Ca\(^{2+}\) Binding and Energy Coupling in the Calmodulin-Myosin Light Chain Kinase Complex

(Received for publication, October 7, 1999, and in revised form, November 12, 1999)

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We have previously shown that 3 Ca\(^{2+}\) ions are released cooperatively and independently from the complex between (Ca\(^{2+}\))\(_4\)-calmodulin and skeletal muscle myosin light chain kinase or a peptide containing its core calmodulin-binding sequence. We now have found that three Ca\(^{2+}\)-binding sites also function cooperatively in equilibrium Ca\(^{2+}\) binding to these complexes. Replacement of sites I and II in calmodulin by a copy of sites III and IV abolishes these cooperative effects. Energy coupling-dependent increases in Ca\(^{2+}\)-binding affinity in the mutant and native calmodulin complexes with enzyme are considerably less than in the peptide complexes, although the complexes have similar affinities. Ca\(^{2+}\) binding to three sites in the native calmodulin-enzyme complex is enhanced; the affinity of the remaining site is slightly reduced. In the mutant enzyme complex Ca\(^{2+}\) binding to one pair of sites is enhanced; the other pair is unaffected. In this complex reversal of enzyme activation occurs when Ca\(^{2+}\) dissociates from the pair of sites with enhanced affinity; more rapid dissociation from the other pair has no effect, although both pairs participate in activation. Ca\(^{2+}\)-independent interactions with calmodulin clearly play a major role in the enzyme complex, and appear to weaken Ca\(^{2+}\)-dependent interactions with the core calmodulin-binding sequence.

The ever present Ca\(^{2+}\)-binding protein, calmodulin (CaM), plays a key role in the subcellular transduction of Ca\(^{2+}\) signals, functioning as a Ca\(^{2+}\)-dependent regulatory subunit for a large array of different target proteins, including protein kinases, ion channels and pumps, nitric-oxide synthases, adenyl cyclases, and phosphodiesterases (1–10). In many cases CaM seems to produce target activation by relieving the effect of an autoinhibitory protein segment adjacent to or overlapping the core CaM-binding sequence in the target (11–15). The myosin light chain kinase family is a well studied example of this type (12, 15). We previously reported that release of Ca\(^{2+}\) from the complex between CaM and skeletal muscle myosin light chain kinase (skMLCK) follows a 3+1 kinetic mechanism: 1 Ca\(^{2+}\) ion is released independently with a rate constant >1000 s\(^{-1}\), and 3 are released in a cooperative manner with a rate constant of 1.6 s\(^{-1}\), which is similar to the rate constant for reversal of CaM-dependent enzyme activation (16). We also reported that a 3+1 Ca\(^{2+}\) release mechanism is preserved in the complex between CaM and a 19 residue peptide (skPEP) that contains the core CaM-binding sequence in the enzyme, although the rates for Ca\(^{2+}\) release are 10–100-fold slower (16). In this study we further investigate the structural basis for the 3+1 Ca\(^{2+}\)-release mechanism, determine its relationship to steady-state Ca\(^{2+}\) binding, and investigate energy coupling in the skMLCK and skPEP complexes with native CaM and with a mutant that no longer exhibits a 3+1 release mechanism.

MATERIALS AND METHODS

Native and mutant CaMs were expressed in Escherichia coli and purified as described by Persechini et al. (17). Skeletal muscle myosin light chain kinase was expressed in S99 cells and purified as described by Fitzsimons et al. (18). A synthetic peptide, KRRWKKNFIVASANN-RFKK-amide (skPEP), based on the core CaM-binding sequence in skMLCK (19), was commercially synthesized (Quality Controlled Biochemicals, Inc.). The concentrations of purified CaMs were determined from their optical absorbances in the presence of 50 μM CaCl\(_2\) using published extinction coefficients (20, 21). Separate extinction coefficients were used to determine the concentrations of the decalcified proteins required for Ca\(^{2+}\) binding studies (21).

