Chapter 4

Calcium-Binding to Greek Key Peptides: \( \beta_\gamma \)-Crystallin Fold is the Calcium-Binding Motif of \( \gamma \)-Crystallin
4.1 Introduction

Our results in Chapter 3, “Calcium binding to γ-crystallin”, clearly show that γ-crystallin is a calcium binding protein with four sites for calcium binding. γ-Crystallin does not have any of the well-known motifs for calcium binding, such as, EF-hand, annexin fold, or C2 domain but it still binds calcium. Hence, there could be an orphan motif for calcium binding in γ-crystallin, which needs to be characterized. γ-Crystallin as mentioned earlier is the prototype and founding member of the βγ-crystallin superfamily and another lens member of this superfamily, β-crystallin and two non-lens members, Protein S and spherulin 3a, were shown to bind calcium. β-Crystallin calcium binding sites were not characterized and Protein S and spherulin 3a were also not shown to have any known calcium-binding motif. The NMR and X-ray structural study on N-terminal domain of Protein S has shown that calcium ion binds at the Greek key and it has two calcium binding sites. Since γ-crystallin is the prototype member of this superfamily and Protein S shows sequence and structural similarities with γ-crystallin and also our results on calcium binding to γ-crystallin show four sites for calcium binding, we thought and proposed that Greek key motif could be the calcium binding motif in γ-crystallin. We attempted to identify the site for calcium-binding using crystal structure of γ-crystallin. We were not able to identify the electron density for calcium. The possible reasons are that the calcium-binding to γ-crystallin is very sensitive to modifications and the pH used for crystallization was also acidic (pH 5.0), which would seriously hamper calcium binding. γ-Crystallin is rich in cysteines, which might get oxidized during exposure to air, thus affecting calcium binding. Therefore, an alternative strategy needs to be applied.

Absence of any known motif for calcium-binding and alignment of the sequence of Greek key motifs of γ-crystallin with Protein S motifs suggest that Greek key could be the calcium-binding motif in γ-crystallin. In order to verify this, we have synthesized peptide corresponding to the third Greek key motif of γ-crystallin and studied its calcium binding properties. We have also synthesized some of the mutants of this peptide, which were expected to disturb the calcium binding ability. Calcium binding studies done on these peptides show that Greek key motif is indeed the calcium-binding motif in γ-crystallin.
4.2 Results

4.2.1 Strategy for the identification of calcium-binding motif in $\gamma$-crystallin: Our attempts to demonstrate calcium-binding motif in $\gamma$-crystallin structure using crystallization of $\gamma$-crystallin in the presence of calcium did not work. Therefore in the absence of structural methods, alternative strategy could have been to show calcium-binding to a peptide of the putative region. Numerous studies have been performed in the past to show calcium-binding characteristics to peptides corresponding to an EF-hand or helix-loop-helix motif of calcium-binding (George et al., 1996; Sharma et al., 1997; Reid, 1990; Julenius et al., 2002; Muralidhar et al., 2004). Calcium-binding to a peptide corresponding to the sequence of Greek key motif of crystallin has not been attempted. We thought that if such a peptide binds calcium, it would show that this could form an independent unit for calcium-binding. We therefore, selected the sequence of $\gamma$-crystallin for synthesizing the peptides.

4.2.2 Sequence alignment of $\gamma$-crystallin with Protein S: Since our results of calcium binding studies of $\gamma$-crystallin shows clearly that $\gamma$-crystallin has four sites for calcium binding. By looking into the sequence homology between the $\gamma$-crystallin and protein S and the similarity between the Greek key motifs, we felt that the calcium could bind at Greek key motif in $\gamma$-crystallin, similar to Protein S.

