinase in the presence of Ca²⁺/CaM is similar to previous reports and may reflect a "thermal instability" of activated enzyme in the absence of ATP (21, 23).

The role of autophosphorylation in the inactivation and formation of sedimentable enzyme was further investigated by altering the conditions of autophosphorylation. Autophosphorylation on ice previously was demonstrated to produce the autonomous form of CaM-kinase without enzyme inactivation (22). (22) suggested that this condition not only slows the kinase reaction, but also modifies the sites autophosphorylated compared to similar conditions at 30°C. Fig. 5, A and B, demonstrates that autophosphorylation at 4°C does not result in the formation of sedimentable enzyme or in a significant loss of activity. Limited autophosphorylation in these experiments was supported by a calculated stoichiometry for autophosphorylation of 0.4 ± 0.04, 0.4 ± 0.06, and 0.4 ± 0.05 mol of phosphate per mol of CaM-kinase after 0, 2, and 5 min, respectively (n = 5, ±S.D.). The absence of mobility shifts for the subunits of CaM-kinase during SDS-PAGE further indicates a restricted state of autophosphorylation (22).

Stoichiometry of autophosphorylation was determined as described under "Experimental Procedures." Percent initial activity (±S.D.) represents syntide phosphorylation, normalized to the time 0 point for enzyme incubated in standard reaction conditions (10 mM MgCl₂, 10 μM ATP, 1 μM CaM, 10 mM MgCl₂, 10 μM ATP, 2 μCi of [γ-³²P]ATP, 10% glycerol and 0.1% Tween-20). Alternative molecular mass forms of the 50- and 60-kDa subunits are indicated with Arrows mark the positions of the 50- and 60-kDa subunits. The enzyme retained 90% of its activity after 5 min in the presence of AMP-PCP. We observed that enzyme incubated in standard conditions at 30°C. Fig. 5, A and B, demonstrates that autophosphorylation at 4°C does not result in the formation of sedimentable enzyme or in a significant loss of activity. Limited autophosphorylation in these experiments was supported by a calculated stoichiometry for autophosphorylation of 0.4 ± 0.04, 0.4 ± 0.06, and 0.4 ± 0.05 mol of phosphate per mol of CaM-kinase after 0, 2, and 5 min, respectively (n = 5, ±S.D.). The absence of mobility shifts for the subunits of CaM-kinase during SDS-PAGE further indicates a restricted state of autophosphorylation (22).
To explore the effects of temperature during autophosphorylation, limited autophosphorylation was accomplished at 30°C by substituting the divalent Mn²⁺ for Mg²⁺ (27). The Western blot in Fig. 5A demonstrates that autophosphorylation in the presence of 0.01 mM Mn²⁺ does not result in the formation of sedimentable enzyme. In addition, no change was observed in the relative mobility of the 50-kDa subunit. The 60-kDa subunit clearly shifted to a 62-kDa form after 2 min and a 64-kDa form after 5 min of autophosphorylation. The stoichiometry of autophosphorylation was approximately 0.3 ± 0.05, 0.8 ± 0.13, and 1.2 ± 0.18 mol of phosphate per mol of CaM-kinase at 0, 2, and 5 min, respectively (n = 5, ±S.D.). Restricted or limited autophosphorylation in Mn²⁺, was supported by a maximal stoichiometry of ~1, whereas in 10 mM Mg²⁺ at 30°C, the maximal stoichiometry was ~2 mol of phosphate per mol of CaM-kinase (see Fig. 4). Similar to autophosphorylation at 4°C, CaM-kinase autophosphorylated in Mn²⁺ retained 90% of its initial activity after 5 min (Fig. 5B). During these experiments, we observed that the specific activity of CaM-kinase measured in the second-stage reaction was influenced by the concentration of Mn²⁺ carried over from the autophosphorylation reaction (0.4 mM Mn²⁺ final second-stage concentration). Although we did not extensively pursue the influence of Mn²⁺ on the catalytic activity of CaM-kinase, the specific activity observed in the second-stage reaction was significantly improved if the concentration of Mn²⁺ was decreased from 10 to 1 mM during the initial autophosphorylation reaction, lowering the final concentration in the second stage reaction to 0.04 mM. Autophosphorylation in 1 mM Mn²⁺ produced similar results to the data reported above for 10 mM Mn²⁺, including a similar stoichiometry and no appreciable changes in the activity or sedimentability of the enzyme over time (data not shown). As seen in Fig. 5B, the inactivation associated with autophosphorylation in standard conditions (10 mM Mg²⁺, 10 μM ATP, pH 6.5, at 30°C) was greater than inactivation in the absence of autophosphorylation, suggesting that additional processes may lead to further enzyme inactivation. Both inactivation and the formation of sedimentable enzyme are influenced by autophosphorylation. Restricted autophosphorylation does not produce sedimentable enzyme nor significant inactivation and may even prevent or protect the enzyme from “thermal inactivation.”

