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Inability of chaperones to fold mutant ζ-crystallin, an aggregation-prone eye lens protein

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Purpose: ζ-crystallin is a quinone oxido-reductase, recruited in the eye lens of hystricomorphic rodents and camels. A deletion mutation constituting the NADPH-binding domain causes congenital cataract in a strain of guinea pigs. The presence of large quantities of α-crystallin, a molecular chaperone, does not provide any protection against this. In order to investigate whether the underlying reason for the lack of protection is the formation of a folding-incompetent protein, we have expressed the mutant protein in a heterologous system along with other known chaperones.
Methods: We expressed the mutant ζ-crystallin in *E. coli* along with other chaperones such as GroEL/ES and DnaK/DnaJ/GrpE and then analyzed whether these chaperones could increase the amount of protein partitioning into the soluble fraction of *E. coli* cells.
Results: These chaperones were unable to rescue the mutant protein from partitioning into inclusion bodies, although they could increase the yield of soluble wild-type ζ-crystallin.
Conclusions: The deletion of 34 amino acids, constituting the NADPH-binding domain of ζ-crystallin, makes the protein incompetent to fold correctly and thus form insoluble aggregates. It perhaps suggests why the mutant strain of guinea pigs have cataract at birth even though their lenses contain high amounts of α-crystallin. This study also shows that certain mutations can render proteins incompetent to fold into soluble molecules despite abundant assistance.

ζ-Crystallin is a taxon-specific crystallin found in the eye lens of two groups of mammals: camelids and some hystricomorphic rodents [1,2]. It is also found in trace amounts in other animals and tissues [3,4]. It belongs to the protein superfamily of medium chain alcohol dehydrogenases [5] and has been characterized as a quinone oxidoreductase [6]. It has been recruited in the lens where it is expressed in large amounts by a lens-specific promoter and may have a structural role to play in maintaining the transparency of the lens ensemble [7]. The presence of ζ-crystallin has been speculated to protect the lens from oxidative damage by acting as an NADPH reservoir [8]. A deletion of 34 amino acids, which results in the omission of the Rossmann fold, makes the protein lose its ability to bind NADPH and leads to an autosomal dominant congenital cataract in a line of strain 13/N guinea pigs. In mutants homozygous for this 34 amino acid deletion in ζ-crystallin, the protein is found mainly in the insoluble fraction of the lens, which can be solubilized only by chaotropic agents such as urea [9]. Only a very small amount is found in the water-soluble fraction, all of which elutes in the high molecular weight peak on a gel permeation column [10], where it could either be in its aggregated form, or in complex with α-crystallin.

It is interesting to note that despite the presence of large amounts of α-crystallin, which is known to act as a chaperone [11] and keep aggregation-prone proteins in solution, the mutant protein forms insoluble aggregates and causes lens opacification. In vitro, α-crystallin does not interact with native proteins, but binds folding and unfolding intermediates and stabilizes them from further aggregation [11-13]. However, the concentration of α-crystallin required to prevent aggregation varies with conditions and the type of target protein [14,15]. Since in the present animal cataract model, α-crystallin failed to prevent aggregation of the mutant ζ-crystallin, we thought it worthwhile to study the effect of other better-characterized chaperones such as GroEL/ES and DnaK/DnaJ/GrpE on the solubility of this protein. The DnaK chaperones recognize short stretches of amino acid sequences of proteins that have a preponderance of certain hydrophobic amino acids on nascent and extended polypeptide chains [16]. The GroE chaperonins are more selective towards hydrophobically collapsed structures [17]. The GroEL/ES and DnaJ/K/GrpE systems are known to cooperate both in vivo [18] and in vitro [19] to ultimately yield a correctly folded protein molecule. The ability of these chaperones to solubilize the ζ-crystallin mutant protein would then indicate whether it is a failure of α-crystallin to chaperone the molecule effectively or the mutation renders ζ-crystallin incompetent to fold into a soluble molecule. In order to study the ability of the chaperones GroEL/ES and DnaK/DnaJ/GrpE to prevent aggregation of the mutant ζ-crystallin in vivo, we decided to clone and express the protein in *E. coli* where these chaperones are known to occur naturally. Earlier reports show that co-overproduction of the above chaperones has been effective in obtaining recombinant proteins in a soluble and active form [20-24]. *E. coli* cells cotransformed with the pET plasmid containing the mutant ζ-crystallin cDNA and another plasmid containing the chaper-

