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Two Roles for μ-Crystallin: A Lens Structural Protein in Diurnal Marsupials and a Possible Enzyme in Mammalian Retinas

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Purpose: μ-Crystallin is a major taxon-specific lens protein in some marsupials. Like other taxon-specific crystallins, it probably has another, non-crystallin role. Here we examine the distribution of μ-crystallin among species and its localization in the eye in placental mammals. We also compare its sequence and ligand binding characteristics with those of known enzymes.

Methods: An antibody (Mup1) was raised against a conserved 21 residue peptide of tammar wallaby μ-crystallin. This was used to detect μ-crystallin immunoreactivity in lens extracts of several species and in the tissues of rat and bovine eyes. PCR methods were used to complete the cDNA sequence of human μ-crystallin. The ability of kangaroo μ-crystallin to bind enzymatic cofactors was tested by blue-sepharose chromatography.

Results: Using Mup1, abundant μ-crystallin was observed in soluble whole lens extracts of diurnal Australian marsupials. Although abundant μ-crystallin was not detectable in whole lens of nocturnal marsupials, other mammals or a bird, lower levels of immunoreactivity were detectable in lens epithelium, retinal pigment epithelium and, particularly, retina of bovine eye. In rat eye the highest levels of Mup1 reactivity were found in retinal photoreceptors. Sequence comparisons of human and kangaroo μ-crystallin reveal a superfamily relationship with enzymes of glutamate and ornithine metabolism. Co-factor binding studies indicate that μ-crystallin, like related glutamyl-tRNA reductases, binds NADPH.

Conclusions: These results suggest that μ-crystallin is a normal component of retina and other tissues which underwent gene recruitment to gain an additional structural role in the lens during the evolution of diurnal marsupial species. μ-crystallin may be an enzyme, possibly of amino acid metabolism, with particular importance for photoreceptors.

In many vertebrates, different enzymes have undergone direct gene recruitment to acquire an additional structural role as a crystallin in the eye lens (1-3). While some taxon-specific crystallins are familiar enzymes, such as lactate dehydrogenase (4), others were discovered first in the lens. These include ζ-crystallin of guinea pig and related species, which is a novel NADPH:quinone oxidoreductase (5, 6); λ-crystallin of rabbits and hares, which is related to various CoA-derivative dehydrogenases (7) but whose function is not yet known; and μ-crystallin, a major component of the eye lens in some Australian marsupials (8, 9).

μ-Crystallin was found to be related in sequence to the ornithine cyclodeaminases (OCD), enzymes in prokaryotes capable of converting ornithine to proline (9-11). In adult kangaroo, μ-crystallin mRNA is most abundant in lens but is also detectable at much lower levels in retina and brain, consistent with the possibility of a non-structural, enzymatic role (9). Furthermore, the gene for μ-crystallin is conserved in human and mouse, even though these species lack abundant μ-crystallin in their lenses (9). A highly conserved partial cDNA clone for human μ-crystallin was obtained from retina (9).

Here we have examined the distribution of μ-crystallin in a collection of Australian marsupials. We have completed the sequence of the human homologue of μ-crystallin, revealing high conservation with that of kangaroo, even though the protein in humans lacks the major structural role in lens. We have localized the protein within mammalian (rat and bovine) eyes. To gain further insight into the non-crystallin role of μ-crystallin, we have examined the relationship of μ-crystallin with known enzymes and its affinities for nicotinamide adenine dinucleotide cofactors. The results suggest that μ-crystallin may be a reductase, possibly involved in metabolism of amino acid derivatives, which may have particular significance for retina.

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METHODS

Antiserum, Western Blots and Immunohistochemistry— A peptide, (ESGDVILSGAEIFAELGEVVK), taken from the original protein microsequencing of *Macropus eugenii* μ-crystallin (8), was synthesized at the UCLA Molecular Biology Institute Peptide Synthesis Facility. The peptide was linked to keyhole limpet hemocyanin carrier protein using glutaraldehyde and polyclonal antiserum was raised in rabbits, as described previously (12). The antiserum was designated Mup1.