Ca\(^{2+}\) binding was determined at 25°C using the flow dialysis technique, as described in detail by Stemmer and Klee (4). The buffer used for these experiments contained 150 mM NaCl, 1 mM MgCl\(_2\), and 10 mM HEPES, pH 7.5, with variable amounts of added Ca\(^{2+}\). The concentration of mutant or native CaM in each experiment was 5–10 μM, and 1.2-fold molar excess of skPEP or skMLCK was included where indicated. Under these conditions Ca\(^{2+}\) binding to free CaM makes a negligible contribution to the observed isotherm. Data were corrected for loss of Ca\(^{2+}\) ion from the dialysis chamber during the experiment (4), and values for free [Ca\(^{2+}\)] and for moles of Ca\(^{2+}\) bound per mole of CaM were fit to one of three different equations,

\[
F_1 = N_1 \frac{x^3 + K_{1}K_{2} + K_{1}K_{2}K_{3}}{1 + K_{1} + K_{2} + K_{3}}, \quad F_2 = N_2 \frac{x^3 + K_{1}K_{2}}{1 + K_{1} + K_{2}}, \quad Y = F_1 + F_2 \quad (\text{Eq. 1})
\]

\[
F_3 = N_1 \frac{x^2 + K_{1}K_{2}}{1 + K_{1} + K_{2}}, \quad F_4 = N_2 \frac{x^2 + K_{1}K_{2}}{1 + K_{1} + K_{2}}, \quad Y = F_3 + F_4 \quad (\text{Eq. 2})
\]

\[
Y = 2F_3 \quad (\text{Eq. 3})
\]

where \(Y\) is moles of Ca\(^{2+}\) ions bound per mole of CaM, \(x\) is the free [Ca\(^{2+}\)], and \(N_1\) and \(N_2\) are binding stoichiometries for the different classes of binding sites. \(F_1\) through \(F_4\) simply refer to different terms in the expressions describing fractional saturation with Ca\(^{2+}\): \(F_1\) is the term for cooperative binding to three sites, \(F_2\) the term for independent
binding to a single site, and F3 and F4 are the terms for cooperative binding to distinct pairs of Ca\(^{2+}\)-binding sites. Equation 1 was derived assuming cooperative binding of Ca\(^{2+}\) to three sites and independent binding to one site (Scheme 1); the others were derived assuming cooperative binding to two independent pairs of Ca\(^{2+}\)-binding sites that are kinetically distinct (Scheme 2) or kinetically indistinguishable (Scheme 3). It is assumed that the individual Ca\(^{2+}\)-binding sites within each EF hand pair are kinetically equivalent, except in the CaM-skPEP and CaM-skMLCK complexes, where one of the N-terminal EF hands is able to form cooperative interactions with the C-terminal lobe. Steps involving the association of CaM with peptide or enzyme are not treated explicitly in these schemes as only Ca\(^{2+}\) binding was monitored experimentally. Nonlinear least squares fits of equations to steady-state binding data were performed using the Prism (GraphPad, Inc.) software package.

Values for \(\Delta G\), the difference between the \(\Delta G\) value for Ca\(^{2+}\) binding to free CaM or CaMCC and to their respective enzyme and peptide complexes, were calculated according to the standard equation,

\[
\Delta G = RT - \sum \frac{\Delta G_i}{K_i}
\]

where the \(F\) superscript designates the dissociation constant for the \(i\)th Ca\(^{2+}\)-binding site in free CaM or CaMCC, and the \(B\) superscript designates the corresponding constant for the peptide or enzyme complex.

Presteady-state measurements of changes in quin-2 MF (Molecular Probes, Eugene, OR) fluorescence were performed using a Kintech (Austin, TX) stopped-flow fluorimeter with a dead time of \(0.5\) ms, and six or more individual stopped-flow time courses were combined to produce each mean fluorescence time course. Observations were obtained by fitting the mean data to standard single or double exponential rate equations using the Prism software package (GraphPad, Inc.). All stopped-flow measurements were performed at 25 °C.

