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Regulation of Ca^{2+}/Calmodulin-Dependent Protein Kinase Kinase α by cAMP-Dependent Protein Kinase: II. Mutational Analysis[1]

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We previously reported that rat brain Ca^{2+}/calmodulin-dependent protein kinase (CaM-kinase) IV is inactivated by cAMP-dependent protein kinase (PKA) [Kameshita, I. and Fujisawa, H. (1991) *Biochem. Biophys. Res. Commun.* 180, 191–196]. In the preceding paper, we demonstrated that changes in the activity of CaM-kinase IV by PKA results from the phosphorylation of CaM-kinase kinase α by PKA and identified six phosphorylation sites, Ser^{24} for autophosphorylation, and Ser^{52}, Ser^{74}, Thr^{108}, Ser^{458}, and Ser^{475} for phosphorylation by PKA. In the present study, a causal relationship between the phosphorylation and change in the activity toward PKIV peptide has been studied using mutant enzymes with amino acid substitutions at the six phosphorylation sites. The following conclusions can be drawn from the experimental results: (i) Phosphorylation of Ser74 and/or unidentified sites causes an increase in activity; (ii) phosphorylation of Thr^{108} or Ser^{458} causes a decrease in the activity; (iii) the inhibitory effect of the phosphorylation of Thr^{108} is canceled by the stimulatory effect of the phosphorylation, but that of Ser^{458} is not; and (iv) the inhibitory effects of Thr^{108} and Ser^{458} are synergistic. In contrast to the activity toward PKIV peptide, the activity toward CaM-kinase IV appears to be decreased by the phosphorylation of Thr^{108}, but not significantly affected by the phosphorylation of Ser^{458}.

Key words: cAMP-dependent protein kinase, Ca^{2+}/calmodulin, CaM-kinase kinase, phosphorylation site, protein phosphorylation.

Our previous finding (*1*) that Ca^{2+}/calmodulin-dependent protein kinase (CaM-kinase) IV is inactivated by incubation with cyclic AMP-dependent protein kinase (PKA) under the phosphorylation conditions provided a valuable example of cross-talk between Ca^{2+}-signaling and cyclic AMP-signaling pathways, because CaM-kinase IV is thought to be a key enzyme in the Ca^{2+}-signaling system in cells (*2*). In the preceding paper (*3*), the inactivation of CaM-kinase IV by PKA was demonstrated to result from the prevention of the activation of CaM-kinase IV through CaM-kinase kinase α by PKA, and several phosphorylation sites on CaM-kinase kinase α, Ser^{24} for autophosphorylation and Ser^{52}, Ser^{74}, Thr^{108}, Ser^{458}, and Ser^{475} for PKA, were identified. However, biochemical studies did not reveal a clear relationship between phosphorylation at these phosphorylation sites and changes in enzyme activity. In the present paper, mutational analyses based on the biochemical data for the phosphorylation sites were performed in an attempt to reveal the molecular mechanism underlying the phosphorylation-activity relationship of CaM-kinase kinase. During the course of the studies, Wayman et al. reported mutational studies of the putative phosphorylation sites of CaM-kinase kinase α, suggesting the importance of Thr^{108} and Ser^{458} in the inactivation by PKA (*4*).

EXPERIMENTAL PROCEDURES

Materials—[γ-^{32}P]ATP (5,000 Ci/mmol) and the Thermo sequenase fluorescent-labelled primer cycle sequencing kit with 7-deaza-dGTP were from Amersham Pharmacia Biotech. Microbial protease inhibitors (pepstatin A, leupeptin, antipain, and chymostatin) were from the Peptide Institute (Osaka). DEAE-cellulose (DE52), phosphocellulose paper (P81), and 3MM paper were from Whatman. FluoroTrans, a polyvinylidene difluoride membrane, was from Pall Gelman Laboratory. NHS-LC-biotin and avidin conjugated with horseradish peroxidase were from Pierce. PKIV peptide (KKKKEHQVLMKTVCGTPGY) (*5*) was synthesized with a Shimadzu PSSM-8 automated peptide synthesizer. GeneEditor™ *in vitro* site-directed mutagenesis system was from Promega. pET11d was from Novagen. Restriction enzymes and other DNA modifying enzymes were purchased from Takara Shuzo, Toyobo, or New England Biolabs. All other reagents were of the highest grade commercially available.

Protein Preparations—Calmodulin was purified from *E. coli* cells transformed with expression vector pET11d carrying a cDNA encoding chicken calmodulin (*6*), essentially as described by Gopalakrishna and Anderson (*7*). The cDNA

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Abbreviations: CaM-kinase, calmodulin-dependent protein kinase; PKA, cyclic AMP-dependent protein kinase; SDS-PAGE, SDS–polyacrylamide gel electrophoresis.

encoding chicken calmodulin was kindly donated by A.R. Means (*8*). Biotinylated calmodulin was prepared from the recombinant chicken calmodulin as described by Mangels and Gnegy (*9*). Recombinant rat CaM-kinase kinase α expressed in *E. coli* (*10*) was purified as described previously (*11*). PKA (catalytic subunit of cyclic AMP-dependent protein kinase) was purified from bovine heart as described previously (*12*). Recombinant rat CaM-kinase IV expressed in Sf9 cells was purified as described previously (*6*). Recombinant rat CaM-kinase IV(K$_{71}$R), in which Lys^{71} (ATP-binding site) was replaced with arginine, was expressed in Sf9 cells and purified as described previously (*11*).

