Activation of Myosin Light Chain Kinase Requires Translocation of Bound Calmodulin*

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A novel translocation step is inferred from structural studies of the interactions between the intracellular calcium receptor protein calmodulin (CaM) and one of its regulatory targets. A mutant of CaM missing residues 2–8 (∆NCaM) binds skeletal muscle myosin light chain kinase with high affinity but fails to activate catalysis. Small angle x-ray scattering data reveal that ∆NCaM occupies a position near the catalytic cleft in its complex with the kinase, whereas the native protein translocates to a position near the C-terminal end of the catalytic core. Thus, CaM residues 2–8 appear to facilitate movement of bound CaM away from the vicinity of the catalytic cleft.

A wide variety of Ca\textsuperscript{2+}-dependent cellular processes are regulated by the ubiquitous Ca\textsuperscript{2+}-binding protein CaM, which interacts with a diverse array of target proteins, including a number of protein kinases (1). Ca\textsuperscript{2+}/CaM-dependent activation of MLCK has provided a number of important insights into the molecular mechanisms underlying CaM-dependent kinase activation. All isoforms of MLCK include a conserved protein kinase catalytic core (2) that is followed immediately by a regulatory segment consisting of autoinhibitory and CaM-bind-

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‡ The abbreviations used are: CaM, calmodulin; cPKA, catalytic subunit of the cAMP-dependent protein kinase; MLCK, myosin light chain kinase; ∆NCaM, deletion mutant of calmodulin missing residues 2–8; skMLCK, skeletal muscle myosin light chain kinase; MOPS, 4-morpholinepropanesulfonic acid; AMPPNP, adenosine 5′-(β,γ-imino)triphosphate.

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MATERIALS AND METHODS

Sample Preparation—The ∆NCaM was expressed in Escherichia coli and purified as described in (16). CaM, skMLCK, and the complexes were prepared as described in (8). All samples contained excess Ca\textsuperscript{2+} so that the CaMs were Ca\textsuperscript{2+} saturated. Buffer conditions were 25 mM MOPS, pH 8.0, 25 mM KCl, 5 mM magnesium acetate, 1 mM dithiothreitol, and 2.5% glycerol.

Scattering Data Acquisition—Scattering data were acquired using the x-ray instruments at Los Alamos (18, 19) and reduced to Q) versus Q as described in (18). Q is (4πsinθ)/λ where θ is half the scattering angle and λ is the wavelength of the scattered radiation. Structural information is derived by calculating the inverse Fourier transform of the autoinhibitory release mechanism (6) for kinase activation in which CaM binding induces a significant movement of the regulatory segment away from the surface of the catalytic core necessary for binding and phosphorylating myosin regulatory light chain. More recently, we showed that substrate binding induces a closure of the catalytic cleft and a reorientation of CaM that allows for more extensive surface contact between the catalytic core and the N-terminal sequence of CaM (8).

CaM has two globular Ca\textsuperscript{2+}-binding lobes connected by a central helix (9) that functions as a “flexible tether” (10, 11). The concept of the flexible tether function obtained its strongest support from structural studies of CaM complexed with isolated peptides containing the core CaM-binding sequences from kinases that show CaM undergoes a dramatic conformational collapse (11–15). Flexibility in the central helix allows the two lobes of CaM to come together, enfolding the helically configured target peptides. A similar conformational collapse of CaM was observed in scattering studies of its complex with a catalytically competent skMLCK (7, 8). The known structures of all the CaM-peptide complexes show little or no interaction between the N-terminal leader sequence of CaM (residues 1–8, ADQLTEEQ) and the bound peptides. Nevertheless, deletion of residues 2–8 results in a CaM mutant (∆NCaM) that binds with high affinity but completely fails to activate skMLCK (16). The mutant protein also has a reduced capacity to activate the smooth muscle form of the kinase but can fully activate neuronal nitric-oxide synthase activity, suggesting that the N-terminal leader sequence plays a specialized role in a subset of CaM-target complexes.