Kangaroo (*Macropus eugenii* and *Macropus fuliginosus*) lens soluble extracts were prepared as described before (8, 13). Adult bovine tissues were obtained from a local slaughterhouse. Lenses from exotic species were obtained as post-mortem samples collected under appropriate permit in Australia (RG) or from The National Zoo, Washington DC.

Tissues were dissected and homogenized in TE buffer (10 mM Tris, pH 7.4, 1 mM EDTA). SDS polyacrylamide gel electrophoresis (PAGE) was performed as described previously (13). Insoluble fractions were removed by microcentrifugation and 10 µg of protein per sample were electrophoresed in 11.3% PAGE gels in Tris-glycine-SDS for 3 hrs at 120 V. Proteins were transferred to nitrocellulose (S & S, Keene, NH) using a Polyblot semi-dry transfer apparatus (ABN, Emeryville, CA). Membranes were stained with Ponceau S (0.2%) to assess electrophoresis and transfer and destained as described previously (13). The blot was blocked in 5% milk powder in PBT (PBS containing 0.1% (v/v) Tween 20) for 1 hr at room temperature (RT). Anti-µ-crystallin serum was diluted 1/5000 in 0.1% milk powder/PBT and the blot was incubated for 1 hr at RT with slow agitation. Pre-immune serum was used as control in the same protocol. Blots were washed twice with PBT at RT and developed using Vectastain ABC kits (horse radish peroxidase; HRP) (Vector Labs, Burlingame CA).

Immunohistochemical and Immunofluorescence localization— Ten micron paraffin sections of adult rat retinas, fixed in formaldehyde, were obtained from Novagen (Madison, WI). Sections were de-waxed in xylene three times for 5 minutes and washed twice in absolute ethanol. Endogenous peroxidases were quenched by incubation for 5 minutes in methanol followed by 15 minute washing in methanol containing 0.3% (v/v) hydrogen peroxide. Sections were rehydrated in consecutive washes of decreasing concentrations of ethanol and finally twice in PBT. Sections were blocked by incubation for 20 minutes in PBT containing 2% rabbit normal serum. Mup1 anti-µ-crystallin antibody at 1/2500 dilution was added in PBT containing 1% normal serum and slides were incubated overnight at 4°C. Pre-immune serum was used as control. Slides were washed 3 times with PBT to remove unbound antibody. Staining was carried out using a Vectastain ABC HRP staining kit, according to manufacturer's instructions.

Immunofluorescent staining was carried out using essentially the same protocol, except that endogenous peroxidases were not quenched. Staining was carried out by the addition of a fluoresceinated secondary antibody (Vector Labs). Again, pre-immune serum was used as control.

Cofactor Binding— Soluble extracts of grey kangaroo (*Macropus fuliginosus*) lenses were prepared by standard homogenization in TE buffer (13). Lens proteins (100 µl volume) were subjected to chromatography using blue sepharose CL-6B (Pharmacia LKB Biotechnology, Milwaukee, WI) essentially as described by Rao et al (14). Columns were washed with TE and then with the same buffer 10 mM in NaCl. Columns were then separately washed with 10 mM solutions of either NAD^+, $NADP^+$, NADPH or NADH (Sigma, St. Louis, MO). Eluates were examined by SDS PAGE.

5' RACE PCR— To complete the partial human cDNA sequence for µ-crystallin, 5' RACE (rapid amplification of cDNA ends) was used (15). 1.5 µg of lyophilized total human retina RNA (Clontech, Palo Alto CA) was resuspended in water with 10 pMol of either oligo(dT) or the specific primer for µ-crystallin described below. The suspension was heated for 3 min at 94°C and then cooled on ice. For reverse transcription, AMV Reverse Transcriptase (RT AMV) (Boehringer Mannheim, Indianapolis IN) was used, following manufacturer's instructions. The resulting cDNA was tailed with dATP using terminal transferase (Boehringer Mannheim), also according to manufacturers protocols. Polymerase chain reaction (PCR) amplification used reagents from Perkin Elmer Cetus (Foster City, CA) with an antisense primer specific for human µ-crystallin (7688:

TGCAGCTGTTCTCTTTGCAGTTAT) and oligo(dT), under the following conditions: 40 cycles of 94°C 30 sec, 50°C 30 sec., 72°C 1 min, followed by a 10 min extension at 72°C.

