Use of Site-directed Mutations in the Individual Ca\textsuperscript{2+}-Binding Sites of Calmodulin to Examine Ca\textsuperscript{2+}-induced Conformational Changes*  

(Received for publication, December 18, 1990)  
Kathy Beckingham  
From the Department of Biochemistry and Cell Biology, Rice University, Houston, Texas 77251

Mutant versions of the calmodulin of *Drosophila melanogaster* have been prepared for use in the study of Ca\textsuperscript{2+} binding and Ca\textsuperscript{2+}-induced conformational changes. In each mutant, a conserved glutamic acid residue indicated to play a critical role in Ca\textsuperscript{2+} binding has been mutated to glutamine in one of the Ca\textsuperscript{2+}-binding sites. Thus a series of four proteins, each with an analogous mutation in one of the four binding sites, has been generated. Here the Ca\textsuperscript{2+}-induced conformational changes in these proteins have been examined by use of the fluorescent hydrophobic reporter molecule, 9-anthroyl choline. These studies confirm earlier work which indicates that the carboxyl-terminal pair of Ca\textsuperscript{2+}-binding sites shows cooperative Ca\textsuperscript{2+} binding to produce a major conformational change in the protein. However, these studies provide evidence that the sites of the amino-terminal pair are more independent in their Ca\textsuperscript{2+} binding properties and contribute individually to the conformational changes associated with Ca\textsuperscript{2+} binding in the amino-terminal half of the protein. This work also indicates that mutation of either of the amino-terminal Ca\textsuperscript{2+}-binding sites can influence the conformational change produced by Ca\textsuperscript{2+} binding to the carboxyl-terminal sites.

Calmodulin is one of the major mediators of Ca\textsuperscript{2+}-induced intracellular regulation in eukaryotes. Calmodulin contains four Ca\textsuperscript{2+}-binding sites of the "EF-hand" type (1), and crystallographic studies (2, 3) have revealed that in the Ca\textsuperscript{2+}-saturated form, the amino- and carboxyl-terminal pairs of binding sites form discrete globular domains which are separated by a long central α-helix. It has long been recognized that Ca\textsuperscript{2+} occupation of the four binding sites produces the conformational changes which permit calmodulin to activate its target proteins. However, conflicting theories as to the contribution of the individual binding sites to these conformational changes have been reported. Data largely from NMR and stopped flow studies provide strong evidence that the two COOH-terminal sites are high affinity sites showing cooperative Ca\textsuperscript{2+} binding which results in a major conformational change in the protein (reviewed in Ref. 4). These methods have not permitted a detailed characterization of Ca\textsuperscript{2+} binding and related structural changes at the NH\textsubscript{2}-terminal sites, however. A completely different view of the Ca\textsuperscript{2+} binding events has been forwarded by Cox and co-workers (5, 6) who argue that the Ca\textsuperscript{2+} binding data for calmodulin support equally well the idea of four independent sites of identical affinity. Under this view the correspondence of a major conformational change in calmodulin with binding of two Ca\textsuperscript{2+} ions is the result of Ca\textsuperscript{2+} binding to any two sites on the protein as opposed to the COOH-terminal pair.

Working with the calmodulin of *Drosophila melanogaster* (7), we have generated a series of site-directed mutants for study of the roles of the individual Ca\textsuperscript{2+}-binding sites of calmodulin in its biological functions. In each of these mutants a conserved glutamic acid residue, which is analogously positioned in all four Ca\textsuperscript{2+}-binding sites, has been mutated in one individual site. A critical role of this glutamic acid residue in Ca\textsuperscript{2+} binding is indicated by the details of the Ca\textsuperscript{2+} coordination system as revealed by crystallographic studies of both mammalian (3) and *Drosophila* calmodulin.1 Uniquely among the six amino acids in each site which provide ligands for Ca\textsuperscript{2+}, this glutamic acid residue provides two ligands for the ion. These are the two carboxylate oxygens of the side chain. In addition, this glutamic acid residue which occupies the "lip edge" of the Ca\textsuperscript{2+}-binding site may be perceived as locking the Ca\textsuperscript{2+} ion into the binding site. Fig. 1 illustrates the role of this glutamic residue in site 1 of the *Drosophila* protein.

In the mutant series used here, glutamine is the replacement amino acid for this glutamic acid residue. This substitution was predicted to hinder Ca\textsuperscript{2+} binding since one of the coordinating oxygens has been lost. However, given the similar properties of glutamic acid and glutamine with respect to hydrophilicity and α-helical propensity (9), this mutational change was expected to generate minimal overall disturbance to protein structure. The two mutants of this series are called: B1:E31Q, B2:E67Q, B3:E104Q, B4:E140Q. These names denote both the exact amino acid and the binding site (B1, B2, etc.) involved. For brevity's sake they are referred to as the B1Q, B2Q, B3Q, and B4Q mutants.

