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X-ray structures of three interface mutants of γB-crystallin from bovine eye lens

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Abstract

γB-crystallin consists of two domains each comprising two "Greek key" motifs. Both domains fold independently, and domain interactions contribute significantly to the stability of the C-terminal domain. In a previous study (Palme S et al., 1996, *Protein Sci 6*:1529–1636) it was shown that Phe56 from the N-terminal domain, a residue involved in forming a hydrophobic core at the domain interface, effects the interaction of the two domains, and therefore, the stability of the C-terminal domain. Ala or Asp at position 56 drastically decreased the stability of the C-terminal domain, whereas Trp had a more moderate effect. In this article we present the X-ray structures of these interface mutants and correlate them with the stability data. The mutations do not effect the overall structure of the molecule. No structural changes are observed in the vicinity of the replaced residue, suggesting that the local structure is too rigid to allow compensations for the amino acid replacements. In the mutants γB-F56A and -F56D, a solvent-filled groove accessible to the bulk solvent is created by the replacement of the bulky Phe side chain. In γB-F56W, the pyrrole moiety of the indole ring replaces the phenyl side chain of the wild type. With the exception of γB-F56W, there is a good correlation between the hydrophobicity of the amino acid at position 56 according to the octanol scale and the stability of the C-terminal domain. In γB-F56W, the C-terminal domain is less stable than estimated from the hydrophobicity, presumably because the ring nitrogen (Nε1) has no partner to form hydrogen bonds. The data suggest that the packing of hydrophobic residues in the interface core is important for domain interactions and the stability of γB-crystallin. Apparently, for protein stability, the same principles apply for hydrophobic cores within domains and at domain interfaces.

Keywords: domain interaction, hydrophobic effect; protein stability; site-directed mutagenesis; X-ray crystallography

Protein structure follows a hierarchical principle in that elements of secondary or super-secondary structure form domains or subdomains that can associate to multi-domain proteins. Furthermore, at the level of quaternary structure, different polypeptides form complex multi-meric proteins. The association of domains or subunits can be advantageous for protein stability, catalytic activity, or allosteric regulation (Jaenicke, 1987, 1996).

In the eye lens both transparency and refraction are dependent on the high concentrations of the crystallin proteins that must, therefore, be highly soluble (Delaye & Tardieu, 1983). It is essential that the association of domains to discrete multi-domain crystallins is not subverted into intermolecular aggregation. As there is little protein turnover especially in the lens core region, crystallins are long-lived, a property often accompanied by high protein stability (DeJong et al., 1989; Steadman et al., 1989).

Although the crystallins are a very heterogeneous group of proteins with many members of vital importance outside the lens, there are only two main families found in the mammalian eye lens: α- and βγ-crystallins (Wistow & Piatigorsky, 1988; Wistow, 1995). The X-ray structures of one member of the β-family (βB2: Bax et al., 1990) and four members of the γ-crystallins (γB: Blundell et al., 1981; γD: Chirgadze et al., 1996; γE and γF: (Norledge et al., 1997) have been solved. These studies revealed a common two-domain architecture with each polypeptide folded into two similar β-sheet domains that are further divided into two Greek key motifs. However, the packing of both domains is *intra*molecular in γ-crystallins, creating a monomeric protein, whereas it is *inter*molecular in β-crystallins, resulting in a homodimer. These alternative modes of domain interaction suggest that the βγ-crystallins exemplify the "domain swapping-" mechanism in oligomeric protein evolution (Bennett et al., 1995). Although no 3D structure is available, there is evidence from NMR spectroscopy

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Abbreviations: 3D, three-dimensional; *B*-factor, isotropic temperature factor; DTT, dithiothreitol; NaOAc, sodium acetate.

that the subunits of oligomeric α-crystallin fold into two domains (Carver et al., 1993). Even the structurally related single domain protein spherulin S3a from the slime mould *Physarum polycephalum* exhibits a tendency to form dimers and tetramers (Rosinke et al., 1997).

γ-Crystallins are among the most abundant crystallins in the bovine lens core region where the protein concentration is the highest. They exhibit a remarkable thermodynamic stability for mammalian proteins (Rudolph et al., 1990; Sen et al., 1992). To investigate this stability, we focused on γB-crystallin, the first crystallin whose structure was solved (Blundell et al., 1981). In contrast to the N-terminal domain, the stability of the C-terminal domain is strongly dependent on pH. Using urea as denaturant at pH 2, both domains unfold independently, reflecting the internal two-domain architecture of the molecule: at 3 M urea, an intermediate is populated that consists of an unfolded C-terminal domain and the N-terminal domain in a native conformation (Rudolph et al., 1990). Experiments using recombinant isolated domains revealed that the less stable C-terminal domain is stabilized by interacting with its N-terminal counterpart in the native molecule (Mayr et al., 1997).