$\gamma$-Crystallin shows 47% sequence homology with Protein S as well as similarity in the structure of 4-fold repeats (Wistow et al., 1985). The motifs 1 and 3 of $\gamma$-crystallin are similar to motif 2 and 4 in Protein S, whereas motif 2 and 4 of $\gamma$-crystallin are similar to motifs 1 and 3 of Protein S (Wistow et al., 1985). The residues involved in calcium binding in Protein S are known from its crystal structure (Wenk et al., 1999). The alignment of individual $\beta_\gamma$ motifs of Protein S and $\gamma$-crystallin was used to identify the residues involved in calcium ligation in $\gamma$-crystallin. This alignment showed that calcium-binding sites of $\gamma$-crystallin are located within Greek key motifs. Based on sequence alignment, we found that the first calcium could ligate at Glu7, Arg31, Asn33 and Ser30, whereas the second calcium at Glu46, Arg76, Ser74 and Asp73 (Figure 4.1). All these residues are located at the surface of the structure and their side chains are available for calcium binding. Similarly, two other calcium-binding sites located at the C-terminal domain were also identified. The residues Glu95, Thr120, Glu121, and His123 form site 3, whereas Glu136, Asn162, Ala163, Gly165 constitute site 4 (Figure 4.1).
4.2.3 Molecular modeling of γ-crystallin: In order to investigate the propensity of these residues identified for calcium binding by sequence alignment, molecular modeling was done on γ-crystallin. The above residues proposed for calcium binding were found to possess bond length and bond angles suitable for forming a bond for calcium binding to the oxygen atoms of the side chains of these residues (Figure 4.2).

4.2.4 Design of Greek key peptides: In order to verify if the Greek key crystallin fold is the calcium ion-binding motif of γ-crystallin, we have synthesized four-stranded 42-
residue $\beta$-sheet peptide (peptide g3) corresponding to the third Greek key motif of $\gamma B$-crystallin. The amino acid sequence of $\gamma$-crystallin is shown in Figure 4.3.

Figure 4.3. Amino acid sequence of bovine $\gamma B$-crystallin (accession number P02526)

This sequence was selected since it has minimum number of interfering amino acids, which hinders during peptide synthesis, such as cysteine. To ascertain if the residues identified by alignment (Figure 4.1) participate in ligation, we have also synthesized mutants of this Greek key peptide (Table 4.1). The two putative residues (Glu95 and Glu121), which were identified for calcium binding by homology with protein S, were modified to Lys in the peptide g3a (Table 4.1). We have also investigated if other acidic residues are involved in calcium binding (such as two aspartates at positions 108 and 109 are replaced by Lys in the peptide g3b).

Table 4.1: Amino acid sequence of the Greek key crystallin fold peptide (corresponding to the third Greek key of bovine $\beta\gamma$-crystallin) and its variants synthesized and studied for calcium binding. Letters in red indicates the mutation of the residues.

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Amino acid sequence</th>
</tr>
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<tbody>
<tr>
<td>g3</td>
<td>90 RMRIYERDDFRQGMSEITDDCPSSLQDRFHVLTEVHSLNVLEG 131</td>
</tr>
<tr>
<td>g3a</td>
<td>90 RMRIYKRDDFRQGMSEITDDCPSSLQDRFHVLTKVHSLNVLEG 131</td>
</tr>
<tr>
<td>g3b</td>
<td>90 RMRIYERDDFRQGMSEITKKCPSSLQDRFHVLTEVHSLNVLEG 131</td>
</tr>
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4.2.5 Secondary structure of Greek key peptides: We have characterized and evaluated the secondary structure of these peptides by CD. The wild type peptide g3 and its mutants are fairly soluble in aqueous medium.

All these peptides show a poorly organized, unfolded structure in aqueous solution. However, in increasing concentration of methanol, these peptides adopt $\beta$-sheet conformation as shown by increasing ellipticity at 216 nm (Figure 4.3). These
peptides form well-defined β-sheet conformation at >80% methanol/water mixture. The designed β-sheet peptides have earlier been shown to possess unfolded structure in aqueous medium, which readily form β-sheet in organic medium (Das et al., 2000).

Figure 4.4: Far-UV CD of Greek key peptides: The peptides (5 μM) were dissolved in water and CD was recorded in various methanol concentrations in 0.5 cm path length cell with 8 accumulations. (a) g3 peptide: (____) 0, (---) 40, (-----) 60, (-----) 80 and (....) 90% methanol; (b) g3a peptide: (____) 0, (---) 30, (....) 50, (-----) 70 and (....) 90% methanol; (c) g3b peptide: (____) 0, (---) 30, (----) 50, (-----) 70 and (....) 85% methanol.

