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Crystal Structure of Calf Eye Lens Gamma-crystallin IIIb at 2·5 Å Resolution: Its Relation to Function

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The crystal structure of γ-crystallin IIIb (gamma C) from calf eye lens has been refined at 2·5 Å resolution. The molecule of about 21 kDa consists of two similar domains. Each domain is composed of two motifs with the 'Greek key' topology which form a pair of four-stranded β-sheets with an antiparallel packing. The molecule has three hydrophobic cores: one within each domain and one between them. Six of the eight functionally important cysteines are located within the N-domain, and only two in the C-domain. Several large clusters of charged residues are at the surface of the molecule. Surface residues Val 101, Met 103 and Leu 155 are important for packing of molecules in crystal medium and possibly in the lens. Features of the γ-crystallin IIIb molecule which may be related to its function in the vertebrate eye lens are briefly discussed. An attempt has been made to correlate molecular characteristics with some general properties of the eye lens such as high density and refractive index gradients and strong stability of the lens during an organism's lifetime.

Key words: eye lens protein; γ-crystallin IIIb; protein structure; protein surface; protein association; charges in proteins.

1. Introduction

Crystallins are structural proteins of the vertebrate eye lens. Their content among all water-soluble lens proteins is about 90%. Their are four main classes of crystallins: α, β, γ and δ (Slingsby, 1985; Wistow and Piatigorsky, 1988). All γ-crystallins and one β-crystallin (β_s) are monomeric proteins. Other crystallins have a tendency to form oligomers and associates with high molecular masses. Sequence homology between β- and γ-crystallins allows their inclusion in one superfamily of related proteins. The relative amounts of β/γ-crystallins vary with age. For example, in rat lens the synthesis of monomeric crystallins with aging falls down and the content of $(\gamma+\beta_s)$-crystallins decreases from 40 to 10% of the total amount in the first 420 days, while the amount of β-crystallins increases from 20 to 50% of total protein in the same period (Siezen et al., 1988).

There are five to seven different γ-crystallin gene products of molecular mass about 21 kDa with chain lengths of 173 or 174 residues. In contrast to other crystallins, γ-crystallins contain many cysteine residues with SH-groups being functionally important. At present about 20 amino acid sequences of γ-crystallins from frog, mouse, rat, calf and human lenses are known (for a review see Chirgadze et al., 1987). Previously, spatial structures of calf lens γ-crystallin were determined for γII at high resolution 1·9 Å (Wistow et al., 1983), for γIIIb at medium resolution 3·0 Å (Chirgadze et al., 1981) and γIVa at resolution 2·3 Å (White et al., 1989). Genes of γ-crystallins were studied in detail (For example see Den Dunnen et al., 1986). All these data reveal peculiarities of the molecular structure which may be responsible for the functioning of the lens in its normal state, and they help us to understand any diseases which may occur. We hope that data presented here on the spatial structure of γ-crystallin IIIb will result in progress in this field.

2. Materials and Methods

Isolation, purification and crystallization of γ-crystallin IIIb have been described previously (Chirgadze et al., 1977). Protein crystals were grown from 1% solution with 50 mM potassium sodium phosphate buffer, pH 7 and 5 mM glutathione (Chirgadze et al., 1986). The crystals belonged to the space group $P2_12_12_1$ with unit cell parameters $58{\cdot}7\times69{\cdot}5\times116{\cdot}9$ Å and two molecules per asymmetric unit. Five heavy atom derivatives from $KAu(CN)_2$, Na-mersalyl, $Hg(SCH_2CH_2NH_3Cl)_2$, $Hg(SCH_2COONa)_2$ and $Hg(SCH_2CH_2OH)_2$ were used (Chirgadze et al., 1981). All derivative crystals diffracted up to 3·0 Å, while the native one reflected up to 2·5 Å on the GX-13 rotating anode generator.

Intensity data were collected from a single native crystal by the oscillation method and treated as described (Chirgadze et al., 1986). All reflections with optical densities less than 2σ were discarded. The final data set contained 13 326 reflections in the range of

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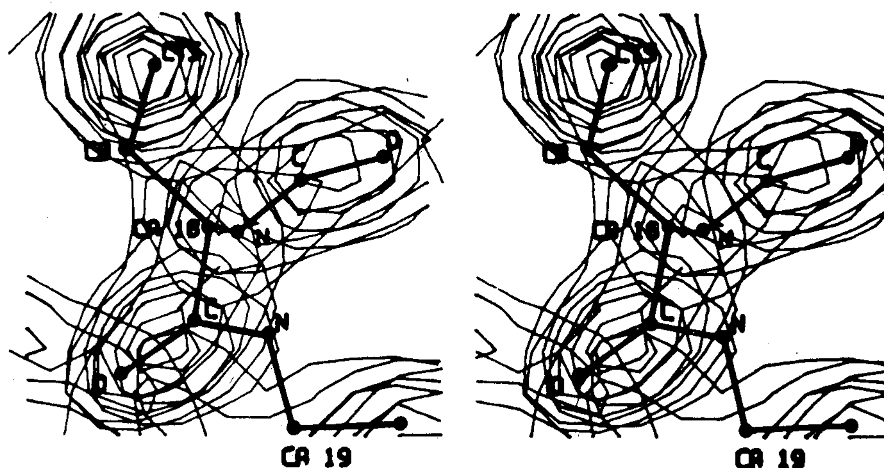

FIG. 1. Fragment of electron density map of calf γ-crystallin IIIb at 2·5 Å resolution.