Lou et al. (22) previously demonstrated that autophosphorylation of CaM-kinase in high (0.5 mM) versus low (0.005 mM) ATP produced a similar stoichiometry of autophosphorylation associated with different sites of autophosphorylation. The functional consequence of autophosphorylation was characteristic of the ATP concentration (i.e. inactivation with little autonomous activity was associated with low ATP, and autonomous activity with little inactivation was associated with high ATP). Fig. 6 demonstrates the effects of ATP concentration (0.01, 0.1, and 1 mM) on the sedimentability of CaM-kinase autophosphorylated at pH 6.5. After 2 min of autophosphorylation in 0.01 mM ATP, the enzyme was completely in the pellet. However, CaM-kinase autophosphorylated at 0.1 mM ATP remained partially in the supernatant after 2 min, and enzyme autophosphorylated for 2 min in 1 mM ATP remained completely in the supernatant. Fig. 6 also illustrates that ATP concentration influenced the formation of the 54-kDa form of the 50-kDa subunit. In 1 mM ATP, the formation of the 54-kDa form was not observed. However, the formation of the 54-kDa form was enhanced with decreased ATP concentration. Autophosphorylation in 1 mM ATP produced changes in the mobility of the 60-kDa subunit without altering the mobility of the 50-kDa subunit. This is reminiscent of mobility changes observed when the enzyme is autophosphorylated in 0.01 mM ATP in the presence of Mn²⁺ (see Fig. 5A). After 2 min of autophosphorylation in 0.01, 0.1, or 1 mM ATP, the percent activity remaining was 53 ± 7.2, 83 ± 6.4, and 103 ± 13.8, respectively (n = 3, ±S.D.). The percent Ca²⁺/calmodulin-independent activity remaining after 2 min of autophosphorylation in 0.01 and 1 mM ATP paralleled changes in the Ca²⁺/calmodulin-dependent activity; 65 ± 6.4 and 122 ± 2.5, respectively (n = 3, ±S.D.). The generation of independent activity in the 0.01 and 1 mM ATP conditions was similar, 53.3 ± 0.6 and 53.3 ± 3.6 (n = 3, ±S.D.), respectively. Autophosphorylation for 2 min in 0.01 mM and 1 mM ATP produced a stoichiometry of 1.2 ± 0.23 and 1.1 ± 0.32 mol of 32PPO₄ per mol of CaM-kinase, respectively (n = 3, ±S.D.). These data indicate that both inactivation and the formation of sedimentable enzyme are not a consequence of the total 32PPO₄ incorporated into CaM-kinase during autophosphorylation. In addition, these data demonstrate that inactivation of the enzyme and the formation of sedimentable CaM-kinase at pH 6.5 are prevented at high concentrations of ATP, while low ATP concentrations appear to promote this process.

