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Review Article

The pathogenic mechanisms of recombinant human αB-crystallin proteins generated by site-directed mutagenesis in *Escherichia coli*

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Abstract

Cataract diseases are known as the most important cause of global blindness. a-crystallin, the most important chaperone of the lenticular tissues, plays a crucial role in the eye lens transparency throughout human life. Missense mutations in the *CRYAB* gene are associated with congenital cataract and various myopathies, primarily by disrupting protein folding, oligomerization, and stability, which in turn compromises chaperone function and enhances protein aggregation. Four pathogenic mutations in this gene -*R69C*, *D109H*, *P20R*, and *A171T*- have been reported to cause cataract and myopathy. In recent studies, after introducing these mutations into the *CRYAB* gene by site-directed mutagenesis, recombinant protein expression in *Escherichia coli* and subsequent purification, the structural and functional changes in recombinant proteins were analyzed using various techniques. Substitutions in the N-terminal domain (P20R), a-crystallin domain (*R69C*, *D109H*), and C-terminal domain (*A171T*) in *CRYAB* alter the secondary, tertiary, and oligomeric structures, diminish *in vitro* and *in vivo* chaperone-like activity, and enhance amyloidogenic propensity of human aB-crystallin protein. Together, these findings elucidate the molecular mechanisms underlying *CRYAB*-related cataract and myopathy. They also highlight the value of recombinant protein models and site-directed mutagenesis in understanding genotype-phenotype relationships. Such insights not only deepen knowledge of disease progression but also provide a framework for developing therapeutic strategies aimed at stabilizing mutant proteins, enhancing chaperone function, and reducing aggregation.

Keywords: *CRYAB* gene, Cataract, Myopathy, Site-directed mutagenesis, Chaperone activity.

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Introduction

The human eye lenses contain a concentration of extremely stable proteins (300-450 mg/mL) known as a-, b- and g-crystallins. These proteins comprise 90% of the total soluble lens proteins, which are required for transparency and a high refractive index (1,2). a-Crystallin (a-Cry) is a stable b-sheet-rich protein with relatively low turnover. It accounts for 40% of the total soluble proteins in the lenticular tissues (3-5). In the lens, this protein belongs to the small heat shock protein (sHSP) family, which binds to unfolded proteins, suppresses stress-induced aggregation, and maintains lens transparency. Expression of the aA-crystallin (aA-Cry) subunit is essentially restricted to the eye lenses, while aB-crystallin (aB-Cry) is a ubiquitous protein that, in addition to the lens, is also expressed in tissues such as retina, brain, lung, kidney, skin, skeletal and cardiac muscle (4-7). In the non-lenticular tissues, besides its chaperone function, aB-Cry is required for the remodeling and protection of the cytoskeleton, serving as a p53 target protein during the apoptosis inhibition (4,6,8-10). High levels of human aB-Cry have been reported to be significantly elevated in certain neurological disorders, such as Parkinson's and Alzheimer's disease (7,8,11).

Cataract causes clouding of normal clear lenses and usually develops over time as lens crystallins undergo post-translational modifications, leading to the development of age-related cataracts (1,12,13). Congenital cataracts account for 10-38% of blindness in children (14). Genes encoding lens crystallins, gap junction proteins (connexins), growth and transcription factors, and beaded filament proteins are developmentally linked to congenital cataracts (14-16). It has also been suggested that about half of the cataract-causing mutations occur in crystallin genes and about a quarter in connexin genes (16). Mutations in *CRYAA* (human aA-Cry gene) are frequently associated with cataract development, whereas those in the *CRYAB* gene are relatively rare (8,15-17). However, mutations in *CRYAB* have been reported to cause desmin-related myopathy, myofibrillar myopathy, and dilated cardiomyopathy. Most hereditary cataracts follow an autosomal dominant pattern, while a small number show autosomal recessive inheritance (11,13,18). This review aims to highlight the pathogenic significance of mutations in the *CRYAB* gene, focusing on their impact on the structure and function of the aB-Cry protein. By reviewing studies on various mutations, particularly R69C, D109H, P20R, and A171T, this research seeks to elucidate the relationship between structural alterations and decreased chaperone activity of the aB-Cry protein. Clarifying these molecular mechanisms provides valuable insight into the basis of cataract and myopathy development.

