Synthetic Fragments of Calmodulin Calcium-binding Site III

A TEST OF THE ACID PAIR HYPOTHESIS*

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The acid pair hypothesis describing the interaction of calcium with the helix-loop-helix conformation of EF hands in calmodulin and related proteins predicts that these calcium-binding sites will have increased affinity for calcium if the anionic amino acid dentates in the loop region which interact directly with the cation are paired on the axial vertices of the resulting octahedral arrangement of chelating residues about the cation. As a test of this hypothesis, synthetic 33 residue analogs of bovine brain calmodulin calcium-binding site III have been prepared by the solid-phase method and analyzed for calcium affinity. The native sequence has a $K_d$ of 735 nM for calcium and contains three anionic ligands which assume the $+x$, $+y$, and $-z$ coordinates of the octahedral arrangement about the cation, thus precluding any pairing of the anionic ligands. This dissociation constant is 26 times weaker than that obtained from a synthetic analog of the sequentially homologous calcium-binding site III of rabbit skeletal TnC ($K_d = 28 \mu M$) which has four anionic ligands paired on the $x$ and $z$ axes. An analog of calmodulin site III with substitutions in the chelating residues at positions 1, 3, 5, 7, 9, and 12 of the 12-residue loop region to make these positions identical to those of rabbit skeletal troponin C site III decreased the calcium dissociation constant of the calmodulin peptide to 19 $\mu M$, similar to the troponin C peptide. Two synthetic analogs of calmodulin site III which contain three anionic ligands with two ligands paired on the $x$ axis and two on the $z$ axis have a $K_d$ for calcium of 524 and 59 $\mu M$, respectively. This study provides strong support for and a better definition of the acid pair hypothesis and further demonstrates the usefulness of synthetic calcium-binding fragments in delineating the mechanism of calcium regulation of calmodulin and related proteins.

Crystal structures of the major calcium-binding regulatory proteins (1-4) have made significant contributions to the knowledge of the events at the molecular level which lead to calcium regulation of biochemical systems. In spite of this abundance of data at the molecular level, several questions remain unanswered. The sequence and structural similarities of calcium-binding sites in the majority of these proteins along with the fact that several orders of magnitude separate the calcium affinity of some of these sites, both between different proteins and within the same protein, invites such questions as: What are the molecular features defining calcium affinity in these proteins? What are the molecular changes resulting from calcium binding which lead to regulation of other biochemical events?

Tufty and Kretsinger (5) noticed the helix-loop-helix conformation of the calcium-binding units in these proteins which he termed the "EF hand." Studies on calcium-induced conformational change in these proteins led to the concept of calcium-induced $\alpha$-helix formation as the first step in the translation of information carried by cellular calcium to biochemical activity (6). The demonstration of high and low affinity calcium sites in rabbit skeletal troponin C (STnC) by Potter and Gergely (7) provided a link between calcium-induced structural change and intracellular levels of calcium. The amphiphilic nature of the $\alpha$-helices induced by calcium led to further refinement of the concept of information transfer and resulted in hypothetical molecular explanations for a variety of calcium-induced interactions between the calcium-binding proteins and other molecules (6, 8).

The fact that the calcium-binding units are a linear sequence of 30-36 amino acids coupled with the fact that the sequences are highly homologous led to the examination of amino acid sequences in search of a sequence factor involved in the description of calcium binding and subsequent events. Vogt and colleagues (9) first noticed a high correlation of $\beta$ turn propensity with the occurrence of a calcium-binding loop region using the $\beta$-turn forming residue probabilities of Lewis et al. (10). The high propensity consisted of two overlapping tetrapeptides in the first 6 residues of the 12-residue loop region of the calcium-binding unit.

An attempt to correlate the amino acid sequence of the loop region of the helix-loop-helix calcium-binding unit with affinity for calcium in an effort to isolate features of the amino acid sequence which may be responsible for high and low calcium affinity recognized two important features of the loop sequence (11). The first concerned the number and arrangement of acidic side chains located in chelating positions 1, 3, 5, 7, 9, and 12 of the 12-residue loop. It was suggested that high affinity sites would have four acidic side chains in chelating positions of the loop, paired on the vertices of the regular octahedral arrangement of liganding atoms. This later became known as the acid pair hypothesis (12, 13). The second feature concerned the hydrophobic nature of the residue in the $-y$ coordinate of the octahedral arrangement of liganding atoms. It was suggested that the sites with high affinity for calcium affinity described in the text.

