Dimerization of β B2-crystallin: The role of the linker peptide and the N- and C-terminal extensions*

SABINE TRINKL, RUDI GLOCKSHUBER,¹ AND RAINER JAENICKE

Institut für Biophysik und Physikalische Biochemie, Universität Regensburg, D-93040 Regensburg, Germany

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Abstract

β B2- and γ B-crystallins of vertebrate eye lens are 2-domain proteins in which each domain consists of 2 Greek key motifs connected by a linker peptide. Although the folding topologies of β B2- and γ B-domains are very similar, γ B-crystallin is always monomeric, whereas β B2-crystallin associates to homodimers. It has been suggested that the linker or the protruding N- and C-terminal arms of β B2-crystallin (not present in γ B) are a necessary requirement for this association. In order to investigate the role of these segments for dimerization, we constructed two β B2 mutants. In the first mutant, the linker peptide was replaced with the one from γ B (β B2 γ L). In the second mutant, the N- and C-terminal arms of 15- and 12-residues length were deleted (β B2 Δ NC). The β B2 γ L mutant is monomeric, whereas the β B2 Δ NC mutant forms dimers and tetramers that cannot be interconverted without denaturation. The spectral properties of the β B2 mutants, as well as their stabilities against denaturants, resemble those of wild-type β B2-crystallin, thus indicating that the overall peptide fold of the subunits is not changed significantly. We conclude that the peptide linker in β B2-crystallin is necessary for dimerization, whereas the N- and C-terminal arms appear to be involved in preventing the formation of higher homo-oligomers.

Keywords: γ B-crystallin; β B2-crystallin; protein association; protein folding; protein stability

Crystallins differ from other proteins mainly by 2 features: First, they exhibit an anomalous long-term stability due to a lack of significant protein turnover in the eye lens, and second, they are soluble at extremely high protein concentrations. Different degrees of oligomerization of these proteins ensure tight packing, which is necessary for both the high refractive index and the transparency of the eye lens; however, unspecific aggregation is one of the physical causes of cataract (Harding & Crabbe, 1984; Wistow & Piatigorsky, 1988).

More than 50% of the soluble lens proteins in vertebrate eye lenses are β - and γ -crystallins. Because of their high structural homology, β - and γ -crystallins are often grouped in 1 subclass, although they share no more than 30% sequence identity and differ in their state of oligomerization. β -Crystallins are subdivided into 2 groups, the basic (β B) and the acidic (β A)

β -crystallins. β B2, the predominant basic β -crystallin, can form homodimers or hetero-oligomers, whereas γ -crystallins are exclusively monomeric. A comparison of the 3-dimensional structures of γ B- (Wistow et al., 1983) and β B2-crystallin (Bax et al., 1990) of the bovine eye lens shows that each β - and γ -polypeptide chain consists of an N- and C-terminal globular domain, each of which comprise 2 topologically equivalent Greek key motifs of antiparallel β -sheets (Fig. 1A). In the native β B2 dimer, the N-terminal domain of each monomer interacts with the C-terminal domain of the other. Hence, the intramolecular domain interactions of the N- and C-terminal domains in γ -crystallin are substituted by intermolecular subunit interactions in the β B2-dimer. In asking why β B2 forms dimers, whereas γ B is monomeric, the obvious difference between the 2 molecules is the conformation of the connecting peptide between the 2 domains. Due to the highly conserved proline and glycine residues, this linker is bent in γ B, whereas it is extended in β B2. β B2 also possesses N- and C-terminal extensions of 16- and 14-amino acid residues length, which are not present in γ B. These "flexible arms" are protruding from the compactly folded domains and could therefore not be defined in the X-ray analysis of β B2. It has been previously suggested that either the N-terminal arm (Berbers et al., 1983) or tryptophan 175, the last structurally well-defined residue in the C-terminal portion of β B2, are indispensable for dimerization. On the other hand, grafting the β B2-linker to γ B-crystallin did not generate a dimer, thus suggesting that the preference for dimer formation of β B2

*This paper is dedicated to the memory of Professor John G.G. Schoenmakers.

Reprint requests to: Rainer Jaenicke, Institut für Biophysik und Physikalische Biochemie, Universität Regensburg, Universitätsstraße 31, D-93053 Regensburg, Germany; e-mail: jaenicke@vax1.rz.uni-regensburg.dbp.de.

