Guinea Pig and Bovine ζ-Crystallins Have Distinct Functional Characteristics Highlighting Replacements in Otherwise Similar Structures[†,‡]

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ABSTRACT: ζ-Crystallin, a major cytosolic protein of guinea pig lens, has been characterized as an NADPH: quinone oxidoreductase (EC 1.6.5.5) [Rao et al. (1992) *J. Biol. Chem. 267,* 97−103]. A bovine lens homolog with 83% sequence identity was found to have very different functional characteristics. While the bovine lens ζ-crystallin exhibits similar physicochemical properties, such as molecular weight, hydropathy profile, and predicted secondary structure, and exhibits strong immunological cross-reactivity with the guinea pig and human lens ζ-crystallins, it shows minimal quinone oxidoreductase activity. On the other hand, bovine lens ζ-crystallin, but not guinea pig ζ-crystallin, showed a strong binding affinity to single-stranded DNA (ssDNA) that could be competed with NADPH, the specific cofactor of ζ-crystallin. NADH and dextran sulfate did not affect this characteristic of bovine ζ-crystallin and the enzyme showed no binding affinity for the heparin−Ultragel A4R. Two-dimensional electrophoresis of bovine lens ζ-crystallin showed a distinct pattern of posttranslational charge modification as compared to the guinea pig protein. Alignment of eight ζ-crystallin sequences, and computer modelling of the bovine and human forms based on the crystallographically analyzed *Escherichia coli* form, suggest that if loss of a functional residue accounts for the lowered catalytic activity of the bovine protein, Tyr 52 of the *E. coli* enzyme, and the equivalent Tyr present in all known mammalian forms except the bovine, is the likely candidate. In the bovine form this tyrosine is replaced by histidine.

Crystallins, the major structural proteins of the eye lens, are the primary determinants of the refractive properties of this tissue. There are two major classes, the ubiquitous crystallins (α and *β*/*γ*) present in all vertebrates and the taxon-specific enzyme-crystallins, which occur in phylogenetically restricted groups (Wistow & Piatigorsky, 1988). The enzyme-crystallins are major soluble lens proteins that are either identical to or related to metabolic enzymes. They have been identified and characterized from a variety of species. The recruitment of enzymes as lens crystallins seems to occur through the modification of gene expression without prior gene duplication (Piatigorsky, 1992; Wistow, 1993).

ζ-Crystallin was first reported as a novel 35-kDa crystallin of the guinea pig (Huang et al., 1987). Subsequently, this crystallin was cloned, sequenced, and characterized as an enzyme-crystallin based on its evolutionary relationship to the alcohol/polyol dehydrogenase superfamily (Rodakanaki et al., 1989). The consensus pyridine nucleotide binding sequence (Rossmann fold) has been conserved in ζ-crystallin and this part of the sequence shows the highest identity to alcohol dehydrogenase (Borrás et al., 1989). Studies of the cofactor binding and catalytic properties of ζ-crystallin revealed that it is an NADPH-dependent quinone oxidoreductase (EC 1.6.5.5) (Rao & Zigler, 1990, 1991; Rao et al., 1992). Quinones such as 1,2-naphthoquinone, 9,10-phenanthrenequinone, and 5-hydroxy-1,4-naphthoquinone are reduced through a one-electron mechanism (Rao et al., 1992). This catalytic activity has been shown to follow Michaelis−Menten kinetics.

ζ-Crystallin is present at high levels (∼10% of total protein) not only in guinea pig lens but also in the lenses of camels and llamas (Garland et al., 1991; Gonzalez et al., 1995). It is present at low levels in various nonlenticular tissues, as well as in the lenses of species outside the hystricomorph rodent and camelid groups (Huang et al., 1990). Guinea pig ζ-crystallin appears to be identical whether isolated from lens or liver (Rao & Zigler, 1992a) and there is apparently a single ζ-crystallin gene which has been partially characterized from several mammalian species (Gonzalez et al., 1994a,b, 1995). Prokaryotic and invertebrate homologs have been characterized from *Escherichia coli* and *Leishmania* (Thorn et al., 1995; Liu & Chang, 1994). Although the physiological significance of ζ-crystallin quinone reductase activity remains unclear, especially in the lens, this is a situation shared by all enzyme-crystallins. However, in species where it is highly expressed in the lens, ζ-crystallin is important as indicated by the development of an autosomal dominant nuclear cataract in guinea pigs which has been associated with a 34-residue deletion at the nucleotide binding domain (Borrás et al., 1990; Rodriguez et al., 1992). The mutant ζ-crystallin fails to bind NADPH

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and is catalytically inactive (Rao & Zigler, 1992b). It is not understood whether lens opacification results from misfolding of the mutant protein or from loss of some specific function. It seems unlikely that loss of catalytic function could be responsible since heterozygotes, which still have enormous amounts of activity, develop cataracts; however, the decreased concentration of NADP(H) in the mutant animals has been proposed as a possible causative factor (Rao & Zigler, 1992b).

Prior to the identification of ζ-crystallin in guinea pig lens, a soluble protein from bovine lens had been isolated and, by use of a DNA−cellulose affinity column, had been characterized as a novel nucleic acid binding protein (Kang et al., 1985). This protein, named RF-36 because of an estimated subunit molecular weight of 36 000, was reported to play a pleiotropic role in gene expression, growth, and differentiation of the lens (Lavers & Chen, 1988). On the basis of a partial sequence, RF-36 was estimated to be ∼70% identical to guinea pig ζ-crystallin (Jörnvall et al., 1993). However, the catalytic and DNA binding properties of guinea pig ζ-crystallin and of the bovine homolog (RF-36) suggest distinct differences. Consequently, we have isolated ζ-crystallin from guinea pig and bovine lenses and compared their structural and functional characteristics, the ultimate goal being to elucidate the physiological function(s) of ζ-crystallin in the lens and the mechanisms involved in the formation of cataracts associated with ζ-crystallin mutation.

EXPERIMENTAL PROCEDURES

Materials. Guinea pig and bovine eyes were obtained from Pel-Freez Biologicals (Rogers, AR) and a local slaughterhouse, respectively. Blue Sepharose CL 6B, Sephadex G200, dextran sulfate sodium salt, and Omega ultrafiltration membranes were purchased from LKB Biotechnology. Dichlorophenolindophenol (DCIP),[1] NADPH, NADH, and Nonidet P40 (NP40) were from Sigma Chemical Co. Heparin−Ultragel A4R was obtained from IBF Biotechnics (France). Single-stranded DNA−agarose was purchased from Gibco−BRL Life Technologies, Inc. DNA sequencing kit Sequenase II was from USB, 9,10-phenanthrenequinone was from Aldrich, and a bovine lens cDNA library (catalog no. BL 1008B) was from Clontech Laboratories, Inc. A plasmid isolation kit was obtained from Qiagen. CM 300 cation-exchange HPLC column was from Synchrome, Inc. All radiolabeled compounds used were purchased from Dupont−NEN.