A study of the Ca\textsuperscript{2+}-induced conformational changes in this mutant series is reported here. Conformational change has been monitored by use of the hydrophobic reporter molecule, 9-anthroyl choline (9AC),2 The fluorescence of 9AC is enhanced upon binding to hydrophobic regions in proteins. A strong fluorescence enhancement for 9AC (10) and several other probes (10, 11) is seen upon addition of Ca\textsuperscript{2+} to calmodulin, providing evidence that the Ca\textsuperscript{2+}-induced changes result in the exposure of hydrophobic surfaces on the protein. Here, conformational changes as free Ca\textsuperscript{2+} levels are increased have been monitored by measuring fluorescence enhancement for 9AC during Ca\textsuperscript{2+} titration of the proteins.

1 J. S. Sack, D. A. Taylor, J. F. Maune, K. Beckingham, and F. A. Quiocio, manuscript in preparation.
2 The abbreviations used are: 9AC, 9-anthroyl choline; EGTA, [ethylenebis(oxyethylenenitrilo)tetraacetic acid; MOPS, 4-morpholinepropanesulfonic acid.
Conformational Change in Calmodulin Ca2+-binding Site Mutants

MATERIALS AND METHODS

Generation of Mutant and Wild-type Calmodulin Proteins—The preparation of an expression vector for wild-type Drosophila calmodulin and the generation of the site-directed mutants are described elsewhere. Proteins were purified using a modification of the procedure of Putkey et al. (12) as described elsewhere and stored at -70 °C. 

Preparation of Decalcified Protein Samples—Proteins were decalcified by passage over a column of Chelex resin (Bio-Rad) that had been washed exhaustively with (i) distilled water and (ii) titration buffer. Fractions containing protein were pooled.

Fluorimetric Titrations—Samples containing the decalcified proteins (50 μM) and 9-anthroyl choline (20 μM) in titration buffer were prepared. A 2-ml aliquot of each sample was transferred to a quartz cuvette, and fluorescence measurements were made as sequential additions of 1-μl aliquots of CaCl2 solutions (varying in concentration from 5 to 100 mM) were added. These CaCl2 solutions were prepared from a commercial 100 mM standard (Orion Research). An SLM fluorimeter (model 4800) was used with excitation at 366 nm and measurement of emission above 418 nm. The fluorimeter was calibrated using a sample of the wild-type protein prepared as for analysis but containing in addition 1 mM EGTA. The pH of the samples was determined at the end of the titrations. In some cases pH was checked both before and after titration to ensure that pH was stable throughout titration. Free Ca2+ concentrations for the titrations were determined using computer programs kindly donated by Dr. W. Dubinsky and Dr. J. P. Thiery. These programs use the dissociation constants for EGTA determined by Fabiato and Fabiato.

RESULTS

In most experiments, fluorescence titrations for the wild-type protein and two mutant proteins were performed. Fig. 2 shows the results of three typical experiments which demonstrate the behavior of all four mutants relative to the wild type. As can be seen, the fluorescence enhancement for the wild-type protein is clearly biphasic with midpoints for the two transitions, as determined from their inflection points, occurring at free Ca2+ concentrations of ~3.1 × 10^-7 M and ~3.7 × 10^-8 M (see Table I). Mutation of either of the COOH-terminal Ca2+-binding sites (mutants B3Q and B4Q) results in the loss of most of the first component of the fluorescence curve seen for the wild-type protein (Fig. 2, A and B). In these mutants, however, the second phase of the wild-type curve is essentially unchanged both in terms of magnitude and the Ca2+ concentration at which the transition midpoint occurs (Fig. 2, A and B, and Table I).

The behavior of the proteins mutated in the binding sites of the NH2-terminal domain (B1Q and B2Q) is distinctly different. In each of these proteins the first phase of the fluorescence enhancement seen for the wild-type protein is only slightly affected (see Fig. 2, A and C). Thus the midpoint for this transition is unaltered (see Table I), but the fluorescence enhancement is decreased somewhat (see Fig. 2, A and C). The major effect of these mutants is on the second phase of the fluorescent enhancement curve. As shown in Fig. 2, A and C, each of these mutants eliminates a component of this second phase rather than the entire transition. For both the B1Q and the B2Q mutants, the midpoint for the residual transition is the same as that seen for the entire transition in the wild-type protein (see Table I).

