βγ-Crystallin is a superfamily with diverse members from vertebrate lens to microbes. However, not many members have been identified and studied. Here, we report the identification of a putative exported protein from *Yersinia pestis* as a member of the βγ-crystallin superfamily. Even though calcium has been known to play an important role in the physiology and virulence of the *Yersinia* genus, calcium-binding proteins have not yet been identified. We have studied the calcium-binding properties of two of the three crystallin domains present in this putative exported protein designated “Yersinia crystallin.” These two domains (D1 and D2) have unique AA and BB types of arrangement of their Greek key motifs unlike the domains of other members of the βγ-crystallin superfamily, which are either AB or BA types. These domains bind two calcium ions with low and high affinity-binding sites. We showed their calcium-binding properties using various probes for calcium and the effect of calcium on their secondary and tertiary structures. Although both domains bind calcium, D1 underwent drastic changes in secondary and tertiary structure and hydrodynamic volume upon calcium binding. Domain D1, which is intrinsically unstructured in the apo form, requires calcium for the typical βγ-crystallin fold. Calcium exerted an extrinsic stabilization effect on domain D1 but not on D2, which is also largely unstructured. We suggest that this protein might be involved in calcium-dependent processes, such as stress response or physiology in the *Yersinia* genus, similar to its microbial relatives and mammalian lens crystallins.

Crystallins are the major structural proteins of the lens. Three major crystallins, α-crystallin, β-crystallins, and γ-crystallins, are known to be present ubiquitously in mammalian lenses (1). β- and γ-crystallins share structural similarities and are classified together as βγ-crystallins (1). Topologically, β- and γ-crystallins are made up of two globular domains; each domain consists of two Greek key motifs arranged as four-stranded antiparallel β-sheets. The domain of these proteins is referred to as the crystallin fold or β motif. Two types of Greek key motifs are described, “A-type” and “B-type,” which are present as either AB or BA combinations.

The βγ, or Greek key, motif was found in several other non-lens proteins, and they were all classified as being within the βγ-crystallin superfamily. Some members of this superfamily are Protein S (2), Spherulin 3a (3, 4), *Streptomyces* killer toxin-like protein (SKLP) (5), cargo proteins from *Tetrahymena thermophila* (6), AIM1 (absent in melanoma) (7), epidermis differentiation-specific proteins (8, 9), yeast killer toxin WmKT (10), and *Streptomyces* metalloproteinase inhibitor (SMPI) (11). These members show structural similarity despite relatively low sequence identity, which reflects the functional diversity found among these proteins.

Two microbial homologues of βγ-crystallins, Protein S and Spherulin 3a, have been studied extensively (for review, see Ref. 12). Protein S is a two-domain protein, whereas Spherulin 3a is one-domain protein present as a dimer. These proteins share the intrinsic thermodynamic stability of γ-crystallins. Under drastic conditions of starvation, desiccation, or heavy metal exposure, the slime mold *Physarum polycephalum* sporulates with a high level expression of Spherulin 3a (13). Similarly, upon stress, soil bacterium *Mycococcus xanthus* differentiates into spores protected by a spore coat containing a high level of Protein S (14). Both proteins are known to bind calcium at the homologous sites (15, 16). The calcium-binding sites are located at the Greek key motif, as depicted in the schematic diagram (Fig. 1). The stability of these proteins is increased severalfold upon the binding of calcium, suggesting a calcium-dependent protective role of these proteins under adverse conditions (12).

Other than Protein S and Spherulin 3a, γ-crystallin and AIM1 are known to bind calcium ions (17, 18), whereas others, such as SKLP (5), WmKT (10), and SMPI (11) have not been shown to bind calcium. The calcium-binding property of members of this protein family is, thus, debated. It is, therefore, of immense interest and importance that new members be identified and analyzed for their calcium-binding properties.

