Evolutionary Remodeling of βγ-Crystallins for Domain Stability at Cost of Ca\textsuperscript{2+} Binding

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The topologically similar βγ-crystallins that are prevalent in all kingdoms of life have evolved for high innate domain stability to perform their specialized functions. The evolution of stability and its control in βγ-crystallins that possess either a canonical (mostly from microorganisms) or degenerate (principally found in vertebrate homologues) Ca\textsuperscript{2+}-binding motif is not known. Using equilibrium unfolding of βγ-crystallin domains (26 wild-type domains and their mutants) in apo- and holo-forms, we demonstrate the presence of a stability gradient across these members, which is attained by the choice of residues in the (N/D)(N/D)XX(S/T)S Ca\textsuperscript{2+}-binding motif. The occurrence of a polar, hydrophobic, or Ser residue at the 1st, 3rd, or 5th position of the motif is likely linked to a higher domain stability. Partial conversion of a microbe-type domain (with a canonical Ca\textsuperscript{2+}-binding motif) to a vertebrate-type domain (with a degenerate Ca\textsuperscript{2+}-binding motif) by mutating serine to arginine/lysine disables the Ca\textsuperscript{2+}-binding but significantly augments its stability. Conversely, stability is compromised when arginine (in a vertebrate-type disabled domain) is replaced by serine (as a microbe type). Our results suggest that such conversions were acquired as a strategy for desired stability in vertebrate members at the cost of Ca\textsuperscript{2+}-binding. In a physiological context, we demonstrate that a mutation such as an arginine to serine (R77S) mutation in this motif of γ-crystallin (partial conversion to microbe-type), implicated in cataracts, decreases the domain stability. Thus, this motif acts as a "central tuning knob" for innate as well as Ca\textsuperscript{2+}-induced gain in stability, incorporating a stability gradient across βγ-crystallin members critical for their specialized functions.

There has been a great deal of interest in understanding the evolution of diversity in protein domains, because similar folds appear to have a common ancestral origin. A βγ-crystallin domain, identified first in lens γ-crystallins (1, 2), is among the diverse domains that probably evolved for high innate stability and provides transparency to the lens (for reviews see Refs. 3–5). Thus, being among the ancient and divergent folds, βγ-crystallin domain is an interesting system for understanding the evolution of domain stability (4, 6). Several proteins from all the three taxa are found to have this fold, forming the basis of the large βγ-crystallin superfamily (6–8). With the availability of more genome sequences, it is of utmost interest to define the molecular basis of diversity and its relationship with domain evolution in the context of the stability.

Recent insights into βγ-crystallins have emerged from two facets as follows: one is from microbial homologues and the other is from vertebrates (both lens and non-lens) (9–15). We believe that all βγ-crystallins can be broadly classified into those possessing the specialized Ca\textsuperscript{2+}-binding motif of six residues with a signature sequence of (N/D)(N/D)XX(S/T)S (13–15), and those without a signature sequence (16). Most proteins of this family with a canonical Ca\textsuperscript{2+}-binding motif are from bacterial sources, whereas vertebrate members and a few microbial members do not have the canonical Ca\textsuperscript{2+}-binding motifs and thus do not bind Ca\textsuperscript{2+} (13–16).

The βγ-crystallin fold serves as a paradigm for the study of protein stability, folding, and molecular evolution, because it has been thought that this domain was evolved from ancestral forms to provide higher stability to a protein (for reviews see Refs. 3–6). Some of the proteins of the superfamily with a canonical Ca\textsuperscript{2+}-binding motif, such as Protein S, Spherulin 3a, and Hahellin gain significant high stability upon Ca\textsuperscript{2+}-binding (17–20). This stabilization is apparently required for their functions, because both proteins seem to perform a protective role in the respective organism after gaining high stability upon Ca\textsuperscript{2+}-binding (21). Conversely, lens β- and γ-crystallins are substantially stable proteins, which is appropriately suited to their functions as long lived proteins with no turnover (4, 6). Although the stability of a βγ-crystallin domain is associated with its function, there is a complete lack of clarity of the molecular basis of Ca\textsuperscript{2+}-dependent (as in case of canonical domains) or Ca\textsuperscript{2+}-independent (as in case of domains with nonfunctional motifs) stability. It would be interesting to understand how a noncanonical vertebrate motif diverged from a canonical Ca\textsuperscript{2+}-binding motif of microbial βγ-crystallins during evolution attaining elevated domain stability. Therefore, mechanistic insights into the stability of βγ-crystallin domains are critical to the understanding of their function and evolution of high thermodynamic stability in members, including those with disabled Ca\textsuperscript{2+}-binding.

In this connection, we studied in detail the molecular basis of evolution of fold stabilization of diverse βγ-crystallin domains.
Stability Tuning Knob in \( \beta\gamma \)-Crystallins

We performed equilibrium unfolding of 26 domains containing either the canonical Ca\(^{2+}\)-binding (N/D)(N/D)XX(S/T)S motif (in the apo- and holo-forms) or nonfunctional motifs. We have decoded the molecular basis of the large variability in stability of \( \beta\gamma \)-crystallin domains as well as the differential stabilization by Ca\(^{2+}\), which is due to the nature of a residue in the motif. By disabling a functional motif, i.e. partial conversion of a microbe-type to a vertebrate-type and vice versa, we demonstrate that although Arg or Lys disables Ca\(^{2+}\)-binding, it augments domain stability. Therefore, we infer that the presence of Arg or Lys in some motifs of lens \( \beta\gamma \)-crystallins is due to adaptive selection where Ca\(^{2+}\)-binding ability was lost, but Ca\(^{2+}\)-independent domain stability was procured. The stability gradient is controlled by the choice of a residue in the (N/D)(N/D)XX(S/T)S motif; thus this motif serves to tune domain stability, which could ultimately have some bearing on the biological functions of the proteins. In a physiological context, we explain why some mutations located in the motif sequence of lens \( \beta\gamma \)-crystallins could ultimately have some bearing on the biological functions.