DISCUSSION

The simplest interpretation of the data shown here for the wild-type calmodulin is that the two phases of the fluorescence enhancement reflect conformational changes in the protein in response to Ca2+ binding at two distinct classes of sites. The dissociation constants for Ca2+ binding at these sites are given by the inflection points for the two phases of the curve. The dissociation constant thus obtained for the binding event inducing the second phase of the conformational change (~3.7 × 10^-7 M) is similar to those reported previously for the lower affinity binding sites of calmodulin. However, the dissociation constant (~3.1 × 10^-7 M) for the change at lower Ca2+ concentrations is an order of magnitude lower than those typically reported for Ca2+ binding to the higher affinity sites of calmodulin. This could reflect an effect of 9AC on Ca2+ binding to calmodulin. It has been shown previously that the binding of hydrophobic drugs (17, 18) including 1-anilino-8-naphthalene sulfonate (17) which, like 9AC, shows fluorescence enhancement upon binding to calmodulin.

If the two phases of the wild-type curve are interpreted as...
suggested above, a major inference of these data is that, as previously proposed, the binding sites of the COOH-terminal domain are the high affinity Ca\textsuperscript{2+}-binding sites and the NH\textsubscript{2}-terminal sites are the lower affinity sites. Thus each of the mutations in the COOH-terminal site affects the conformational change seen at low Ca\textsuperscript{2+} concentrations, and each of the mutations in the NH\textsubscript{2}-terminal sites primarily affects the conformational change seen at higher levels of Ca\textsuperscript{2+}.

In proposing that the four Ca\textsuperscript{2+}-binding sites of calmodulin have identical affinities for Ca\textsuperscript{2+}, Burger et al. (5) argued that the structural changes in calmodulin appear sequential and ordered because they follow the appearance of one particular calmodulin-Ca\textsuperscript{2+} species (for example, calmodulin with two Ca\textsuperscript{2+} bound) rather than the binding of Ca\textsuperscript{2+} to particular sites on the protein. This hypothesis is hard to reconcile with the data presented here. In the mutant calmodulins used in this study, an exactly analogous mutation has been made in each of the binding sites of calmodulin in turn. Thus, if the ligand binding properties of the four sites are identical in the native protein, this mutation should alter Ca\textsuperscript{2+} binding at each site in exactly the same manner. As a result, the conformational changes resulting from Ca\textsuperscript{2+} binding should be altered identically by each mutant of this series. This is clearly not the case.

Previous studies have indicated that Ca\textsuperscript{2+} binding at the two pairs of sites within the two halves of the molecule is cooperative in character (16). The data presented here support this possibility for Ca\textsuperscript{2+} binding to the two COOH-terminal sites. Mutation of either site prevents almost entirely the conformational change occurring at low Ca\textsuperscript{2+} concentration. This must mean that Ca\textsuperscript{2+} binding at both of these sites is required for this conformational change and suggests both binding events can be prevented by mutation of either binding site.

Interestingly the data for mutants B1Q and B2Q present a different view of Ca\textsuperscript{2+} binding at sites 1 and 2. Mutation of either of these sites eliminates only a component of the second phase of the conformational change seen with the wild-type protein. This indicates that under the conditions used here, sites 1 and 2 show more or less independent Ca\textsuperscript{2+} binding with each binding event contributing in an additive manner to the total conformational change produced by binding in this domain of the protein. Studies with synthetic peptides representing individual EF-hands have reinforced the idea that a single EF-hand cannot execute tight Ca\textsuperscript{2+} binding (19). Thus a peptide representing the third Ca\textsuperscript{2+}-binding site of calmodulin has a very poor affinity for Ca\textsuperscript{2+} (K\textsubscript{D} = 878 \mu M). The third Ca\textsuperscript{2+}-binding site of skeletal troponin C as a synthetic peptide has a much higher Ca\textsuperscript{2+} binding affinity (K\textsubscript{D} = 28 \mu M), but NMR studies now indicate that this peptide dimerizes to form a pair of EF-hands in the presence of Ca\textsuperscript{2+} (20). No studies of the Ca\textsuperscript{2+} binding properties of the NH\textsubscript{2}-terminal sites of either calmodulin or troponin C as individual peptides have been performed so the behavior of these sites in isolation cannot be directly addressed. The structure of cardiac troponin C, however, leaves no doubt that at least in the context of the entire protein molecule, the individual EF-hand binding sites of its NH\textsubscript{2}-terminal pair can act independently of another, both in terms of Ca\textsuperscript{2+} binding and generation of conformational change (21).

