NAD kinase is a Ca\(^{2+}\)/calmodulin (CaM)-dependent enzyme capable of converting cellular NAD to NADP. The enzyme purified from pea seedlings can be activated by highly conserved soybean CaM, SCaM-1, but not by the divergent soybean CaM isoform, SCaM-4 (Lee, S. H., Kim, J. C., Lee, M. S., Heo, W. D., Seo, H. Y., Yoon, H. W., Hong, J. C., Lee, S. Y., Bahk, J. D., Hwang, I., and Cho, M. J. (1995) J. Biol. Chem. 270, 21806–21812). To determine which domains were responsible for this differential activation of NAD kinase, a series of chimeric SCaMs were generated by exchanging functional domains between SCaM-4 and SCaM-1. SCaM-1111, a chimeric SCaM-1 that contains the first domain of SCaM-4, was severely impaired (only 40% of maximal) in its ability to activate NAD kinase. SCaM-1444, a chimeric SCaM-4 that contains the first domain of SCaM-1 exhibited nearly full (~70%) activation of NAD kinase. Only chimeras containing domain I of SCaM-1 produced greater than half-maximal activation of NAD kinase. To define the amino acid residue(s) in domain I that were responsible for this differential activation, seven single residue substitution mutants of SCaM-1 were generated and tested for NAD kinase activation. Among these mutants, only K30E and G40D showed greatly reduced NAD kinase activation. Also a double residue substitution mutant, K30E/G440D, containing these two mutations in combination was severely impaired in its NAD kinase-activating potential, reaching only 20% of maximal activation. Furthermore, a triple mutation, K30E/M368/G440D, completely abolished NAD kinase activation. Thus, our data suggest that domain I of CaM plays a key role in the differential activation of NAD kinase exhibited by SCaM-1 and SCaM-4. Further, the residues Lys\(^{30}\) and Glu\(^{40}\) of SCaM-1 are critical for this function.

Calmodulin (CaM)\(^1\) is a major intracellular calcium receptor in both animal and plant cells. CaM undergoes conformational change upon binding calcium and then interacts with a variety of target enzymes to modulate their activities (for reviews, see Ref. 1). In plants, several CaM-dependent enzymes such as NAD kinase, glutamate decarboxylase, elongation factor-1α, nucleoside triphosphatase, and Ca\(^{2+}\)-ATPase have been shown to be regulated by Ca\(^{2+}\)/CaM complex (for reviews, see Ref. 2; see also Refs. 3–8).

Among them, pea NAD kinase, the enzyme capable of converting cellular NAD to NADP, is a strict CaM-requiring enzyme for its activity. Since NAD and NADP are utilized as coenzymes by a variety of enzymes involved in cellular metabolic processes, it is very important for organisms to precisely regulate their cellular NAD(H)/NADP(H) ratio. Therefore, control of NAD kinase activity by CaM is crucial for cellular metabolic homeostasis. For example, trimethylation of lysine 115 in CaM reduces its ability to activate NAD kinase 4–5-fold (9). Thus, the perturbation of NAD kinase regulation by overexpression of a nonmethylatable K115R mutant CaM resulted in a decreased growth rate, reduced seed production, and reduced pollen viability in transgenic plants (10). Despite the importance of NAD kinase in plant cells, currently it is not well understood how CaM binds and activates the enzyme. To resolve this question, it would be a prerequisite to purify NAD kinase homogeneously and/or to isolate the corresponding gene. However, attempts at this have not been successful, mainly because of the intrinsic instability of the enzyme (11).

In an effort to learn about the CaM and target protein interaction mechanism, crystal and solution structures of CaM and CaM-binding target peptide complexes have been resolved (12–14). These studies provided important information about the mechanism of CaM binding to target peptides and the structural changes that CaM undergoes upon binding. In these structures, the central helix of CaM bends and twists to envelope CaM-binding peptide in a hydrophobic tunnel. However, these structures alone cannot reveal how the surface of CaM interacts with specific functional domains of target enzyme to result in their activation.

