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# Activation of Type II Calcium/Calmodulin-dependent Protein Kinase by $Ca^{2+}$/Calmodulin Is Inhibited by Autophosphorylation of Threonine within the Calmodulin-binding Domain*

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**It is now well established that autophosphorylation of a threonine residue located next to each calmodulin-binding domain in the subunits of type II $Ca^{2+}$/calmodulin-dependent protein kinase causes the kinase to remain active, although at a reduced rate, after $Ca^{2+}$ is removed from the reaction. This autophosphorylated form of the kinase is still sensitive to $Ca^{2+}$/calmodulin, which is required for a maximum catalytic rate. After removal of $Ca^{2+}$, new sites are autophosphorylated by the partially active kinase. Autophosphorylation of these sites abolishes sensitivity of the kinase to $Ca^{2+}$/calmodulin (Hashimoto, Y., Schworer, C. M., Colbran, R. J., and Soderling, T. R. (1987) *J. Biol. Chem.* 262, 8051–8055). We have identified two pairs of homologous residues, $Thr^{305}$ and $Ser^{314}$ in the $\alpha$ subunit and $Thr^{306}$ and $Ser^{315}$ in the $\beta$ subunit, that are autophosphorylated only after removal of $Ca^{2+}$ from an autophosphorylation reaction. The sites were identified by direct sequencing of labeled tryptic phosphopeptides isolated by reverse-phase high pressure liquid chromatography. $Thr^{305-306}$ is rapidly dephosphorylated by purified protein phosphatases 1 and 2A, whereas $Ser^{314-315}$ is resistant to dephosphorylation. We have shown by selective dephosphorylation that the presence of phosphate on $Thr^{305-306}$ blocks sensitivity of the kinase to $Ca^{2+}$/calmodulin. In contrast, the presence of phosphate on $Ser^{314-315}$ is associated with an increase in the $K_{act}$ for $Ca^{2+}$/calmodulin of only about 2-fold, producing a relatively small decrease in sensitivity to $Ca^{2+}$/calmodulin.**

Type II $Ca^{2+}$/calmodulin-dependent protein kinases (CaM kinases)[1] are a family of closely related enzymes that phosphorylate several substrate proteins in different tissues (1–4). The brain CaM kinase is a hetero-oligomer of molecular weight 600,000 to 700,000 (5–8). It is expressed at high concentrations in many forebrain neurons and constitutes approximately 1% of total brain protein (9). The isozyme purified from the forebrain is composed of structurally similar $\alpha$ (54 kDa) and $\beta$ (58–60 kDa) subunits in an average ratio of approximately 9 $\alpha$ to 3 $\beta$ (5). Both subunits are catalytic, and can be autophosphorylated at several sites (5, 6).

The CaM kinase is regulated in a complex way by autophosphorylation. When purified kinase is activated in the presence of CaM, a threonine residue adjacent to the calmodulin-binding domain ($Thr^{286}$ in $\alpha$ and $Thr^{287}$ in $\beta$) is rapidly autophosphorylated (10–12). The presence of phosphate at this site allows the kinase to remain active, at a reduced rate, when free $Ca^{2+}$ is removed by chelation with EGTA. This mechanism may allow the CaM kinase to remain active *in vivo* after an initial activating calcium transient is over (13). The rate of calcium-independent phosphorylation of exogenous substrates by kinase autophosphorylated at $Thr^{286-287}$ is 20–80% of the fully calcium-stimulated rate of nonphosphorylated kinase. The activity can be stimulated to the maximum rate by the readdition of CaM (13–16).

Upon removal of $Ca^{2+}$, new sites become accessible for autophosphorylation by the partially active form of the kinase (13, 17–19). Autophosphorylation of these new sites inhibits stimulation of kinase activity by CaM (17, 19). We report here the identification of two of these new sites. One, $Thr^{305}$ in $\alpha$ and $Thr^{306}$ in $\beta$, is located within the calmodulin-binding domain in a five-amino acid segment found previously to be essential for high affinity binding of CaM (20). The other, $Ser^{314}$ in $\alpha$ and $Ser^{315}$ in $\beta$, is located at the carboxyl end of the calmodulin-binding domain. By selective dephosphorylation, we have found that only autophosphorylation of $Thr^{305-306}$ correlates closely with the loss of sensitivity to CaM. When this site is maximally phosphorylated, stimulation of kinase activity is inhibited even at concentrations of CaM 30 times higher than the $K_{act}$ of nonphosphorylated kinase. The inhibition is reversed when this site is dephosphorylated with protein phosphatases 1 or 2A (44). In contrast, the sensitivity of the kinase to CaM is reduced only about 2-fold by the presence of phosphate on $Ser^{314-315}$.

## EXPERIMENTAL PROCEDURES[2]

## RESULTS

*New Sites Autophosphorylated after Removal of $Ca^{2+}$ from the Autophosphorylation Reaction*—To identify new sites that are autophosphorylated in type II CaM kinase after removal of $Ca^{2+}$ from the autophosphorylation reaction, purified kinase was autophosphorylated with [$\gamma$-$^{32}$P]ATP for 5 and 35 s in the presence of $Ca^{2+}$ or for 5 s in the presence of $Ca^{2+}$

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[1] The abbreviations used are: CaM, $Ca^{2+}$/calmodulin; CaM kinase, type II CaM-dependent protein kinase; SDS, sodium dodecyl sulfate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; MLCK, myosin light chain kinase; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; PTH, phenylthiohydantoin; S.E., standard error of the mean.

[2] Portions of this paper (including "Experimental Procedures" and Figs. 2–4) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

followed by 30 s in its absence. Tryptic phosphopeptide maps of kinase labeled under each condition were compared (Fig. 1, *A*, *C*, and *E*). As we reported previously (10), α-Thr$^{286}$ (Fig. 1*A*), β-Thr$^{287}$, and β-Thr$^{382}$ were autophosphorylated after incubation for 5 s in the presence of $Ca^{2+}$ (10–12). One or more additional identified sites in both subunits were autophosphorylated at a slower rate (Fig 1*C*; Ref. 10). Two new sets of phosphopeptides appeared only when autophosphorylation in the presence of $Ca^{2+}$ was followed by addition of EGTA to chelate free $Ca^{2+}$ (Fig. 1*E*). One set eluted early from the HPLC column and contained phosphoserine; the other set eluted later and contained phosphothreonine. The same new phosphopeptides were also observed when EGTA was added after 60 s of autophosphorylation in the presence of $Ca^{2+}$ (data not shown).