We present here the results of scattering experiments on ∆NCaM and native CaM in their respective complexes with a catalytically competent skMLCK (17) that reveal the dramatic structural basis for the inability of ∆NCaM to activate skMLCK catalysis.
CaM-dependent Activation of MLCK

**Table I**

| Protein       | $R_g$ | $d_{\text{max}}$ | $I_0$ | $c$ | $I_0/eM$ |
|---------------|-------|------------------|------|----|----------|
| CaM          | 21.2 ± 0.2 | 66 ± 3 | 40.8 ± 0.4 | 13.7 | 1.00 ± 0.01 |
| ΔNCaM        | 19.5 ± 0.2 | 60 ± 3 | 10.5 ± 0.1 | 3.2 | 1.11 ± 0.01 |
| CaM:skMLCK   | 29.7 ± 0.3 | 90 ± 5 | 12.2 ± 0.2 | 1.2 | 1.00 ± 0.02 |
| ΔNCaM:skMLCK | 24.6 ± 0.2 | 70 ± 5 | 10.7 ± 1.0 | 1.0 | 1.06 ± 0.01 |

$M$ is the molecular weight of scattering particle, $c$ is the protein concentration. $I_0/eM$ are relative values, scaled so that the native CaM data give a value of 1.0. Under the conditions of these experiments we know that CaM is monodisperse (24). The values of $I_0/eM$ are calculated assuming $M$ is the molecular weight of the individual proteins or expected complexes. A value of 1.0 for each data set is what is expected for monodisperse particles with molecular weights equal to those of the individual proteins or the 1:1 complexes.

**Two-Ellipsoid Model of the Complexes Reveals Sizes and Dispositions of Components**—To gain further insights to the relative sizes and dispositions of CaM or ΔNCaM and skMLCK, we simulated x-ray scattering data using SASMODEL (see “Materials and Methods”). Each complex was modeled as two uniform density ellipsoids, representing CaM or ΔNCaM and skMLCK. During this procedure the ellipsoids were required to be in contact, and their dimensions were loosely constrained to be in a range consistent with the dimensions for CaM and skMLCK determined in previous scattering studies (8). Specifically, the ellipsoid semi-axis lengths were constrained to be in the ranges 38–48 Å, 23–35 Å, and 16–22 Å for skMLCK and 22–28 Å, 18–24 Å, and 14–19 Å for CaM. For the ΔNCaM complex, we also allowed the bound ΔNCaM to assume the more elongated shape associated with its uncomplexed state, to test the possibility that the ΔNCaM might not collapse upon binding. In each calculation, $10^4$ different models were tested, with randomly chosen ellipsoid dimensions, relative orientations, and contact points. The best-fit models are depicted in Fig. 2 (green crosses), and their respective fits ($\chi^2$) and structural parameters are given in Table II. As expected, the model for the native CaM:skMLCK complex is similar to the one proposed previously based on neutron contrast variation data for the complex with deuterated CaM (8). Contrast variation allows us to readily distinguish CaM and skMLCK in the complex, and we therefore were able to unequivocally assign the smaller ellipsoid to the CaM. The model for the ΔNCaM:skMLCK complex indicates individual ellipsoid dimensions similar to those for the native CaM:skMLCK complex. We therefore conclude that, like native CaM, ΔNCaM collapses about the core CaM-binding sequence of the kinase in this complex. A most significant difference between the complexes is that bound ΔNCaM is more centrally located with respect to the long axis of the kinase than the bound native protein. **Higher Resolution Structural Model for the Complex Reveals Position of the Catalytic Cleft with Respect to the CaM-binding Site**—To evaluate how sensitive the scattering data are to the position of CaM or ΔNCaM relative to the catalytic cleft in the kinase, we performed additional calculations using higher resolution structural models. We modeled the catalytic core of skMLCK using the crystal structure of the catalytic subunit of the cAMP-dependent protein kinase, cPKA (Ref. 3, PDB accession number 2cpk) and SwissModel, an automated homology