4.2.6 Aggregational properties of Greek key peptides: We have characterized and evaluated the aggregational propensity of these peptides by HPLC. The wild type peptide g3 and its mutants are fairly soluble in aqueous medium. These peptides form aggregates of various sizes as seen in their gel filtration profiles on HPLC. The chromatogram of g3, g3a and g3b are shown in the Figure 4.4a, b and c respectively. The fractionation range of the 1-125 gel filtration column is 2-80 kDa. For the lower side molecular weight marker, insulin (5.7 kDa), was used and for higher side molecular weight marker, albumin (67 kDa), was used. Insulin eluted at 14 ml and albumin at 6.8 ml. All these peptides eluted higher than monomer mass and they eluted between albumin and insulin elution volumes, which indicates that they all form higher oligomers. If they could have existed as monomers then they could have eluted as a single peak near 14-15 ml. In g3 peptide a peak is seen at this value indicating that it exists as both monomer and oligomer. This result is in agreement with the earlier suggestions of non-
existence of single Greek key motifs. Greek key motifs always associate to form a domain. Generally single isolated domains are also not found, they form dimers as in spherulin 3a.

Figure 4.5: Gel filtration HPLC of g3, g3a and g3b peptide on Protein-Pak I-125 column in 50 mM Tris buffer, pH 6.8 containing 50 mM NaCl.

4.2.7 Probing calcium binding to Greek key peptides by direct $^{45}$Ca-binding: The calcium-binding to these Greek key peptides was performed by direct calcium-binding using $^{45}$Ca overlay method. Figure 4.6 shows the peptide dot-blot for three peptides, g3, g3a and g3b. To validate the procedure, we have used the peptide, corresponding to the EF4 of neuronal calcium sensor-1 (36 residues, residues 144-179) as positive control and BSA as negative control. It is seen from the Figure 4.5 that wild type peptide g3 binds calcium as strongly as EF-hand peptide of neuronal calcium sensor-1 does. However, the two modified peptides (g3a and g3b) do not show detectable binding of $^{45}$Ca. These results show that an individual peptide corresponding to a Greek key is able to bind calcium and forms a complete unit in the same way as an EF-hand does, though the binding site geometry is different. The g3a peptide in which Glu95 and Glu121
residues were modified to Lys does not show any binding indicating their participation in
the binding. These residues were also investigated on the molecular model and were
found to be able to show the geometry suitable for binding. The other peptide, g3b, also
does not show any significant signal since the affinity was decreased due to the
modification of aspartic acid residues 108 and 109 to Lys (Figure 4.6).

![Figure 4.6: Calcium-binding to Greek key peptides by calcium overlay method. The wild
type peptide g3, its variants (g3a and g3b) were spotted onto a PVDF membrane and detected by
$^{45}$Ca overlay. For a positive control, EF-hand peptide corresponding to site 4 of rat neuronal
calcium sensor-1 was used; bovine serum albumin (BSA) was used as a negative control.]

**4.2.8 Probing calcium-binding to Greek key peptides by calcium probe Stains-all:**
The calcium-binding to individual Greek key peptides was further evaluated using the
more sensitive assay of Stains-all binding. This assay is suitable and convenient for
comparing closely related calcium-binding proteins and peptides. A similar approach
was used previously to study and compare the calcium-binding properties of individual
EF-hand peptides of calmodulin (Sharma et al., 1997). The magnitude of the induced J
or $\gamma$ band is a direct indicator of the affinity towards calcium. The g3 peptide binds
Stains-all and induces a strong J band (Figure 4.7a). The addition of calcium decreases
the J band intensity. There is no induction of CD band in the peptide g3a at the
dye:peptide ratio used for g3. However, J band induction was seen in g3a when higher
concentrations of peptide (4 times more) were used (Figure 4.7b). Thus the binding of
Stains-all to modified peptide g3a is decreased to several fold indicating the role of these
two amino acid residues in calcium-binding (Figure 4.7b). When dye-binding was
performed with peptide g3b, we found that this peptide binds the dye and induces the $\gamma$
band indicating that it has not lost the calcium-binding ability completely though the
geometry and microenvironment of the binding site is altered due to modifications of
acidic residues to basic residues (Figure 4.7c). The addition of calcium in g3a and g3b peptides decreased the CD band intensity indicating the replacement of the dye. The addition of calcium to g3 peptide Stains all complex did no decrease the J band intensity significantly, when compared to g3a and g3b peptide Stains all complex, indicating the stronger affinity of the g3 peptide compared to its variants. These results show that though the $^{45}$Ca binding was not seen in the dot-blot assay for peptides g3a and g3b, we were able to see the dye binding indicating the decreased affinity. The affinities of these peptides calculated based on induced CD band are in the order of g3> g3b> g3a.