Isolation of ζ-Crystallin. ζ-Crystallin was isolated from bovine lenses using Sephadex G200 gel filtration, Blue Sepharose affinity chromatography, and ion-exchange (Synchrome CM 300) chromatography. Frozen bovine lenses were homogenized in 50 mM Tris-HCl buffer, pH 7.5, containing 1.0 mM EDTA, 10 mM mercaptoethanol, and 2 mM sodium azide. The soluble fraction (20000*g*) was separated on a Sephadex G200 column (80 × 3.75 cm) at 4 °C. Fractions (5 mL) containing ζ-crystallin, which coeluted with βH-crystallin, were pooled and dialyzed for 18 h at 4 °C against 20 mM Tris-HCl buffer, pH 7.4, containing 0.5 mM EDTA and 5 mM β-mercaptoethanol (buffer A). After centrifugation to remove any precipitate, the soluble fraction was applied to a Blue Sepharose CL 6B affinity column (25 × 2 cm). The column was washed with buffer A alone and with buffer A containing 0.1 M NaCl, and finally the strongly bound protein was eluted with buffer A containing 2 M NaCl. The protein eluted was dialyzed against buffer A at 4 °C and concentrated by ultrafiltration using an Omega 30 K membrane. The clear protein sample was equilibrated with 10 mM sodium phosphate buffer, pH 7.2, using a PD-10 desalting column, and was then separated on a Synchrome CM 300 HPLC column. After injection onto the column, the sample was washed for 10 min isocratically with 10 mM sodium phosphate buffer, pH 7.2, at 0.5 mL/min flow rate at room temperature (23 °C), followed by elution with a linear gradient (20 min) from 0 to 1.0 M NaCl in the same buffer. Fractions containing ζ-crystallin were tested by SDS−PAGE and dot blot analysis using ζ-crystallin antibody. Guinea pig lens ζ-crystallin was isolated by the same protocol except that the initial Sephadex G200 gel-filtration step was omitted.

Enzyme Assay. ζ-Crystallin catalytic activity was measured by the procedure described (Rao et al., 1992). The standard assay system contained, in a final volume of 1.0 mL, 0.1 M Tris buffer, pH 7.8, 0.2 mM EDTA, appropriate amounts of enzyme protein, 0.1 mM NADPH, and either 25 μM 9,10-phenanthrenequinone or 0.1 mM DCIP. Reactions were carried out at 23 °C using a Beckman DU 50 recording spectrophotometer. With 9,10-phenanthrenequinone, activity was monitored by recording the NADPH oxidation at 340 nm, while assays with DCIP were monitored by following the decrease in absorbance at 600 nm. One unit of enzyme activity was defined as the amount of enzyme that oxidizes either 1 μmol of NADPH (with phenanthrenequinone as substrate) or 1 μmol of DCIP (with 2,6-dichlorophenolindophenol as substrate) per minute at 23 °C using $\Delta\epsilon$ of 6.22×10^3 M^{-1} cm^{-1} or 21×10^3 M^{-1} cm^{-1}, respectively.

ssDNA Binding Assay. Lenses were homogenized in 10 mM Tris-HCl buffer, pH 7.4, and the 20000*g* soluble fraction was loaded onto the ssDNA−agarose affinity column (10 × 1 cm). The column was washed with 50 mL of buffer, followed by 50-mL washings with the same buffer containing 0.1 M NaCl, the buffer containing 0.5 mg/mL dextran sulfate sodium salt, and finally with the buffer containing 2 M NaCl to elute strongly bound protein. This bound protein fraction was analyzed by SDS−PAGE subsequent to dialysis at 4 °C against 10 mM Tris buffer, pH 7.4.

Two-Dimensional Electrophoresis. The Blue Sepharose-bound protein fraction containing partially purified ζ-crystallin was subjected to 2D electrophoresis (Datiles et al., 1992). 2D samples were prepared in 9.0 M urea containing 2% NP-40, 2% ampholytes (p*I* range 3.5−10.0), and 10 mM DTT. The first dimension, isoelectric focusing, was carried out using Pharmacia LKB immobilized nonlinear pH gradients (pH 3−10) and was run for 32 000 V·h. The second dimension was run on 18 × 18 cm, 15−18% gradient acrylamide SDS slab gels using the ISO-DALT system of Hoefer Scientific Instruments. Gels were stained with colloidal Coomassie Blue G.

Circular Dichroism. Analyses were carried out using a Jasco Model 600 spectropolarimeter as described (Rao et

[1] Abbreviations: ssDNA, single-stranded DNA; DCIP, 2,6 dichlorophenolindophenol; NP40, Nonidet P40; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; CD, circular dichroism; QOR, quinone oxidoreductase; MDR, medium-chain dehydrogenases/reductases; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPLC, high-performance liquid chromotography.

al., 1994). The far-UV CD was scanned using 1.5 mg of purified ζ-crystallin/mL in 0.1 M sodium phosphate buffer, pH 7.5, with 0.2 mM path length and 0.5 s time constant. The near-UV CD was recorded using 3.0 mg of ζ-crystallin/mL in the presence and absence of 0.1 mM NADPH, with a 1 cm path length and a 0.5 s time constant. Both CD spectra were determined at 25 °C.

Fluorescence Quenching Studies. These measurements were carried out essentially as described (Rao & Zigler, 1990) using an Aminco SPF-500 spectrofluorometer. Corrected fluorescence emission spectra were recorded with excitation at 280 nm using 0.3 nmol of ζ-crystallin/mL in the presence and absence of NADPH in 10 mM Tris-HCl buffer, pH 7.2, at 25 °C.

Metabolic Labeling of Lens Proteins with [^{35}S]Methionine. Fresh guinea pig and bovine lenses were organ-cultured for 4 h in a medium containing 100 μCi mL^{-1} [^{35}S]methionine as described (Huang et al., 1990). After incubation, lenses were rinsed with phosphate-buffered saline and homogenized in 20 mM Tris-HCl buffer, pH 7.4, containing 1.0 mM EDTA and 10 mM β-mercaptoethanol. In the case of guinea pig, the whole lens was homogenized, whereas for bovine samples, only the epithelium and outer cortex were used. The soluble extracts were dialyzed overnight against the same buffer. After this, ζ-crystallin was isolated using Blue Sepharose affinity chromatography as described above.

cDNA Library Screening, Subcloning, and Sequencing. A λgt11 oligo(dT)-primed bovine lens library obtained from Clontech (catalog no. BL 1008B) was screened as described (Gonzalez et al., 1993). The screening was performed using the insert of the human CRYZ cDNA full-length clone HL103. About 10^5 plaques were screened using nitrocellulose filters (S & SNC, Schleicher & Schuell). The inserts of the positive clones were released with *Eco*R1 and subcloned into the vector Bluescript KS II ± (Stratagene). The recombinant plasmids were purified with Qiagen columns and both strands were sequenced by the dideoxy chain-termination method using [^{35}S]ATP and Sequenase II.