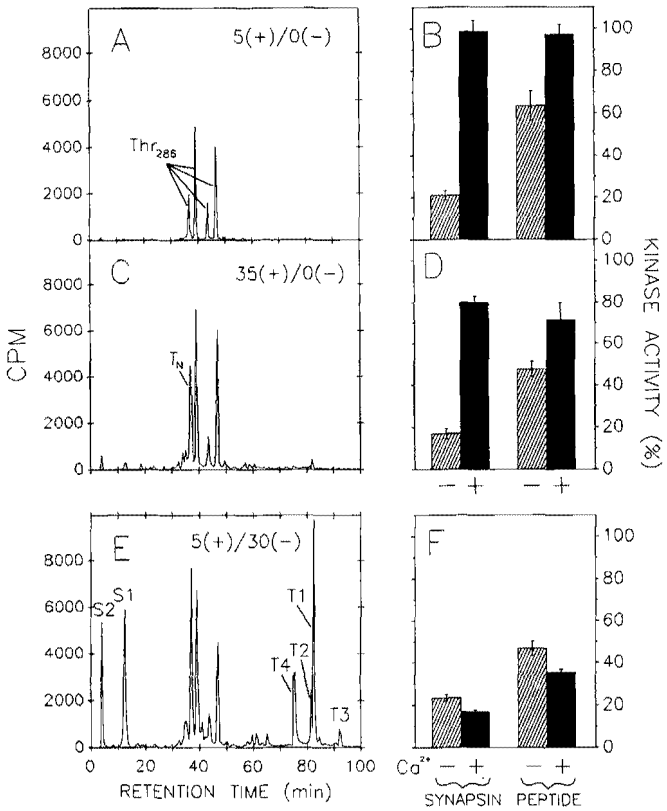


FIG. 1. **Autophosphorylation of new sites after removal of $Ca^{2+}$ is associated with loss of sensitivity of type II CaM kinase to CaM.** Type II CaM kinase (5 μg) was autophosphorylated for 5 s (*A* and *B*) or 35 s (*C* and *D*) in the presence of $Ca^{2+}$, calmodulin, and [γ-$^{32}$P]ATP, or for 5 s in the presence of $Ca^{2+}$ followed by 30 s in the absence of $Ca^{2+}$ (*E* and *F*), as described under "Experimental Procedures." An aliquot (1 μg) was removed and kinase activity was measured in the presence and absence of $Ca^{2+}$ with both a synthetic peptide and synapsin I as substrates (*B*, *D*, and *F*). The activity of unautophosphorylated kinase (100%; not shown) was 9.9 ± 1.1 μmol/min/mg ($n$ = 4) with peptide as substrate and 6.5 ± 0.4 μmol/min/mg ($n$ = 5) with synapsin I as substrate. Values are the average ± S.E. In each experiment, activity was measured in triplicate. Labeled tryptic phosphopeptides were prepared from the remaining 4 μg of kinase and fractionated by chromatography on a C4 reverse-phase HPLC column as described under "Experimental Procedures." Only peptide maps from the α subunit are shown in the figure (*A*, *C*, and *E*). *A* and *C* represent sites autophosphorylated in the presence of $Ca^{2+}$. The peptides containing Thr$^{286}$ were identified previously (10). A peptide containing phosphothreonine that appeared after 35 s of autophosphorylation in the presence of $Ca^{2+}$ ($T_N$; *C*) has not been described previously. This peptide is resolved from those containing Thr$^{286}$ in other HPLC runs. It was blocked at the $NH_2$ terminus upon Edman sequencing and was identified by amino acid analysis as a tryptic peptide derived from the $NH_2$ terminus of the α subunit (data not shown). It is not discussed further in this study. *E* represents sites autophosphorylated after the removal of $Ca^{2+}$. Peptides S1 and T1–T4 also appeared in maps of the β subunit (data not shown). Peptide S1 from the β subunit had a slower mobility than S1 from the α subunit, eluting near the peaks containing Thr$^{286-287}$. Peptides T1–T4 from the β subunit had mobilities identical to those of T1–T4 from the α subunit. The chemical identity of phosphorylated residues in each peak was determined as described under "Experimental Procedures." Maps similar to those in *A* and *E* were obtained in eight separate experiments. Maps similar to that in *C* were obtained in two separate experiments. *S*, peak containing phosphoserine; *T*, peak containing phosphothreonine.

Autophosphorylation of Thr$^{286-287}$ produced a significant $Ca^{2+}$-independent kinase activity toward exogenous substrates (Fig. 1*B*; Refs. 10 and 13–16). When the substrate was a synthetic peptide, the $Ca^{2+}$-independent activity was 64% of the activity of unphosphorylated kinase in the presence of calcium (control activity). When the substrate was synapsin I, $Ca^{2+}$-independent activity was 21% of control activity. Thus, the rate of $Ca^{2+}$-independent activity relative to control is highly dependent on the substrate protein. Activity declined slightly upon further autophosphorylation in the presence of $Ca^{2+}$, apparently due to thermal instability (14); however, the $Ca^{2+}$-independent activity remained approximately 67% (peptide substrate) and 21% (synapsin I) of the activity in the presence of $Ca^{2+}$ (Fig. 1*D*).When autophosphorylation was allowed to continue in the absence of $Ca^{2+}$ after 5 s of autophosphorylation in the presence of $Ca^{2+}$, sensitivity of the kinase to CaM was abolished (Ref. 17, Fig. 1*F*). This was evident when kinase activity was measured with either of the exogenous substrates. Calcium-independent activity remained unchanged. Loss of sensitivity to CaM could not be overcome by addition of concentrations of CaM as high as 4.5 μM, 30 times higher than the $K_{act}$ for CaM of nonphosphorylated kinase (data not shown). However, the sensitivity rapidly reappeared when the kinase was dephosphorylated with protein phosphatase 2A (Fig. 8) or with protein phosphatase 1 (Ref. 17; data not shown).

*Identification of Sites Autophosphorylated at Serine after Removal of Calcium*—Material from the peak labeled *S1* in Fig. 1*E* was purified in sufficient quantity for automated gas-phase sequencing, as described under "Experimental Procedures." After the second step of purification, C18 reverse-phase chromatography, the peptide was obtained as a single symmetrical peak containing 560 pmol of radioactive phosphate (Fig. 2*A*). The major sequence obtained from this peak, Asn-Phe-Ser-Gly-Gly-Lys (Table I), corresponds to the predicted sequence of a tryptic peptide in the α subunit containing residues Asn$^{312}$ to Lys$^{317}$ (43, Fig. 9). The recovery of 512 pmol of PTH-Asn in the first cycle is consistent with a stoichiometry of 1 mol of phosphate/mol of peptide. Fewer

TABLE I

*Sequences of α-S1 and β-S1 peptides*

Recoveries of PTH-derivatives were consistent with the moles of $PO_4$ submitted, assuming a stoichiometry of 1 mol of $PO_4$/mol of peptide.

| Cycle | α-S1 | | β-S1 | | |
|---|---|---|---|---|---|
| | Residue | Yield[a] | Residue | Yield[b] | Yield[c] |
| 1 | Asn | 512 | Asn | 46 | 74 |
| 2 | Phe | 347 | Phe | 37 | 26 |
| 3 | Ser | 390[d] | Ser | | 38[d] |
| 4 | Gly | 183 | Val | 9 | 22 |
| 5 | Gly | 116 | Gly | 30 | 48 |
| 6 | Lys | 57 | | | |

[a] Sample contained 560 pmol of $PO_4$.
[b] Sample contained 200 pmol of $PO_4$.
[c] Sample contained 150 pmol of $PO_4$.
[d] >90% recovered as PTH-dehydroalanine.

than 5 pmol of any other PTH-derivative was recovered in each cycle. Nearly all of the serine (90%) at the third position was recovered as PTH-dehydroalanine, as expected if it were phosphorylated (28, 29). During sequencing on the gas-phase sequencer, one-third to one-half of unmodified serine is recovered as PTH-serine (10), the rest as PTH-dehydroalanine. Therefore, if the serine in the major sequence were unmodified, we would have expected 130–195 pmol of PTH-serine. We conclude that the phosphorylated serine in peak S1 is $\alpha$-Ser$^{314}$.

The other phosphoserine-containing peak generated from the $\alpha$ subunit, labeled *S2*, eluted in the void volume of both C4 and C18 reverse-phase columns. Electrophoresis of material from this peak on cellulose sheets revealed that over 90% of the radioactivity was associated with a peptide, rather than with free phosphoserine. The S2 peptide has not been sufficiently purified to determine its sequence. Its rate of autophosphorylation and its rate of dephosphorylation by protein phosphatase (data not shown) were essentially identical to that of peptide S1 (Figs. 5*A* and 7*A*). It may be an alternatively cleaved tryptic peptide containing Ser$^{314}$ or a peptide containing phosphorylated $\alpha$-Ser$^{318}$ (10).

Tryptic peptide maps of the $\beta$ subunit autophosphorylated under the same set of conditions revealed only a single new phosphoserine-containing peak that eluted near the peaks containing Thr$^{287}$ ($\beta$-S1; data not shown). Material from this peak was purified after two separate large scale autophosphorylation reactions. In both cases, the radioactive peak eluted from the C18 column was associated with one major peptide peak (Fig. 2*B*). The $NH_2$-terminal sequence of this peptide was determined. The major sequence, Asn-Phe-Ser-Val-Gly (Table I), corresponds to the predicted sequence of a tryptic peptide in the $\beta$ subunit containing residues Asn$^{313}$ to Arg$^{318}$ (Fig. 9). This peptide contains only one potential phosphorylation site, Ser$^{315}$. During the sequencing of both peptides, minor PTH-amino acid derivatives were recovered in one cycle, but the identity and position of these amino acids differed for the two peptides. Neither of the alternative sequences that included these amino acids was found in the sequence of the CaM kinase. In each case, the recovery of PTH-derivatives was consistent with a stoichiometry of 1 mol radioactive phosphate/mol of peptide (Table I). Again, the failure to recover any PTH-serine in the third cycle is consistent with the presence of phosphorylated serine at this position. We conclude that the phosphorylated serine in peak $\beta$-S1 is Ser$^{315}$. The autophosphorylation sites containing $\alpha$-Ser$^{314}$ and $\beta$-Ser$^{315}$ are homologous and are located at the carboxyl-terminal end of the calmodulin-binding domain (Ref. 43 and Fig. 9).

