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Chemical Quenched Flow Kinetic Studies Indicate an Intraholoenzyme Autophosphorylation Mechanism for Ca^{2+}/Calmodulin-dependent Protein Kinase II*

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Autophosphorylation of α-Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) at Thr-286 generates Ca^{2+}-independent activity that outlasts the initial Ca^{2+} stimulus. Previous studies suggested that this autophosphorylation occurs between subunits within each CaM kinase II holoenzyme. However, electron microscopy studies have questioned this mechanism because a large distance separates a kinase domain from its neighboring subunit. Moreover, the recently discovered ability of CaM kinase II holoenzymes to self-associate has raised questions about data interpretation in previous investigations of autophosphorylation. In this work, we characterize the mechanism of CaM kinase II autophosphorylation. To eliminate ambiguity arising from kinase aggregation, we used dynamic light scattering to establish the monodispersity of all enzyme solutions. We then found using chemical quenched flow kinetics that the autophosphorylation rate was independent of the CaM kinase II concentration, results corroborating intraholoenzyme activation. Experiments with a monomeric CaM kinase II showed that phosphorylation of this construct is intermolecular, supporting intersubunit phosphorylation within a holoenzyme. The autophosphorylation rate at 30 °C was ~12 s^{-1}, more than 10-fold faster than past estimates. The ability of CaM kinase II to autophosphorylate through an intraholoenzyme, intersubunit mechanism is likely central to its functions of decoding Ca^{2+} spike frequency and providing a sustained response to Ca^{2+} signals.

Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II)[1] is involved in numerous cellular processes including cell cycle regulation, apoptosis, protein secretion, and gene expression (1–3). In the brain, CaM kinase II is essential for long term potentiation, a hypothesized cellular mechanism for learning and memory (4, 5). Autophosphorylation plays a central role in the function of CaM kinase II. In the basal state, the enzyme is inactive because of the intramolecular binding of an autoinhibitory sequence to the kinase active site (Fig. 1*A*). As the intracellular Ca^{2+} concentration rises above 200 nM, Ca^{2+}-bound calmodulin (Ca^{2+}/CaM) begins to bind CaM kinase II, displacing the autoinhibitory segment, and allowing the kinase to phosphorylate itself at Thr-286 (α-subunit; 287 for other subunits). This autophosphorylation has several important functional consequences: 1) it allows CaM kinase II to retain enzymatic activity in the absence of CaM (6); 2) it causes a greater than 1,000-fold decrease in the dissociation rate of CaM from the enzyme, a phenomenon referred to as "CaM trapping" (7–10); 3) it gives CaM kinase II the ability to decode the frequency of Ca^{2+} oscillations and translate this information into different levels of enzymatic activity (11). The biological significance of Thr-286 autophosphorylation has been established using a "knock-in" mouse mutant in which the endogenous CaM kinase II contains Ala instead of Thr at residue 286 (12). Although the mutation does not alter the level of kinase protein or the ability of the kinase to be activated by Ca^{2+}/CaM, the mutant mouse does not display hippocampal long term potentiation and is deficient in spatial learning.

A striking feature of CaM kinase II is that it assembles to form a ~600-kDa holoenzyme consisting of 12 subunits, each containing a kinase domain. This molecular arrangement suggests three possible mechanisms by which autophosphorylation of Thr-286 might occur: 1) within the same polypeptide chain (intraholoenzyme, intrasubunit autophosphorylation), 2) by a different polypeptide chain within the same molecular assembly (intraholoenzyme, intersubunit autophosphorylation), and 3) by a kinase domain from a different assembly (interholoenzyme autophosphorylation). Previous studies have attempted to distinguish among these mechanisms. It has been found that the rate of autophosphorylation is not dependent on CaM kinase II concentration, a result that would be expected only if autophosphorylation were intraholoenzyme (13–15). Further experiments employing CaM kinase II holoenzymes that have been engineered to contain both kinase-active and kinase-inactive subunits suggest that autophosphorylation occurs in an intersubunit fashion within each holoenzyme (15–17).

Although biochemical studies of CaM kinase II have supported an intraholoenzyme, intersubunit autophosphorylation mechanism, structural evaluations of the enzyme do not readily suggest how this type of autophosphorylation might occur. Although no crystal structure of CaM kinase II has yet been obtained, several studies have probed the structure using transmission electron microscopy (18–21). A recent three-dimensional view of CaM kinase II has provided considerable detail regarding the architecture of the enzyme (Fig. 1, *B* and *C*) (20). Here it was demonstrated that the association domains of CaM kinase II form a gear-shaped core for the holoenzyme, and the kinase domains emanate as foot-like processes outward

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[1] The abbreviations used are: CaM kinase II, Ca^{2+}/calmodulin-dependent protein kinase II; Ca^{2+}/CaM, Ca^{2+}-bound calmodulin; α-CaM kinase II, α-subunit of CaM kinase II; mCaMKII, monomeric CaM kinase II; PIPES, 1,4-piperazinediethanesulfonic acid.

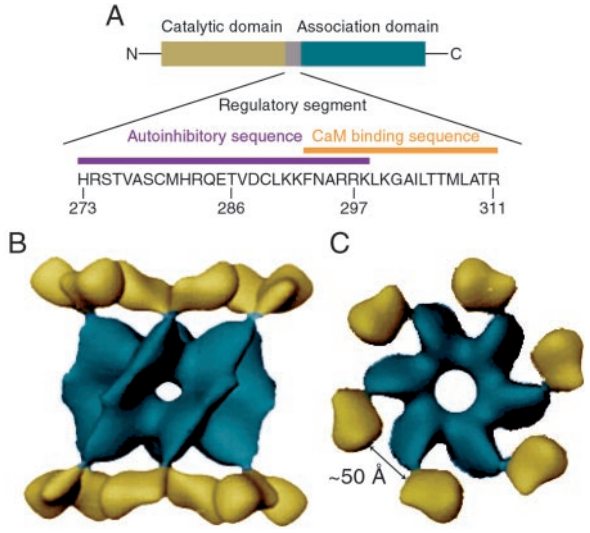

FIG. 1. **Structure of the CaM kinase II holoenzyme.** *A*, domain organization of α-CaM kinase II. The catalytic domain (*gold*), regulatory segment (*gray*), and association domain (*blue*) are shown. The regulatory segment comprises a stretch of amino acids which contains both an autoinhibitory sequence (*purple*) and a CaM binding motif (*orange*). *B*, shaded surface view from the side of α-CaM kinase II (20). Each holoenzyme consists of 12 CaM kinase II polypeptide chains. The association domain of each polypeptide chain (shown in *blue*) forms a phalange-shaped structure; each catalytic domain (shown in *gold*) emanates outward from the center of the structure as a foot-like process. The structure is arranged so that the catalytic domains are grouped into two sets of six with one ringed set of catalytic domains at the "top" of the assembly and the other at the "bottom." Overall, the structure has 622 symmetry. *C*, top view of the holoenzyme. This representation shows only the domains comprising the top part of the holoenzyme (20).