The structure of the protein suggests that the driving force for this domain interaction is a hydrophobic cluster or core at the domain interface, consisting of six residues conserved among the γ-crystallins. These residues are M43, F56, and I81 of the N-terminal domain, and the topologically equivalent residues V132, L145, and V170 of the C-terminal domain (Fig. 1). In an earlier study (Palme et al., 1997), we investigated this interaction in detail by site-directed mutagenesis, replacing the N-terminal F56 with amino acids varying in size (Ala and Trp) and polarity (Asp). These mutations had a similar impact on the intrinsic stability of the N-terminal domain itself, but effected the C-terminal domain to a very different extent. The exchange of F56 for a smaller or polar residue (Ala and Asp, respectively) caused a significant destabilization of the C-terminal domain, suggesting that these mutations reduced hydrophobic domain–domain interactions. In contrast, the exchange of F56 for Trp had a minor effect on the stability of the molecule. These findings emphasize the notion that specific hydrophobic interactions between domains are an important factor in stabilizing the native tertiary fold of these eye lens proteins, thus preventing aggregation and cataract.

In the present study, we describe the crystal structures of these mutants and correlate the structural information with the available biophysical data.

Results

In previous studies, crystals of γB-crystallin (space group $P4_12_12$) were obtained using a batch crystallization method at neutral pH (Blundell et al., 1981; Najmudin et al., 1993). As the amount of material available from heterologous expression in *E. coli* was limited (Palme et al., 1997), we screened for more economical crystallization conditions. The interface mutants and a wild-type reference were crystallized in space group C2 from hanging drops by salting out using alkaline chlorides at acidic pH. The structures of one mutant (γB-F56A) and the wild-type reference were solved by molecular replacement using the original γB-structure as a search model. The crystals of the other mutant proteins were isomorphous to γB-F56A and showed identical cell dimensions; thus, their structures could be solved by difference Fourier methods. The structures were refined to 1.85 Å (γB-F56A and -F56W) and 2.0 Å (γB-F56D), respectively (Table 1). In comparison with the wild-type protein in $P4_12_12$, the different packing in space group C2 imposes a slight twist in the relative orientation of the two domains. Therefore, the mutant proteins were compared with the wild-type protein in space group C2. A detailed investigation of

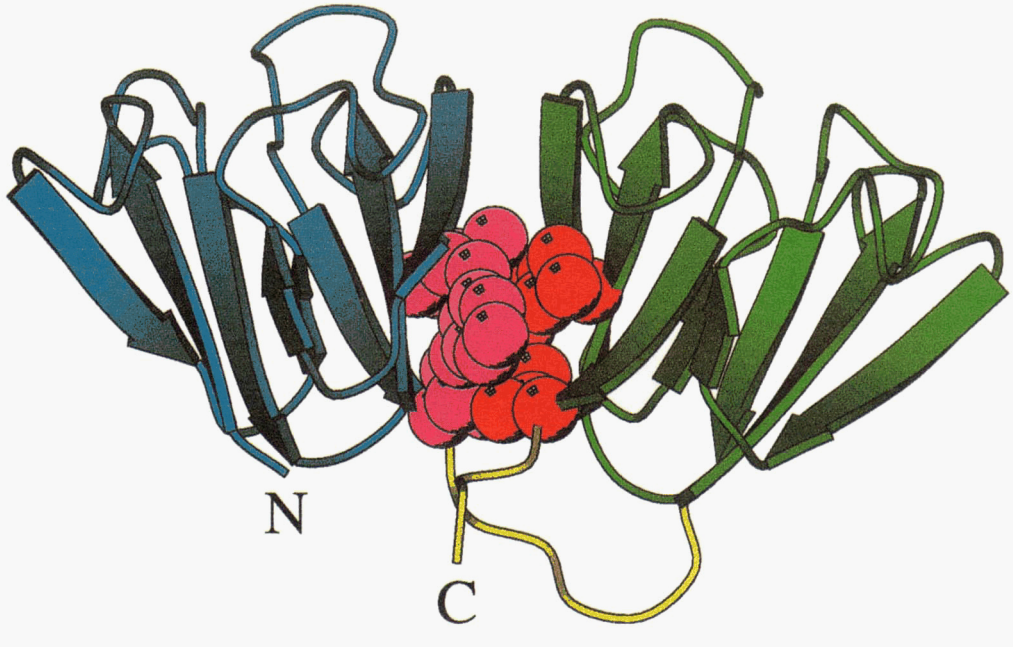

Fig. 1. γB-crystallin. C_α-backbone of the N-terminal domain (residues 1–81) in blue, C-terminal domain (88–170) in green, connecting peptide (82–87), and C-terminal extension (171–174) in yellow. The van der Waals spheres of side chains involved in the hydrophobic core in the domain interface are highlighted in pink (M43, F56, I81) and red (V132, L145, V170).