The formation of sedimentable CaM-kinase suggested dramatic alterations in the physical characteristics of the soluble enzyme. Enzyme autophosphorylated in conditions that either promote (0.01 mM ATP at pH 6.5) or prevent (1 mM ATP at pH 6.5) the formation of sedimentable enzyme was visualized using TEM. CaM-kinase autophosphorylated in 0.01 mM ATP for 5 min produced distinct uranyl acetate-staining structures that were uniformly distributed over the grid (Fig. 7A, × 5000). At a higher magnification (Fig. 7B, × 33,000), particles ranging from 100 nm to 300 nm in diameter were evident and appeared to interconnect and associate to form branched structures, termed enzyme complexes. Under these conditions (0.01 mM ATP at pH 6.5) enzyme complexes were not observed after pre-autophosphorylation on ice (time 0), yet were evident after 2 min of autophosphorylation at 30°C (data not shown). In contrast, after 5 min of autophosphorylation in 1 mM ATP at pH 6.5, the grid was void of uranyl acetate-staining structures, and increased magnification revealed only the sizing beads (data not shown). The TEM conditions utilized in these experiments did not generate sufficient contrast to expose the 20–30-nm structure of individual CaM-kinase holoenzymes (34). In addition, we directly analyzed the sedimented form of CaM-kinase following centrifugation (15,000 × g for 30 min) by TEM and as expected, the enzyme complexes observed following centrifugation and resuspension were more tightly packed than enzyme complexes deposited directly to the grid (data not shown).

Throughout this study we have used immunostaining to identify the soluble and sedimentable enzyme. Fig. 7C demonstrates the Coomassie Blue staining pattern of enzyme autophosphorylated in standard conditions for 0 or 5 min. The
In this study we observed that the conditions of autophosphorylation influence both the formation of sedimentable CaM-kinase and a time-dependent loss of substrate phosphorylation. Autophosphorylation at 4 °C or in Mn2⁺ at 30 °C did not result in a significant loss of enzyme activity (less than 10%) nor in the formation of sedimentable enzyme, indicating that limited autophosphorylation either prevents or is not sufficient for these changes. Lou and Schulman demonstrated that these conditions restrict the sites of autophosphorylation and maintain the autonomous form of the enzyme without its inactivation (27). Furthermore, the substitution of Mn2⁺ for Mg2⁺ was reported to increase the affinity of the kinase for ATP (27). The K_m for Mg2⁺/ATP is reported to be within the range of 1.6–20 μM for both substrate phosphorylation and autophosphorylation (4, 15, 18). The low ATP concentration used in this study, 10 μM, is within the range of the calculated K_m for ATP and, therefore, may not be saturating. The potential consequence or role of an unoccupied ATP binding site on the inactivation and formation of sedimentable CaM-kinase is currently under investigation.

We observed that ATP concentration influenced both enzyme inactivation and the formation of sedimentable enzyme. The inactivation associated with autophosphorylation in low ATP (10 μM) was further influenced by pH, with maximal inactivation and formation of sedimentable enzyme occurring at pH 6.5, whereas autophosphorylation in high ATP (1 mM) prevented these changes. The stoichiometry of autophosphorylation was comparable between low and high ATP conditions, indicating that formation of sedimentable enzyme and/or inactivation were not due to the extent of 32PO4 incorporation. Lou et al. (22) also reported a similar ATP sensitivity to the inactivation of CaM-kinase associated with autophosphorylation and similar stoichiometries of autophosphorylation in high (500 μM) and low (5 μM) ATP. These authors also reported that tryptic mapping of CaM-kinase autophosphorylated in low ATP produced unique phosphopeptides compared to high ATP (22).

In addition, Hanson et al. (9) reported that Thr-253 on CaM-kinase was autophosphorylated preferentially at low ATP. Site-specific autophosphorylation-associated alterations in the properties of CaM-kinase are complex and well documented (e.g. Thr-286 phosphorylation and autonomous activity); however, it is currently unknown whether the differential autophosphorylation associated with low ATP induces the inactivation and/or formation of sedimentable enzyme or is merely a consequence of these processes.

Inactivation and the formation of sedimentable enzyme during autophosphorylation in low ATP were influenced by pH. Autophosphorylation at pH 6.5 in low ATP produced robust inactivation and formation of sedimentable enzyme. Autophosphorylation at pH 7.5 was the only condition observed that produced enzyme inactivation, yet was not accompanied by the formation of sedimentable enzyme, indicating that inactivation

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DISCUSSION

The majority of the non-CaM-kinase-staining protein remains in the supernatant after 5 min of autophosphorylation. The 50- and 60-kDa subunits of CaM-kinase were the major constituents of the sedimentable protein following autophosphorylation. These data indicate that the formation of enzyme complexes is due primarily to interactions between soluble holoenzymes, presumably through self-association; however, a minor constituent of the sedimentable protein was detected at approximately 75 kDa, and it remains possible that proteins that co-purify with CaM-kinase could influence the formation and/or associate with sedimentable enzyme. We feel that this is an unlikely possibility because the formation of sedimentable enzyme during autophosphorylation also was observed using a recombinant preparation of the alpha subunit of CaM-kinase that is greater than 95% pure by Coomassie staining of SDS-PAGE analyzed protein.2