For measurements of Ca\(^{2+}\) dissociation from native and mutant CaM-skPEP or CaM-skMLCK complexes, syringe A of the stopped flow apparatus contained 5 \(\mu\)M CaM, a 1.5-fold molar excess of enzyme or peptide, and 50 \(\mu\)M CaCl\(_2\). Enzyme or peptide was omitted for measurements of Ca\(^{2+}\) dissociation from mutant or native CaM alone. Syringe B contained 200 \(\mu\)M quin-2 MF. Both syringes also contained 150 mM NaCl and 20 mM MOPS, pH 7.0. In all cases reactions were initiated by mixing equal volumes from syringes A and B, so that the final concentrations of protein, CaCl\(_2\), and quin-2 MF were half their initial values.

Calibration of the photomultiplier voltage with respect to increases in Ca\(^{2+}\) was performed as described previously (16). quin-2 has been reported to bind Ca\(^{2+}\) ion with an association rate constant of \(7.5 \times 10^8\) M\(^{-1}\) s\(^{-1}\), so at the final concentration of 100 \(\mu\)M used in stopped-flow experiments the rate of association between Ca\(^{2+}\) and indicator would exceed any measurable rate for Ca\(^{2+}\) dissociation from a binding site in native or mutant CaM by at least a factor of 100 (22). Experiments to monitor reversal of CaM-dependent activation of skMLCK activity were performed at 25 °C using a pheumatically driven three-syringe mixing device built and operated as described by McCarthy et al. (23). Reactions containing 1 \(\mu\)M CaM, 50 \(\mu\)M skMLCK, and 100 \(\mu\)M peptide substrate were initiated by adding [\(\gamma\)-\(^{32}\)P]ATP (\(10^{-10}\) cpm/pmol; NEN Life Science Products) to a final concentration of 1 \(\mu\)M. The reaction buffer contained 50 mM MOPS, 150 mM NaCl, 1 mM dithiothreitol, 200 \(\mu\)M CaCl\(_2\), and 10 mM MgCl\(_2\), pH 7.0. After addition of EGTA to a final concentration of 5 mM, phosphorylation reactions were quenched at the indicated times by adding 1 volume of 150 mM phosphoric acid. Quenched samples were processed and analyzed to determine \(^{32}\)P incorporation into the peptide substrate as described previously (16).

**RESULTS**

As reported previously, 3 Ca\(^{2+}\) ions are released from the CaM-skPEP complex with a rate constant of 0.15 s\(^{-1}\), and 1 is released with a rate constant of 1.9 s\(^{-1}\); the corresponding values for the CaM-skMLCK complex are 1.6 and >1000 s\(^{-1}\) (Fig. 1; Table I). All 4 Ca\(^{2+}\) ions are released with a single apparent rate constant of 0.11 s\(^{-1}\) from the complex between skPEP and CaMCC, in which the N-terminal EF hand pair has been replaced by a copy of the C-terminal pair. In the CaMCC-skMLCK complex one pair of Ca\(^{2+}\)-binding sites releases 2 Ca\(^{2+}\) ions with a similar rate constant of 0.33 s\(^{-1}\), while the other releases 2 Ca\(^{2+}\) ions with a much faster rate constant of
rate constant for release from the other (Fig. 2; Table I). Studies in the CaMCC-skMLCK complex, but
unique determinants2 are not present in the C-terminal EF hand pair, since it has been replaced by a copy of the N-terminal pair in this CaM mutant (Table I). Furthermore, the CaM14C and CaM32C complexes with skPEP, respectively, follow 3 + 1 and 2+2 release mechanisms, suggesting that unique determinants in the N-terminal lobe are confined to EF hand I (Table I). In CaM32C, residues 9–42 (EF hand I) are replaced by a duplication of residues 82–115; in CaM14C, residues 43–75 (EF hand II) are replaced by a duplication of residues 116–148. Since Ca2+ release from the both the CaM-skPEP and CaM-skMLCK complexes follows a 3+1 kinetic mechanism, some of these experiments were conducted using only the peptide complex (Table I).