¹Present address: Institut für Molekularbiologie und Biophysik, Eidgenössische Technische Hochschule Zürich-Hönggerberg, CH-8093 Zürich, Switzerland.

Abbreviations: IPTG, isopropyl β -thiogalactoside; NTA, nitrilotriacetic acid; PMSF, phenylmethanesulfonyl fluoride; Tris, Tris(hydroxymethyl)aminomethane.

A

Fig. 1. β B2 wild type and mutants. **A:** α -Carbon backbone diagram of the polypeptide chains of β B2- (—) and γ B-crystallin (—). **B:** Amino acid sequence of rat β B2-crystallin (Aarts et al., 1989). Amino acids deleted in the β B2 Δ NC mutant are marked in bold. **C:** Amino acid sequence of the linker region in β B2, γ B, and β B2 γ L. Exchanged residues are marked in bold. The amino acid residues are numbered following the alignment of β B2- with γ B-crystallin (Bax et al., 1990).

B

N-terminal domain:

-16**MASDHQTQAGKPQPLN**¹PKIIIFEQENFQGHSELSGPCPNLKETGMEKAGSV
LVQAGPWVGYEQANCKGEQVFVEKGEYPRWDSWTSSRRTDSLSSLR⁷⁹

Linker:

⁸⁰PIKVDSQEH⁸⁸

C-terminal domain:

⁸⁹KIILYENPNFTGKKMEIVDDDVPSFHAHGYQEKVSSVRVQSGTWVGYQYPGYRGL
QYLLEKGDYKDNSDFGAPHPQVQSVRRIRDMQWHQRGAFHPSS¹⁸⁵

C

β B2-crystallin (rat): ... L R ⁸⁰**P I K V D S Q E H K I** ...

γ B-crystallin (bovine): ... C R ⁸⁰**L I P Q H T G T F R M**...

β B2 γ L-crystallin: ... L R ⁸⁰**L I P Q H T G T H K I** ...

should be assigned to differences in the domain interface rather than to the extended connecting peptide (Mayr et al., 1994).

In order to investigate the molecular basis for the dimerization of β B2 further, a β B2 mutant was constructed in which the straight linker peptide of β B2 was replaced with the v-shaped linker of γ B (β B2 γ L). Furthermore, the effect of the N- and C-terminal extensions of β B2 on its quaternary structure was studied, making use of a truncated β B2 mutant, lacking the N- and C-terminal arms (β B2 Δ NC). The mutants and the wild-type β B2 were expressed in *Escherichia coli* as native proteins and purified to homogeneity. The states of association of β B2 and its mutants were determined by analytical ultracentrifugation

and their stabilities against denaturants were compared by spectral analysis.

Results

Cloning, expression, and purification of β B2 and its mutants

Recombinant rat β B2 and its mutants were expressed in *E. coli* BL21 (DE3) using derivatives of the expression plasmids pET3d and pET11a, where the β B2 genes are under control of the T7-promoter (Dubendorff & Studier, 1991a, 1991b; Studier, 1991).

Fig. 2. A: Silver-stained SDS-PAGE illustrating the purification of β B2 and the isolated β B2, β B2 γ L, and β B2 Δ NC mutants. Lane 1, molecular mass standards; lane 2, soluble fraction of a cell extract of induced *E. coli* BL21(DE3) pET β B2; lane 3, β B2 containing fractions after NTA chromatography; lane 4, β B2 after Q-Sepharose; lane 5, molecular mass standards; lane 6, β B2; lane 7, β B2 γ L; lane 8, β B2 Δ NC1; lane 9, β B2 Δ NC2. **B:** Elution profile of β B2 Δ NC in degassed 0.1 M sodium phosphate buffer, pH 7.0, applied onto a Superose 12 size-exclusion column. Peak 1, β B2 Δ NC1; peak 2, β B2 Δ NC2. Arrows indicate the proteins used as molecular mass standards: bovine serum albumin (66 kDa), hen egg albumin (45 kDa), equine myoglobin (18 kDa), and hen egg lysozyme (14 kDa). Fifty microliters of a 0.5-mg/mL protein solution were applied to the column and eluted at a flow rate of 0.2 mL/min.

The gene of the β B2 Δ NC mutant lacking the N- and C-terminal arms was amplified by the polymerase chain reaction using primers eliminating residues -16--2 and 174-185 (Fig. 1B). The mutant containing the γ B-linker peptide (β B2 γ L) was constructed by replacing residues 80-87 with the corresponding octapeptide sequence of γ B using site-directed mutagenesis (Fig. 1C).