Preparation of Extracts of Nuclear Protein. Crude nuclear extract from bovine lens epithelium was isolated as described (Digham et al., 1983), with certain modifications. Briefly, the epithelium isolated from fresh bovine lenses was minced in phosphate-buffered saline and centrifuged at 250*g* for 10 min. The tissue was then suspended in 5 volumes of 10 mM Hepes buffer, pH 7.9, containing 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM DTT. The sample was kept on ice for 20 min and centrifuged at 250*g* at 4 °C. The pellet was suspended in 2 volumes of the Hepes buffer containing 0.05% NP40 detergent and was homogenized with 12−15 strokes using an all-glass Dounce homogenizer. The nuclei were pelleted by centrifugation at 2500 rpm for 10 min at 4 °C and were washed three times with 2 volumes of buffer. Finally, the sample was spun at 25000*g* for 20 min at 4 °C to remove residual soluble material. The nuclei were resuspended in 2 volumes of 20 mM Hepes buffer, pH 7.9, containing 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and 0.5 mM PMSF and were homogenized with 8 strokes. The resulting suspension was gently stirred on a magnetic stirrer for 30 min in an ice bath and centrifuged at 25000*g* for 20 min. The clear supernatant was dialyzed against 20 mM Hepes buffer, pH 7.9, containing 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT, and 0.5 mM PMSF. The dialyzed sample was used to test for the presence of ζ-crystallin by SDS−PAGE followed by Western blot analysis. Protein content was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

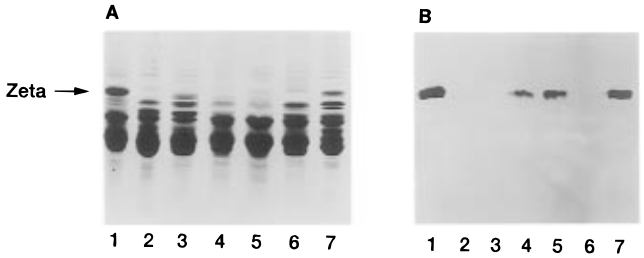

FIGURE 1: SDS−polyacrylamide gel electrophoresis of lens soluble proteins from different species (A) and a Western blot showing the immunoreactivity of ζ-crystallin in the same samples with a peptide antibody against guinea pig ζ-crystallin (B). All samples were loaded with equal amounts of protein. Samples 1−7 represent lens soluble proteins from guinea pig, rat, rabbit, cow, bison, pig, and llama, respectively.

SDS−PAGE and Western Blot Analysis. Sodium dodecyl sulfate−polyacrylamide slab gel electrophoresis was performed according to Laemmli (1970) using a Hoefer Mighty Small apparatus. Gels contained 12.5% acrylamide and were stained with Coomassie Blue R-250.

Immunoblots were developed according to the Bio-Rad protocol using antibodies raised against ζ-crystallin synthetic peptides (Huang et al., 1990). Transfer of proteins from SDS−polyacrylamide gels to nitrocellulose membrane was accomplished using a Bio-Rad transfer unit, and protein bands were visualized with the peroxidase reaction.

Protein Analysis, Modeling, and Alignment. Analysis of the DNA and protein sequences was performed using the GCG package (FastA, Peptide Sort, and Pep Plot). Models of human and bovine ζ-crystallin were calculated based upon the X-ray structure of the *E. coli* enzyme (Thorn et al., 1995) using the program ICM (version 2.5, Molsoft LLC, Metuchen, NJ, 1996). The multiple sequence alignment was created according to a Needleman and Wunsch (1970) algorithm followed by a neighbor-joining procedure (Saitou & Nei, 1987). The model building involved several steps. First, tethers were imposed between residues of the *E. coli* template structure and residues of the structure to model, after which these tethers were minimized. Subsequently, all methyl groups were rotated to minimize clashes, followed by iterative combined geometry and energy optimization. After adjustments of polar hydrogen positions, the whole molecule was subjected to free minimization to obtain the final structure.

RESULTS

ζ-Crystallin Expression in the Bovine Lens. The relative level of ζ-crystallin in the ocular lens of different species was evaluated by separation of lens soluble homogenates on SDS−PAGE followed by Western blot analysis with an antibody against a synthetic peptide matching the N-terminal 14-residue sequence of guinea pig ζ-crystallin (Figure 1). As compared to rat, rabbit, and pig, lens homogenates of cow and bison showed strong immunoreactivity. However, as shown by Coomassie Blue staining, the abundance of ζ-crystallin in the bovids is still much below that in guinea pig and llama lens, where the protein is considered to be an enzyme-crystallin. Bovine lens ζ-crystallin was purified to

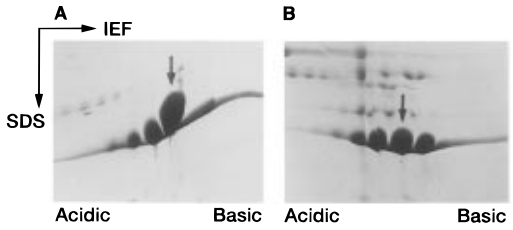

FIGURE 2: Coomassie blue stained two-dimensional electrophoresis of ζ-crystallin from guinea pig (A) and bovine (B) lenses. ζ-Crystallin preparations were partially purified by Blue Sepharose chromatography from lenses that had been incubated in [^{35}S]-methionine-containing medium for 4 h. Samples from guinea pig and bovine lenses were separated initially by isoelectric focusing, followed in the second dimension by SDS−PAGE. Arrows indicate the single ζ-crystallin spot in each sample that was labeled following organ culture with [^{35}S]methionine.

homogeneity in three steps. Fractionation of bovine lens soluble homogenate on Sephadex G200 showed that ζ-crystallin coeluted with βH-crystallin, a major soluble protein with molecular mass of 150−200 kDa. Subsequently, Blue Sepharose affinity chromatography of the βH-crystallin/ζ-crystallin fractions showed strong binding of a 35-kDa polypeptide to the column. Subsequent to elution with high-salt (2 M NaCl) buffer, Western blot analysis confirmed that the 35-kDa protein present in the Blue Sepharose-bound fraction is ζ-crystallin. Finally, the gradient elution of the preparation from Blue Sepharose on a Synchrome CM 300 cation-exchange column showed elution of ζ-crystallin at 0.45 M NaCl. In each of these chromatography steps, guinea pig ζ-crystallin behaved virtually identically to the bovine protein. While ζ-crystallin isolated from the bovine lens showed a strong immunological cross-reactivity with guinea pig ζ-crystallin antibody against a 14-residue N-terminal segment and with antibody raised against isolated intact guinea pig ζ-crystallin, it showed no reactivity with antibodies against a sequence (residues 295−308) from near the C-terminal end of guinea pig ζ-crystallin.

