The X-ray structure of a mutant eye lens β B2-crystallin with truncated sequence extensions

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Abstract

β -Crystallins are oligomeric eye lens proteins that are related to monomeric γ -crystallins by domain swapping: like γ -crystallins, they are comprised of two similar domains but they differ in having long sequence extensions. β B2, a major component of β -crystallin oligomers, self-associates to a homodimer in solution. In two crystal structures of native β B2, the protein is a 222-symmetric tetramer of eight domains. It has previously been shown that a mutant of rat β B2-crystallin, in which the bulk of the N- and C-terminal sequence extensions has been deleted, assembles into dimers and tetramers. Here we present the 3.0 Å resolution X-ray structure of the tetramer, β B2 Δ NC1. The mutant tetramer has a very similar set of domain interactions to the native structure. However, the structures differ in the relative orientation of the two sets of four domains. The paired N- and C-terminal domain interface, which is at the heart of the dimer structure, is very similar to the native structure. However, the truncation of the C-terminal extension removes an important tryptophan residue, which prevents the extension from acting as a (non-covalent) linker, as it does in native β B2. There is a knock-on structural effect that removes a contact between extension and covalent linker, and this appears to cause a small twist in the linker that is amplified into a 20° rotation between sets of paired domains.

Keywords: β B2-crystallin; domain interactions; extension; linker

Transparency and refraction of eye lenses are dependent on the lens crystallin structural proteins being sufficiently concentrated and evenly distributed over distances comparable to the wavelength of light (Benedek, 1971). The proteins have to last a lifetime, as the lens cells lose their nuclei and cells are never shed (Harding & Crabbe, 1984). It is, therefore, important to define the structural features that contribute towards crystallin stability and their interactions. There are two major protein classes in the mammalian lens: the small heat shock related protein α -crystallin of unknown structure, and the β γ -crystallin super-family of β -sheet proteins (Wistow & Piatigorsky, 1988; de Jong et al., 1994). β γ -Crystallin polypeptides fold into two similar domains (comprised of two "Greek key" motifs) connected by a linker and domains assemble into monomers, dimers and higher oligomers (Blundell et al., 1981; Bax et al., 1990). The X-ray structure of β B2-crystallin showed a novel way of making oligomers from a two-domain monomer by a process now known as domain swapping, and similar examples are found in many oligomeric structures (Bennett et al., 1995; Fletterick & Bazan, 1995). A major sequence

difference between oligomeric β -crystallins and monomeric γ -crystallins is that β -crystallins have long terminal extensions. There is interest in the structural role played by sequence extensions in maintaining protein–protein interactions within the lens, as early truncation of β -crystallin sequence extensions is associated with loss of solubility and cataract in rodent models (David et al., 1993; Tumminia et al., 1996).

β B2-Crystallin is a dimer in solution, and the ordered part of the bovine structure, as visualized in the I222 X-ray crystal structure (Bax et al., 1990), is shown in Figure 1. The structure of each subunit has an extended linker in contrast with the bent linker of monomeric γ -crystallins. In β B2 crystallin there is a 15-residue N-terminal extension, but in this crystal structure only two residues (–1 and –2) that were adjacent to the domain were visible. In the β B2 X-ray structure the first five residues of the C-terminal extensions (shown in yellow in Fig. 1) cross the interface between the paired domains and insert Trp 175 into a hydrophobic pocket in the partner N-terminal domain, rather like a non-covalent linker, and so contribute to stabilizing the dimer. The additional 10 C-terminal residues were not visualized (Lapatto et al., 1991). Using 2-D NMR spectroscopy, those regions found disordered in the crystal structure were found to be mobile in solution (Carver et al., 1993), which was in agreement with the protein engineering studies show-