Table 1. *Crystallographic data*[a]

	γB wild type[b]	γB-F56W	γB-F56A	γB-F56D
Crystals				
Cell dimensions (Å) or (°)	$a = 61.05$	$a = 60.94$	$a = 61.45$	$a = 61.02$
	$b = 57.80$	$b = 57.80$	$b = 57.84$	$b = 57.82$
	$c = 47.50$	$c = 47.50$	$c = 47.27$	$c = 47.40$
Monoclinic angle (°)	$\beta = 93.03$	$\beta = 92.68$	$\beta = 92.74$	$\beta = 92.91$
Solvent content (%)	38.8	38.7	39.6	39.4
Data collection				
Maximum resolution (Å)	2.50	1.85	1.85	2.00
No. of reflections	8,610	29,331	37,259	17,073
No. of unique reflections	5,310	12,010	14,053	8,494
Redundancy	1.6	2.4	2.7	2.0
Completeness (%)	87.4	86.5	98.0	82.6
$I/\sigma(I)$	8.7	7.2	7.3	6.7
R_{merge}	6.6	6.2	6.2	7.7
Refinement				
R-factor (%)	16.6	18.5	18.3	19.8
R_{free}-factor (%)	25.7	23.0	22.4	24.5
No. of water molecules	84	91	136	50
RMSD bond lengths (Å)		0.01	0.01	0.01
RMSD bond angles (°)		1.24	1.16	1.22
RMSD ΔB bonded atoms (Å^2)		1.93	2.04	2.09

[a]In all cases the spacegroup is C2 with one molecule per asymmetric unit.
[b]Glen Wright and Ajit Basak (pers. obs.).

crystal packing and lattice contacts in both space groups will be published elsewhere.

The point mutations did not change the overall structure of the molecule, as determined by a superposition of the C_α-backbone (Fig. 2); the RMS deviations (RMSDs) between mutants and wild-type reference (≈0.3 Å) are within the experimental error of the coordinates (Table 2). A more detailed investigation of the local structure around the mutated residue F56, especially of other hydrophobic residues involved in the interface core, also failed to reveal any significant conformational changes (Table 2; Fig. 3).

In γB-F56W, the pyrrole moiety of the indole ring of W56 occupies the position of the F56 side chain. The ring plane of W56 is rotated around χ_2 by ≈30° relative to the wild-type phenyl ring (Fig. 3). A detailed inspection revealed no violations of standard side chain conformational parameters indicating that no steric strain is imposed by the bulkier indole ring. The nitrogen atom of the indole ring (Nε1) does not form any hydrogen bonds. There are no potential partners from the protein and there is no space for solvent molecules at suitable hydrogen bond distances (<3.5 Å).

In γB-F56A and -F56D, a residue located near the interface cleft, R168, moves slightly toward the N-terminal domain (≈1 Å), presumably as a consequence of the smaller side chain at position 56. In addition, solvent molecules fill the space created by the replacement of the phenyl side chain. In γB-F56D, two water molecules, HOH 224 and 228, are defined in the $2F_o - F_c$-map and exhibit moderate B-factors of 26.7 Å^2 and 24.0 Å^2, respectively (average B-factor for solvent molecules in this mutant: 18.2 Å^2). HOH 228 forms hydrogen bonds to the carboxyl oxygen Oδ1 of D56 (2.6 Å), to the backbone oxygen of L57 (3.2 Å) and to HOH 224 (3.2 Å). In γB-F56A, a water molecule (HOH 245) is found