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one expression system were therefore used in this study to see if the mutant protein could be solubilized by the above mentioned chaperone systems. Since expression of heterologous proteins in *E. coli* itself can lead to aggregation of the protein and its deposition into inclusion bodies [25,26], wild type ζ-crystallin was used as control in all experiments.

METHODS

Cloning of guinea pig wild-type and mutant ζ-crystallin: Total RNA was isolated from lenses of normal and the mutant strain of 13/N guinea pigs using Trizol reagent (Life Technologies Inc., Rockville, MD). The first strand cDNA was synthesized using an oligo (dT) primer and Moloney murine leukemia virus-reverse transcriptase (Stratagene, La Jolla, CA) according to the manufacturer's guidelines. Gene-specific primers having engineered *NdeI* and *HindIII* sites were then used to amplify the ζ-crystallin cDNA by polymerase chain reaction. The amplified products of both the wild-type and mutant ζ-crystallins were cloned into a T-vector pCR2.1 (Invitrogen, Carlsbad, CA) to generate pCR2.1-ζ and pCR2.1-mζ plasmids.

Sequencing and subcloning of guinea pig wild-type and Mutant ζ-crystallins: The wild-type and the mutant ζ-crystallin cDNAs were sequenced with the help of M13 forward and reverse primers using the Big Dye™ terminator cycle sequencing kit (Perkin Elmer International Inc., Rotkruez, Switzerland) in an Applied Biosystems (Perkin Elmer, Foster City, CA) automated DNA sequencer. The sequences matched with the earlier published data. The coding regions were excised from pCR2.1 by digestion with *NdeI* and *HindIII* and ligated into pET21a (Novagen) which had been linearized by digestion with *NdeI* and *HindIII* to generate pET21a-ζ and pET21a-mζ, respectively.

Co-overexpression of wild-type and mutant ζ-crytallins with chaperones GroEL/ES and DnaK/DnaJ/GrpE: The expression plasmids pOFx-T7-SL3 and pOFx-T7-KJE3, which encode the cDNAs of the GroE operon and DnaK/DnaJ/GrpE respectively under a T7-promoter, were obtained as a kind gift from Dr. Olivier Fayet, Microbiologie et Genetique, Toulouse, France. These were transformed into competent *E. coli* BL21(DE3) cells into which pET21a-ζ or pET21a-mζ had been earlier transformed. The pET21a vector possesses a pBR322 origin of replication which is compatible with the p15A origin of replication of the pOFX-T7-SL3 and pOFX-T7-KJE3. Thus both plasmids could be stably maintained in the same bacterial cell. Also, the two plasmids within a cell carried different antibiotic resistance genes by which only those bacteria which possessed both plasmids would be selected. The co-transformed cells were cultured overnight and a 1% inoculum was used to subculture 15 ml of Luria Broth with appropriate antibiotics. The subcultured flasks were grown at 37 °C to mid exponential phase (A_{600} about 0.6), induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and either maintained at 37 °C or shifted to 30 °C. Aliquots of 3 ml were withdrawn at 1, 2, and 3 h after induction and the A_{600} recorded. The cells were pelleted at 6000 x g for 20 min and resuspended in 300 µl of 50 mM potassium phosphate buffer pH 7.8. The cells were disrupted by sonication on a Branson cell sonifier and centrifuged at 10000 x g for 30 min. The supernatant was separated as the soluble fraction. The pellet comprised the insoluble fraction and was resuspended in 300 µl of SDS sample buffer. Total cell lysates were obtained by taking 1 ml aliquots at the same time intervals, pelleting down the cells and resuspending them into 100 µl of SDS sample buffer.