Vibrillin and Flavollin mutants) and 0 M urea and eluted using a linear gradient of 0–1.5 M NaCl. The Clostrillin and its mutants were purified from the soluble fraction on a Q-Sepharose column using 50 mM Tris–HCl, pH 9.9, 100 mM KCl. \( \gamma \)-Crystallin was purified by pre-equilibrated SP-Sepharose column by 50 mM BisTris–HCl buffer, pH 5.5. The final purification of these proteins was performed on a Superdex-75 or Sephacryl-200 (Amersham Biosciences) gel filtration column.

Circular Dichroism and Fluorescence Spectroscopy—Far-UV CD spectra of proteins (0.1 mg/ml) were recorded on a Jasco J-815 spectropolarimeter in various concentrations of GdmCl\(^5\) (0–6 M) in 50 mM Tris buffer, pH 7.0, containing 100 mM KCl. Samples were scanned between 200 and 250 nm using a 0.1-cm path length quartz cuvette. Intrinsic fluorescence emission spectra were recorded on a F-4500 fluorescence spectrophotometer (Hitachi Inc., Japan) with proteins in 0.1–1.0 mg/ml concentration range in 50 mM Tris–HCl, pH 7.4, and 100 mM KCl. The spectra were recorded from 300 to 450 nm at an excitation wavelength of 295 nm using excitation and emission band passes of 5 nm each.

Equilibrium Unfolding—Equilibrium unfolding was studied for 26 proteins using GdmCl in the range of 0–6 M concentration with a step of 0.1 M (a total of 70 data points for each unfolding transition) as required for best fit (24) in the apo (Ca\(^{2+}\)-free, with 1 mM EDTA) and holo (with 5 mM CaCl\(_2\)) in 50 mM Tris buffer, pH 7.0, containing 100 mM KCl. Unfolding was monitored by following the changes in the intrinsic Trp emission fluorescence by exciting at 295 nm. Unfolding was also monitored by measuring far-UV CD spectra at varying concentrations of GdmCl.

Data Analysis—The ratio of fluorescence intensity (at 360/320 nm) was plotted as a function of GdmCl concentration, and the data were fitted to the following two-state Equation 1. Best fits were analyzed by GraphPad Prism software. The \( c_{\text{is}} \) value (where \( c_{\text{is}} \) indicates the midpoint of unfolding transition) was calculated from fractions of folded and unfolded states of protein, plotted versus GdmCl concentration, and was used for comparing the stability. The \( c_{\text{is}} \) value is a concentration of denaturant where fractions at unfolded and folded states of protein are equal (\( F_{\text{u}} = F_{\text{f}} \)). The free energy of unfolding (\( \Delta G_{\text{fu}} \)) determined from the ratio of native to denatured protein at each GdmCl concentration, was plotted against GdmCl concentration (25).

Equation 1 was used for two-state model,

\[
Y_{\text{obs}} = \frac{\{Y_u + Y_e \exp(-\Delta G_{\text{fu}}^o - m[1D]/RT)\}}{\{1 + \exp(-\Delta G_{\text{fu}}^o - m[1D]/RT)\}}
\]

where \( R \) is the universal gas constant; \( T \) is the absolute temperature; \( [D] \) is the molar concentration of denaturant; \( \Delta G_{\text{fu}}^o \) is the free energy of unfolding; \( m \) is the dependence of \( \Delta G_{\text{fu}}^o \) on denaturant concentration; \( Y_{\text{obs}} \) is observed structural signal change; \( Y_u \) and \( Y_e \) are the spectroscopic signals at the native and denatured state, respectively.

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The abbreviations used are: GdmCl, guanidinium chloride; BisTris, 2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)propane-1,3-diol; ITC, isothermal titration calorimetry.
Isothermal Titration Calorimetry (ITC)—Ca\(^{2+}\)-binding isotherms of proteins were determined using a Microcal VP-ITC (Microcal Inc.). The protein solutions and calcium chloride (10 mM) were prepared in Chelex-treated 50 mM Tris, pH 7.0, and 100 mM KCl buffer. 1.4 ml of protein solutions in the concentration range of 70–100\,\mu M were used for the binding experiment at 30 °C. Aliquots of 3\,\mu l of the CaCl\(_2\) as ligand solution were injected for each titration. Blanks were obtained by titrating the buffer with identical concentrations of Ca\(^{2+}\). The curve fittings were performed using the Origin software (Version 7.0) supplied by Microcal after subtraction with the appropriate buffer blank.