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Fig. 1. The structure of native bovine β B2-crystallin dimer. The two subunits are related by a twofold (P axis) perpendicular to the plane of the diagram. The linker peptide (dark blue) between domains is extended, and N- and C-terminal domains (light blue and red, respectively) from different molecules pair to form the dimer. The C-terminal extension (yellow) makes extensive interactions with the partner N-terminal domain. Of particular importance is the side chain of Trp 175 (also shown in yellow): this is the last residue in the extension that can be seen in the electron density, and makes an important interaction with a hydrophobic pocket at the bottom of the N-terminal domain. The search model used in molecular replacement consisted of a pair of interacting N- and C-terminal domains with the linkers and extensions removed.

ing that neither of the extensions were necessary for dimerization (Kroone et al., 1994; Trinkl et al., 1994). Bovine β B2 has also been crystallized in space group C222 (Nalini et al., 1994), and in both crystal forms the unit of structure in the lattice is a tetramer. Although in both tetrameric structures the bulk of the extensions do not contribute to stabilizing the tetramer, in the C222 form the N-terminal extension mediates tetramer–tetramer interactions (Nalini et al., 1994).

To investigate more closely the role of the extensions in β -crystallin stability and interactions, a mutant rat β B2-crystallin (β B2 Δ NC) was made in which the bulk of the N- and C-terminal extensions was truncated (Trinkl et al., 1994). Although the mutant lacks the important interface residue Trp 175, solution studies showed that it associates to form not only dimers (β B2 Δ NC2) but also tetramers (β B2 Δ NC1) (Trinkl et al., 1994). Here we present the X-ray structure of the tetrameric form of rat β B2 Δ NC.

Results and discussion

Structure solution

Crystals of the tetrameric form of truncated rat β B2, residues –1 to 173 (Table 1), grew in the presence of 0.2 M magnesium acetate in space group C2 with cell dimensions (Å) $a = 90.2$, $b = 68.6$, $c = 77.6$, $\alpha = \gamma = 90.0^\circ$, $\beta = 98.6^\circ$. There are two identical

subunits (A and B), or one dimer, in the asymmetric unit based on a calculated solvent content of 58.1% (Matthews, 1968). Examination of the diffraction data (3.0 Å) showed pseudo-orthorhombic symmetry along both the [100] and [010] projections. The structure was solved by molecular replacement using paired N- and C-terminal domains of bovine β B2 as a search molecule comprising residues –1 to 81 and residues 88 to 170, in which the linker and the whole of the C-terminal extension were deleted (Table 1; Fig. 1). Following rigid body refinement, positional refinement was performed with X-PLOR (Brunger et al., 1987) using non-crystallographic restraints and the missing linker and extension residues were gradually built into the new density. The final crystallographic R -factor was 25%. The electron density for the linker is shown in Figure 2.

As can be expected at 3.0 Å resolution, not all the side chains could be modeled satisfactorily, but the overall fit to the electron density is good. Side chains of His 16 and Gln 86 in both molecules, and Lys 89 in the B chain, could not be modeled at any stage, and alanines were used in refinement. The final Ramachandran plot, determined with PROCHECK (Laskowski et al., 1993), has residues 28, 47, 69, 71, 71A, and 79 in “generously allowed” regions; only the insertion Asp 106A is in a “disallowed region,” which was also an outlier in the native structures. The RMS deviations (RMSDs) from ideal bond lengths and angles are 0.013 Å and 2.07° , respectively.

Table 1. The sequence of rat β B2-crystallin with the eight residues where bovine differs from rat shown underneath^a

N-terminal extension	
-15	-1
<u>ASDHQTQAGKPQPLN</u>	
Motif 1	
1	10
PKIIIFEQENFQGHSELSGPCPNLKETGMEKAGSVLVQA	
	N
	V
Motif 2	
40	50
GPWVGYEQANCKGGEQFVFEKGEYPRWDSWTSSRRTDSLSSLRPI	
Linker	
82	87
KVDSQE	
Motif 3	
90	100
HKIILYENPNFTGKKMEIVDDDVPSFHAHGYQEKVSSVRVQS	
T	VI
Motif 4	
130	140
GTWVGYYQYPGYRGLQYLLEKGDYKDNSDFGAPHPQVQSVRRRI	
	SG
	Q
C-Terminal extension	
180	
<u>RDMQWHQRGAFHPSS</u>	

^aThe sequence has been broken up into the component modules. The numbering is based on γ B-crystallin. The extensions deleted in β B2 Δ NC1 are underlined.