1) Molecular and structural properties of aB-Cry protein

The secondary structure of a-Cry, is primarily made by b-sheet. Circular dichroism (CD) and Fourier-transform infrared (FTIR) measurements indicated that the secondary structure of a-Cry consists of 40% b-sheet, 15% α-helix and the remainder, random coil and turns (19). a-Cry is a polydisperse hetero-oligomer made from aA- and aB- subunits (ratio of 3:1, respectively), forming non-covalent aggregates with molecular masses ranging from 300 to 1000 kDa (1,2,4,5). In humans, aB (175 amino acid residues) is encoded by the *CRYAB* gene, which is located on chromosome 11. The molecular mass of this protein subunit is 20 kDa (4,8). Each subunit comprises three structural domains:

an N-terminal domain (NTD), a conserved central a-Cry domain (ACD) with an immunoglobulin-like fold, and a flexible C-terminal domain (CTD). The ACD (90 residues) is the hallmark of sHSP and, through its chaperone activity, prevents the self-assembly of protein aggregates. The hydrophobic NTD is involved in oligomerization and chaperone-like activity, while the flexible CTD enhances solubility of the protein-substrate complex (5,20-23). The tertiary and quaternary structures of a-Cry due to highly dynamicity and the polydispersity have not been identified so far (4). The chaperone activity of a-Cry subunits is vital to the maintenance of eye lens transparency and for cataract prevention. This chaperone is capable to bind partially unfolded b- and g-Cry proteins at the onset of denaturation, preventing their further precipitation which at last culminated in the inhibition of eye lens opacification (24). Moreover, human a-Cry inhibits inactivation of different enzymes (4). Moreover, a-Cry is a dynamic oligomer which exhibits a subunit exchange feature (constantly exchanged subunits by association and dissociation). This feature of a-Cry is important for its chaperone activity (1).

2) Overview of pathogenic mutations in *CRYAB*

Mutations in *CRYAB* include cataract and myopathy-associated variants distributed across the three domains. According to the results, no particular domain has been reported to be responsible for the different types of cataracts or myopathies (Figure 1) (8).

Until now, various mutations in the *CRYAB* gene have been reported, which are associated with congenital cataract and myopathy diseases. Cataract-related mutations in *CRYAB* comprise R11H (25), R11C, R12C (26), P20S (27),

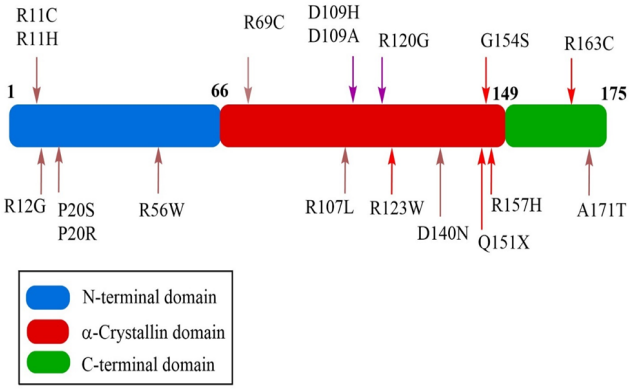

Fig 1. Domain organization and some mutation sites in the primary structure of human aB-Cry. The human aB-Cry protein consists of 175 amino acids organized into three domains. The red arrows describe the phenotype of mutations that cause myopathy, brown arrows cause cataract, and purple arrows cause myopathy and cataract.

P20R (28), R56W (29), R69C (14), R107L (30), D109H (6), D109A (17), R120G (31), D140N (27), A171T (32) and X176W (33). Moreover, D109H, D109A, D109G (34), R120G, R123W (35–37), Q151X (38), G154S (39), R157H (40), R163W (41,42) and X176W mutant aB-Cry are myopathy-causing mutations, and D109H, D109A, R120G, and X176W mutations result in cataract as well as myopathy development (Table 1 and Figure 1).

R120G, the first discovered missense mutation in aB-Cry, has been discovered in a French family possessing association with cataract disease, desmin-related myopathy (co-aggregation of the mutant protein with desmin filaments) and cardiomyopathy (31). The amino acid residue R116 in aA-Cry corresponds to R120 in aB-Cry. The R116C cataract mutation of aA-Cry demonstrated noticeably reduced chaperone-like activity similar to R120G mutation in aB-Cry (46-48).

The important cataract-associated mutations, D140N and R107L, have been reported in the functional ACD of human aB-Cry protein (27,30). The first case of restrictive cardiomyopathy associated with a mutation in

Table 1. Cataract and myopathy-causing mutations of the *CRYAB* gene.