cDNAs for mutants of CaM-kinase kinase α in which threonine and/or serine residues identified as phosphorylation sites by autophosphorylation or PKA (*3*) were replaced with other amino acids, such as alanine, aspartate, or glutamate, were prepared by site-directed mutagenesis as follows. cDNAs for the CaM-kinase kinases (S$_{24}$A), (S$_{24}$D), (S$_{52}$A), (S$_{52}$D), (S$_{74}$A), and (S$_{74}$D) were prepared using the single-stranded DNA obtained from pUC118, into which the *Xba*I–*Xba*I fragment (288 bp) of pETCaMKKα (*10*) was inserted, as a template, and appropriate synthetic oligonucleotides as primers, essentially according to the method of Kunkel *et al.* (*13*). The mutations were confirmed by the dideoxynucleotide chain-termination method (*14*) using a DNA sequencer, LI-COR model 4000L. The pET11d carrying each cDNA containing the entire coding sequence of the mutant CaM-kinase kinase α was constructed by ligation of three fragments, the *Nco*I–*Xba*I fragment (250 bp) of the pUC118 containing the mutated segment, and the *Xba*I–*Sfi*I fragment (702 bp) and *Sfi*I–*Nco*I fragment (6.2 kbp) of pETCaMKKα. cDNAs for the CaM-kinase kinases (S$_{458}$A), (S$_{458}$D), (S$_{475}$A), and (S$_{475}$D) were prepared similarly using the single-stranded DNA obtained from pUC118, into which the *Pst*I–*Bam*HI fragment (282 bp) of pETCaMKKα was inserted, as a template, and appropriate synthetic oligonucleotides as primers. After the mutations were confirmed, the pET11d carrying the cDNA for the mutant enzyme was constructed by ligation of the *Pst*I–*Bam*HI fragment (282 bp) of the pUC118 containing the mutated segment and the *Bam*HI–*Sfi*I (6.6 kbp) and *Sfi*I–*Pst*I segments (318 bp) of pETCaMKKα. cDNAs for the CaM-kinase kinases (T$_{108}$A), (T$_{108}$D), (T$_{108}$E), (S$_{458}$E), and (S$_{475}$E) were prepared using the GeneEditor™ *in vitro* site-directed mutagenesis system (Promega) with pETCaMKKα (*10*) as a template and appropriate synthetic oligonucleotides as primers. A pET11d carrying cDNA for the mutant enzyme (S$_{24}$D/S$_{52}$D/S$_{475}$D) was constructed by ligating the *Bam*HI–*Xma*I fragment (5.8 kbp) of the pET11d carrying the cDNA for the enzyme (S$_{24}$D), the *Xma*I–*Sfi*I fragment (814 bp) of pET11d carrying the enzyme (S$_{52}$D), and the *Sfi*I–*Bam*HI fragment (600 bp) of pET11d carrying the enzyme (S$_{475}$D). A pET11d carrying cDNA for the enzyme (S$_{52}$D/S$_{74}$D) was constructed by ligating the *Nco*I–*Hga*I fragment (193 bp) of the pET11d carrying the enzyme (S$_{52}$D), the *Hga*I–*Bam*HI fragment (1,359 bp) of pET11d carrying the enzyme (S$_{74}$D), and the *Bam*HI–*Nco*I fragment (5.6 kbp) of pETCaMKKα. pET11d carrying cDNAs for the enzymes (S$_{52}$D/S$_{74}$D/S$_{458}$D) and (S$_{52}$D/S$_{74}$D/S$_{475}$D) were constructed by ligating the *Bam*HI–*Sfi*I fragment (6.6 kbp) of the pET11d carrying the enzyme (S$_{52}$D/S$_{74}$D) and the *Sfi*I–*Bam*HI fragment (600 bp) of the pET11d carrying the enzymes (S$_{458}$D) and (S$_{475}$D), respectively. A pET11d carrying a cDNA for the enzyme (S$_{52}$D/S$_{74}$D/S$_{458}$D/S$_{475}$D) was constructed by ligating the *Bgl*II–*Bgl*II fragment (1,519 bp) of the pET11d carrying the enzyme (S$_{52}$D/S$_{74}$D/S$_{458}$D) and the *Bgl*II–*Bgl*II fragment (5.7 kbp) of pET11d carrying the enzyme (S$_{475}$D). pET11d carrying cDNA, for the mutant enzymes (S$_{52}$D/S$_{458}$D) and (T$_{108}$D/S$_{458}$D) were constructed by ligating the *Bam*HI–*Sfi*I fragment (6.6 kbp) of the pET11d carrying the enzymes (S$_{52}$D) and (T$_{108}$D), respectively, and the *Sfi*I–*Bam*HI fragment (600 bp) of the pET11d carrying the enzyme (S$_{458}$D). pET11d carrying cDNA, for the enzymes (S$_{52}$D/S$_{475}$D), (S$_{74}$D/S$_{475}$D), and (T$_{108}$D/S$_{475}$D) were constructed by ligating the *Bam*HI–*Sfi*I fragment (6.6 kbp) of the pET11d carrying the enzymes (S$_{52}$D), (S$_{74}$D), and (T$_{108}$D), respectively, and the *Sfi*I–*Bam*HI fragment (600 bp) of the pET11d carrying the enzyme (S$_{475}$D). All mutations were confirmed by the dideoxynucleotide chain-termination method (*14*) with a DNA sequencer (LI-COR model 4000L). *E. coli* strain BL21(DE3) (*15*) was transfected with a pET11d carrying cDNA for a mutated enzyme and grown to an A_{600} value between 0.6 and 1.0 at 30°C in 50 ml of M9ZB medium containing 200 μg/ml ampicillin. Isopropyl β-D-thiogalactoside (IPTG) was then added to a final concentration of 1 mM. After 2.5 h, the bacteria were harvested by centrifugation, washed in buffered saline, suspended in 1 ml of 20 mM Tris-HCl (pH 7.5 at 4°C) containing 1 mM dithiothreitol, 1 mM EGTA, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of microbial protease inhibitors (pepstatin A, leupeptin, antipain, and chymostatin), and then disrupted by sonic oscillation. The supernatants were obtained by centrifugation, and the mutated enzymes were purified to apparent homogeneity by SDS-PAGE, as shown in Fig. 1, by streptomycin treatment, and by chromatographies on DE52 and calmodulin-Sepharose columns as described previously (*11*).

Phosphorylation Reaction—The phosphorylation of proteins or peptides by protein kinases was carried out at 30°C in the standard phosphorylation mixture comprising 50 mM Mops-NaOH (pH 7.0 at 30°C), 2 mM dithiothreitol, 5 mM $Mg(CH_3COO)_2$, 0.1 mM nonradioactive or [γ-^{32}P]ATP, 1 μM calmodulin, 0.1 mM EGTA, 0.2 mM $CaCl_2$, and the indicated amounts of proteins or peptides. After incubation for the indicated times, the incorporation of [^{32}P]phosphate into the protein substrates was determined by the 3MM paper method of Corbin and Reimann (*16*), except that the filter papers were washed with ice-cold 10% trichloroacetic acid containing 2 mM ATP. The incorporation of [^{32}P]phosphate into the peptide substrates was determined by the phosphocellulose paper method of Roskoski (*17*).

Other Procedures—The concentration of calmodulin was determined spectrophotometrically using an absorption coefficient at A_{280} (1 mg/ml) of 0.21 (*18*) and a molecular weight of 16,700 (*19, 20*). Other proteins were determined by the method of Lowry *et al.* (*21*), as modified by Peterson, (*22*) using bovine serum albumin as a standard. The amount of CaM-kinase IV(K$_{71}$R) was corrected for an overestimation by a factor of 1.15 made by the Lowry's method (*23*). SDS-PAGE was carried out according to the method of Laemmli (*24*). Gel overlay assay by biotinylated calmodulin was performed as described by Kincaid *et al.* (*25*).

RESULTS

As reported in the preceding paper (*3*), the activity of CaM-kinase kinase α toward the PKIV peptide is increased by incubation with PKA in the presence of Ca^{2+}/calmodulin under phosphorylation conditions, but decreased by incubation with PKA in the absence of Ca^{2+}/calmodulin, and six phosphorylation sites, one for autophosphorylation (Ser^{24}) and five for phosphorylation by PKA (Ser^{52}, Ser^{74}, Thr^{108}, Ser^{458}, and Ser^{475}) are present in CaM-kinase kinase α. The phosphorylation-activity relationship was investigated by the biochemical study, but a definite conclusion could not be drawn. To specify which of the six phosphorylation sites are involved in changes in enzyme activity, 25 mutant enzymes, in which the phosphorylatable serine and/or threonine residues were replaced with alanine to prevent phosphorylation or with aspartate or glutamate to mimic phosphorylation, were prepared by site-directed mutagenesis, and the respective mutant enzymes expressed in *E. coli* were purified to apparent homogeneity by SDS-PAGE, as shown in Fig. 1. Among the 25 mutant enzymes, 22 gave single protein bands at a position corresponding to the wild-type enzyme, and the other three, CaM-kinase kinases ($S_{24}D$), ($S_{24}D/S_{458}D$), and ($S_{24}D/S_{52}D/S_{475}D$), in which Ser^{24} was replaced with aspartate, gave single bands that migrated more slowly. SDS-PAGE analysis followed by autoradiography of the wild-type enzyme autophosphorylated by incubation with [γ-^{32}P]ATP in the presence of Ca^{2+}/calmodulin under the phosphorylation conditions gave a similar slower migrating radioactive band (data not shown), indicating that mutant enzymes in which Ser^{24} is replaced with aspartate mimic the autophosphorylated enzyme in their electrophoretic behavior. On the other hand, replacing phosphorylation sites other than Ser^{24} with aspartate caused no significant shift in the electrophoretic mobility.

Table I shows the specific activities of the wild-type and 25 mutant CaM-kinase kinases α toward PKIV peptide before and after preincubation for 30 min at 30°C with or without PKA in the presence or absence of Ca^{2+}/calmodulin under phosphorylation conditions. None of the 26 CaM-kinase kinases showed a significant change in activity following incubation in the absence of Ca^{2+} and PKA, indicating that the enzymes were stably maintained under the experimental conditions.