A superposition of the two molecules in the asymmetric unit of the final model using the program BESTFIT (H.P.C. Driessen, unpubl. obs.), using the method of Kearsley (1989), gave the RMSD for all identical atoms, over all residues, as 0.85 Å. The rotation

angle between subunits is 179.7°. The RMSD and rotation angles between the coordinates of N-terminal domains (residues 1–81) only, and between C-terminal domains (residues 88–170) only, were 0.86 Å, 178.4°, and 0.64 Å, 178.4°, respectively.

Fig. 2. Stereo diagram of the linker region in the final model. Because of the twist in the relative orientation of domain pairs at either end of the dimer, the linker region was not included in the initial model and had to be built in during refinement. The electron density omit map, shown here with $2F_o - F_c$ electron density contoured at 1σ , was calculated with the final linker residues excluded. The map has good continuous density for the main chain, and the side chains of Val 83, Asp 84, and Glu 87 are all well defined, whereas Gln 86 was not visible and so was built as an alanine.

Comparison of the mutant truncated crystallin with native β B2: a "twist" in the linker peptide

Figure 3 shows how the pairs of domains in the mutant dimer have a different relative orientation from that between them in the native protein. The transformation of one pair of β B2 Δ NC1 domains onto one pair of native β B2 domains, taken from the I222 structure, was calculated with BESTFIT. This transformation was then applied to the other pair of β B2 Δ NC1 domains. It is clear from Figure 3b that the change in relative orientation is largely due to a rotation about the Q -axis of the tetramer (as defined in Fig. 5). Calculating the exact transformation with BESTFIT shows the rotation angle to be 20.6° with a small translational component of 1.2 Å.

The big difference in the subunit interactions forming the dimer is in the C-terminal extension. In both crystal forms of native β B2 (C222 and I222) residues 171–175 of this extension make extensive interactions with the partner N-terminal domain (Fig. 4a). Of particular importance is Trp 175, which binds into a hydrophobic pocket on the N-terminal domains (mainly formed from prolines 1 and 80, but also involving Pro 41 and Ala 39 to some extent), causing the extension to be stretched out along the bottom of the domain interface. Met 173 is also important in this interaction, as its side chain packs against the outside surface of Trp 175, effectively excluding solvent (Fig. 4a). In β B2 Δ NC1, without Trp 175, there is nothing to pull the extension across to the N-terminal domain, and Met 173 interacts instead with Ser 85 of the partner linker (Fig. 4b). In doing so, a small change in the configuration of this region of the linker brings about a small reorientation of the covalently connected N- and C-terminal domains, which gives rise to a relative re-orientation of the two sets of domain pairs in the dimer (see Fig. 3). This, in turn, causes the same degree of twist in the tetramer.

Rat and bovine structures are very similar

The sequence differences between rat and bovine β B2 shown in Table 1 have no significant effect on the structure of the protein, or its oligomerization properties. On the basis of the solvent accessibilities, and analysis of lattice contacts using MODEL (H.P.C.

Driessen & P.F. Lindley, unpubl. obs.), none of these residues is involved in interactions between subunits to form the dimer, and only one (at position 161) is involved peripherally in dimer–dimer interactions to form the tetramer.

Subunit interactions forming the dimer

The truncated protein differs from native in the relative orientation of the two sets of paired domains. The two sets of paired domains in the β B2 Δ NC1 dimer are twisted by 20.6° relative to each other when compared to the I222 form of the native β B2 structure, and by a small screw component of 1.2 Å. Relative to the C222 form, the distortion is slightly less (15.2° with a 1.1 Å screw). It is considered that these differences are brought about by conformational changes in the linker and truncated C-terminal extension.