Nucleotide change	Amino acid change	Inheritance pattern	Cataract phenotype	Myopathies	References
31C>T	R11C	AR	Nuclear		(26)
32G>A	R11H	AD	Nuclear		(25)
34C>T	R12C	AR	Nuclear		(26)
58C>T	P20S	AD	Posterior polar		(43)
59C>G	P20R	AD	Posterior polar		(28)
166C>T	R56W	AR	Nuclear		(29)
205C>T	R69C	AD	N/A		(14)
320G>T	R107L	AD			(30)
325G>C	D109H	AD	Posterior polar	Myofibrillar myopathy	(6)
326A>C	D109A	AD	Unilateral	Myofibrillar myopathy	(17)
326A>G	D109G			Restrictive cardiomyopathy with skeletal myopathy	(34)
358A>G	R120G	AD	Discrete	Desmin-related myopathy	(31)
367C>T	R123W			Hypertrophic cardiomyopathy	(35-37)
418G>A	D140N	AD	Lamellar		(43)
451C>T	Q151X			Myofibrillar myopathy	(38)
460G>A	G154S			Dilated cardiomyop-athy and myofibrillar myopathy	(39,44,45)
470G>A	R157H			Dilated cardiomyop-athy	(40)
487C>T	R163C			Dilated Cardiomyopathy	(41,42)
511G>A	A171T	AD	Lamellar		(32)
527A>G	X176W	AD	Posterior polar	Adult onset cardiomyopathy	(33)

AD: Autosomal dominant, AR: Autosomal recessive, N/A: Not available.

CRYAB (D109G), which was identified and reported in 2017, is also linked to skeletal myopathy (34). In this mutation, a charged residue (aspartate) at position 109 is substituted with glycine. The R123W mutation has been recently reported in *CRYAB* in middle-aged monozygotic twins with cardiomyopathy (36,49).

Clinical genetics studies have also identified R69C, D109H, P20R, and A171T mutations in the *CRYAB* gene. The development of posterior polar cataract (a rare form of congenital cataract with an autosomal dominant inheritance), myofibrillar myopathy,

and cardiomyopathy has been attributed to the D109H mutation in human αB-Cry, whereas the R69C mutation is associated with congenital cataract. These autosomal dominant mutations affect highly conserved residues in the ACD of human aB-Cry (6,14). The cataract-causing P20R mutation (in exon 1 of the *CRYAB* gene) is linked to autosomal dominant hereditary posterior polar cataract at a highly conserved residue in the NTD of human aB-Cry. The autosomal dominant mutation A171T at the conserved position in the C-terminal extension (CTE, the last highly variable 12 amino acids of CTD) of human aB-Cry causes lamellar pediatric congenital cataract (26,28,32,50).

3) Site-directed mutagenesis to generate different mutations

Mutagenesis is an influential method to investigate regions that are essential for the chaperon function (51). Different mutations were created using site-directed mutagenesis (52-56). The basic procedure of site-directed mutagenesis is shown in Figure 2.

To perform this method, a double-stranded DNA (dsDNA) vector with an insert of the interested cDNA and two mutagenic oligonucleotide primers were used. These primers possess a complementary segment to opposite strands of the vector, except for a specific mismatch to introduce the mutation. Moreover, the primers should have the following properties: (1) 25 to 45 bases in length, (2) melting temperature ³78 °C, and (3) locating of desired mutation at the middle of primer (10-15 bases distances from both sides). The oligonucleotide primers are hybridized to the target vector and extended during the polymerization reaction by Phusion high-fidelity DNA polymerase. This DNA