The activities of the 25 mutants of CaM-kinase kinase α toward PKIV peptide measured without preincubation are compared with that of the wild-type enzyme, as shown in Fig. 2. Among the 25 mutant enzymes, 9 showed significantly lower activities than the wild-type enzyme. Among them, 8 mutants contained aspartate or glutamate as a substitute for Thr^{108}, aspartate for Ser^{458}, or both. The fact that all of mutants containing aspartate or glutamate for Thr^{108}, or aspartate for Ser^{458} showed low activities as compared with the wild-type enzyme suggests that the phosphorylation of Thr^{108} or Ser^{458} causes a decrease in the enzyme activity toward PKIV peptide. The activity of the double mutant ($T_{108}D/S_{458}D$), in which both Thr^{108} and Ser^{458} were replaced with aspartate, was only 28% of that of

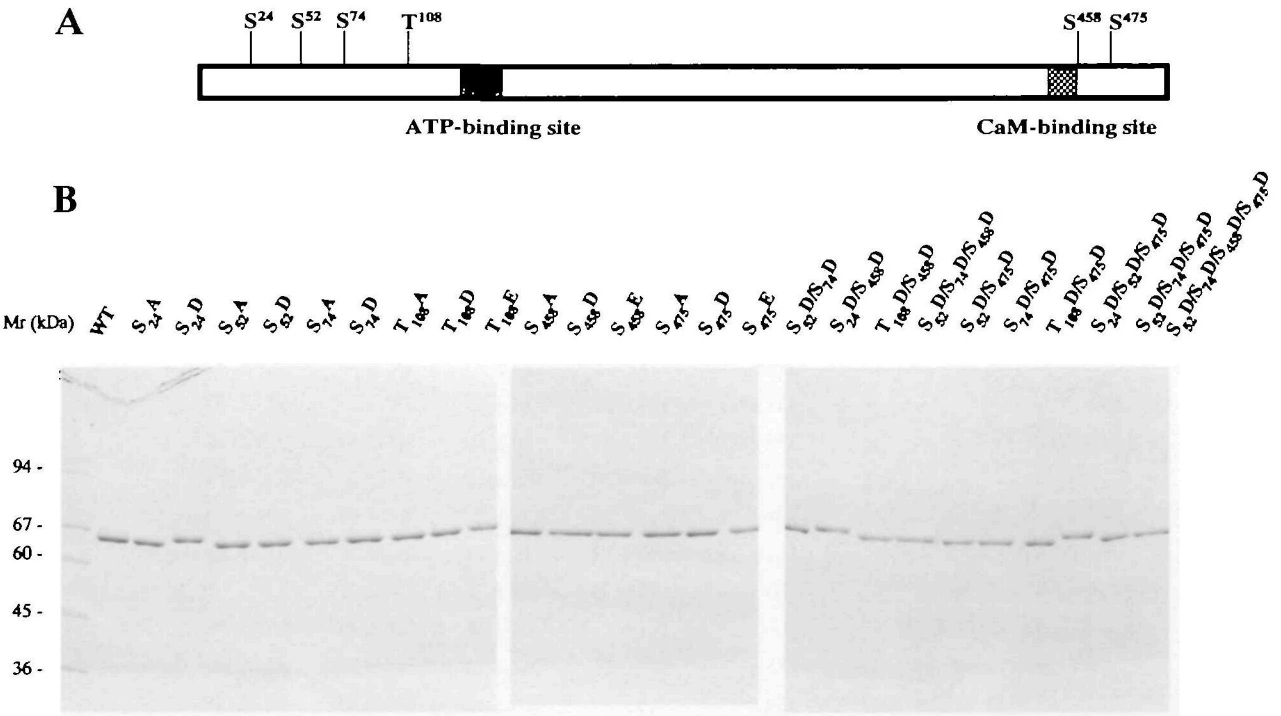

Fig. 1. **SDS-PAGE of mutants of CaM-kinase kinase α.** (A) The positions of the phosphorylatable serine and threonine residues of CaM-kinase kinase α (*3*) are shown schematically. (B) Approximately 0.5 μg of wild-type (WT) and mutated CaM-kinase kinase α, in which alanine (A), aspartate (D), and glutamate (E) residues were substituted for the phosphorylatable serine (S) or threonine (T) residues, ($S_{24}A$), ($S_{24}D$), ($S_{52}A$), ($S_{52}D$), ($S_{74}A$), ($S_{74}D$), ($T_{108}A$), ($T_{108}D$), ($T_{108}E$), ($S_{458}A$), ($S_{458}D$), ($S_{458}E$), ($S_{475}A$), ($S_{475}D$), ($S_{475}E$), ($S_{52}D/S_{74}D$), ($S_{24}D/S_{458}D$), ($T_{108}D/S_{458}D$), ($S_{52}D/S_{74}D/S_{458}D$), ($S_{52}D/S_{475}D$), ($S_{74}D/S_{475}D$), ($T_{108}D/S_{475}D$), ($S_{24}D/S_{52}D/S_{475}D$), ($S_{52}D/S_{74}D/S_{475}D$), and ($S_{52}D/S_{74}D/S_{458}D/S_{475}D$), were purified as described under "EXPERIMENTAL PROCEDURES," subjected to SDS-PAGE on a 7.5% acrylamide gel, and then stained with Coomassie Brilliant Blue R-250.

the wild-type enzyme, much lower than the activities of the respective single mutants ($T_{108}D$) and ($S_{458}D$), whose activities were 73 and 69%, respectively, of the wild-type enzyme. These data indicate the synergistic effect of the phosphorylation of Thr^{108} and Ser^{458}. In contrast to the $S_{458}D$ mutant, $S_{458}E$ and $S_{458}A$ showed almost the same activity as the wild-type enzyme. This suggests that the substitution of glutamate for Ser^{458} does not mimic the phosphorylation of Ser^{458}, although the substitution of aspartate does. The $S_{74}A$, $S_{74}D$, and $S_{74}D/S_{475}D$ mutants showed significantly higher activities than the wild-type enzyme, suggesting that the phosphorylation of Ser^{74} causes an increase in enzyme activity, and that the activation is probably due to the conversion of this serine to another residue because replacing Ser^{74} not only with aspartate, but also with alanine caused an increase in the enzyme activity. The fact that the activity of the enzyme ($S_{52}D/S_{74}D/S_{458}D$) was almost the same as that of the enzyme ($S_{458}D$) suggests that the inhibi-

TABLE I. **Specific activities of CaM-kinase kinase α mutants toward PKIV peptide.** Approximately 20 μg/ml of purified wild-type and mutated CaM-kinase kinase α, in which alanine (A), aspartate (D), and glutamate (E) residues were substituted for the phosphorylatable serine (S) or threonine (T) residues, were preincubated at 30°C in the standard phosphorylation mixture containing nonradioactive 0.1 mM ATP with or without 0.5 μg/ml PKA in the presence or absence of Ca^{2+}/calmodulin, as indicated. Before and after preincubation for 30 min, a 10-μl aliquot was incubated at 30°C for 1 min in a final volume of 50 μl of phosphorylation mixture containing 0.2 mM PKIV peptide and 0.1 mM [γ-^{32}P]ATP (about 200 cpm/pmol). The incorporation of [^{32}P]phosphate into the peptide was determined by the phosphocellulose paper method. The results are expressed as the specific activities (nmol/min/mg) and each value represents the mean ± SD of four or five independent determinations.

Substitutions						Specific activity (nmol/min/mg)				
							Preincubation			
S^{24}	S^{52}	S^{74}	T^{108}	S^{458}	S^{475}	(-)	(-Ca^{2+})	(-Ca^{2+}/+PKA)	(+Ca^{2+})	(+Ca^{2+}/+PKA)
S	S	S	T	S	S	283 ± 22	276 ± 32	186 ± 26	365 ± 32	546 ± 67
A	-	-	-	-	-	293 ± 25	307 ± 39	193 ± 24	352 ± 30	565 ± 40
D	-	-	-	-	-	279 ± 18	266 ± 25	170 ± 16	317 ± 15	447 ± 48
-	A	-	-	-	-	319 ± 50	342 ± 54	203 ± 25	430 ± 97	564 ±117
-	D	-	-	-	-	222 ± 32	225 ± 25	133 ± 16	279 ± 37	399 ± 34
-	-	A	-	-	-	366 ± 21	381 ± 38	201 ± 20	464 ± 51	562 ± 49
-	-	D	-	-	-	397 ± 23	387 ± 55	214 ± 9	514 ± 45	634 ± 53
-	-	-	A	-	-	281 ± 34	276 ± 30	246 ± 21	406 ± 50	551 ± 46
-	-	-	D	-	-	207 ± 28	201 ± 25	183 ± 21	387 ± 47	547 ± 70
-	-	-	E	-	-	169 ± 11	180 ± 18	164 ± 14	334 ± 23	467 ± 23
-	-	-	-	A	-	328 ± 16	324 ± 30	317 ± 33	416 ± 75	631 ± 74
-	-	-	-	D	-	195 ± 21	201 ± 21	123 ± 7	287 ± 29	352 ± 22
-	-	-	-	E	-	299 ± 31	321 ± 25	253 ± 23	392 ± 35	559 ± 42
-	-	-	-	-	A	335 ± 25	329 ± 37	216 ± 9	433 ± 26	674 ± 37
-	-	-	-	-	D	330 ± 27	311 ± 49	190 ± 19	416 ± 44	613 ± 62
-	-	-	-	-	E	265 ± 19	269 ± 29	165 ± 17	331 ± 28	515 ± 27
-	D	D	-	-	-	301 ± 37	314 ± 33	177 ± 21	402 ± 61	504 ± 56
D	-	-	-	D	-	188 ± 13	189 ± 12	119 ± 11	248 ± 20	322 ± 29
-	-	-	D	D	-	80 ± 8	73 ± 8	84 ± 8	175 ± 11	228 ± 9
-	D	D	-	D	-	179 ± 23	161 ± 33	92 ± 16	306 ± 48	262 ± 38
-	D	-	-	-	D	285 ± 33	271 ± 26	168 ± 10	344 ± 36	515 ± 23
-	-	D	-	-	D	369 ± 42	379 ± 37	209 ± 38	491 ± 65	649 ± 48
-	-	-	D	-	D	191 ± 13	178 ± 24	144 ± 20	338 ± 22	441 ± 59
D	D	-	-	-	D	298 ± 23	300 ± 31	177 ± 11	347 ± 8	487 ± 18
-	D	D	-	-	D	273 ± 20	281 ± 39	149 ± 19	349 ± 33	435 ± 48
-	D	D	-	D	D	156 ± 22	146 ± 5	81 ± 8	244 ± 22	232 ± 25