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FIG. 7. Electron micrographs of sedimentable CaM-kinase and SDS-PAGE analysis of sedimented enzyme. A and B, CaM-kinase autophosphorylated in standard reaction conditions was processed for TEM as described under “Experimental Procedures.” A, a field of uranyl acetate-staining structures at × 5000 following autophosphorylation in 0.01 mM ATP for 5 min at pH 6.5. B, the same field as in A but at a higher magnification (× 33,000). Arrows indicate polystyrene sizing beads 90 nm in diameter. Grid fields at high and low magnification are representative of the entire grid. C, analysis of sedimentable enzyme by SDS-PAGE and Coomassie Blue staining was performed on enzyme autophosphorylated in standard reaction conditions, with the exception of a decrease in reaction volume (50 μl) and an increase in the amount of enzyme (2.0 μg). Complete reactions were terminated at 0 and 5 min with EDTA (100 mM final). Centrifugation and processing of autophosphorylated enzyme was performed as described in Fig. 2 through SDS-PAGE. The gel was processed as described under “Experimental Procedures.” Arrows indicate the 50- and 60-kDa subunits of CaM-kinase. Supernatant (S) and pellet fractions (P) fractions are indicated. Positions of molecular mass standards are indicated on the right.
and the formation of sedimentable enzyme may occur by dis-
tinct processes. Enzyme inactivation could proceed at elevated
pH, yet potential protein-protein associations necessary for the
formation of sedimentable enzyme may be unstable or may not
form. A potential limitation of this interpretation is that
smaller complexes may still form during autophosphorylation
at pH 7.5 but are simply not detected by the sedimentation
criteria used in this study. Interestingly, we also observed that
increased pH slowed the generation of maximal independent-
activity in low ATP. Maximal independent activity at higher
pH values (pH 7.5) required increasing the time period of
preautophosphorylation on ice.

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we observed that both autophosphorylation in low ATP with
Mn2+ and in high ATP with Mg2+ prevented the inactivation of
CaM-kinase associated with autophosphorylation at 30 °C, in-
dicating that autophosphorylated enzyme is differentially sus-
ceptible to thermal inactivation. The time course of inactiva-
tion following autophosphorylation in low ATP at pH 6.5 was
correlated temporally with the incorporation of 32P into the
enzyme (see Fig. 4). Interpreting the role of autophosphoryla-
tion in the inactivation of CaM-kinase is complicated by a loss
of enzyme activity in the presence of Ca2+/CaM and in the
absence of autophosphorylation (see Fig. 5A). This process is
temperature-sensitive and also is attributed to thermal lability
(21, 23). Ishida et al. (35) recently reported that this mode of
inactivation may be produced via Ca2+/CaM interaction with a
low affinity CaM-binding domain. The relationship of this au-
tophosphorylation-independent inactivation to the inactivation
observed during autophosphorylation is unclear. Determining
the role of autophosphorylation in enzyme inactivation is fur-
ther complicated by potential alterations in the structure of
CaM-kinase associated with the formation of sedimentable en-
zyme. Stearic constraints due to enzyme aggregation may also
contribute to the loss of substrate phosphorylation observed.

Interestingly, the enzyme complexes formed during auto-
phosphorylation of purified CaM-kinase in our study (Fig. 7B)
appeared similar in ultrastructure to preparations of isolated
cytoskeletal complexes enriched in CaM-kinase previously de-
scribed (36). The formation of the enzyme complexes in our
study is apparently due to the self-association of soluble CaM-
kinase holoenzymes. The results of our study were consistent
with multiple CaM-kinase preparations, with enzyme purities
ranging from 70–90% of the total protein, and although poten-
tial interactions and associations with proteins that co-purify
with CaM-kinase cannot be excluded completely, they do not
significantly co-sediment with sedimentable enzyme (Fig. 7C).
Whether CaM-kinase self-association has any role in the for-
mation of cytoskeletal specializations such as postsynaptic den-
sities or the cytoskeletal complexes described by Sahyoun et al.
(36) is unknown.

