The acid pair hypothesis predicts the calcium affinity of the helix-loop-helix calcium-binding motif based on the number and location of acidic amino acid residues in chelating positions of the calcium-binding loop region. This study investigates the effects of the number and position of acidic residues in the loop region on calcium affinity and selectivity using 33-residue synthetic models of single helix-loop-helix calcium-binding motifs.

Increasing the number of acidic residues in the octahedrally arranged chelating positions of the loop region from 3 to 4 by replacing an asparagine in the +y position with an aspartic acid increases the calcium affinity of the models between 2- and 38-fold. Differences in affinities are more pronounced in the models containing an x axis acid pair.

The calcium affinities of peptide models containing 3 or 4 acidic residues in chelating positions of the loop region and an x axis acid pair are reduced when the residue in the +z position is changed from asparagine to serine. A similar reduction in calcium affinity occurs in the z axis acid paired peptides when the +x chelating residue is changed from serine to asparagine.

Models with 3 acidic residues in chelating positions containing an x axis acid pair have greater calcium affinity than comparable peptide models with an x axis acid pair. The presence of x or z axis acid pairs in comparable peptide models containing 4 acidic residues in chelating positions does not greatly alter calcium affinity. Calcium selectivity resides in x axis acid paired peptides, whereas z axis acid paired peptides exhibit both magnesium- and calcium-induced structural changes. This ion selectivity may be explained by postulating that the z axis residue side chains produce the initial, rate-limiting interactions with the cation, causing hydration shell destabilization and initiating the subsequent ligand interactions.

The hlh1 calcium-binding motif (EF hand), first characterized in carp parvalbumin (Kretsinger and Nockholds, 1973), has been found in the crystal structures of other calcium-binding proteins including calmodulin (Babu et al., 1985), bovine intestinal calcium-binding protein (Szepesty et al., 1981), skeletal muscle troponin C (Herzberg and James, 1985; Sundaralingam et al., 1989), and oncomodulin (Ahmed et al., 1990). This study investigates the effects of the number and position of acidic residues in the loop region on calcium affinity and selectivity using 33-residue synthetic models of the helix-loop-helix calcium-binding motif.

Increasing the number of acidic residues in the octahedrally arranged chelating positions of the loop region from 3 to 4 by replacing an asparagine in the +y position with an aspartic acid increases the calcium affinity of the models between 2- and 38-fold. Differences in affinities are more pronounced in the models containing an x axis acid pair.

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The relevance of analyzing structural requirements for cation binding in terms of the residues in the chelating positions of the loop region has been demonstrated using a hybrid peptide model of the third calcium-binding site in troponin C and calmodulin (Reid, 1990). This study showed that the nature of the amino acids in the six chelating positions of the loop region is one factor dictating the calcium affinity of the hlh calcium-binding motif. The acid pair hypothesis proposed that high affinity calcium-binding sites should have 4 acidic amino acid residues paired on the x and z coordinates of the octahedral cation coordination sphere (see Fig. 1) (Reid, 1987a). This hypothesis was tested to determine whether the difference in calcium affinity of hlh calcium-binding motifs was the result of the number and/or location of acidic amino acid residues occupying chelating positions of the loop region (Reid, 1990). The test results suggested that an hlh motif with an acid pair results in greater calcium affinity than an hlh motif with no acid pairs. It also appeared that calcium affinity was greater if the acid pair was on the z axis as opposed to the x axis.

To define further the structural parameters that distinguish high and low calcium affinity in the helix-loop-helix calcium-binding motif, this study uses analogs of the third calcium-binding site of calmodulin to investigate the differential calcium affinities observed between x and z axis acidic peptides. Since it is generally accepted that the high affinity calcium-binding sites are also magnesium-binding sites and that the low affinity calcium-binding sites are selective for calcium, this study also examines the magnesium interaction with the synthetic calcium-binding model peptides to determine if it is possible to use the criteria of magnesium binding for further definition of a high affinity calcium-binding site.