tory effect of the phosphorylation of Ser^{458} is dominant over the stimulatory effect of the phosphorylation of Ser^{74}. Mutant enzymes in which two other phosphorylatable sites, Ser^{24} and Ser^{475}, were each replaced with aspartate, showed almost the same activity as the wild-type enzyme, suggesting that the phosphorylation of Ser^{24} or Ser^{475} causes no significant change in the enzyme activity. The activity of the mutant (S$_{52}$D), in which another of the six residues identified as phosphorylation sites (*3*), Ser^{52}, was replaced with aspartate, was somewhat lower than that of the wild-type enzyme, but the activities of the double and triple mutants (S$_{52}$D/S$_{74}$D), (S$_{52}$D/S$_{475}$D), (S$_{24}$D/S$_{52}$D/S$_{475}$D), and (S$_{52}$D/S$_{74}$D/S$_{475}$D) containing aspartate in place of Ser^{52} were almost the same as that of the wild-type enzyme, suggesting that the phosphorylation of Ser^{52} does not always result in a decrease in enzyme activity.

Figure 3 shows the effects of the substitutions of alanine, aspartate, or glutamate residues for the phosphorylatable serine and/or threonine residues on the increase in en-zyme activity upon autophosphorylation. The wild-type en-zyme was approximately 1.3-fold activated after incubation in the presence of Ca^{2+}/calmodulin under the phosphorylation conditions, and 19 of the 25 mutated enzymes examined were activated to the same extent as the wild-type enzyme. The other 6 mutants were activated to a greater extent than the wild-type enzyme and four of them contained aspartate or glutamate substitution at Thr^{108}. The specific activities of the mutant enzymes (T$_{108}$D) and (T$_{108}$E) after incubation for autophosphorylation were almost the same as that of the wild-type enzyme (Table I), indicating that the decrease in the enzyme activity caused by the substitution of aspartate or glutamate for Thr^{108} is canceled out by autophosphorylation, and the autophosphorylation accordingly results in a larger increase in enzyme activity. The mutants (S$_{74}$D) and (S$_{74}$D/S$_{475}$D) showed the highest specific activities after autophosphorylation (Table I), indicating that the increase in enzyme activity caused by the substitution of aspartate for Ser^{74} (Fig. 2) continues after autophos-

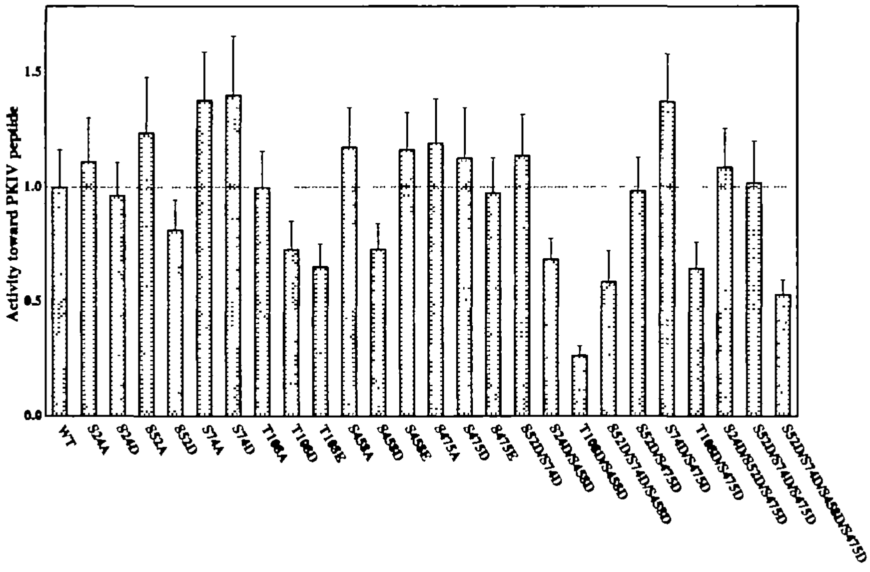

Fig. 2. **The effects of substitutions of Ala, Asp, or Glu for the phosphorylatable Ser or Thr residues of CaM-kinase kinase α on the activity toward PKIV peptide.** The ratios of the specific activities toward PKIV peptide of the indicated enzymes to that of the wild-type enzyme (283 ± 22 nmol/min/mg) were calculated from the results shown in Table I.

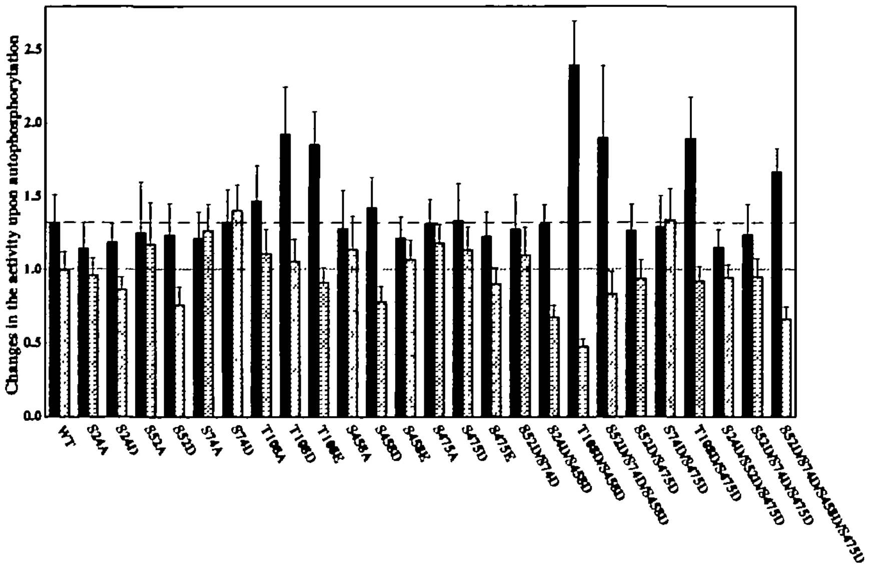

Fig. 3. **Changes in the activities of the CaM-kinase kinase α mutants by autophosphorylation.** The ratios of the specific activities toward PKIV peptide of the indicated wild-type and mutant enzymes preincubated in the presence of Ca^{2+}/calmodulin [fourth column (+Ca^{2+}) of Table I] to those in its absence [second column (−Ca^{2+}) of Table I] (solid bars), and the ratios of the activities of the indicated enzymes (+Ca^{2+} column of Table I) to that (365 nmol/min/mg) of the wild-type enzyme preincubated in the presence of Ca^{2+}/calmodulin (dotted bars) were calculated from the results shown in Table I.

phorylation. Thus, the effect of the phoshorylation of Ser^{74} on the activation of the enzyme appeared to be additive to that of the autophosphorylation. The activities of the mutated enzymes containing aspartate in substitution for Ser^{458}, such as the enzymes (T$_{108}$D/S$_{458}$D), (S$_{52}$D/S$_{74}$D/S$_{458}$D/S$_{475}$D), (S$_{24}$D/S$_{458}$D), and (S4$_{58}$D), after autophosphorylation were much lower than that of the wild-type enzyme (Table I), indicating that the decrease in enzyme activity caused by the substitution of aspartate for Ser^{458} continues after autophosphorylation. Thus, the phosphorylations of Thr^{108} and Ser^{458} appear to cause a decrease in the enzyme activity toward PKIV peptide, and that the decrease caused by the phosphorylation of Thr^{108} is canceled out by autophosphorylation of the enzyme while that of Ser^{458} is not.