We have previously reported that the rate constants are similar for reversal of CaM-dependent kinase activation and for cooperative release of Ca2+ from three binding sites in the CaM-skMLCK complex (16). In this study we performed a similar analysis for CaMCC-dependent kinase activity, and determined a rate constant for reversal of 0.2 s$^{-1}$, similar to the rate constant for release of Ca2+ from one of the EF hand pairs in the CaMCC-skMLCK complex, but ~60-fold slower than the rate constant for release from the other (Fig. 2; Table I). Studies with tryptic fragments of CaM indicate that both lobes in CaMCC must be bound to produce activation of skMLCK activity, but the more rapid release of Ca2+ ion from one of the lobes in the complex appears to have no effect on its catalytic activity (24).

Equilibrium Ca2+ binding to the peptide and enzyme complexes with CaM, which exhibit a 3+1 Ca2+ release mechanism, and CaMCC, which exhibit a 2+2 release mechanism, was investigated using flow dialysis (Fig. 3). The results are completely consistent with the presteady-state kinetic data. Equilibrium data for the CaM complexes are fit by Equation 1, which is based on independent binding to one site and cooperative binding to three sites (Scheme 1). Mamar-Bachi and Cox (25) have also reported what appears to be cooperative binding to three Ca2+-binding sites in the complex between CaM and smooth muscle myosin light chain kinase. Data for the CaMCC-

Ca2+ Binding and Energy Coupling

TABLE I

<table>
<thead>
<tr>
<th>Ligand</th>
<th>skMLCK</th>
<th>skPEP</th>
<th>None</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca2+</td>
<td>1.6 (2.8)</td>
<td>&gt;1000 (1)</td>
<td>0.15 (2.8)</td>
</tr>
</tbody>
</table>
| CaMNN  | ND	extsuperscript{a} | ND       | 1.2 (3.1) | 5.3 (0.9) | >1000 (4) | —
| CaMCC  | 0.33 (2.3) | 12.5 (1.8) | 0.11 (4) | — | 16.6 (4) | — |
| CaM14C | ND       | ND       | 0.31 (3.2) | 4.3 (1) | 12.96 (2) | >1000 (2) |
| CaM32C | ND       | ND       | 0.8 (2.1) | 3.6 (1.9) | 10.8 (2) | 3.4 (2) |

	extsuperscript{a} Data for CaM have been published elsewhere (16).
	extsuperscript{b} ND, not determined.

	extsuperscript{c} Not applicable.

A unique functional determinant is defined here as any amino acid or group of amino acids in one CaM EF hand pair that cannot be functionally replaced by its structural analog in the other pair.

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Fig. 2. The rate for reversal of kinase activation in the CaMCC-skMLCK complex. Reactions containing 1 µM CaM, 50 nM skMLCK, and 100 µM peptide substrate were initiated by adding [γ-32P]ATP to a final concentration of 1 mM. After addition of EGTA to a final concentration of 3 mM at t = 0, phosphorylation reactions were quenched at the indicated times and analyzed to determine 32P incorporation. The dashed line indicates that time course for 32P incorporation in the absence of added chelator. A value of 0.2 s$^{-1}$ for the rate constant for reversal of enzyme activation was determined by a nonlinear least squares fit of the data to an integrated rate equation (16). Error bars represent the S.E. (n = 3).
FIG. 3. Steady-state Ca\(^{2+}\) binding to CaMCC and CaM alone and in their complexes with skMLCK or skPEP. Symbols: ○, CaM; □, CaMCC; ■, CaM-skMLCK; △, CaMCC-skMLCK; ▲, CaM-skPEP; and ∆, CaMCC-skPEP. The CaM or CaMCC concentrations used were between 5 and 10 μM, and a 1.2-fold molar excess of skPEP or skMLCK was added where indicated. Curves were generated by a least squares fit of the data to Equation 1 (CaM-skPEP, CaM-skMLCK), Equation 2 (CaM, CaMCC-skMLCK), or Equation 3 (CaMCC, CaMCC-skPEP).