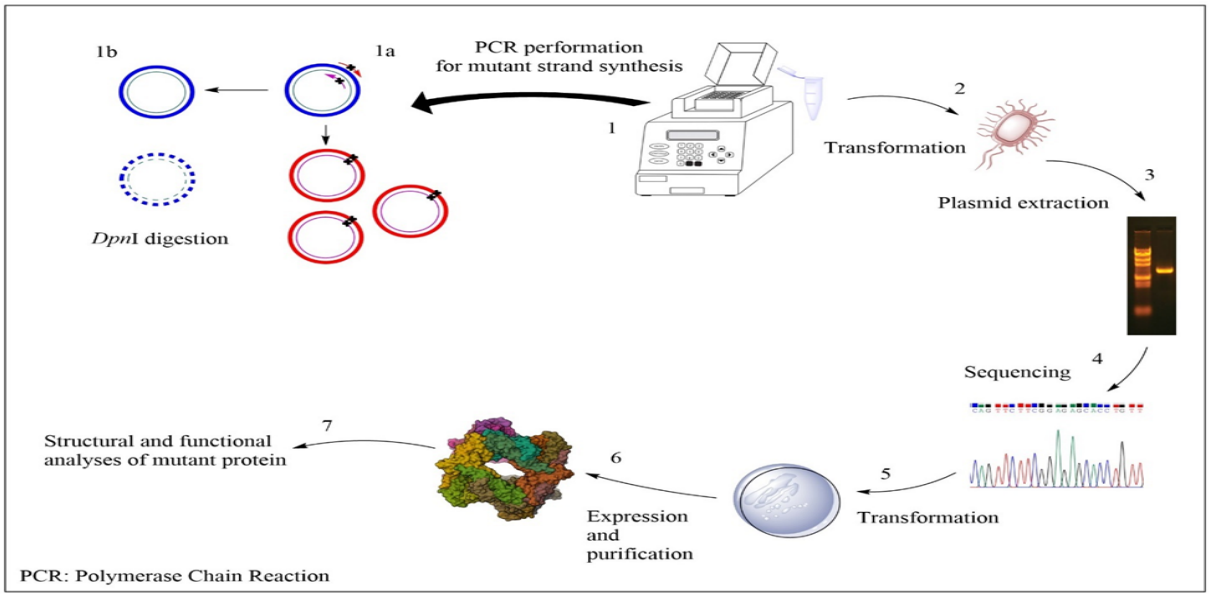

Fig 2. Schematic representation of the site-directed mutagenesis procedure (1). Mutagenic oligonucleotides with defined mismatches anneal to complementary strands of the plasmid and are extended by a high-fidelity DNA polymerase (1a). The methylated parental template is selectively digested by *Dpn*I (1b). (2) The mutant plasmid is transformed into ultra-competent cells. (3) After plasmid extraction and (4) confirmation of the confirmed mutation by sequencing, (5) the mutagenic plasmid is transformed into the BL21 (DE3) strain of *E. coli* for expression, followed by (6) purification of the recombinant protein. This approach is used to generate mutation variants for structural and functional characterization (7).

polymerase is a *Pyrococcus*-like proofreading enzyme with a processivity-enhancing domain. Then, the mutated product was digested with *Dpn*I. The *Dpn*I endonuclease was used to digest the methylated and hemimethylated parental DNA template (sequence 5′-Gm6ATC -3′), which is susceptible to the *Dpn*I enzyme. Finally, the selected nicked vector with the desired mutation was transformed into ultra-competent cells (52,57).

The recent mutations R69C, D109H, P20R, and A171T were introduced into the *CRYAB* gene using Site-Directed Mutagenesis. In these studies, the wild-type (Wt) human aB-Cry cDNA was cloned into a pET-28b (+) vector between *Nco*I and *Not*I restriction sites. This vector is kanamycin resistant. After transformation of the mutated gene into XL 10-Gold ultra-competent cells, expression was carried out in the BL21 (DE3) strain of *E. coli* cells, and the purified mutant proteins were subjected to various spectroscopic techniques (Fig 1) (58,59). This review summarizes the findings of these studies about structural and functional properties of the mutant proteins and the mechanisms by which these mutations contribute to the development of cataract and myopathy.

4) Structural and functional consequences of various mutant aB-Cry proteins

According to the Clinvar database, 168 missense mutations in αB-Cry have been linked to cataracts, cardiomyopathy, or myopathy, although many have uncertain pathogenicity and lack clinical evidence (60). This highlights the need for functional studies on these variants. According to earlier studies, a range of detrimental alterations in the structure and function of human aB-Cry caused by various mutations may explain their contribution to the development of cataract and myopathy disorders (9,27,54,56,61-65). Numerous pathogenic aB-Cry mutations demonstrate loss the chaperone activity and are linked with the increased protein aggregation (9,63-68). Although, several pathogenic mutant aB-Cry demonstrated an increased *in vitro* chaperone activity (56,60, 69-72). Additionally, specific mutations in aB-Cry change the subunit exchange rate (66,73). The mechanism of cataract formation by mutations in a-Cry genes has been investigated by several studies. One mechanism is based on increased protein aggregation by a-Cry mutations, causing a low affinity for substrate protein. These changes occur by the alteration in the structure of the chaperone protein and loss of the oligomeric structural regulation of a-Cry due to mutation, resulting in higher co-aggregation of mutant protein and client proteins (74).