Figure 4 shows the effects of the substitutions on the increase in enzyme activity caused by incubation with PKA in the presence of Ca^{2+}/calmodulin under the phosphorylation conditions, where the enzymes should be phosphorylated by itself as well as by PKA. The wild-type enzyme was activated about 2-fold by incubation with PKA under these conditions. Among the 25 mutated enzymes, all four mutants that contained aspartate or glutamate in substitution for Thr^{108}, (T$_{108}$D), (T$_{108}$E), (T$_{108}$D/S$_{458}$D), and (T$_{108}$D/S$_{475}$D), were activated to a greater extent than the wild-type enzyme, and the three mutants not containing aspartate for Ser^{458} showed almost the same activity as the wild-type enzyme after incubation with PKA. Another mutants, (T$_{108}$D/S$_{458}$D), containing aspartate for Ser^{458}, showed much lower activity than the wild-type enzyme. This, together with the above results (Fig. 3), indicates that the effect of the phosphorylation of Thr^{108} is also canceled out by incubation in the presence of Ca^{2+}/calmodulin both will and without PKA. The other 21 mutants without aspartate or glutamate substitutions for Thr^{108} were activated to a similar or lesser extent than the wild-type enzyme, and among them, mutants containing alanine or aspartate substitutions for Ser^{74}, (S$_{74}$A), (S$_{74}$D), (S$_{52}$D/S$_{74}$D), (S$_{52}$D/S$_{74}$D/S$_{458}$D), (S$_{74}$D/S$_{475}$D), (S$_{52}$D/S$_{74}$D/S$_{475}$D), and (S$_{52}$D/S$_{74}$D/S$_{458}$D/ S$_{475}$D), showed a tendency to be less activated by incubation with PKA in the presence of Ca^{2+}/calmodulin than the wild-type enzyme. This result, together with the previous observation that a significant amount of phosphate is incorporated into Ser^{74} upon incubation with PKA in the presence of Ca^{2+}/calmodulin (*3*), indicates that the phosphorylation of Ser^{74} may be involved to some extent in the activation of CaM-kinase kinase α by PKA in the presence of Ca^{2+}/calmodulin. The fact that the activities after incubation with PKA in the presence of Ca^{2+}/calmodulin of all mutants containing aspartate for Ser^{458}, (S$_{458}$D), (T$_{108}$D/S$_{458}$D), (S$_{52}$D/S$_{74}$D/S$_{458}$D), and (S$_{52}$D/S$_{74}$D/S$_{458}$D/S$_{475}$D), were much lower than that of the wild-type enzyme, indicates that the decrease in enzyme activity that occurs upon the phosphorylation of Ser^{458} is not canceled by incubation with PKA in the presence of Ca^{2+}/calmodulin, as well as without PKA as described above. The activations of mutant enzymes (S$_{52}$D/S$_{74}$D/S$_{458}$D) and (S$_{52}$D/S$_{74}$D/S$_{458}$D/S$_{475}$D) by incubation without PKA in the presence of Ca^{2+}/calmodulin were significantly higher than that of the wild-type enzyme (Fig. 3), but the activation by incubation with PKA were almost the same as that of the wild-type enzyme (Fig. 4), supporting the above discussion that the phosphorylation of Ser^{74} is involved in the activation of the enzyme by PKA in the presence of Ca^{2+}/calmodulin.

Figure 5 shows the effects of the mutations on the decrease in the enzyme activity caused by incubation with PKA in the absence of Ca^{2+}/calmodulin under the phosphorylation conditions, where autophosphorylation should not occur. The activity of the wild-type enzyme decreased to two-thirds of its original activity upon phosphorylation by PKA under the experimental conditions. Among the 25 mutants, 5 did not show significant decreases in enzyme activity after incubation: (T$_{108}$D/S$_{458}$D) showed an increase, (S$_{458}$A) showed no change, and (T$_{108}$E), (T$_{108}$D), and (T$_{108}$A) showed little, if any, decrease. Four of the 5 mutants, (T$_{108}$A), (T$_{108}$D), (T$_{108}$E), and (T$_{108}$D/S$_{458}$D), contained substitutions of nonphosphorylatable amino acids for Thr^{108}. These results, together with the previous observation that a significant amount of phosphate is incorporated into Thr^{108} upon incubation with PKA (*3*), indicate the involvement of the phosphorylation of Thr^{108} in the decrease in enzyme activity caused by incubation with PKA in the absence of Ca^{2+}/calmodulin. The other mutant, (S$_{458}$A),

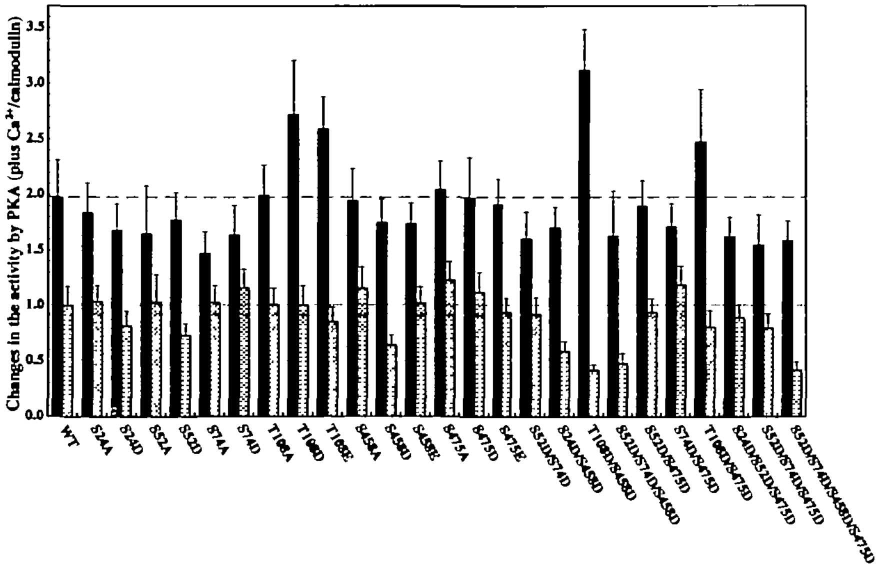

Fig. 4. **Changes in the activities of the CaM-kinase kinase α mutants by PKA (plus Ca^{2+}/calmodulin).** The ratios of the specific activities toward PKIV peptide of the indicated enzymes preincubated in the presence of PKA and Ca^{2+}/calmodulin [last column (+Ca^{2+}/+PKA) of Table I] to those in their absence [second column (−Ca^{2+}) of Table I] (solid bars), and the ratios of the activities of the indicated enzymes (+Ca^{2+}/+PKA column of Table I) to that (546 nmol/min/mg) of the wild-type enzyme preincubated in the presence of PKA and Ca^{2+}/calmodulin (dotted bars) were calculated from the results shown in Table I.

which contained no Thr^{108} substitution, not only showed no significant decrease in activity, but also the highest activity after incubation among the wild-type and 25 mutant enzymes. Another mutant enzyme containing a substitution for Ser^{458}, ($S_{458}E$), showed the second highest activity, although its activity decreased somewhat after incubation with PKA. In contrast to these two mutants, ($S_{458}D$) was inactivated to the same extent as the wild-type enzyme by incubation with PKA. These results, together with the above finding that the substitution of glutamate for Ser^{458} does not mimick the phosphorylation of Ser^{458} while substitution by aspartate does, indicate that blocking the phosphorylation of Ser^{458} inhibits the inactivation of the enzyme by PKA in the absence of Ca^{2+}/calmodulin. Thus, the phosphorylation of Ser^{458} appears to promote either the phosphorylation of Thr^{108} by PKA or the inactivation of the enzyme by the phosphorylation of Thr^{108}, or both. As shown in the preceding paper (*3*), CaM-kinase kinase α undergoes phosphorylation at Ser^{458} by PKA in the absence of Ca^{2+}/calmodulin, but little, if any, phosphorylation at Ser^{458} in its presence, and the rate of the phosphorylation of Thr^{108} by PKA in the absence of Ca^{2+}/calmodulin is one-fifth of that in its presence, supporting the concept that the phosphorylation of Ser^{458} promotes the phosphorylation of Thr^{108} by PKA. On the other hand, the fact that the activity of the double mutant ($T_{108}D/S_{458}D$), which is approximately 26% that of the wild-type enzyme, is much lower than the activities of the respective single mutants ($T_{108}D$) and ($S_{458}D$),