Rapid increase in the number of genomes sequenced in the recent past led us to look for more βγ-crystallin members. Because the genome sequence of *Yersinia pestis* was completed (19), we were interested in knowing whether microbial homologues of βγ-crystallins are present in this pathogen. *Y. pestis* is the causative agent of the dreaded disease plague. *Yersinia* genus consists of three species pathogenic to humans and animals, namely *Y. pestis*, *Yersinia enterocolitica*, and *Yersinia pseudotuberculosis*. *Y. pestis* is the most fatal of all three and has caused more numbers of deaths than any other disease (20) and is a threat because of its potential use as a bioweapons agent. It has been known for a long time that *Y. pestis* is unable to grow at 37 °C in calcium-depleted medium (21), which is known as a low calcium response. Loss of this property makes the bacterium avirulent. Calcium level also affects the physiology and metabolism of *Y. pestis* during growth at 37 °C (22, 23); still no calcium-binding protein from this pathogen has been identified.
The calcium-binding microbial homologues of crystallins, if any, in \textit{Y. pestis}, with analogy to microbial \(\beta\)-crystallins, could be a possible candidate for stress response encountered during the various phases of the life cycle in animals and humans. We, therefore, searched the genome sequence of \textit{Y. pestis} CO92 and found a putative exported protein (locus tag YPO2884) and a hypothetical protein (locus tag YPO0466) as \(\beta\)-crystallins, containing three and two \(\beta\)\textit{y} domains, respectively.

In this context, we studied two of the three \(\beta\)-crystallin domains present in the sequence of a putative exported protein, which we refer to as “Yersinia crystallin.” Yersinia crystallin domains show novel Greek key motif combinations (AA and BB types), which have not been found in other crystallin domains. Our data showed that domains of Yersinia crystallin bind calcium with micromolar affinity. We report that calcium was required for the proper fold and stability of one of the domains. We also report the presence of a homologue and a paralogue of Yersinia crystallin that we have identified in this pathogen and another microorganism.

**EXPERIMENTAL PROCEDURES**

Identification and Selection of \(\beta\)-Crystallin Domains from \textit{Y. pestis}—Using \(\gamma\)-crystallin and other members of the crystallin superfamily as a template sequence, we performed a BLAST program (24) search against the genome sequence of the \textit{Y. pestis} strain CO92. The signature sequence XXXXX/FYYXG forms the beginning of a crystallin-type Greek key motif about 35 residues in length with a conserved Ser at the 34th position in a stretch of (D/N)X...XXG forms the beginning of a crystallin-type Greek key motif occurring in pairs. We obtained two proteins with genuine Greek key motifs occurring in pairs. These proteins were annotated as the putative exported protein (GenBank accession number NP_406389, locus tag YPO2884) and the hypothetical protein (GenBank accession number NP_404108, locus tag YPO0466). Two single domains of Yersinia crystallin, D1 (first) from residue 21 to 112 (92 residues) and D2 (second) from amino acid 110 to 215 (107 residues) were selected.

Molecular Modeling—Molecular modeling was performed either using the web-based Swiss Modeler server (25) or the three-dimensional PSSM fold recognition server (26). The nearest homologue for D1 was the N-terminal domain of Protein S (Protein Data Bank (PDB) code 1hdf) (27), and the nearest homologue for D2 was Spherulin 3a (PDB code 1hdf) (32% identity and 49% similarity) (27). The initial coordinates generated were used as a template for molecular modeling. Similarly, the final model of D2 had residues from Lys-2 to Phe-104 and the root mean square deviation at C\textalpha{} atoms was 1.873 \(\AA\) compared with the template structure. The final model of D2 was decalcified with Chelex-100-treated buffer. All the buffers used in the studies were treated with Chelex-100 (Bio-Rad) to remove calcium.

**Ca Overlay Assay**—The assay was performed as described earlier (30) with minor modifications. 50 \(\mu\)g of protein was spotted on a nitrocellulose paper and washed with buffer containing 20 mM Tris (pH 7.0) and 50 mM NaCl. The blots were incubated in this buffer containing 1 \(\mu\)Ci \(^{45}\text{Ca}^\text{Cl}_2\) (PerkinElmer Life Sciences) for 10 min at room temperature. The blot was then washed three times with 50% ethanol, air-dried, and scanned in a phosphorimaging device (Fuji FLA-3000).