The enzyme complexes shown in the micrographs (Fig. 7, A
and B) are representative of fields throughout the entire grid
and were observed only in autophosphorylation conditions that
produced sedimentable enzyme. However, what percentage of
the sedimentable enzyme has adopted this macromolecular
structure is difficult to ascertain. As suggested by the reviewer,
we attempted to quantify these structures by modeling the
volume for both holoenzymes and the enzyme complexes and
determining the theoretical number of holoenzymes required to
construct the enzyme complexes observed in Fig. 7. Based on
the assumptions and analysis provided under “Appendix,” we
estimated that 7% of the holoenzymes in the reaction were
recovered as enzyme complexes. Of the many assumptions
required to complete the analysis, the 7% estimation was
highly dependent upon the assumed volume of the holoenzyme.
This includes the values chosen for determining the volume of
holoenzyme as well as potential alterations in the holoenzyme's
volume associated with formation of enzyme complexes (i.e.
packing and compression due to aggregation). For example, if
the packing of the holoenzyme causes a decrease in their ap-
parent diameter from 30 to 20 nm, the enzyme recovered in-
creased to 15%, whereas an additional decrease in the diameter
to 10 nm produces a recovery of over 60%. One interpretation
from our calculations is that the formation of the enzyme com-
plexes could be associated with significant packing and conden-
sation of the holoenzymes, and structural alterations of the
enzyme over time could represent an important contribution to
the time-dependent losses in the enzyme's activity.

Although the role of autophosphorylation in the formation of
sedimentable enzyme is unknown, this phenomena was not
observed in the absence of autophosphorylation in the reaction
conditions of this study. However, in the absence of 10% gly-
cerol, we observed that 20–30% of the enzyme sedimented when
incubated at 30 °C in the presence of Ca2+/CaM at pH 6.5 for 5
or 10 min (data not shown). The formation of sedimentable
enzyme in the absence of autophosphorylation was not as rapid
nor as complete as the formation of sedimentable enzyme ob-
served with autophosphorylation, and was not detected in the
standard assay conditions containing 10% glycerol. Glycerol
and ethylene glycol are used routinely to stabilize purified
CaM-kinase and one possibility suggested from these data is
that these agents decrease enzyme aggregation.

CaM-kinase is isolated from both cytosolic and particulate
fractions (36–39). How CaM-kinase interacts or associates
within these subcellular compartments, and whether the en-
zeine's distribution is regulated is unknown. The changes in
the sedimentability of purified CaM-kinase observed during
conditional autophosphorylation in this study may reflect con-
formational changes in the enzyme that enable or promote
protein interactions with itself and/or other proteins. Subcel-
lular translocation of particulate to soluble CaM-kinase during
autophosphorylation has been reported in the Aplysia ganglia
(6), and the redistribution of the cytosolic enzyme into the
particulate fraction has been reported during neuronal activa-
tion (40, 41) and following epileptic (42) and ischemic condi-
tions (25, 26, 43). Cerebral ischemia is one well documented
example in which dramatic and rapid fluctuations occur to both
pH and ATP. Silver and Erecinska (44) demonstrated that,
within minutes, an ischemic insult altered the intracellular pH
in hippocampal neurons from approximately pH 7.4 to pH 6.2.
Folbergrova et al. (45) reported that the physiological ATP
concentration is approximately 2.8 mmol/kg of cortical tissue,
and that during ischemic insult, ATP stores are virtually de-
pleted within minutes. It is currently unknown whether auto-
phosphorylation plays a role in the inactivation and redis-
tribution of soluble CaM-kinase into the particulate fraction
during ischemia.