To understand more of the structure-function relationship of CaM in the activation of target enzymes, a number of mutant CaMs were generated by in vitro mutagenesis and then tested for target enzyme activation to examine the effect of substituted residue(s). Most of these studies focused on the central helix (15–17), the Ca\(^{2+}\)–binding domains (18–20), or on highly conserved residues outside the Ca\(^{2+}\)–binding domains (21). Recently, CaM and cardiac troponin C chimeric proteins were generated and investigated with regard to target enzyme activation (22, 23). These studies were based on the fact that cardiac troponin C is a close structural homolog of CaM but that it could not activate CaM target enzymes (24). With these CaM-troponin C chimeras, the researchers successfully identified important regions (domains) or residues in CaM that were important in the activation of several target enzymes such as smooth muscle myosin light chain kinase, PDE and nitric-oxide...
synthase (25, 26).

Previously, we characterized two new divergent CaM isoforms from five soybean cDNAs encoding CaM (27). The proteins produced in bacteria transformed with two of these cDNAs, SCaM-1 and SCaM-4, differed significantly in their abilities to activate two CaM target enzymes, PDE and NAD kinase. Both of the SCaM isoforms similarly activated cAMP-PDE. However, SCaM-4, the divergent CaM isoform, did not activate NAD kinase at all, while SCaM-1 was a good activator of NAD kinase. In the present study, we investigated the region of CaM that is functionally important for this differential activation of NAD kinase by exchanging domains of SCaM-1 and SCaM-4 and examining these chimeric proteins for their ability to activate NAD kinase. From the domain exchanges between these two naturally occurring CaM isoforms, we could map an essential domain of CaM for NAD kinase activation. We further analyzed single, double, and triple residue-substituted mutants, we could identify two critical residues in NAD kinase activation. We describe here the construction of these chimeric and mutant SCaMs and the effect of the introduced changes on NAD kinase activation. The possible functioning of the identified domain and residues in the NAD kinase activation is also discussed with reference to the CaM-target peptide complex structure.

EXPERIMENTAL PROCEDURES

Construction of SCaM-1/SCaM-4 Chimeric CaM Expression Plasmids—To exchange domains between SCaM-1 and SCaM-4, CaM was divided into four functional domains on the basis of the four Ca2+-binding regions called EF hands. Three new restriction sites, StuI, HindIII, and BclI, which were intrinsically present at the relevant positions in the SCaM-4 cDNA, were created in the cDNA sequence of SCaM-1 (see Fig. 1). In generating these restriction enzyme sites, we used the U-DNA mutagenesis method (28). To prepare a single-stranded DNA template, the Ncol-BamHI fragment of SCaM-1/pET3-d plasmid (27) was subcloned into the vector pBluescript SK+ (Stratagene) and introduced into Escherichia coli CJ236. Single-stranded DNA was obtained after infection with helper phage VCSM13 (Stratagene). Oligonucleotides CMO-1, CMO-3, and CMO-4 were used to generate a HindIII site, StuI site, and BclI site in SCaM-1, respectively. The nucleotide sequences of these oligonucleotides were as follows: CMO-1, 5'-ATCGAATCCATCTGGATGTTG-3'; CMO-3, 5'-CTGGAAGCCCTCTTGACGAAAC-3'; CMO-4, 5'-CCAAGGTTGATCATACAGG-3'. Domain I exchanges were done by exploiting SacI sites of SCaM-1 and SCaM-4 cDNAs. After swapping the domains as desired, the chimeric SCaM coding sequences were reintegrated to a T7 expression vector, pET-3d. The integrity of each expression construct was verified by nucleotide sequencing. The amino acid sequences of these chimeric proteins are as follows: SCaM-1114, Ala1-Thr110 from SCaM-1 and Asn111-Arg149 from SCaM-4; SCaM-1144, Ala1-Phe99 from SCaM-1 and Lys90-Arg149 from SCaM-4; SCaM-1444, Ala1-Ile46 from SCaM-1 and Gln49-Arg149 from SCaM-4; SCaM-1N4, Ala1-Ala15 from SCaM-1 and Phe16-Ala149 from SCaM-4; SCaM-4N1, Ala1-Ile46 from SCaM-4 and Gln90-Lys149 from SCaM-1; SCaM-4411, Ala1-Phe99 from SCaM-4 and Arg90-Lys149 from SCaM-1; SCaM-4441, Ala1-Ile46 from SCaM-4 and Asn111-Lys149 from SCaM-1; SCaM-4N1, Ala1-Ala15 from SCaM-4 and Phe16-Lys149 from SCaM-1.