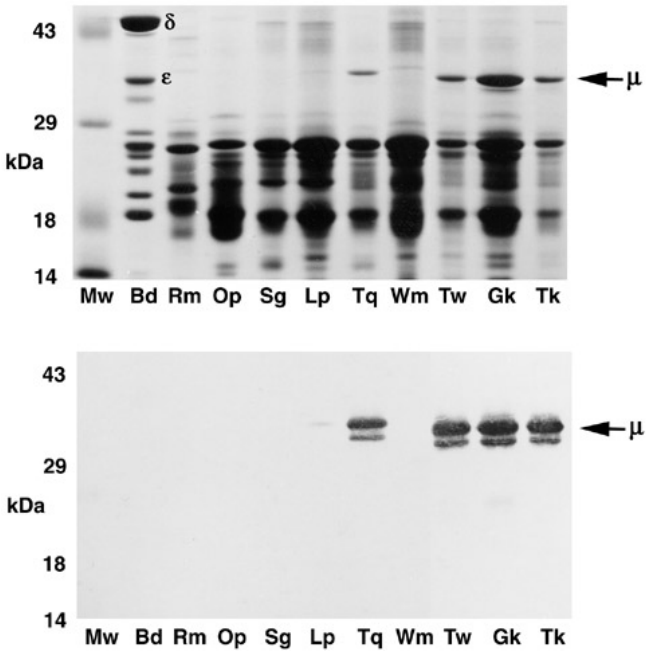

Figure 1. Species distribution of µ-crystallin as an abundant lens protein. Upper panel shows SDS PAGE of soluble lens extracts, stained with coomassie. Position of µ-crystallin (molecular size 34 kDa) and, for comparison, two avian taxon-specific crystallins, δ and ε (3) are indicated. Lower panel shows the results of western blotting with Mup1 for a similar gel. Species are: Bd, Black duck (*Anas rubripes*); Rm, Rhesus monkey (*Macaca mulatta*); Op, American Opossum (*Didelphis virginiana*); Sg, sugar glider (*Petaurus breviceps*); Lp, Leadbeater's possum (*Gymnobelideus leadbeateri*); Tq, tiger quoll or spotted native cat (*Dasyurus maculatus*); Wm, common wombat (*Vombatus ursinus*); Tw, tammar wallaby *(Macropus eugenii*); Gk, western grey kangaroo (*Macropus fuliginosus*); Tk, tree kangaroo (*Dendrolagus sp.*).

RESULTS

Antiserum to µ-crystallin— Antiserum was raised in rabbits against a 21 residue peptide of tammar wallaby (*Macropus eugenii*) µ-crystallin. The antiserum, Mup1, was tested on western blots of soluble lens extracts of several species (Figure 1). Strong immunoreactivity was observed in several Australian marsupials, but not in other mammals, a bird, or an American

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                                                  M  S  R  V  P  A  F  L  S  A  A  E  V  E  E  H  L  R  S  S  S   21

agactgaggttagaaggcacaggtggcgagATGAGCCGGGTACCAGCGTTCCTGAGCGCGGCCGAGGTGGAGGAACACCTCCGCAGCTCCAGC  93

 L  L  I  P  P  L  E  T  A  L  A  N  F  S  S  G  P  E  G  G  V  M  Q  P  V  R  T  V  V  P  V   52
CTCCTCATCCCGCCTCTAGAGACGGCCCTGGCCAACTTCTCCAGCGGTCCCGAAGGAGGGGTCATGCAGCCCGTGCGCACCGTGGTGCCGGTG 186

 T  K  H  R  G  Y  L  G  V  M  P  A  Y  S  A  A  E  D  A  L  T  T  K  L  V  T  F  Y  E  D  R   83
ACCAAGCACAGGGGCTACCTGGGGGTCATGCCCGCCTACAGTGCTGCAGAGGATGCACTGACCACCAAGTTGGTCACCTTCTACGAGGACCGC 279