**RESULTS**

Scattering Data—The scattering data (Fig. 1A) for free ΔNCaM indicate it has a slightly more compact structure than native CaM (Table I). Fig. 1C compares the vector length distribution functions, $P(r)$, for the two proteins. Based on calculations of model $P(r)$ functions using the crystal structure for CaM (not shown), this difference can be accounted for solely by the deletion of the N-terminal residues in the mutant protein. The scattering data thus indicate that ΔNCaM has a two lobed structure, similar to native CaM. In contrast, the solution structures of the ΔNCaM and CaM complexes with skMLCK differ dramatically. The experiments on the complexes were performed with and without substrates; a peptide containing the phosphorylation sequence from myosin regulatory light chain (pMLC, sequence KGKRAARATSNVPS) and a nonhydrolyzable analog of adenosine-triphosphate (AMPPNP). The same differences were observed between the native CaM and ΔNCaM complexes in the presence or absence of substrates. We used data collected in the presence of the substrates for our analyses, because the native CaM:skMLCK complex is less prone to aggregation under these conditions. The $I_0$ values indicate that the protein solutions contain monodisperse particles with the molecular weights expected for 1:1 complexes (Table I). As we have reported previously (7, 8), the $P(r)$ function and $R_g$ value for the CaM:skMLCK complex (Fig. 1D) indicates an elongated structure (Table I). In contrast, data for the ΔNCaM:skMLCK complex indicate a more symmetrical structure, evident in its significantly smaller $d_{\text{max}}$ and $R_g$ values (Table I).
are generated. The N terminus of CaM in both models is indicated with P to calculate model representations random points that fill the ellipsoid shapes and are used dots red binding peptide (MLCK-I) are superimposed with ribbon representations of the respective higher resolution models. The ribbons for skMLCK and the CaM-binding peptide (MLCK-I) are red and for CaM, cyan. The pale green dots represent random points that fill the ellipsoid shapes and are used to calculate model Pr functions from which model scattering profiles are generated. The N terminus of CaM in both models is indicated with N.

Table II

| Two ellipsoid models | $\chi^2$ | $R_z$ values (Å) | Ellipsoid semi-axis dimensions
|---------------------|---------|------------------|-----------------|
| CaM-skMLCK          | 1.04    | 30.05            | CaM (25,18,15)  |
| $\Delta$CaM-skMLCK  | 2.00    | 26.35            | skMLCK (41,24,19) |
| High resolution models |       |                  | $\Delta$CaM (26,20,16) |
| CaM-skMLCK          | 1.00    | 29.8 (29.7)$^b$  | skMLCK (41,24,19) |
| $\Delta$CaM-skMLCK  | 1.00    | 24.5             |                 |

$^a$The dimensions obtained in these calculations compare well with the values obtained for CaM-skMLCK, with bound substrates, using neutron contrast variation and deuterated CaM; CaM (26.4, 22.4, 16.5) and skMLCK (41.3, 24.0, 16.4) (8).

$^b$The results obtained for both the random and systematic searches gave identical parameters for the CaM-skMLCK complex, and for $\Delta$CaM-skMLCK the $R_z$ values were within 0.1 Å (the value in parentheses was obtained for the systematic search).

The cPKA and skMLCK catalytic cores have 29.4% amino acid sequence identity and 61% sequence similarity, making cPKA a good template for homology modeling. For sequences with 30–40% sequence identity, homology modeling can yield structures that give backbone root mean square deviation (RMSD) values from experimentally derived structures as low as 1.5 Å (23). To model bound CaM or $\Delta$NCaM, we used the NMR structure for CaM complexed with a peptide based on the core CaM binding sequence in skMLCK (MLCK-I, Ref. 14, PDB accession number 2bbm), deleting residues 2–8 for the complex with $\Delta$NCaM. The skMLCK model does not account for 32 N-terminal residues and 52 C-terminal residues in the kinase, as they have no corresponding sequences in cPKA. However, of the C-terminal residues, 23 are accounted for by the core CaM-binding sequence. Thus, the structural models for the complexes account for ~90% of the skMLCK sequence.

Simulated scattering data for the $\Delta$NCaM or CaM complexes with skMLCK were generated by positioning the modeled components relative to each other and calculating the expected scattering profiles using PRPDB (see "Materials and Methods"). Two approaches were used. The first was to randomly orient and position the components with respect to each other while loosely constraining the distance between their centers of mass to be no more than 60 Å (22,400 models tested). Using this approach, ideal $\chi^2$ values ($\chi^2 = 1.00$) were obtained for both complexes. In the second approach, the two components were placed in the same arbitrary relative positions, and the CaM-MLCK-I component was systematically moved through every position on a 5 Å grid extending ±50 Å in each of the x, y, and z directions (9,261 possible x, y, z coordinates tested). The best-fit model identified from the 5 Å grid search was further refined by performing a finer grid search (1Å resolution, ±5 Å in each direction, 1,331 possible x, y, z coordinates tested). In a final refinement step, the two components were randomly rotated about their origins in all directions (125,000 different orientations tested). Using this approach we also were able to obtain $\chi^2$ values of 1.00 for both the CaM-skMLCK and $\Delta$NCaM-skMLCK complexes. The relative positions of skMLCK and CaM based on the random search and the systematic grid search were equivalent, giving RMSD main chain deviations between the differently derived models of 0.22 and 0.26 Å for the CaM-skMLCK and $\Delta$NCaM-skMLCK complexes, respectively. Fig. 2 shows the backbone traces of the best-fit higher resolution model for each complex, overlaid with its corresponding best-fit ellipsoid model. For both complexes, there is excellent agreement in the positions of each component determined using the two modeling approaches. Similar to our previous analysis based on neutron scattering data, the higher resolution model for the skMLCK component does not completely fill the ellipsoid dimensions calculated using the x-ray scattering data, which is to be expected given the N- and C-terminal sequence segments omitted from the homology model.