*Identification of Sites Autophosphorylated at Threonine after Removal of Calcium*—Four new tryptic peptides containing phosphothreonine appeared in maps of both the $\alpha$ and $\beta$ subunits after removal of $Ca^{2+}$ from the autophosphorylation reaction (T1–T4, Fig. 1*E*; data not shown for the $\beta$ subunit). Peptide T1 was the major phosphothreonine containing peptide and was the only one of the four autophosphorylated at a rate that correlated closely with the rate of loss of sensitivity to CaM (Fig. 5*A*).

Peak T1 from the $\beta$ subunit was purified twice from large scale autophosphorylation reactions as described under "Experimental Procedures." In both cases, the purified radioactive peak was associated with one major peptide peak (Fig. 3*A*). The $NH_2$-terminal sequence of this peptide was determined. The major sequence, Gly-Ala-Ile-Leu-*X*-Thr-Met-Leu-Ala (Table II), corresponds to that of the predicted tryptic peptide containing residues Gly$^{302}$ to Arg$^{312}$ in the $\beta$ subunit (Fig. 9). During the sequencing of one peptide, there were no other contaminating PTH-derivatives. During the sequencing of the second peptide, there were some contaminating PTH-amino acid derivatives at each cycle. However, none of the alternative sequences that included these amino acids was found within the sequence of the CaM kinase. For both of these peptides, the recovery of PTH-derivatives was consistent with a stoichiometry of 1 mol of radioactive phosphate/mol of peptide (Table II). There are two predicted tryptic peptides within the $\beta$ subunit that contain threonine residues and might be blocked at the $NH_2$ terminus; Met$^1$ to Arg$^9$ and Gln$^{319}$ to Lys$^{342}$ (Gln can cyclize to pyroglutamate). It was possible that these peptides could be present as contaminants of the major peptide in peak T1 and could be the source of the phosphothreonine. Therefore, in a separate experiment, the T1 peptide was purified by one cycle of chromatography on a C4 reverse-phase HPLC column and then digested with thermolysin as described under "Experimental Procedures." Thermolysin hydrolyzes peptide bonds on the $NH_2$-terminal side of hydrophobic residues. Therefore it would be expected to remove three to four amino acids, including Thr$^{311}$, from the carboxyl terminus of peptide T1. The major thermolytic phosphopeptide was purified by C18 reverse-phase chromatography. The purified radioactive peak was associated with one major peptide peak (Fig. 3*B*), which eluted earlier than the parent peptide and contained 90 pmol of radioactive phosphate. The $NH_2$-terminal sequence of this peptide was determined and the major sequence was Gly-Ala-Ile-Leu-*X*-*X*-Met (Table II). During the sequencing, small amounts of contaminating PTH-amino acid derivatives were recovered in the first and fourth through sixth cycles. However, none of the alternative sequences that included these amino acids were found in the sequence of the CaM kinase. The sequence of the thermolytic peptide confirms the sequence of the parent tryptic phosphopeptide, T1, and indicates that the phosphothreonine was in fact contained in that sequence.

Although peptide T1 contains three potential threonine autophosphorylation sites, we have concluded that the phosphorylated threonine is most likely Thr$^{306}$ (homologous to Thr$^{305}$ in the $\alpha$ subunit) for the following reasons. No PTH-threonine and only traces of PTH-dehydrothreonine (30) were

TABLE II

*Sequence of peptides containing $\beta$-Thr$^{306(307)}$*

Recoveries of PTH-derivatives were consistent with the moles of $PO_4$ submitted, assuming a stoichiometry of 1 mol of $PO_4$/mol of peptide.

| Cycle | Tryptic peptides | | | Thermolytic peptide | |
|---|---|---|---|---|---|
| | Residue | $\beta$-T1 yield[a] | $\beta$-T1 yield[b] | Residue | Yield[c] |
| 1 | Gly | 30 | 50 | Gly | 43 |
| 2 | Ala | 27 | 24 | Ala | 21 |
| 3 | Ile | 21 | 15 | Ile | 11 |
| 4 | Leu | 15 | 31 | Leu | 14 |
| 5 | | —[d] | — | | — |
| 6 | Thr | 2[e] | 2.5 | | — |
| 7 | Met | 3[e] | 2 | Met | <2 |
| 8 | Leu | 4[e] | | | |
| 9 | Ala | 4[e] | | | |
| 10 | | — | | | |
| 11 | Arg | <0.5 | | | |

[a] Sample contained 85 pmol of $PO_4$.
[b] Sample contained 80 pmol of $PO_4$.
[c] Sample contained 91 pmol of $PO_4$.
[d] Trace quantities of dehydrothreonine recovered.
[e] Approximately equal recovery in the following cycle.

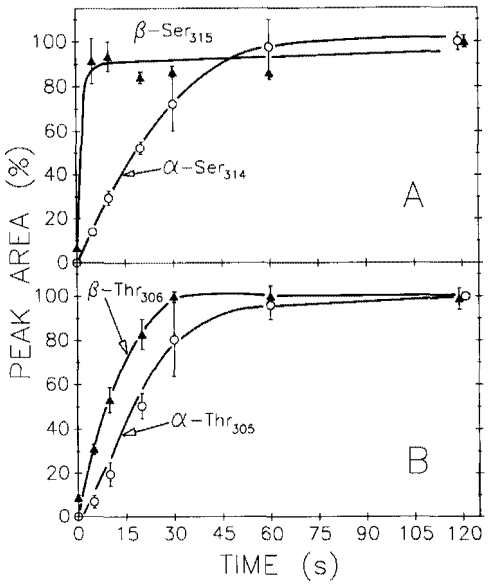


FIG. 5. **Time course of autophosphorylation of Ser$^{314-315}$ and Thr$^{305-306}$.** Kinase (5 μg) was autophosphorylated for 5 s in the presence of $Ca^{2+}$, calmodulin and [γ-$^{32}$P]ATP, then for the indicated times in the absence of $Ca^{2+}$ as described under "Experimental Procedures." Labeled phosphopeptides were prepared from 3.6 μg of kinase from each reaction and fractionated by HPLC chromatography as described under "Experimental Procedures." Radioactivity recovered in peaks containing phosphorylated α-Ser$^{314}$ (○) and β-Ser$^{315}$ (▲), (*A*), and in those containing phosphorylated α-Thr$^{305}$ (○) and β-Thr$^{306}$ (▲) (*B*), was determined at each time point and normalized as described under "Experimental Procedures." Values are the average, ± the range, of the results of two separate experiments.

recovered from the tryptic peptides in the fifth cycle, corresponding to Thr$^{306}$. In contrast, PTH-threonine was recovered in significant amounts in the sixth cycle, corresponding to Thr$^{307}$ (Table II). Finally, removal of Thr$^{311}$ from the peptide by thermolytic digestion did not remove the radioactive phosphate.

Purified peptide T1 from the α subunit (α-T1) was not recovered in sufficient quantity for sequencing. However, several lines of evidence indicate that it is identical to T1 from the β subunit (β-T1), as expected from the sequences of α and β (Fig. 9). First, α-T1 coeluted with β-T1 during reverse-phase HPLC chromatography (Fig. 4, *A-C*). Second, when either α-T1 or β-T1 were chromatographed a second time under the same conditions, the original single peak split into three peaks (Fig. 4, *D* and *E*). The retention times of the new peaks were identical for T1 derived from either subunit. Third, if dithiothreitol was added to α-T1 or β-T1 immediately following the first chromatographic separation, recovery of the parent peak during the second separation increased to 70%. Dithiothreitol may stabilize the parent peptide by inhibiting oxidation of Met$^{307}$ in α and Met$^{308}$ in β. Finally, proteolysis of α-T1 and β-T1 with thermolysin produced identical new peptide peaks upon fractionation by HPLC (data not shown).