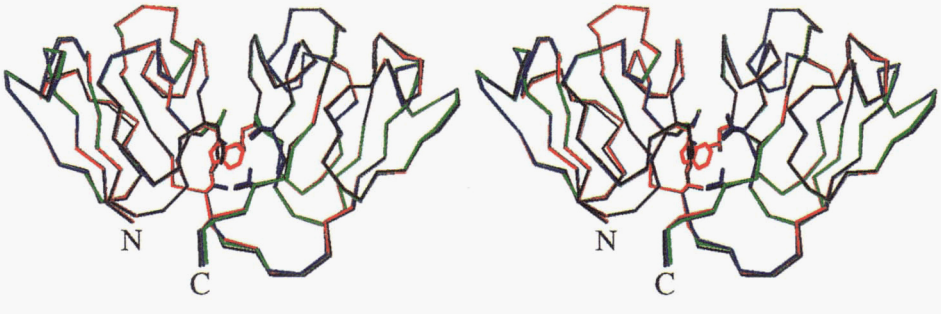

Fig. 2. Stereo view of the C_α-backbone of γB-wild type (in black) and its mutants γB-F56W (red), γB-F56A (green), and γB-F56D (blue).

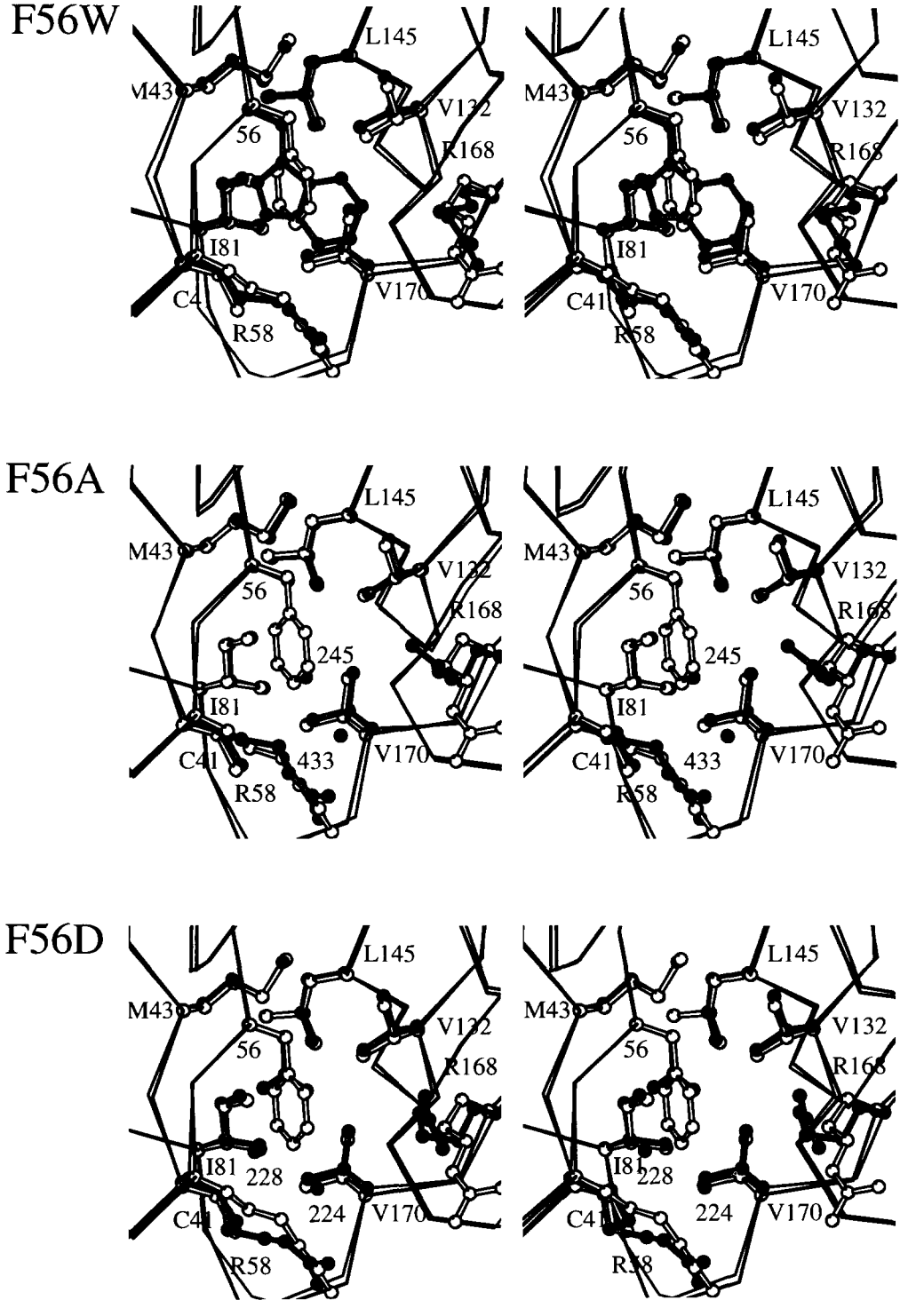

Fig. 3. Detailed view of the hydrophobic interface core around residue 56. The stereo pairs show superpositions of the wild type (white atoms and bonds) with the respective mutant (black atoms, grey bonds). Upper pair: γB-F56W, middle: γB-F56A, lower: γB-F56D. For clarity, only the C_α-backbone and some side chains are indicated.