RESULTS

Canonical (N/D)(N/D)X\(_1\)X\(_2\)(S/T)S and Noncanonical Motifs of \(\beta\gamma\)-Crystallin Domains

This specialized Ca\(^{2+}\)-binding motif of \(\beta\gamma\)-crystallins is composed of six amino acids with the sequence (N/D)(N/D)X\(_1\)X\(_2\)(S/T)S (13–15). Two such stretches interlock to form a site, where the 3rd and 5th residues of one stretch (X\(_1\) and Ser/Thr respectively) and the 2nd residue of the other stretch ligate calcium ion. Similarly, Ca\(^{2+}\) at another site is reciprocally coordinated. The 1st residue of (N/D)(N/D)XX(S/T)S motif is involved in indirect coordination via water, whereas the 2nd and 5th residues coordinate Ca\(^{2+}\) with their side chain. The X\(_1\) (3rd residue) ligates via the backbone. This sequence signature distinguishes the Ca\(^{2+}\)-binding \(\beta\gamma\)-crystallins from those that are not likely to bind Ca\(^{2+}\). Vertebrate homologues, such as AIM1, Crybg3, lens \(\gamma\)-crystallins, etc., and a few diverged domains, such as Nitrollin from Nitrosospira multiformis, do not have the canonical sequence, except the conservation of the structural Ser (at the 6th position) (Fig. 1; Table 1).

Selection of \(\beta\gamma\)-Crystallin Domains for Equilibrium Unfolding

To gain the mechanistic insights into domain stability, we undertook conventional but comprehensive GdmCl-based equilibrium unfolding of a selection of proteins containing \(\beta\gamma\)-crystallin domains with or without canonical motifs (Table 1). To avoid complexities that could arise from domain connectivity, linkers, and inter-domain interactions, we chose proteins with only single domains of similar size (about 10 kDa) (Fig. 1). The proteins studied were as follows: Vibrillin from V. cholerae, Flavollin from F. johnsoniae, Clostrillin from C. beijernickii (all three having domains with two canonical motifs), Centillin from R. centenum (with one canonical and one noncanonical motif), nitrollin from N. multiformis (both noncanonical motifs), and \(\gamma\)-crystallin (both noncanonical motifs) from eye lens (B. taurus) (Fig. 1). Mutations were selectively performed at the first, third, and fifth positions (Ca\(^{2+}\)-coordinating positions) in the Ca\(^{2+}\)-binding motif, i.e. \(^1\)(N/D)(N/D)X\(_1\)X\(_2\)(S/T)S\(^6\) (Table 1) as follows: (i) Asp at the 1st position, as Asn/Asp occupies this position often; (ii) either a polar or a hydrophobic amino acid at X\(_1\) (3rd) position; and (iii) Ser or Thr at the 5th position, as this position is occupied in most domains by Ser and in a few cases by Thr. We also undertook the partial conversion of a microbe-type domain to a vertebrate-type \(\beta\gamma\)-crystallin (the 5th position was converted to Arg or Lys, thus disabling the site for Ca\(^{2+}\)-binding). The protein domains were also analyzed by far-UV circular dichroism for any conforma-
The affinity and stoichiometry of Ca\textsuperscript{2+} domains and their mutants typically driven with an average dissociation constant (K\textsubscript{d}) of 11 μM was calculated considering Vibrillin as monomer (Fig. 2A; supplemental Table S1). The affinity and stoichiometry of Ca\textsuperscript{2+} binding to Centillin WT and its mutants is enthalpically driven, which is shown by the negative sign in binding isotherms. Centillin is a low affinity binding protein to Ca\textsuperscript{2+} as guessed by presence of one disabled and one functional motif (NDRARS and FRQISS). The dissociation constant (K\textsubscript{d}) of wild-type Centillin is 515 μM, which increases to 56 μM in F75D mutant (affinity increased by 10 times, from 515 to 56 μM). The data for Centillin F75D was fitted in one set of binding sites. In Centillin, the first site is naturally disabled as Arg (at the 5th position) does not allow side chain ligation required for Ca\textsuperscript{2+}-binding. Substituting this Arg to Ser in F75D mutant (Centillin R36S,F75D) in NDRARS motif further increased the affinity to 28 μM, comparable with βγ-crystallin domains with moderate affinity (Fig. 2B; supplemental Table S1). The binding to Centillin R36S,F75D mutant was entropically unfavored as entropy change (ΔS) for Ca\textsuperscript{2+}-binding to one site was positive. The binding of Ca\textsuperscript{2+} to Clostrillin T41K mutant was enthalpically driven with a dissociation constant of 775 μM. Clostrillin T41K has a very low affinity (~100-fold less than that of WT) as compared with Clostrillin WT (4 μM), although the binding was entropically favored (Fig. 2E; supplemental Table S1).

Stability Gradient across βγ-Crystallins

Equilibrium unfolding of this extensive set of 26 proteins was performed under identical conditions in the presence or absence of Ca\textsuperscript{2+} with at least 70 data points to allow a good fit of data (24). We describe below our data in the order of increasing stability, demonstrating high diversity in domain stability as well as variability in the extent of the gain of stability by Ca\textsuperscript{2+}.

We first describe the GdmCl-induced unfolding of an uncharacterized βγ-crystallin domain of Vibrillin with the canonical motif of NDTLSS and NDVISS sequence that binds Ca\textsuperscript{2+} with dissociation constant (K\textsubscript{d}) of 11 μM (Table 1). As seen from the data, c\textsubscript{50} for the apoprotein is 0.9 M GdmCl (Fig. 3, A and B; Table 1; supplemental Fig. S1A). Ca\textsuperscript{2+} does not influence the stability of the domain (c\textsubscript{50} for the holo-form is 1.0 M), and thus Ca\textsuperscript{2+} plays no role on the stability. Flavollin, which binds Ca\textsuperscript{2+} with moderate affinity (K\textsubscript{d} ~33 μM), is relatively more stable than Vibrillin (c\textsubscript{50} for the apoprotein is 1 M). A minor gain in the domain stability by Ca\textsuperscript{2+} (c\textsubscript{50} 1.3 M) suggests a weak stabilization by Ca\textsuperscript{2+}-binding (Δc\textsubscript{50} = 0.3 M) (Fig. 3, C and D; supplemental Fig. S1B). These fluorescence data were well supported by far-UV CD spectra, both in the apo- and holo-forms.