*Functional Effects of Autophosphorylation of the New Sites Exposed after Removal of $Ca^{2+}$*—Thr$^{305-306}$ is located near the center of the CaM binding domain, and Ser$^{314-315}$ is located at its carboxyl-terminal end. Therefore, autophosphorylation of either of these sites might be expected to influence binding of CaM to the kinase. We examined the functional effects of autophosphorylation of these sites by comparing the kinetics of their autophosphorylation with the kinetics of loss of sensitivity to CaM. The rates of autophosphorylation of α-Ser$^{314}$ (Fig. 5*A*), α-Thr$^{305}$, and β-Thr$^{306}$ (Fig. 5*B*) were all similar to the rate of loss of sensitivity to CaM (Fig. 6). However, we found that the rates of dephosphorylation of these sites by protein phosphatase 2A were quite different from each other (Fig. 7). α-Thr$^{305}$ and β-Thr$^{306}$ were dephosphorylated rapidly, whereas α-Ser$^{314}$ and β-Ser$^{315}$ were relatively resistant to dephosphorylation. S2, the serine-containing peptide from the α-subunit that eluted at the void volume during reverse-phase HPLC (Fig. 1*C*), was similarly resistant to dephosphorylation (data not shown).

The rate of dephosphorylation of Thr$^{305-306}$ (Fig. 7*B*) was similar to the rate of recovery of sensitivity to CaM (Fig. 8*C*). In contrast, at the end of 3 min, long after the kinase had again become fully responsive to CaM, less than half of the phosphate on Ser$^{314-315}$ had been removed (Fig. 7*A*). Similar results were obtained with protein phosphatase 1 (data not shown). To test whether the presence of phosphate on Ser$^{314-315}$ had any affect on the sensitivity of the kinase to CaM, the apparent $K_{act}$ for CaM of unphosphorylated kinase was compared with that of kinase phosphorylated only at Ser$^{314-315}$. The latter was prepared by dephosphorylating autophosphorylated kinase for 60 s as described in the legend to Fig. 7. The apparent $K_{act}$ for CaM of unphosphorylated kinase was 127 ± 5.7 nM ($n$ = 3) and for kinase phosphorylated only at Ser$^{314-315}$ was 206 ± 16 nM ($n$ = 2). Thus, we conclude that autophosphorylation of Thr$^{305-306}$ inhibits activation of CaM kinase by CaM completely; whereas autophosphorylation of Ser$^{314-315}$ alone produces only a 1.6-fold increase in the apparent $K_{act}$ for CaM. Slower autophosphorylation of other threonine residues represented by peptides T2–T4 may contribute to loss of sensitivity to CaM (see "Discussion").

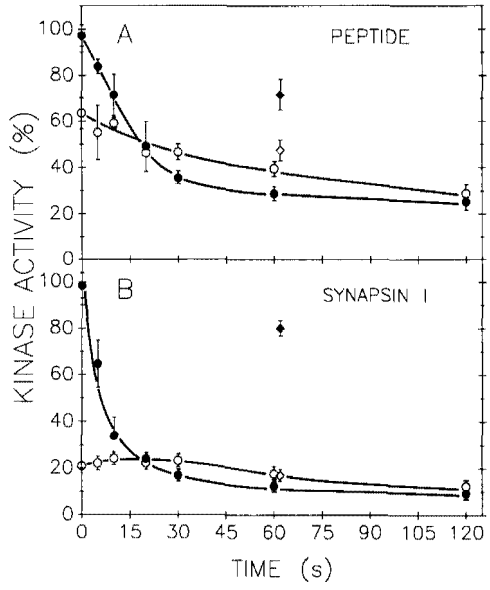


FIG. 6. **Time course of changes in kinase activity produced by autophosphorylation in the absence of $Ca^{2+}$.** Kinase (5 μg) was autophosphorylated for 5 s in the presence of $Ca^{2+}$ and then for the indicated times in the absence of $Ca^{2+}$ as described under "Experimental Procedures." Aliquots (1 μg) were removed at the indicated times and kinase activity was measured with either peptide (*A*) or synapsin I (*B*) as substrate as described under "Experimental Procedures." Values are plotted as percent of the activity of unphosphorylated kinase in the presence of $Ca^{2+}$, which was 6.4 ± 0.7 μmol/min/mg with synapsin I as substrate and 9.2 ± 1.7 μmol/min/mg with peptide as substrate. In a separate set of control incubations (◆, ◇) EDTA was added after 5 s of autophosphorylation in the presence of $Ca^{2+}$, and the incubation was continued for an additional 60 s. Values for each substrate are the average ± S.E. of the results of three separate experiments in which activity was measured in triplicate. ●, ◆, activity in the presence of $Ca^{2+}$; ○, ◇, activity in the absence of $Ca^{2+}$.

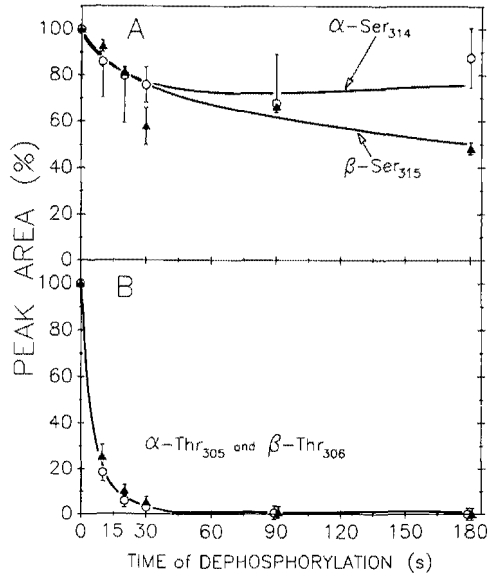


FIG. 7. **Time course of dephosphorylation of α-Ser$^{314}$, β-Ser$^{315}$, α-Thr$^{305}$, and β-Thr$^{306}$.** Kinase (5 μg) was autophosphorylated with [γ-$^{32}$P]ATP for 5 s in the presence of $Ca^{2+}$ and then for 30 s in the absence of $Ca^{2+}$, as described in the legend to Fig. 5. Dephosphorylation was initiated by addition of protein phosphatase 2A as described under "Experimental Procedures" and continued for the indicated times. Labeled phosphopeptides were prepared from 3.6 μg of kinase from each time point and fractionated by HPLC chromatography as described under "Experimental Procedures." Radioactivity recovered in peaks containing α-Ser$^{314}$ (○) and β-Ser$^{315}$ (▲) (*A*) and in those containing α-Thr$^{305}$ (○) and β-Thr$^{306}$ (▲) (*B*) was determined at each time point and normalized as described under "Experimental Procedures." The amount of radioactive phosphate recovered in each site before dephosphorylation was taken as 100%. Values are the average ± range of the results from two separate experiments.