Construction of Single, Double, and Triple Residue Mutants—The mutant SCaMs were generated in a procedure similar to that for the domain exchanges described above. The names of the oligonucleotides, their respective amino acid residue substitutions, and nucleotide sequences used are as follows: CMO-5 for K30E, 5'-GGTTGATCACAACAAAGGAGG-3'; CMO-7 for G40D, 5'-ACTGTTATGGCTTGTTTGATCAGATCCA-3'; CMO-9 for T92V, 5'-AAGGCTTTATGTTGAGTGAACA-3'; CMO-10 for M36I, 5'-CAACGAACTTAAAGTGTCACACCG-3'; CMO-11 for G33A, 5'-CATACAGTCGCAGCTTGTTGGT-3'; CMO-12 for A46E, 5'-TTGGACCTCTCCTCTTGGATTT-3'; CMO-13 for S19G, 5'-ATCTCTATGCAACAAAGGAGG-3'.

Protein Expression and Purification—Expression plasmids encoding the chimeric CaMs or mutant SCaMs were introduced into E. coli BL21(DE3)pLysS by means of electroporation (29). A single colony of a transformant was inoculated into 8 ml of LB medium containing 100 μg/ml ampicillin and 33 μg/ml chloramphenicol and grown overnight at 37 °C. Five milliliters of the overnight culture was transferred to a fresh 37 °C. Five milliliters of the overnight culture was transferred to a fresh 37 °C. Five milliliters of the overnight culture was transferred to a fresh 37 °C. Five milliliters of the overnight culture was transferred to a fresh
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RESULTS

Construction and Purification of Chimeric SCaMs—To investigate the structure-function relationship between CaM and NAD kinase, we generated chimeric SCaMs by functional domain exchanges between SCaM-1 and SCaM-4. CaM was divided into four functional domains on the basis of the four Ca²⁺-binding domains. To facilitate domain exchanges, three new restriction enzyme sites were created in the cDNA sequence of SCaM-1 without changing codons by the U-DNA mutagenesis method (28). Construction of the chimeric SCaM expression plasmids and their expression and purification from E. coli are described in detail under “Experimental Procedures.” A schematic illustration of chimeric SCaMs and restriction enzyme sites used or created in domain exchanges is shown in Fig. 1. When the purified chimeric proteins were analyzed by SDS-polyacrylamide gel electrophoresis in the presence of either 5 mM CaCl₂ or 5 mM EGTA, all of the chimeras exhibited characteristic Ca²⁺-dependent electrophoretic mobility shifts as shown in Fig. 2A. These results suggest that all chimeras retain their Ca²⁺-binding ability and undergo Ca²⁺-dependent conformational changes, although the evidence is indirect (35, 36). In the presence of Ca²⁺, SCaM-4 showed faster migration than SCaM-1, and this property was maintained in SCaM-1444, SCaM-4111, and SCaM-4441. Two chimeras, SCaM-1114 and SCaM-4111, had intermediate electrophoretic mobilities. Among the slow migrating chimeras, including SCaM-1, the mobility of SCaM-1N4 is particularly interesting, because this chimera contains all four domains of SCaM-4 except its extreme N-terminal portion (from Ala¹ to Ala¹⁵). In the presence of EGTA, most of chimeric SCaMs displayed similar electrophoretic mobilities to SCaM-1 except for SCaM-1114 and SCaM-1444, which showed faster migration than SCaM-1 and SCaM-4.