TABLE II
Parameters for equilibrium binding of Ca\(^{2+}\) to complexes between CaM or CaMCC and skPEP or skMLCK

<table>
<thead>
<tr>
<th></th>
<th>skPEP</th>
<th>skMLCK</th>
<th>None&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(N)</td>
<td>3.0</td>
<td>1.0</td>
<td>3.0</td>
</tr>
<tr>
<td>(K)</td>
<td>0.22</td>
<td>1.0</td>
<td>29.5</td>
</tr>
<tr>
<td>(K)</td>
<td>0.05</td>
<td>1.0</td>
<td>29.5</td>
</tr>
<tr>
<td>(K)</td>
<td>0.10</td>
<td>1.0</td>
<td>29.5</td>
</tr>
<tr>
<td>(K)</td>
<td>0.18</td>
<td>1.0</td>
<td>29.5</td>
</tr>
<tr>
<td>(\Delta G)</td>
<td>8.9</td>
<td>2.2</td>
<td>4.7</td>
</tr>
</tbody>
</table>

| CaMCC          |            |        |                  |
| \(N\)          | 4          | 1.0    | 3.0              |
| \(K\)          | 0.17       | 1.0    | 29.5             |
| \(K\)          | 0.03       | 1.0    | 29.5             |
| \(K\)          | 0.18       | 1.0    | 29.5             |
| \(\Delta G\)   | 8.9        | 2.2    | 4.7              |

<sup>a</sup>These data have been published elsewhere (21).

DISCUSSION

We have found that a 3+1 Ca\(^{2+}\) release mechanism is retained in the CaMNN and CaM14C complexes with skPEP, but not in the CaMCC and CaM32C complexes. Since only EF hand I cannot be functionally replaced by its structural analog in the opposite EF hand pair, it appears to be the only one containing unique determinants for the Ca\(^{2+}\) release mechanism. This does not mean that it contains all determinants for the Ca\(^{2+}\) release mechanism, but residues in EF hand I that differ from those in analogous positions in EF hand III must include at least an important subset of determinants. If we consider that EF hand I is nearest to EF hands III and IV in the NMR structure for the CaM-skPEP complex (Fig. 4), the simplest interpretation of our results is that interactions between these three sites and the core CaM-binding sequence determine the release mechanism. However, if we map the locations of nonconserved positions in the sequence alignment of EF hands I and III to the structure for the CaM-skPEP complex it is evident that, except for Phe-12, none involve side chains in EF

FIG. 4. Localization of amino acid differences between EF hands I and III in the CaM-skPEP complex. Except for these amino acids, CaM and the peptide are represented by ribbon traces. The ribbon representing bound peptide is colored yellow. Dashed lines connect the positions of nonconserved side chains in a sequence alignment of EF hands I and III with their positions in EF hand I in the NMR structure for the CaM-skPEP complex (36). Bound Ca\(^{2+}\) ions are labeled according to the number of the EF hand they are associated with. Highlighted positions in the alignment indicate where there are differences in charge between the two sequences. The numbering shown above the alignment is for the amino acid sequence in EF hand I.
hand I directly interacting with the bound peptide or with the C-terminal CaM lobe (Fig. 4). There are several significant differences at amino acid positions in the connecting loop between EF hands I and II, suggesting that interactions between the EF hands may determine whether they can be induced to function independently (Fig. 4). On the other hand, it is possible that replacement of EF hand I by EF hand III, or of the N-terminal EF hand pair by the C-terminal pair, alters the complex in a way that simply cannot be deduced from the NMR structure for the native complex. Further structure-function studies will be required to resolve these possibilities.