This indication that the NH\textsubscript{2}-terminal binding sites of calmodulin can act independently to produce different components of the Ca\textsuperscript{2+}-induced conformational change is reinforced by proton NMR and enzyme activation studies with
this mutant series.\textsuperscript{4,5} Studies of the activation of skeletal myosin light chain kinase, for instance, indicate that the conformations of the binding site 1 and 2 mutants in the presence of excess Ca\textsuperscript{2+} are distinctly different since their capacities to activate the enzyme are very different. If all Ca\textsuperscript{2+} binding to the NH\textsubscript{2}-terminal domains (and hence all the resultant conformational change) were eliminated by each of these mutants, they would be expected to have similar activation capacities for this enzyme. In addition to addressing the interactions between the paired Ca\textsuperscript{2+}-binding sites in each half of calmodulin, the data presented here provide information concerning the possibility of interaction between the two halves of the molecule. The second phase of the fluorescent enhancement seen for the wild-type protein (which appears to result from Ca\textsuperscript{2+} binding to sites 1 and 2) is essentially unaffected in the COOH-terminal mutants. Thus under the conditions used here, it would appear that elimination of Ca\textsuperscript{2+} binding at sites 3 and 4 has little effect on the NH\textsubscript{2}-terminal domain. In contrast, mutation of the NH\textsubscript{2}-terminal sites does appear to affect the conformational change initiated by Ca\textsuperscript{2+} binding to sites 3 and 4. That the B1Q and B2Q mutants alter the structural change resulting from Ca\textsuperscript{2+} binding to the COOH-terminal domain, not Ca\textsuperscript{2+} binding itself, is indicated by the fact that the first phase of the fluorescent enhancement for these mutants is decreased in intensity but the midpoint for the transition is unchanged (see Table I). In order for the NH\textsubscript{2}-terminal mutations to affect the conformational change induced by Ca\textsuperscript{2+} binding at sites 3 and 4 it is necessary to hypothesize that these mutations have structural consequences for the protein that are unrelated to Ca\textsuperscript{2+} binding at these sites.

Acknowledgments—I thank Dai-rong Su and John F. Maune for protein preparations, Dr. Jonathan S. Sack for the preparation of Fig. 1, and Drs. John S. Olson and R. Bruce Weissman for helpful discussions. The generosity of Dr. Florante A. Quiocio in allowing me use of his fluorimetry facilities is gratefully acknowledged.

REFERENCES
1. Kretsinger, R. H., and Nockolds, C. E. (1973) J. Biol. Chem. 248, 3313–3320
2. Babu, Y. S., Sack, J. S., Greenough, T. J., Bugg, C. E., Means, A. R., and Cook, W. J. (1988) Nature 315, 37–40
3. Babu, Y. S., Bugg, C. E., and Cook, W. J. (1988) J. Mol. Biol. 204, 191–204
4. Forsén, S., Vogel, H. J., and Drakenburg, T. (1986) in Calcium and Cell Function (Cheung, W. Y., ed) Vol. 6, pp. 113–157, Academic Press, New York
5. Burger, D., Cox, J. A., Comte, M., and Stein, E. A. (1984) Biochemistry 23, 1966–1971
6. Cox, J. A. (1988) Biochem. J. 249, 621–629
7. Smith, V. L., Doyle, K. E., Maune, J. F., Munjaal, R. P., and Beckingham, K. (1987) J. Mol. Biol. 196, 471–485
8. Yamanaka, M. K., Saugstad, J. A., Hanson-Pointon, O., McCarthy, B. J., and Tobin, S. L. (1987) Nucleic Acids Res. 8, 3335–3348
9. Schulz, G. E., and Schirmer, R. H. (1979) Principles of Protein Structure, pp. 108–112, Springer-Verlag, New York
10. LaPorte, D., C., Wierman, B. M., and Storm, D. R. (1980) Biochemistry 19, 3814–3819
11. Tanaka, T., and Hidaka, H. (1980) J. Biol. Chem. 255, 11078–11080
12. Putkey, J. A., Slaughter, G. R., and Means, A. R. (1985) J. Biol. Chem. 260, 4704–4712
13. Toda, H., Yazawa, M., Kondo, K., Honma, T., Narita, K., and Yagi, K. (1981) J. Biochem. (Tokyo) 90, 1493–1505
14. Yazawa, M., Sakuma, M., and Yagi, K. (1980) J. Biochem. (Tokyo) 87, 1313–1320
15. Fabiato, A., and Fabiato, F. (1975) J. Physiol. (Lond.) 249, 497–517
16. Klei, C. B. (1988) in Calmodulin (Cohen, P., and Klei, C. B., eds) Vol. 5, pp. 35–56, Elsevier Science Publishing Co., Inc., New York
17. Keller, C. H., Olwin, B. B., Heideman, W., and Strom, D. R. (1982) in Calcium and Cell Function (Cheung, W. Y., ed) Vol. 3, pp. 103–127, Academic Press, New York
18. Inagaki, M., Tanaki, T., and Hidaka, H. (1983) Pharmacology 27, 125–129
19. Reid, R. E. (1987) Biochemistry 26, 6070–6073
20. Shaw, G. S., Hodges, R. S., and Sykes, B. D. (1990) Science 249, 280–283
21. Putkey, J. A., Sweeney, H. L., and Campbell, S. T. (1989) J. Biol. Chem. 264, 12370–12379
22. Gariépy, J., and Hodges, R. S. (1983) FEBS Lett. 160, 1–6