Activation of NAD Kinase by Chimeric SCaMs—When the chimeras were tested for their ability to activate NAD kinase, two chimeric SCaMs with domain I exchanges differed remarkably from their parents, SCaM-1 and SCaM-4 (see Fig. 3 and Table I). The exchange of domain I of SCaM-1 with that of SCaM-4, SCaM-4111, resulted in a drastic decrease in NAD kinase activation. SCaM-4111 showed only 38% of maximal activation and 3-fold increased Kₘ value when compared with SCaM-1. Conversely, SCaM-1444, when domain I of SCaM-1 was put into SCaM-4, was able to activate NAD kinase. SCaM-1444 activated NAD kinase up to 68% of the maximal activation exhibited by SCaM-1 and had a similar Kₘ value (6.75 nm) as SCaM-1. Furthermore, all chimeric SCaMs carrying domain I of SCaM-1 (SCaM-1114 and SCaM-1444) were able to activate NAD kinase (see Table I). Chimeric SCaMs that did not carry this domain were similar to SCaM-4 and unable to activate NAD kinase. Since SCaM-1444 was able to activate NAD kinase but SCaM-4111 was not, either the N-terminal helix or the first EF hand in domain I of SCaM-4 must be responsible for its failure to activate this enzyme. To more clearly define whether the N-terminal helix or Ca²⁺-binding site 1 was responsible, we substituted just the N-terminal helix of SCaM-4 (Ala¹-Ala¹⁵) into SCaM-1 (SCaM-4N1; see Fig. 1). SCaM-4N1 exhibited 87% maximal activation of NAD kinase with a Kₘ value that was only 2-fold higher than SCaM-1 (Table I and Fig. 3). Further, substitution of the N-terminal helix of SCaM-1 into SCaM-4 (SCaM-1N4), did not produce a chimera that could activate NAD kinase (Table I). Thus, the N terminus of SCaM-4 does not appear to be responsible for its inability to activate this kinase. It follows that the region responsible for the differential activation of NAD kinase exhibited by SCaM-1 compared with SCaM-4 must be the first EF hand (amino acids Phe¹⁶-Leu¹⁸) of these proteins. These results strongly suggest that this first EF hand of CaM plays a decisive role in the differential activation of NAD kinase exhibited by SCaM-1 and SCaM-4.

Activation of cAMP-Phosphodiesterase by Chimeric SCaMs—We have previously shown that SCaM-1 and SCaM-4 are equally effective activators of PDE (27). We therefore determined whether any of the chimeric constructs were also affected in their ability to activate PDE or whether the changes made affected specifically the NAD kinase activation. As shown in Table I, SCaM-1, SCaM-4, and all of the chimeras were equally effective in the activation of PDE. Further, SCaM-1444 and SCaM-4111 that showed severe impairment of NAD kinase activation showed similar Kₘ values for PDE (3.7, 6.8, 13.6, and 3.3 nm for SCaM-1, SCaM-1444, SCaM-4111, and SCaM-4, respectively). Thus, the domain exchanges between SCaM-1 and SCaM-4 had no impact on the ability of these chimeric SCaMs to activate PDE.

Construction and Enzyme Activation of Single Substitution Mutant SCaMs—The exchanged first EF hand region, which was responsible for differential NAD kinase activation included the C-terminal region of helix 1, the first Ca²⁺-binding loop, helix 2, linker, and the N-terminal portion of helix 3 of CaM. This spans the region from Phe¹⁶ to Leu¹⁸. As shown in Fig. 4, SCaM-4 has seven amino acid substitutions in domain I when compared with SCaM-1. Among these substitutions, three are nonconservative exchanges between differently charged amino acids of K30E, G40D, and A46E. Since these substitutions may have an effect on the interaction of CaM with target enzymes, we tried to define residue(s) critical for NAD kinase activation by creating individual single residue mutants of SCaM-1, each.

FIG. 1. Schematic illustration of chimeric SCaMs generated by domain exchanges between SCaM-1 and SCaM-4. SCaM-1 sequences are in white and SCaM-4 sequences are in black. The four Ca²⁺-binding loops are shown as half circles. Arrows indicate restriction enzyme sites used (closed arrowheads) or created (open arrowheads) for the domain exchanges.
of which had one residue within the domain I substituted by the one that occurred in SCaM-4.