The R120G mutation reduces the chaperone-like properties of aB-Cry and induces structural irregularities. Structural analyses show alterations in secondary, tertiary, and quaternary structures, accompanied by an increase in oligomeric mass (9,67). Both R120G and D140N mutations, located within the conserved ACD, significantly affect chaperone activity (27). Site-directed mutagenesis studies indicate that a-Cry can tolerate many amino acid substitutions in its primary sequence (75). Unlike R120G, D140N does not markedly change the secondary structure but alters the tertiary structure and surface hydrophobicity, increases aggregation propensity, and reduces thermal stability compared to Wt protein. (Table 2).

The Q151X mutation removes CTD, reducing oligomerization but enhancing chaperone activity. The mutant has a high tendency to self-aggregation, predominantly localizing in

Table 2. Structural and functional effects of different mutations on human aB-Cry.

Mutation	Disease	Domain	Structural effects	Functional effects	References
R120G	Cataract, myopathy	ACD	Altered secondary, tertiary, quaternary structure, increased oligomer mass	Reduced chaperone activity, increased protein aggregation	(9)
D140N	Cataract	ACD	Altered tertiary structure, increased surface hydrophobicity, reduced thermal stability	Increased aggregation	(27)
Q151X	Myopathy	CTD	C-terminal deletion, reduced oligomerization	Enhanced chaperone activity, increased self-aggregation	(70)
P20S	Cataract	NTD	No change in oligomer size	Decreased chaperone activity, increased nuclear import, apoptosis	(61)
A171T	Cataract	CTD	—	Increased apoptosis	(62)
G154S	Myopathy	CTD	Reduced thermal stability	Reduced chaperone activity, increased amyloid formation	(55)
R157H	Myopathy	CTD	Smaller oligomer size	Similar or enhanced chaperone activity, reduced enzyme refolding	(55)
D109G	Myopathy	ACD	Significant structural changes	Reduced chaperone activity, protein aggregation, increased amyloid propensity	(62,65)
P39L	Cataract, cardiomyopathy	NTD	Changes in secondary, tertiary, quaternary structure, altered stability	Increased chaperone activity, aggregation propensity	(72)
R123W	Cardiomyopathy	ACD	Significant structural alterations, abnormal oligomerization	Decreased chaperone activity, impaired interaction with calcineurin	(64)
R107L	Cataract	ACD	Larger oligomer formation, decreased stability	Reduced chaperone activity	(63)
K90N	Myopathy	ACD	Structural alterations, increased oligomer	Reduced chaperone activity, impaired protein aggregation prevention, increased amyloid formation	(76)
R11G	Cataract, Myopathy	NTD, Mini chaperone	Significant structural changes	Increased chaperone activity, altered interaction with target proteins, increased amyloid fibril propensity	(71)
R157C	Cardiomyopathy	CTD, IXI motif	—	Enhanced chaperone activity, increased amyloid fibril formation	(56)
R163C	Dilated cardiomyopathy	CTD	Larger oligomers, reduced stability	Increased chaperone activity, higher amyloid formation	(68)
R56Q	Cardiomyopathy	NTD, Mini chaperone	Larger oligomers, reduced stability	Increased chaperone activity, amyloid formation, increased interaction with desmin and aA-Cry	(60)

the insoluble fraction of transfected cells. Based on these findings, Hayes and colleagues suggested that Loss of the CTD increases the propensity of aB-Cry to self-aggregate (70).

The P20S mutation is linked to autosomal dominant posterior polar congenital cataract in a Chinese family. The oligomeric size of this mutant remained unchanged. However, the

mutation significantly reduced chaperone function and subunit exchange of a-Cry oligomers. P20S also enhances nuclear import and induces apoptosis in lens epithelial cells, contributing to cataract development (66). Moreover, the protein aggregation and cell death of several cataract and cardiomyopathy causing mutants were investigated by Raju and Abraham in 2013. In this study, the separately transfected HeLa cells with the D109H, R120G, D140N, and R157H mutants showed considerably more aggregation. Also, transfected cells by the mutant proteins D109H and A171T exhibited a markedly increased level of apoptotic rate. Moreover, in these cases, the apoptosis induction might be considered as the mechanism for the development of cataract and myopathy (62).