SDS-PAGE: Soluble and insoluble fractions from the cell lysates were normalized with respect to cell density and loaded on 12% SDS polyacrylamide gels under reducing conditions and stained with Coomassie Brilliant Blue R250 (Sigma, St. Louis, MO). Gels were scanned and analyzed densitometrically to quantify the amount of ζ-crystallin partitioning into the soluble fraction.

Enzyme activity: ζ-Crystallin activity [6] was monitored in the soluble fraction of the cell lysates using 9,10-phenanthrenequinone as substrate. The reaction mixtures contained 0.1 mM NADPH, 0.1 mM EDTA, 25 µM 9,10-phenanthrenequinone, 50 mM potassium phosphate buffer, pH 7.8 and appropriate amounts of enzyme in a final volume of 1.0 ml. The reaction was initiated by addition of substrate and the oxidation of NADPH monitored at 340 nm using a Hitachi

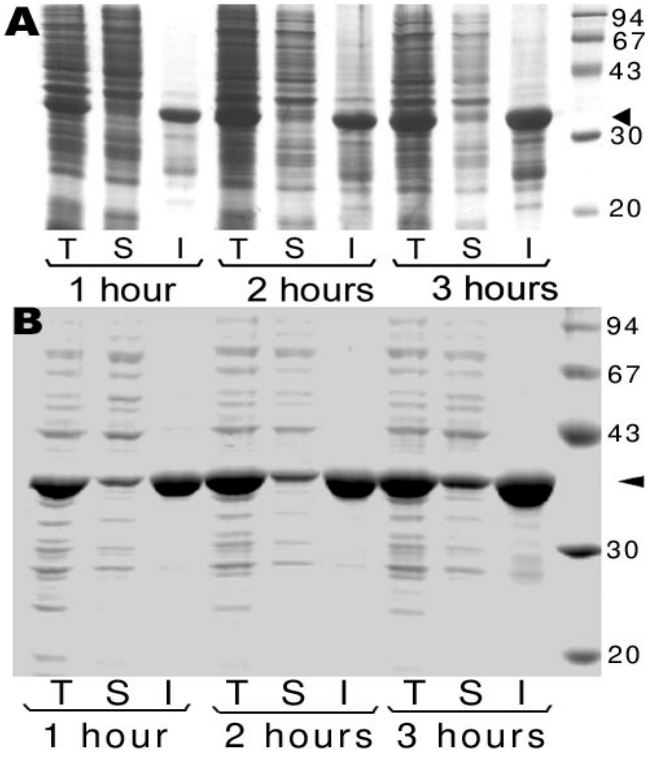

Figure 1. Time dependent accumulation of ζ-crystallins in E. coli cells. Time dependent accumulation of mutant (**A**) and wild-type (**B**) ζ-crystallins into the soluble and insoluble fractions of E. coli cells. Aliquots were drawn 1, 2, and 3 h after induction and the total cell lysate (T), soluble (S) and insoluble (I) fractions run on SDS-polyacrlamide gels. Molecular weight markers are indicated. The positions of ζ-crystallins and mutant ζ-crystallins are marked by arrows.

U2000 spectrophotometer (Hitachi Ltd., Tokyo, Japan). Blanks lacking the substrate or enzyme were run routinely. One unit of enzyme activity was defined as oxidation of 1 µmol of NADPH min^{-1} mg^{-1} protein.

RESULTS & DISCUSSION

Cloning of guinea pig lens wild-type and mutant ζ-crystallins: The coding regions of wild-type and mutant ζ-crystallins were subcloned into the expression vector pET21a to yield pET21a-ζ and pET21a-mζ. The expression plasmids were transformed into *E. coli*BL21(DE3). Induction of the plasmids by IPTG resulted in the expression of recombinant ζ-crystallin proteins which migrated on SDS-PAGE at about 35 kDa, the mutant having a slightly greater electrophoretic mobility due to its lower molecular mass.