Our in vitro data predict that both a decrease in pH and ATP
concentration would result in alterations in the physical and
catalytic properties of CaM-kinase analogous to the changes
observed in vivo and in situ during an ischemic insult (25, 26,
43). Our in vitro data further predict that activation of cellular
CaM-kinase in conditions of neutral pH and high ATP would
result in the production of a predominantly soluble enzyme
with little corresponding inactivation. In contrast, the activa-
tion of CaM-kinase in conditions of neutral pH and low ATP
ultimately would result in the production of a predominantly
inactive soluble enzyme. The data presented in this report
suggest that different intracellular environments will modify
the functional output of CaM-kinase through autophosphorylation-associated changes in the activity and/or association of the enzyme with itself and potentially other proteins. The reversibility of autophosphorylation-associated inactivation and/or self-association is unknown. Bidirectional regulation is a hallmark of physiological regulatory mechanisms and the potential to yield active, soluble enzyme from sedimentable enzyme possibly through dephosphorylation and/or other conditions warrants future investigation.

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APPENDIX

In an attempt to estimate the number of holoenzymes recovered as enzyme complexes (see Fig. 7, A and B), the micrographs were digitized and the electron dense particles quantified using Image software (version 1.44, Wayne Rasband at NIMH). The steps in analysis are given in Table I. Several assumptions were required to complete the calculations.

First Assumption: Holoenzyme Is a Cylinder—The diameter of a holoenzyme has been estimated to be approximately 30 nm (34). The “dumbbell” shaped models proposed by Kanaseki et al. (34) appear cylindrical; however, no published information is available concerning the holoenzyme’s height. We utilized a uniform height of 5 nm, a size slightly smaller value than the predicted size of the “lollipops” (6 nm) described by Kanaseki et al. (34).

Second Assumption: Enzyme Complexes Are Spherical—The average diameter of an enzyme complex is roughly 200 nm. However, two-dimensional projections make it impossible to determine whether the volume occupied is spherical, elliptical or discoidal. A spherical volume is the easiest geometry to model in the absence of any three-dimensional information.

Third Assumption: Holoenzymes Packed into Complexes Are Modeled as Nonoverlapping 30-nm Diameter Spheres—The packing density of the holoenzymes within the enzyme complexes is unknown (i.e. whether the holoenzyme’s volume is altered by aggregation). We hypothesize that the space occupied by aggregating holoenzymes condenses; however, we have no information to model these changes. Therefore, we have taken the simplest possibility and assumed that the holoenzymes within the enzyme complex retain their initial starting volume.

Fourth Assumption: Total Number of Enzyme Complexes Is Uniform over the Entire Grid—We have assumed a uniform protein distribution over the entire grid because we have no way to determine whether the copper mesh and frame of the grid influences the distribution of the deposited protein.

Fifth Assumption: 27% of the Starting Protein Is Deposited on the Grid—Although we were unable to quantify the protein deposited on the carbon-coated grid, the difference in the protein recovered in the microconcentrator in the presence and absence of a grid was quantifiable. Approximately 73% ± 12% (n = 4, with 2 microconcentrators per n) of the protein was indicating that only 27% of the protein applied to the microconcentrator was potentially deposited on the grid. Whether all of this protein (27% of the total or 0.27 μg) is actually deposited on the grid or whether staining and washing further alters this value is unknown. This corrected value was used as the amount of starting enzyme for the analysis.