Bovine ζ-Crystallin Characteristics. The absorption and fluorescence spectra of bovine ζ-crystallin were virtually identical to those of guinea pig ζ-crystallin, exhibiting a 280 nm maximum absorption and a 312 nm emission maximum when excited at 280 nm. Hydropathy plots for bovine and guinea pig lens ζ-crystallins also showed only minor differences.

Two-dimensional electrophoretic patterns of bovine and guinea pig ζ-crystallins were somewhat different. As reported from one-dimensional isoelectric focusing (Huang et al., 1987), the guinea pig protein has three charge variants (Figure 2A). The major species is the most basic one, which has a p*I* consistent with the theoretical p*I* calculated from the amino acid composition (p*I* = 7.8). The pattern for bovine ζ-crystallin is shown in Figure 2B and consists of four species of more equal intensity. This group is more basic than the ζ-crystallin from guinea pig lens, as expected from its theoretical p*I* of 8.61. Each of the protein spots from both species was identified as ζ-crystallin by reactivity with antibodies to ζ-crystallin. The lower intensity spots visible above the ζ-crystallin grouping in each panel are other enzymes which also bind to Blue Sepharose. They are much stronger in the bovine sample because bovine ζ-crystallin is not a major protein as in guinea pig lens, and the bovine preparation was concentrated to make the ζ-crystallin concentration comparable to that of the guinea pig sample.

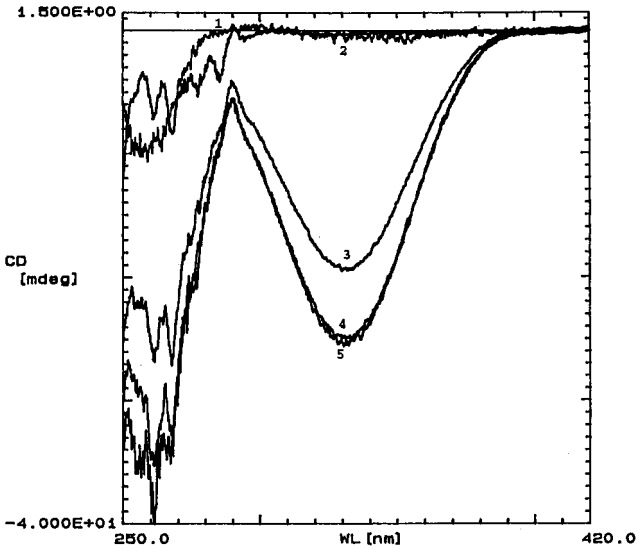

FIGURE 3: Near-UV CD spectra of bovine ζ-crystallin with and without NADPH. Titration of ζ-crystallin with NADPH: (1) 100 μM NADPH alone, (2) apo ζ-crystallin (A_{280} = 1.34) alone, (3) after the addition of 50 μM NADPH, (4) after the addition of 100 μM NADPH, and (5) after the addition of 150 μM NADPH.

Since in both species there is only a single ζ-crystallin gene, studies were undertaken to identify which of the charge variants represented the unmodified gene product. Guinea pig and bovine lenses were placed in organ culture with [^{35}S]-methionine for 4 h. The whole guinea pig lenses and the outer cortical region of the bovine lenses were then homogenized in buffer and the soluble extracts were run on 2D gels. After staining, the gels were dried and autoradiograms were developed. In each case, label was found in only a single spot. For the guinea pig sample this was the major spot, i.e., the most basic species. Presumably the more acidic species represent charge variants generated postsynthetically. This sort of pattern is typical of many of the long-lived proteins of the lens (Datiles et al., 1992; Harding & Crabbe, 1984) and also of MDR (medium-chain dehydrogenase/reductase) enzymes in general (Danielsson et al., 1994). Surprisingly, the results with the bovine sample were different. The only labeled spot was the second most basic one, indicating that in this case not only is there the typical pattern of posttranslational acidification, but also some of the ζ-crystallin also undergoes modification to a more basic form.

The far-UV circular dichroism spectrum of bovine ζ-crystallin was essentially identical to that of guinea pig ζ-crystallin (Huang et al., 1987; Rao et al., 1994), exhibiting a mixture of α-helix and β-sheet secondary structure with nearly 40% α-helix. ζ-Crystallin from guinea pig lens has a conserved consensus pyridine nucleotide binding site and has been shown by fluorescence quenching and near-UV CD to bind NADPH (Rao & Zigler, 1990; Rao et al., 1994). Here, we have applied similar techniques to demonstrate that bovine ζ-crystallin also binds NADPH specifically. NADPH strongly quenched the intrinsic fluorescence of bovine ζ-crystallin, whereas NADH, NAD^+, $NADP^+$, and ATP showed very minimal effect (data not shown). Figure 3 depicts the near-UV CD of bovine ζ-crystallin in the presence and absence of NADPH. Neither ζ-crystallin nor NADPH alone had any optical activity above 300 nm. However, upon mixing the two together, a negative CD band was induced

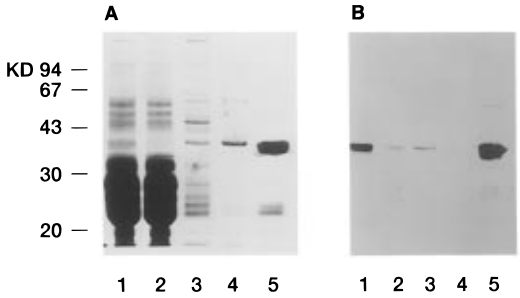

FIGURE 4: Single-stranded DNA binding affinity of bovine ζ-crystallin. Panel A shows SDS−PAGE analysis of bovine lens fractions eluted from an ssDNA−agarose affinity column. Lane 1 contains the total lens soluble fraction that was loaded onto the column, and lanes 2−5 represent protein eluted sequentially with the following buffers: 10 mM Tris (buffer A), buffer A + 0.1 M NaCl; buffer A + 0.5 mg/mL dextran sulfate, and buffer A + 2 M NaCl. Panel B shows immunoblot analysis of the same fractions using antibody to guinea pig ζ-crystallin.