 G  I  T  S  V  V  P  S  H  Q  A  T  V  L  L  F  E  P  S  N  G  T  L  L  A  V  M  D  G  N ... 113
GGCATCACCTCGGTCGTCCCTTCCCACCAGGCTACTGTGCTACTCTTTGAGCCCAGCAATGGCACCCTGCTGGCGGTCATGGATGGAAATG.. 370
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Figure 2a. Human μ-crystallin: The 5' end of the human cDNA obtained by 5' RACE. Figure shows the partial cDNA sequence from multiple RACE-derived clones. Genbank Accession number L02950. Conceptual translation is shown in italics.

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MSRVPAFLSAAEVEEHLRSSSLLIPPLETALANFSSGPEGGVMQPVRTVV  50
  WS    RSED  RY G   I L A  K        S    V      I

PVTKHRGYLGVMPAYSAAEDALTTKLVTFYEDRGITSVVPSHQATVLLFE 100
  A  Q F  I  V   S             GMSP  TA    T   F D

PSNGTLLAVMDGNVITAKRTAAVSAIATKFLKPPSSEVLCILGAGVQAYS 150
    S  SI    I

HYEIFTEQFSFKEVRIWNRTKENAEKFADTVQGEVRVCSSVQEAVAGADV 200
     K               K      Q  K D           T

IITVTLATEPILFGEWVKPGAHINAVGASRPDWRELDDELMKEAVLYVDS 250
     M  K                                I  NC
      ......................
QEAALKESGDVLLSGAEIFAELGEVIKGVKPAHCEKTTVFKSLGMAVEDT 300
R          I             V       R               A

VAAKLIYDSWSSGK 314
     V
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Figure 2b. Alignment of complete deduced protein sequences for human retina and grey kangaroo (*Macropus fuliginosus*) lens μ-crystallin. The human sequence is shown and below it are non-identical positions from kangaroo. Dots indicate the location of the peptide used to raise antiserum.

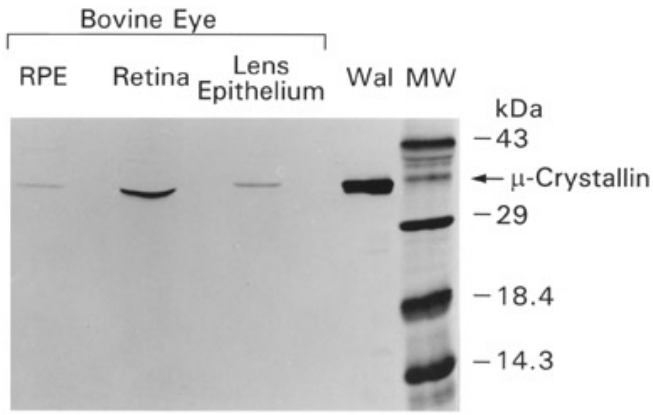

Figure 3. Detection of μ-crystallin in bovine eye tissues. Western blot of adult bovine eye tissues positive for μ-crystallin immunoreactivity; retinal pigment epithelium (RPE), retina and lens epithelium. Wal shows results for tammar wallaby (*Macropus eugenii*) lens protein as positive control. μ-Crystallin is detected at a size of 34 kDa. In this gel the separation of the major and minor μ-crystallin bands is closer than in Figure 1. Bovine tissues show only a single band. Other eye tissues, lens fibers, cornea, iris and sclera were negative (not shown).

marsupial (Virginia opossum, *Didelphis virginiana*). AmongAustralian species, μ-crystallin was detected abundantly in all macropods, including tree kangaroo (*Dendrolagus sp.*) and, as identified previously by microsequencing, in a dasyurid (tiger quoll or native spotted cat, *Dasyurus maculatus*). A weak positive reaction was also seen in one petaurid (Leadbeater's possum, *Gymnobelideus leadbeateri*). No reactivity was detectable in whole lens extracts of other species, including wombat (*Vombatus ursinus*). The dasyurid μ-crystallin exhibited a slightly higher apparent molecular size than that of macropods. In both dasyurid and macropod lens extracts, the μ-crystallin antiserum recognized a close doublet of bands. The minor band is only observed in the presence of the major μ-crystallin band in lens extracts. It may represent an age-related post-translational modification of μ-crystallin in lens, since lens proteins are subject to very low turnover and accumulate throughout life.