Time dependent accumulation of wild-type and mutant ζ-crystallin in the soluble/aggregated form: In order to determine the comparative amount of protein partitioning into the insoluble fraction over time, aliquots of the *E. coli*culture containing the pET21a-ζ and pET21a-mζ plasmids were collected 1, 2 and 3 h after induction with IPTG. The cells were pelleted and fractionated into soluble and insoluble fractions. These fractions and the total cell lysate, normalized with respect to cell density, were analyzed on a 12% SDS-polyacrylamide gel. On the stained gels, the 35 kDa band corresponding to ζ-crystallin (Figure 1B) and a slightly lower moving band corresponding to the mutant ζ-crystallin (Figure 1A) could be clearly distinguished from other *E. coli* proteins. The expression levels of the wild-type and the mutant protein increased with time, reaching maximum at 3 h after induction. All of the expressed mutant protein partitioned into the insoluble fraction even at 1 h after induction, when only low levels of the protein had accumulated within the bacterial cell (Figure 1A). As the protein expression increased with time, the accumulation of the mutant protein in the insoluble fraction also increased. On the other hand, the level of the wild-type protein in the insoluble fraction was much less during the first hour after induction and progressively increased with time, concomitant with the accumulation of the expressed protein (Figure 1B). The deletion of 34 amino acids constituting the NADPH-binding domain of the mutant appears to alter its folding pathway resulting in its deposition into inclusion bodies even when expressed at low levels. In contrast, the wild-type protein goes into inclusion bodies only as its concentration in the cell increases. This might be explained by off-pathway aggregation of folding intermediates being favored over on-pathway folding reactions at the relatively high concentrations of the newly synthesized polypeptides within the cells [27]. Mutant ζ-crystallin appears to be unstable, having a tendency to go into inclusion bodies even at low concentrations. Thus, it appears that it is the inherent instability of protein that causes aggregation. This tendency to aggregate and precipitate may lead to lens opacity in the 13/N strain of guinea pigs harboring a mutation in the ζ-crystallin gene [28].

Effect of overproducing chaperones GroEL/ES or DnaK/DnaJ/GrpE along with the wild-type and mutant ζ-crystallin: Plasmids pOFX-T7-SL3 and pOFX-T7-KJE3 were transformed separately into *E. coli* BL21(DE3) and the expression

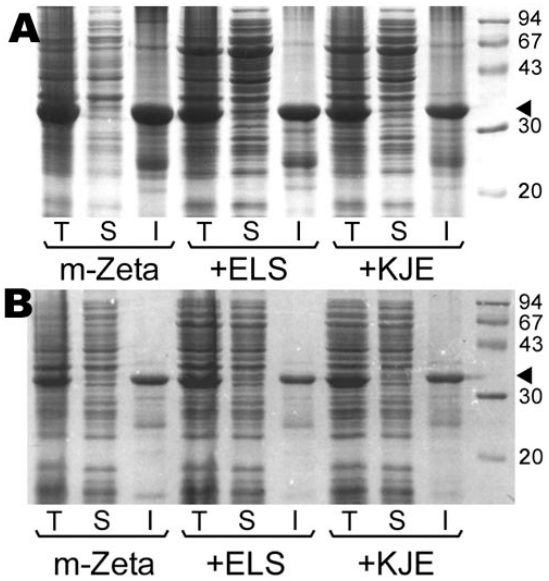

Figure 2. Effect of chaperones on the solubility of mutant ζ-crystallin. Effect of co-expression of specific molecular chaperones on the solubility of mutant ζ-crystallin. Aliquots were drawn at (**A**) 3 h after induction with IPTG at 37 °C and (**B**) 1 h after induction with IPTG at 30 °C. Cells were fractionated into the soluble (S) and insoluble (I) fractions and run on SDS-polyacrylamide gels. Molecular weight markers are indicated. The position of the mutant ζ-crystallin is marked by an arrow. (m-ζstands for the mutant protein, ELS stands for GroEL/ES, and KJE stands for DnaK/DnaJ/GrpE.)