from the center forming two hexameric ring structures. Importantly, a large distance (~50 Å) separates neighboring kinase domains. Because intraholoenzyme, intersubunit autophosphorylation would require that neighboring kinase domains be in close proximity and this is not seen in the structure, the recent electron microscopy analysis does not favor an intraholoenzyme, intersubunit mechanism.

How might the seemingly contradictory structural and biochemical data be reconciled? One hypothesis derives from the propensity of CaM kinase II to form supramolecular assemblies of many holoenzymes. This property of CaM kinase II has been characterized recently *in vitro* (22, 23) and has also been shown to occur within neurons (24, 25). If CaM kinase II was in a supramolecular form in previous biochemical evaluations of the autophosphorylation mechanism, the results obtained in these studies may be ambiguous: it is possible that the autophosphorylation was occurring between different holoenzymes but appeared to be intraholoenzyme because of the supramolecular, or aggregated, state of the kinase.

In this work we investigated the autophosphorylation mechanism of CaM kinase II in light of the recent three-dimensional electron microscopy structure. To eliminate experimental ambiguity arising from the state of aggregation of the kinase, dynamic light scattering was used to establish that CaM kinase II is monodisperse during autophosphorylation. The autophosphorylation process was then characterized using chemical quenched flow kinetics, the first application of this approach to study CaM kinase II. It was determined that the rate of autophosphorylation of a monodisperse CaM kinase II holoenzyme solution was independent of CaM kinase II concentration, supporting an intraholoenzyme autophosphorylation mechanism. Further studies with a monomeric version of CaM kinase II (mCaMKII) demonstrated that this molecule phosphorylates through an intermolecular process, suggesting that autophosphorylation within the holoenzyme is intersubunit. The rate constant for autophosphorylation at near physiological temperatures was ~12 s^{-1}, more than 10-fold faster than previous estimates of the CaM kinase II autophosphorylation rate.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—The rat α-CaM kinase II cDNA in the vector pBakPak9 (CLONTECH) and virus containing the cDNA were gifts from Dr. Neal Waxham (8). The α-CaM kinase II gene was subcloned previously into the vector pFasBak for expression in the baculovirus Sf21 cell expression system (Invitrogen) (10). Virus containing the α-CaM kinase II cDNA was first produced in monolayer Sf21 cultures as described by the manufacturer. For protein expression, virus was added to a 500-ml Sf21 suspension culture for 60–72 h; cells were then harvested and either stored at −80 °C or used immediately for protein purification (10).

α-CaM kinase II was purified as described by Singla *et al*. (10) with a few modifications. Briefly, cell pellets were resuspended, lysed by Dounce homogenization and sonication, and clarified by centrifugation. The supernatant was loaded onto a 10-ml phosphocellulose column (P-11 cation exchange resin, Whatman) and eluted in a 120-ml gradient from 0.1 to 0.5 M NaCl. The appropriate fractions were pooled. For one light scattering experiment, the material was purified further using a CaM-Sepharose column as described (10). In all other experiments, the material was then applied at 0.8 ml/min to a Sephacryl S-300 Sepharose gel filtration column (Amersham Biosciences) that had been equilibrated in previously 20 mM Hepes pH 7.4, 200 mM KCl, 0.1 mM EDTA, 1 mM β-mercaptoethanol. Pure fractions were pooled and stored on ice until further use. If necessary, the enzyme was concentrated at 4 °C in a Centriprep centrifugal filter device (Amicon) with a YM-50 membrane. Both monomeric α-CaM kinase II and α-CaM kinase II (T286A) were purified in the same manner as wild type. CaM was purified as described (10).

The concentration of α-CaM kinase II was evaluated using an extinction coefficient of 66,350 M^{-1} cm^{-1} obtained from the protein sequence (26, 27). All reported concentration units for CaM kinase II represent the concentration of single CaM kinase II polypeptide chains, not the concentration of holoenzymes. An extinction coefficient of 44,890 M^{-1} cm^{-1} was used for the mCaMKII construct, which is formed from residues 1–326 of CaM kinase II.

Dynamic Light Scattering—Dynamic light scattering experiments were performed with a DynaPro 801 instrument (Protein Solutions, Ltd.). Protein samples were either centrifuged at 10,000 × *g* or sterile filtered to remove dust particles before analysis. The samples were then illuminated at 780 nm using a solid state laser. Each sample was evaluated at least 20 times, with the quoted values being the mean of these independent evaluations.

Dynamic light scattering experiments provide a direct determination of the diffusion coefficient (*D*) of the particles in solution (www.proteinsolutions.com). Using the diffusion coefficient, the hydrodynamic radius (R_H) of the particles can be calculated from the Stokes-Einstein equation

$$D = kT/6\pi\eta R_H \qquad \text{(Eq. 1)}$$

where *k* is the Boltzmann constant, *T* is the absolute temperature, and η is the solvent viscosity. Approximate molecular weights for each of the species were determined from R_H by the analysis software using a reference standard curve. For experiments in which CaM kinase II holoenzymes were autophosphorylated before analysis, the autophosphorylation reaction time was 1 min.