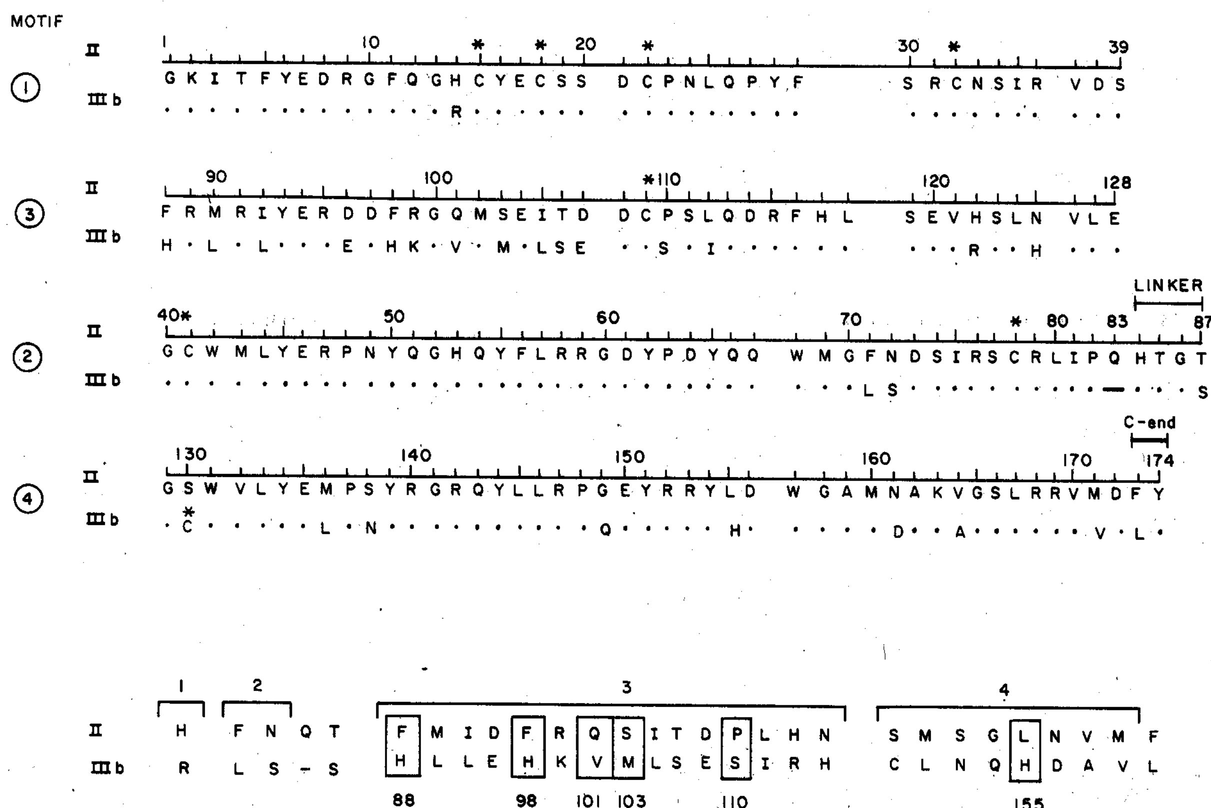

FIG. 2. Sequences of calf γ-crystallins II and IIIb from eye lens (see Bhat and Spector, 1984; Chirgadze et al., 1987). Common residue numeration for all γ-crystallins of vertebrates is used. The same residues are designated by points, deletion, by dash. Bottom: Replacement positions grouped in motifs, significant replacements are boxed.

20–2·5 Å, which makes 76% of all possible reflections. R_{merge} expressed as $\Sigma(I_i - \langle I \rangle)/\Sigma\langle I \rangle$ was equal to 6·9%, where $\langle I \rangle$ is the intensity of ith reflection averaged from different films.

Initial phases up to 3·0 Å resolution (Chirgadze et al., 1981) were improved and extended to 2·7 Å using a mixed electron density model (Lunin et al., 1985). After a detailed inspection of the electron density map, an initial model was constructed with about 95% of total non-hydrogen atoms using the interactive graphic program ISOCUB (Nevskaya et al., 1986). Since the exact sequence of γIIIb was unknown, we used the tentative sequence of calf γIIIb (Chirgadze et al., 1987) deduced on the basis of the preliminary chemical sequence data of γ-crystallin III (Croft and Slingsby, 1973) and closely related rat and human γ-crystallin sequences (Den Dunnen et al., 1986; Meakin et al., 1987).