Both native β B2 and its mutants accumulated in *E. coli* cells as soluble proteins at concentrations >5% of the total cellular protein after induction of protein synthesis by IPTG (Fig. 2A).

Natural β B2-crystallin, as well as the β B2 γ L mutant, are rich in histidine residues within their N- and C-terminal extensions (Fig. 1B). We assumed that these regions are accessible to the solvent and thus attempted metal-chelate affinity chromatography as the initial chromatographic step in the purification scheme of these proteins. Both β B2 and β B2 γ L are selectively bound to a nickel-nitrilotriacetic acid matrix and obtained >85% pure after elution with an imidazole gradient (Fig. 2A). After anion-exchange chromatography on Q-Sepharose, homogeneous β B2 and β B2 γ L were obtained in yields of 10 and 5 mg/L bacterial culture, respectively (Fig. 2A).

For the purification of the β B2 Δ NC mutant, the NTA column could not be used because the protein lacks the histidine-rich extensions. Therefore, β B2 Δ NC was purified by sequential chromatography, using (1) Q-Sepharose anion exchange, (2) cation-exchange chromatography on S-Sepharose, and (3) preparative

size-exclusion chromatography on Superdex 75. As shown in Figure 2B, gel filtration yielded 2 different oligomeric forms. Both peaks, β B2 Δ NC1 and β B2 Δ NC2, were reproducibly obtained at a ratio of approximately 1:4. No interconversion of the 2 forms was observed after incubating the isolated proteins for more than 20 days at 4 °C as shown by rechromatography on Superose 12. The determination of the N-terminal sequences of both association forms revealed identical N-termini (NPKII). Also, identical migration characteristics on SDS-polyacrylamide gels were observed (Fig. 2A). Thus, the 2 recombinant β B2 Δ NC variants supposedly represent 2 distinct states of oligomerization of the same polypeptide chain.

Determination of the state of association

The molecular masses of native β B2-crystallin and its mutants were determined by analytical ultracentrifugation and compared with the calculated molecular weights of the corresponding monomers (Table 1). As expected, sedimentation equilibrium runs revealed that natural rat β B2-crystallin, like the bovine protein, forms homodimers that are also observed in the case of β B2 Δ NC2. In contrast, the high molecular weight fraction β B2 Δ NC1 is found to be a homotetramer. The linker mutant β B2 γ L represents a homogeneous monomer, even at the high protein concentrations accumulating at the bottom of the cen-

Table 1. Determination of the molecular mass of β B2 and its mutants in 0.1 M sodium phosphate buffer, pH 7.0^a

Protein	Molecular mass calculated for the monomer (kDa)	Molecular mass by analytical ultracentrifugation (kDa)	$s_{20,w}^0$ (S)	State of association
β B2	23.38	42.6 $\pm$ 1.9	4.23	Dimer
β B2 γ L	23.33	22.0 $\pm$ 1.8	2.53	Monomer
β B2 Δ NC1	20.05	72.2 $\pm$ 2.8	5.34	Tetramer
β B2 Δ NC2	20.05	35.5 $\pm$ 2.3	3.42	Dimer

^a The monomeric molecular masses were calculated from the amino acid sequences using the "uwgcg" program.

trifuge cell (~ 1 mg/mL). The sedimentation coefficients obtained from sedimentation velocity experiments are consistent with the masses calculated from the equilibrium runs (Table 1). All proteins migrated as symmetrical bands, and no heterogeneity could be detected upon linearization of the high-speed sedimentation equilibria.

Spectral properties

In order to characterize the recombinant β B2-crystallin and the various mutants, CD and fluorescence spectroscopy techniques were used (Fig. 3). As one would predict from the 3-dimensional structures of β - and γ -crystallins, the far-UV CD spectra are typical for β -sheet proteins with negligible helix content; the molar residual ellipticities are remarkably low ($-10,000$ deg $\cdot$ cm 2 $\cdot$ dmol $^{-1}$ at 218 nm) compared to other β -sheet proteins ($-5,000$) (Brahms & Brahms, 1980). The far-UV CD spectra show clearly that the overall polypeptide fold of β B2 is maintained in the mutants (Fig. 3A).