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* The abbreviations used are: STnC, rabbit skeletal troponin C; Tn, Troponin; Ac, acetyl; CaM, calmodulin; Ag$^{8+}$STnC, Ac-$^8$STnC(90-123)amide; L$^{10+}$CaM, Ac-L$^{10+}$CaM(81-113)amide; N[(X3CaM, Ac-N[(X3CaM(81-113)amide; NDL/3XCaM, Ac-N[DL/3XCaM(81-113)amide; NDL/3XCaM, Ac-N[DL/3XCaM(81-113)amide; HPLC, high pressure liquid chromatography; NTA, nitrilotriacetic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; TPE, trifluoroethanol; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

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Acid Pair Hypothesis

Circular Dichroism Spectroscopy—Calcium interaction with the synthetic peptides was examined using CD spectroscopy on a Jasco J500 CD spectrophotometer as previously described (22).

Free calcium concentrations were determined using a modified Perrin and Sayce computer program (23) with log of the association constant (24) for the differing species of nitrolotriacetic acid (NTA) oct.: H⁺ to NTA⁻, 0.79; H⁺ to HNTA⁻, 2.40; H⁺ to H₂NTA⁻, 1.89; Ca²⁺ to NTA⁻, 6.50. To correct the free metal concentration for peptide-bound calcium, it is assumed that 1 mol of calcium is bound/mole of peptide, and an estimate of this association constant is inserted into the modified Perrin and Sayce program for two ligands. Free calcium concentrations are calculated, and these values are used to determine an association constant for calcium as described below. This value is then used to revise the estimated association constant in the modified Perrin and Sayce program. The procedure is repeated until the calculated association constant equals that inserted in the Perrin and Sayce program. The H⁺ to peptide logₐ association constant was set at 4.00 to correspond roughly to the pKa of the acid side chains involved in calcium chelation.

The association constant of peptide for calcium was calculated using a nonlinear regression computer program which fit the CD calcium titration data to an equation of the form:

\[
f = \frac{K_a[Ca^{2+}] \cdot [l] + K_c[Ca^{2+}]}{[l] + K_c[Ca^{2+}]}
\]

where \(f\) is the fraction of peptide molecules in the calcium-chelated state and is determined as the ratio of the change in ellipticity at 222 nm to the maximum change in ellipticity which can be elicited by calcium at 222 nm. \(K_a\) is the apparent association constant of the peptide for calcium and \([Ca^{2+}]\) is the concentration of free calcium calculated as described above. This calculation assumes that all activities are equal to concentrations and that the peptide has a single independent calcium-binding site.

It is acknowledged that the deviations seen in the calcium-binding curves at high and low calcium concentrations may suggest some degree of heterogeneity in the binding process. However, the fact that the calcium-induced UV difference spectrum of one of the peptides (A⁎StrNC) (22) is blue-shifted, which may indicate the movement of tyrosine into a more hydrophilic environment plus the fact that all the calcium titration data fit the theoretical curve of a single independent calcium-binding site with correlation coefficients greater than 0.996, are taken to indicate that calcium-induced aggregation of the peptides is unlikely in these experiments.

A previous study (25) has shown the \(\alpha\)-helix content obtained from \([\Omega]_{222}\) for a pure \(\alpha\)-helix with an added refinement allowing for differences in the lengths of helical segments (26) can be used to determine the calcium-induced \(\alpha\)-helix content for a fragment of CaM in the presence of a change in difference between these two values is in good agreement with the value derived from the CD difference spectrum by the curve fitting procedure. Hence, this simplified procedure was adopted to estimate the helical content of the synthetic peptides. The fraction of \(\alpha\)-helix (\(f_\alpha\)) present was calculated as follows:

\[
f_\alpha = \frac{[\Omega]_{222}([\Omega]_\alpha - k/n)}{[\Omega]_{222}}
\]

where \([\Omega]_\alpha\) and \(k\) are calculated constants (26) which are 39,560 and 2.57, respectively, and \(n\) is the average helical length taken as 9 in these cases (22, 25).