After purification, the single residue mutant proteins were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 2B). Only the K30E mutant exhibited an electrophoretic mobility that was distinct from other single residue mutants and SCaM-1. The K30E mutant had an extremely high electrophoretic mobility in the presence of Ca\(^{2+}\), which was even slightly higher than SCaM-4. However, in the presence of EGTA, the electrophoretic mobility was similar to that of SCaM-4. All other single residue mutants had mobilities similar to that of SCaM-1 in the presence or absence of Ca\(^{2+}\).

While all of these single residue mutant SCaMs could maximally activate PDE, only K30E and G40D had greatly reduced ability to activate NAD kinase (Table II, Fig. 5). K30E could activate NAD kinase only to 51% of maximal activation, and the \(K_{\text{act}}\) value was increased 3-fold to 22.9 nM. The maximal activation and \(K_{\text{act}}\) value for the G40D mutant were 68% and 9.3 nM, respectively. The K30E substitution had, therefore, a more detrimental effect on the NAD kinase activation than G40D. Other single residue mutants of SCaM-1, S17G, T29V, G33A, M36I, and A46E, showed relatively good activation of NAD kinase with activations higher than 80% of the maximum.

The NAD kinase assay was performed as described under "Experimental Procedures." The activity of NAD kinase (percentage of maximal activation) is expressed relative to the maximal activation by SCaM-1. Phosphodiesterase activity was measured using cAMP as substrate in the presence of saturating concentration (100 nM) of activator CaMs. For both assays, values represent fitted data using nonlinear regression analyses from at least triplicate assays. \(K_{\text{act}}\) is the concentration of activator CaM needed to obtain half-maximal activation of NAD kinase. Relative \(K_{\text{act}}\) is calculated by dividing the \(K_{\text{act}}\) by the \(K_{\text{act}}\) for SCaM-1 (7.4 nM).

| Chimeric SCaMs | NAD kinase | Phosphodiesterase |
|----------------|------------|-------------------|
|                | \(K_{\text{act}}\) | Relative \(K_{\text{act}}\) | Maximal activation | Maximal activation |
| SCaM-1          | 7.4        | 1.00              | 100.00            | 100.0             |
| SCaM-1114       | 10.4       | 1.41              | 83.36             | 101.1             |
| SCaM-1144       | 37.9       | 5.12              | 114.36            | 107.8             |
| SCaM-1444       | 6.7        | 0.83              | 67.89             | 102.4             |
| SCaM-4111       | 14.1       | 1.91              | 87.45             | 109.6             |
| SCaM-4111       | 23.8       | 3.22              | 38.18             | 104.6             |
| SCaM-4411       | ND         | ND                | ND                | ND                |
| SCaM-4441       | ND         | ND                | ND                | 101.2             |
| SCaM-1N4        | ND         | ND                | ND                | 110.5             |
| SCaM-441        | ND         | ND                | ND                | 100.8             |
| SCaM-4          | ND         | ND                | ND                | 104.3             |