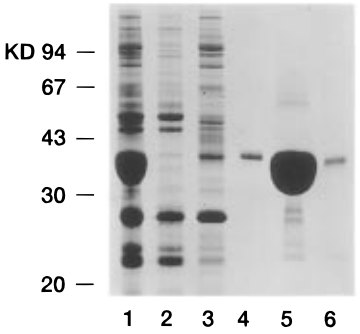

FIGURE 5: Competitive displacement of ζ-crystallin bound to ssDNA by NADPH. SDS−PAGE analysis of bovine lens fractions eluted from the ssDNA−agarose column with different buffer washes. Lane 1, Blue Sepharose-bound fraction from bovine lens total soluble protein applied to the column. Lanes 2−6, protein eluted sequentially with the following buffers, respectively: buffer A, buffer A + 0.1 M NaCl, buffer A + 10 mM NADH, buffer A + 10 mM NADPH, and buffer A + 2 M NaCl.

with a maximum at 330 nm, confirming that bovine ζ-crystallin binds NADPH.

Since bovine ζ-crystallin binds NADPH, its possible catalytic function as a quinone reductase was tested using 9,10-phenanthrenequinone and 2,6-dichlorophenolindophenol as electron acceptors. The bovine protein exhibited only 0.55 unit/mg of protein, or 6.0% of the guinea pig ζ-crystallin activity (9.1 units/mg of protein), in the presence of 50 μM 9,10-phenanthrenequinone. In agreement with earlier reports (Garland et al., 1991), the bovine ζ-crystallin (10 μg/mL) exhibited no activity with DCIP, whereas ζ-crystallin from guinea pig lenses showed specific activity of 0.714 unit/mg of protein. Bovine ζ-crystallin quinone reductase activity was also tested using other quinone substrates, such as 1,2-naphthoquinone and 5-hydroxy-1,4 naphthoquinone (juglone)-and activity was found to be less than 5% of that of guinea pig ζ-crystallin.

We also compared the binding affinity of ζ-crystallin from bovine and guinea pig lenses to ssDNA. Bovine lens soluble homogenate was prepared in 10 mM Tris-HCl buffer, pH 7.4, containing 0.5 mM EDTA and 5 mM mercaptoethanol and was applied to a ssDNA−agarose affinity column. The protein fraction that eluted with 2 M salt contained a major polypeptide of 35 kDa (Figure 4). Immunoblot analysis using a specific antibody revealed it to be ζ-crystallin. On the other hand, ζ-crystallin from guinea pig lens homogenate prepared identically showed no binding affinity to ssDNA and eluted completely with 0.1 M NaCl buffer (data not shown). From the literature, it is known that some pyridine nucleotide binding proteins such as glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase (Grosse et al., 1986) bind ssDNA through the Rossmann nucleotide fold. Glyceraldehyde-3-phosphate dehydrogenase also has other bioactivities, as an uracil DNA glycosylase (Meyer-Siegler et al., 1991) and an Ap_4A binding protein (Baxi & Vishwanatha, 1995). We have tested the effect of NADPH on the affinity of bovine ζ-crystallin for ssDNA. For this experiment, 4 mg of ζ-crystallin isolated from bovine lens using Blue Sepharose affinity chromatography was loaded onto an ssDNA−agarose affinity column. The column was washed stepwise with buffer alone, buffer containing 0.1 M salt, buffer containing 10 mM NADH, and finally with buffer containing 10 mM NADPH. Figure 5 shows the effect of pyridine nucleotides on the binding of ζ-crystallin to ssDNA. Bovine lens ζ-crystallin, as stated above, bound to the column. It did not elute with 0.1 M NaCl, nor with 10 mM NADH, but was eluted completely with 10 mM NADPH. This experiment demonstrates displacement of bovine ζ-crystallin from the ssDNA affinity column by NADPH, suggesting the possibility of a shared binding site for DNA and NADPH. Bovine ζ-crystallin did not bind to heparin−Ultragel, to which many double-stranded DNA binding proteins show strong binding affinity. The affinity for ssDNA confirms findings that RF-36 (bovine ζ-crystallin) is a DNA binding protein (Kang et al., 1985). In view of the strong affinity for DNA, nuclei were isolated from bovine lens epithelial cells and nuclear extract was prepared as described above. When tested with antibody to ζ-crystallin, this preparation exhibited no immunoreactivity on Western blots. Likewise, a commercially obtained nuclear extract from HeLa cells gave no evidence of ζ-crystallin when tested in the same manner.

Cloning of Bovine ζ-Crystallin cDNA. The bovine and guinea pig ζ-crystallin preparations have many similarities, yet certain functional properties differ markedly. In order to gain insight into possible structural correlates for this divergence of functional characteristics, the bovine ζ-crystallin cDNA was isolated by screening 10^5 clones from a λgt 11 bovine lens library with the human liver ζ-crystallin full-length cDNA insert. A number of positive clones were found, consistent with the high level of expression in the lens. A cDNA insert containing 1200 nucleotides was ligated into Bluescript and sequenced in both directions (clone BL5). The open reading frame contained 987 residues and included an ATG initiation site at the 5′ end and a TGA stop codon at the 3′ end. The cDNA sequence reported here is the complete sequence for bovine ζ-crystallin (RF-36).

Figure 6 illustrates the homology of the bovine ζ-crystallin sequence with those determined for ζ-crystallins from human, mouse, llama, guinea pig, *E. coli*, *Pseudomonas*, and *Leishmania*. It should be noted that this is the first complete sequence of bovine ζ-crystallin. An earlier report was based on a partial sequence containing some gaps and several inaccuracies (Jörnvall et al., 1993). Using the alignment in Figure 6 and the coordinates for the crystallographically analyzed *E. coli* (QOR) quinone oxidoreductase (Thorn et al., 1995), the catalytically largely inactive bovine protein and the human protein were independently modeled into the