Chemical Quenched Flow and Bench Top Kinetic Experiments—Chemical quenched flow experiments were performed with a model RQF-3 chemical quenched flow device from Kintek Corporation (Austin, TX). The instrument was maintained at the experimental temperature using either a circulating, temperature-controlled bath or an ice water bath. Before the quenched flow analysis, all solutions were maintained on ice. To initiate a quenched flow experiment, Ca^{2+}/CaM was first added to CaM kinase II (in gel filtration buffer: 20 mM Hepes pH 7.4, 200 mM KCl, 0.1 mM EDTA, 1 mM β-mercaptoethanol). The solution was immediately added to sample loop A. Then, a buffered Mg^{2+}/ATP solution was added to loop B. In our experimental setup, 11.5 μl of sample from loop A was mixed rapidly with 11.5 μl from loop B, and the solution was then quenched with 67 μl of the quench solution (20 mM Hepes pH 7.4, 200 mM KCl, 0.1% bovine serum albumin, 50 mM EDTA, 1 mM EGTA). The solution conditions during the autophosphorylation reaction were (except where noted) 20 mM Hepes pH 7.4, 200 mM KCl, 500

TABLE I
Dynamic light scattering data

The dynamic light scattering device provided the hydrodynamic radius (R_H ± S.D.), approximate molecular mass (molecular mass ± S.D.), and base-line parameter for solutions of CaM kinase II holoenzyme (CaMKII) and monomer (mCaMKII). If the base-line value falls in the range of 0.997–1.001, the solution is monomodal. If it is greater than 1.005, the solution is polydisperse. If the value is in the range 1.002–1.005, the solution is primarily monomodal but contains a few large particles.

Kinase form	Temperature	R_H	Approximate molecular mass[a]	Expected	Base line
	°C	*nm*	*kDa*	*kDa*	
CaMKII	0	9.12 ± 0.2	590 ± 35	649	1.000
CaMKII, CaM-bound	0	10.5 ± 0.2	825 ± 48	851	1.001
CaMKII, CaM-bound, +Mg^{2+}/ATP	0	12.1 ± 0.3	1,150 ± 52	851	1.001
CaMKII, CaM-bound, +Mg^{2+}/ATP	23	12.0 ± 0.4	1,127 ± 72	851	1.001
CaMKII, CaM-bound, +Mg^{2+}/ATP	30	12.8 ± 0.4	1,438 ± 69	851	1.002
CaMKII (T286A)	0	9.42 ± 0.2	645 ± 35	649	1.000
mCaMKII	0	3.26 ± 0.2	53.1 ± 10	36.8	1.002
mCaMKII, CaM-bound	0	3.38 ± 0.2	58.2 ± 11	53.6	1.000
mCaMKII, CaM-bound, +Mg^{2+}/ATP	0	3.75 ± 0.2	74.3 ± 13	53.6	1.001
CaMKII, CaM-Sepharose purified[b]	0	16.7 ± 2.6	2,621 ± 370	649	1.009
CaMKII, CaM-bound, +Mg^{2+}/ADP	0	316 ± 29	2,390,000 ± 250,000	851	1.001
CaMKII, CaM-bound, +Mg^{2+}/ATP[c]	0	76 ± 9	19,700 ± 2,500	851	1.079

[a] The approximate molecular mass is determined experimentally from the R_H value; the expected molecular mass is calculated from the actual molecular masses of CaMKII (649 kDa), CaM (16.8 kDa), and mCaMKII (36.8 kDa).
[b] For this experiment, material was purified using a CaM-Sepharose rather than a gel filtration column.
[c] The solution for this experiment was 50 mM PIPES pH 7.0. For all other experiments, the solution was 20 mM Hepes pH 7.4, 200 mM KCl.

μM Ca^{2+}, 10 μM CaM, 500 μM ATP, and 2 mM Mg^{2+}.

Experiments at 0.58, 0.14, and 0.035 μM mCaMKII were slow enough to be evaluated with bench top experiments. Here, 11.5 μl of both the Ca^{2+}/CaM/CaM kinase II mixture and the Mg^{2+}/ATP solution were pipetted together to initiate autophosphorylation. After varying times, the reaction was quenched with 67 μl of quench buffer and evaluated for autonomous activity as described below.

Autonomous Activity Assays—After quenching of the autophosphorylation reaction, the level of autonomous activity of the enzyme solution was evaluated immediately. Here, 10 μl of the quenched solution was added to 40 μl of the autonomous kinase assay mixture. For all experiments, the Ca^{2+}/CaM activity of the quenched solution was also evaluated. The solution conditions of the assay mixtures were 50 mM PIPES pH 7.0, 20 mM Mg^{2+}, 200 μM ATP, 0.1% bovine serum albumin, 50 μM autocamtide-2 kinase substrate, and 1 μCi of [^{32}P]ATP; in addition, the autonomous assays contained 1 mM EGTA, whereas the Ca^{2+}/CaM assays contained 500 μM Ca^{2+} and 2 μM CaM. For both autonomous and Ca^{2+}/CaM assays, experiments were performed in duplicate. When high concentrations of CaM kinase were used, solutions were first diluted in quench buffer before addition to kinase assays so that the final enzyme concentration was ~10 nM. Enzyme activity was evaluated for 1 min at 30 °C. 20-μl aliquots of kinase assay were then spotted on P-81 phosphocellulose paper, placed in 0.5% phosphoric acid, washed in water, dried, and evaluated with scintillation counting. The specific Ca^{2+}/CaM activity of the holoenzyme was typically 5–20 μmol/min/mg, whereas that of the mCaMKII was 4–15 μmol/min/mg.

^{32}P Incorporation Experiments—For the experiments in which Thr-286 autophosphorylation was monitored by ^{32}P incorporation, the initial experimental setup was identical to the chemical quenched flow experiments described above except that the 11.5 μl of Mg^{2+}/ATP solution also contained 0.5 μCi of [^{32}P]ATP. After quenching, 20 μl of the solution was added to 10 μl of SDS-PAGE loading buffer and run on 10% SDS-PAGE. Protein bands corresponding to CaM kinase II were excised and evaluated using scintillation counting.