The core of the interface between N- and C-terminal domains, and their relative orientation to each other, remains largely unchanged in all three structures. The total buried area in the dimer interface of native β B2 (I222 form) is $2,040 \text{ Å}^2$ per subunit, whereas that of β B2 Δ NC1 is only $1,660 \text{ Å}^2$ per subunit. However, Gln 174 and Trp 175 make extensive interactions with the partner N-terminal domain in native β B2, and both are absent from the interface in β B2 Δ NC1. If these two residues are removed from the calculations, the buried area in the native β B2 dimer interface drops to $1,680 \text{ Å}^2$ per subunit, nearly identical with that of β B2 Δ NC1. The absence of Trp 175 from the dimer interface thus explains the ready dissociation of truncated dimers into folded monomers (Trinkl et al., 1994). The interface tryptophan can best be described as a sticky surface patch adhering to the N-terminal domain rather than a plug.

A least-squares fit of the paired domains of bovine β B2 onto those of β B2 Δ NC1 also underlines the similarity of the N- and C-terminal domain interaction. Taking the N-terminal domain as residues 1–81 and the C-terminal domain as 88–170, fitting paired N- and C-terminal domains with BESTFIT gives the RMSD between main-chain atoms of the two structures at only 0.59 Å. The RMSD between main-chain atoms of C-terminal domains alone is 0.64 Å, and that between N-terminal domains is 0.49 Å, so the interfaces are clearly very similar. Close analysis of the contacts

Fig. 3. The relative reorientation of domain pairs in β B2 Δ NC1. If one pair of domains in the β B2 Δ NC1 dimer (gray) is super-imposed on one pair of the native dimer (green), the other pair can clearly be seen to be in a different orientation. (b) An alternative view of the bottom pair of domains in (a) is shown parallel to the Q axis, demonstrating that the difference is predominantly a rotation about Q .

Fig. 4. Different roles for Met 173 in dimer contacts. (a) In native β B2-crystallin Met 173 makes contacts with Trp 175, helping to bury that residue. (b) In the truncated mutant, β B2 Δ NC1, Met 173 interacts with Ser 85 of the partner linker, introducing a change in the conformation of the partner linker.

between paired N- and C-terminal domains using the program MODEL, and viewed graphically with O (Jones & Kjeldgaard, 1994), confirms that the interfaces are essentially identical, except in the C-terminal extension.

Dimer-dimer interactions forming the tetramer

The truncated β B2 Δ NC1 forms a very similar tetramer to that observed in the lattices of the native crystallin (Fig. 5). As with residues involved in dimer formation, so those involved in tetramer formation are unchanged between β B2 Δ NC1 and native bovine β B2. A comparison of the area buried in tetramer formation shows that there is no significant difference between the two structures. The surface area of each dimer buried in β B2 Δ NC1 is 1350 Å², while that in native β B2 is 1340 Å² per dimer. Analysis of the contacts using MODEL confirms the similarity between the structures, the only major difference being due to a change in the conformations of the side chains of Arg 71 and Arg 140 giving rise to different hydrogen bonding for Arg 140 N ϵ in the two structures.

Crystal lattice effects

A recent comparison of three different crystal structures of the cell-cycle control protein p13^{suc1}, a strand-exchange dimer, has

Fig. 5. The structure of β B2 Δ NC1 tetramer. The two truncated dimers (red and blue) interact about the non-crystallographic Q and crystallographic R axes to form a tetramer. The putative cation binding site is shown in blue chicken wire. The spatial relationship between the upper and lower set of four domains differs from the native tetramer due to a twisting of paired domains about the Q axis.

suggested that residues involved in strand-exchange (equivalent to the linker in β B2) represent a flexible hinge (Khazanovich et al., 1996). It is likely that the β B2 linker is a relatively flexible region of the protein and therefore sensitive to changes in its local environment or to the overall crystal packing. On consideration of the tetramer-tetramer interactions in the three β B2 structures, there is a total of 240 contacts per truncated tetramer compared to 269 in the native C222 lattice, and 208 in the native I222 form, which roughly parallels the differences in solvent content. In the more densely packed native C222 form, the N-terminal extensions make extensive tetramer-tetramer interactions (Nalini et al., 1994), and yet the domain organization is very similar to the native I222 form. This indicates that in the mutant structure it is the truncated C-terminal extension of one subunit that causes the linker to twist in the partner subunit rather than crystal lattice effects.