The species distribution reflects a general correlation between the presence of abundant μ-crystallin and some degree of diurnal habit. While nocturnal activity is common amongst marsupials, some daylight activity is seen in macropods and even in dasyurids (16). In contrast, wombat, sugar glider (*Petaurus breviceps*) and Leadbeater's possum appear to be more strictly nocturnal (16). This is consistent with the idea that taxon-specific crystallins are mainly of selective value to diurnal species of terrestrial vertebrate in which they may help to "soften" the lens for better accommodation or, through bound cofactors, to protect the eye by filtering harmful UV radiation (3, 17). The low but detectable level of μ-crystallin in Leadbeater's possum might represent residual expression of a gene which was expressed at higher levels in a diurnal ancestor.

Human μ-Crystallin cDNA— To complete the partial cDNA sequence of human μ-crystallin and to map the transcription start site, 5' RACE (15) was used to amplify RNA using a specific antisense primer designed from the partial cDNA (9). Human retina RNA was used as template and a single PCR product was obtained and subcloned. Multiple clones were sequenced to complete the 5' end of the human cDNA and to deduce the N-terminal region of the protein sequence (Figure 2a).

The complete human sequence represents the first for a placental mammal. As such, it allows comparison of

orthologous proteins, one of which (the kangaroo μ-crystallin) has acquired an additional role as a crystallin. As shown in Figure 2b, in spite of this difference in function, the human and kangaroo protein sequences are very closely related. The two proteins, from species separated by over 120 Myr of evolution, are 83% identical. Most of the sequence differences, 35 out of 52 total, are located in the first 114 residues of the two proteins. This means that the C-terminal two-thirds of μ-crystallin contains only one-third of the sequence variability, suggesting that this is the location of functionally important, conserved sites.

Localization of μ-crystallin protein expression— Outside the lens, kangaroo μ-crystallin mRNA was previously detected only in retina and brain in adult tissues (9). A partial human μ-crystallin cDNA was cloned from adult retina and μ-crystallin mRNA was also detected in brain, muscle, and kidney (9). To examine the localization of μ-crystallin protein within the eye of placental mammals, in which gene recruitment of μ-crystallin has not occurred, western blots were performed with dissected bovine eye tissues (Figure 3). The human sequence shows that the peptide used to raise the Mup1 antiserum to μ-crystallin is well conserved in mammals (Figure 2b) and indeed the antiserum was able to detect a band of the expected size in parts of the bovine eye.

When comparing equal amounts of proteins, the strongest immuno-reaction in bovine eye was observed in retina (Figure 3). Immunoreactivity was also detected in dissected adult bovine lens epithelium (representing a small fraction of total lens) and in retinal pigment epithelium (RPE). Since the RPE preparation is likely to contain some photoreceptor outer segments, the signal in RPE may have been from this source. No reactivity was detected in iris (including ciliary body), lens fibers (representing most of the protein in the bovine lens), cornea nor sclera (not shown).

To examine the cellular localization of μ-crystallin in the retina in placental mammals, immunohistochemistry was performed on adult rat retina using peroxidase staining (Figure 4a). Compared with pre-immune serum, the Mup1 antiserum gave staining in several retinal cell types. However, the most intense reaction was in the photoreceptors, particularly in the outer segments (OS). This was confirmed by immunofluorescence (Figure 4b). Compared with pre-immune serum, which gave barely visible levels of staining in the same cells (not shown), μ-crystallin immunoreactivity was again seen throughout adult rat retina with the strongest reaction in photoreceptors. Using this technique, there was clear evidence for preferential localization of μ-crystallin protein in rod cell OS.