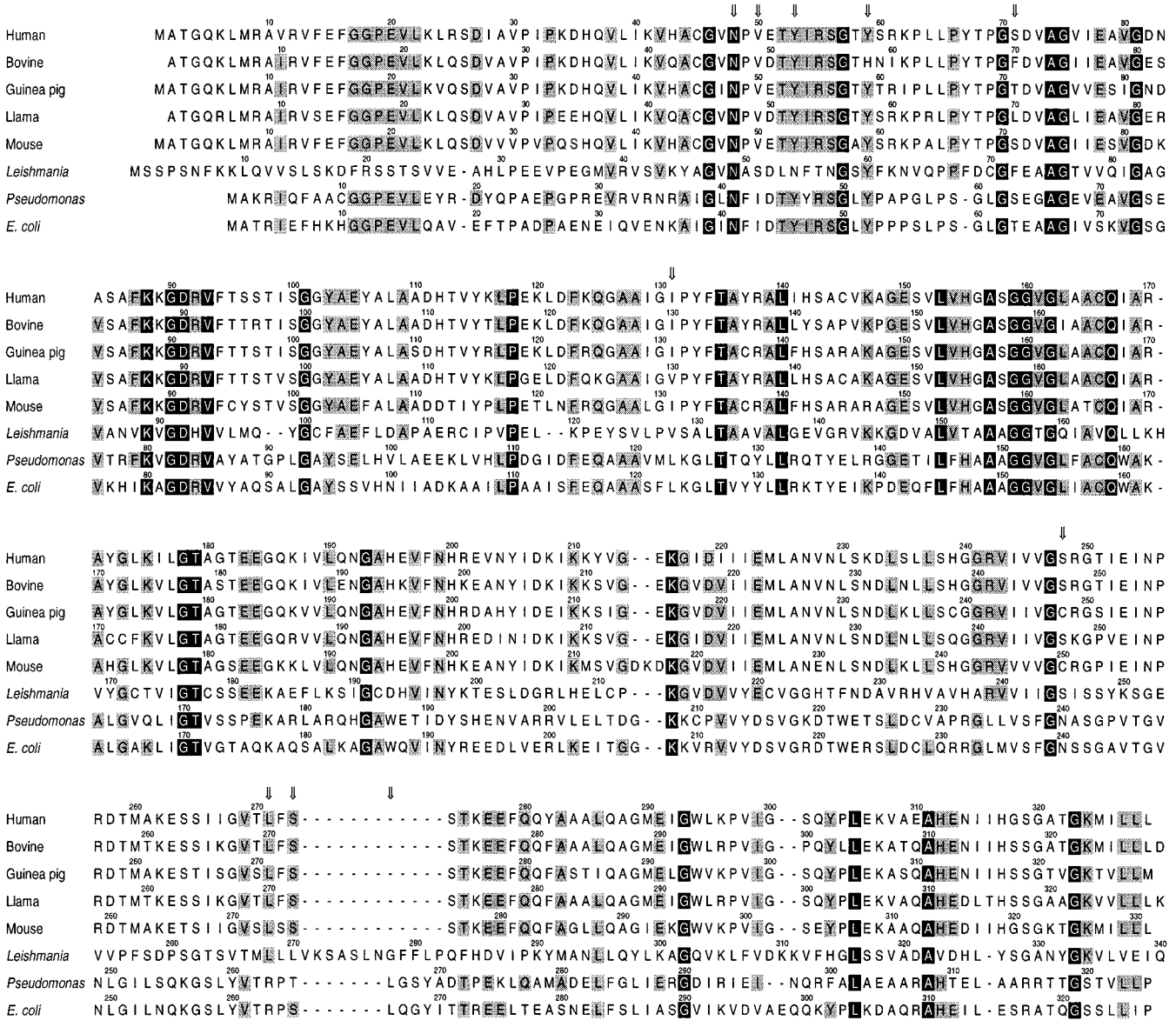

FIGURE 6: Alignment of the novel ζ-crystallin structure (bovine, second line from top in each set) with the human (top line), the crystallographically analyzed *E. coli* (bottom line), and all other characterized ζ-crystallins. Residues in white against a black background denote strictly conserved residues, while a stippled background denotes residues present in 6 or more of the structures. Note that glycines (G) represent the majority of the strictly conserved residues and that positions approximately 215−290 contain few conserved residues and a long and variable gap segment. Residues ascribed as active-site positions (Edwards et al., 1996) are denoted by arrows, and as shown, all are not strictly conserved. (*Pseudomonas* sequence has been taken from EMBL databank, Accession No. P43903).

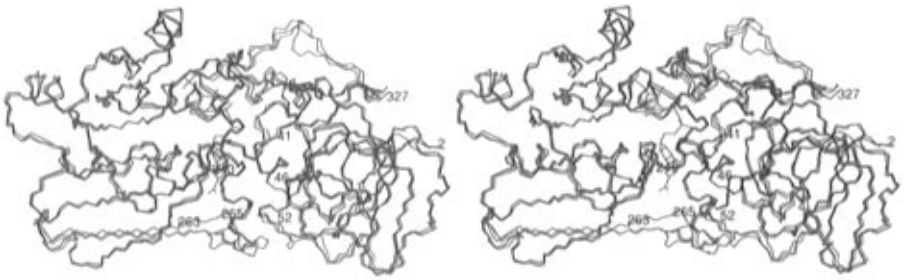

FIGURE 7: Stereo outlines of the modeled structures of the polypeptide conformation of the human (blue) and bovine (red) ζ-crystallins versus that of the crystallographically analyzed *E. coli* (black) enzyme. The coenzyme is shown in magenta (center). Numbers denote start (2) and end (327) positions, as well as residues 41, 46, 52, 240, 263, and 265, which have been ascribed positions at the active site, compatible with their central location adjacent to the nicotinamide part of the coenzyme. Note the generally close folding similarity between all three structures, except for local deviations, including that at the functionally important positions 263 and 265, where chain locations do not match.

E. coli protein structure. Overall relationships are shown in Figure 7, and active-site relationships are shown in Figure 8.

Three points are of special interest in these comparisons: One is the fairly extensive differences between the forms, with less than 10% of all residues strictly conserved (Figure

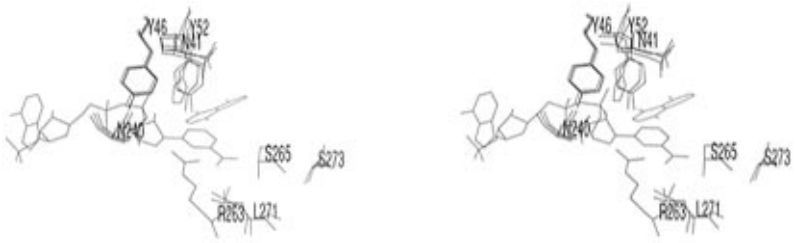

FIGURE 8: Enlarged part of the active-site segment, showing the coenzyme (magenta), the substrate (5-hydroxy-2,4-naphthoquinone, green), two adjacent tyrosine residues (residues 46 and 52), and an asparagine (41), an arginine/lysine (263/271), and a serine (265/273) residue. The parts in black denote the crystallographically analyzed true structure of the *E. coli* enzyme, blue denotes the modeled human enzyme, and red denotes the modeled bovine enzyme. Note that position Tyr 46 is closely matching in all three structures, while position 52 matches in the human and *E. coli* forms (Tyr) but deviates in the bovine form (His), possibly correlated with its altered enzymatic activity. Residues more distant from the active site but reported to be important (Arg 263 and Ser 265 in *E. coli*) also deviate between the mammalian and *E. coli* forms but do so similarly for the human and bovine forms, therefore probably not correlating with the altered enzyme activity of just the bovine form.