Conclusion

The mutant protein β B2 Δ NC1 presented here, which lacks the N-terminal extension and has a truncated C-terminal extension, forms a tetramer in the crystal lattice similar to the native tetramer, but with a twist of around 20°. No role for the N-terminal extension in dimer or tetramer formation has been found, but the C-terminal extension, particularly Trp 175, is involved in native dimer formation (Fig. 1). The truncated C-terminal extension of β B2 Δ NC1 lacks Trp 175, and makes different interactions from those in native β B2 (Fig. 4). In particular, Met 173 in β B2 Δ NC1 interacts with the linker peptide of the partner molecule, appearing to bring about the change in relative orientation of domains by changing the conformation of the linker peptide. Although a putative weak binding cation site has been identified (see Experimental), it is considered that the truncation of the C-terminal extension is the most likely source of the altered linker conformation. Only a small change in the linker conformation is needed to effect a large change in orientation between sets of paired domains, which in turn, will bring about changes in the packing of tetramers in the crystal and in the lens. A recent study showing how truncation of the C-terminal domain extension of γ β -crystallin by one tyrosine residue has a radical effect on paired domain interactions (Norledge et al., 1996) underlines the critical role of these C-terminal extensions in mediating longer range order in the eye lens β γ -crystallins.

Experimental

Expression and crystallization

The β B2 Δ NC construct (residues -1 to 173, see Table 1) was expressed and purified as described by Trinkl et al. (1994), and the tetrameric (β B2 Δ NC1) and dimeric (β B2 Δ NC2) forms were separated by gel filtration.

β B2 Δ NC1 was subjected to crystallization trials by the hanging drop method using Crystal Screen™, Hampton research. Best results were found with solution 18 of the crystal screen, which consists of 0.2 M magnesium acetate trihydrate in a 0.1 M sodium cacodylate buffer, pH 6.5. Hanging drops of 4 μ L protein solution and 1 μ L well solution were suspended at room temperature over a well containing 0.5 mL of solution 18 and 0.5 mL distilled water. The protein solution used was 13 mg/mL protein in 5 mM morpholinopropane sulfonic acid (MOPS) buffer (pH 7.0) with 2 mM sodium azide. Rhomboid-shaped crystals grew within a few days.

Data collection and processing

A first X-ray dataset was collected for β B2 Δ NC1 on a MAR Research 18 cm Image Plate, mounted on a Siemens XP-18 rotating anode generator running at 40 kV and 100 mA using CuK α radiation. Data to 3.5 Å resolution were collected from two crystals at two different times. REFIN (Kabsch, 1993) and DENZO (Otwinowski, 1993) were used to determine the spacegroup as C2 with cell dimensions $a = 90.2$ Å, $b = 68.6$ Å, $c = 77.6$ Å, $\alpha = \gamma = 90.0^\circ$, $\beta = 98.6^\circ$. For the first dataset, profile fitted intensities were measured using DENZO, and the data were scaled and merged using SCALEPACK with postrefinement to improve the estimates of mosaicity and the unit cell parameters. A total of 5,732 reflections were merged to 3,785 unique reflections with an R_{merge} of 9.1% and 81.2% of reflections had $I/\sigma(I) > 3$ (46.6% in the highest resolution bin: 3.5–3.63 Å). The average redundancy was therefore 1.51 (1.38 in the highest resolution bin). The second set

of data collected in-house (again to 3.5 Å) was processed similarly to the first, merging 15,227 reflections to 5,549 unique reflections (average redundancy 2.74 overall, 2.44 in the highest bin) with $R_{\text{merge}} = 10.0\%$. These data were 91.4% complete (61.8% at 3.5–3.63 Å) with 83.9% of reflections having $I/\sigma(I) > 3.0$ (34.1% at 3.5–3.63 Å). The two 3.5 Å datasets were then merged with each other as one batch giving a total of 5,640 unique reflections (93.4% complete) to 3.5 Å (69.7% complete at 3.5–3.63 Å).