Kinetic Data Analysis—Data analysis was performed with the program Scientist (Micromath, Salt Lake City, UT). For experiments with the holoenzyme, the ratio of autonomous to Ca^{2+}/CaM activity was determined at each data point. This ratio multiplied by 100 was then fit to the following increasing exponential function to determine the first order rate constant for autophosphorylation.

$$A = M(1 - e^{-k_1 t}) \quad \text{(Eq. 2)}$$

Here A is the percent autonomy, M is the maximal autonomy, k_1 is the first order rate constant for intraholoenzyme autophosphorylation, and t is time.

Unlike intraholoenzyme autophosphorylation, intermolecular autophosphorylation requires that the kinetic data be fit with the dependent variable in units of concentration rather than percent autonomy. Hence, for experiments with mCaMKII, the percent autonomy was first determined at each data point. Then this value was multiplied by the concentration of monomer in the experiment to determine the concentration of phosphorylated monomer (P) at each data point. The values of P were then fit to the following expression to determine the second order rate constant (k_2) for intermolecular autophosphorylation.

$$P = C^2 k_2 t/(Ck_2 t + 1) \quad \text{(Eq. 3)}$$

Here, C is the total concentration of CaM kinase II monomer in the experiment.

To evaluate the temperature dependence of CaM kinase II autophosphorylation, the data were analyzed in terms of the Arrhenius equation

$$k_1 = Be^{-E_a/RT} \quad \text{(Eq. 4)}$$

where E_a is the activation energy of the process, R is the gas constant, T is the absolute temperature, and B is a constant.

Decay of Enzyme Activity with Temperature—To monitor the decay of enzymatic activity with temperature after autophosphorylation, 9.2 μl of 0.5 μM CaM kinase II was mixed with a 2.3-μl mixture of 2.5 mM Ca^{2+} and 10 μM CaM and incubated at the various temperatures. A 11.5-μl solution of 4 mM Mg^{2+} and 1 mM ATP was added, and autophosphorylation was allowed to occur for 15 s, 1 min, or 3 min. The quench solution was then added, and the Ca^{2+}/CaM activity was assessed.

RESULTS

Evaluation of the Self-association State of CaM Kinase II Holoenzymes—Dynamic, or quasi-elastic, light scattering was used to evaluate the state of aggregation α-CaM kinase II holoenzymes and α-CaM kinase II mutants (29, 30). This technique monitors the fluctuation in scattering intensity of particles in solution illuminated using a monochromatic light source (www.protein-solutions.com). Through an autocorrelation analysis of the scattering intensity fluctuations, one can determine whether the particles in solution are all of uniform size (monodisperse) or varying sizes (polydisperse). This is evaluated using the base-line parameter (See Table I) (www.protein-solutions.com). In addition, dynamic light scattering also allows a determination of the particle hydrodynamic radius, and this provides an approximate molecular weight of the scattering particles. In the following analysis, solutions were concluded to be free of aggregation only if they contained an approximate molecular weight appropriate for the particle under investigation and also a base-line parameter within (or very close to) the monodisperse range.

Table I summarizes the dynamic light scattering data for wild type and mutant forms of α-CaM kinase II. α-CaM kinase II purified as described under "Experimental Procedures" was monodisperse. If 500 μM Ca^{2+} and equimolar CaM were added to α-CaM kinase II, the material remained monodisperse but

now gave a slightly higher approximate molecular weight, consistent with the molecular weight of the α-CaM kinase II holoenzyme plus 12 CaM molecules. If, in addition to Ca^{2+}/CaM, a solution of 2 mM Mg^{2+} and 500 μM ATP was added to facilitate autophosphorylation, the solution also remained monodisperse as evidenced by the base-line parameter. The concentration of CaM kinase II in these experiments was typically 3–5 μM; however, experiments at 10 μM CaM kinase II gave nearly identical results. Solutions of a CaM kinase II (T286A) mutant and mCaMKII were also established to be monodisperse.

Several conditions produced solutions that did not contain monodisperse holoenzymes. If CaM kinase II was purified using a CaM-Sepharose column (rather than the standard purification with a gel filtration column), a monodisperse preparation was not obtained. Furthermore, if ADP replaced ATP in the autophosphorylation reaction, particles with a substantially larger radius of hydration than found in single holoenzymes were formed. Finally, it was observed that whether or not α-CaM kinase II remained monodisperse during autophosphorylation was dependent on experimental solution conditions. If experiments were performed at pH 7.0 and no added salt, rather than at the standard conditions of pH 7.4 and 200 mM KCl, α-CaM kinase II aggregated during autophosphorylation (Table I). These findings are consistent with previous results demonstrating that aggregation of α-CaM kinase II during autophosphorylation is influenced by both pH and ionic strength (23).

Characterization of the Time Course of CaM Kinase II Autophosphorylation by Chemical Quenched Flow Kinetics—The dynamic light scattering experiments described above established experimental conditions under which CaM kinase II does not aggregate during autophosphorylation. The kinetics of CaM kinase II autophosphorylation were then explored under the same conditions. Two different methods of monitoring Thr-286 autophosphorylation were utilized. The first was the autonomous activity of CaM kinase II (the activity in the absence of Ca^{2+}/CaM), which has previously been determined to reflect the level of Thr-286 autophosphorylation (31). The second was the level of ^{32}P incorporation into the enzyme after autophosphorylation with [^{32}P]ATP (32).

Fig. 2 compares the time courses of autonomous activity generation and ^{32}P incorporation for chemical quenched flow experiments performed under identical solution conditions (0 °C, 2.5 μM ATP). It was observed that the time courses of autonomy generation and ^{32}P incorporation were nearly identical, suggesting that both methods are reflecting accurately the level of Thr-286 autophosphorylation under these conditions. Fitting the data to an exponential function to obtain the autophosphorylation rate constant for each experiment gave values of 0.064 and 0.056 s^{-1} for the autonomy and ^{32}P incorporation experiments, respectively. The autonomous activity saturated at a level that was ~70% of the activity in the presence of Ca^{2+}/CaM, a maximal autonomous activity similar to that observed in previous studies (11). The maximal ^{32}P incorporation approached 1 mol of phosphate/1 mol of subunit. Control autophosphorylation experiments that employed a CaM kinase II mutant (CaM kinase II (T286A)) had <1% of both the maximal autonomous activity and maximal ^{32}P incorporation of the wild type enzyme, further indicating that both probes reflect the level of Thr-286 autophosphorylation under these conditions accurately.