at the position of the terminal carbon atom (Cζ) of the wild-type phenyl ring (Figs. 3, 4). This solvent molecule is situated at slightly more than van der Waals distance from surrounding side chains, and it has no hydrogen bonding partners from the protein within a distance of 3.5 Å. Instead, HOH 245 forms a hydrogen bond to HOH 433 (2.7 Å), which is located toward the outside of the interface cleft and is itself in hydrogen-bond distance to main chain N and O of M171 (3.0 and 2.7 Å). The *B*-factor of HOH 245 (34.4 $Å^2$) is comparable to the average *B*-factor of all solvent molecules in this mutant (31.2 $Å^2$).

Table 2. *Superposition of γB-crystallin and its point mutants*[a]

Mutant	Complete molecule		Interface all atoms
	All atoms	α-Backbone	
γB-F56W	0.94	0.29	0.19
γB-F56A	0.87	0.28	0.15
γB-F56D	1.01	0.28	0.16

[a]Mutants were superposed on γB wild type by using LSQKAB. Calculations were based on the entire molecule (using either all atoms or the α-backbone only) and for the interface residues M43, I81, V132, L145, and V170 only. These residues are involved in forming the hydrophobic core in the interface. All RMSDs are in Å.

Equilibrium unfolding experiments performed at pH 2 showed that the stability of the C-terminal domain is markedly effected by its interaction with the N-terminal domain (Mayr et al., 1997). To ascertain if there is a correlation between this domain interaction and the hydrophobicity of the amino acid at position 56, we compared the stability of the C-terminal domain with the hydrophobicity of the mutated amino acid in the N-terminal domain making use of hydrophobicity scales (for a review, see Ponnuswamy, 1993). The transition midpoint, $c_{1/2,urea}$, of the first transition of the two-step unfolding of γB-crystallin at pH 2 was used as a qualitative measure for the stability of the C-terminal domain because no free energies of stabilization are available (Palme et al., 1997). Using $c_{1/2,urea}$ is reasonable as the cooperativity ("m-value") of the transition is comparable in all proteins investigated. Using the octanol scale (Fauchere & Pliska, 1983) we find a linear correlation between amino acid hydrophobicity and domain stability (correlation coefficient $R = 0.83$, Fig. 5A). A similar result is obtained when the ethanol-scale (Tanford, 1962) is applied ($R = 0.85$, data not shown).

According to the octanol-scale a stabilization of the C-terminal domain is expected in γB-F56W due to the much higher hydrophobicity of Trp compared to Phe. A comparison of different hydrophobicity scales revealed that alcohol-based solvent systems have an increased affinity for amino acid side chains capable of forming hydrogen bonds (Radzicka & Wolfenden, 1988; Karplus, 1997). We therefore analyzed the data using the scale based on cyclohexane (Radzicka & Wolfenden, 1988), a solvent that makes no polar interactions. Following the argumentation of Karplus (1997) that polar or charged amino acids should be omitted in the analysis, we find a perfect linear correlation between hydrophobicity and domain stability for Ala, Phe, and Trp ($R = 1.00$, Fig. 5B). Attempts to correlate the stability with the solvent accessible surface area buried by the amino acid side chain at position 56 were less satisfactory, as the correlation does not take into account H-bonds or the polarity of the residue ($R = 0.71$, data not shown).

Discussion

Domain interactions contribute significantly to the stability of the C-terminal domain in γB-crystallin (Mayr et al., 1997). In a previous study, we exchanged F56 from the N-terminal domain, one of six conserved hydrophobic residues forming a hydrophobic core at the domain interface, for various amino acids (Palme et al., 1997). While a smaller or a more polar residue (Ala or Asp) destabilized the C-terminal domain drastically, the substitution by Trp had a more moderate effect. The stability of the C-terminal domain thus depends on direct interactions with the N-terminal domain and, therefore, perturbations of the hydrophobic interface can have a substantial impact on the domain interactions and, hence, on the stability of the two-domain protein.