6), but still with overall folds that are compatible with largely similar conformations (Figure 7), except for localized segments of dissimilarity (Figures 6−8). In particular, one dissimilarity involves the segments around positions 260−270, where internal deletions/insertions cause missing/extra residues (Figure 6), visible also as a deviating chain conformation in this region (Figure 7). Furthermore, in a 75-residue segment (positions 216−291 in the bovine sequence) around the deletion/insertion part of the molecules, only one residue (Gly 246) is strictly conserved (Figure 6) and the *Leishmania* protein is largely nonidentical to the others. Nevertheless, remaining folds show similarity (Figure 7). The residue distribution, with glycine by far the most conserved (50%; i.e., 15 of 30 strictly conserved residues are Gly), is typical of proteins with conserved function (Jörnvall et al., 1995). Therefore, these proteins, although widely divergent, as expected from the extensive species spread, are still related in structures and functions.

The second aspect of interest concerns the actual positions of the deviations. Thus, the above-mentioned segment of dissimilarity and gaps involves part of the active-site residues as defined from the crystallographic analysis of the *E. coli* protein (Edwards et al., 1996). Residues 263, 265, and 266 in the *E. coli* sequence, previously assigned important functions in substrate binding from their alignments with other MDR enzymes, are in this segment and are not strictly conserved (Figure 6). In addition, they occupy highly deviant conformations in the three proteins (Figure 7). Nevertheless, core regions with glycine residues are largely conserved and alignments (Figure 6) are continuous. Therefore, modeling is still possible, although with some uncertainty for the most deviating segments, one of which includes positions ≈250−300, confirming the previous conclusion from analysis of other ζ-crystallins that this segment is variable (Jörnvall et al., 1993). While such differences at the active site are not typical of species variants, they have been noted also in alcohol dehydrogenase of class I (Luque et al., 1994).

Finally, among the few residues conserved in both alignment position and conformation, and among the residues reported to have catalytic importance (Edwards et al., 1996), Tyr 52 of the *E. coli* sequence (residue 58 or 59 of the mammalian sequences) shows the most interesting features (Figures 7 and 8) at the active site. This is one of the few positions where the bovine protein differs from all others (Figures 6 and 8). Hence, provided that the deviating functional properties of the bovine enzyme result from a single replacement at the active site, position 52 appears to constitute the most likely candidate, with histidine being present in the bovine protein and tyrosine at the equivalent position in all other reported ζ-crystallin sequences.

DISCUSSION

ζ-Crystallin, a major lens protein of guinea pig, camel, and llama, is an enzyme-crystallin with a novel NADPH: quinone oxidoreductase activity (Rao et al., 1992). In this study, ζ-crystallin from bovine lens has been isolated and compared structurally and functionally to other homologs. Three previously reported results led us to undertake this comparison. The first was our observation that bovine ζ-crystallin failed to show significant reduction of DCIP in the presence of NADPH (Garland et al., 1991), the second was the discovery that bovine ζ-crystallin is identical to the bovine lens protein RF36 (Kang et al., 1985) that binds DNA and exhibits other properties possibly related to regulation of lens fiber differentiation, and the third was the fact that all ζ-crystallins are clearly related (Jörnvall et al., 1993).

All mammalian ζ-crystallins that have been analyzed exist as homotetramers of 35-kDa subunits. The five mammalian sequences available exhibit >80% identity in pairwise comparisons, with the human and bovine sequences being 87% identical Since the most extensive analyses have been done with the guinea pig protein, it was used for comparison with bovine ζ-crystallin. The absorption and fluorescence spectra of the two preparations were found to be essentially identical. The same was true for the far-UV CD spectra with approximately 40% α-helical secondary structure, and hydropathy plots showed a close similarity, indicating no gross structural differences between the two proteins. As in all known mammalian ζ-crystallins, bovine ζ-crystallin retained the consensus sequence (GxxGxxG) of the pyridine nucleotide binding site. As has been shown with guinea pig and human ζ-crystallin, bovine ζ-crystallin binds to Blue Sepharose and is eluted specifically with NADPH.

Interestingly, in contrast to these close similarities in structure, the two proteins also have distinct functional differences. Unlike guinea pig (and human) ζ-crystallin, bovine ζ-crystallin showed very little catalytic activity as a quinone oxidoreductase. On the other hand, while bovine ζ-crystallin binds to ssDNA, possibly through the NADPH binding site, guinea pig ζ-crystallin, which also binds NADPH, shows no affinity for ssDNA. Two-dimensional electrophoresis of ζ-crystallin isolated from these two species

revealed a clear difference in terms of their migration and charge modification. Bovine ζ-crystallin has a more basic p*I* (>8.5) and resolved into four major charge species, whereas guinea pig ζ-crystallin resolved into three major spots. Further, the distribution of protein into these different charge variants was different. By use of metabolic labeling with [^{35}S]methionine, a single, labeled polypeptide was identified in each species, consistent with the presence of a single gene. In the guinea pig sample, the posttranslational modification that produces the other two polypeptides generates only more acidic forms, whereas in the bovine lens a more basic polypeptide is produced, as well as more acidic ones. Although neither the biochemical mechanism responsible for generation of the additional basic polypeptide in the bovine sample nor its functional significance is understood, the increased positive change could contribute to the high affinity of the bovine form for ssDNA.

Our earlier studies of ζ-crystallin isolated from guinea pig, human, and llama have shown that it is catalytically active as a quinone oxidoreductase. Genomic analysis of these species revealed the presence of a single gene responsible for the expression of ζ-crystallin. Further, the recent studies of an *E. coli* homolog of ζ-crystallin, which has 31% sequence identity to guinea pig lens ζ-crystallin, have shown that it is also catalytically active as a quinone reductase (Thorn et al., 1995). Therefore, in order to understand the structural and functional differences between bovine and guinea pig lens ζ-crystallin, the complete sequence of bovine ζ-crystallin has been deduced by cloning the lens cDNA. The sequence revealed that bovine ζ-crystallin is strictly homologous to the other mammalian ζ-crystallin sequences available but also that extensive differences exist to other forms (Figure 6). Preliminary analysis of bovine ζ-crystallin genomic DNA revealed the presence of only one gene (unpublished results).