\(\text{ND}\) no detectable NAD kinase activation.
Construction of Double and Triple Residue Mutant SCaMs—Although two of the single residue mutants, K30E and G40D, showed greatly reduced NAD kinase-activating abilities, these single residue substitutions alone could not duplicate the complete inability of SCaM-4 to activate NAD kinase. Also, previous studies on the structure-function relationship between CaM and target enzymes commonly showed that more than two amino acid residues in the critical domain were necessary for the target enzyme activation (25, 26, 37). We therefore examined the effect of combining the two individual single residue substitutions, K30E and G40D. Two additional double residue mutants, K30E/M36I and M36I/G40D, were also generated to compare effectiveness. The M36I mutation was selected because the mutation had the third greatest effect on NAD kinase activation (see Table II). For triple residue mutants, the M36I mutation was combined with the K30E/G40D double mutation. Two other triple residue mutants, S17G/K30E/G40D and K30E/G40D/A46E, were also prepared to evaluate the combined effect of M36I and K30E/G40D mutations. The S17G and A46E mutation were selected because they posed no significant effect on NAD kinase activation as single mutations (see Table II). All of the multiple residue mutant SCaMs behaved similarly during the purification procedure with regard to heat stability and elution profile on a phenyl-Sepharose column. When the multiple residue mutants were analyzed by SDS-polyacrylamide gel electrophoresis, all had electrophoretic mobilities that were intermediate between SCaM-1 and -4 in the presence of Ca\textsuperscript{2+}. However, in the presence of EGTA, all multiple residue mutants that had both K30E and G40D substitutions (K30E/G40D, K30E/M36I/G40D, K30E/G40D/A46E, and S17G/K30E/G40D) exhibited extremely retarded electrophoretic mobilities with an apparent molecular mass of 23 kDa (see Fig. 2C). Both substitutions, K30E and G40D, made the net charge of CaM more negative. The more acidic nature of these mutants may interfere with their binding to SDS, which may then be reflected in the retarded mobility of these mutants. However, we cannot rule out the possibility that the combined substitutions, K30E/G40D, could cause the secondary structure of CaM to assume a more relaxed conformation in the absence of Ca\textsuperscript{2+}. Activation of NAD Kinase and PDE by Double and Triple Substitution Mutant SCaMs—The combined effect of K30E and G40D was additive insofar as K30E/G40D could activate NAD kinase only up to 22%, while K30E and G40D single mutants activated 52 and 68% of maximal level, respectively (see Fig. 5). In addition, the relative \( K_{\text{act}} \) increased to approximately 18-fold for the double mutant, indicating that the relative affinity for NAD kinase was greatly reduced. In contrast, the K30E/M36I double residue mutant did not show significant additional inhibition of NAD kinase activation when compared with the single residue mutant, K30E (Table II). However, the combination of M36I with G40D reduced NAD kinase activation to 23% of maximal level. Interestingly, both double mutants, K30E/G40D and M36I/G40D, had greatly increased \( K_{\text{act}} \) values. While the K30E/G40D double mutant showed greatly decreased ability to activate NAD kinase, the mutant could maximally activate PDE (90.9%), and the \( K_{\text{act}} \) value for PDE did not increase significantly (10.9 as compared with 3.7 nM for SCaM-1).

Among the triple residue mutants, K30E/M36I/G40D showed the greatest impairment of its ability to activate NAD kinase. This mutant showed almost no activation of NAD ki-
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nase (0.2% of maximal level), but it still maximally activated PDE (98.8% maximal activation and 12.4 nM $K_{eq}$). Thus, the triple residue mutant K30E/M36I/G40D recapitulated the phenotype of SCaM-4. However, S17G/K30E/G40D and K30E/G40D/A46E also exhibited severe impairment in their ability to activate NAD kinase (5–7% of the maximal activation), although individual S17G or A46E single mutations had no detrimental effect on NAD kinase activation.

SCaM-4 and K30E/M36I/G40D Retained the Ability to Bind NAD Kinase—Several lines of evidence indicated that the binding and activation mechanism of CaM to target enzymes can be separated; i.e. some mutant CaMs bind target enzymes but cannot activate them (17, 22, 23). To investigate whether the inability of SCaM-4 and the K30E/M36I/G40D to activate NAD kinase is due to their failure to bind NAD kinase, CaM overlay assays were performed using HRP-conjugated SCaM-1, SCaM-4, and K30E/M36I/G40D. As shown in Fig. 6A, a single band of approximately 55 kDa was evident in all three SCaM overlays. The 55-kDa protein band is thought to represent NAD kinase, since no other CaM-binding protein band except for the 55-kDa band was observed in our NAD kinase preparation. Further, other workers who intensively purified NAD kinase employing CaM-Sepharose affinity chromatography showed a 55-kDa band to be the main protein band (38). When overlays were performed with HRP-conjugated SCaM-1 either in the presence of a 50-fold excess of unlabeled SCaM-4 or K30E/G40D, the binding of HRP-conjugated SCaM-1 to both NAD kinase and CaN was competitively abolished by these unlabeled CaMs (Fig. 6B). These results suggest that SCaM-4 and K30E/M36I/G40D retain the ability to bind NAD kinase but are unable to activate NAD kinase.