We have previously reported that the rate constants are similar for reversal of CaM-dependent kinase activation and for the cooperative release of Ca$^{2+}$ from three binding sites in the CaM-skMLCK complex (16). In this study we found that the rate constant for reversal of CaMCC-dependent kinase activation is similar to the rate constant for Ca$^{2+}$ release from one lobe, most likely the C-terminal, and much slower than the rate constant for release from the other. Equilibrium binding and Ca$^{2+}$ release by the CaM and CaMCC complexes with enzyme or peptide are respectively described by $3\pm 1$ and $2\pm 2$ kinetic mechanisms. As we might expect, reversal of CaM- or CaMCC-dependent enzyme activation is associated with release of 3 or 2 Ca$^{2+}$ ions, respectively. These observations suggest that (Ca$^{2+}$)$_3$CaM-skMLCK and (Ca$^{2+}$)$_2$CaMCC-skMLCK are the minimal Ca$^{2+}$-liganded species exhibiting CaM-dependent kinase activity. This seems at odds with studies of the Ca$^{2+}$-dependence for enzyme activation suggesting that (Ca$^{2+}$)$_3$CaM is responsible (28). However, the degree of cooperativity seen in the Ca$^{2+}$ dependence of skMLCK activation is determined by the Ca$^{2+}$-liganded CaM species in equilibrium with the activated complex. Because of energy coupling effects on Ca$^{2+}$ binding these could easily differ from the species present in the complex itself, which are what determine its equilibrium Ca$^{2+}$-binding characteristics.

The striking difference in equilibrium Ca$^{2+}$ binding to the enzyme and peptide complexes we have observed was implied by our earlier studies showing that Ca$^{2+}$ is released much more slowly from the peptide complexes (Ref. 16; Table I). The $\Delta G$ values calculated for the peptide complexes with CaMCC and CaM are $-10.2$ and $-11.1$ kcal/mol, while the values calculated for the corresponding enzyme complexes are $-2.8$ and $-4.2$ kcal/mol. There are two possible explanations for this difference: 1) the peptide is bound much more tightly than the enzyme, and 2) a greater fraction of $\Delta G$ is generated by Ca$^{2+}$-independent interactions in the enzyme complex. Peptides based on the core CaM-binding sequences in skeletal and smooth muscle myosin light chain kinase have been reported to bind CaM with dissociation constants similar to those for the intact enzymes, so the second of these two possibilities must apply (19, 29). This means that interactions between CaM and the core CaM-binding sequence must also differ in the enzyme and peptide complexes, consistent with the slower Ca$^{2+}$ association rates derived for the latter. Site-directed mutagenesis studies have shown that amino acids in CaM that do not interact with skPEP in the NMR structure of the CaM-peptide complex play critical roles in the enzyme complex (21, 30, 31). A good example is our own finding that deletion of a small region in the N-terminal leader sequence in CaM abolishes its ability to activate skMLCK activity (31). Our results suggest that these secondary interactions somehow weaken the association between CaM and the core CaM-binding sequence, while at the same time preserving the overall affinity of the complex.