As seen from the unfolding transitions, which binds Ca\textsuperscript{2+} with a relatively high affinity (K\textsubscript{d} 4 μM), Clostrillin is more stable than both Vibrillin and Flavollin (c\textsubscript{50} for apo and holo-Clostrillin is 1.9 and 2.5 M respectively, Δc\textsubscript{50} increased from 3.6 kcal/mol for apoprotein to 5.2 kcal/mol for holo-form) (Fig. 3, E and F; Table 1; supplemental Fig. S1C). Thus, Clostrillin is more stable, and Ca\textsuperscript{2+} aids in enhancing its stability considerably, a phenomenon not observed in Flavollin and Vibrillin. We next present the stability profile of Centillin, which is even more stable than all three domains described above (highly noncooperative unfolding with c\textsubscript{50} 2.3 M) (Fig. 3, G and H; supplemental Fig. S1D). Interestingly, the gain of stability in the presence
of Ca\(^{2+}\) is significantly higher in Centillin (with \(c_{1/2}\) of 3.3 M, \(\Delta G\) 3.6 kcal/mol, which is less than that is seen in case of Clostrilllin because of low cooperative index, \(m\)). In all the four domains described above, there is either no (Vibrillin), very weak (Flavollin), strong (Clostrillin), or very strong (Centillin) stabilization of the domain upon Ca\(^{2+}\)-binding, thus demonstrating a gradient of stability. The molecular determinants of this high variability in the stability of \(\beta\gamma\)-crystallins domains, as well as differential stabilization by Ca\(^{2+}\), are not known. To understand if this differential stability in structurally similar domains is due to the diversity of sequence of the \(\text{(N/D)(N/D)XX(S/T)S}\) motif (both in canonical as well as noncanonical forms), we determined stabilities of the domains carrying mutations (which are natural substitutions found in other orthologues) in the motif (Table 1).

**Domains with Canonical Ca\(^{2+}\)-binding Motifs and Control of Their Stability**

We first focus our attention on all the four Ca\(^{2+}\) co-coordinating positions of the canonical motif.

**Ser (5th Position) Augments the Domain Stability over Thr**

We examined how the presence of Thr at the 5th position in the \(\text{(N/D)(N/D)XX(S/T)S}\) motif in Clostrilllin (T41S or T82S) influences the stability as well as gain by Ca\(^{2+}\)-binding (Fig. 4; Table 1).

Mutating Thr to Ser in either site does not alter domain stability in the apo-form significantly (\(c_{1/2,\text{apo}} 2.1\) M) but enhances the Ca\(^{2+}\)-induced gain in stabilization, more by the mutation at the 1st site, Clostrilllin T41S (\(c_{1/2,\text{holo}} 3.2\) M, \(\Delta G\) 5.9 kcal/mol, \(K_d\) 22.5 \(\mu\)M) (Fig. 4, A and D), than the 2nd site, Clostrilllin T82S (\(c_{1/2,\text{holo}} 2.9\) M, \(\Delta G\) 5.2 kcal/mol, overall \(K_d\) 10 \(\mu\)M), more than that seen in clostillin WT (\(c_{1/2,\text{holo}} 2.5\) M, \(\Delta G\) 5.2 kcal/mol) (Fig. 4, B and E; Table 1). Mutating Thr to Ser at both sites (T41S and T82S) \(K_d\) 12 \(\mu\)M provides greater stability as well as gain in stabilization by Ca\(^{2+}\) (\(c_{1/2,\text{holo}} 3.4\) M, \(\Delta G\) changes from 4.3 (apo) to 6.3 (holo) kcal/mol) (Fig. 4, C and F; Table 1; supplemental Fig. S2, A–C). These data were further supported by far-UV CD experiments (supplemental Figs. S3 and S4).

In the reverse case, mutating Ser to Thr (in Flavollin) decreases domain stability, with more pronounced reduction in mutation at the 1st site (Flavollin S40T), although a greater reduction in Ca\(^{2+}\)-binding affinity is seen in Flavollin S81T \(K_d\) 134 \(\mu\)M) than in Flavollin WT \(K_d\) 33 \(\mu\)M), confirming the above data (Fig. 4, G–I). Domains with Thr (at the 5th position) are less stable, although the presence of Ser elevates the same, suggesting that Ser is favored over Thr for better stability of a domain, and thus it appears to be selected evolutionarily at this position, providing a rationale for why Ser is preferred over Thr in most domains in the natural state.

\(X_1\) Residue and Domain Stability

\(X_1\) residue (the 3rd residue) in the motif coordinates Ca\(^{2+}\) via the backbone carbonyl and is a more variable residue of the
motif. We present here two cases for assessing the role of the nature of this residue in Clostrillin (W39D), where this is Trp (NDWMTS), and in Flavollin (D38V), where this residue is polar Asp (NDDISSS) (Table 1; Fig. 5).

Polar Asp Residue (at 3rd Position) Is Likely to Be Related with Less Stable Domains—Mutating W39D (at 3rd position) in Clostrillin not only results in a drastic reduction of stability \((c_{1/2} \sim 1.1 \text{ M})\) compared with WT Clostrillin \((c_{1/2} \sim 1.9 \text{ M})\) in the apo-form but also abolishes the \(Ca^{2+}\)-induced gain in stability \((\Delta G \sim 0.2 \text{ M}, \Delta G\) changes from 1.5 for apo to 3.0 kcal/mol for holo) (Fig. 5, A and E; supplemental Fig. S2D), despite the fact that \(Ca^{2+}\)-binding is fairly strong in Clostrillin W39D mutant \((K_{d} \sim 31 \mu\text{M})\). Far-UV CD data further support this observation (supplemental Fig. S5, A and B).