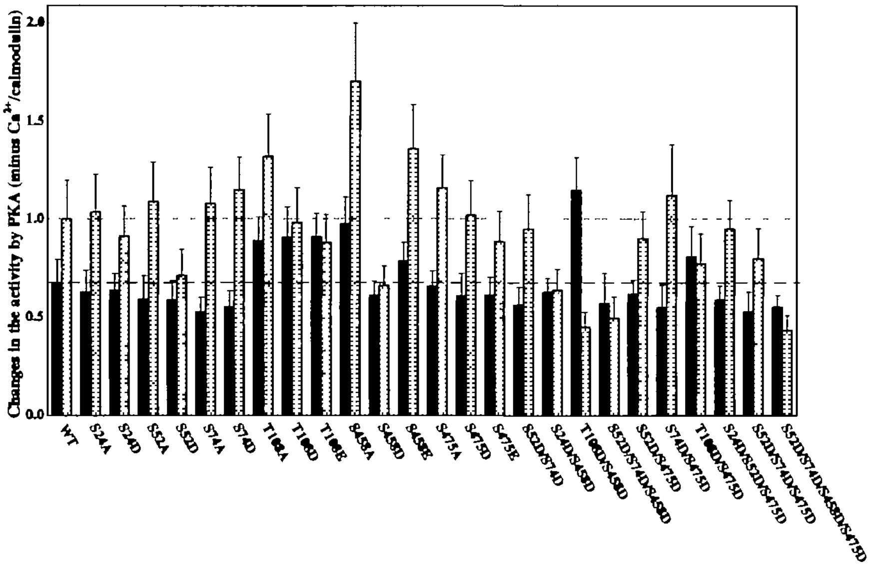

Fig. 5. **Changes in the activities of the CaM-kinase kinase α mutants by PKA (minus Ca^{2+}/calmodulin).** The ratios of the specific activities toward PKIV peptide of the indicated enzymes preincubated in the presence of PKA [third column (–Ca^{2+}/+PKA) of Table I] to those in its absence [second column (–Ca^{2+}) of Table I] (solid bars), and the ratios of the activities of the indicated enzymes (–Ca^{2+}/+PKA column of Table I) to that (186 nmol/min/mg) of the wild-type enzyme preincubated in the presence of PKA (dotted bars) were calculated from the results shown in Table I.

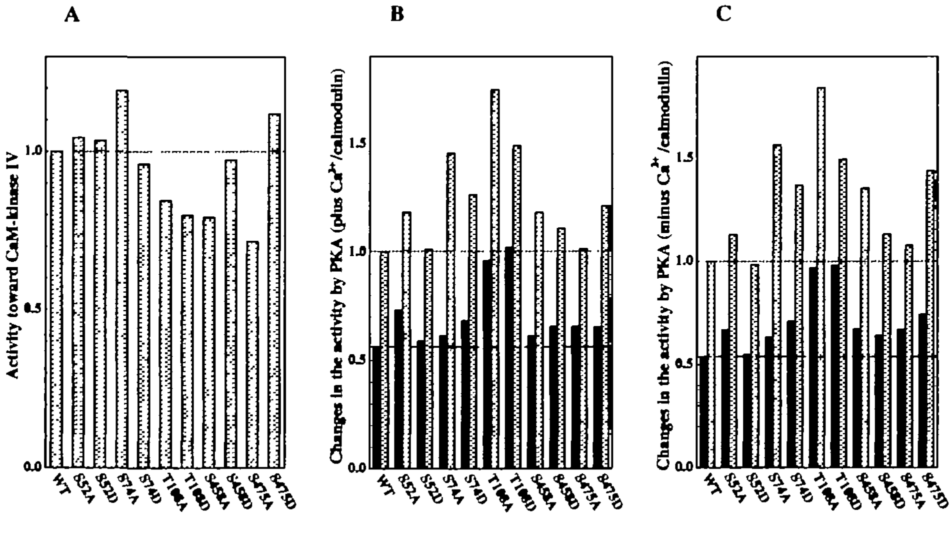

Fig. 6. **The effects of the substitutions on the activity of CaM-kinase kinase α toward CaM-kinase IV.** Approximately 5 μg/ml of wild-type and the indicated mutant enzymes was preincubated at 30°C in the standard phosphorylation mixture containing 0.1 mM nonradioactive ATP with 0.5 μg/ml of PKA in the presence or absence of Ca^{2+}/calmodulin. After incubation for 10 min, the mixtures were immediately cooled by immersion in ice water and diluted 3.3-fold with 10 mM Hepes-NaOH (pH 7.2 at 4°C) containing 10% glycerol, 0.05% Tween 80, 0.1 mM dithiothreitol, and 0.1 mM EDTA. Aliquots of 2-μl were incubated at 30°C for 1 min in a final volume of 50 μl of the standard phosphorylation mixture containing 2 μM calmodulin, 0.1 mM [γ-^{32}P]ATP (about 1,000 cpm/pmol), and 50 μg/ml of CaM-kinase IV($K_{71}R$), and the incorporation of [^{32}P]phosphate into the CaM-kinase IV was determined by the 3MM paper method. (A) The ratios of the activities of the indicated enzymes to that of the wild-type enzyme without preincubation. (B) The ratios of the activities of the indicated enzymes preincubated in the presence of PKA and Ca^{2+}/calmodulin to those in their absence (solid bars), and the ratios of the activities of the indicated enzymes to that of the wild-type enzyme preincubated in the presence of PKA and Ca^{2+}/calmodulin (dotted bars). (C) The ratios of the activities of the indicated enzymes preincubated in the presence of PKA to those of the enzymes preincubated in its absence (solid bars), and the ratios of the activities of the indicated enzymes to that of the wild-type enzyme preincubated in the presence of PKA (dotted bars).

which are 73 and 69%, respectively, of the wild-type enzyme (Fig. 2). This supports the concept that the phosphorylation of Ser^{458} promotes the inactivation of the enzyme when Thr^{108} is phosphorylated by PKA. Thus, the phosphorylation of Ser^{458} appears not only to promote the rate of the Thr^{108} phosphorylation by PKA, but also to enhance the extent of the inactivation of the enzyme by the Thr^{108} phosphorylation by PKA. The preceding paper (*3*) demonstrates that the rate of phosphorylation of Ser^{458} in CaM-kinase kinase α by PKA (0.008 mol phosphate/mol of CaM-kinase kinase α/min) in the presence of Ca^{2+}/calmodulin is much slower (about 36-fold) than that in its absence (0.29 mol phosphate/mol of enzyme/min). Thus, a primary factor in determining whether the activity of CaM-kinase kinase α increases or decreases after phosphorylation by PKA appears to be the phosphorylation of Ser^{458} by PKA, which occurs in the absence but not the presence of Ca^{2+}/calmodulin.