Although the profiles of the near-UV CD spectra for β B2 and its mutants appear to be similar, certain differences should be noted: the spectra of natural β B2 and the tetrameric β B2 Δ NC1 mutant coincide, whereas the spectrum of the dimeric β B2 Δ NC2 closely resembles the one of the monomeric linker mutant β B2 γ L (Fig. 3B).

Fluorescence emission spectra also reveal significant differences between wild-type β B2 and its mutants. The emission maximum is shifted from 325 nm for β B2 to 320 nm for β B2 Δ NC1/2 and 330 nm for β B2 γ L. Although the fluorescence spectra were

Fig. 3. Spectral properties of β B2 and its mutants in 0.1 M sodium phosphate buffer, pH 7.0. β B2, (—); β B2 γ L, (---); β B2 Δ NC1, (---); β B2 Δ NC2 (— · —). **A:** Far-UV CD spectra. **B:** Near-UV CD spectra. **C,D:** Fluorescence emission spectra (excitation wavelength: 280 nm) of native proteins (**C**) and denatured proteins (in 5 M urea) (**D**). Protein concentration: 17 μ M for CD spectra and 0.85 μ M for fluorescence spectra.

measured at identical molar concentrations of the proteins, the fluorescence intensities at the emission maxima are found to be different. Compared with the β B2 dimer, the β B2 Δ NC1 tetramer shows almost unchanged fluorescence, whereas for the β B2 γ L monomer and the β B2 Δ NC2 dimer, a decrease in fluorescence emission is observed. In the presence of 5 M urea, identical spectra typical for denatured proteins were obtained for β B2 and all its mutants; both the decrease in intensity and the red shift point to extensive denaturation. The fluorescence properties of denatured β B2 and the linker mutant β B2 γ L are identical; both proteins have the same number of tryptophan and tyrosine residues. β B2 Δ NC1 and β B2 Δ NC2 contain 1 less tryptophan; therefore, their fluorescence intensity is lower in the denatured state. The identical fluorescence properties of denatured β B2 Δ NC1 and β B2 Δ NC2 further support the previous assumption of identical polypeptide chains with alternative association modes.

Folding and stability

In order to compare the stabilities of β B2 and its mutants against pH and denaturants with the anomalously high stability of γ B-crystallin, the proteins were unfolded at acidic pH or in the presence of chaotropic denaturants. Measurements were performed using fluorescence spectroscopy at the maximum of the difference spectra obtained for the native and unfolded proteins (315 nm).

In contrast to γ B-crystallin, which is stable between pH 1 and 9 (Rudolph et al., 1990), β B2 and its mutants denature irreversibly at pH values below pH 4.5 and above pH 9. Also, the stability against urea is surprisingly low for β B2 and its variants compared with γ B that cannot be unfolded by urea at neutral pH (Sharma et al., 1990); the half-concentration of the urea-dependent denaturation transitions of β B2 and β B2 Δ NC1 and β B2 Δ NC2 is 1.7 M, the one of the linker mutant β B2 γ L, 2.1 M. With increasing protein concentrations, the denaturation transition for β B2 and β B2 Δ NC1 is shifted to higher urea concentrations, indicating stabilization due to quaternary structure formation (Fig. 4). At urea concentrations close to the denaturation transition, no aggregation was detectable by light scattering at 360 nm. Except for the tetramer, the urea-dependent denaturation transition is found to be fully reversible (Fig. 4). As shown by fluorescence emission, gel filtration, and ultracentrifugation, renaturation of the urea-denatured proteins leads back to the correctly folded and associated state. This does not hold for β B2 Δ NC1: evidence gained from analytical ultracentrifugation and gel filtration clearly shows that only a dimeric form is regenerated upon refolding and reassociation that is indistinguishable from β B2 Δ NC2. This holds even at high protein concentrations beyond 2 mg/mL in the renaturation assay, where gel filtration clearly proves that only the dimer is formed. At all concentrations investigated (20 μ g/mL–2 mg/mL), renaturation of β B2 Δ NC1 is independent of protein concentration.