Is μ-Crystallin an Enzyme? The sequence of μ-crystallin revealed a superfamily relationship with OCD, a bacterial enzyme that converts ornithine to proline with NAD^+ as cofactor (9-11). To determine whether μ-crystallin itself has any propensity for cofactor binding, we employed the procedure previously used by Rao et al to examine another taxon-specific lens protein, guinea pig ζ-crystallin (14). We chose to use kangaroo lens soluble extract as a convenient source of highly abundant μ-crystallin. The soluble lens extract was passed over a blue-sepharose column (Figure 5) and, while other crystallins did not bind or were released by low salt washes, μ-crystallin was substantially retained on the column at 100 mM salt. The ability of solutions of NAD^+, NADH,

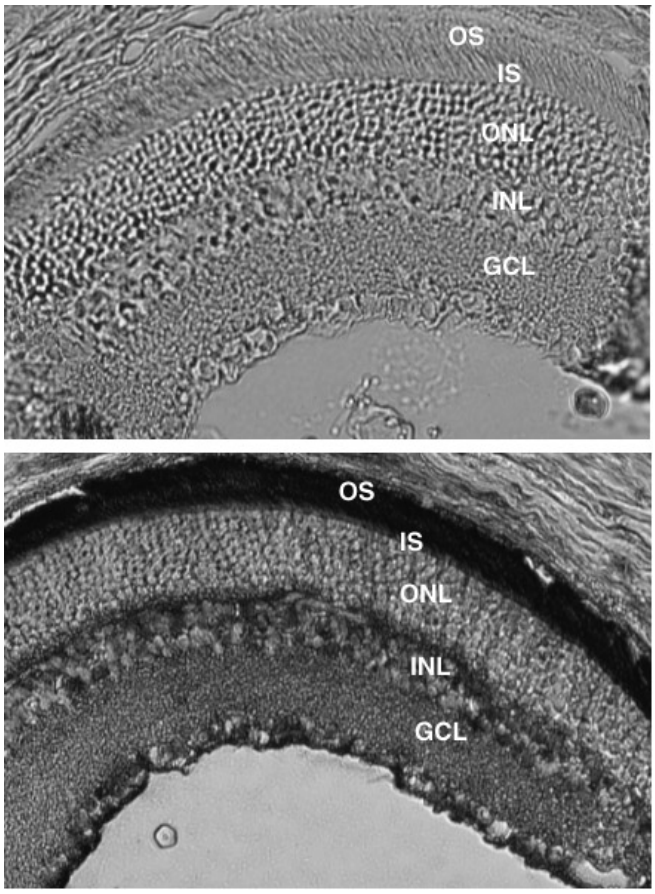

Figure 4a. Localization of μ-crystallin in adult rat retina. Immunohistochemistry using peroxidase staining. Above, rat retina with pre-immune serum. Below, similar section with μ-crystallin antiserum.

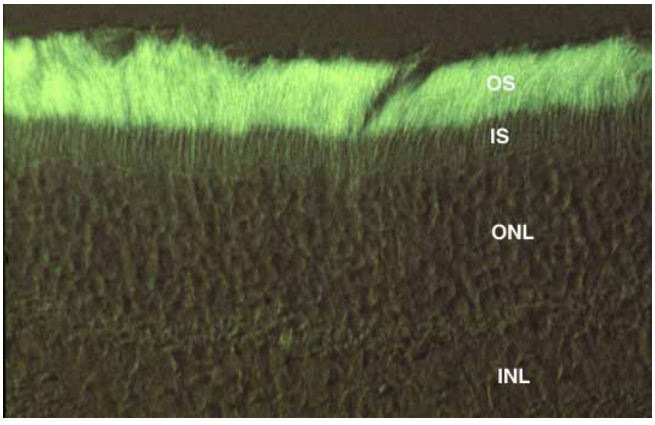

Figure 4b. Immunofluorescence using μ-crystallin antiserum. Retina layers are indicated; OS, photoreceptor (rod) outer segments; IS, photoreceptor inner segments; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

$NADP^+$, and NADPH (separately) to elute μ-crystallin was then tested. As summarized in Figure 5, 10 mM NADPH efficiently eluted μ-crystallin from blue-sepharose, while the other cofactors were ineffective. The other cofactors also failed to elute the bound protein at 100 mM concentration (not shown). The μ-crystallin again resolved as a doublet, but both forms bound the column and were similarly eluted by NADPH. These results suggest that μ-crystallin, like ζ-crystallin (14), is an NADPH-binding protein.