Higher resolution data (to 3.0 Å) were collected from a third crystal at station 9.6 of the Synchrotron Radiation Source (SRS), SERC Daresbury Laboratory, using a 30 cm MAR Research Image Plate and a radiation wavelength of 0.88 Å. This dataset was also auto-indexed and the intensities measured using DENZO. Scaling and merging with SCALEPACK resulted in 11,492 reflections being reduced to 6,601 with an R_{merge} of 5.2% (23.5% in the range 3.11–3.0 Å). These data were 66.4% complete (66.7% at 3.0–3.11 Å) with 82.3% (47.5% at 3.11–3.0 Å) having $I/\sigma(I) > 3.0$. Finally, all three datasets were scaled together yielding a grand total of 8,554 unique reflections (82.5% complete) with an overall average redundancy of 3.8 (1.65 at 3.11–3.0 Å) and 82.5% of reflections having $I/\sigma(I) > 3.0$.

Pseudo-symmetry

The diffraction data, after scaling and merging, were examined using HKLVIEW. Along both the [100] and [010] projections there is pseudo-orthorhombic symmetry ($p2mm$). This does not lead to a spacegroup ambiguity, as the β -angle is undoubtedly far removed from 90° and so one of the pseudo-mirror planes in the 010 projection is at an angle of 8.6° (β -90) to the axis. It does, however, strongly imply the existence of non-crystallographic 222 symmetry in the crystal. The third special projection ([001]) has the expected $c1m1$ symmetry, but the pseudosymmetry is not apparent. This is because the pseudo-orthorhombic special projection will lie in the pseudo-mirror plane perpendicular to both [100] and [010]. Pseudo-222 symmetry is not surprising, because the native β B2 forms a tetramer with 222-symmetry in both crystal forms and β B2 Δ NC1 is a tetramer in solution.

Molecular replacement

The initial 3.5 Å dataset was used to solve the rat structure by molecular replacement, using native bovine β B2-crystallin (Brookhaven PDB ID: 2bb2) as a search model (the sequence identity between rat and bovine β B2-crystallin in the residue range -1 to 173 is 95% (Table 1). Using the method of Matthews (1968), and a relative molecular mass of 20,371, the solvent content of the crystals was found to be 58.1% for two molecules in the asymmetric unit of the crystal, or 79.1% for one molecule in the asymmetric unit. The solvent content of the crystals of native bovine β B2-crystallin was 67.8% in the I222 form and 55.5% in the C222 form. Seventy-nine percent is close to the upper limit for solvent content of protein crystals, and the pseudo-symmetry is consistent with there being two molecules (half a tetramer) in the asymmetric unit, so it was presumed there were two subunits (i.e., one dimer) in the asymmetric unit.

On considering the structure of β B2-crystallin (Fig. 1), there is no interaction between the N- and C-terminal domains of the same molecule, other than they are connected to either end of a 15 Å long connecting peptide. A change in the relative orientation of these two domains would make this search model ineffective. How-

ever, in the dimer, N- and C-terminal domains from different molecules interact about a pseudo-twofold axis (Fig. 1), burying a large proportion of their surface areas. A significant change in the relative orientation of these domains would be much less likely. A search model was therefore constructed, consisting of paired N- and C-terminal domains, with no linker. The N-terminal domain was taken from residue Asn -1 to Ile 81, while the C-terminal domain consisted of residues His 88 to Val 170 (Table 1; Fig. 1).