DISCUSSION

In this study, we investigated the structure-function relationship of CaM in the activation of NAD kinase by using chimeric constructs made between SCaM-1 and SCaM-4. Interestingly, despite the relatively even distribution of the 32 amino acid substitutions in SCaM-4 relative to SCaM-1, only chimeric CaMs possessing the first EF hand of SCaM-1 could activate NAD kinase to greater than half-maximal level. Furthermore, exchanging the first EF hand region in SCaM-4 for the equivalent region in SCaM-1 was sufficient to confer the ability to activate NAD kinase on SCaM-4. Thus, the striking difference between SCaM-1 and SCaM-4 with regard to NAD kinase activation is confined to just the first EF hand region.

In comparison with SCaM-1, the first EF hand region of SCaM-4 has seven amino acid substitutions, and only three of them could be regarded as nonconservative exchanges. When these substitutions were duplicated in SCaM-1 in their respective positions, only the K30E and G40D substitutions significantly decreased NAD kinase activation. The Lys$^{30}$ residue is located within the first Ca$^{2+}$-binding EF hand structure. The positively charged side chain of Lys$^{30}$ protrudes to the outside and is not involved in the interaction with CaM-binding peptide in the three-dimensional structures formed by CaM complexed with CaM-binding domain peptides (see Fig. 7; Refs. 12–14). The Lys$^{40}$ residue may interact with the corresponding portion of NAD kinase by charge interaction. The exchange of the positively charged Lys$^{30}$ with an acidic and negatively charged Glu could therefore, interfere with the electrostatic interaction that might be necessary for the conformational change of NAD kinase to an active state. The Gly$^{40}$ residue is located in the linker region that connects the C-terminal helix of loop I to the N-terminal helix of the second Ca$^{2+}$-binding loop of CaM. The Gly$^{40}$ residue has been strictly conserved during evolution and makes a sharp bending possible due to its small size (39). Thus, the exchange of Gly$^{40}$ for a relatively bulkier and charged Asp could interfere with this bending of the linker. Interestingly, in Paramecium, the exchange of Gly$^{40}$ with Glu was shown to be responsible for a behavioral aberration mutant phenotype (39). As proposed by Kink et al. (39), the Gly$^{40}$ residue was shown to be located in the bending region of linker in the three-dimensional structure of CaM-target peptide com-

![Fig. 5. Activation of NAD kinase by single and multiple residue mutant SCaMs.](http://www.jbc.org/)

![Fig. 6. Binding of SCaM-1, SCaM-4, and K30E/M36I/G40D to NAD kinase.](http://www.jbc.org/)
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The three-dimensional structure of bovine CaM and the CaM-binding domain peptide of CaM-dependent protein kinase II complex (14) is shown. CaM is displayed as a ribbon diagram, and the 26-residue peptide comprising the CaM-binding domain of multifunctional CaM-dependent protein kinase II (residues 577–602) is shown by an α-carbon backbone structure in blue. The critical residues (Lys30, Met36, and Gly40) for NAD kinase activation are shown as ball and stick structures in red, green, and yellow, respectively. This figure was generated from the x-ray coordinate data deposited in the Brookhaven Protein Data Bank (code name 1CDM) by using the molecular visualization program, RasMol 2.5.

During evolution, SCaM-4 may have undergone minimal residues by a hydrophobic interaction. It is important for stabilization of the CaM-target peptide complex together with other Met residues (13). Therefore, the bulkier hydrophobic side chain of Ile may prevent proper positioning of the CaM-binding domain of NAD kinase and thereby interfere with the conformation of the CaM-NAD kinase complex needed for activity.

It is interesting that the detrimental effect of double and triple residue mutations in domain I was more severe than the entire exchange of domain I (SCaM-4111). Thus, it seems that some of other substituted residues in SCaM-4, such as Val30 and/or Ala33, may compensate for the detrimental effect of Glu30 and Asp40 in the activation of NAD kinase. Given the multiple regulatory role of CaM for a variety of target enzymes, SCaM-4 may have evolved a primary structure that can accommodate different regulatory effects on multiple target enzymes. During evolution, SCaM-4 may have undergone minimal residue substitutions to acquire regulatory functions different from SCaM-1, such as the inability to activate NAD kinase, while retaining the ability to activate PDE. To verify this notion, the effect of combinations of Glu30 or Asp40 with Val30 and/or Ala33 should be examined. Also, the back mutation on SCaM-4 focusing on these critical residues should be evaluated. Such experiments are currently in progress.