Since the incubation of CaM-kinase kinase α with PKA in the presence of Ca^{2+}/calmodulin results in an increase in activity toward PKIV peptide, but a decrease in activity toward CaM-kinase IV (*3*), the effects of the substitutions on the activity toward CaM-kinase IV were examined as shown in Fig. 6. The enzymes ($S_{74}A$) showed a higher activity and ($T_{108}D$) a lower activity toward CaM-kinase IV (Fig. 6A) as well as PKIV peptide (Fig. 2) than did the wild-type enzyme. On the other hand, ($S_{458}D$), which showed much lower activity toward PKIV peptide than the wild-type enzyme (Fig. 2), had almost the same activity toward CaM-kinase IV as the wild-type enzyme, suggesting that the phosphorylation of Ser^{458} does not play an important role in controlling the activity toward CaM-kinase IV, in contrast to the activity toward PKIV peptide. In contrast to the activity toward PKIV peptide, the activity of CaM-kinase kinase α toward CaM-kinase IV is not significantly affected by incubation in the presence of Ca^{2+}/calmodulin (autophosphorylation), and decreased by incubation with PKA in both the presence and absence of Ca^{2+}/calmodulin (*3*). Hence, the effects of substitutions for the phosphorylatable serine or threonine residues on changes in the enzyme activity toward CaM-kinase IV through phosphorylation by PKA in the presence (Fig. 6B) and absence (Fig. 6C) of Ca^{2+}/calmodulin were examined. The activity of the wild-type enzyme decreased to approximately one-half of its original activity after incubation with PKA in both the presence and absence of Ca^{2+}/calmodulin, and the changes in the activities of the wild-type and mutant enzymes after incubation with PKA in the presence of Ca^{2+}/calmodulin were almost identical to those in its absence. The activities of ($T_{108}A$) and ($T_{108}D$) did not decrease significantly after incubation with PKA in the presence or absence of Ca^{2+}/calmodulin, indicating that the phosphorylation of Thr^{108} is involved in the decrease in the activity toward CaM-kinase IV by the incubation with PKA in the presence or absence of Ca^{2+}/calmodulin. The fact that the activity of ($T_{108}D$) after incubation with PKA was much higher than that the wild-type enzyme, although lower than that of ($T_{108}A$), indicates that aspartate in place of Thr^{108} only partly mimics phosphorylated Thr^{108} in enzyme action catalyzing the phosphorylation of CaM-kinase IV. The mutant ($S_{458}D$) showed essentially the same activity, even after incubation with PKA in the presence or absence of Ca^{2+}/calmodulin, as the wild-type enzyme. Thus, the phosphorylation of Ser^{458} by PKA, which is important for controlling the PKIV peptide-phosphorylating activity as described above, appears not to be important for controlling the CaM-kinase IV-phosphorylating activity. An alternative possibility is that aspartate in place of Ser^{458} does not mimic phosphorylated Ser^{458} in the phosphorylation of CaM-kinase IV. None of the 10 mutated enzymes in which each of the five serine or threonine residues identified as PKA phosphorylation sites (*3*) was replaced by asparate or alanine was inactivated more by incubation with PKA, and all showed significantly lower activities after incubation with PKA than did the wild-type enzyme.

To obtain a clue as to the mechanism for the changes in

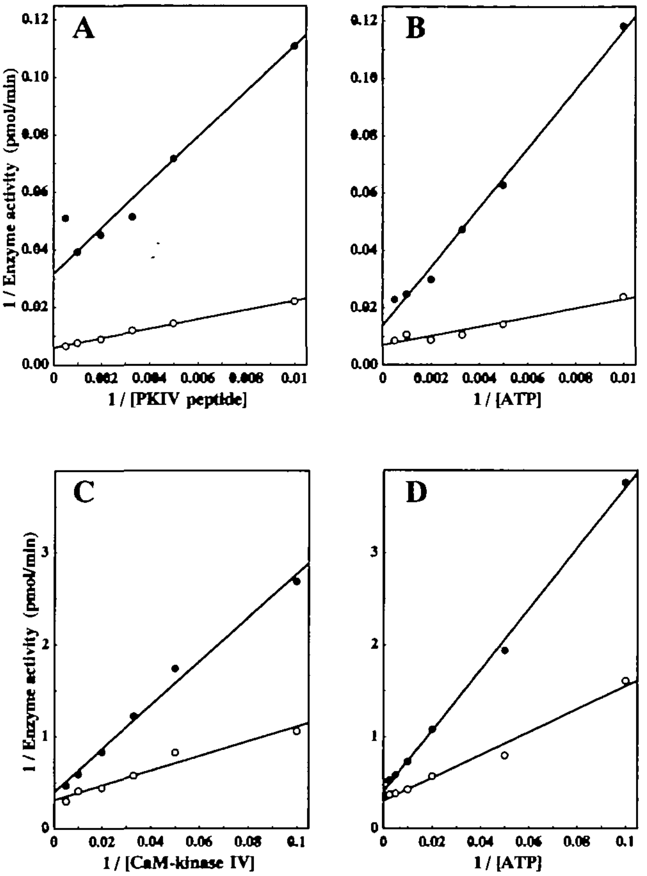

Fig. 7. **The effects of the concentrations of peptide or protein substrate and ATP on the activity of CaM-kinase kinase α ($T_{108}D/S_{458}D$).** (A and B) Approximately 200 ng of wild-type CaM-kinase kinase α (○) or mutant ($T_{108}D/S_{458}D$) (●) was incubated in a final volume of 50 μl of the standard phosphorylation mixture containing 0.2 mM [γ-^{32}P]ATP (about 50 cpm/pmol) and various concentrations of PKIV peptide (A), or 0.2 mM PKIV peptide and various concentrations of [γ-^{32}P]ATP (about 50 cpm/pmol) (B) at 30°C for 1 min, and the incorporation of [^{32}P]phosphate into the peptide was determined by the phosphocellulose paper method. (C and D) Approximately 2.4 ng of wild-type CaM-kinase kinase α (○) or mutant ($T_{108}D/S_{458}D$) (●) was incubated in a final volume of 50 μl of the standard phosphorylation mixture, except that the concentration of calmodulin was 5 μM, containing 0.1 mM [γ-^{32}P]ATP (about 1,000 cpm/pmol) and various concentrations of CaM-kinase IV($K_{71}R$) (C), or 50 μg/ml (0.94 μM) of CaM-kinase IV($K_{71}R$) and various concentrations of [γ-^{32}P]ATP (about 1,000 cpm/pmol) (D) at 30°C for 1 min, and the incorporation of [^{32}P]phosphate into CaM-kinase IV was determined by the 3MM paper method.

TABLE II. **Summary of kinetic parameters of CaM-kinase kinase α ($T_{108}D/S_{458}D$).** Kinetic parameters were obtained from the double-reciprocal plots shown in Fig. 7. The V_{max} values were calculated from the apparent V_{max} values on the basis of the Michaelis equation $v = V/(1 + K_m/[S])$.

	K_m for substrate (μM)	K_m for ATP (μM)	V_{max} (nmol/min/mg)
	Assayed with PKIV peptide		
Wild type	280	250	1,840
$T_{108}D/S_{458}D$	280	770	880
	Assayed with CaM-kinase IV(K_{71}R)		
Wild type	0.46 (24 μg/ml)	40	1,910
$T_{108}D/S_{458}D$	1.2 (63 μg/ml)	87	2,270

the activity of CaM-kinase kinase α induced by PKA, the kinetic properties of ($T_{108}D/S_{458}D$), which always showed the lowest activity toward PKIV peptide (Table I and Figs. 2–5), were examined in comparison with the wild-type enzyme using PKIV peptide and CaM-kinase IV as substrates, as shown in Fig. 7, and the kinetic parameters are summarized in Table II. The substitutions of aspartate for both Thr^{108} and Ser^{458} caused an approximately 3-fold increase in the K_m value for ATP but no change in the K_m for PKIV peptide, and a decrease in the V_{max} by about one-half, when the enzyme was assayed with PKIV peptide as a substrate, and 2- to 3-fold increases in the K_m values for both ATP and CaM-kinase IV and an increase in the V_{max}, when assayed with CaM-kinase IV(K_{71}R) as a substrate. As reported in the preceding paper (*3*), incubation of CaM-kinase kinase α with PKA in the absence of Ca^{2+}/calmodulin results in a significant increase in the K_m for ATP but no increase in the K_m for the peptide substrate, and a slight decrease in the V_{max}, when the enzyme is assayed with PKIV peptide, and 3- to 4-fold increases in both K_m values for ATP and the protein substrate and a 1.7-fold increase in the V_{max}, when assayed with CaM-kinase IV(K_{71}R). In contrast, incubation of the enzyme with PKA in the presence of Ca^{2+}/calmodulin results in a marked increase (approximately 3-fold increase) in V_{max} when the enzyme is assayed with PKIV peptide (*3*). Thus, the kinetic properties of the mutant enzyme ($T_{108}D/S_{458}D$) are very similar to those of the enzyme phosphorylated by PKA in the absence of Ca^{2+}/calmodulin, providing further support that the phosphorylations of Ser^{458} and Thr^{108} are important contributors to the change in activity caused by incubation with PKA in the absence of Ca^{2+}/calmodulin.