**Determination of Macroscopic Binding Constants**—Chromophoric calcium chelator BAPTA tetra potassium salt (Molecular Probes) was used to determine the macroscopic calcium-binding constants for the domains as described earlier (31). Chelex-100-treated buffer (10 mM Tris-Cl (pH 7.0) containing 10 mM KC\textalpha{}) was used at 25 °C in a 1-ml quartz cuvette. Protein concentrations used were 20–30 \(\mu\)M. Aliquots from stock calcium solution were added, and absorbance at 263 nm was read in a Shimadzu UV-visible 1601 spectrophotometer. The titration data were analyzed using the Caligator software (32), which uses a Levenberg-Marquardt non-linear curve-fitting routine. The data were best fit to a two-site model. The macroscopic dissociation constants from the best fit to data were used.

**Fluorescence Spectroscopy**—Fluorescence emission spectra were recorded on a Hitachi F-4500 spectrofluorometer. The cuvettes were soaked in EDTA solution and were rinsed with Chelex-100-treated MQ-water (Millipore) and dried before use. The spectra were recorded in the correct spectrum mode of the instrument using excitation and emission band passes of 5 nm. All measurements were done at room temperature.

**Circular Dichroism (CD) Spectroscopy**—Far- and near-UV CD spectra of both domains were recorded at room temperature on a Jasco-715 spectropolarimeter using 0.01 and 1 cm path-length cuvettes, respectively. Secondary structure fractions from far-UV CD spectra were calculated using the CDP software package (33), which uses multiple algorithms and CDNN based on neural networks (34).

**Terbium Binding**—Terbium binding to both the domains was carried out using a terbium nitrate solution in water. The stock solution was used at 25 °C in a 1-ml quartz cuvette. Protein concentrations used were 20–30 \(\mu\)M. Aliquots from stock calcium solution were added, and absorbance at 263 nm was read in a Shimadzu UV-visible 1601 spectrophotometer. The titration data were analyzed using the Caligator software (32), which uses a Levenberg-Marquardt non-linear curve-fitting routine. The data were best fit to a two-site model. The macroscopic dissociation constants from the best fit to data were used.

\(^1\)The abbreviations used are: DTT, dithiothreitol; CD, circular dichroism; BAPTA, 1,2-bis-(2-aminophenoxy)ethane-N,N,N',N''-tetra-acetic acid; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol.
A type motif

| Protein | Sequence | N-terminal | Ref. | 
|---------|----------|------------|------|
| YPO2884 | 25 KVCFTMDYGESLCAAQGNSVASIDK----------WDRRISSISIPHG | **D1** | 65 |
| YPO2884 | 69 VVTYTEDVFLGASRSPFAEDVLVSDKLDLYL----------NNDISAFKIKK | | 115 |
| YPO2884 | 269 KVKNYKLFDFGKEKHEQGFLFNS----------KSSLNSFLRIPNGG | | 308 |
| YPO4066 | 82 KYKFCLETFQGSKYQGQDJCQKQGFMPSL----------NDKVTAIRLYG | | 122 |
| PBBR0866 | 125 YVKYVEYHGQWNGSTWVQDTPYTRLRL----------SDISSSFLLERT | | 165 |

B type motif

| Protein | Sequence | N-terminal | Ref. | 
|---------|----------|------------|------|
| YPO2884 | 112 AVCFYGSEGFTGDACLCSLSGQFDLYRGNPEPKSKSHLVNPINDVEYSIKIPPP | **D2** | 165 |
| YPO2884 | 167 QQHVEYDDNYKYFVFVTD-----EDY---TPDLDLII----------RNNKNISSMSVQSGDE | | 210 |
| YPO2884 | 222 KSIFKIKQSKGSPSWIRDPFRR-----IKY--RDILDFQGSLSDTDDYISIKFDEG | | 256 |
| YPO4066 | 113 AVCFYEDTFQGSDALCSQMSQHDLYSDAQFANSNRTVP3HNSIQSKVPFLG | | 167 |
| YPO4066 | 169 MTTYMKFDQPMNPFPTL-----ENINLLETKLMNSDQMTLKSASEK | | 213 |
| PBBR0866 | 168 FACLQYAMGYDGTPWCMAM-----GGEIDMGMMAELFT----------NEMSSVFLSANA | | 211 |
| PBBR0866 | 215 SALTTRIVTMNRVSUTLIRL-----GMLKGDGPNDEANSFRYVTRQPST | | 256 |