EXPERIMENTAL PROCEDURES

Synthetic Protocol—The peptides were synthesized semiautomatically using a Vega 1000 peptide synthesizer, except for CaM:3x(NSD), and CaM:3x(DDN), which were synthesized on an Applied Biosystems Model 430A peptide synthesizer. All syntheses used the t-butyloxycarbonyl protecting group. Side chains were protected as the cyclohexyl ester, serine as the benzyl ether, tyrosine as the t-butyloxycarbonyl derivative, aspartic acid as the benzyl ester, and asparagine as the N-benzyl derivative. The peptides were quantitated by amino acid analysis of an aliquot of the synthetic peptides to determine if it is possible to use the criteria of magnesium binding for further definition of a high affinity calcium-binding site.

RESULTS AND DISCUSSION

The acid pair hypothesis is an attempt at an explanation for the various affinities for cations demonstrated by the hlh calcium-binding motifs found in a number of calcium-binding proteins including calmodulin, troponin C, calbindin, parvalbumin, and calbindin D9K. The structural similarity and amino acid sequence homology among the calcium-binding sites in these proteins belie the diversity of cation affinities and selectivities demonstrated by these sites. The acid pair hypothesis assumes that the calcium affinity of the hlh calcium-binding motif can be explained in terms of the number and location of acidic amino acid residues at positions 1, 3, 5, 9, and 12 of the 12-residue loop region of the motif. These five positions are located at the +1, +2, +3, -1, -2, and -3 coordinates of an octahedral coordination shell (Fig. 1). Regardless of cation affinity, the +1 and +2 coordinates positions (i.e. positions 1 and 12 of the loop) are invariably aspartic acid and glutamic acid, respectively. Since the side chain of position 7 does not interact directly with the cation, the variation of the 3 residues at positions 3, 5, and 9 may provide a means of changing the cation affinity of all hlh calcium-binding motifs. It is our contention that the acid pair hypothesis is required but not sufficient for a complete description of cation binding to the hlh calcium-binding motif.
Helix-Loop-Helix Cation-binding Motif

be an acidic residue, so it is replaced in our models with either serine or asparagine. The effects of serine or asparagine at this position on cation affinity are found to be different; therefore, for comparison purposes, the residues are not freely interchangeable. CaM:3z(NDN), which has a serine residue in the +z chelating position, shows a 29-fold lower affinity for calcium than CaM:3z(NDN), with an asparagine residue at the same position (K_c(a) = 15.4 mM versus 524 μM, respectively) (Fig. 2 and Table I). Similarly, CaM:4z(DSD) shows a 10-fold lower affinity for calcium than CaM:4z(DDN) (K_c(a) = 407 versus 42.1 μM, respectively) (Fig. 2 and Table I). All other variables being equal, the asparagine residue in the +z position results in a model motif with significantly greater cation affinity than an otherwise identical model motif with serine at that position.

The differences in calcium affinities may be attributed to different conformations of the calcium-binding sites that are influenced by the ϕ and ψ angles of the residue occupying the +z position. The common ψ angle of serine residues in proteins ranges from 150 to 180°, which is considerably greater than the ψ angle of asparagine and aspartic acid residues, which ranges from 75 to 135° (Matthews, 1993). In the absence of calcium, both CaM:3z(NSD) and CaM:4z(DSD) show very little α-helical structure (Fig. 3 and Table I). Five residues make up the α-helical portion of both apopeptides compared to 6–8 residues in hlh calcium-binding motifs showing high affinity for calcium (see CaM:4z(DND), CaM:3z(NDS), and CaM:4z(DDS) in Fig. 3 and Table I). It is possible that a certain amount of preformed structure in the +z position results in a model motif with significantly greater cation affinity than an otherwise identical model motif with serine at that position. In the presence of trifluoroethanol (Table I), high affinity peptides show 27 residues of α-helical structure in the presence of the structure-forming solvent, whereas CaM:3z(NSD) and CaM:4z(DSD) show 21–22 residues of α-helical structure in the same solvent (Fig. 3 and Table I).