In the other studies, the physico-chemical properties of two aB-Cry CTD mutations, G154S and R157H, associated with myopathy, have been examined. R157H displays smaller oligomeric size, whereas G154S has a molecular weight similar to the Wt protein. Both mutations reduce thermal stability. G154S decreases chaperone activity toward substrates such as the S1 fragment of myosin, βL-Cry, and γ-Cry, and shows increased amyloid fibril formation, while R157H maintains or enhances chaperone activity, likely due to its smaller oligomeric size. Both mutants exhibit reduced enzyme refolding and *in vivo* chaperone function. The pathogenicity of G154S is linked to impaired chaperone function, decreased stability, and higher amyloidogenic propensity, potentially disrupting interactions with target proteins. Additionally, CTD mutations can affect IXI motif binding to the ACD; R157H, positioned near this motif, may alter aB-Cry interactions, contributing to disease (53-55) (Table 2).

Asp109 is highly conserved. The D109G mutation increases amyloid aggregation, reduces stability, and may disrupt interactions with partners like desmin, leading to protein aggregates and contributing to restrictive cardiomyopathy and skeletal myopathy (65). The substitution of leucine to proline at position 39 (P39L) in human aB-Cry has been associated with conflicting interpretations of pathogenicity in cataracts and cardiomyopathy. The P39L variant in NTD, resulting from a substitution of C to T at nucleotide position 116 in the human αB-Cry gene, was initially found in an individual with left ventricular non-compaction. P39L alters secondary, tertiary, and quaternary structures, stability, chaperone activity, and aggregation propensity. This mutation increases chaperone activity, potentially affecting apoptosis and contributing to cataract and cardiomyopathy pathogenesis (72).

Desmin, titin, and calcineurin are key cardiac protein targets for aB-Cry, and proper interactions with these proteins are critical for heart function. The R123W mutation has been implicated in cardiomyopathy, likely due to disrupted interaction with calcineurin. Site-directed mutagenesis and recombinant expression studies show that substituting the conserved arginine with tryptophan at position 123 alters the structure of aB-Cry, reduces chaperone activity, modifies oligomerization, and increases aggregation propensity. These structural and functional changes may impair the ability of the mutant protein to interact with target proteins, contributing to cardiac pathology (64). The other mutation, R107L, in aB-Cry occurs in the inter-domain loop connecting β-strands within the immunoglobulin-like fold. The Arg107, together with Asp80, is essential for forming a stabilizing salt bridge and the

protein's dimeric structure. The R107L mutation disrupts these interactions, leading to larger oligomer formation, reduced stability, and diminished chaperone activity, which likely contributes to cataract development (63).
The K90N mutation in aB-Cry replaces a lysine, which can form ionic bonds, with a neutral asparagine, reducing local electrostatic potential and increasing hydrophobicity. The mutation causes notable structural changes, promotes the formation of larger oligomers and amyloid fibrils, and enhances thermal stability. These alterations reduce chaperone activity and impair the protein's ability to prevent aggregation, likely contributing to cell death and myopathy (76). The R11G mutation has also been associated with various pathologies, including cataracts, myofibrillar myopathy, and dilated cardiomyopathy. The R11 residue of aB -Cry, part of the conserved WX(R/K)R sequence in the NTD, plays a key role in the 'mini-chaperone' region essential for chaperone activity. This mutation induces significant structural changes, increasing protein stability and amyloid fibril formation. These alterations may disrupt normal interactions with target proteins in the heart and lens. Additionally, R11G enhances chaperone activity and increases the ability of protein to inhibit cell death under oxidative stress (71).
R157C mutation, near the IXI motif in the CTD, enhances chaperone activity and amyloid fibril formation, possibly impairing interactions with cardiac proteins such as desmin and calcineurin, contributing to cardiomyopathy (56). Furthermore, the R163C mutation in the conserved palindromic sequence of the aB-Cry CTD has been linked to dilated cardiomyopathy. This mutation promotes larger oligomer formation and enhances chaperone activity, which may protect against cell death but could also cause excessive client protein sequestration or coaggregation, leading to cytotoxicity. Additionally, R163C decreases stability and increases amyloid fibril formation, likely to affect interactions with cardiac proteins (68). Besides, R56Q mutation in the mini-chaperone sequence (residues 43-58) alters structure, forms larger oligomers, increases chaperone activity, reduces stability, and promotes amyloid-like aggregation. It binds desmin and aA-Cry more strongly, which may paradoxically disrupt apoptosis and contribute to cardiomyopathy (60).