Hydrophobic Residue (at 3rd Position) Enhances Domain Stability—A hydrophobic residue augments the stability, however, as seen in the equilibrium unfolding of Flavollin D38V mutant, demonstrating a significantly high domain stability in the apo-form \((c_{1/2} \sim 2 \text{ M})\) when compared with apo-Flavollin WT \((c_{1/2} \sim 1.0 \text{ M})\). Furthermore, we observed that there is no significant gain in stability of the D38V mutant upon binding \(Ca^{2+}\) \((\Delta c_{1/2} \sim 0.1 \text{ M})\) (Fig. 5, B and F; Table 1; supplemental Figs. S2E and S5, C and D). The role of the hydrophobic residue was validated in Centillin, where Arg to Val (R34V) mutation augments the stability in comparison with Centillin WT \((c_{1/2} \sim 3.3 \text{ M})\) for apoprotein and 4.8 M for holoprotein. The stability of the mutant was increased by 3.4 kcal/mol upon \(Ca^{2+}\)-binding \((\Delta G \sim 9.4 \text{ kcal/mol})\) (Fig. 5, D and H; supplemental Fig. S2F). The \(Ca^{2+}\)-binding affinity of Centillin F75D mutant \((K_{d} \sim 56 \mu\text{M})\) also increased 10-fold when compared with Centillin WT \((K_{d} \sim 515 \mu\text{M})\) (Fig. 2, B and C).

Therefore, it is the choice of a residue in the sequence of the \((N/D)(N/D)XX(S/T)S\) motif that is responsible for high or low stability. Because this motif is degenerated in a large majority of proteins expressed in vertebrates (lens \(\beta\gamma\)-crystallin has one functional and three noncanonical motifs), we next scrutinized this nonfunctional motif for its potential as a stability regulator.

**FIGURE 3. Stability gradient across \(\beta\gamma\)-crystallin domains.** Equilibrium unfolding of \(\beta\gamma\)-crystallin domains in the absence or presence of \(CaCl_{2}\). Change in the \(\lambda_{\text{max}}\) and best fits in the two-state unfolding process with [GdmCl] of Vibbrillin (A and B), Flavollin (C and D), Clostrillin (E and F), and Centillin (G and H) proteins. There is a gradual increase in stability (or stability gradient) of the selected domains in apo-form; similar gradient is also seen in terms of gain of stability by \(Ca^{2+}\).
Domains with Noncanonical Motifs and Control of Their Stability

We have seen above how the sequence of the canonical motif dictates domain stability. We now focus on the domains that have the degenerate motifs and thus do not bind Ca\(^{2+}\).

Conversion of Functional Motif (Microbial-type) to Disabled Motif (Vertebrate-type), Ser/Thr to Arg or Lys Augments Domain Stability

Thr to Arg mutation at the 5th position (N/D)(N/D)XX(S/T)S motif) in either site in Clostrillin (T41R or T82R) enhances the stability of the domain significantly (c\(_{1/2}\) 2.6 M, \(\Delta G\), 5.7 kcal/mol) but abolishes the Ca\(^{2+}\)-dependent stability gain (affinity of both mutants with one functional Ca\(^{2+}\)-binding site, \(K_d\) 270 \(\mu M\) for T41R and 47 \(\mu M\) for T82R) (Fig. 6, A, B, E, and F). Converting a canonical to a noncanonical motif by mutating Thr to Arg at both sites (T41R and T82R) abolished Ca\(^{2+}\)-binding as expected, but it increased domain stability to a level par with that seen in WT holo-Clostrillin (c\(_{1/2}\) 2.6 M, \(\Delta G\), 5.7 kcal/mol) (Fig. 6, C and G; Table 1; supplemental Fig. S7, A–C). We confirmed this enhancement of stability when Ser-78 in Centillin was mutated to Arg (FRQ-ISS to FRQIRS) (c\(_{1/2}\) increased to 2.6 M from 2.3 in WT) (Fig. 6, D and H). To examine if this stability is not Arg-specific, we determined the stability of domains with lysine mutations in the place of Arg. Mutating Thr to Lys at the 5th position in the Ca\(^{2+}\)-binding motif of Clostrillin (Clostrillin T41K) also increases the domain stability (c\(_{1/2}\) 2.2 M, \(\Delta G\), 5.5 kcal/mol) as compared with the domain with Arg at the same position (Fig. 7, A and B; supplemental Fig. S7D).

This demonstrates that the stability of a \(\beta\gamma\)-crystallin domain increased when converting from a functional to disabled motif and is thus governed by the motif despite being nonfunctional for Ca\(^{2+}\)-binding. This enhancement in stability of a domain is not Arg-specific; the same was achieved by Lys while impairing Ca\(^{2+}\)-binding. We next examined if converting a nonfunctional motif to a partially functional motif (such as mutating Arg to Ser) would reverse the stability.