The calcium-binding sites in troponin C and calmodulin that contain serine in the +z chelating position of the loop region do not help in the interpretation of these data. Rabbit skeletal troponin C site II is a low affinity calcium-binding site and contains a serine residue in the +z position. While this is a plausible explanation for the low affinity of this site in STnC based on this study, Shaw et al. (1991), using synthetic peptide analogs of STnC, have shown that the low affinity of this site may be due to the nonchelating glutamic acid residue at position 2 of the loop region. These authors, working with the chicken STnC sequence, substituted the chelating residues of the high affinity site III (see SCIII in Fig. 4) with the chelating residues of the low affinity site II (LII) and found that this new hybrid site (LIIIL) has an affinity for calcium on par with the affinity of a synthetic model of the high affinity site III. This suggests that the nonchelating residues of site III are responsible for the high calcium affinity of STnC site III. The hybrid peptide (LIIIL) discussed by these authors has a loop sequence very similar to that of CaM:4z(DSD) (Fig. 4), which has a low affinity for calcium (K_c(a) = 407 μM). Therefore, the suggestion that replacing the lysine residue at nonchelating position 2 of the loop region with glutamic acid residue is the cause of low cation affinity in STnC site II does not correlate with the low affinity of CaM:4z(DSD). It is difficult to reconcile the difference in cation affinities between the hybrid peptide of Shaw et al. and CaM:4z(DSD), and further studies are needed to solve this problem.

The CD calcium-binding site of carp parvalbumin also demonstrates anomalous behavior when compared with the model studies described above. The CD site contains serine in the +z chelating position (Fig. 4) and has high calcium affinity (K_c(a) = 0.4–2.0 mM) (Moeschler et al., 1980; Haiech et al., 1979). In view of the detrimental effect of serine in the +z position on calcium binding, there may be compensation in the parvalbumin site for this anticipated detrimental action of serine, but this is only speculation and is currently under investigation.

The −x coordinating residue (position 9) in the hlh calcium-binding motif is most frequently found to be aspartic acid, which is paired with the invariant aspartic acid residue in the +x position to give an x axis acid pair. The second and third most frequently occurring residues in this position are serine and glutamic acid, respectively, whereas asparagine is the fourth most common residue in this position. For studies on the hlh calcium-binding motifs with a single z axis acid pair, we require a nonacidic residue in the −x position, which would make serine or asparagine the best substitution. Studies using synthetic motifs with serine or asparagine in this position indicate that the effects of these residues in this position on calcium affinity are not identical; therefore, for comparison purposes, these residues are not freely interchangeable. Replacement of a serine residue in the −x chelating position of CaM:3z(NDS) with an asparagine residue to give CaM:3z(NDN) results in a 17-fold decrease in affinity for calcium (K_c(a) = 458 versus 1000 μM) (Table I). Similarly, CaM:4z(DNN), with an asparagine residue in the −z chelating position, has a 19-fold lower affinity for calcium than CaM:4z(DDS), which has a serine residue in the −x chelating position (K_c(a) = 542 versus 29.2 μM, respectively) (Fig. 2 and Table I). CaM:4z(DDD), with 4 acidic residues in chelating positions, has a 9-fold lower calcium affinity than CaM:3z(NDS), with only 3 acidic residues in chelating positions (K_c(a) = 542 versus 58.8 μM). This may be due to the detrimental effect of the asparagine in the −x chelating position of CaM:4z(DDN).

This detrimental effect of asparagine in the −x chelating position of the loop region may be of a similar nature to that of serine in the +z position, i.e. an alteration in conformation of the calcium-binding site due to the greatly different ψ angle between serine and asparagine residues. The apopeptides CaM:3z(NDN) and CaM:4z(DDN) both show the least amount of α-helical structure compared to the other hlh motifs with greater calcium affinity (4 residues compared to 6–8 residues) (Fig. 3 and Table I). These peptides also show among the least amount of trifluoroethanol-induced α-helical structure (19–22 residues) (Table I). Thus, a certain amount of structure in the apopeptide may result in more efficient calcium chelation, and the peptides with asparagine in the −x position are lacking that structure even in structure-inducing solvents such as trifluoroethanol.