**FIG. 2. A- and B-type Greek key motif.** The sequence of D1 and D2 is aligned with the Greek key crystallin motif found in the paralogue and homologue. Locus tags YPO2884, YPO4066, and PBBR0866 represent a hypothetical protein (Y. pestis), and hypothetical protein (P. profundum) respectively. Underlined sequences represent residues with high homology. Putative residues involved in calcium binding are indicated by an asterisk. a, b, c, and d represent the four β strands of a Greek key motif.

out on a Hitachi F-4500 spectrofluorometer. The excitation wavelength was 285 nm with band passes of 5 nm for both excitation and emission. The titration buffer consisted of 10 mM BisTris (pH 6.7) and 10 mM KCl. Dissociation constants of the protein for terbium were calculated by non-linear curve fitting to the following equation (35) using the program Microcal Origin 6.0.

\[
F = \frac{(F_{\text{max}} - F_{\text{min}})[\text{Tb}]}{[\text{Tb}]} + F_{\text{min}}
\]  

where \(F, F_{\text{max}}, \) and \(F_{\text{min}}\) represent the fluorescence intensity at a given point, at saturation and without terbium, respectively. \([\text{Tb}]\) is the free terbium concentration at a given point, which was calculated using the following equation.

\[
[Tb]_t = \frac{[Tb]_0 - ([P][F - F_{\text{min}}])}{(F_{\text{max}} - F_{\text{min}})}
\]

\(P\) is the total protein concentration.

Hydrodynamic Volume—Hydrodynamic volumes of the domains were determined using a Superdex G-75 (Amersham Biosciences) column, calibrated using standard molecular weight markers. Each domain was run with EDTA or with calcium added to a final concentration of 1 and 10 mM respectively. The buffer used was 50 mM HEPES (pH 7.5), 100 mM KCl for cross-linking reaction.

Glutaraldehyde Cross-linking—Glutaraldehyde (Sigma) was used for cross-linking studies. 100 mM Tris-Cl (pH 7.5), 100 mM KCl, 1 mM EDTA and 1 mM DTT. EDTA was replaced with 10 mM CaCl2 for holoproteins.

Molecular Modeling—Our attempts to crystallize these domains for structure determination failed, and therefore we cooling was quenched by the addition of 1M Tris-Cl (pH 7.5) at the end of incubation. SDS-PAGE sample buffer was added and the sample boiled for 5 min at 100 °C. The samples were analyzed on a 15% SDS-polyacrylamide gel, and the gel was stained using Coomassie Brilliant Blue R-250.

**RESULTS AND DISCUSSION**

**Sequence Analysis and Identification of Yersinia Crystallin**—We identified a putative exported protein (locus tag YPO2884) from Y. pestis strain CO92 as a member of the β-crystallin superfamily. This 806 amino acid protein has a 22 amino acid long secretion signal at the N-terminal, suggesting possible localization in periplasm. We identified six Greek key motifs in their N-terminal ends organized as three β-crystallin domains. The last two Greek key motifs are variants. This is the first multidomain microbial crystallin. The long C-termina

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2 A. Vezzi, S. Campanaro, M. D’Angelo, F. Simonato, N. Vitulo, F. Lauro, A. Cestaro, G. Malacrida, B. Simionati, N. Cannata, D. Bartlett, and G. Valle, genome analysis of *Photobacterium profundum* revealing the complexity of high pressure adaptations; direct submission to the EMBL/GenBank™/DDBJ data bases, 2004.
could not pursue solving their three-dimensional structure. We
built homology models of both of the domains. Modeled struc-
tures of D1 and D2 were typical of $\beta\gamma$-crystallin domain, having
four anti-parallel $\beta$ strands (Fig. 3). We calculated the sec-
dondary structure composition from our models using the MOLE-
MAN 2 software program, which indicated the prevalence of sheet (47 and 49%) and loop (46 and 47%) in D1 and D2,
respectively, with the remainder constituting a helical region
(5.5 and 3.2%, respectively). D2 has a short stretch of $\alpha$-helix in
the loop. Spherulin 3a also has a helical segment (GLNDVV
from amino acids 43–48) in its first Greek key motif. A homol-
ogous sequence PLNDEV from amino acids 152–157 is present
in this domain forming an $\alpha$-helical stretch. It is seen that D1
and D2 share structural characteristics with $\beta\gamma$-crystallins
from vertebrate eye lens.