FIGURE 4. Stability change in a \(\beta\gamma\)-crystallin domain with minor change in microenvironment of Ca\(^{2+}\)-binding motif. Equilibrium unfolding of Clostrillin mutants (Thr to Ser) and Flavollin mutants (Ser to Thr) in the absence or presence of CaCl\(_2\). Changes in \(\lambda_{max}\) with [GdmCl] concentration are as follows: A, Clostrillin T41S; B, Clostrillin T82S; and C, Clostrillin T41S,T82S; compared with Clostrillin WT. Best fits at two-state transitions and fractions of unfolded states for Clostrillin mutants are as follows: D, Clostrillin T41S; E, Clostrillin T82S, and F, Clostrillin T41S,T82S. Changes in wavelength maxima with increasing [GdmCl] of Flavollin S40T (G), Flavollin S81T (H), and Flavollin S40T,S81T (I) are shown.
Conversion of Disabled Motif (from Microbials) to Functional Motif, Arg to Ser Mutation (at 5th Position) Attenuates Stability

For this purpose, we selected Arg to Ser mutation in two proteins, i.e. Centillin F75D mutant and in nitrollin domains. As seen above (Fig. 5, D and H), in the case of Centillin F75D, a domain with high stability can be designed by mutating the 1st residue to aspartate. This site is nonfunctional because of the presence of Arg (the 5th position in NDRARS motif) at a position generally occupied by Ser in the functional motif. We examined the role of the R36S mutation on stability of Centillin F75D. A sharp decrease in the stability (both apo- and Ca$^{2+}$-dependent) greater than seen in F75D mutant was found in R36S (c$_{1/2}$ decreased to 2.3 from 4.8 m in F75D mutant) which was equal to that of WT protein (Fig. 7, C and D; supplemental Fig. S7E).

For further confirmation of the above results, it was necessary to demonstrate a similar decrease in stability in a more diverged $\beta\gamma$-crystallin. We selected nitrollin because it is highly diverged in a sequence than a microbial $\beta\gamma$-crystallin (more...
similar to a vertebrate-type) and does not have any canonical Ca\textsuperscript{2+}-binding sites (16). After mutating Arg to Ser at the 5th position in one or both ENKVRS and FDNFRS motifs of nitrollin, we observed a significant attenuation of domain stability ($c_{1/2}$ 2.8 M) in comparison with nitrollin WT ($c_{1/2}$ 3.3 M, $\Delta G$ decreased from 6.2 (WT) to 4.6 (mutant) kcal/mol) (Fig. 7, E and F; supplemental Fig. S7). This demonstrates that the stability of a $\beta$-crystallin domain is governed by the noncanonical motif despite being nonfunctional for Ca\textsuperscript{2+}-binding.

Partial Conversion of Vertebrate Nonfunctional Motif to Microbial-type Motif, Arg-77 to Ser Mutation at 5th Position (Cataract Mutation in $\gamma$-Crystallin) Attenuates Stability

To understand the biological consequences of these data in pathophysiology, we explored the role of noncanonical Ca\textsuperscript{2+}-binding sites (16). After mutating Arg to Ser at the 5th position in one or both ENKVRS and FDNFRS motifs of nitrollin, we observed a significant attenuation of domain stability ($c_{1/2}$ 2.8 M) in comparison with nitrollin WT ($c_{1/2}$ 3.3 M, $\Delta G$ decreased from 6.2 (WT) to 4.6 (mutant) kcal/mol) (Fig. 7, E and F; supplemental Fig. S7). This demonstrates that the stability of a $\beta$-crystallin domain is governed by the noncanonical motif despite being nonfunctional for Ca\textsuperscript{2+}-binding.

To examine the effect of R77S mutation, we prepared individual N-terminal domain and its mutant (converted the sequence of the vertebrate-type motif NDSIRS to NDSISS which is a microbial-type). Mutant domain was significantly less stable ($c_{1/2}$ 2.2 M) than its wild-type domain ($c_{1/2}$ 3.0 M, $\Delta G$ 8.3 kcal/mol) (Fig. 8, A–D), suggesting that the conversion of Arg to Ser leads to drastic attenuation in domain stability; thus it should be a reason for its implication in hereditary cataract.

FIGURE 7. Attenuation of stability upon conversion of a naturally disabled Ca\textsuperscript{2+}-binding motif to functional motif. Equilibrium unfolding of Clostrillin T41K, Centillin R36S,F75D, nitrollin R50S,R95S mutants. Comparison of changes in the $\lambda_{\text{max}}$ and their best fits in to two-state unfolding process as follows: A and B, Clostrillin T41K; C and D, Centillin R36S,F75D; E and F, and nitrollin R50S,R95S mutants with [GdmCl] in the absence or presence of CaCl\textsubscript{2}. The $c_{1/2}$ value of Clostrillin T41K was comparable with the holo-form of Clostrillin WT. The significant change in the $c_{1/2}$ value in the holo-state of Centillin F75D mutant (4.8 M) was lost in Centillin R36S,F75D mutant (2.3 M). Similarly $c_{1/2}$ value decreases in nitrollin R50S,R95S (Arg to Ser) (2.8 M) as compared with nitrollin WT (3.3 M).