Given a dissociation constant of 1 nm, the $\Delta G$ value for the CaM-skMLCK complex is $-12$ kcal/mol. If we subtract the $-8$ kcal/mol $\Delta G$ value determined for this complex, we are left with $-8$ kcal/mol, which suggests a Ca$^{2+}$-independent $K_d$ value of $1.2 \mu M$ for the complex. However, literature values indicate that Ca$^{2+}$-free CaM binds skMLCK with a dissociation constant $\approx 25 \mu M$, so intermediates with greater Ca$^{2+}$ dependence must be involved in complex formation (25, 32, 33). Figure 5 presents a hypothetical three-step model for the association of CaM and skMLCK to illustrate how this might occur. In the model, binding of the N- and C-terminal CaM lobes to the enzyme occurs sequentially in steps 1 and 2, consistent with our earlier studies with CaM fragments (24). Exposure of the Trp side chain in the core sequence is depicted based on the crystal structure for CaM-dependent kinase I (11). If we assume that the core sequence is loosely associated with the enzyme, consistent with its susceptibility to proteolysis, then steps 1 and 2 would be expected to approximate interactions with skPEP, accounting for the Ca$^{2+}$ dependence of complex formation. Significant Ca$^{2+}$-independent secondary interactions are not formed until step 3, and they involve residues in the enzyme that are not exposed until CaM is bound. This is consistent with structural modeling and biochemical data, indicating that a stretch of amino acids flanking the core CaM-binding sequence is pulled out of a groove in the surface of the enzyme when CaM is bound (12). In support of the type of structural change depicted in step 3, recent small angle x-ray scattering studies published by Krueger et al. (15) suggest that in the CaM-skMLCK complex CaM, in association with the core CaM-binding sequence, is held away from the catalytic site (34, 35).

The apparent reduction in energy coupling seen in the CaM-skMLCK complex is physiologically important. If the enzyme complex had the same dissociation constants for Ca$^{2+}$ ions as the peptide complex, it would be $-50\%$ saturated at a typical resting free Ca$^{2+}$ concentration of $0.1 \mu M$ (Fig. 3). However, the reduced level of energy coupling in the enzyme complex produces Ca$^{2+}$-binding affinities that are tuned to the normal range of values for the free Ca$^{2+}$ concentration: below a value of $0.2 \mu M$ no Ca$^{2+}$ is bound to the complex, while at a free Ca$^{2+}$ concentration of $1 \mu M$, formation of the active (Ca$^{2+}$)$_2$CaM-skMLCK complex is half-maximal (Fig. 3). Since Ca$^{2+}$ binding to only one N-terminal EF hand is enhanced in the complex, formation of (Ca$^{2+}$)$_2$CaMCC-skMLCK is expected to occur only at relatively high free Ca$^{2+}$ concentrations, but binding of Ca$^{2+}$ to the lower affinity sites seems not to have a significant effect on the catalytic activity of the complex (16).

Energy coupling effects in the two CaM lobes are distinct, and this is most apparent in the CaMCC complexes. The $\Delta G$ value for both EF hand pairs in the CaMCC-skPEP complex is $-5.1$ kcal/mol, but in the CaMCC-skMLCK complex one pair has a value of $-2.6$ kcal/mol and the other a value of $-0.2$ kcal/mol.
kcal/mol. The EF hand pair with the more negative $\Delta \Delta G$ value is almost certainly in the C-terminal lobe since its Ca$^{2+}$ release and steady-state binding properties in the complexes with skPEP or skMLCK are similar to those for the C-terminal lobe in CaM (Tables I and II). Thus, interaction of skMLCK with the skPEP or skMLCK are similar to those for the C-terminal lobe and steady-state binding properties in the complexes with CaM-skMLCK complexes, as well as for the analogous smooth muscle kinase complexes, raises an important question: what is the source of the energy required to move the enzyme into its catalytically active conformation? In the simplest case, where binding to the core CaM-binding sequence and activation are directly coupled, we would expect the $\Delta G$ value for the enzyme complex to be reduced relative to the value for the peptide complex, which also would result in a higher $K_d$ value. However, evidence indicating a requirement for secondary interactions in the enzyme complex suggest that enzyme activation and binding to the core sequence are not directly coupled. The studies presented here further suggest that enzyme activation is, in effect, driven by a transfer of binding energy from predominantly Ca$^{2+}$-dependent interactions with the core CaM-binding sequence to predominantly Ca$^{2+}$-independent secondary interactions.

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doi: 10.1074/jbc.275.6.4199

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