5) Molecular pathomechanisms of mutations in human aB-Cry proteins

5.1) R69C and D109H recombinant aB-Crys:
The Asp residue at position 109 is considered a hot spot, participating in a network of intra-molecular interactions with other residues (17). D109 is a highly conserved residue that forms an inter-subunit salt bridge with R120 at the dimer interface, playing a significant role in dimer stability (6,77). Dimer formation in aB-Cry is crucial for its chaperone function and consequently prevents aggregation of various client proteins (5,78,79). Moreover, the D109 residue is located adjacent to motifs implicated in microtubule assembly (FISREFHR, 113-120) and in the prevention of protein aggregation (80). Additionally, this residue, positioned within the HGKHEERQDE sequence, displays a significant role in inhibiting amyloid-beta (Ab) fibril formation (81). R69 is located adjacent to a motif important for oligomeric assembly and subunit interactions as a mini-chaperone (residues 73-92), which exhibits anti-aggregation ability (82,83). Therefore, the substitution of R69 with a cysteine residue alters the chaperone function of human aB-Cry. The arginine residue is expected to participate

in salt bridges and hydrogen bonding. Earlier, substituting Arg with Cys (R116C mutation in aA-Cry) was shown to disrupt the salt-bridge and hydrogen bonding, which is considered an important cause of cataract development (46,48). A similar assumption has been proposed regarding the cataractogenic nature of the R69C mutant aB-Cry.

According to recent studies, the R69C and D109H mutations induce structural alteration in human aB-Cry (58). These results were obtained using various spectroscopic assessments, including fluorescence spectroscopies (Contour plots and 3D fluorescence excitation emission spectra), CD, FTIR, and Raman analyses. These results show the β-structure components of the R69C mutant protein shift toward an a-helix and an unordered structures. Moreover, the D109H mutant protein induces secondary and tertiary structural alterations, which are often more pronounced than those observed in the R69C aB-Cry protein (58). The chaperone-like activity of lens a-Cry is highly important to reduce the risk of cataract development. a-Cry exhibits a substrate-specific chaperone-like activity (5,24); therefore, different client proteins are used to assess its chaperoning (anti-aggregation) function. As reported previously, a-Cry is able to refold denatured proteins to their native states and can restore enzyme activity under stress (5,24). Thus, the reactivation and refolding ability of the mutant aB-Cry proteins on thermally inactivated and unfolded a-glucosidase are being assessed. Moreover, the protective effect of mutant forms of aB-Cry *in vivo* was evaluated using cell survival experiments at 50 °C. BL21 (DE3) *E. coli* cells transformed with pET-28b (+) plasmid vector or the recombinant constructs, either carrying Wt or mutant human aB-Cry proteins, were grown separately at 37 °C and 50 °C. The ratio of colony-forming units under heat shock (50 °C) to those under normal conditions (37 °C) was measured, which reflects the cell survival response (52). The R69C mutant protein exhibits reduced anti-aggregation activity against multiple client proteins. The R69C protein shows slightly reduced refolding and reactivation efficacy compared to Wt protein and provides moderate protection of *E. coli* cells under thermal shock (~77% for R69C compared to ~93% for Wt), indicating a functional decline of the mutant protein. The D109H protein shows the weakest chaperone-like activity among the four mutant variants. This mutant protein exhibits weak refolding ability, reduced thermal inactivation, and poor heat-survival protection in cells (~38%, similar to the vector control), compared to Wt, R69C, P20R, and A171T aB-Cry proteins. Dynamic light scattering (DLS) and Small-angle X-ray scattering (SAXS) analyses of oligomeric size reported a major oligomer population larger than that of Wt, with increased Rg and triaxial ellipsoid volume for R69C and D109H mutant proteins (Table 3). These results confirm a significant increase in oligomeric size distribution and polydispersity of human αB-Cry. Studies report that the poor chaperone-like activity of D109H aB-Cry is related to its tendency to form larger oligomers (58). Under thermo-chemical stress, transmission electron microscopy (TEM) analyses show that R69C aB-Cry forms long, branched fibrils, while D109H protein exhibits a strong tendency to form large amyloid plaques. Thus, both R69C and D109H mutations significantly increase the amyloidogenic propensity of human αB-Cry protein (Table 3). Overall, R69C and especially D109H proteins disrupt the aB-Cry protein

Table 3. Comparison of the structural and functional properties of four mutant aB-Cry proteins.

aB-Crys	Domain (important role)	Oligomeric size compared to Wt protein	Chaperone-like activity	Amyloidogenesis
R69C	ACD; interaction motif	↑ ~23.5 nm by DLS; Rg ~7.8 nm)	↓ (Moderate loss; client-dependent); α-Gls refolding similar to Wt	↑↑ (Long, branched fibrils)
D109H	ACD; D109–R120 salt bridge	↑↑↑ ~23.5 nm; Rg ~6.6 nm)	↓↓↓ (Most reduction among ACD mutation pairs); poor *in vivo* protection	↑↑↑ (Long, unbranched plaques)
P20R	NTD; interactive sequence residues 9–20, near 21 SRLFDQFFG29 motif, Ser19	↑↑ (Marked increase)	↓↓↓ (Lost most activity among the NTD/CTE mutation pairs)	↑
A171T	CTE; dimer–dimer interaction	Unchanged/ ↑	↓	↑

structure, increase oligomer size, and diminish chaperone function, which could be considered molecular pathomechanisms in the development of congenital cataract and myopathy (58).