In order to investigate whether β B2 unfolding is a 2-state process, the equilibrium transitions were monitored at different protein concentrations and the data were fitted according to Sandberg and Terwilliger (1989). As mentioned, the stability of β B2 against urea denaturation increases with increasing protein concentrations. However, the data cannot be fitted according to a 2-state model, thus excluding a direct interconversion of folded dimers into unfolded monomers ($D \leftrightarrow 2 U$). If

unfolding would follow a 2-state model, the transition curves should be shifted symmetrically, which is not the case (Fig. 4A). In spite of the fact that β B2 Δ NC2 is also a dimer, the urea transitions are found to be independent of protein concentration. As shown by analytical ultracentrifugation in the presence of 0.8 M urea, i.e., at urea concentrations close to the beginning of the denaturation curve (Fig. 4D), β B2 Δ NC2 is already completely dissociated to monomers before the change in fluorescence allows the denaturation transition to be detected. Therefore, the observed unfolding reaction refers to the monomer whose transition principally does not depend on protein concentration. The dissociation is thus spectroscopically silent in β B2 Δ NC2. In contrast, β B2 and the tetrameric β B2 Δ NC1 remain native and correctly associated at low urea concentrations. In the case of the linker mutant, the urea-dependent transition is again independent of protein concentration, as one would expect for a monomeric protein (Fig. 4B).

In summary, refolding of β B2 Δ NC1 is fully identical with refolding of β B2 Δ NC2. Both transitions are independent of protein concentrations and coincide (Fig. 4C,D). Therefore, the dimeric and tetrameric forms of β B2 Δ NC obtained after overproduction in *E. coli* indeed appear to represent 2 different oligomeric forms of the same protein.

Discussion

We have found that the state of association of β B2-crystallin depends on both the connecting peptide between the 2 domains and the N- and C-terminal arms protruding from the compactly folded molecule. Analytical ultracentrifugation experiments indicate that exchanging the straight linker of β B2 against the v-shaped connecting peptide characteristic for γ B-crystallin leads to monomeric β B2 γ L. Apparently the "grafted peptide" enforces intramolecular interactions between the 2 domains instead of the intersubunit interactions stabilizing the natural dimeric β B2 molecule.

On the other hand, deleting the N- and C-terminal arms of β B2 favors a dimeric and tetrameric form of the recombinant protein. Both are not interconvertible; instead, refolding of both the dimer and the tetramer exclusively yields the native dimer. There are a number of reasons that may explain why the expression of the recombinant protein in *E. coli* leads to an additional quaternary tetramer structure, differing from the authentic dimeric form. First, the high local concentration of the β B2 Δ NC mutant during translation and folding in the host cell may favor tetramer formation. In vitro refolding experiments are limited here to a maximum concentration of $\sim 100 \mu$ M (2.5 mg/mL) which is about 1–2 orders of magnitude below the cellular concentrations in *E. coli* or the eye lens; at the upper limit of 2.5 mg/mL, no tetramer was observed. Second, accessory proteins may be involved in quaternary structure formation in vivo. In this context, the tetramer as a product of association may be considered as a consequence of overexpression. The observation that reconstitution under all conditions investigated yields the native dimer favors the latter explanation.

The dichroic absorption in the far UV indicates that the overall polypeptide fold of β B2 is conserved in all the mutants. The proteins also exhibit comparable stability against urea, with midpoints of the denaturation transition at 1.7 M (for β B2, β B2 Δ NC1 and 2) and 2.1 M (for β B2 γ L), respectively. The pH range of conformational stability between pH 4.5 and 9

Fig. 4. Urea-induced equilibrium transitions of β B2 and its mutants. Denaturation and renaturation were performed in 0.1 M sodium phosphate buffer, pH 7.0, $\pm$ urea, incubating the protein solutions for 24 h at 25 $^{\circ}$ C. Unfolding at varying protein concentrations (c_p): symbols ($\Delta/\square/\circ$) refer to high, medium, and low c_p , respectively; $\bullet$ refers to renaturation at given c_p . **A:** β B2-crystallin. c_p (unfolding), 200/20/3.5 μ g/mL; c_p (refolding), 20 μ g/mL. **B:** β B2 γ L. c_p (unfolding), 80/20/10 μ g/mL; c_p (refolding), 20 μ g/mL. **C:** β B2 Δ NC1. c_p (unfolding), 200/20/5 μ g/mL; c_p (refolding), 200 μ g/mL. **D:** β B2 Δ NC2. c_p (unfolding), 200/50/20 μ g/mL; c_p (refolding), 20 μ g/mL.