The initial γ-crystallin IIIb model was refined crystallographically with the FROG program (Urzhumtsev et al., 1989) which uses the fast Fourier transform and fast differentiation algorithms (Lunin and Urzhumtsev, 1985). Three stages of refinement were performed with two manual corrections. Each

stage consisted of about 80–100 cycles. The final *R*-factor was about 25% for the 6–2·5 Å resolution range which included 12300 reflections. Both molecules of the asymmetric unit were refined independently. Average deviations from ideal values were 0·023 Å for bond distances and about 3·0 degrees for bond angles. Isotropic temperature factors of all the protein atoms had an average value of 34 Å². All peptide torsion angles ϕ and ψ were in allowed regions on the Ramachandran plot. At this limit of resolution it is not a common practice to introduce water molecules in the crystal structure of protein. So we have not added water molecules, although some of them were well resolved on the electron density map after final cycles of refinement. It can only be done at the next stage when the diffraction data of a much higher limit of resolution will be available. Some details of the refinement procedures have been presented elsewhere (Chirgadze et al., 1986).

The majority of the peptide groups of the main chain and the side groups of γ-crystallin IIIb were well resolved. Some problems arose with those parts of the electron density map where heavy-atom sites were located but they were overcome at the final stages of refinement. There was no evidence in the electron density map of glutathione molecules. To confirm the existence of functionally important cysteine residues we have carefully examined the electron density map in the corresponding regions. The theoretical ratio of electron densities assigned to the CS side group of cysteine and to the CO peptide group is 1·61. The mean value of this ratio was about 1·5 for all the groups in both molecules. Gamma-crystallin IIIb contains extra Cys 130 compared to γII. The mentioned ratio for this cysteine was 1·7 in both molecules of the asymmetric unit. An example of an electron density map at 2·5 Å resolution is represented in Fig. 1, where one can see a cysteine 18 density spot in the side chain region.

3. Results and Discussion

Sequences of Calf Gamma-crystallin II and IIIb

A tentative amino acid sequence of γ-crystallin IIIb is compared with an exact sequence of γ-crystallin II (Bhat and Spector, 1984) in Fig. 2. We use a common numeration for the whole γ-crystallin family (Chirgadze et al., 1987). We can refer calf γ-crystallin IIIb to type C proteins belonging to the class γ_{ABC} of non-cryoproteins according to the nomenclature proposed by Siezen et al. (1988). γ-Crystallin IIIb has 173 amino acid residues and consists of four repeated motifs. They have 39, 43, 41 and 44 residues respectively. There is a deletion at position 83 of γIIIb as compared with γII. Homology between these two sequences is about 84%. There are 28 amino acid replacements, the majority of them occurring in the variable third motif. At six positions, hydrophobic residues are replaced by hydrophillic ones or vice versa, and we consider them as significant replacements.

Domain Structure and Main Chain Topology of Gamma-crystallin IIIb

The molecule of γIIIb is composed of two very similar spherical domains each about 25 Å in diameter (Fig. 3) and related by a pseudo twofold rotation axis. Each domain has a hydrophobic core with the interdomain region also being hydrophobic which seems to restrict the mobility of domains relative to each other. Both domains consist of two similar motifs related by another pseudo twofold axis. Motifs are positioned consecutively along the chain. However, motifs 1 and 3 are located spatially in opposite parts of the molecule, while motifs 2 and 4 touch each other within the molecule. It should also be mentioned that the sequence homology, as well as the similarity of the main chain structure is much higher for corresponding motifs of different domains than that for motifs within each domain.

The unique topology of the domain structure is

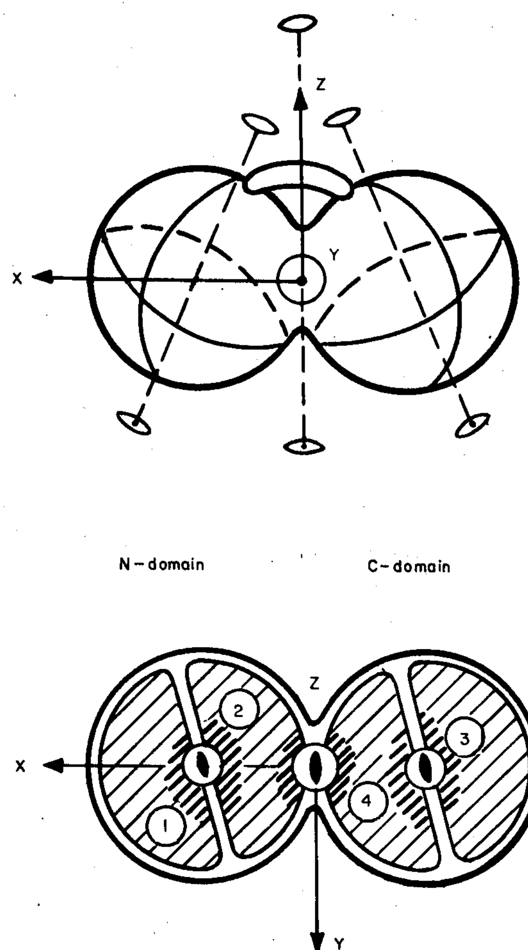

FIG. 3. Schematic three-dimensional organization of γ-crystallin IIIb. Top: Disposition of principal and twofold symmetry rotation axes in the molecule. Bottom: Location of four repeated motifs in the domains. Intra- and inter-domain hydrophobic cores are heavily hatched.