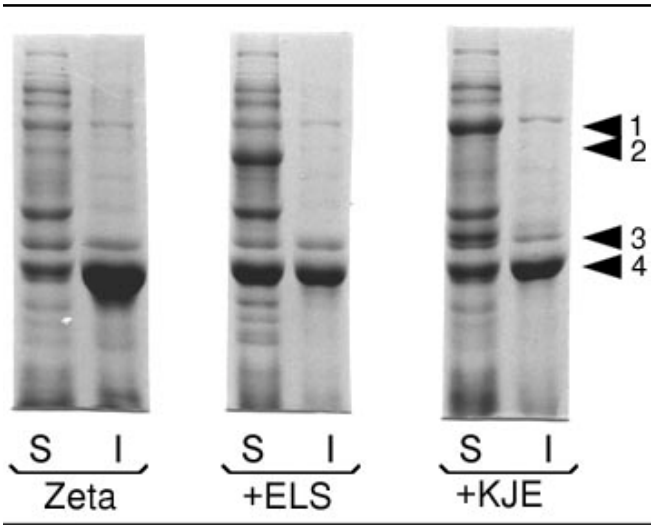

Figure 3. Effect of chaperones on the solubility of wild type ζ-crystallin. Effect of co-expression of specific molecular chaperones on the solubility of wild type ζ-crystallin 3 h after induction by IPTG at 37 °C. Cells co-expressing DnaK/DnaJ/GrpE along with ζ-crystallin and those expressing GroEL/ES along with ζ-crystallin were fractionated into the soluble (S) and insoluble (I) fractions and run on SDS-polyacrylamide gels. The arrows indicate the positions of (1) DnaK, (2) GroEL, (3) DnaJ, and (4) ζ-crystallin. (ELS stands for GroEL/ES and KJE stands for DnaK/DnaJ/GrpE.)

of the chaperones they encode was induced with IPTG. The cellular levels of both chaperones increased approximately 4 fold (data not shown). These plasmids were then transformed into BL21(DE3) cells already harboring pET21a-ζ or pET21a-mζ. When ζ-crystallin and the GroEL/ES proteins were co-expressed, there was no detectable change in the expression rates of ζ-crystallin.

To determine whether the chaperones GroEL/ES or DnaK/DnaJ/GrpE increased the solubility of the wild-type and mutant ζ-crystallins, the *E. coli* cells harboring both expression plasmids (pET21a-ζor pET21a-mζ and pOFX-T7-SL3 or pOFX-T7-KJE3) were grown to mid-exponential phase at 37 °C, induced by IPTG and maintained at 37 °C. Three h post induction, the total cell lysate as well as the soluble and insoluble fractions of the above cultures were analyzed on SDS-polyacrylamide gels. As evident from Figure 2A, co-expression with GroEL/ES or DnaK/DnaJ/GrpE had no effect on the solubility of the mutant protein. Even when the cells were cultured at 30 °C (Figure 2B) in order to slow their growth rate and hence their protein synthesis machinery, thus providing time for the newly synthesized polypeptides to fold properly, the mutant protein still partitioned entirely into the pellet fraction. Here again the chaperones could not rescue the mutant from going into inclusion bodies. For the above experiment, cells were harvested within 1 h of induction by IPTG when the relative protein concentrations were low in order to avoid aggregation simply due to crowding. In contrast, the solubility of the wild-type protein was increased significantly (Figure 3) when co-expressed with GroEL/ES (3-fold), and DnaK/DnaJ/GrpE (2.4-fold). There was a concomitant recovery of the quinone oxido-reductase activity which the ζ-crystallin possesses (Figure 4). In the control strain (containing no ζ-crystallin or chaperone plasmids), grown at 37 °C for 3 h, there was a basal activity from the *E. coli*genome-encoded quinone oxido-reductase. The activity increased slightly when pET21a-ζ alone was expressed. The activity almost doubled in the cells containing both pET21a-ζ and pOFx-T7-SL3. The co-expression of plasmid-encoded DnaK/DnaJ/GrpE also enhanced the yield of active ζ-crystallin by 2.1 fold. Our results show that overproduction of either GroEL/ES or DnaK/DnaJ/GrpE facilitates folding and assembly of the ζ-crystallin into its native structure in *E. coli* whereas the mutant protein fails to fold despite the presence of chaperones.