Figure 4.7: Stains-all binding and the effect of calcium on Stains-all peptide complex of (a) g3 peptide, (b) g3a peptide and (c) g3b peptide. Dye concentration in all experiments was 43 μM. The ellipticity data are represented in millidegrees. Protein concentration used was 60 μM for g3 and g3b whereas 250 μM for g3a. Calcium was added to the dye-peptide mixture and calcium concentrations used were: no calcium (---), 0.1 mM calcium (- - - -), and 1 mM calcium (..........).

4.2.9 Effect of calcium on the conformation of Greek key peptides: Some EF-hand peptides undergo conformational changes upon addition of calcium. So we were interested to see whether calcium induces any conformational change in these peptides. All these peptides are random coil in aqueous solution. On calcium addition, as shown in the Figure 4.8 there was no change in the conformation. Some calcium binding peptides
bind calcium if they have some partial folded structure, and induce proper structure formation. So we checked the effect of calcium on these peptides in 60% methanol solution. There was no change in the conformation as seen in the Figure 4.9. These results taken together, show that these peptides, similar to the whole gamma protein, do not induce any change in conformation upon calcium addition. This kind of observations could also be because of the binding of the calcium on the surface of the Greek key.

Figure 4.8: Calcium titration of Greek key peptides in aqueous solution. Calcium was added to the peptide solution, mixed and incubated for 5 min and then the CD spectra were recorded. Peptide concentration used was 10 μM. Path length of the cell used was 0.5 cm. Calcium added was 0.1 mM (dashed line) and 1 mM calcium (dotted line).

4.3 Discussion

For the first time, we have shown that the four-stranded Greek key β-sheet peptide corresponding to crystallin fold forms an individual calcium binding site. These peptides adopt β-sheet conformation in water/methanol mixture, and form aggregates producing anti-parallel β-sandwich motif as shown by far UV-CD (Figure 4.4). Our results of alignment of relevant regions of proteins that contain this fold, and of four-stranded Greek key peptides used in this study (Table 4.1) suggest that the first calcium ligates at the Z residue next to the conserved aromatic amino acid of the sequence
Y/F/WZXXXXXG, which is located at the end of the first β-strand ('a' strand). The amino acid at this position is generally Asp, Asn, Glu, Gln, Ser, Tyr and rarely Lys. Other three residues needed for calcium ligation lie just before the beginning of the fourth β-strand (before the conserved Ser) and are usually Asp, Asn, Ser, Thr, Val, or Ala (Figure 4.1).

![Graphs](image)

Figure 4.9: Calcium titration of Greek key peptides in 60% methanol solution. Calcium was added to the peptide solution, mixed and incubated for 5 min and then the CD spectra were recorded. Peptide concentration used was 8 μM. Path length of the cell used was 0.5 cm. Calcium added was 0.1 mM (dashed line) and 1 mM calcium (dotted line).

Clout et al., (2001) have shown similarity in the calcium binding sites in spherulin 3a and Protein S, and pointed out the role of these residues in ligation. Our results with Greek key peptides directly demonstrate the importance of these residues in ion binding. The role of conserved Ser in calcium ligation has already been shown earlier (Teintze et al., 1988). Though there is a stretch of acidic residues in the 'b' and 'c' strands, they do not directly participate in the ion binding as shown in our results with peptide g3b. However, modifying the aspartate pair (Asp108 and Asp109) partially decreases the affinity towards calcium and alters the microenvironment of the peptide g3b as shown by Stains-all binding, since these residues are known to form a part of the cluster of alternate sign affecting the molecular surface (Chirgadze and Tabolina, 1996).
Our results demonstrate that Greek key crystallin fold forms a motif for calcium ion binding in $\beta\gamma$-crystallin superfamily. The calcium-binding properties of Protein S, $\beta$-crystallin and spherulin 3a have already been reported (Teintze et al., 1988; Sharma et al., 1989a; Rosinke et al., 1997). Our results suggest that other proteins of the superfamily, which have not been shown so far to bind calcium, would also bind the cation. The presence of topologically homologous residues in members of the superfamily makes us suggest that this family represents a novel class of calcium-binding proteins. Conformationally, these are all-beta proteins, and the binding site is located within the Greek key topology. As the geometry of this motif is distinct from that of the other calcium-binding motifs, we propose that the crystallin fold is a novel calcium-binding motif and $\beta\gamma$-crystallin superfamily represents a novel class of calcium-binding protein family.

4.4. Conclusions

Using the $\gamma$-crystallin sequence, it was possible to design 4-stranded $\beta$-sheet peptides. Like an EF-hand peptide, Greek key peptide binds calcium, suggesting that Greek key motif forms a unit for calcium ion binding.