The CD studies were carried out using a 100 mM MOPS, pH 7.2, 150 mM KCl, 1 mM NTA buffer and a 3:1 (v/v) mixture of trifluoroethanol with this buffer. The calcium-free solutions were prepared from demineralized water and Nalgene laboratory equipment was used in place of glassware to avoid contamination of solutions with calcium leached from glass. The concentration of the calcium solutions used in the titrations were determined by titration with EGTA as the primary standard using murexide as indicator. The peptides were quantitated from amino acid analysis of an aliquot of the solutions taken prior to the assay.

RESULTS AND DISCUSSION

The acid pair hypothesis relates calcium affinity to chemical properties of the amino acid residues in positions 1, 3, 5, 7, 9, and 12 of the loop region in the helix-loop-helix calcium-binding unit. Previous studies on synthetic calcium-binding units from rabbit skeletal TnC (13) and bovine brain calmodulin (12) indicated a 26-fold difference in calcium affinity. In order to determine if this large difference in calcium affinity
could be attributed to the above residues in the loop region, a hybrid of the two peptides was prepared and examined for calcium affinity. The hybrid (NDL/4XZCaM) differed from the calmodulin peptide (L\(^{109}\)CaM) in positions 3 (Asp changed to Asn), 5 (Asn changed to Asp), and 9 (Ser changed to Asp) (Fig. 1). These changes produced a loop region identical to the rabbit skeletal TnC peptide (A\(^{88}\)STnC) in positions 1, 3, 5, 7, 9, and 12 (Fig. 1). This hybrid has an affinity for calcium (K\(_d = 19\) \(\mu M\)) similar to that of the A\(^{88}\)STnC peptide (K\(_d = 28\) \(\mu M\)) and 39-fold greater than the L\(^{109}\)CaM peptide (K\(_d = 735\) \(\mu M\)) (Table I and Fig. 2).

It is possible that the difference in calcium affinity (Fig. 3) as well as the differences in structure (Table I and Fig. 2) between the apo-, calcium saturated, and TPE calmodulin analogs (NDL/4XZ and L\(^{109}\)CaM) may be due to the difference in the number of anionic chelating amino acid residues and not the location of the acidic residues in the loop region. This problem is addressed through the synthesis of two peptide analogs of calmodulin which have 3 acidic residues in chelating positions of the loop region (Fig. 1; NDL/3XCaM and NDL/3ZCaM).

NDL/3XCaM was prepared by changing Asp\(^ {95}\) of L\(^{109}\)CaM to Asn and Ser\(^ {101}\) to Asp. The NDL/3XCaM peptide has 3 acidic residues in chelating positions with one acid pair on the x axis (Fig. 1). The calcium affinity of this peptide is 524 \(\mu M\) which is slightly better than the natural L\(^{109}\)CaM sequence (K\(_d = 755\) \(\mu M\)). It would appear that the cation affinity is not greatly affected by pairing the acid residues on the x axis, and if we compare the affinity of NDL/3XCaM (K\(_d = 524\) \(\mu M\)) with NDL/4XZCaM (K\(_d = 19\) \(\mu M\)) we find that the latter has 28-fold greater affinity for calcium than the former (Fig. 3, Table I). This suggests that the high calcium affinity of NDL/4XZCaM is due to the number of acidic residues in chelating positions as opposed to positioning of the acidic residues on x and z axes. However, if we compare the calcium affinity of L\(^{109}\)CaM and NDL/4XZCaM with the NDL/3ZCaM peptide (K\(_d = 59\) \(\mu M\)), we find that the calcium affinity of NDL/3ZCaM to be 12-fold higher than the natural L\(^{109}\)CaM sequence and only three times less than the NDL/4XZCaM peptide (Fig. 3; Table I). The NDL/3ZCaM sequence is also provided the -x position is occupied by a charged aspartic acid residue or uncharged polar residue. The x axis acid pair would explain the high affinity of both calcium sites in the helix-loop-helix unit would require four acidic residues paired on the x and z coordinate axes of the tetrahedral arrangement of chelating ligands. It would now appear that high affinity can be had by helix-loop-helix units with 3 acid residues provided 2 of the residues are paired on either an x or z coordinate axis. Under these circumstances the -x dentate will not be mediated by a water molecule and the