DISCUSSION

The activity of CaM-kinase kinase α toward PKIV peptide is altered when the enzyme undergoes autophosphorylation or is phosphorylated by PKA, and six phosphorylation sites, one for autophosphorylation (Ser^{24}) and five for phosphorylation by PKA (Ser^{52}, Ser^{74}, Thr^{108}, Ser^{458}, and Ser^{475}) have been identified (*3*). Changes in activity produced by amino acid substitutions at the six phosphorylatable serine and threonine residues (Fig. 2) suggest that the phosphorylation of Ser^{74} causes an increase and that the phosphorylations of Thr^{108} and Ser^{458} synergistically cause a decrease in activity. The replacement of Ser^{24}, which has been identified as the major autophosphorylation site (*3*), by amino acids such as alanine or aspartate had no significant affect either the enzyme activity (Fig. 2) or the extent of activation of the enzyme through autophosphorylation (Fig. 3), indicating that the phosphorylation of Ser^{24} is not involved in the activation of the enzyme. These results, together with the previous observation that the amount of phosphate incorporated into Ser^{24} upon autophosphorylation accounts for only 30% of the total phosphate incorporated into the enzyme (*3*), provide further support for the contention that the gradual activation of CaM-kinase kinase α upon autophosphorylation is due to a conformational change induced by phosphorylation at many unidentified sites on the enzyme (*3*). The enzyme ($S_{74}D$), whose activity was 1.4-fold higher than that of the wild-type enzyme (Fig. 2), was further activated upon autophosphorylation to the same extent (about 1.3-fold) as the wild-type enzyme (Fig. 3 and Table I), indicating that the activation of the enzyme that occurs upon phosphorylation of Ser^{74} is additive with the activation produced by autophosphorylation. The mutant enzymes containing the substitutions at Ser^{74} were less activated upon phosphorylation by PKA in the presence of Ca^{2+} and calmodulin than the wild-type enzyme (Fig. 4). Therefore, the phosphorylation of Ser^{74} appears to be involved in the activation of the enzyme by PKA, since a significant amount of phosphate has been demonstrated to be incorporated into Ser^{74} upon phosphorylation by PKA in the presence of Ca^{2+}/calmodulin (*3*).

Since the lowers activity produced by the substitution of Thr^{108} with aspartate in ($T_{108}D$) was increased much more greatly upon autophosphorylation (Fig. 3) and upon incubation with PKA in the presence of Ca^{2+}/calmodulin (Fig. 4), and more weakly decreased upon incubation with PKA in the absence of Ca^{2+}/calmodulin (Fig. 5), the decrease in the activity toward PKIV peptide caused by the phosphorylation of Thr^{108} appears to be canceled by autophosphorylation as well as by phosphorylation by PKA in the presence or absence of Ca^{2+}/calmodulin. In contrast, the lower activity produced by the substitution of Ser^{458} with appartate in ($S_{458}D$) increased upon autophosphorylation (Fig. 3) or upon incubation with PKA in the presence of Ca^{2+}/calmodulin (Fig. 4), and decreased upon incubation with PKA in the absence of Ca^{2+}/calmodulin (Fig. 5) to the same extent as the wild-type enzyme. Thus, the decrease in activity produced by the phosphorylation of Ser^{458}, in contrast to Thr^{108}, appears to continue after autophosphorylation or phosphorylation by PKA. The double mutants ($T_{108}D/S_{458}D$) almost always showed the lowest activities (Table I), which were significantly lower than the activities of ($S_{458}D$), even after autophosphorylation (Fig. 3) or phosphorylations by PKA in the presence (Fig. 4) or absence (Fig. 5) of Ca^{2+}/calmodulin, suggesting that the inhibitory effect of the phosphorylation of Thr^{108} in an enzyme phosphorylated at Ser^{458} was not canceled by autophosphorylation or phosphorylation by PKA. The rate of phosphorylation of Ser^{458} by PKA in the absence of Ca^{2+}/calmodulin was more than 1 order of magnitude greater than that in the presence of Ca^{2+}/calmodulin (*3*), indicating that binding of Ca^{2+}/calmodulin to the enzyme inhibits the phosphorylation of Ser^{458} by PKA. Conversely, it has been reported that the phosphorylation of CaM-kinase kinase α by PKA in the absence of Ca^{2+}/calmodulin inhibits the binding of calmodulin to the enzyme, and that this effect is lost when Ser^{458} is replaced by alanine (*4*). Thus, the phosphorylation of Ser^{458} in CaM-kinase kinase α by PKA appears strongly to suppress the binding of Ca^{2+}/calmodulin to the CaM-kinase kinase, and *vice* *versa*, presumably because Ser^{458} is located in the calmodulin-binding domain (Fig. 1A). Our attempts to confirm that the phosphorylation of Ser^{458} in CaM-kinase kinase α by PKA abolishes the binding of calmodulin were unsuccessful, because it was difficult to phosphorylate only Ser^{458} sufficiently among the five PKA phosphorylation sites. On the other hand, the substitution of aspartate for Ser^{458}, as in the case of the phosphorylation of Ser^{458}, apparently abolishes the binding of Ca^{2+}/calmodulin in the calmodulin overlay assay, as shown in Fig. 8. However, the mutant ($S_{458}D$), as well as the wild-type or other mutant enzymes, was purified by affinity chromatography on calmodulin-Sepharose, and it showed no significant activity in the absence of Ca^{2+}/calmodulin (data not shown) but significant activity in its presence as shown above, indicating that the ($S_{458}D$) can bind calmodulin. Furthermore, this indicates that the calmodulin overlay assay does not necessarily reflect the binding of calmodulin.

In contrast to the results obtained with PKIV peptide as a substrate, when the activity of CaM-kinase kinase α was measured with CaM-kinase IV as a substrate, only the substitution of aspartate for Thr^{108} among the five phosphorylation sites for PKA resulted in a significant decrease in activity (Fig. 6A), and only mutant enzymes in which Thr^{108} was replaced with other amino acids such as aspartate or alanine, underwent no significant changes in activity upon incubation with PKA in the presence (Fig. 6B) and absence (Fig. 6C) of Ca^{2+}/calmodulin, while the wild-type and other mutant enzymes underwent significant decreases in activity. These results indicate that the phosphorylation of Thr^{108} is most important for regulating the activity toward CaM-kinases IV and I of CaM-kinase kinase α by PKA, because the activities of CaM-kinase kinase α toward both CaM-kinases IV and I are regulated in a similar manner by PKA (*3*). Thus, it appears that the phosphorylations of Thr^{108} and Ser^{458} in CaM-kinase kinase α by PKA play a key role in the regulation of the activity toward PKIV peptide, but that the phosphorylation of only Thr^{108} plays a key role in the regulation of the activity toward CaM-kinases IV and I.

A comparison of the kinetic properties of the wild-type enzyme and the mutant ($T_{108}D/S_{458}D$), which almost always showed the lowest activity toward PKIV peptide, revealed that the substitutions of aspartate for both Thr^{108} and Ser^{458} caused a large decrease in the V_{max} for PKIV peptide, but an increase in the V_{max} for CaM-kinase IV, and caused no change in the K_m for PKIV peptide but a significant increase in the K_m for CaM-kinase IV. These kinetic properties of ($T_{108}D/S_{458}D$) are very similar to those of the enzyme phosphorylated by PKA in the absence of Ca^{2+}/calmodulin (*3*). Thus, the mechanism of the changes in the activity of CaM-kinase kinase α caused by PKA appears to vary with the substrate used for assay.

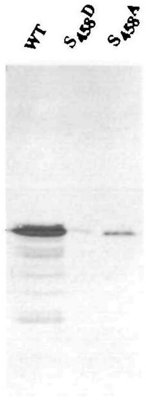

Fig. 8. **Analysis of mutants of CaM-kinase kinase α by calmodulin overlay after SDS-PAGE.** Approximately 0.05 μg samples of the wild type, mutant ($S_{458}D$), and mutant ($S_{458}A$) CaM-kinase kinase α were subjected to SDS-PAGE on a 7.5% acrylamide gel, and the separated proteins were transferred onto a polyvinylidene difluoride membrane (FluoroTrans, Pall Gelman Laboratory). The membrane was blocked with 5% nonfat milk for 30 min at 24°C, then incubated with 25 μg/ml biotinylated calmodulin for 60 min, followed by 2 μg/ml avidin conjugated with peroxidase for 60 min, in 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 1 mM $CaCl_2$. The positive bands that bound calmodulin were detected with diaminobenzidine tetrahydrochloride and H_2O_2 in the presence of $CoCl_2$.

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