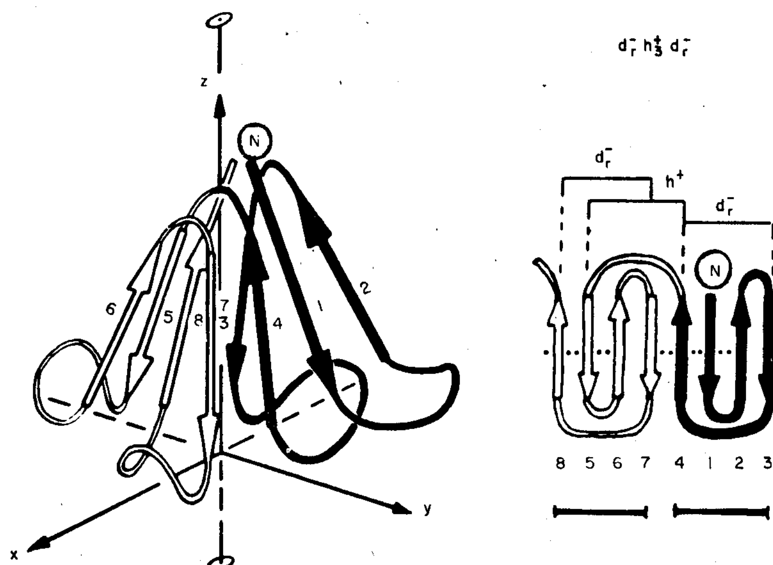

FIG. 4. Symmetry and topology of C_α-pathway in domains of γ-crystallin IIIb. One of two Greek key motifs is emphasized in black. Nomination of simple topological submotifs is given in the paper of Chirgadze (1987).

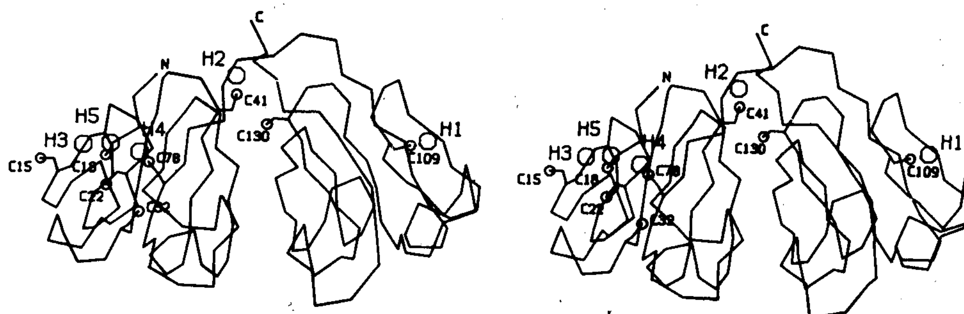

FIG. 5. Stereodrawing of the alpha carbon skeleton of calf γ-crystallin IIIb. Cysteine side chains are shown. Enlarged H-circles denote heavy atom-binding sites.

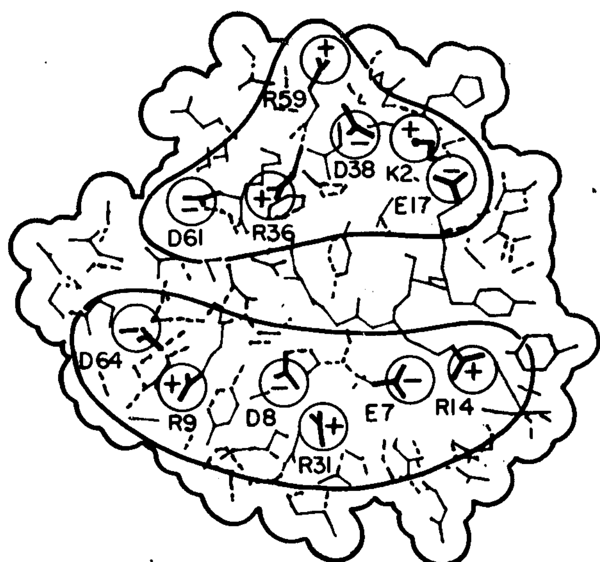

FIG. 6. Clusters of charged side groups on the N-domain surface of γ-crystallin IIIb as viewed approximately along the X-axis of the molecule.

given in Fig. 4. According to the nomenclature (Chirgadze, 1987), two similar 'Greek key' motifs of the d_r^- type form a pair of four-stranded β-sheets with an antiparallel packing. Each sheet is composed of three strands from one motif and one strand from another. The strands of both motifs with numbers from 4 to 7 form a hairpin of the h^+-type. An overlapping of the hairpin with two Greek key motifs makes the whole domain structure with the $d_r^- h_3^+ d_r^-$ topology more stable.

The spatial structure of γ-crystallin IIIb is rather similar to the known structure of γ-crystallin II. Root mean square (rms) deviations of C_α-atom positions of γ-crystallin IIIb from those of γ-crystallin II are equal to 0·87 and 0·93 Å for the two γIIIb molecules. In both cases, the peptide linker 84–87 has much greater C_α-atom deviations of about 3 Å. It should be mentioned that the similarity of corresponding domains of γII and γIIIb molecules is slightly higher than that of whole molecules: a domain-to-domain comparison gives a rms C_α-atom deviation equal to 0·72 Å for N-domains and 0·60 Å for C-domains. It should be noted, however, that all these values depend on coordinate errors. Nevertheless, the above-mentioned rms deviations indicate the existence of a slightly different disposition of domains in two different γ-crystallins. Atoms of side chains are displaced to a greater extent. It concerns mostly surface residues with long side chains and residues which take part in intermolecular interactions in the crystal medium.