To study the mechanism of CaM kinase II autophosphorylation, conditions must first be established in which ATP binding to the enzyme is not the rate-limiting step of autophosphorylation. To ascertain these conditions experimentally, the rate of autophosphorylation of CaM kinase II was evaluated at

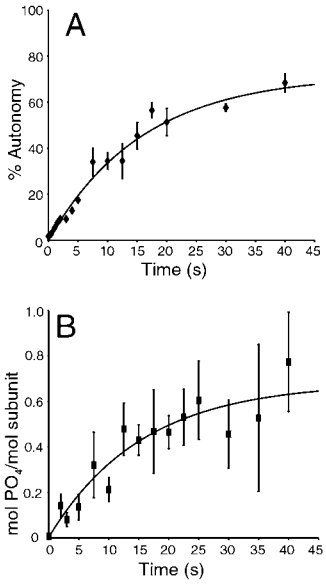

FIG. 2. **Time course of CaM kinase II autophosphorylation monitored by chemical quenched flow kinetics.** *A*, generation of autonomous activity as a function of autophosphorylation time. Plotted is the percentage of autonomous enzymatic activity compared with Ca^{2+}/CaM activity (±S.D., $n = 2$) *versus* time (in seconds). *B*, incorporation of phosphate into CaM kinase II monitored using [^{32}P]ATP as a function of time. Plotted is the mol ratio of phosphate to CaM kinase II subunit (±S.D., $n = 4$) *versus* time. For both experiments, the autophosphorylation conditions were 20 mM Hepes pH 7.4, 200 mM KCl, 500 μM Ca^{2+}, 10 μM CaM, 2.5 μM ATP, 2 mM Mg^{2+}, 0.46 μM CaM kinase II, 0 °C.

different concentrations of ATP (Fig. 3). At the lowest concentration of ATP examined (2.5 μM), the rate of autophosphorylation was 0.062 s^{-1}. However, at concentrations of 100, 500, and 2,500 μM ATP, an autophosphorylation rate of ~0.4 s^{-1} was observed (Table II). Hence, at ATP concentrations greater than 100 μM, the experimentally observed rate constant reflects an event involved in the autophosphorylation process, not ATP binding. These results are consistent with the previously determined ATP K_m for autophosphorylation of ~20 μM (33).

Concentration Dependence of the CaM Kinase II Holoenzyme Autophosphorylation Rate—To determine whether autophosphorylation of CaM kinase II is intra- or interholoenzyme, we examined how the rate of autophosphorylation varied with the CaM kinase II concentration. Intraholoenzyme and interholoenzyme autophosphorylation reactions would be expected to show different kinetic signatures (34, 35). If autophosphorylation was intraholoenzyme, it would be best modeled as a first order transition governed by the first order rate constant k_1. In this case, the reaction time course would be an exponential that would be independent of the enzyme concentration. However, if autophosphorylation were interholoenzyme, it would require interaction of two holoenzymes and hence be a second order process. Here, the reaction time course would be parabolic and strongly dependent on enzyme concentration.

Chemical quenched flow experiments were performed at seven different concentrations of CaM kinase II between 0.05 and 12.72 μM. It was observed that the time courses of autonomy generation were similar at all concentrations (Fig. 4*A*), demonstrating only slight differences in their level of maximal autonomy. Each time course fit well to an exponential function, and the rate constant for the autophosphorylation process was between 0.33 and 0.63 s^{-1} for all experiments. The plot of the observed rate constant *versus* CaM kinase II concentration in Fig. 4*B* illustrates that the rate of autophosphorylation is independent of holoenzyme concentration.

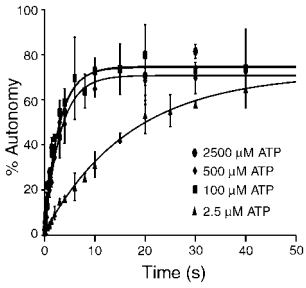

FIG. 3. **Time course of CaM kinase II autophosphorylation at different ATP concentrations.** Plotted is the percentage of autonomous enzymatic activity compared with Ca^{2+}/CaM activity (±S.D., n = 2) *versus* time (in seconds). The *solid lines* are nonlinear least squares best fits of each data set to Equation 2. The *ovals*, *diamonds*, *squares*, and *triangles* represent experiments at 2,500, 500, 100, and 2.5 μM ATP, respectively.

We probed the mechanism of autophosphorylation further by asking whether addition of a high concentration of α-CaM kinase II (T286A), a form of the enzyme which is just as active as wild type but does not display autonomous activity, increased the rate of autophosphorylation of wild type α-CaM kinase II. If autophosphorylation is interholoenzyme, it would be expected that wild type α-CaM kinase II would be phosphorylated more rapidly in a solution that also contained a high concentration of α-CaM kinase II (T286A) compared with one that did not. However, if autophosphorylation is intraholoenzyme, the addition of α-CaM kinase II (T286A) would be expected to have no effect on the rate of wild type α-CaM kinase II phosphorylation at Thr-286. Table II shows the results of a quenched flow experiment with a 0.46 μM solution of α-CaM kinase II plus 3.4 μM α-CaM kinase II (T286A). Under these conditions the autonomous activity is generated at the same rate as in the absence α-CaM kinase II (T286A). These results, together with the lack of concentration dependence of the autophosphorylation rate, indicate that autophosphorylation of CaM kinase II occurs intraholoenzyme.

Concentration Dependence of the Autophosphorylation Rate for a Monomeric CaM Kinase II—We studied the concentration dependence of the autophosphorylation rate for mCaMKII to determine whether CaM kinase II autophosphorylation within holoenzymes occurs by an inter- or intrasubunit reaction. In this form of the protein, the oligomerization domain has been deleted, but the kinase domain and regulatory segment have been retained. Previous studies have found that this construct is nearly identical to wild type in its activation by calmodulin, its activity toward peptide substrates, and the site specificity at several autophosphorylation sites (15). Here it was observed that the time course of autophosphorylation was strongly dependent on enzyme concentration. This is best illustrated by examining the half-times of the reactions ($t_{1/2}$), the time it takes for half of the molecules to be autophosphorylated (Table III). The $t_{1/2}$ for autophosphorylation of the mCaMKII varied from 17.8 to 899 s depending on mCaMKII concentration, whereas those for the CaM kinase II holoenzyme were constant at around 2 s.