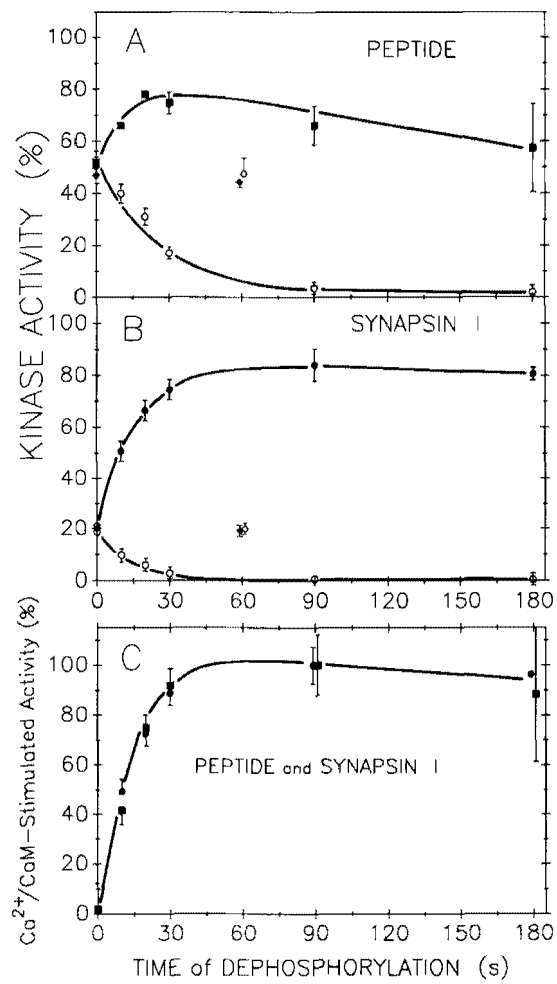


FIG. 8. **Time course of reversal of insensitivity to CaM by dephosphorylation.** Kinase (5 μg) was autophosphorylated for 5 s in the presence of $Ca^{2+}$ and then for 30 s in the absence of $Ca^{2+}$ as described in the legend to Fig. 5. Dephosphorylation was initiated by addition of protein phosphatase 2A as described in the legend to Fig. 7. Aliquots (1 μg) were removed at the indicated times and kinase activity was measured with either peptide (■, ○; *A* and *C*) or synapsin I (●, ○; *B* and *C*) as substrate as described under "Experimental Procedures." Values in *A* and *B* are plotted as the percent of the activity of unphosphorylated kinase in the presence of $Ca^{2+}$, which was 5.4 ± 0.9 μmol/min/mg ($n$ = 3) with synapsin I as substrate and 10.8 ± 2.6 μmol/min/mg ($n$ = 2) with peptide as substrate. To control for thermal instability, autophosphorylated kinase was incubated for 60 s, at 30°C, in the absence of protein phosphatase 2A (◆, ◇). In *C*, the difference between activity in the presence of $Ca^{2+}$ and in its absence is plotted for both substrates as a percent of the maximum difference, which is set at 100%. ■, ●, ◆, activity in the presence of $Ca^{2+}$ and calmodulin; ○, ◇, activity in the absence of $Ca^{2+}$ and calmodulin. Values are the average ± S.E. In each experiment, activity was measured in triplicate.

## DISCUSSION

Activation of type II CaM kinase in the presence of CaM is accompanied by rapid autophosphorylation of a pair of homologous residues, Thr$^{286}$ in the α subunit and Thr$^{287}$ in the β subunit, that are adjacent to the calmodulin binding domain in each subunit (10–12). Autophosphorylation of two to three of these sites in a dodecameric holoenzyme switches it to a new state in which all the subunits continue to phosphorylate themselves as well as exogenous substrates, at a reduced rate, after $Ca^{2+}$ is removed from the reaction (10, 13–16). When the kinase is in this state, it is still sensitive to CaM which is required for the maximum catalytic rate. However, 15–30 s after removal of $Ca^{2+}$ from the autophosphorylation reaction additional sites become autophosphorylated that cause the kinase to become insensitive to stimulation by CaM (17, 19).

In this study we have identified autophosphorylation sites that regulate sensitivity to CaM. Two homologous sites in each of the kinase subunits are autophosphorylated only after removal of calcium from an ongoing autophosphorylation reaction. They are Thr$^{305}$ and Ser$^{314}$ in the α-subunit and Thr$^{306}$ and Ser$^{315}$ in the β-subunit (Fig. 9). All of these sites are located within the calmodulin-binding domain (32–35). We have shown by selective dephosphorylation that autophosphorylation of Thr$^{305-306}$ abolishes sensitivity of the kinase to CaM even at concentrations of CaM 30 times higher than the $K_{act}$ of nonphosphorylated kinase. Autophosphorylation of Ser$^{314-315}$ alone has a much smaller effect on the requirement for CaM, increasing the apparent $K_{act}$ for CaM only about 2-fold.

Lou and Schulman (19) reported that calcium-independent phosphorylation of protein substrates was inhibited after autophosphorylation in the absence of $Ca^{2+}$. However, in our experiments, calcium-independent autophosphorylation of synapsin I and a synthetic peptide substrate were not inhibited after autophosphorylation in the absence of $Ca^{2+}$, in agreement with the results of Hashimoto *et al.* (17). Autophosphorylation of Ser$^{314-315}$ may account for the enhanced incorporation of phosphate into serine-containing tryptic peptides after removal of $Ca^{2+}$ from the autophosphorylation reaction reported by Lickteig *et al.* (18). These workers did not observe phosphopeptides T1 through T4, perhaps because the kinase subunits were not alkylated prior to trypsinization. We have observed that alkylation dramatically alters the

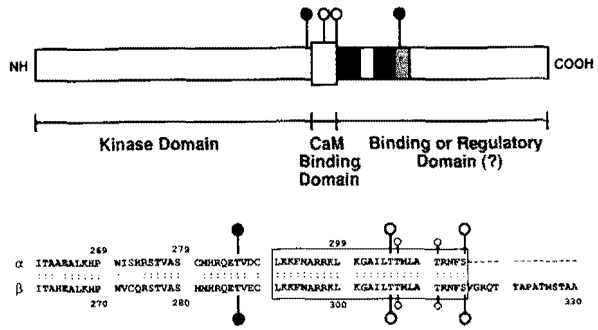


FIG. 9. **Summary of new sites autophosphorylated after removal of $Ca^{2+}$.** *A*, schematic representation of sites autophosphorylated in the absence of $Ca^{2+}$ and functional domains in the CaM kinase subunits. The largest subunit (shown) is the $\beta$ subunit. The *shaded region* is removed from the $\beta'$ subunit by alternative splicing (43). The *filled* and *shaded* regions are not present in the $\alpha$ subunit. All other regions are identical in the $\beta$ and $\beta'$ subunits and similar, but not identical in the $\alpha$ subunit (43). The locations of sites autophosphorylated rapidly in the presence of $Ca^{2+}$ are marked by *black ball and stick symbols*. The locations of sites autophosphorylated only after $Ca^{2+}$ is removed from an autophosphorylation reaction are marked by *open ball and stick symbols*. B, specific location of these sites in the sequences of the $\alpha$ and $\beta$ subunits. (The full sequences are reported in Ref. 43.) The calmodulin binding domain (32–35) is enclosed in a *box*. The *small open ball and stick symbols* mark the location of additional threonine residues in the calmodulin binding domain that may be autophosphorylated at a slower rate than $Thr^{305-306}$ or $Ser^{314-315}$ (see "Discussion").

pattern of cleavage of the subunits by trypsin (data not shown).

We were a bit surprised to observe autophosphorylation of $Thr^{305-306}$, because it is not preceded by the sequence Arg-*X*-*X*, which is a minimal requirement for phosphorylation of serines and threonines in exogenous proteins by type II CaM kinase (36). However, this is the second example of an autophosphorylation site in the CaM kinase that is not contained within a consensus sequence. The first to be identified was $Thr^{382}$ in the $\beta$ subunit, which is autophosphorylated rapidly in the presence of $Ca^{2+}$ (10). The rapid autophosphorylation of these "nonconsensus" sites implies that the three-dimensional structure of the kinase places them near the active site. $Thr^{305-306}$ is located within a sequence of 5 hydrophobic residues, $Thr^{305-306}$-Thr-Met-Leu-$Ala^{309-310}$, that are essential for high-affinity binding of calmodulin to type II CaM-kinase (34). Autophosphorylation of this threonine would therefore be expected to inhibit calmodulin binding perhaps by disrupting an important hydrophobic interaction or by changing the secondary structure of the calmodulin binding domain in a critical region.

In contrast to $Thr^{305-306}$, $Ser^{314-315}$ is situated within a substrate consensus sequence, $Arg^{311-312}$-Asn-Phe-$Ser^{314-315}$. The location of this site within the calmodulin binding domain is homologous to the location of $Ser^{512}$ in smooth muscle myosin light chain kinase (MLCK; Refs. 37 and 38). In the absence of bound CaM, $Ser^{512}$ in MLCK can be phosphorylated by the cAMP-dependent protein kinase (38, 39). This phosphorylation produces a 10–20-fold increase in the apparent $K_{act}$ for CaM, thus dramatically reducing the sensitivity of MLCK to stimulation by CaM (39). The homology between $Ser^{314-315}$ in type II CaM kinase and $Ser^{512}$ in MLCK caused several labs to propose that autophosphorylation of $Ser^{314-315}$ controls the sensitivity of type II CaM kinase to CaM (19, 40, 41). However, we find that autophosphorylation of $Ser^{314-315}$ has only a small effect on affinity for CaM, whereas autophosphorylation of $Thr^{305-306}$ appears to block calmodulin binding completely. The diminished effect of autophosphorylation of $Ser^{314-315}$ compared with $Ser^{512}$ in MLCK may be explained by differences in the sequences of the calmodulin binding domains of the two kinases that could produce different orientations of the phosphoserine residues. It may also be related to the observation that nonphosphorylated type II CaM kinase has a lower affinity for CaM ($K_{act}$ of 30–125 nM; Refs. 2, 4, and 7) than does nonphosphorylated MLCK ($K_{act}$ of 1–6 nM; Refs. 2 and 39). Thus, the conformation of the calmodulin binding domain of type II CaM kinase may produce a relatively low affinity for CaM even before $Ser^{314-315}$ becomes autophosphorylated.