Arrangement of Cysteine Thiol Groups and Heavy-atom Binding Sites

A peculiarity of all vertebrate γ-crystallins is the large amount of cysteine residues. According to our data, calf γ-crystalline IIIb contains eight cysteines. Six of them at positions 15, *18*, *22*, *32*, *41*, *78* are situated in the N-terminal domain while cysteines *109* and 130 are in the C-terminal domain (Fig. 5). Five of the cysteines are conserved in all-known γ-crystallin sequences (Chirgadze et al., 1987) and they are given in italics. Cysteines 22, 109 and 41, 130 are located at symmetry equivalent positions in different domains of γ-crystallin IIIb. All cysteine residues are situated near tryptophan or tyrosine side chains and may serve for protection of these residues from photo-oxidation and contribute to the stability of the molecule (Wistow et al., 1983).

The accessibility of thiol groups to the solvent is quite different in γ-crystallin IIIb. We have found that Cys 15 is highly accessible, cysteines 18, 22, 41, 109 and 130 are partly accessible and Cys 32 and 78 are completely buried. Therefore, thiol groups of γ-crystallin IIIb have different reduction potentials. Thiols at positions 18, 22 and 78 are close enough to form intramolecular —S—S bonds and under certain conditions they may be oxidized. Such bonds may be formed with little conformational change and one of them was observed in old crystals of γ-crystallin II (Wistow et al., 1983). In contrast to the γII molecule, γIIIb has an additional cysteine at position 130. This residue is observed in the inter-domain region together with Cys 41. The formation of a disulfide bond between these two residues may be very important for the stabilization of an inter-domain contact if the connecting peptide is cleaved.

It has been found that heavy atom reagents, most of which are mercurials, have four common binding sites H1, H2, H3 and H4. Na-mersalyl has an additional fifth site H5 with a relatively low occupancy. Site H1 is situated near Cys 109, site H2 near Cys 41 and sites H3, H4, H5 are located in the same region near Cys 18 and Cys 22. There is no heavy atom reagent close to other cysteine residues. Residues 15 and 130 are inaccessible due to the molecular contact in the crystal of γ-crystallin IIIb (Sergeev et al., 1988). Common binding sites for different heavy atom compounds may testify to their universal character.

In the eye lens there are γ-crystallin-glutathione mixed disulphides (Harding, 1970). It was shown that γ-crystallin IIIb has at least one binding site for glutathione (Slingsby and Miller, 1985). A glutathione molecule has carboxylic groups on both ends approximately 11 Å apart. The same groups are about 12 Å apart in one of the used mercurial derivatives, namely $Hg(SCH_2COONa)_2$. Therefore, we suggest that binding sites of glutathione appear to be near sites H1, H2 or H3, which correspond to heavy-atom binding sites with high occupancies.

Surface of Calf Gamma-crystallin IIIb

An essential part of the molecular surface of γIIIb is occupied by charged side groups. Their total number is 46. We included the charged group of the carboxyl end, but did not consider the histidine side chains. The majority of these groups are arranged in four clusters, including four to eight alternating charges of the opposite sign, the distances between which are less than 7–8 Å. Such clusters contain mainly residues conservative for all known γ-crystallins (Chirgadze et al., 1987). For example, one cluster of the N-domain consists mainly of conservative residues Glu 7, Asp 8, Arg 9, Arg 31 and Asp 64. Another includes conservative residues Lys 2, Glu 17, Arg 36 and Arg 59 (Fig. 6). Similar charged clusters are observed in the C-domain. Five arginine residues are located in the inter-domain region, and they take part in protecting the inter-domain hydrophobic core from the solvent. Two oppositely charged groups are considered to be an ion-pair if the distance between them is less than 4–5 Å. There are about 10 ion-pairs. Intramolecular ion-pairs and clusters of oppositely charged groups on the molecular surface contribute to molecular stability. Only a few charged groups are involved in intramolecular ion-pairs in γIIIb crystals (Sergeev et al., 1988).

The total charge surface density of γ-crystallin IIIb is equal to 0·5 charged groups per 100 $Å^2$. This value was calculated according to the method of Barlow and Thornton (1986) taking the value of the total accessible surface area of the γIIIb molecule to be 9300 $Å^2$ (Sergeev et al., 1988). The total charge surface density of γIIIb is the lowest value of the 32 proteins. For this set of proteins the discussed values range from 0·5 to 2·5 charged groups per 100 $Å^2$, with the mean value of 1·2 charged groups per 100 $Å^2$ (Barlow and Thornton, 1986). On the other hand, γ-crystallin is related to the few proteins with a rather high content of ion-pairs. The specific amount of ion-pairs in γIIIb is equal to 5·7 charged groups per 100 residues in comparison to the mean value of 3·9 groups per 100 residues for 38 considered proteins (Barlow and Thornton, 1983). We can see that in comparison with many other proteins eye lens γ-crystallin IIIb, as well as other crystallins, has a low total charge density and a high ion-pair content. It should be mentioned that the local charge density in the charge clusters of this protein is 8–10 times higher than the mean total value.