The +y coordinating residue (position 3) in the loop region is not involved in acid pairing since the −y chelating position does...
not involve the amino acid side chain as discussed above. To maintain the single x or z axis acid pair but to increase the number of acidic residues in the loop region that interact with the cation, the only change that can be made is to alter the +y residue from asparagine to aspartic acid. Therefore, the +y position is utilized in the synthetic models to increase the number of acidic residues in the loop region for comparison of cation affinities. The synthetic peptide hlh calcium-binding motifs that contain 3 acidic residues in chelating positions, 2 of which form an acid pair, will have acidic residues at positions 1 and 12 and at either position 5 or 9 for a z axis acid pair or at position 9 for an x axis acid pair. The +y position will contain an asparagine residue, which is the second most frequently occurring amino acid in this position next to aspartic acid. Any other change to chelating positions in the loop region (i.e. changing the residues at position 5 or 9) would not only increase the number of acidic residues, but would also increase the number of acid pairs, which would further complicate the interpretation of the results.

Both the x and z axis acid paired hlh motifs (Fig. 2) show a higher affinity for calcium when an aspartic acid residue, rather than an asparagine residue, occupies the +y chelating position. The x axis acid paired peptides with an aspartate residue in the +y chelating position (CaM:4x(DSD), $K_{Ca} = 407 \mu M$; and CaM:4x(DNN), $K_{Ca} = 42.1 \mu M$) show 38- and 12-fold greater affinities for calcium than the respective peptides containing an asparagine residue in the +y chelating position (CaM:3x(NSD), $K_{Ca} = 15.4 \mu M$; and CaM:3x(NND), $K_{Ca} = 524 \mu M$). The z axis acid paired peptides with aspartate in the +y chelating position (CaM:4x(DDN), $K_{Ca} = 542 \mu M$; and CaM:4x(DDS), $K_{Ca} = 29.2 \mu M$) both display a 2-fold greater affinity for calcium than their respective peptides having an asparagine residue in the +y chelating position (CaM:3z(NDN), $K_{Ca} = 1000 \mu M$; and CaM:3z(NDS), $K_{Ca} = 58.8 \mu M$).

If one accounts for the detrimental effects of the nonacidic residues in the +z position of the x axis acid paired peptides (CaM:3x(NSD) and CaM:4x(DSD)) (Fig. 2 and Table I) and in the +z position of the z axis acid paired peptides (CaM:3z(NND) and CaM:4z(DNN)) (Fig. 2 and Table I) by comparing x axis acid paired peptides with the detrimental residue in the +z position to z axis acid paired peptides with the detrimental residue in the +x position (compare CaM:3x(NSD) with CaM:3z(NND) and CaM:4x(DSD) with CaM:4z(DNN)) (Fig. 2 and Table I) and x axis acid paired peptides with nondetrimental residues to z axis acid paired peptides with nondetrimental residues (compare CaM:3x(NND) with CaM:3z(NDS) and CaM:4x(DND) with CaM:4z(DDS)) (Fig. 2 and Table I), substituting asparagine in the +y position with aspartate causes a greater difference in calcium affinity with the x axis acid paired peptides than with the z axis acid paired peptides. This result is interesting since
the 3z axis acid paired peptides have calcium affinities that are much lower than the corresponding 3z axis acid paired peptides (compare CaM:3x(NSD) with CaM:3x(NND) and CaM:3x(NDS) with CaM:3x(NDS)) (Table 1), but addition of aspartate to the +y position produces 4x and 4z axis acid paired peptides with similar calcium affinities (compare CaM:4x(DSD) with CaM:4z(DDN) and CaM:4x(DDS)) (Table 1). It would appear that the z axis plays a more important role in calcium chelation than the x axis in the motifs containing 3 acidic residues with one acid pair; however, that role is much less important when there are 4 acidic residues with one acid pair in chelating positions. A possible explanation is that the z axis residues are the first to interact with the calcium cation, which produces the initial, rate-limiting outer-sphere hydration shell interaction and destabilization, followed by x and y axis binding (Eigen, 1961).