Based on the amino acid sequence, the calculated p*I* for bovine ζ-crystallin was 8.61, while guinea pig ζ-crystallin gave a theoretical p*I* of 7.81. These values are consistent with the 2D gel results and the difference is largely accounted for by the presence of four more lysine residues in bovine ζ-crystallin. It should be noted, however, that the native bovine protein eluted at the same position as guinea pig ζ-crystallin when separated on cation-exchange chromatography as described above. Comparison of the bovine sequence with the other four mammalian sequences (Figure 6, top five lines) indicates that sequence identity is extensive throughout but that there are a number of unique substitutions in the bovine sequence. Of particular interest with regard to the functional differences between bovine ζ-crystallin and the other mammalian forms are 11 residues that are completely conserved in the human, guinea pig, llama, and mouse sequences but are different in the bovine one. These positions as found in the human sequence (Figure 6, top line) are Tyr 59, Arg 61, Ser 97, His 142, Ala 148, Leu 163, Gly 181, Gln 191, Glu 196, Ser 301, and Pro 304. Each of these residues has been replaced in the bovine sequence as indicated in Figure 6. In the case of four of these substitutions (Tyr 59, Leu 163, Gly 181, and Pro 304) even the *E. coli* homolog of ζ-crystallin, which is catalytically active as a quinone reductase (Thorn et al., 1995), has retained at the equivalent position the residue conserved in all the mammals except cow. While there are no large regions of the bovine sequence which have undergone major modifications, there are several specific areas which could be suspected as possible sites contributing to functional differences between the bovine protein and other ζ-crystallins. For example, residues 59−61 are Tyr-Ser-Arg in the human, mouse, and llama sequences and Tyr-Thr-Arg in the guinea pig. In contrast, these positions are His-Asn-Ile in the bovine ζ-crystallin. Likewise, there are several potentially significant substitutions in the sequence of residues 142−148. Specifically, the bovine sequence has Tyr at position 142 and Pro at 148 while all other known mammalian ζ-crystallins have His and Ala at these positions, respectively. The bovine sequence also has Pro 145 instead of either Cys or Arg present in the other species. Thus, three very bulky residues are introduced into this region of the bovine sequence and could be expected to have an effect on conformation. The regions 180−195 and 300−303 also have three and two conserved residues changed in the bovine sequence.

Since no mammalian ζ-crystallin has been crystallized there is no firm 3D structural data for the molecule. However, using the *E. coli* QOR structure (Thorn et al., 1995) to model ζ-crystallin provides some insight. QOR has catalytic activity as a quinone reductase and has 31% residue identity to ζ-crystallin (Thorn et al., 1995). Edwards et al. (1996) have demonstrated from crystal structures that three members of the MDR superfamily, *E. coli* quinone oxidoreductase, alcohol dehydrogenase, and glucose dehydrogenase, are structurally highly similar although the extent of sequence identity is low. With a structure-based sequence alignment, these comparisons were extended to ζ-crystallin. The comparisons reveal an atypical pattern with variability at parts of the presumed active site but with typical Gly conservation and folding similarities overall (Figure 7). This analysis indicates that among the residues cited above that are uniquely different in the bovine protein relative to all the other mammalian sequences, Tyr 52 (*E. coli* QOR sequence) is the only one present at the active site. The exchange of this residue for histidine at the equivalent position in the bovine ζ-crystallin sequence (Figure 6) may explain the functional difference between the bovine enzyme and the other mammalian forms. In spite of the consistent pattern and overall correlations, it should be noted that species differences are large at the active site, limiting model building, and that therefore other explanations are also possible.

The physiological significance of ζ-crystallin expression in the lens remains obscure. However, abundant evidence shows that metabolic enzymes have frequently been recruited as crystallins through modification in gene expression (Wistow & Piatigorsky, 1988; Piatigorsky, 1992; Wistow, 1993; de Jong et al., 1989). Some of the crystallins recruited through this mechanism are known to be catalytically active, while others may be inactive or the proper substrates have yet to be identified. Interestingly, most of the enzyme-crystallins identified so far seem to be non-flavin-containing pyridine nucleotide binding oxidoreductases (Wistow, 1993; de Jong et al., 1989; Zigler & Rao, 1991). Unlike other enzyme-crystallins, the expression of ζ-crystallin as a major protein is not restricted to a single phylogenetic group. It is a major crystallin both in some hystricomorph rodents (guinea pig) and in camelids (camel and llama). Further, the recruitment of ζ-crystallin in these two groups has been shown to have occurred independently through the acquisi-

tion of unique alternative lens-specific promoters, suggesting that ζ-crystallin has a function in the lens that confers selective advantage (Gonzalez et al., 1995). This view is supported by the fact that mutation of the ζ-crystallin gene in guinea pigs causes hereditary cataracts (Rodriguez et al., 1992). ζ-Crystallin expression is relatively high also in the lenses of bovids (cows and bison) when compared to other species such as rat, rabbit, cat, human, and monkey. While the abundance of ζ-crystallin in bovine lens is much less than in guinea pigs or camelids, the higher levels relative to most species may be indicative of a different functional role. Such a function could relate to its unique DNA binding properties. Alternatively, it has previously been suggested that oxidoreductases may have been recruited as enzyme-crystallins because they markedly increase the levels of pyridine nucleotides in the lens, which might be beneficial in protecting the lens from oxidative stress (Zigler & Rao, 1991). Thus, the somewhat increased amounts of ζ-crystallin present in bovine lens might reflect a greater need for NADP(H), which has been reported to be at higher concentration in bovine lens than in rat, rabbit, or human lenses (Stewart & Augusteyn, 1984).

ζ-Crystallin from bovine lens binds ssDNA. Furthermore, the specific cofactor, NADPH, will competitively displace the ssDNA from the protein. This suggests that NADPH and ssDNA may compete for the same binding site, as has been reported for some other pyridine nucleotide binding proteins including glyceraldehyde-3-phosphate dehydrogenase (GAPDH), lactate dehydrogenase, glutamate dehydrogenase, isocitrate dehydrogenase, thymidylate synthetase, dihydrofolate reductase, and catalase (Preiss et al., 1993; Elzinga et al., 1993; Chu et al., 1993a,b). GAPDH has also been reported to be involved in tRNA transport and to bind to AUUUUA-rich regions of untranslated mRNA sequences (Nagy & Rigby, 1995; Singh & Green, 1993). On the basis of studies with pyridine nucleotide binding proteins and their binding affinity to DNA and RNA, Hentze (1994) suggested a bifunctional role for the nucleotide binding domain (Rossmann fold) of metabolic enzymes in catalysis and in gene regulation. Although it has been reported that GAPDH is expressed in the cell nucleus (Singh & Green, 1993), our attempts to detect ζ-crystallin in bovine lens cell nuclei were unsuccessful.

In conclusion, it is clear that the homologous ζ-crystallins isolated from bovine and guinea pig lenses exhibit very different functional characteristics and that RF-36, originally identified as a DNA binding protein from bovine lens, is actually ζ-crystallin. The fact that there appears to be only a single gene for ζ-crystallin in all species studied and that the protein is expressed in numerous tissues suggests that it has an important function in all species, perhaps related to the quinone oxidoreductase activity or the ssDNA binding properties outlined above. Further, it is interesting to note that ζ-crystallin homologs from plants have been shown to be induced by oxidative stress (Babiychuk et al., 1995), a finding that is consistent with a possible role for ζ-crystallin in the antioxidative defenses of the lens.

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