X-ray structures

In contrast to the biophysical characteristics, neither the overall structure of the molecule nor the conformation of side chains at the hydrophobic interface are altered significantly as a consequence of the above mutations. Evidently, there is no structural response that might minimize or compensate the effect of the mutations. Numerous studies on protein stability using site-directed mutagenesis revealed that the impact of a mutation strongly depends on the packing density in the vicinity of the mutated residue and the conformational flexibility of surrounding residues (Alber et al., 1987; Serrano et al., 1992; Jackson et al., 1993; Matthews, 1995). The six residues that form the hydrophobic core at the domain

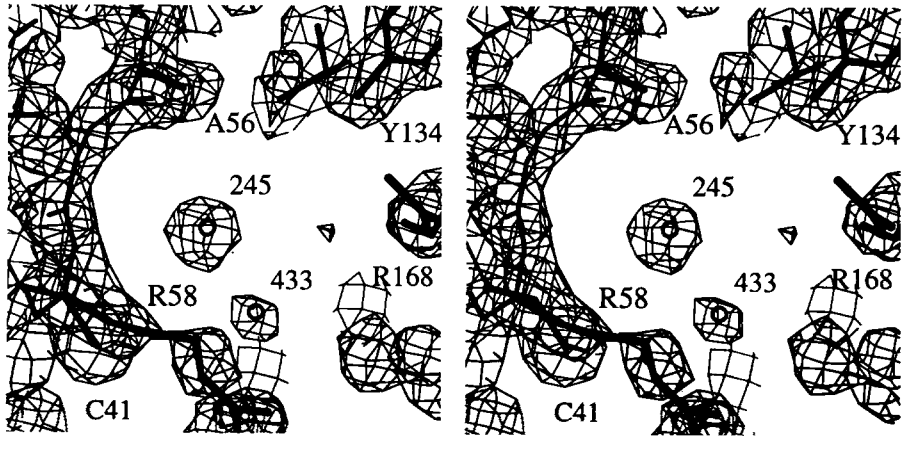

Fig. 4. Stereo picture of the $2F_o - F_c$-electron density map of HOH 245, HOH 433, and surrounding residues. The map was calculated omitting the solvent molecules (contour level 1σ).

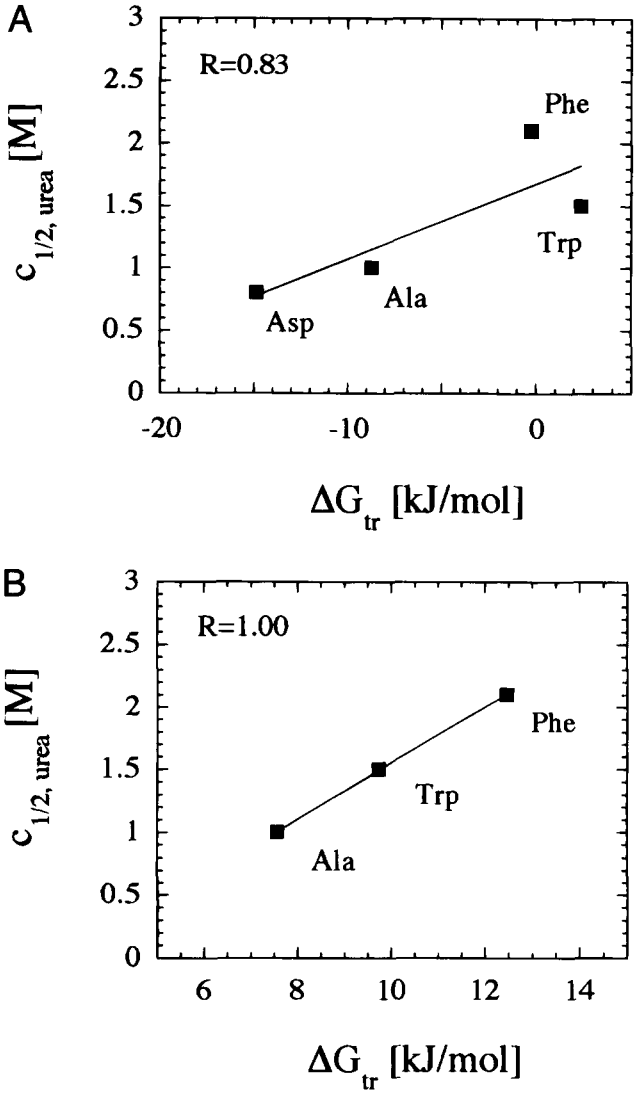

Fig. 5. Correlation between the stability of the C-terminal domain and the hydrophobic properties of residue 56. The stability of the C-terminal domain was estimated from the mid-point of the first urea-induced equilibrium unfolding transition at pH 2 (Palme et al., 1997). *R*: correlation coefficient. **A:** Hydrophobicity classified according to the free energy of transfer (ΔG_{tr}) between octanol and water (Fauchere & Pliska, 1983). **B:** Hydrophobicity classified according to the cyclohexane scale (Radzicka & Wolfenden, 1988). Mutant γB-F56D is omitted; if the mutant is included, *R* drops to 0.70 ($\Delta G_{tr,Asp} \approx 36$ kJ/mol).