Seeking more clues to the possible function of μ-crystallin, sequence databases were searched for related proteins using BLAST (Basic Local Alignment Search Tool) programs (18, 19). As previously observed (9), OCD gave a significant match (about 30% identity). A sequence recently identified as a lysine cyclodeaminase (20) also showed similarity. More distant similarity also was found for another family of enzymes, the glutamyl-tRNA reductases (gluTR), enzymes of porphyrin synthesis in bacteria and plants, which use glutamyl-tRNA as substrate and produce a glutamate semialdehyde as product (21). Interestingly, the conventional two-step pathway for the reaction catalyzed by OCD, conversion of ornithine to proline, also passes through a glutamate semialdehyde intermediate. No substrate or product for μ-crystallin has yet been identified (data not shown); however, these superfamily relationships suggest that glutamate derivatives may be good candidates for any future efforts at substrate screening.

Dot matrix analysis shows that the similarity between μ-crystallins and gluTR is concentrated in the C-terminal half of the μ-crystallin sequence (Figure 6a), corresponding roughly to the region of μ-crystallin most highly conserved between human and kangaroo (Figure 2b). A sequence alignment within this region (Figure 6b) shows that the similarity spans two blocks of sequence (VLXXGAG and DIIIXST) that have been previously identified as highly conserved and possibly of functional significance in the gluTR family (21). We suggest that this may define a functional domain common to μ-crystallins, ornithine/lysine cyclodeaminases, and gluTR, perhaps including cofactor binding or active sites.

DISCUSSION

It seems probable that, like other taxon-specific crystallins (1, 3), μ-crystallin is an enzyme, but one previously unknown in animals. While in most species μ-crystallin maintains only its original function in retina, brain and other tissues, in some marsupials it has followed the path of gene recruitment (1, 3), acquiring an additional role as a major component of the lens.

Localizations using antiserum to μ-crystallin show that in placental mammals the protein is expressed at low levels in lens epithelium but at much higher levels in retina. Within retina μ-crystallin immunoreactivity is particularly abundant in photoreceptor outer segments, suggesting a role important for photoreceptor function. It is also present at lower levels in several other retinal cell types. The non-lens expression of μ-crystallin in both marsupial and placental mammals is consistent with the idea that the protein has a non-structural, probably enzymatic, role in retina and other tissues.

μ-Crystallin is related in sequence to ornithine and lysine cyclodeaminases and, more distantly, to glutamyl-tRNA reductases. These cyclodeaminases and reductases represent a diverse superfamily of enzymes that share a theme of unusual catalytic pathways for amino acids, including ornithine and glutamate or their derivatives. The membership of μ-crystallin in this enzyme superfamily suggests that it may have a broadly similar function. Indeed, kangaroo μ-crystallin, like gluTR (21), shows evidence for preferred binding of the enzyme

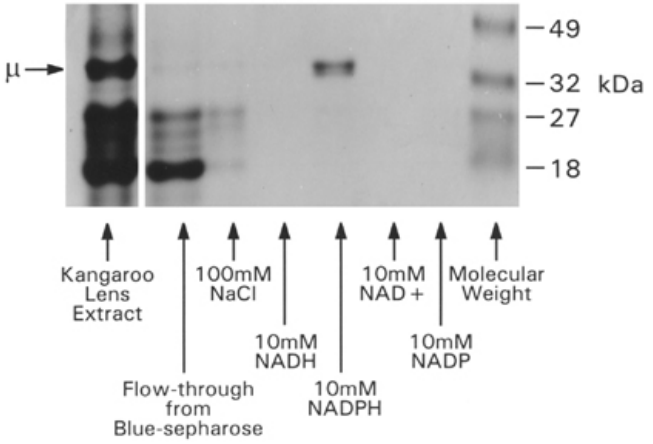

Figure 5. NADPH binding of kangaroo lens μ-crystallin. Summary of results for binding soluble extract of kangaroo lens to blue-sepharose followed by elution with salt and (separately) with 10 mM concentrations of oxidized and reduced nicotinamide adenine dinucleotide cofactors. μ-Crystallin is specifically retained on blue sepharose and is preferentially eluted by NADPH. All lanes are from the same gel. The kangaroo lens extract lane was moved to improve clarity of the figure.