The low total charge density of γIIIb is conditioned by the high content of ion-pairs and charge clustering. This should cause a relatively low hydrophillic charge potential because some charge groups are shielded from the solvent water molecules by adjacent cluster charge groups. We suggest that clusters of charged groups with opposite signs reduce the polarity of these surface spots and decrease their solvation ability as previously suggested for the analogous three-

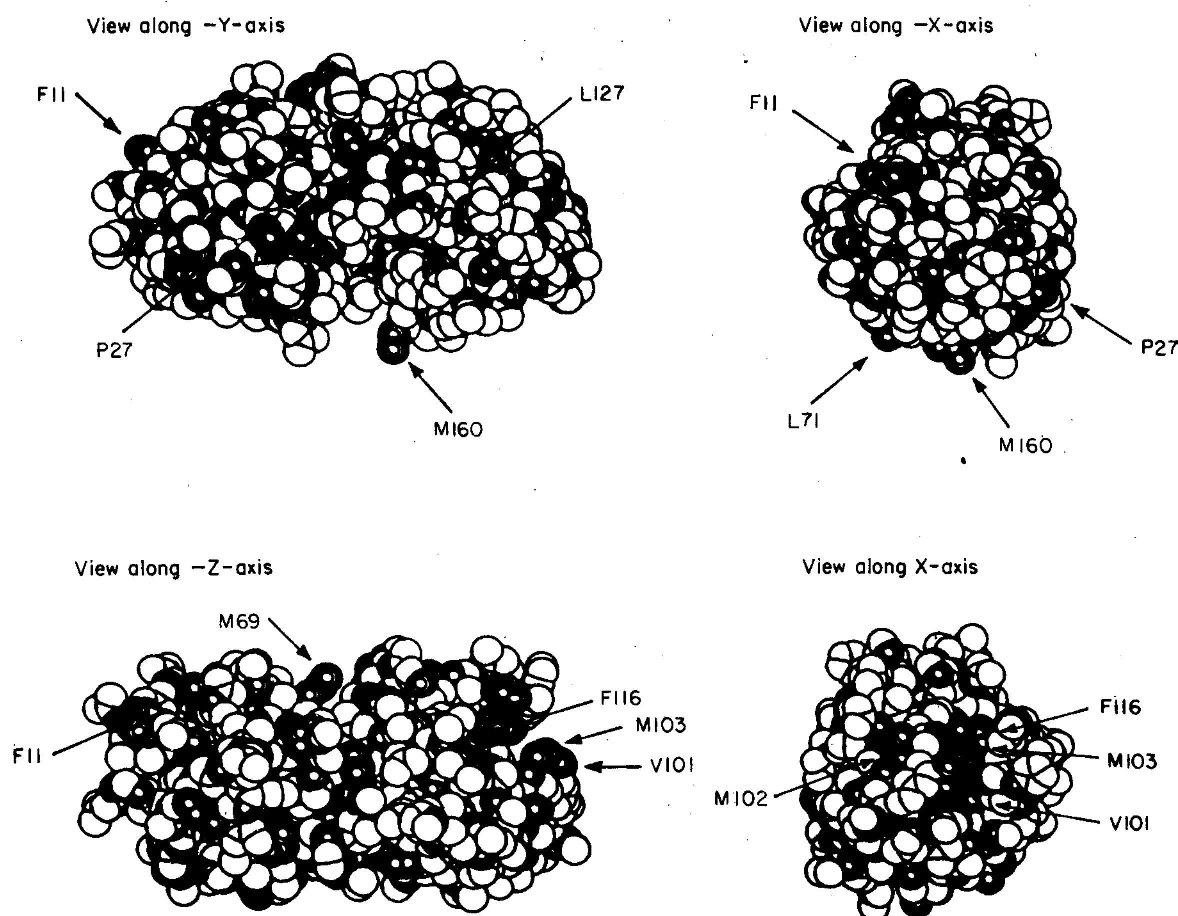

FIG. 7. Space-filling model of calf γ-crystallin IIIb. Atoms of non-polar groups are blackened. Principal axes are presented on the scheme of the molecule in Fig. 3.

dimensional structure of calf γ-crystallin II (Wistow et al., 1983). However, the total hydrophillic potential of γ-crystallins includes both the charged groups and all the other polar groups exposed on the protein surface. That is why an exact estimation of this potential is difficult.

An approximate representation of the molecular surface may be calculated as the projection of a space-filling atomic model, namely its accessible surface (Fig. 7). The surface of γIIIb has both hydrophilic and partly exposed hydrophobic side chains. This is a usual case for such a colouring of the protein surface. Another important surface property is the relief.

Analysis of intramolecular contacts of γ-crystallins in crystal packing allows specific contact points on the surface which could be functionally important for the packing behaviour of different gene products in the lens to be selected. We examined the γIIIb surface using corresponding maps (Chirgadze et al., 1990). Such maps are presented in Fig. 8. One of them is a relief surface map of γIIIb. Two other maps show the crystal contact regions for each of two γ-crystallin IIIb molecules in an asymmetric unit. The crystal of γIIIb consists of molecular layers. Within these layers the protein molecules are joined into long associates, forming a 'head-to-tail' contact between sites A and B located on the N- and C-domain of the molecule (Sergeev et al., 1988).