**Acid Pair Hypothesis**

The majority of crystal structures of calcium-binding proteins with an acid pair on the x axis indicate that the -x coordinating residue interaction with the cation is mediated by a water molecule, and there are not two negative charges interacting with the cation to stabilize the complex but one negative charge from the +x coordinating residue and a water molecule which is expected to be a less efficient interaction due to the incomplete dehydration of the cation. Such an inefficient interaction of the acid pair with the cation may lead to a repulsive interaction during the cation-binding process between the +x acidic residue and other acidic residues located in any of the remaining five chelating positions. This would explain the high affinity of both calcium sites in the parvalbumin calcium-binding proteins. The crystal structure of parvalbumin (4) indicates that a water molecule is not involved in the -x coordination of the calcium cation in site II but is involved in the -x coordinate of site III. In the case of site III, high affinity can be attributed to the fact that there is a z axis acid pair which interacts directly with the cation resulting in a stable, efficient interaction (Fig. 4). The high affinity of site II can be attributed to the fact that a Glu residue is located in the -x coordinating positions of all known parvalbumin site II sequences, and therefore the side chain is long enough to interact directly with the cation and mediation by water is not needed to maintain proper structure in the site. Therefore, parvalbumin site II will interact directly with the cation through an acid pair on the x coordinate axis which results in complete dehydration and a stable interaction with the cation.

This study has provided considerable refinement of the acid pair hypothesis. Originally, the hypothesis stated that maximum calcium affinity in the helix-loop-helix unit would require four acidic residues paired on the x and z coordinate axes of the tetrahedral arrangement of chelating ligands. It would now appear that high affinity can be had by helix-loop-helix units with 3 acid residues provided 2 of the residues are paired on either an x or z coordinate axis. Under these circumstances the -x dentate will not be mediated by a water molecule and the...
Acid Pair Hypothesis

TABLE I

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Apoprotein</th>
<th>Cal2+ peptide</th>
<th>TFE peptide</th>
<th>Kd μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aⁿ-STnC</td>
<td>8.079 ± 273 (9)</td>
<td>17.223 ± 248 (20)</td>
<td>20.872 (24)</td>
<td>28.2 ± 1.7</td>
</tr>
<tr>
<td>NDDL/4ZCaM</td>
<td>6.573 ± 98 (8)</td>
<td>17.187 ± 183 (20)</td>
<td>20.902 (24)</td>
<td>19.1 ± 0.1</td>
</tr>
<tr>
<td>L⁰⁰CaM</td>
<td>5.476 ± 145 (6)</td>
<td>13.755 ± 40 (16)</td>
<td>19.760 (20)</td>
<td>735 ± 61</td>
</tr>
<tr>
<td>NDL/3ZCaM</td>
<td>7.095 ± 291 (8)</td>
<td>19.540 ± 423 (23)</td>
<td>23.450 (27)</td>
<td>58.8 ± 0.1</td>
</tr>
<tr>
<td>NDL/3XCaM</td>
<td>5.715 ± 247 (7)</td>
<td>15.251 ± 197 (18)</td>
<td>27.599 (32)</td>
<td>524 ± 16</td>
</tr>
</tbody>
</table>

* Mean residue ellipticity at 222 nm is expressed in millidegrees ± S.D. (n = 3).
* Kd is the calcium dissociation constant expressed as μM concentration ± S.D. (n = 3).
* The data indicated are the result of a single experiment. The TFE is 3:1 (v/v) in 100 mM MOPS, pH 7.2, 150 μM KCl, 1 mM NTA.
* The number in the brackets is the calculated number of residues in the α-helix.

Glutamate side chain will interact directly with the cation. This difference involving the −x coordinate is illustrated by comparing the known sequences of TnC site II which are low affinity for calcium with the majority of known parvalbumin site II sequences which are high affinity for calcium (11). The sites in both proteins have identical residues in the chelating positions except for the −x dentate which is Asp in TnC site II and Glu in parvalbumin site II (the −y dentate is assumed to be the peptide carbonyl oxygen of residue 7 in the loop region in all examples).

In order to examine the applicability of the acid pair hypothesis to the natural calcium-binding proteins, an examination of the 12 residue loops from bovine brain calmodulin (Fig. 4) utilizing the premises of the acid pair hypothesis was undertaken. Analysis shows that the four sites in this protein will have two high and two low affinity sites for calcium. Sites I and IV will have high affinity for calcium based on the four acidic residues present in chelating positions and one acid pair on the z axis. These sites reside in different pairs of sites and therefore will be independent but need not be identical...
The acid pair hypothesis to the amino acid sequences of the loop regions in the calcium-binding sites of calmodulin can be used to predict the temporal order of structural features induced in the protein by calcium. Assuming positive cooperativity between which acid residues are paired are also listed.