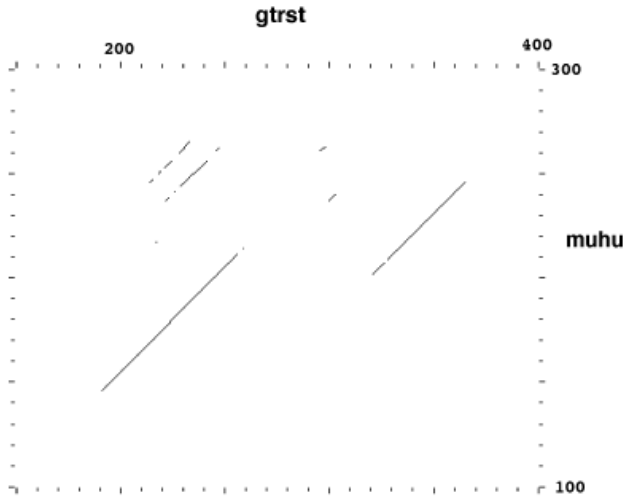

Figure 6a. Membership of μ-crystallin in an enzyme superfamily. Dot matrix comparison of amino acid sequences for gluTR from *Salmonella typhimurium* (26) (gtrst) and human μ-crystallin (muhu). Diagonal shows regions of similarity. Comparison drawn using DOTPLOT in the GCG package (27), window size 60, stringency 24.

Figure 6b. Sequence comparison of the core conserved regions of gluTR, μ-crystallins and ornithine and lysine cyclodeaminases. Compared regions correspond to residues 122-208 of human and kangaroo μ-crystallin (see Figure 2b). GluTR sequences are: gtrec, *Escherichia coli* (28); gtrst, S. typhimurium (26); gtrbs, *Bacillus subtilis* (29); gtrvib, *Chlorobium vibrioforme* (30). μ-Crystallins are: muhu, human (9 and present study); muka, Grey kangaroo (*Macropus fuliginosus*) (9). Cyclodeaminase are: ocdach5, *Agrobacterium tumefaciens*, plasmid Ach5 (11); ocdc58, *A.tumefaciens*, plasmid C58 (10); lcd, lysine cyclodeaminase of *Streptomyces hygroscopicus* (20); ocdr, *Rhizobium meliloti* (31). Regions conserved among members of all three protein families are boxed. Other regions show more local conservation between pairs of families.

cofactor NADPH. Considering the expression pattern of μ-crystallin, the similarity with enzymes of ornithine and glutamate is interesting since glutamate is a major neurotransmitter in retina, specifically of the photoreceptors (22), while photoreceptors also are peculiarly sensitive to systemic ornithine toxicity in the disease gyrate atrophy (23).

As for the lens role of μ-crystallin, western blotting shows that although it is undetectable in fibers or in whole lens, it can be detected at low levels in the dissected epithelial cells of the bovine lens. This low expression in lens is consistent with the idea that taxon-specific crystallins have been recruited from enzymes already expressed in lens (1, 3, 8) that may confer additional benefits when over-expressed as part of the refractive structure of the lens. Such benefits may include protective roles in osmoregulation, detoxification/ antioxidation, UV filtering, or stabilization of cytoskeleton (3).

Although the functional role of μ-crystallin in retina, lens, and other tissues of placental mammals remains to be determined, it is intriguing that the human gene for μ-crystallin is localized to chromosome 16p13.11-p12.3 (24) in the same region as a translocation associated with microphthalmia and cataract in man (25).

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