The fact that the $t_{1/2}$ values were highly dependent on the mCaMKII concentration suggested that the data should be analyzed in terms of an intermolecular process (see "Experimental Procedures"). Fig. 5 shows the concentration of phosphorylated mCaMKII as a function of time for four different experiments. Fitting each data set to the parabolic time course expected for an intermolecular process provided a good fit and gave a value of ~25,000 M^{-1} s^{-1} for the second order rate constants for each data set. Fitting each data set to an exponential function did not provide a better fit to the data. The fact that mCaMKII phosphorylates in an intermolecular fashion supports the notion that autophosphorylation within the holoenzyme occurs intersubunit.

Temperature Dependence of CaM Kinase II Autophosphorylation—The experiments described thus far were performed at 0 °C to ensure monodispersity of the enzyme solution even during long incubation periods. We next wanted to ascertain how quickly autophosphorylation occurs near physiological temperature.

We first established that CaM kinase II holoenzymes remained monodisperse during 1-min autophosphorylation reactions at temperatures as high as 30 °C (Table I). We then explored whether the autonomous activity of CaM kinase II could still be used as a quantitative probe for Thr-286 autophosphorylation at temperatures greater than 0 °C because the enzyme has been shown to lose some enzymatic activity during autophosphorylation at higher temperatures (22). CaM kinase II was autophosphorylated for varying times at 23, 30, and 37 °C and then assayed for Ca^{2+}/CaM activity to test for temperature-dependent instability (Fig. 6*A*). The data indicate that enzyme activity can be used as a probe for Thr-286 autophosphorylation at temperatures as high as 30 °C as long as reaction times are kept under 1 min.

CaM kinase II autophosphorylation was examined at 12, 23, and 30 °C (Fig. 6*B*). As expected, the rate of autophosphorylation increased with increasing temperature (Table II). At 30 °C, k_1 was 12 s^{-1}, 30-fold greater than the value of 0.4 s^{-1} at 0 °C. A plot of ln k_1 *versus* 1/*T* (Arrhenius plot) revealed a straight line (Fig. 6*C*). From the slope, an activation energy of 18.0 kcal/mol was calculated for the autophosphorylation process (see "Experimental Procedures"), indicating that a significant energy barrier must be overcome for autophosphorylation to take place. Extrapolating the straight line fit to 37 °C suggests that autophosphorylation would occur at a rate of ~20 s^{-1} at this temperature. This value is greater than an order of magnitude faster than previous estimates of the autophosphorylation rate of CaM kinase II at physiological temperature (15, 36, 37).

It is conceivable, albeit unlikely, that at temperatures closer to physiological temperature interholoenzyme autophosphorylation could contribute more significantly to the autophosphorylation mechanism of CaM kinase II than it does at 0 °C. To explore whether interholoenzyme autophosphorylation occurs at higher temperatures, the rate of autophosphorylation at 23 °C was explored at both 0.5 and 8.6 μM CaM kinase II. At both protein concentrations the rate constant for autophosphorylation was ~5 s^{-1} (Table II), indicating that interholoenzyme autophosphorylation is not significant at 8.6 μM CaM kinase II even at temperatures well above 0 °C.

DISCUSSION

The notion that CaM kinase II autophosphorylation is an intraholoenzyme reaction occurring between two neighboring subunits has been challenged by structural studies that indicate that the distance between subunits is too large to permit such autophosphorylation. In this study, we have characterized the CaM kinase II autophosphorylation process using dynamic light scattering and chemical quenched flow kinetics under conditions that address ambiguities in the previous analyses. The results support an intraholoenzyme, intersubunit mechanism for autophosphorylation and also reveal that autophosphorylation is a relatively rapid process, occurring with at a rate of ~12 s^{-1} at near physiological temperature.

Several previous biochemical studies had also suggested that CaM kinase II autophosphorylation occurs intraholoenzyme (13, 14, 17). However, the observation that CaM kinase II could readily be induced to form aggregates during autophosphoryl-

TABLE II
Kinetic data for the CaM kinase II holoenzyme

Shown are the concentration of CaM kinase II, concentration of ATP, and temperature of quenched flow experiments. The best fit parameters were the first order rate constant (k_1) and the maximal autonomy. Error values represent the S.D. of the nonlinear least squares best fit to Equation 2. The autophosphorylation conditions were 20 mM Hepes pH 7.4, 200 mM KCl, 500 μM Ca^{2+}, 10 μM CaM, and 2 mM Mg^{2+}.

Exp. no.	[CaM kinase II]	[ATP]	Temperature	k_1	Maximal autonomy
	μM	μM	°C	s^{-1}	%
1	0.76	500	0	0.42 ± 0.02	65 ± 2
2	0.76	2,500	0	0.39 ± 0.02	71 ± 2
3	0.76	100	0	0.40 ± 0.80	71 ± 5
4	0.76	2.5	0	0.062 ± 0.04	71 ± 3
5	12.7	500	0	0.41 ± 0.04	65 ± 3
6	10.6	500	0	0.63 ± 0.10	64 ± 3
7	4.61	500	0	0.43 ± 0.02	74 ± 1
8	1.61	500	0	0.37 ± 0.04	65 ± 2
9	0.46	500	0	0.44 ± 0.03	69 ± 2
10	0.16	500	0	0.33 ± 0.03	63 ± 2
11	0.05	500	0	0.52 ± 0.03	68 ± 2
12	0.46 + 3.4 μM T286A	500	0	0.36 ± 0.03	59 ± 2
13	0.5	500	12	2.1 ± 0.4	66 ± 4
14	0.5	500	23	4.9 ± 0.8	78 ± 3
15	0.5	500	30	11.9 ± 1.5	77 ± 3
16	0.50 + 8.4 μM T286A	500	23	4.4 ± 0.9	68 ± 5