This search model was moved to the origin, and structure factors calculated in a P1 cell of dimensions (Å) $a = 75$, $b = 55$, $c = 55$, $\alpha = \beta = \gamma = 90.0^\circ$. The structure factors were normalized with ECALC, and then used for the cross-rotation function in AMoRe, with a range of search radii and a 3 Å cutoff at the origin. The results consistently yielded two peaks $\sim 5.4\sigma$ and 4.3σ above the rest. Furthermore, these peaks are related to each other by a twofold axis, as expected from a dimer in the asymmetric unit. The top 10 peaks were then input to the AMoRe translation function to ascertain the position of the first molecule in the asymmetric unit. The best solutions for the top two rotation function peaks gave solutions with correlation coefficients of 20% and 16.5%, compared to around 10% for the other rotations tried. To find the position of the second molecule in the asymmetric unit, each of the two molecules was fixed, in turn, in its new-found position, and the translation function repeated for the second orientation. This improved the correlation coefficient to 38% compared to 29% for the next highest combination. Because of the internal symmetry of the search model, a second (incorrect) solution was expected with the N-terminal domain in the position of the C-terminal domain (and vice versa). This was discovered with a correlation coefficient of 27%, well below that of the correct solution. Rigid body refinement of these solutions was then carried out using the AMoRe option FIT-FUN. The packing of these two final solutions was then examined using MOLPACK (Wang et al., 1991) and found to be self-consistent with no major clashes. Furthermore, it agreed with the observed pseudosymmetry in the diffraction pattern. The packing of other combinations of orientations that scored high in the translation function was also examined, and found to be unsatisfactory.

Refinement

Rigid body refinement

The program PDBSET was used to rotate and translate the model coordinates to the correct positions as determined by the molecular replacement. Five percent of the reflections were flagged as not to be refined against, to calculate the free R -factor during refinement. Refinement with each domain as a separate rigid body was then carried out in X-PLOR (Brunker et al., 1987), over the resolution range 8.0–3.5 Å, resulting in a free R -factor of 45.7% and a refined R -factor of 44.7%. Following rigid-body refinement the degree of rotation between each end of the dimer was recalculated with BESTFIT, and found to be a 20° rotation and a 1.4 Å translation. Any change in the relative orientation of paired N- and C-terminal domains was also measured by the same method, and found to be very small.

Addition of linker and extension

Positional refinement (Powell minimization) was then performed with X-PLOR, using the NCS-restraints option. The equivalent regions were defined as being the two N-terminal domains and the two C-terminal domains found in the asymmetric unit. At this stage neither the linkers (residues 82–87) nor the short

C-terminal extensions (residues 171–173) were present in the model. After 300 cycles the free and refined R -factors were 35.7% and 26.9%, respectively, over the resolution range 8.0–3.5 Å. Only an overall isotropic temperature factor was refined. This model was then rebuilt using the O graphics program (Jones & Kjeldgaard, 1994), during which eight bovine β B2 residues were replaced by those of rat β B2 (Table 1).

After the first round of refinement, the 3.0 Å data became available, and were included in all later stages of refinement. Five percent of the new reflections (in the range 3.5–3.0 Å) were also excluded from refinement for the calculation of a free R -factor. Maps were calculated using the programs SFALL to calculate structure factors and SIGMAA to generate weights. During the next seven rounds of refinement and rebuilding using O and X-PLOR, linker residues were only added to the model when electron density was available in the $2F_o - F_c$ and $F_o - F_c$ maps calculated after refinement. The missing residues of the linker and C-terminal extension were added in to the electron density by adding them one or two at a time to the ends of the current model, as summarized in Table 2. The linker region was at first ill-defined in both the $2F_o - F_c$ and $F_o - F_c$ density maps.

During the early course of refinement (without the linker) the electron density for the missing linker residues improved, and residues could be built in to this density using the linker peptide of the native bovine β B2 structure as a guide to orienting the new residues. This was done on the assumption that the change in conformation of the linker would be due to an accumulation of small changes along its length rather than a sudden twist at one particular point. The task was also facilitated by the fact that the linker had obviously to be in an extended conformation, like in the native protein. The final electron density for the linker, calculated from an omit map in which the linker residues were omitted from the model, is shown in Figure 2.