Our results are in good agreement with the earlier observation that CaMs from various sources such as bovine, mung bean, mushroom, and Tetrahymena could efficiently activate pea NAD kinase (11). In all of the CaM sequences identified so far, Lys30, Met36, and Gly40 are extremely well conserved. The only exceptions known are SCaM-4 and SCaM-5 from plants, a CLP from humans (40), and two CaMs from yeasts (41, 42). In both budding and fission yeast, the Gly40 residue is conserved, but the Lys30 residue is exchanged for Asn and Ser, respectively. The human CLP is also conserved at Gly40, but it has an Arg residue in place of Lys30. Therefore, it would be very intriguing to see whether these CaMs could activate NAD kinase.

When compared with other studies of the CaM structure-target enzyme activation mechanism, our data of the structure-function relationship of CaM with regard to NAD kinase activation show the closest similarity to the smooth muscle myosin light chain kinase activation model. Others using an approach similar to ours have suggested that domain I of CaM plays a critical role in the activation of smooth muscle myosin light chain kinase (22). The exchange of three amino acid residues in domain I of CaM (Glu14, Thr34, and Ser38) could prevent the activation of the enzyme (37). Two of the residues, Thr34 and Ser38, are located on helix 2 of CaM, a region in which Lys30, Met36, and Gly40 also reside. Thus, it is plausible that NAD kinase may interact with CaM in a similar manner as smooth muscle myosin light chain kinase. Domain I of CaM has also been observed to play an important role in activation of CaM kinase II (22). Substitution of domain I with that of cardiac troponin C severely impaired activation of CaM kinase II. Interestingly, SCaM-4 was far less effective in the activation of CaM kinase II, while SCaM-1 was as effective as bovine CaM.2

The different regulatory effects of SCaM-1 and SCaM-4 on target enzyme activation are in agreement with recent genetic and biochemical studies of the different requirements for specific CaM domains. Ohya and Botstein (43) showed that a different N-terminal lobe or C-terminal lobe of CaM is required for a specific function in yeast such as actin organization, nuclear division, or bud emergence. Also Persechini et al. (44) reported that the EF hand pairs in both the C- and N-terminal lobes of CaM contain distinct but overlapping sets of determinants for binding and stimulation of target enzymes and also that CaM-modulated enzyme activation requires specific determinants in CaM outside of the EF hand pairs. In our study, the critical region of CaM in the activation of NAD kinase is domain I in the N-terminal lobe of CaM.

While we were preparing this manuscript, Liao et al. (45) reported different stimulation of NAD kinase by plant CaM isoforms from Arabidopsis. Based on the observation of two N-terminal and C-terminal CaM mutants, they suggested that the C-terminal hydrophobic domain of CaM is critical for NAD kinase activation. Since both the N-terminal and C-terminal lobes of CaM are essential for maximal activation of CaM target enzymes, our data for the role of domain I of CaM in NAD kinase are not contrary to the conclusion drawn by Liao et al. but may complement it. In this study, we investigated the region responsible for the differential activation of NAD kinase by SCaM isoforms. We found that the first EF hand is critical for this differential activation and therefore must also be important for NAD kinase activation. In our hands, SCaM-4111 retained approximately 40% NAD kinase activation ability, and SCaM-1444 reached only 68% of the maximal activity. These results suggest that the C-terminal half of CaM also plays a role in the activation of NAD kinase. Thus, domain I in the N-terminal lobe of CaM may play a critical role in concert
with the C-terminal end in the C-terminal lobe to maximally activate NAD kinase. This agrees well with the hypothesis proposed by Su et al. (26), in which they emphasized the functional importance of latch domain of CaM in target enzyme activation.

Our effort to investigate the structure-function relationship of CaM in NAD kinase activation by exploiting two naturally occurring CaM isoforms and exchanging domains between them was successful and promising. The strategy used in this study, therefore, should be extendible to studies of other enzymes. These chimeric CaMs may also be applied to the study of how a small molecule CaM could adapt itself for the interaction with and regulation of a variety of target enzymes/proteins during its molecular and biochemical evolution.

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