Cloning and Purification of Individual $\beta\gamma$-Crystallin Do-
 mains (D1 and D2) of Yersinia Crystallin—The domain D1
 corresponding to 22–112 amino acids of Yersinia crystallin was
cloned, expressed, and purified as a soluble protein of 10.5 kDa
in E. coli (Fig. 4). The D2 domain corresponding to residues
110–215 of Yersinia crystallin was cloned and purified as a
12.7 kDa protein from inclusion bodies. We have also purified
the domain D2 from soluble fraction for comparison, although
the yield was very poor.

The $\beta\gamma$-Crystallin domain is an independent fold, and there
are many single domain proteins, such as SMPI, WmKT, and
Spherulin 3a. As seen from sequence analysis, D1 and D2
 appeared to have different features. To understand the prop-
eties at domain level, we studied them separately as they
exhibited novel Greek key combination. Their domain-domain
interaction and folding could have been affected in a multi-
domain protein. Moreover, only single domain studies would
establish that individual domains bind calcium, which could not
have been ascertained from a two or three domain protein. To
examine whether these domains bind calcium, we performed a
number of assays for studying calcium-binding properties.

Direct Calcium Binding to D1 and D2 by $^{45}$Ca Overlay As-
say—We investigated calcium binding by dot-blot calcium over-
lay method (30). Direct assay using radioactive $^{45}$Ca revealed
that both domains, D1 and D2, bind calcium as shown in Fig. 5.
Bovine serum albumin was used as a negative control and
exhibited novel Greek key combination. Their domain-domain
interaction and folding could have been affected in a multi-
domain protein. Moreover, only single domain studies would
establish that individual domains bind calcium, which could not
have been ascertained from a two or three domain protein. To
examine whether these domains bind calcium, we performed a
number of assays for studying calcium-binding properties.

Calcium Dissociation Constants of D1 and D2—We have
determined the dissociation constants (kDa) of calcium for
these domains using the chromophoric calcium chelator
BAPTA. The titration data for both domains were best fit to a
two-site model using Caligator software (32). Both domains
bind calcium in submicromolar and submillimolar range. There
are high ($K_d$ for D1 is $3.923 \times 10^{-7}$ M, and for D2, $K_d$ is
$9.504 \times 10^{-6}$ M) and low affinity site ($K_d$ for D1 and D2 are
$1.681 \times 10^{-4}$ M and $3.298 \times 10^{-4}$ M, respectively) in both do-
 mains (Table I). Such low and high affinity sites have also been
described for Protein S and Spherulin 3a (27, 42, 43).

Calcium-induced Conformational Changes of D1 and D2 by
Fluorescence—The lone Trp of D1 was found to be solvent-
exposed showing an emission maximum at 352 nm. Upon the
addition of calcium, the emission maximum blue shifted from
352 to 338 nm accompanied by a $>2$-fold increase in the emis-
sion intensity (Fig. 6a). Sequence alignment with Spherulin 3a
reveals that this Trp-54 is in the vicinity (WNDRISS) of the
first putative calcium-binding site (Fig. 2). Domain D2 also
shows an emission maximum of 352 nm, suggesting that the
engineered Trp is solvent-exposed, and the protein is likely to
be unstructured. Unlike the D1, there was no shift in the
emission maxima of D2 upon the addition of calcium. However,
there was an 8% decrease in the fluorescence intensity (Fig.
6b). magnesium ions did not affect the fluorescence spectra
of D1 and D2, suggesting ionic specificity (data not shown).
A second derivative UV light absorption spectrum of calcium-
bound D1 also showed a red shift, suggesting that Tyr and Trp
are buried in the holoform of D1 (data not shown). Thus,
calcium binding affects the microenvironment of Trp in D1 but not in D2.