The best-fits to the scattering data obtained using the higher resolution structural models predict a specific orientation of the kinase catalytic cleft with respect to bound CaM or $\Delta$NCaM (see Fig. 2). To evaluate the sensitivity of the scattering data to the relative orientations of the CaM and kinase components,
we performed additional calculations in which they were rotated about the three perpendicular axes defined by their associated best-fit ellipsoids. The rotations allowed us to test different orientations of the kinase and CaM structures while leaving the ellipsoid envelopes unperturbed. At each rotation angle (sampled in 30° steps) a translation search of ± 5 Å in the x, y, and z directions with a 1 Å step size was performed to optimize the translation for each rotation angle. The best-fit obtained after this translational search was considered the best one for that rotation angle. Fig. 3 presents a plot of the best χ² values obtained for the calculated scattering profiles and the experimental data at each rotation angle. All rotations of the kinase or CaM components away from their positions in the starting best-fit models result in significantly poorer fits for both complexes. The sensitivity to rotation angle is greatest for the kinase component, which shows the narrowest and deepest minimum with respect to this parameter, presumably because of its more irregular shape. In contrast, fits for the CaM and ∆NCaM structures go through shallower and broader minima in χ² as a function of rotation angle. In the best-fit models for both complexes, the CaM-binding peptide of MLCK is positioned with its N terminus pointing toward the kinase, which allows it to be connected with this sequence. In addition, the N-terminal helix of CaM in the native complex is in contact with the kinase, as was observed for the model derived from the neutron contrast variation data (8). The I(Q) functions derived from the best-fit models for each complex are compared with the experimental I(Q) and P(r) data in Fig. 1, B and D, respectively.

Because of the potential importance of the changes in CaM residues 1–8 in the activation mechanism (16), three peptides were synthesized for competitive binding studies; the native sequence and Glu residues substituted for Ala, and a scrambled native sequence. None of these peptides affected the catalysis or CaM activation properties at 1 mM (data not shown). This result suggests that the N-terminal sequence is not simply functioning as a latch to hold bound CaM to the native sequence from CaM results in a complex with skMLCK in which both lobes likely interact with the core CaM-binding sequence in the kinase. These results are consistent with the fact that although native CaM and ∆NCaM bind skMLCK with equal affinities only the native protein is capable of activating the kinase catalytic function (16). The inability of ∆NCaM to activate the kinase would appear to be due at least in part to its blocking of the catalytic cleft. In this position, it may also fail to disrupt auto-inhibitory interactions with the catalytic core. In contrast, a translocation of bound native CaM exposes the catalytic cleft and likely also disrupts interactions between the catalytic core and the autoinhibitory sequence, thus allowing catalysis to proceed. The precise role of the N-terminal leader sequence in facilitating translocation of bound CaM is not fully resolved. The simplest interpretation is that CaM residues 2–8 facilitate displacement of an autoinhibitory region flanking the core CaM-binding sequence in the kinase, allowing translocation of bound CaM to a position at the end of the C-terminal lobe of the enzyme, thereby allowing substrate binding to the exposed catalytic cleft.

DISCUSSION

Our results indicate that removal of the N-terminal leader sequence from CaM results in a complex with skMLCK in which the mutant protein blocks the catalytic cleft. This result is in stark contrast with the complex with native CaM, in which bound CaM appears to translocate so that the catalytic cleft is exposed, and the N-terminal helix of CaM interacts with the C-terminal portion of the catalytic core. The higher resolution modeling of the native CaMΔCMLCK complex also resolves the ambiguity that remained in the interpretation of the original two ellipsoid modeling of the neutron data regarding which side of the kinase, with respect to the catalytic cleft, is the CaM-binding site (8). In both native and mutant complexes CaM adopts a collapsed structure in which both lobes likely interact with the core CaM-binding sequence in the kinase.

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