The peptide represented by peak T1 contains 2 threonine residues in addition to $Thr^{305-306}$ (Fig. 9; $Thr^{306-307}$ and $Thr^{310-311}$). Our data suggest that autophosphorylation of $Thr^{306-307}$ and/or $Thr^{310-311}$ may also contribute to the inhibition of sensitivity to CaM. In addition to peak T1, three smaller unindentified phosphothreonine-containing peaks (T2–T4) appear in tryptic digests of kinase when autophosphorylation occurs in the absence of calcium (Fig. 1*C*). They all have a mobility similar to T1, but they appear at a significantly slower rate. We have not obtained sufficient quantities of peaks T2–T4 for sequencing; however, we believe they may contain the same tryptic peptide as T1, each phosphorylated at one or more of the other threonines. Peaks T2 and T3 appear slowly, then decrease in size after about 20 s. By 60 s they are nearly gone (data not shown). Peak T4 rises after a brief lag and is maximal by 60 s. This behavior is consistent with a model in which the peptide in peak T2 is autophosphorylated at $Thr^{306-307}$, the peptide in peak T3 is autophosphorylated at $Thr^{310-311}$, and the peptide in peak T4 is autophosphorylated at both. This model predicts that when the sites are dephosphorylated (Fig. 7), peak T4 will give rise to peaks T2 and T3, which will then slowly disappear. This predicted relationship was observed (data not shown). Furthermore, the rate of autophosphorylation of $Thr^{305-306}$ is consistently slightly slower than the rate of loss of sensitivity to CaM (Figs. 5*B* and 6) and the rate of dephosphorylation of this site is slightly faster than the rate of recovery of sensitivity to CaM (Figs. 7*B* and 8). When peaks T2–T4 are summed with peak T1 the summed rate of autophosphorylation correlates more closely with the rate of loss of sensitivity to CaM. Similarly, the summed rate of dephosphorylation correlates more closely with the rate of recovery of sensitivity to CaM. These observations suggest that slow autophosphorylation of the $Thr^{306-307}$ or $Thr^{310-311}$ may contribute to loss of sensitivity to CaM.

We have proposed previously that autophosphorylation of $\alpha$-$Thr^{286}$ and $\beta$-$Thr^{287}$, which switches type II CaM kinase to a partially calcium-independent state, may play an important physiological function by prolonging the effects of elevated intracellular $Ca^{2+}$ that accompanies neuronal activity (13). A second stage of regulation of the kinase by autophosphorylation is the suppression of sensitivity to CaM that occurs after removal of $Ca^{2+}$ from an autophosphorylation reaction (17). We show here that autophosphorylation of one or more threonine residues within the calmodulin binding domain occurs only after $Ca^{2+}$ is removed and causes this suppression of sensitivity to CaM. The importance, in intact neurons, of these two stages of regulation will depend upon the abundance, specificity, and subcellular location of neuronal protein phosphatases. The catalytic subunits of protein phosphatase 1 and phosphatase 2A can rapidly dephosphorylate all autophosphorylation sites on the kinase except $Ser^{314-315}$. Shields *et al.* (42) have shown that these two classes of phosphatase appear to contribute most of the phosphatase activity towards the CaM kinase in synaptosomes. It will be interesting to

learn whether there are additional highly specialized phosphatases that participate in regulation of the kinase. It will also be important to study the steady state level of phosphorylation of $Thr^{286-287}$, $Thr^{305-306}$, and $Ser^{314-315}$ in living neurons under different physiological conditions.

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Supplemental Material To

**Activation of Type II CaM Kinase by $Ca^{2+}$/Calmodulin is Inhibited By Autophosphorylation of Threonine Within The Calmodulin Binding Domain**

Bruce L. Patton, Stephen G. Miller, and Mary B. Kennedy

## EXPERIMENTAL PROCEDURES

**Materials** - Iodoacetamide, caffeine, bovine serum albumin, and phosphorylase b, were purchased from Sigma and dithiothreitol was purchased from Schwarz/Mann Biotech. SDS was purchased from Biorad and [$\gamma$-$^{32}$P]ATP was purchased from ICN Biochemicals. The synthetic peptide substrate for CaM kinase (Calmodulin-dependent protein kinase substrate analog) was purchased from Peninsula Laboratories. This substrate is a 10 residue synthetic peptide, Pro-Leu-Arg-Arg-Thr-Leu-Ser-Val-Ala-Ala, derived from phosphorylation site-2 of rabbit skeletal muscle glycogen synthase (21). It was dissolved at a concentration of 0.5 mM in 40 mM Tris-HCl (pH 7.0) and stored in aliquots at -80°C. Trypsin treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone was purchased from Worthington and thermolysin from Boehringer-Mannheim Biomedical. C4 and C18 reverse-phase HPLC columns (0.46 x 25 cm) were purchased from Vydac and acetonitrile (HPLC/UV Grade, Burdick and Jackson) from Baxter Healthcare. Trifluoroacetic acid (sequenal grade) and hydrochloric acid (constant boiling, sequenal grade) were purchases from Pierce Biochemical and thin layer cellulose sheets from J. T. Baker. Type II CaM kinase was purified from rat brain as described previously (7, 10). Calmodulin was purified from bovine brain by the method of Watterson *et al.* (22) and synapsin I was prepared by a modification of the method of Ueda and Greengard (23) as described previously (5). The catalytic subunit of protein phosphatase-2A was purified from rabbit skeletal muscle by the method of Tung, *et al.* (24) and was activated as described previously (10). Purified protein phosphatase-2A had a specific activity of 7.0 µmol $PO_4$ released from phosphorylase a per min per mg enzyme.

**Autophosphorylation** - Autophosphorylation was performed in a mixture (typically in a final volume of 25 µl) containing 5 µg kinase, 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 5 µg calmodulin, 5 mM 2-mercaptoethanol, 25 mM dithiothreitol, 100 µM [$\gamma$-$^{32}$P]ATP (3.0-6.0 x $10^5$ cpm/pmol), 0.6 mM EGTA, and 0.9 mM $CaCl_2$. Reaction mixtures were pre-warmed at 30°C for 60 s and autophosphorylation was initiated by the addition of kinase. Autophosphorylation in the presence of $Ca^{2+}$ was terminated by the addition of 12.5 µl of ice-cold 0.4 M EDTA. 25 mM Tris-HCl (pH 7.0) (EDTA Stop-Buffer) followed by 2.5 µl of 33 mM EGTA. When autophosphorylation in the presence of $Ca^{2+}$ was followed by autophosphorylation in the absence of $Ca^{2+}$, 2.5 µl of 33 mM EGTA was added after 5 s of autophosphorylation, and the reaction was allowed to continue. It was terminated by addition of 12.5 µl of ice-cold EDTA Stop-Buffer. Ten µl of $H_2O$ or buffer (next section) was added to bring the volume to 50 µl. A 10 µl aliquot of each reaction containing 1 µg of kinase was immediately diluted 50-fold into ice-cold 40 mM Tris-HCl (pH 8.0), 1 mg/ml bovine serum albumin, and its activity was measured with exogenous substrate in the absence and presence of CaM. Forty µl of 6% (w/v) SDS, 10% (w/v) glycerol, 4% (v/v) 2-mercaptoethanol, 120 mM Tris-HCl (pH 6.7), and a trace of bromphenol blue (2X gel sample buffer) were added to the remaining quenched autophosphorylation reaction in preparation for gel electrophoresis, tryptic digestion and separation of phosphopeptides by reverse-phase HPLC.