**Effect of Calcium on Far-UV CD spectra of D1 and D2**—Far-UV CD of apoD1 showed a large negative ellipticity peak at 200 nm, suggesting that the protein is intrinsically unstructured (44), which disappeared on calcium addition and emerged as a positive peak (Fig. 7a). This CD spectrum of holo-D1 was in the β-sheet conformation with a characteristic negative peak at about 215 nm. CDPro program analysis showed that D1 in the apo form had 9.85% helix, 22.23% β-sheet, 24.93% turns, and 44.16% unordered region (Table II). Calcium binding to D1 increased β-sheet content by 2-fold to 42.4%, largely at the expense of the unordered fraction, which reduced to 29.66%. CDNN output showed that this increase was largely in the antiparallel β-sheet content of the protein (data not shown). The β-sheet content of the holoform of D1 correlates with the secondary structure fractions calculated from the model. Tertiary structure class analysis using the CLUSTER program (45) also predicts that apoD1 is denatured, and calcium-loaded D1 is an all-β protein also supported from the homology model. Far-UV CD spectra of domain D2 showed a negative trough at ~200 nm with a negative peak at 220 nm (Fig. 7b). The secondary structure analysis of the spectra showed that the protein had 31.83% β-sheet, 35.2% unordered, and 23% turns, with 9.8% helix (Table II). Unlike D1, there were no significant changes in the CD spectra upon titration with calcium, suggesting that calcium does not induce any perturbation in the conformation of D2 protein (Fig. 7b).

**Effect of Calcium on Near-UV CD Spectra of D1 and D2**—Near-UV CD of apoD1 showed a very weak signal, confirming that the protein was in an unstructured state. Upon the addition of calcium, two strong bands emerged in the spectra of the holoprotein, one at ~290 nm for the $\lambda_{\text{em}}$ band of Trp and a broad peak ~255–270 nm, with peaks at 262 and 268 nm characteristic of Phe (Fig. 8). Near-UV CD of the D2 domain showed a broad band between 280 and 290 nm for Trp and Tyr with weak ellipticity (data not shown). The addition of calcium had no significant effect on the near-UV CD of D2, suggesting that this protein did not undergo conformational changes upon cation binding (data not shown), similar to the γ-crystallin (17) and AIM1-g1 domain (18). Because we purified D2 from an inclusion body, it is likely that this domain failed to refold. To rule out this possibility, we also used the protein purified from the soluble fraction and obtained similar results.

**Terbium-binding properties of D1 and D2**—The luminescent calcium probe terbium was used to study calcium binding to both domains. When excited at 285 nm, terbium bound to D1 showed two fluorescence peaks, at 491 and 547 nm, due to energy transfer from the excited Trp and Tyr (Fig. 9a). However, Trp fluorescence decreased to 80% of the maximum with a blue shift of ~10 nm to 340 nm. When calcium was added to the terbium-saturated D1, it decreased the terbium fluorescence at 547 nm, whereas Trp fluorescence increased by 47%. In an independent experiment, terbium was not able to displace calcium bound to D1 (data not shown).

Terbium binding to D2 enhanced fluorescence at 547 and 491 nm (Fig. 9b). Terbium binding to D2 shifted the wavelength maxima of Trp fluorescence from 350 to 342 nm. The change of fluorescence intensity by terbium at 547 nm was 8-fold for D2 compared with 3-fold for D1, which could be attributed to the presence of Tyr-157 in one of the calcium-binding sites of D2. This Tyr-157 possibly contributes side chain oxygen for liganding cation, also indicated by sequence alignment (Fig. 2). The dissociation constant, $K_D$ of D1 and D2 for terbium was calculated to be 124 and 3.1 μM, respectively (Table I). The addition of calcium to terbium-saturated D2 resulted in the reduction of intensity at 547 nm to ~30% of the highest value. Unlike D1, terbium was able to displace calcium bound to D2 (data not shown). This displacement of calcium by terbium might be due to the lower affinity of D2 for calcium compared with that of D1. The lower affinity of D2 for calcium was also reflected in the macroscopic binding constant (Table I).

**Hydrodynamic Volume and Chemical Cross-linking Studies**—To study the effect of calcium on the hydrodynamic volume...
of Yersinia crystallin domains, we performed analytical gel filtration chromatography. In the apo form, D1 eluted at a volume higher than expected for a monomer (Fig. 10a). The presence of calcium drastically decreased the hydrodynamic volume, and the elution volume corresponds to a monomer (Fig. 10a). Thus, there is significant change in the Stokes radius of the D1 upon calcium binding, which could be attributed to the transition of the protein from an unstructured to a structured state and the resulting compaction (44, 46). There was no significant change in the hydrodynamic volume of D2 upon calcium addition (data not shown).