interface (M43, F56, I81, V132, L145, V170) are as densely packed as residues involved in the hydrophobic core of both domains. In γB-crystallin, their average *B*-factor (11.3 Å^2) is comparable to the average *B*-factor of the four Trp-residues (10.3 Å^2) that are buried within the cores of the domains. We conclude that the rigid scaffold provided by the β-sheet secondary structure and the tight packing of residues involved in the hydrophobic interface prevent structural adjustments.

In the absence of structural adaptation, the exchange of the bulky phenylalanine side chain for smaller residues (Ala or Asp) leaves cavities. In both γB-F56A and -F56D they are filled with water molecules that are not present in the wild-type structure. As these are in contact with solvent molecules from higher hydration shells, the space is better described by a groove or cleft filled with water. In γB-F56D, one of these solvent molecules (HOH 228) is held in place by hydrogen bonds to the carboxylic acid side chain. In γB-F56A, HOH 245 in the hydrophobic groove is well defined in the electron density map with a low *B*-factor, but has no hydrogen bond partner from the protein; instead, it is only connected to a water molecule further outside the interface cleft. Apart from this hydrogen bond, the restricted size of the groove probably accounts for the low mobility of the water molecule: surrounding protein atoms lie within slightly more than van der Waals distance (4–4.5 Å). This interpretation is in line with findings of Buckle and co-workers (Buckle et al., 1996), who identified a buried water molecule by X-ray crystallography in an enclosed cavity in the I76A mutant of barnase. They argue that spatial restriction of mobility and a partner for hydrogen bonding are necessary for a water molecule to be detected.

Protein stability

The stabilization of a protein by the hydrophobic effect is based on the unequal distribution of polar and non-polar side chains between the surface and the interior of the molecule. According to the "like dissolves like" rule, bringing a non-polar residue into the hydrophobic core of a protein has a stabilizing effect, whereas polar side chains tend to be exposed on the molecular surface. In γB-crystallin, tightly packed hydrophobic side chains form an additional core-like structure at the domain interface. Similar to the cores within the domains, this hydrophobic cluster is expected to effect both the domain interactions and the stability of the individual domains. There is a correlation between the measured stability of the C-terminal domain and the hydrophobicity of the exchanged residue 56 at the interface. According to the scale, the C-terminal domain in γB-F56W is less stable than expected from the higher hydrophobicity of Trp compared to Phe. A similar deviation from the linear relationship between the hydrophobicity of the aromatic residues and protein stability was reported for T4 lysozyme. In this case, substitution of I3 by Phe, Tyr, and Trp did not allow the bulky aromatic residues to be buried completely, so that the side chains could not exhibit their full hydrophobic potential (Matsumura et al., 1988). In the γB-mutants presented here, this explanation does not apply because the solvent exposure of W56 and F56 is similar.

A detailed inspection of the structure around W56 in γB-F56W suggests a reason for the poor correlation with hydrophobicity: the ring nitrogen (Nε1) of the side chain does not make any hydrogen bonds with protein or solvent atoms. It has been shown that the burial of a potential H-bond forming atom in the interior of a protein is destabilizing (Serrano et al., 1992; Pace et al., 1996). This interpretation is supported by the perfect linear correlation between domain stability and hydrophobicity if the data are fitted using the cyclohexane scale. Because this solvent forms no hydrogen bonds and does not contain water (Radzicka & Wolfenden, 1988) it should be better suited to describe the interior of a protein in the absence of any polar interactions or hydrogen bonds. The observation underlines the fact that structural parameters have to be considered when interpreting protein stability in terms of simple hydrophobicity.