5.2) P20R and A171T mutant aB-Cry proteins:

The P20R mutation occurs at a highly conserved residue in the NTD of human aB-Cry, which participates in oligomer assembly and subunit exchange and is important for the chaperone-like activity (28,84). It has been suggested that, due to their close proximity, mutation of the Pro20 residue may affect phosphorylation of the Ser19 residue and subsequently reduce the anti-apoptosis function of human aB-Cry (61,85). The conserved A171 residue is located in the CTE, which serves an electrostatic solubilizing function (32,86). The autosomal dominant A171T mutation can disrupt dimer–dimer interactions in aB-Cry oligomers, which are important for chaperone activity and anti-aggregation ability (87).

Congenital-cataract mutations P20R and A171T demonstrate significant changes in the amyloidogenic feature, structure, and chaperone-like activity compared to Wt aB-Cry (Table 3). Both substitutions alter secondary structure content and modify oligomeric assembly, as analyzed by spectroscopic techniques, client-anti aggregation suppression, and TEM (59). The P20R or A171T mutant proteins exhibited significantly reduced chaperone-like activity. This reduction in chaperone-like activity was particularly pronounced for the P20R mutant protein, which was explained due its tendency to form larger oligomers. An increased amyloid fibril formation was observed for P20R and A171T mutant proteins. Thus, both mutations increase the propensity for amyloidogenesis under thermochemical stress (analyzed by fluorescence and TEM), consistent with the results observed for R69C and D109H proteins, suggesting a common biochemical mechanism in cataract pathogenesis that leads to protein aggregation and lens opacity (58,59). Some structural and functional properties of human aB-Cry remained relatively unchanged upon the A171T mutation (55). However, cells expressing the A171T mutant protein were more prone to apoptosis compared to those expressing Wt protein, which may contribute to the cataract pathogenesis caused by this mutant variant of human aB-Cry (62). Overall, the structural and functional changes associated with the P20R or A171T mutations explain the participation of these pathogenic mutants in the development of human congenital cataract diseases.

Conclusions

Pathogenic aB-Cry mutations impair protein folding, oligomerization, and chaperone activity, resulting in aggregation, loss of cellular homeostasis, and the onset of cataracts and myopathies. Four pathogenic mutations associated with these disorders induce marked structural and functional changes, including enhanced amyloid fibril formation, weakened chaperone-like activity, and an increased oligomeric size distribution compared with the Wt protein. Among these variants, D109H has the most detrimental impact on human aB-Cry protein structure and function, while A171T exhibits relatively minor effects. These findings demonstrate a direct link between the structural and functional changes caused by these mutations and the development of cataract and myopathy disorders in human patients. Therapeutic strategies aimed at preventing protein aggregation, enhancing chaperone activity, or stabilizing mutant aB-Cry offer promising avenues for intervention. Recombinant protein models provide a powerful platform for mechanistic studies and drug discovery, enabling detailed evaluation of mutant protein behavior and screening of potential therapeutic compounds. Future research, combining site-directed mutagenesis, molecular techniques, and genetic analyses, will further elucidate genotype–phenotype relationships and guide the development of targeted therapies. Overall, the strong correlation between aB-Cry structural perturbations, functional impairment, and disease underscore the importance of mechanistic insights in guiding the design of effective treatments. Future studies could employ *E. coli* expression systems to investigate compound *CRYAB* mutations (e.g., introducing both D109H and A171T mutations simultaneously) to assess their synergistic or counteractive effects on aB-Cry structure and function. Additionally, aB -Cry exhibits an anti-apoptotic role, and transfection of eukaryotic cell lines with mutant variants could be used to evaluate how these mutations alter the protein's protective function against apoptosis. Together, these approaches would provide a more comprehensive understanding of mutation-driven pathogenicity.

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Conflict of interest

The author declares that they have no conflicts of interest with the contents of this article.

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