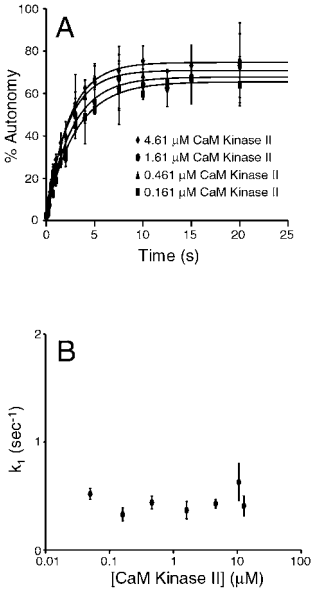

FIG. 4. **Effect of different CaM kinase II concentrations on autophosphorylation.** *A*, time course of CaM kinase II autophosphorylation at different CaM kinase II concentrations. Plotted is the percentage of autonomous enzymatic activity compared with Ca^{2+}/CaM activity (±S.D., $n = 2$) *versus* time (in seconds). For clarity, only four of seven experiments are shown. The *solid lines* are nonlinear least squares best fits of each data set to Equation 2. The *diamonds*, *ovals*, *triangles*, and *squares* represent experiments at 4.61, 1.61, 0.461, and 0.161 μM CaM kinase II, respectively. *B*, concentration dependence of CaM kinase II autophosphorylation rate. Plotted is the first order rate constant for autophosphorylation (k_1) *versus* CaM kinase II concentration (μM). *Error bars* represent the S.D. of the best fit to the time course data.

TABLE III
Kinetic data for the monomeric CaM kinase II

Shown are the concentration of monomeric CaM kinase II in the quenched flow experiments and the half-time ($t_{1/2}$) of the autophosphorylation time course. The best fit parameter was the second order rate constant (k_2). Error values represent the S.D. of the nonlinear least squares best fit to Equation 3. The autophosphorylation conditions were 20 mM Hepes pH 7.4, 200 mM KCl, 500 μM Ca^{2+}, 10 μM CaM, 500 μM ATP, 2 mM Mg^{2+}, and 0 °C.

Exp. no.	[mCaMKII]	$t_{1/2}$	k_2
	μM	s	$M^{-1}\ s^{-1}$
1	2.15	17.8	19,500 ± 4,000
2	0.58	99.8	23,800 ± 4,000
3	0.14	292	32,000 ± 10,000
4	0.035	899	26,100 ± 11,000

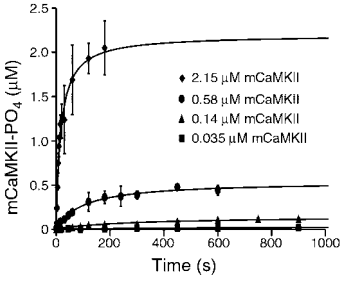

FIG. 5. **Time course of mCaMKII autophosphorylation at different mCaMKII concentrations.** Plotted is the concentration of phosphorylated mCaMKII (±S.D., $n = 2$) *versus* time (in seconds). The *solid lines* are nonlinear least squares best fits of each data set to Equation 3. The *diamonds*, *ovals*, *triangles*, and *squares* represent experiments at 2.15, 0.58, 0.14, and 0.035 μM mCaMKII, respectively.

ation (22–25) created significant doubt about the experiments used to identify this mechanism. For instance, it had been observed that the rate of CaM kinase II autophosphorylation is independent of CaM kinase II concentration (13, 14), suggesting intraholoenzyme autophosphorylation if CaM kinase II were monodisperse. Monodispersity was not experimentally established, however, and if the holoenzymes were self-associated, interholoenzyme autophosphorylation would also not show a concentration dependence because the phosphorylation would be occurring within an assembly of holoenzymes. Likewise, experiments in which active CaM kinase II holoenzymes were mixed with inactive holoenzymes did not show phosphorylation of the inactive CaM kinase II molecules (16, 17), suggesting that autophosphorylation occurs intraholoenzyme. However, if the holoenzymes were self-associated here, few active and inactive holoenzymes would gain access to one another, thus leaving open the possibility that autophosphorylation might still occur interholoenzyme. Given that the recent electron microscopy structure that shows a ~50 Å distance between subunits also does not readily indicate how intraholoenzyme, intersubunit autophosphorylation might take place (20), it was reasonable in this work to explore the mechanism of CaM kinase II autophosphorylation further. By providing data that support an intraholoenzyme, intersubunit autophosphorylation mechanism under conditions in which CaM kinase II is monodisperse, the uncertainty regarding intraholoenzyme au-

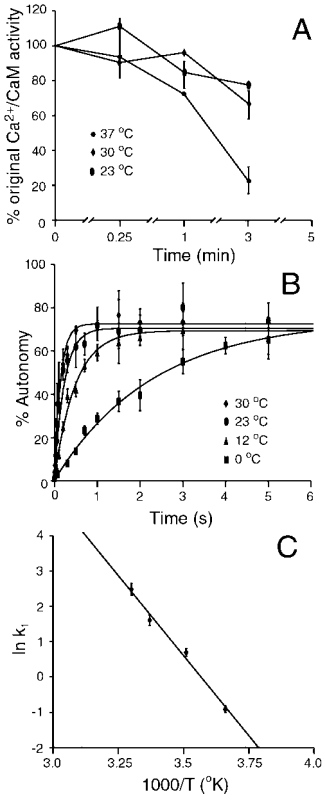

FIG. 6. **Effect of temperature on CaM kinase II autophosphorylation.** *A*, loss of Ca^{2+}/CaM enzyme activity with autophosphorylation time. Plotted is the percentage of original Ca^{2+}/CaM activity (±S.D., $n = 2$) *versus* autophosphorylation time (in minutes). The *circles*, *diamonds*, and *ovals* represent experiments performed at 37, 30, and 23 °C, respectively. *B*, time course of CaM kinase II autophosphorylation at different temperatures. Plotted is the percentage of autonomous enzymatic activity compared with Ca^{2+}/CaM activity (±S.D., $n = 2$) *versus* time (in seconds). The *solid lines* are nonlinear least squares best fits of each data set to Equation 2. The *diamonds*, *ovals*, *triangles*, and *squares* represent experiments at 30, 23, 12, and 0 °C, respectively. *C*, Arrhenius plot of CaM kinase II autophosphorylation rate. Plotted is the natural log of the first order rate constant for autophosphorylation (k_1) *versus* 1,000/temperature (K). *Error bars* represent the S.D. of the best fit to the time course data. The *solid line* is the linear best fit of the data.

tophosphorylation that arose from the characterization of CaM kinase II supramolecular aggregation has been eliminated.