FIGURE 8. Comparison of stability of N-terminal domain of lens $\gamma$-crystallin and its cataract-related R77S mutant. A, location of Arg-77 in the crystal structure of N-terminal domain of $\gamma$-crystallin (Protein Data Bank code 1hko) in second Greek key motif as marked in figure. B, changes in $\lambda_{\text{max}}$ with [GdmCl] concentration of $\gamma$-crystallin WT and R77S mutant. C, change in ratios of fluorescence intensity at fixed wavelengths (360 and 320 nm) with increasing concentrations of denaturant (GdmCl) of N-terminal domain of $\gamma$-crystallin and its R77S mutant. D, best fit and fractions of unfolded states for the $\gamma$-crystallin WT (single domain) and R77S mutant. The $c_{1/2}$ value 3.0 M, $\Delta G$ decreased from 8.3 ($\gamma$-crystallin WT) to 2.2 ($\gamma$-R77S mutant) kcal/mol. The data were fitted in a two-state transition.
**DISCUSSION**

Evolution of Stability in βγ-Crystallin Superfamily—The vertebrate lens βγ-crystallins are evolved as innately stable proteins (3). Although various factors, such as domain-domain interaction via β-strands and stacking of aromatic pairs, contribute to additional stability in the case of two-domain βγ-crystallins (30–32), the molecular basis and adaptive evolution of this high thermodynamic stability in the vertebrate members of the superfamily are not known. In this comprehensive analysis of chemical stability of βγ-crystallins, we have reported the presence of a stability gradient in domains, ranging from low to ultra-stable domains (Fig. 9A).

Functional Motif-operated Stability in Microbial βγ-Crystallins—We have demonstrated that high variability in stability between members is due to differences in the sequence of the (N/D)/(N/D)/XX(S/T)S motif. In addition, there is a large variation in the extent of the gain of domain stability (from no gain to high gain) upon binding Ca2+. Thus, in some members, Ca2+ acts as an extrinsic stabilizer. The design of a domain with the required stability is facilitated by the choice of residues in the motif sequence; the presence of Asp at the 1st position or a hydrophobic residue at the 3rd position is more likely associated with a more stable domain (for scheme see Fig. 9B). We have also demonstrated that the frequent choice of Ser over Thr at the 5th position in most canonical motifs is more appropriate for higher domain stability. A polar residue at the 1st position (1st N/D residue) is associated with ultra-high stability (Fig. 9). This residue participates in indirect coordination with Ca2+ via water in the opposite Greek key motif. When Ca2+ forms a complex with protein, the solvent (water) must be displaced from the binding site, which would require more energy, and thus the domain would be more stable in holo-form. Similarly, we have shown how a stable domain could be converted to a domain with comparatively low stability using an appropriate residue.

Therefore, it is possible to speculate that such replacements in the motif may have been selected for desired stability required for their functions. This would account for the occurrence of βγ domains in nature with the entire range of stability. This stability gradient would be in accordance with the dissimilar functions of a domain in the species, leading us to compare this feature to a stability switch that is off in some domains, while being on in other domains.

Vertebrate βγ-Crystallins Acquire High Innate Stability at the Cost of Ca2+-Binding—High variability in domain stability in microbial homologues is created by minor differences in the canonical sequence of the (N/D)/(N/D)/XX(S/T)S motif of Ca2+ binding. Because most vertebrate members do not have the canonical motif and appeared later in evolution, it would be of interest to know if a similar control in the stability of the domains with nonfunctional motifs would be observed when there is no Ca2+ binding. It is noteworthy to mention that there is a high degree of variations in the sequence of noncanonical motifs. In lens β2-crystallin, one motif out of the four is functional, which is responsible for the weak Ca2+-binding seen in this protein. In noncanonical motifs, the 5th position, occupied by Ser/Thr in the canonical motif, is often occupied by Arg, and thus no binding of Ca2+ is seen. Therefore, we can equate that replacing the 5th Ser by Arg not only results in a degenerate motif but also leads to the partial conversion of a microbial-type to a vertebrate-type domain. In converting a functional motif to a degenerate motif by mutating Ser to Arg or Lys, Ca2+ binding is disabled, but the stability of the domain is augmented significantly (to almost equal that seen in the case of the Ca2+ bound form). The converse is also true as Arg to Ser replacement at the same position decreases the domain stability.

Mutations in the Stability Knob (Degenerate Noncanonical (N/D)/(N/D)/XXRS Motif) of Lens βγ-Crystallins, Stability and Cataract—We propose a model for how stability has evolved in vertebrate βγ-crystallins over their microbial relatives, including the eye lens crystallins (Fig. 9B). The additional stability in βγ-crystallins following a gene duplication event was acquired at the cost of Ca2+ binding, such as the selection of arginine in place of serine at the 5th position of the motif. The importance of such replacements (at the 5th position of the motif) that can be seen as the mutation of the highly conserved Arg-77 to Ser in

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**FIGURE 9.** A, domain stability controlled by the choice of residues in the 1(N/D)/(N/D)/XX(S/T)S motif. Changes in transition midpoint (ΔC1/2) of βγ-crystallin domains calculated as the difference between c1/2 of mutant and its WT protein for both apo- (gray) and holo (lines) forms (Table 1). B, schematic representation demonstrating the (N/D)/(N/D)/XX(S/T)S motif as the stability tuning knob in βγ-crystallins. Hydrophobic residue at X(1) position augments the stability, although a polar residue turns off the stability. Ser residue at the 5th position in most canonical motifs is more appropriate for higher domain stability. A polar residue at the 1st position enhances the stability. Arg at the 5th position in noncanonical motifs (in vertebrate members) provides more stability to a domain.
lens γD-crystallin leads to autosomal dominant cataracts apparently by decreasing solvent accessibility (28). Our results confirm the importance of this position because this is among the arginines that were recruited in vertebrates as replacements for serine in microbial homologues, thus changing the crystallin-like properties such as domain stability (Fig. 9). We are not contesting other factors contributing to the stability of two-domain proteins, such as inter-domain interactions, etc. (31). Our analysis of known mutations in lens βγ-crystallins implicated in cataracts (reviewed in Refs. 27–29) demonstrates startling facts; many of these mutations leading to cataracts are located in this motif of six amino acid residues. The cause of the cataract can be easily explained based on our work because a βγ domain with a mutation in this region would at least drastically influence the stability.