The above assignments based on the application of the acid pair hypothesis to the amino acid sequences of the loop regions in the calcium-binding sites of calmodulin can be used to predict the temporal order of structural features induced in the protein by calcium. Assuming positive cooperativity between sites within the pairs (28) (that is, sites I and II are cooperative as are III and IV) and no interaction between pairs, the first site to bind calcium would be either site I or site IV, site IV being the more likely candidate as described above. If the positive cooperative interaction between IV and III is sufficient to increase the calcium affinity of site III above that of site I, then the second cation will bind to site III, and we have then established a binding order in the conventional high affinity sites of calmodulin. A possible mechanism of cooperativity between site IV and III which may account for the increased affinity of site III over site I is a modified version of one described previously (11) and involves calcium-induced movement of the aromatic side chain of residue 10 in the loop region of site IV to a position in site III where it can assist the aromatic residue side chain in position 7 of site III in the dehydration of the calcium cation during binding to site III. Binding of calcium to the second, lower affinity pair should proceed from site I to IV through a cooperative mechanism, however, there are no aromatic residues in positions 7 or 10 of site I, the side chains of which can move into site II to aid the aromatic residue in position 10 of that site in dehydration of the cation. The cooperative mechanism is not easily described for the paired low affinity sites, and this may account for some of the difficulty in getting accurate experimental data on cooperativity of these two sites in isolation (28, 29).

An alternative temporal order of calcium binding is also suggested by the acid pair hypothesis and the low affinity of site III. If cooperativity between site IV and III is insufficient to raise the calcium affinity of site III above that of site I, then the second cation will bind to site I first. Assuming cooperativity enhances site III calcium affinity above that of site II, the resulting sequence of calcium binding would be IV > I > III > II. If cooperativity does not enhance site III affinity above site II, the following binding sequence would result: IV > I > II > III. This scenario of calcium binding to calmodulin is similar to the four equivalent interactive site model described by Iida and Potter (30) and Cox et al. (31, 32).

Although the acid pair hypothesis is ineffective in suggesting which of the two models (the paired cooperative sites model or the four interactive sites model) is more likely to prevail in calcium interaction with calmodulin, it is useful in suggesting the order in which the sites are filled by calcium in both models.

The situation in bovine brain calmodulin is similar to that occurring in the four calcium-binding sites of rabbit skeletal troponin C. According to the acid pair hypothesis and in agreement with current dogma, STnC will have two high and two low affinity calcium sites in a situation similar to the paired cooperative sites model in calmodulin. The two high affinity sites are paired on the C-terminal half of the molecule and carry both x and z acid pairs (Fig. 4). There is potential for cooperative interactions between the two high affinity sites in TnC although it is not readily apparent which of the two sites will bind calcium first. The two low affinity sites in TnC are not identical by the acid pair hypothesis. Although both sites contain 4 acid residues in chelating positions, site I carries an Asp residue in both coordinates which does not interact with the cation via the amino acid side chain. If we adhere to the hypothesis, then site I will have 3 acid residues which interact with the cation and none of the anionic residues are paired on a chelating axis (Fig. 4).

Therefore site I will be lower in affinity for calcium than site II which has 4 acid residues with an acid pair on the x axis and the sequence of filling the TnC sites should proceed IV = III > II > I.
Acid Pair Hypothesis

The synthetic calcium-binding peptide analogs described in this study are useful tools for the investigation of calcium interaction with calmodulin and related calcium-binding proteins. They have been used to provide strong support and to further elaborate upon the major premise of the acid pair hypothesis which is that cation affinity of the helix-loop-helix calcium-binding unit will be increased if the anionic amino acid dentates in the loop region which interact directly with the cation are paired on the axial vertices of the resulting octahedral arrangement of chelating residues about the cation. Although the evidence thus far suggests that the synthetic peptides bind calcium identically to the sites in the natural proteins, the definitive study on this point will conclude when the crystal structure of the synthetic peptides has been solved (33).

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