REFERENCES
1. Hanson, P. I., and Schulman, H. (1992) Annu. Rev. Biochem. 61, 559–601
2. Colbran, R. J. (1992) Neurochem. Int. 21, 469–497
3. Bennett, M. K., Erdondu, N. E., and Kennedy, M. B. (1983) J. Biol. Chem. 258, 12735–12744
4. Kuret, J., and Schulman, H. (1984) Biochemistry 23, 5495–5504
5. Dunkley, P. R. (1991) Mol. Neurobiol. 5, 179–202
6. Saitoh, T., and Schwartz, J. H. (1985) J. Cell Biol. 100, 835–842
7. Miller, S. G., and Kennedy, M. B. (1986) Cell 44, 861–870
8. Fong, Y. L., Taylor, W. L., Means, A. R., and Soderling, T. R. (1989) J. Biol. Chem. 264, 16759–16763
9. Hanson, P. I., Kaploff, M. S., Lou, L. L., Rosenfeld, M. G., and Schulman, H. (1989) Neuron 3, 59–70
10. Washam, M. N., Aronowski, J., Westgate, S. A., and Kelly, P. T. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 1275–1277
11. Hashimoto, Y., Schwer, R. K., Colbran, R. J., and Soderling, T. R. (1987) J. Biol. Chem. 262, 8051–8055
12. Patton, B. L., Miller, S. G., and Kennedy, M. B. (1990) J. Biol. Chem. 265, 11204–11212
13. Colbran, R. J., and Soderling, T. R. (1990) J. Biol. Chem. 265, 11213–11219
14. Hanson, P. I., and Schulman, H. (1992) J. Biol. Chem. 267, 17216–17224
15. Colbran, R. J. (1993) J. Biol. Chem. 268, 7163–7170
16. Meyer, T., Hanson P. I., Stryer, L., and Schulman, H. (1992) Science 256, 1199–1202
17. Hanson, P. I., Meyer, T., Stryer, L., and Schulman, H. (1994) Neuron 12, 943–956
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18. Kuret, J., and Schulman, H. (1985) J. Biol. Chem. 260, 6427–6433
19. Yamauchi, T. and Fujisawa, H. (1985) Biochem. Biophys. Res. Commun. 129, 213–219
20. Bronstein, J. M., Farber, D. B., and Wasterlain, C. G. (1986) FEBS Lett. 196, 135–138
21. Lai, Y., Nairn, A. C., and Greengard, P. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 4253–4257
22. Lou, L. L., Lloyd, S. J., and Schulman, H. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 9497–9501
23. Rostas, J. P., Brent, V., and Dunkley, P. R. (1987) Neurosci. Res. Commun. 1, 3–8
24. Lickteig, R., Shenolikar S., Denner, L., and Kelly, P. T. (1988) J. Biol. Chem. 263, 19232–19239
25. Aronowski, J., Grotta, J. C., and Waxham, M. N. (1992) J. Neurochem. 58, 1743–1753
26. Kolb, S. J., Hudmon, A., and Waxham, M. N. (1995) J. Neurochem. 64, 2147–2152
27. Lou, L. L., and Schulman, H. (1989) J. Neurosci. 9, 2020–2032
28. Gopalakrishna, R., and Anderson, W. B. (1982) Biochem. Biophys. Res. Commun. 104, 830–836
29. Kelly, P. T., and Shenolikar, S. (1987) Methods Enzymol. 139, 690–714
30. Laemmli, U. K. (1970) Nature 227, 680–685
31. Waxham, M. N., Aronowski, J., and Kelly, P. T. (1989) J. Biol. Chem. 264, 7477–7482
32. Scholz, W. K., Baitinger, C., Schulman, H., and Kelly, P. T. (1988) J. Neurosci. 8, 1039–1051
33. Roskoski, R., J. r. (1983) Methods Enzymol. 99, 3–6
34. Kanaseki, T., Ikeuchi, Y., Sugiura, H., and Yamauchi, T. (1991) J. Cell Biol. 115, 1049–1060
35. Ishida, A., Kitani, T., Okuno, S., and Fujisawa, H. (1994) J. Biochem. (Tokyo) 115, 1075–1082
36. Sahyoun N., LeVine, H. I., Bronson D., Siegel-Greengstein, F., and Cuatrecasas, P. (1985) J. Biol. Chem. 260, 1230–1237
37. Kennedy, M. B., McGuiness, T., and Greengard, P. (1983a) J. Neurosci. 3, 818–831
38. Kennedy M. B., Bennet, M. K., and Erondou, N. E. (1983b) Proc. Natl. Acad. Sci. U.S.A., 80, 7357–7361
39. Goldenring, J. R., Casanova, J. E., and Delcorno, R. J. (1984) J. Neurochem. 43, 1669–1679
40. Bronstein, J. M., Wasterlain, C. G., Lasher, R., and Farber, D. B. (1989) Brain Res. 495, 83–88
41. Willmund, R., Mitschulat, H., and Schneider, K. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 9789–9793
42. Bronstein, J. M., Farber, D. B., and Wasterlain, C. G. (1993) Brain Res. Rev. 18, 125–147
43. Shackelford, D. A., Yeh, R. Y., and Zivin, J. A. (1993) J. Neurochem. 61, 738–747
44. Silver, I. A., and Erecinska, M. (1992) J. Cereb. Blood Flow Metab. 12, 759–772
45. Folbergrova, J., Minamisawa, H., Ekholm, A., and Siesjo, B. K. (1990) J. Neurochem. 55, 1690–1696