is rather narrow compared to the structurally homologous monomeric γ B-crystallin. γ B shows long-term stability at pH 1–9 and does not unfold in 10 M urea at neutral pH (Sharma et al., 1990). At pH 2.0, urea causes sequential unfolding of the N- and C-terminal domains in a fully reversible biphasic 3-state $N \leftrightarrow I \leftrightarrow U$ transition (Rudolph et al., 1990). In contrast to this “folding-by parts” mechanism (Wetlaufer, 1981), the denaturation transitions of β B2 and its mutants are monophasic under all conditions investigated. Even the monomeric β B2 γ L unfolds in a highly cooperative way in 1 single transition. Evidently, the unfolding of one of the domains destabilizes the whole molecule, suggesting that the stability of the domains in β B2 is similar, and that the domains mutually stabilize each other. Experiments using the separate single domains are presently being performed in order to determine the free energies of stabilization and to characterize the residues involved in the specific domain and subunit interactions.

In contrast to the close similarity in the overall structure and stability of β B2 and its mutants, both near-UV CD and fluorescence emission exhibit significant differences. The local en-

vironment of the aromatic residues must be closely similar in the β B2 γ L monomer and the β B2 Δ NC2 dimer, on the one hand, and in the β B2 dimer and the β B2 Δ NC1 tetramer, on the other. The differences may reflect either topological variants with respect to the interactions of domains and/or subunits, or local differences in the positions of the chromophores relative to each other or to the solvent. A quantitative interpretation cannot be given. However, 2 conclusions can be drawn: (1) The fact that natural β B2 and its truncated tetrameric form (β B2 Δ NC1) show identical chromophore interactions supports the idea that the tetramer as a dimer-of-dimers is an association form trapped close to the native state of β B2. As far as aromatic residues are involved, the association in both forms buries the same surfaces. From the distribution of chromophores in the 3-dimensional structure, it is obvious that in the tetramer the QR interface must be the same as in β B2 (Bax et al., 1990). (2) The similarity in the spectra of the monomeric β B2 γ L and the dimeric β B2 Δ NC2 points to a relatively weak coupling of the subunits in β B2 Δ NC2 compared to the compact complete β B2-crystallin. The reduced stability of β B2 Δ NC2 toward urea seems to confirm this as-

sumption. However, it remains to be shown what is the effect of specific residues in the N- and C-terminal extensions, especially Trp¹⁷⁵, regarding the tertiary and quaternary contacts between the domains and subunits. Preliminary X-ray data collected for the C-terminal domain of γ B clearly show that the "loose ends" of the polypeptide chain may have a strong impact on the intermolecular interactions in the crystal lattice (C. Slingsby, H. Driessen, E.-M. Mayr, & R. Jaenicke, unpubl. results).

The principle underlying the different quaternary structures of γ B- and β B2-crystallin has been discussed ever since the structures were unraveled (Blundell et al., 1981; Bax et al., 1990; Lapatto et al., 1991). From the high-resolution X-ray data, no well-defined interactions for the N-terminal extension could be detected; in contrast, in the C-terminal arm, Trp¹⁷⁵ was found to interact with 2 residues in the N-terminal domain, thus contributing to interdomain contacts that may stabilize the dimer via conserved interfaces.

The truncated β B2 Δ NC mutants lack Trp¹⁷⁵. The fact that they still form dimers and higher oligomers sheds some doubt on the assumption that specific Trp interactions are a necessary requirement for the dimerization of β B2. In contrast to this conclusion, earlier proteolytic cleavage experiments seemed to indicate that the first 7 N-terminal residues of β B2-crystallin are essential for the formation of the dimer, whereas the last 7 residues of the C-terminal extension were found to be dispensable (Berbers et al., 1983). In these experiments, obviously, protease treatment leads to conformational distortions of the N-terminal domain: as shown by NMR spectroscopy, crucial quaternary contacts are affected by limited proteolysis (Carver et al., 1993). In agreement with the present findings, the NMR spectra indicated further that both terminal extensions in β B2 show high conformational flexibility, precluding their involvement in subunit association.