How might an intraholoenzyme, intersubunit autophosphorylation mechanism be reconciled with the three-dimensional electron microscopy structure of α-CaM kinase II? One idea is that upon CaM binding, a small part of the regulatory segment traverses the distance between kinase domains and becomes autophosphorylated. This could occur without a global rearrangement of the holoenzyme structure, and if the segment of protein that traverses the distance is small, it would be invisible to electron microscopy.

A second possibility is that CaM kinase II has dynamic properties that allow it to change conformation rapidly. This could allow two neighboring kinase domains to come into close proximity transiently so that autophosphorylation could occur. If the overall amount of time in the conformation with the kinase domains close together is much less than the time spend in the conformation with the kinase domains far apart, this conformational change would not be visible in the structural analyses of CaM kinase II. This type of dynamic conformational change could take several forms, including a "swiveling" of the foot-like kinase domains to bring them into proximity. The biochemical demonstration of intersubunit autophosphorylation described here would suggest that the gear-like shape of the holoenzyme, with a hole at its center (20), may be designed to enable a large movement of subunits.

The rate constant for autophosphorylation showed no concentration dependence between 0.05 and 12.72 μM CaM kinase II (Fig. 4). Two conclusions can be drawn from this information: 1) α-CaM kinase II is capable of phosphorylating itself via an intraholoenzyme mechanism, and 2) intraholoenzyme autophosphorylation is the dominant mechanism of autophosphorylation at concentrations up to about 10 μM CaM kinase II. Our findings do not exclude the possibility that interholoenzyme autophosphorylation could potentially take place. However, this process must be significantly slower than intraholoenzyme autophosphorylation. Furthermore, because the concentration of CaM kinase II studied here was nearly as high as the estimated concentration of CaM kinase II in hippocampal dendritic spines (predicted to be up to 50 μM (38)), interholoenzyme autophosphorylation likely contributes little to CaM kinase II activation even at the high concentrations found in neurons. Our data indicate that a viable structural model of CaM kinase II autophosphorylation needs to at least account for the ability of the kinase to autophosphorylate between subunits of the same holoenzyme if it also incorporates autophosphorylation through an interholoenzyme process.

An intraholoenzyme, intersubunit autophosphorylation mechanism bestows CaM kinase II with many biologically advantageous properties. For instance, the intersubunit nature of autophosphorylation is central to the ability of CaM kinase II to decode the frequency of Ca^{2+} oscillations (11). This is physiologically important because increases in cellular Ca^{2+} do not usually take place in a graded manner but rather occur as spikes of increased concentration which occur with a given frequency (39, 40). Frequency detection is believed to arise molecularly from the requirement of two CaM molecules for each autophosphorylation event: one to a "kinase" subunit that catalyzes autophosphorylation and the other to a "substrate" subunit that becomes autophosphorylated (15, 17). Computer modeling suggests that the requirement for coincident binding of CaM to two neighboring subunits of a holoenzyme is critical for proper spike frequency detection (41). If autophosphorylation occurred intrasubunit, rather than intersubunit, two CaM molecules would not be required, and CaM kinase II presumably would not be able to detect Ca^{2+} spike frequency. An interholoenzyme reaction would require two CaM molecules, one on each holoenzyme, but such a scheme would likely show a reduced dependence on the frequency of Ca^{2+} spikes. CaM kinase II is not unique in utilizing CaM binding to both a kinase and substrate molecule to facilitate phosphorylation of the substrate. Phosphorylation of CaM kinase IV by CaM kinase kinase also employs this mechanism (42, 43). However, CaM kinase II is unique in that its signaling cascade is self-contained within a single, self-associated molecular machine.

Studies of the temperature dependence of α-CaM kinase II autophosphorylation revealed that the rate increased with temperature from 0.4 s^{-1} at 0 °C to 12 s^{-1} at 30 °C. Based on this trend, it was estimated that autophosphorylation would occur at ~20 s^{-1} at 37 °C. No carefully determined value for the autophosphorylation rate of CaM kinase II had been reported previously. However, most estimates had placed the rate considerably slower, usually about 0.5–1.0 s^{-1} (15, 36, 37, 44, 45).

The ability of CaM kinase II to autophosphorylate rapidly is especially biologically significant if, as evidence suggests (28, 46), the extent of CaM kinase II activation is limited by the amount of CaM available in the cell. In this case, CaM kinase II would be only partially saturated with CaM during each

spike in cellular Ca^{2+} because CaM kinase II has a lower affinity for Ca^{2+}/CaM than most other CaM-binding proteins (7). Rapid autophosphorylation would ensure that each CaM kinase II subunit available for autophosphorylation becomes autonomous during the limited time before CaM dissociates. Because CaM dissociates from unphosphorylated CaM kinase II with a rate constant of $\sim 2\ s^{-1}$ (10), a rate of autophosphorylation of $\sim 20\ s^{-1}$ indicates that autophosphorylation will occur nearly every time CaM molecules bind the holoenzyme in the proper orientation.

The elucidation in this work of the rate constant and mechanism of CaM kinase II autophosphorylation should provide the foundation for further mechanistic studies of CaM kinase II. Additional kinetic studies of CaM and ATP recognition, as well as substrate binding and phosphorylation, will be required to provide a more complete understanding of the activation mechanism of CaM kinase II. Other issues regarding autophosphorylation, such as whether it is propagated in a defined direction within each hexameric ring of the holoenzyme, also still remain unresolved. Future studies will probe these issues, and in so doing will provide a better understanding of the role of CaM kinase II in long term potentiation and other signaling processes.

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Chemical Quenched Flow Kinetic Studies Indicate an Intraholoenzyme Autophosphorylation Mechanism for Ca^{2+}/Calmodulin-dependent Protein Kinase II

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