It is generally believed that the high affinity hlh calcium-binding motifs in naturally occurring proteins such as troponin C and parvalbumin also bind magnesium (Potter and Gergely, 1975). Magnesium-induced structural change in the synthetic peptide model hlh calcium-binding motifs was examined in order to better delineate calcium selectivity in the model sites.
great. The nonsigmoidal titration curves, the smaller amount of magnesium dissociation constant of 17 mM (Table I). Three of the peptide models demonstrated magnesium-induced structural change detected by CD spectroscopy as α-helix formation. These peptides have one feature in common in that they all contain a z axis acid pair (see peptides CaM:3z(NDS), CaM:4z(DDN), and CaM:4z(DDS) in Fig. 3). However, the CD-monitored magnesium titration curves in Fig. 5C show that only CaM:4z(DDS) displays the typical sigmoidal curve, has the greatest amount of magnesium-induced structural change (Δθ_{222} = -11,294 degrees cm²/dmol), and has a magnesium dissociation constant of 17 mM (Table I). It is possible that this peptide binds magnesium in a manner similar to the binding of calcium, although the structural change is not as great. The nonsigmoidal titration curves, the smaller amount of magnesium-induced structural change, and the larger magnesium dissociation constants of CaM:3z(NDS) (Δθ_{222} = -2356 degrees cm²/dmol; K_{Mg} = 81 mM) and CaM:4z(DDN) (Δθ_{222} = -1790 degrees cm²/dmol; K_{Mg} = 54 mM) may represent a metal ion complex different from that of CaM:4z(DDS) and involving the z axis residues only.

The fact that CaM:3z(NDN) did not bind magnesium can be explained by the presence of only 3 acidic residues in chelating positions along with the presence of the detrimental asparagine residue in the x axis chelating position as shown previously for calcium binding. This effect was compensated somewhat when the asparagine residue in the z position was replaced with an aspartate residue, as occurs in CaM:4z(DDN). As a result of the increased charge, the hkh motif is now capable of binding magnesium (K_{Mg} = 54 mM) (Table I). Similarly, the substitution of the asparagine residue in the x position of CaM:3z(NDS) with aspartate to give CaM:4z(DDS) resulted in an hkh motif that showed a 5-fold increase in magnesium affinity (K_{Mg} = 81 versus 17 mM, respectively). Therefore, the hkh calcium-binding motifs containing aspartate in the x position and a single acid pair on the z axis will bind magnesium if the site contains aspartate in the x position and/or serine in the –x chelating position.

In conclusion, the results of this study indicate that the acid pairs on the x and z axes have different effects on cation affinities, with the implication being that the z axis is more efficient in cation binding than the x axis when 3 acidic residues interact directly with the cation. This differential effect disappears for calcium binding when 4 acidic residues interact directly with the cation. Affinity for magnesium is demonstrated by z axis paired model peptides only. The affinity is characterized by both ligand charge (as reflected by the presence or absence of an aspartate in the z position) and conformational flexibility (as reflected in the type of amino acid residue found in the –x position) in the loop region of the hkh calcium-binding motif. There is no correlation between magnesium binding and high calcium affinity in the peptide models studied. We suggest that the acid pair hypothesis is an excellent source of experimental approaches to determine the structure-function relationships.
in the hlh calcium-binding motifs. This study indicates that the hypothesis is not sufficient to describe the cation affinity and selectivity of these motifs in the natural proteins. It does, however, provide a basis on which the synthetic models and natural sites can be compared in a effort to determine additional factors dictating cation selectivity and affinity in these calcium-binding sites.

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