As there are two molecules in an asymmetric unit, we observe two slightly different contacts A_1B_2 and A_2B_1. The layers are packed together with a C_1D_2 contact. Area D_2 is composed of three separate parts D_2', D_2'' and D_2'''. All contact regions are clearly seen on the surface maps where residues of contact regions are identified. After comparison of the surface relief and crystal contact area we can conclude that almost all contact regions include protrusions of the molecular surface of γ-crystallin IIIb.

Crystal packing is quite different for highly homologous γII and γIIIb molecules (Sergeev et al., 1988). As crystallization conditions were practically the same and crystal polymorphism was not observed, it appears that the difference in the crystal packing is caused by amino acid replacements. The influence of these replacements should be taken into account as a whole. But we assume that a considerable contribution can be made by the six hydrophobic/hydrophilic replacements at 88, 98, 101, 103, 110 and 155.

Indeed, we have shown that the difference in the crystal packing of calf γIIIb and γII appears to be

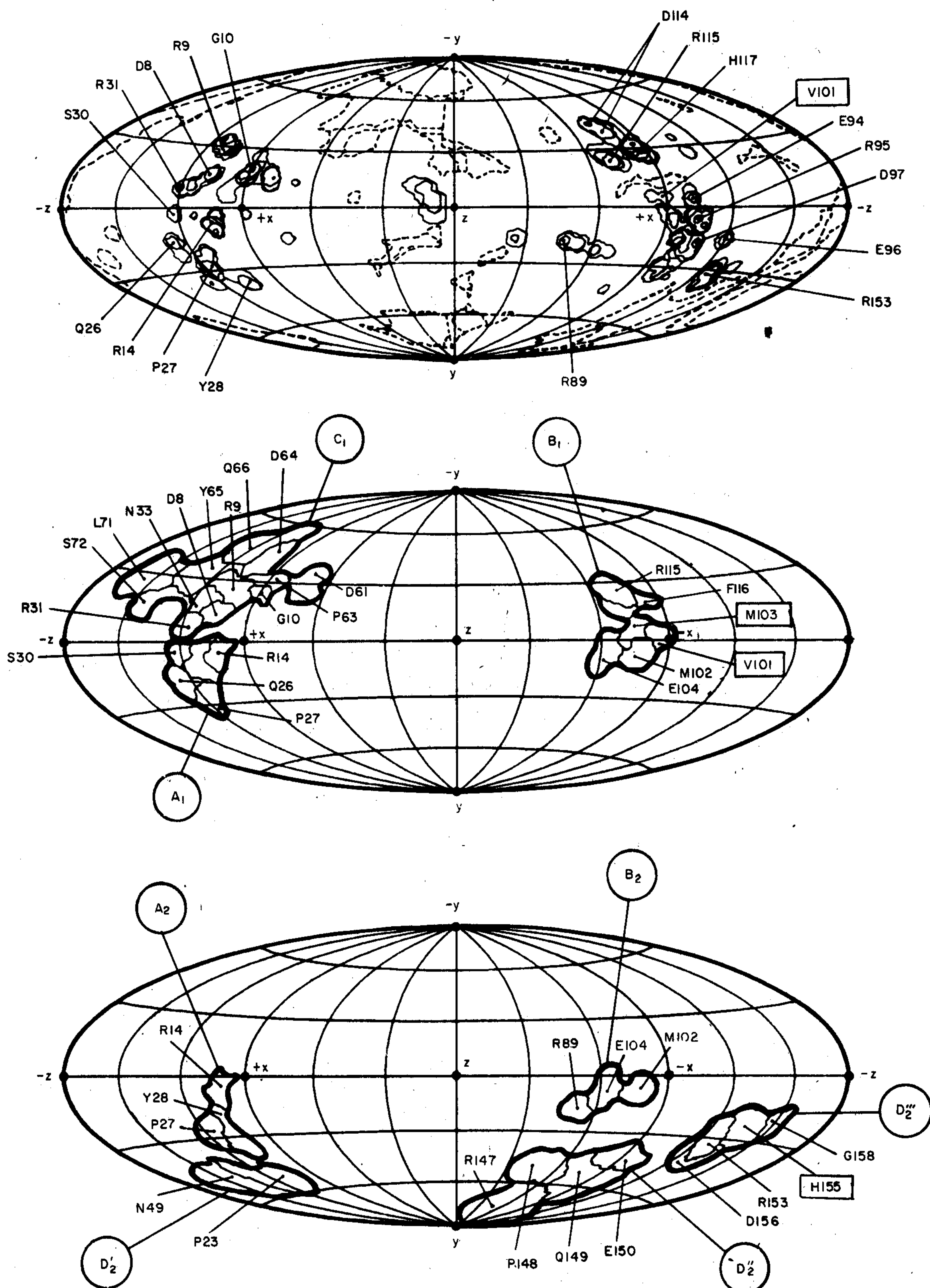

FIG. 8. Molecular surface maps of γ-crystallin IIIb. Top: Relief map. Solid lines show protrusions with levels from 8 to 14 Å with an increment of 1 Å upper 'sea' level, dotted lines denote hollows with levels from −3 to −7·5 Å and an increment −1·5 Å lower 'sea' level. Middle and bottom: Contact surface maps for molecules 1 and 2 of an asymmetric unit. Residues involved in intermolecular contact areas in crystal medium are joined in the heavily sized zones. Residues mainly responsible for specific crystal packing are selected by boxes.