**Dephosphorylation of Autophosphorylated Kinase** - Protein phosphatase-2A was diluted with phosphatase buffer (50 mM Tris-HCl [pH 7.0], 30 mM dithiothreitol, 5 mM caffeine, 1 mg/ml bovine serum albumin). Type II CaM kinase was autophosphorylated as described above, and reactions were terminated with EDTA stop-buffer (30°C). Dephosphorylation was initiated 5 s later by the addition of 0.33 µg protein phosphatase-2A in 10µl phosphatase buffer. Control incubations were diluted with 10µl of phosphatase buffer. At the times indicated in figure legends, 10 µl aliquots were diluted 50-fold into ice-cold 50 mM Tris-HCl (pH 8.0), 1 mg/ml bovine serum albumin. Kinase activity was measured immediately with exogenous substrate in the presence and absence of CaM. Dephosphorylation reactions were terminated, at the same times, by the addition of 50 µl of 2X gel sample buffer, in preparation for gel electrophoresis, tryptic digestion and phosphopeptide mapping.

**Assays of Kinase Activity** - Kinase activity was measured in a reaction mixture (final volume 50 µl) containing 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.2 mM [$\gamma$-$^{32}$P]-ATP (1.5-3.0 x $10^3$ cpm/pmol), either 10 µg synapsin I (2.5 µM), or 2.16 µg CaM kinase peptide substrate (40 µM), and either 0.4 mM EGTA (minus calcium) or 0.4 mM EGTA/0.9 mM $CaCl_2$ (plus calcium). Assays in the presence of $Ca^{2+}$ also contained 2.5 µg calmodulin. Reactions were initiated by addition of 5 µl of diluted kinase (10 ng) to pre-warmed (30°C) reaction mixtures and incubated for 15 sec. Reactions were terminated by the addition of 2X SDS gel sample buffer. Incorporation of phosphate into substrates was determined after separation of $^{32}$P-labeled product from [$\gamma$-$^{32}$P]ATP by SDS-PAGE, as described previously (25). $^{32}$P-labeled synapsin I was separated in 8% polyacrylamide gels and peptide substrate in 21% polyacrylamide gels. Separation of peptide product by gel electrophoresis produced a higher signal to noise ratio than separation of peptide product by binding to phosphocellulose paper (26). The 21% acrylamide gels often polymerized unevenly at the surface, which caused streaking of the peptide during electrophoresis. This problem was prevented by adding a 2 cm layer of 13% acrylamide while the 21% acrylamide was polymerizing. Mixing at the boundary was prevented by the addition of 5% glycerol (w/v) to the 21% acrylamide solution.

**Tryptic Peptide Mapping of Phosphokinase** - Duplicate aliquots (0.2 µg kinase) of each quenched autophosphorylation and dephosphorylation reaction were subjected to SDS-PAGE and incorporation of labeled phosphate into the subunits was measured as described previously (10, 13). Fifty µl of 0.15% (w/v) deoxycholic acid, and 400 µl of ice-cold 12% (w/v) trichloroacetic acid were added to the remaining phosphokinase (3.6 µg), and the mixture was incubated for 15 min on ice. Precipitated protein was sedimented by centrifugation at 13,000 x g for 15 min, and the supernatants removed by aspiration. SDS was removed from the pellets by two washes with 1 ml of -20°C acetone. Protein was then reduced with dithiothreitol, and carboxamidomethylated by treatment with iodoacetamide as described previously (10), except that reduction was performed in 10 mM dithiothreitol, and carboxamidomethylation proceeded for 90 min in the dark at 4°C. The subunits were then separated by SDS-PAGE on 10% polyacrylamide gels, and digested with trypsin as described previously (10).

After elution from the gel pieces, tryptic peptides were filtered through 0.2 µm filters, lyophilized and resuspended in 250-300 µl of 0.07% (v/v) TFA (pH 2.3). In the elution step, the recovery of radioactivity from the gel pieces was 86% ± 8% for the α-subunit, and 76% ± 8% for the β-subunit. The peptides were fractionated on a C4 reverse-phase column (0.46 x 25 cm) at 1.0 ml/min, as described previously (10). Briefly, the sample was injected onto a column equilibrated for 30-40 min with Buffer A (0.07% (v/v) trifluoroacetic acid in $H_2O$). The column was developed with the following gradient of Buffer B (70% (v/v) acetonitrile, 30% (v/v) $H_2O$, 0.07% (v/v) trifluoroacetic acid): 0-5 min, 0% Buffer B; 5-95 min, 0 to 35% Buffer B; 95-100 min, 35% to 100% Buffer B. Column effluent was collected in 200 fractions of 0.5 ml. Radioactivity was detected as Cerenkov radiation (49% efficiency). The total recovery of labeled peptides from the column was 63-85%. An additional 9-18% eluted in a broad peak when the column was washed with 100% buffer B.

**Time Courses of Phosphorylation and Dephosphorylation of Sites** - The time courses of phosphorylation and dephosphorylation of particular sites were plotted in the following way. For each time point, the areas under peaks of radioactivity were calculated with an integration program. Then, the fraction of radioactivity in each peak was calculated by dividing the area of the peak by the total area of all radioactive peaks. The amount of radioactivity in peptides containing a particular site was calculated by multiplying the fraction of radioactivity in those peptides by the total radioactivity in the kinase subunit before digestion with trypsin (measured for each time point in the gel piece after gel electrophoresis). To plot the time course of autophosphorylation of a particular site, the radioactivity in that site at each time point was plotted as a percent of the radioactivity in the site at the longest time point. To plot the time course of dephosphorylation of a site, the radioactivity in the site at each time point was plotted as a percent of the radioactivity in the site at time zero. The molar amount of phosphate incorporated into each site was estimated by multiplying the fraction of radioactivity in the site by the total moles of phosphate per mole of subunit (measured independently, as described above). The calculated maximal molar amount of phosphate in a given site was variable among different experiments, even under apparently identical conditions. For example, the maximum incorporation of phosphate into some sites, calculated in this way, varied from 0.1 moles per mole subunit to 0.7 moles per mole subunit. The determination of total moles phosphate per mole subunit was the source of the largest variance. This appeared to result from variable recovery of protein subunits during gel electrophoresis, and we were unable to correct for it. Chemical instability of certain peptides may have been an additional source of variance. Nevertheless, the specific catalytic activity of the kinase, the time courses of phosphorylation of each site, and the time to reach maximum phosphorylation were highly reproducible from experiment to experiment (Figs. 1, 3, 4 and 5).

**Phosphoamino Acid Analysis** - Several labeled phosphopeptides were subjected to partial acid hydrolysis in 6 N HCl for 2 hr at 110°C *in vacuo*. The hydrolysates were lyophilized and resuspended in electrophoresis buffer (acetic acid/formic acid/$H_2O$ [78:25:897], pH 1.9). Phosphoamino acid standards (phosphoserine, phosphothreonine, and phosphotyrosine) were added and the hydrolysates were electrophoresed on thin layer cellulose sheets for 2 hr at 750 V as described by Darbre (27). The phosphoamino acid standards were visualized with ninhydrin and the positions of $^{32}$P-labeled phosphoamino acids were determined by autoradiography.

**Preparation of Phosphopeptides for Sequencing** - To obtain quantities of phosphopeptides sufficient for sequencing, autophosphorylation reactions were scaled up. Reactions were performed in 1 ml batches, each containing 200 µg kinase, as previously described (10). Autophosphorylation was performed for 30 to 120 sec in the presence of calcium before addition of 100 µl of 22 mM EGTA. The reactions continued after the removal of calcium for an additional 60 sec and were terminated by addition of 100 µl of 100% (w/v) trichloroacetic acid. Reduction, carboxamidomethylation, SDS-PAGE, tryptic digestion, and recovery of peptides were performed as described above, except that 100 µg of kinase was fractionated per lane in 10% polyacrylamide gels and the separated subunits from each lane were digested with a total of 50 µg of trypsin. Peptides from all 1 ml reactions were pooled (typically 3 to 5 1 ml reactions) and fractionated by HPLC on a C4 reverse-phase column in two or three batches, as described above, to avoid overloading the column. Fractions containing the peptides to be sequenced were pooled and the volume was reduced to approximately 100 µl in a Speed-Vac concentrator. Final purification of each peak was achieved by chromatography on a C18 reverse-phase column equilibrated in Buffer A. Immediately prior to injection, each sample was brought to 300 µl by addition of Buffer A. The peptide containing α-$Ser_{40}$ was eluted with the gradient: 0-10 min, 0% buffer B; 10-90 min, 0% to 10% buffer B; 90-100 min, 10% to 100% buffer B. The peptide containing β-$Ser_{31}$ was eluted with the gradient: 0-10 min, 0% buffer B; 10-15 min, 0% to 10% buffer B; 15-95 min, 10% to 15% buffer B; 95-100 min, 15% to 100% buffer B. The peptide containing β-$Thr_{30}$ was eluted with the gradient: 0-10 min, 0% buffer B; 10-15 min, 0% to 18% buffer B; 15-120 min, 18% to 25% buffer B; 120-150 min, 25% to 100% buffer B. Peptide peaks were detected by monitoring absorbance of the effluent at 214 nm, and 0.5 ml fractions were collected by hand. The pure phosphopeptides were sequenced on an Applied Biosystems automated gas-phase sequencer, or subjected to total hydrolysis and amino acid analysis, as previously described (10).