Because the N- and C-terminal extensions evidently do not play a significant role in the stabilization of the dimeric state of β B2, the intersubunit interfaces and/or the connecting peptide between the domains must be essential. From the X-ray data it was argued that sequence differences between β - and γ -crystallins in the linker region are primarily responsible for the different arrangements (Bax et al., 1990). The present study clearly confirms that the β B2 linker is of crucial importance in determining the state of association. As has been shown, mounting the straight connecting peptide of β B2 between the domains of γ B (γ B β) does not alter its monomeric state (Mayr et al., 1994). Obviously, in this case, the domain interactions dominate over the optimum conformation of the linker. One potentially crucial residue in determining dimerization is Leu⁸⁰. In the γ B β linker mutant, Leu⁸⁰ was not exchanged; this residue is a conserved proline in all β -crystallins and a leucine in all vertebrate γ -crystallins (Bax et al., 1990). The characterization of alternative linker mutants of γ B and β B2, where the residues at position 80 are also exchanged, may allow us to resolve the structural role of this residue.

In discussing the evolution of the various eye lens crystallins, it has been stressed that in the β - and γ -crystallin families, a single structural pattern is used as a building block. Different extensions and linkers are used in order to generate a wide variety of highly symmetrical molecules with different states of oligomerization. In the present study, we were able to demonstrate that it is possible to generate a whole array of oligomerization

states of β B2 ranging from monomers to tetramers by merely varying the linker or deleting the extensions. The physiological role of the extensions was previously discussed in connection with the formation of hetero-oligomers with other acidic β -crystallins (Slingsby & Bateman, 1990; Cooper et al., 1993). On the other hand, it was shown that removing the extensions caused aggregation in whole-lens cell extracts, suggesting that the extensions may be essential in enhancing the solubility of β -crystallins (David & Shearer, 1993). Thus, the extensions may not only provide specific intermolecular contacts, but also serve as "spacers" in order to prevent wrong association at high concentrations to homotetramers or insoluble higher aggregates.

Materials and methods

Molecular cloning procedures and expression

The expression plasmid pET β B2 was kindly provided by Dr. Nicolette Lubsen (University of Nijmegen). The mutant β B2 Δ NC was generated by amplifying the shortened gene from amino acid -1 to 174 using PCR. The sequences of the PCR primer of the N- (Δ NC-N-ter) and C- (Δ NC-C-ter) termini are:

Δ NC-N-ter: 5' GAAGGAGATATACATATGAACCCCTAAGATC 3'

Δ NC-C-ter: 5' GTTAGCAGCCGGATCCAAGCTTACATGTCAACGGATTC
GAC 3'.

β B2 γ L was obtained by site-specific mutagenesis according to Kunkel et al. (1987). Molecular cloning procedures were based on Sambrook et al. (1989). The sequence of the mutagenesis primer (γ L) is as follows:

γ L: 5' CTCAGTTCTCTGAGACTGATCCCGCAGCACACCGGTACCCACAA
GATCATCTTA 3'.

For the bacterial expression of the recombinant proteins, *E. coli* BL21(DE3) cells harboring the corresponding plasmids were grown in LB-medium containing ampicillin (100 μ g/mL). At an optical density at 550 nm of 1.0, crystallin overproduction was induced by adding IPTG (1 mM final concentration). β B2 and β B2 Δ NC were grown at 37 °C and harvested by centrifugation. For the mutant β B2 γ L it was necessary to lower the growing temperature to 21 °C in order to obtain the protein in the soluble fraction of the *E. coli* extract. The cells were grown overnight after induction with IPTG.

Protein purification

The cells were lysed by French press treatment in 50 mM Tris/HCl buffer, pH 8.0, containing 100 μ M PMSF. All purification steps were carried out at 4 °C.

β B2

The soluble fraction of the *E. coli* extract was dialyzed against 50 mM Tris/HCl buffer, pH 7.5, 1 M NaCl, and applied to an NTA affinity chromatography column (Diagen) equilibrated with the same buffer. β B2-Crystallin was eluted using a gradient from 0 to 0.3 M imidazole. The pooled fractions were dialyzed against 50 mM Tris buffer, pH 8.5, 2 mM EDTA, and

injected onto a Q-Sepharose fast-flow anionic ion-exchange column (Pharmacia) equilibrated with the dialysis buffer. The elution through a linear 0–0.5 M NaCl gradient yielded 98% pure β B2-crystallin as judged by densitometric measurements of silver-stained SDS-PAGE.