TABLE 1

Connection of functionally important qualities of vertebrate eye lens with molecular structure of β/γ-crystallins

Eye lens qualities	Properties of β/γ-crystallins
High density and high refractive index	Specific contact regions on the molecular surface cause close packing of molecules in the lens medium (Wistow et al., 1983; White et al., 1989; Chirgadze et al., this paper).
Gradients of density and refractive index from lens centre to the periphery	Differential expression of different γ-crystallin gene products. Two-domain structure which makes possible different molecular packings. Surface residue variations supply difference in protein association (Siezen et al., 1988; Sergeev et al., 1988; White et al., 1989).
Lens transparency	Lack of density fluctuations. All gene products have a similar structure (Clark and Benedek, 1980; Blundell et al., 1983; Chirgadze et al., 1987).
High 'stability' of the lens	Destruction and aging is prohibited by a large amount and specific arrangement of thiol cysteine groups which provides a protective mechanism. Reduction of these groups by means of glutathione in metabolic processes (Harding, 1970, 1972; East et al., 1978; Spector and Roy, 1978; Bours, 1980; Zigler, et al., 1981).

determined by a few key positions of surface residues (Sergeev et al., 1987, 1988). In region B_1 of γIIIb there are two such residues, Val 101 and Met 103. In γII molecules these residues are replaced by Gln 101 and Ser 103 which are not involved in any interactions in the crystal. Therefore, residues Val 101 and Met 103 may be responsible for long associates of γ-crystallin IIIb in crystal layers. The third significant surface residue is 155. Polar His 155 in γIIIb is replaced by hydrophobic Leu 155 in γII. In both γ-crystallins this residue is involved in crystal interactions but in different ways. Therefore, residue 155 may also contribute to differences in the crystal packing. The remaining three significant positions are occupied by hydrophilic residues His 88, His 98, Ser 110 in γIIIb and by hydrophobic ones Phe 88, Phe 98, Pro 110 in γII. These replacements slightly increase the total hydrophilic surface potential of γ-crystallin IIIb.

4. Conclusions

We can consider two aspects of the γ-crystallin IIIb structure: properties common for the whole γ-crystallin family and its individual characteristics.

Recently we have examined the sequence homology of many γ-crystallins of vertebrates and have concluded that their spatial structures must be very similar (Chirgadze et al., 1987). Therefore all the molecules of γ-crystallin:

(a) are highly symmetrical and consist of four repeated motifs,
(b) are composed of two similar domains,
(c) contain a large amount of cysteines, mainly in the N-domain,
(d) contain charged surface groups, in the form of charge-compensated clusters,
(e) seem to have specific binding sites for glutathione.

The characteristic features of calf γ-crystallin IIIb as an individual gene product of eye lens are as follows:

(a) three key residues in positions 101, 103 and 155 which determine its specific associative ability,
(b) as compared with calf γII there are three residues in positions 88, 98 and 110 with hydrophillic side chains which seem to increase slightly the total surface hydrophilic potential,
(c) as compared with calf γII, there is an extra Cys 130 which helps to preserve the two-domain structure under degradation conditions.

An essential feature of the γ-crystallin IIIb surface is the hydrophobic spot formed by residues Val 101, Met 102, Met 103, Phe 116 and Leu 118.

The unique structure of the eye lens medium is obviously dependent on the spatial organization of individual crystallin molecules and their interactions. The two-domain organization is a common property of all the proteins from the β/γ-crystallin superfamily (Slingsby et al., 1988) and it appears to cause different molecular packing arrangements. Such differences depend, to a great extent, on point mutations at key positions. The involvement of the majority of charged groups in ion-pairs and clusters with an almost complete compensation of alternative charges of the opposite sign increases the molecular stability, preventing the molecule from unfolding at partial degradation.

Maintaining the unique lens ultrastructure during the whole lifetime of an organism seems to depend in part on the functioning of γ-crystallins. This depends on the reduction potentials of cysteine thiol groups which are directly related to their arrangement in the three-dimensional protein structure. Degradation of the native structure of γ-crystallin causes a sequential increase in the number of accessible SH groups which can interact with glutathione or form special S—S bonds (Harding, 1972; East et al., 1978). In any case, dangerous non-specific protein aggregation is warded off, and the lens medium preserves its transparency.

For a complete explanation of all lens qualities in normal or pathological states, we must have knowledge of all crystallins and lens ultrastructure. However, even now many qualities of the lens, such as high density, high refractive index and its gradient, lens transparency and its unusually high 'stability' during an organism's life may be connected with the three-dimensional structure of crystallins. They are summarized in Table I. It should be mentioned that both common and individual properties of γ-crystallins are equally important.

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