In some instances, peptide T1 recovered from the C4 column was proteolysed with thermolysin. Fractions containing the peak were concentrated to 25-50 µl. Thermolysin (1µg) in 400 µl of 0.1 M $NH_4HCO_3$ (pH 8.0) was added and digestion continued for 4 hrs at 37° C. The digest was applied to a C18 reverse-phase HPLC column equilibrated in buffer A. Peptides were eluted with the following gradient of Buffer B: 0-10 min, 0% buffer B; 10-90 min, 0 to 15% buffer B; 90-100 min, 15 to 100% buffer B. Fractions containing radioactive phosphopeptides were identified by counting Cerenkov radiation.

**Other Methods** - Protein was measured by the method of Peterson (31) with bovine serum albumin as standard.

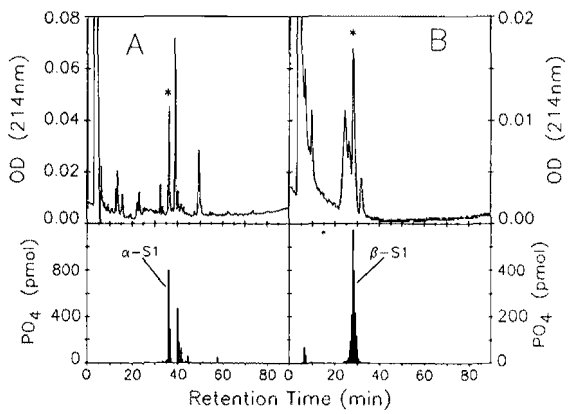


**Figure 2. Purification of Peptides α-S1 and β-S1 by HPLC C18 Column Chromatography.** Kinase holoenzyme with a 1:1 ratio of α to β subunits (3.2 mg) was autophosphorylated for 120 s in the presence of $Ca^{2+}$, calmodulin, and [$\gamma$-$^{32}$P]ATP, then for 60 s after the addition of EGTA to chelate free $Ca^{2+}$, as described in the legend to Fig. 1. The α and β subunits were reduced, alkylated, purified, and digested with trypsin as described under Experimental Procedures. The resulting phosphopeptides were fractionated by chromatography on a C4 column. Fractions containing phosphopeptides α-S1 (Figure 1) and β-S1 were concentrated and fractionated on a C18 column as described under Experimental Procedures. Absorbance of the eluate was monitored at 214 nm and fractions containing radioactivity were identified by measuring Cerenkov radiation. Fractions corresponding to peaks marked by asterisks were concentrated and submitted for N-terminal sequence analysis (Table I). (A) Peptide α-S1; The fraction eluting at 36 min was submitted for sequencing. (B) Peptide β-S1; The central fraction eluting at 28 min was submitted for sequencing.

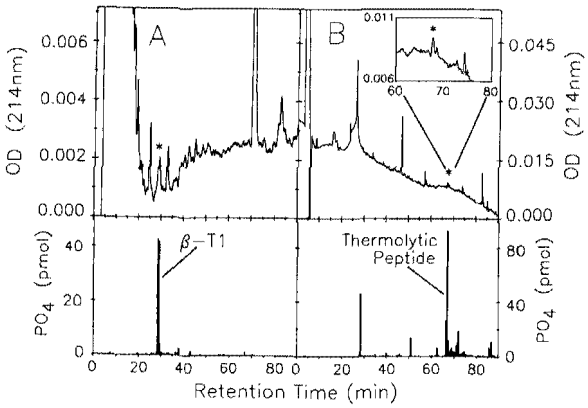


**Figure 3. Purification of Peptide β-T1 and a Thermolytic Peptide Derived from β-T1 by HPLC on a C18 Column.**

Kinase holoenzyme with a 1:1 ratio of α to β subunits (600 μg) was autophosphorylated for (A) 10 s in the presence of $Ca^{2+}$, calmodulin and [γ -$^{32}$P]ATP, then for 90 s in the absence of $Ca^{2+}$ or for (B) 120 s in the presence of $Ca^{2+}$, calmodulin and [γ -$^{32}$P]ATP and 60 s in the absence of $Ca^{2+}$ as described in the legend to Fig. 1. In each case, kinase was reduced and alkylated, and the β subunit was purified and digested with trypsin as described under Experimental Procedures. The resulting phosphopeptides were fractionated by chromatography on a C4 reverse-phase HPLC column. (A) Fractions containing peptide β-T1 were concentrated and fractionated by chromatography on a C18 column (see Experimental Procedures). Absorbance of the eluate was monitored at 214 nm, and fractions containing radioactivity were identified by measuring Cerenkov radiation. The two fractions eluting at 28.5 min, each of which contained approximately equal amounts of radioactivity, were pooled, concentrated and submitted for N-terminal sequence analysis (Table 2).

(B) In a separate experiment, fractions from the C4 column, containing the β-T1 peptide, were concentrated, then further proteolysed with thermolysin (1 μg) as described under Experimental Procedures. The digest was fractionated on a C18 column. The ratio of the two major thermolytic peptides varied with the hydrolysis conditions, suggesting that they are alternative thermolytic products of the same peptide. The digestion conditions were optimized to produce one major peak which was sequenced. The fraction eluting at 67 min and containing most of the radioactivity in the peak was concentrated and submitted for N-terminal sequence analysis (Table 2).

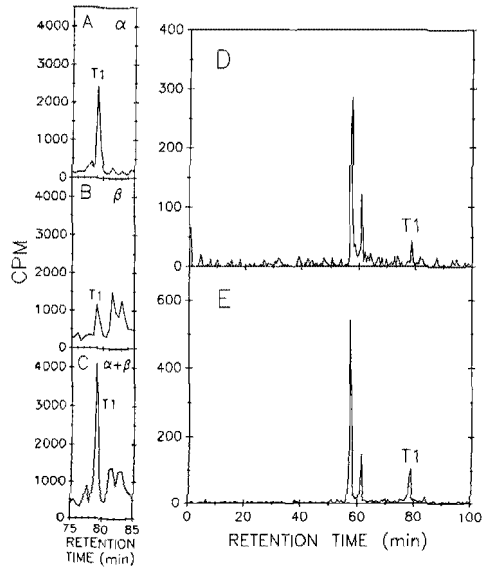


**Figure 4. T1 peptides from the α and β subunits are identical.**

Type II CaM kinase (7.5 μg) was autophosphorylated for 10 s in the presence of $Ca^{2+}$, calmodulin, and [γ-$^{32}$P]ATP, then for 50 s in the absence of $Ca^{2+}$. Tryptic phosphopeptides were prepared and aliquots containing 7 x $10^4$ cpm of labeled peptides from each subunit were fractionated by HPLC chromatography as described in Fig. 1. (A) Peptide T1 from the α subunit. (B) Peptide T1 from the β subunit. (The peaks that eluted later than T1 contain β-$Ser_{91}$ and β-$Thr_{93}$, respectively (10).) (C) Peptide T1 from a sample in which equal amounts of phosphopeptides from the α and β subunits were mixed prior to application to the HPLC column. In panels A-E the retention time of the T1 peptide was 79 min.

In a separate experiment, fractions containing the T1 peptide from the α or β subunits were reduced in volume after elution from a C4 HPLC column, and then chromatographed a second time on the same column. (D) T1 peptide originally prepared from the α subunit. (E) T1 peptide originally prepared from the β subunit.