Through this analysis, we have not only provided a rationale for the existence of high diversity in the stability in the structurally similar βγ-crystallin domains but also how evolutionary remodeling came about to provide the innate stability seen in vertebrate crystallins. The logic is that the vertebrates attained stability without involving Ca2+-binding. It is concluded here that this (N/D)(N/D)XX(S/T)S motif not only in canonical form but also in degenerate form acts as a stability regulatory switch. Any modification (mutation) in the motif sequence in lens βγ-crystallins would cause cataract, since domain properties (such as stability) are likely to be directly affected. Divergence in the motif sequence during evolution gave rise to new proteins (i.e. vertebrate βγ-crystallins) with elevated stabilities. We, for the first time, elucidate the conservation of elements controlling domain stability between canonical Ca2+-binding βγ-crystallins from microbial and vertebrate homologues with the noncanonical motif.

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REFERENCES
1. Blundell, T., Lindley, P., Miller, L., Moss, D., Slingsby, C., Tickle, I., Turnbull, B., and Wistow, G. (1981) Nature 289, 771–777
2. Wistow, G., Turnell, B., Summers, L., Slingsby, C., Moss, D., Miller, L., Lindley, P., and Blundell, T. (1983) J. Mol. Biol. 170, 175–202
3. Jaenicke, R. (1994) Naturwissenschaften 81, 423–429
4. Jaenicke, R., and Slingsby, C. (2001) Crit. Rev. Biochem. Mol. Biol. 36, 435–499
5. Bloemendal, H., de Jong, W., Jaenicke, R., Lubsen, N. H., Slingsby, C., and Tardeul, A. (2004) Prog. Biophys. Mol. Biol. 86, 407–485
6. Wistow, G. J., and Piatigorsky, J. (1988) Annu. Rev. Biochem. 57, 479–504
7. Lubsen, N. H., Aarts, H. J., and Schoenmakers, J. G. (1988) Prog. Biophys. Mol. Biol. 51, 47–76
8. Kappé, G., Purkiss, A. G., van Genesken, S. T., Slingsby, C., and Lubsen, N. H. (2010) J. Mol. Biol. 71, 219–230
9. Wistow, G., Summers, L., and Blundell, T. (1985) Nature 315, 771–773
10. Lapatto, R., Nalini, V., Bax, B., Driessen, H., Lindley, P. F., Blundell, T. L., and Slingsby, C. (1991) J. Mol. Biol. 222, 1067–1083
11. Bagby, S., Harvey, T. S., Eagle, S. G., Inouye, S., and Ikura, M. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 4308–4312
12. Clout, N. J., Basak, A., Wieligmann, K., Bateman, O. A., Jaenicke, R., and Slingsby, C. (2000) J. Mol. Biol. 304, 253–257
13. Clout, N. J., Kretschmar, M., Jaenicke, R., and Slingsby, C. (2001) Structure 9, 115–124
14. Shimeld, S. M., Purkiss, A. G., Dirks, R. P., Bateman, O. A., Slingsby, C., and Lubsen, N. H. (2005) Curr. Biol. 15, 1684–1689
15. Aravind, P., Mishra, A., Suman, S. K., Jobby, M. K., Sankaranarayanan, R., and Sharma, Y. (2009) Biochemistry 48, 12180–12190
16. Aravind, P., Suman, S. K., Mishra, A., Sharma, Y., and Sankaranarayanan, R. (2009) J. Mol. Biol. 385, 163–177
17. Wenk, M., and Mayr, E. M. (1998) Eur. J. Biochem. 255, 604–610
18. Wenk, M., and Jaenicke, R. (1999) J. Mol. Biol. 293, 117–124
19. Kretschmar, M., Mayr, E. M., and Jaenicke, R. (1999) J. Mol. Biol. 289, 701–705
20. Srivastava, A. K., Sharma, Y., and Chary, K. V. (2010) Biochemistry 49, 9746–9755
21. Kaiser, D., Manoil, C., and Dworkin, M. (1979) Annu. Rev. Microbiol. 33, 595–639
22. Roshan, M., Vijaya, P. H., Lavanya, G. R., Shama, P. K., Santhiya, S. T., Graw, J., Gopinath, P. M., and Satyamoorthy, K. (2010) Mol. Vis. 16, 887–896
23. Barnwal, R. P., Jobby, M. K., Devi, K. M., Sharma, Y., and Chary, K. V. (2009) J. Mol. Biol. 386, 675–689
24. Hung, H. C., Chen, Y. H., Liu, G. Y., Lee, H. J., and Chang, G. G. (2003) Bull. Math. Biol. 65, 553–570
25. Pace, C. N. (1986) Methods Enzymol. 131, 266–280
26. Mishra, A., Suman, S. K., Srivastava, S. S., Sankaranarayanan, R., and Sharma, Y. (2011) J. Mol. Biol. doi: 10.1016/j.jmb.2011.10.037
27. Kannabiran, C., and Balasubramanian, D. (2000) Indian J. Ophthalmol. 48, 5–13
28. Hejtmanek, J. F. (2008) Semin. Cell Dev. Biol. 19, 134–149
29. Graw, J. (2009) Exp. Eye Res. 88, 173–189
30. Kong, F., and King, J. (2011) Protein Sci. 20, 513–528
31. Das, P., King, J. A., and Zhou, R. (2010) Protein Sci. 19, 131–140
32. Palme, S., Slingsby, C., and Jaenicke, R. (1997) Protein Sci. 6, 1529–1536