β B2 Δ NC

The protein was first chromatographed on Q-Sepharose ff, using the same conditions as for wild-type β B2 (see above). After dialysis against 0.1 M acetic acid/NaOH, pH 4.8, the protein was injected onto an S-Sepharose cationic exchange column, equilibrated with the dialysis buffer. The purified protein eluted as 1 peak in a 0–1 M NaCl gradient. The resulting mixture of the 2 different association forms, β B2 Δ NC1 and β B2 Δ NC2, was further purified by size-exclusion chromatography on a Superdex 75 fast protein liquid chromatography (FPLC) column (Pharmacia). This chromatographic step was carried out at room temperature in 0.1 M sodium phosphate buffer, pH 7.0. Analytical gel filtration was performed on a Superose 12 FPLC column. Proteins used as molecular mass standards were bovine serum albumin (66 kDa), hen egg albumin (45 kDa), equine myoglobin (18 kDa), and hen egg lysozyme (14 kDa).

β B2 γ L

The protein was first applied onto the NTA column following the same procedure as described before, omitting the 1 M NaCl in the dialysis buffer. As a second purification step, the protein was applied onto a Q-Sepharose fast-flow anionic-exchange column, after dialysis against 25 mM Tris/HCl buffer, pH 8.5. The elution with a 0–0.25 M NaCl gradient yielded homogeneous protein. To avoid aggregation and proteolysis of the protein, NaCl and glycerol were added to final concentrations of 0.3 M NaCl and 5% glycerol (v/v).

Proteins were dialyzed against 0.1 M sodium phosphate buffer, pH 7.0, and stored for up to 1 week at 4 °C. For long-term storage of small portions, the proteins were shock frozen with liquid nitrogen and stored at –20 °C.

The success of the purification procedures was followed by silver-stained SDS-PAGE (Fling & Gregerson, 1986). The concentrations of the native proteins were determined using molar absorption coefficients ($A_{280\text{nm}, 1\text{mg/mL}, 1\text{cm}}$) calculated according to Gill and von Hippel (1989): 1.9 for β B2, 1.83 for β B2 γ L, 1.82 for β B2 Δ NC1, and 1.89 for β B2 Δ NC2.

Determination of the molecular mass by analytical ultracentrifugation

Sedimentation velocity and sedimentation equilibrium measurements were performed in a Beckman Spinco model E analytical ultracentrifuge equipped with a high-intensity light source and a UV scanning system. Double sector cells (12-mm path) with sapphire windows were used in an AnG rotor. To detect possible concentration-dependent association or heterogeneity, the meniscus depletion technique was applied (Yphantis, 1964). Experiments were performed at 0–2 °C with initial protein concentrations of ~0.2 mg/mL, using a scanning wavelength of 280 nm. The partial specific volumes of the proteins were calculated from the amino acid composition and corrected for 20 °C and water viscosity (Durchschlag, 1986). *s*-Values were de-

termined at 44,000 rev/min by plotting $\ln r$ versus t and correcting for 20 °C and water viscosity. Sedimentation equilibria were obtained at 20,000 rev/min and evaluated from $\ln c$ versus r^2 plots using a computer program provided by Dr. Gerald Böhm (University of Regensburg).

Spectroscopic characterization

Spectral properties of native and denatured proteins

Fluorescence emission spectra were recorded at an excitation wavelength of 280 nm at 25 °C. The proteins were dissolved in 0.1 M sodium phosphate buffer, pH 7.0, with or without 5 M urea at a protein concentration of 0.85 μ M.

Near-UV and far-UV CD spectra were accumulated using a Jasco J 500 A CD spectropolarimeter at protein concentrations of 17 μ M in 0.1 M sodium phosphate buffer, pH 7.0, at 25 °C. Ten-millimeter cuvettes were used for the near-UV CD (350–250 nm) and 0.1-mm cuvettes for the far-UV CD spectra (250–190 nm).

Unfolding/folding equilibrium transitions

The urea-induced unfolding equilibrium transitions of β B2 and its mutants were determined by monitoring the relative change in the fluorescence intensities at 315 nm (excitation wavelength: 280 nm). For unfolding, the proteins were incubated at different urea concentrations in 0.1 M sodium phosphate buffer, pH 7.0, for 24 h at 25 °C. Renaturation of the unfolded proteins was accomplished by dilution (1/100) in buffers containing different urea concentrations. As in the case of the unfolding experiments, the proteins were incubated for 24 h at 25 °C.

Measurements of dichroic absorption were performed using the same samples and monitoring the relative change in ellipticity at 225 nm. Cuvettes of 1 mm or 10 mm were used, depending on the protein concentration. Urea concentrations were calculated from the refractive index of the solutions (Pace, 1986).

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