To identify the protomers of D1, we carried out chemical cross-linking studies. Fig. 10b shows the glutaraldehyde cross-linked D1 domain. As seen in lanes 2 and 4, in the absence of
calcium, D1 is largely a monomer, whereas the addition of calcium resulted in the formation of a predominantly monomeric species in addition to dimer, trimer, and higher forms to a lesser extent (Fig. 10b, lanes 3 and 5). Thus, apoD1 is actually a monomer with a large hydrodynamic volume expected for an intrinsically unstructured protein (44), whereas the domain is compact in calcium-bound form; the holo-D1 has low hydrodynamic volume as also seen from Fig. 10a. The higher mobility of apoD1 could be due to intramolecular cross-linking. The discrepancy between the results of gel filtration and chemical cross-linking can be attributed to the way in which both techniques separate protomers.

Putative Calcium-binding Sites—Based on the similarities in the sequences of D1 and D2 with Protein S and Spherulin 3a, we propose homologous calcium-binding sites in D1 and D2 located in the Greek key motifs (Fig. 2). The first residue that coordinates calcium is from the “a” strand (residue next to the first conserved aromatic residue); the other three residues are from the loop between strands “c” and “d” (near the conserved Ser at the stretch ((D/N)-X-X-S) of the Greek key motif. We strongly suggest that all paralogues and homologues of this protein would bind calcium at the putative sites shown in Fig. 2.

Our studies established that both the domains showed properties not predicted from their modeled structure. From UV, fluorescence, and CD spectral studies, we conclude that the homology model of D1 holds good only for the holoform, whereas biophysical properties of D2 do not match with those expected from the model. This large deviation from the predicted structure and properties might be attributed to the unique Greek key combination and sequence variation.

Calcium Binding, Domain Stability, and Possible Functions—β- crystallins have high intrinsic stability, which these domains lack in the apo form. Removal of calcium from D1 destabilized the protein, suggesting its extrinsic stabilizing effect, whereas D2 showed no such characteristics. Two microbial homologues, Protein S and Spherulin 3a, also show the high extrinsic stabilization effect by calcium ions (42, 47), but they have intrinsic stability.
The life cycle of *Y. pestis* involves phases in flea, rat, and humans. During the colonization in these hosts, the bacterium is exposed to various drastic conditions before it can establish its niche. Under these circumstances, the bacterium needs to be protected. Based on our results, we suggest the possible functions of *Yersinia* crystallins in stress protection and ionic homeostasis. Earlier workers have suggested that the presence of high levels of calcium is a type of physiological stress unique to *Yersinia*, which results in growth inhibition of *Yersinia* species at 37 °C (48, 49). Some proteins, such as Gsr A (50), Omp R (51), Rpo S (52), and response regulator PhoP (40) have been shown to protect the organism from stresses encountered during colonization of phagocytes. We suggest that, by similarity with other crystallins, *Yersinia* crystallin might be protecting the bacterium during adverse conditions, because domain D1 is stabilized by calcium, and D2 can act as a calcium buffer. On the basis of genomic differences between virulent and non-virulent strains, Golubov et al. (37) reported the presence of *Yersinia* crystallin and its parologue in *Y. enterocolitica* with possible impact on virulence. Therefore, a possible role of *Yersinia* crystallin in virulence cannot be ruled out completely.

**Conclusions**—In summary, we have described *Yersinia* crystallin from *Y. pestis* as another microbial relative of β-crystallins, possessing some common as well as several distinct properties. To our knowledge, this is the only calcium-binding protein studied from *Y. pestis*. These domains are intrinsically unstructured. Domain D1 requires calcium for its structural stabilization, whereas domain D2 does not show any such effect by calcium binding. Our results suggest that members of the β-crystallin superfamily are calcium-binding proteins. The paralogues of this protein in other *Yersinia* genera are also calcium-binding proteins and might play important roles in calcium-regulated processes in this pathogen. Our study should lead to the more detailed in vivo study of the roles played by these proteins in the physiology and host-parasite interaction of this deadly pathogen. Such studies would also improve our limited understanding of the crystallin superfamily.

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