Putative magnesium binding

After the sixth cycle of refinement, when all the residues had been added into the density, a large region of density at a high level in both the $2F_o - F_c$ and $F_o - F_c$ maps had not been accounted for. This density lay near the middle of the tetramer, on the crystallographic twofold R axis relating dimers in the tetramer, but slightly removed from the non-crystallographic twofolds (Fig. 5). The buffer solution contained Mg^{++} , Na^+ , cacodylate, and acetate ions, and

Table 2. Refinement of β B2 Δ NC1^a

Run no.	Resol range	R-Factors		New residues included	NCS refined
		Free	Refined		
1	8–3.5	35.7	26.9		Yes
2	8–3.0	42.0	31.2	83,171,172	Yes
3	8–3.0	40.8	28.3	173	Yes
4	8–3.0	39.9	26.8	84,87	No
5	8–3.0	38.0	25.5	85,86	No
6	8–3.0	36.1	25.0		No
7	8–3.0	36.7	24.9		No
8	8–3.0	35.8	24.8		No
9	8–3.0	N/A	25.3		No

^aThe New residues included column lists the numbers of the residues that were built into the coordinates before that round of refinement.

so each of these was considered as a possibility to explain this density. Cacodylate is a much bigger molecule than the other three, and it is doubtful whether it could physically fit into the space. Furthermore, the arsenic should have led to a much higher peak in the $F_o - F_c$ map, and so cacodylate was discounted. The nearest neighbour residues to this density were two aspartates (106A and 107) and their symmetry related equivalents, and so it seemed improbable that a negatively charged ion would fit in. At this resolution (3.0 Å) and with an R -factor of 25%, it is a matter of some debate as to whether water molecules can be identified. However, water molecules were placed into the density and refined. Placing a water molecule in the position left some residual positive density in the difference map, suggesting a larger species might belong. The difference map showed that replacing this water with Mg^{++} gave no negative density, and less positive density, suggesting it explained the density slightly better. However, the coordination of a magnesium ion at this position is very low (two aspartate residues at a distance of 3.3 Å and two water bridges to two more aspartates), so the more diffuse Na^+ was also considered. This gave similar results in refinement to the magnesium.

The electron density for the putative Mg^{2+} ions is far from unambiguous. It nestles close to the heart of the tetramer (Fig. 5), near the highly negatively charged region formed by two aspartate residues (106A and 107), and their symmetry related equivalents. However, a larger reorientation of domains than that seen would be necessary to bring all of these residues close enough to properly coordinate calcium or magnesium, and only one side of the tetramer could bind at a time. Crystals of native β B2 grown in the presence of lower levels of Ca^{2+} (Bax & Slingsby, 1989; Bax et al., 1990) showed no calcium binding. Low affinity binding of Ca^{++} to β B2-crystallin has been reported (Sharma et al., 1989), and so Mg^{++} was deemed the most likely candidate although having poor coordination, and therefore low occupancy. Much higher resolution data are needed to determine the nature of this area of the structure. It is possible the density represents an ion picked up from the *E. coli* expression system that is responsible for stabilising the mutant tetramer in solution and might explain why on unfolding and re-folding, the tetramer is exclusively converted to a dimer (Trinkl et al., 1994).

The C-terminus of the protein

There was evidence of extra density beyond Met 173 pointing into the solvent in both molecules in the asymmetric unit. The calculated mass of the reported sequence is $M_C = 20,371$ Da, whereas that measured by mass spectrometry is significantly larger at $M_{MS} = 20,503$ (measurements made by O. Bateman, Birkbeck College, on a VG-Platform mass spectrometer). Although the measured mass agrees well with the calculated mass of a protein containing the subsequent Gln 174 of the native sequence (Table 1), this was not supported by the sequence of the expression clone. At this resolution it is, therefore, not possible to explain the origin of this density.

Secondary structure, accessibilities, and lattice contacts

Surface accessibilities were calculated using the CCP4 program AREAIMOL, which uses the method of Lee and Richards (1971), and analyzed using RESAREA and DIFFAREA. Ion pair interactions and lattice contacts were calculated with the program MODEL (H.P.C. Driessen & P.F. Lindley, unpubl. obs.), and further examined using O (Jones & Kjeldgaard, 1994). All programs, unless otherwise stated, are from CCP4 (1994).

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