Although both domains are able to fold independently (Rudolph et al., 1990; Mayr et al., 1997), the hydrophobic core at the domain interface provides an additional increment to the stability of the

molecule. Replacement of the bulky F56 by Ala or Asp disrupts the tight packing of the interface side chains and creates cavities that reduce stability by reducing van der Waals interactions. A statistical analysis of various protein structures has also confirmed that the hydrophobic effect is a dominant force in protein–protein association (Tsai et al., 1997). The fact that the pairing geometry was unaltered indicates that other factors are involved in this interface interaction. It is likely that both the surrounding polar contacts and the covalent linker contribute to this stable orientation of domains. The burial of non-hydrogen-bonded polar atoms (Nε1 in Trp) or the polarity of the side chain (Asp) also have to be taken into account to explain the specific effects of amino acids replacements at the domain interface. Our results indicate that many of the principles governing the stability of protein domains, such as optimal packing of hydrophobic groups, also apply to the association of folding units at domain interfaces. It is particularly important in the eye lens that domain pairing is optimized, as unpairing might lead to random intermolecular aggregation causing light scattering.

Materials and methods

Crystallization

All recombinant mutants of γB-crystallin were heterologously overexpressed in *E. coli*, purified, and characterized as described previously (Palme et al., 1997). Crystallization was performed in hanging drops at room temperature. Initial conditions were obtained by using the Hampton Screen Kits 1 and 2 (Hampton Research, Laguna Hills, California). The mutants γB-F56A, -F56D, and -F56W were crystallized from 1.5–2.0 M CsCl, 10 mM DTT, 100 mM NaOAc, pH 4.6 to 5.0, initial protein concentration 4–5 mg/mL. Crystals of 300–800 μm length along the longest axis were obtained after three to five days.

Structure determination

All programs, unless otherwise stated, are from the CCP4 suite (CCP4, 1994). Data for wild type γB and its mutants were collected at the Synchrotron Radiation Source (SRS), SERC Daresbury Laboratory, at wavelengths of 1.488 Å (station 7.2) or 0.87 Å (station 9.6) and in house using a rotating anode generator (CuKα wavelength 1.5418 Å). Crystals were mounted in glass capillaries, and data were collected at room temperature using MAR research image plates. Data were processed using the Program DENZO (Otwinowski, 1993). Scaling and merging were carried out using the programs ROTAVATA and AGROVATA. Data were further reduced with TRUNCATE. The data processing statistics are summarized in Table 1.

Molecular replacement was used to solve the structure of γB-F56A (program AMORE; Navaza, 1994). γB wild type (Najmudin et al., 1993) was used as search model, giving $\Delta S/\sigma = 8.6$ for the rotation function and $\Delta S/\sigma = 4.4$ for the translation function. After rigid body refinement using both domains as rigid bodies (program X-PLOR; Brünger, 1992), the shifted model had an *R*-factor of 40.1%. The packing was examined graphically using MOLPACK (Wang et al., 1991). As the other mutants crystallize isomorphously, they were solved by difference Fourier methods using the refined structure of γB-F56A.

Refinement was performed by *simulated annealing* or conventional *least squares* refinement with X-PLOR (Brünger, 1992). All protein atoms and solvent molecules were refined at unit occupancy. Electron density maps were calculated using the CCP4 suite (CCP4, 1994) with coefficients $2|F_o| - |F_c|$ or $|F_o| - |F_c|$. Rebuilding and adding of solvent molecules were done graphically using a Silicon Graphics workstation and the program O (Jones et al., 1991). Final *R*- and R_{free}-factors are listed in Table 1. The solvent content of the crystals was calculated according to Matthews (1968).

Structure analysis

Analysis of the structures using the program PROCHECK (Laskowski et al., 1993) showed that all residues lie within the allowed regions of the Ramachandran plot. Structures were superposed graphically using the program O (Jones et al., 1991). The rms deviations were calculated with LSQKAB. Figures were drawn using the programs MOLSCRIPT (Kraulis, 1991), BOBSCRIPT (Esnouf, 1997), and Kaleida Graph (Abelbeck Software).

Calculation of buried solvent-accessible surface area

The accessible surface area was calculated using the rolling-sphere algorithm (Lee & Richards, 1971) with the program SURFACE and a probe radius of 1.4 Å. Surface accessibilities per residue were calculated by adding atomic values obtained by SURFACE with the program RESAREA. The surface area buried by the side chain of residue 56 was calculated as follows (cf. Chen et al., 1995): first, the accessible surface area of the complete molecule lacking the side chain of residue 56 was determined (value A). Second, the accessible surface area of the side chain within a tripeptide Tyr-X-Leu (in extended conformation) was calculated (value B). The sum of A + B gives the total accessible surface area of the truncated molecule and the isolated side chain. From this number the accessible surface area of the intact molecule (value C) was subtracted (A + B − C) to yield the solvent accessible surface area that is buried by the side chain.

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