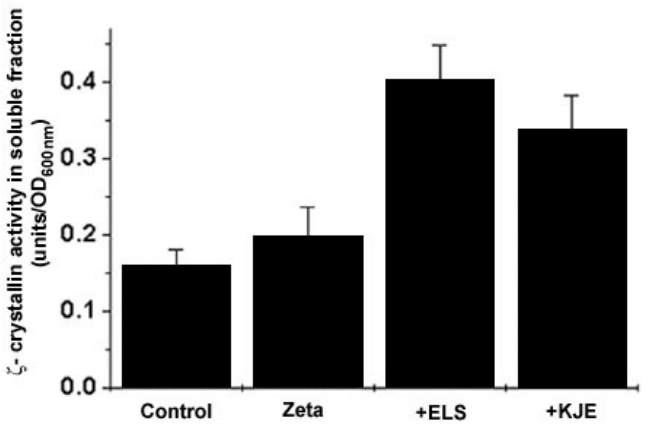

Figure 4. Activity of wild-type ζ-crystallin in the soluble fractions. Aliquots were drawn 3 h post-induction and the cells were fractionated into the soluble and insoluble fractions. The soluble fractions, normalized with respect to cell density (A_{600}), were used for activity measurements. (ELS stands for GroEL/ES and KJE stands for DnaK/DnaJ/GrpE.)

Under normal circumstances of in vivo folding, molecular chaperones which modify the kinetic partitioning between productive folding and off-pathway reactions [19] might fall short to prevent a large number of nascent polypeptides from sticking to each other during overexpression of proteins in *E. coli*. Increasing the chaperone supply may help to solve this problem to some extent. This explains why the recombinant wild-type ζ-crystallin, which was partitioning into the insoluble fraction due to its high levels in the overexpressing *E. coli* cells, could be solubilized by DnaK/DnaJ/GrpE and by GroEL/ES.

However, if the covalent structure of the protein is extensively modified due to various reasons such as truncation or deletion of domains, it might not be possible for them to attain stability in the form of a folded end-product. Chaperones, in such cases can not provide any assistance. The co-expression studies of ζ-crystallin or its mutant form with chaperones GroEL/ES and DnaK/DnaJ/GrpE reinforce the fact that all aggregation-prone proteins cannot be assisted to fold by chaperones. The mutant ζ-crystallin could not be solubilized by either of the chaperone systems used, even at low concentrations of the ζ-crystallin.

The only difference between the wild type and the mutant protein is the deletion of the cofactor-binding Rossmann fold, which appears to be essential in the folding pathway of the protein. Deletion of a 34 amino acid stretch which accounts for more than 10% of the entire protein must be important in maintaining the 3-dimensional structure of the protein and thus the mutant is unable to form a stable end-product. The observation that chaperones could not aid the mutant ζ-crystallin to become soluble, highlights the fact that chaperones only assist proteins with folding competent sequences to reach their native conformations by stabilizing intermediates in their folding pathway. Chaperone systems may not be able to help if a given sequence is inherently incapable of achieving a stable conformation despite providing ample space and time.

Congenital Cataract in the strain 13/N guinea pigs may be attributable to mainly three possible reasons: (a) loss of enzyme activity, (b) loss of ability to bind the cofactor which decreases the NADPH levels in these lenses making them prone to oxidative damage or, (c) destabilization of the protein structure which causes it to aggregate and result in opacity. The first reason can be ruled out as heterozygous lenses also have cataract, where 50% of the active protein, a considerably large quantity for enzymatic function, is still retained. The second reason also seems less likely as oxidative damage due to decreased NADPH reserves in the eye lens might take some time for the onset of disease. However, these guinea pigs are afflicted with cataract at birth. Thus the main reason

for congenital cataract in the 13/N guinea pigs appears to be the loss of native structure of the ζcrystallin. The deletion mutation results in a folding incompetent protein which becomes insoluble.

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