Biochimie 142 (2017) 168–178

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

Some properties of three αB-crystallin mutants carrying point substitutions in the C-terminal domain and associated with congenital diseases

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ARTICLE INFO

Article history:
Received 22 August 2017
Accepted 12 September 2017
Available online 14 September 2017

Keywords:
αB-Crystallin
Small heat shock proteins
Heterooligomers
Chaperone-like activity
Congenital human diseases

ABSTRACT

Physico-chemical properties of G154S, R157H and A171T mutants of αB-crystallin (HspB5) associated with congenital human diseases including certain myopathies and cataract were investigated. Oligomers formed by G154S and A171T mutants have the size and apparent molecular weight indistinguishable from those of the wild-type HspB5, whereas the size of oligomers formed by R157H mutant is slightly smaller. All mutants are less thermostable and start to aggregate at a lower temperature than the wild-type protein. All mutants effectively interact with a triple phosphomimicking mutant of HspB1 and form large heterooligomeric complexes of similar composition. All mutants interact with HspB6 forming heterooligomeric complexes with size and composition dependent on the molar ratio of two proteins. The wild-type HspB5 and its G154S and A171T mutants form only high molecular weight (300–450 kDa) heterooligomeric complexes with HspB6, whereas the R157H mutant forms both high and low (~120 kDa) molecular weight complexes. The wild-type HspB5 and its G154S and A171T mutants form two types of heterooligomers with HspB4, whereas R157H mutant effectively forms only one type of heterooligomers with HspB4. G154S and A171T mutants have lower chaperone-like activity than the wild-type protein when subfragment S1 of myosin or β_L-crystallin are used as a model substrates. With these substrates, the R157H mutant shows equal or higher chaperone activity than the wild-type HspB5. We hypothesize that the mutations in the C-terminal region modulate the binding of the IP(I/V) motif to the core α-crystallin domain. The R157H mutation is located in the immediate proximity of this motif. Such modulation could cause altered interaction of HspB5 with partners and substrates and eventually lead to pathological processes.

1. Introduction

Small heat shock proteins (sHsp) form a large family of widespread proteins expressed in practically all phyla including viruses, bacteria, fungi, plants and animals [1,2]. These proteins form the first stress defense line protecting the cells against accumulation of improperly or partially unfolded proteins [2]. Monomers of sHsp have a comparatively small molecular weight ranging from 12 up to 42 kDa and a conserved primary structure [3–5]. All sHsps contain an α-crystallin domain (ACD) of about 90 amino acid residues, being a hallmark of this protein family [6]. This domain is flanked by the N-terminal part of variable length and the C-terminal part containing the so-called C-terminal domain and the C-terminal extension [3]. The α-crystallin domain participates in intersubunit interactions, stabilizes the formation of sHsp dimer and is involved in the interaction of sHsp with their protein targets [7]. The C-terminal region is multifunctional and is involved in inter- and intrasubunit interaction as well as in the interaction of sHsp with their targets [8,9]. The N-terminal domain can be up to one-half of the sHsp length and is intrinsically disordered for the most part. This domain is also involved in intra- and intersubunit

Abbreviations: DTT, dithiothreitol; DLS, dynamic light scattering; PMSF, phenylmethane sulfonylfluoride; SDS, sodium dodecyl sulfate; SEC, size exclusion chromatography; sHsp, small heat shock proteins; TPCK, tosyl phenylalanyl chloromethyl ketone.

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http://dx.doi.org/10.1016/j.biochi.2017.09.008
0300-9084/

interactions and further plays an important role in the formation of large sHsp oligomers and their interaction with client proteins [7,10]. Highly flexible structure of the N- and C-terminal regions provides for dynamic formation of homo- and heterooligomers with a variable number and different nature of sHsp subunits [11,12].

The human genome contains 10 genes encoding small heat shock proteins [13,14]. Some of these proteins (e.g. HspB1 (Hsp27), HspB5 (αB-crystallin), HspB6 (Hsp20), HspB8 (Hsp22)) are expressed in practically all human tissues, whereas the other members of sHsp family are tissue-specific [15]. Ubiquitously expressed αB-crystallin (HspB5) is involved in many vital cellular processes. It possesses chaperone-like activity and prevents aggregation of partially unfolded proteins, protects the cell against oxidative stress, stabilizes the cytoskeleton and demonstrates antiapoptotic activity [16,17]. Correspondingly, α-crystallin and its peptides are considered as potential therapeutic agents [18]. Since HspB5 is involved in multiple cellular processes, any changes of its structure can potentially induce detrimental effects. Indeed, about 15 mutations of HspB5 were thus far associated with different human diseases such as congenital cataract, dilated cardiomyopathy and desmin-related myopathy (myofibrillar myopathy) [19–21]. Three of these mutations are localized in the C-terminal region of HspB5. The mutation G154S is associated with rare cases of dilated cardiomyopathy [22] and myofibrillar myopathy [23]. The point mutation R157H is associated with late onset dilated cardiomyopathy [24]. Finally, the point mutation A171T located in the C-terminal extension of HspB5 is associated with lamellar cataract detected in a South-Indian family [25]. As a first step towards understanding the molecular mechanisms of these diseases, here we have investigated the physico-chemical properties of G154S, R157H and A171T mutants of HspB5. The functioning of sHsps is dependent on their stability, oligomeric state, chaperone-like activity and ability to form heterooligomeric complexes with other small heat shock proteins. Correspondingly, here we have investigated these properties of the G154S, R157H and A171T mutants of HspB5, in particular including their interaction with HspB1, HspB4 and HspB6 all of which are natural partners of HspB5 [9,26,27].

2. Materials and methods

2.1. Proteins

Coding sequence of human HspB5 was cloned in the pET23b vector at *Nde*I and *Xho*I sites as described [28]. Constructs encoding G154S, R157H and A171T mutants of HspB5 were prepared in Eurogen (Moscow). Expression construct encoding human HspB4 (αA-crystallin) was kindly provided by Dr. S. Weeks (KU Leuven, Belgium). Coding sequence was confirmed by DNA sequencing. Expression of the wild-type HspB4, HspB5 and its mutants was performed in *Escherichia coli* BL21 by the auto-induction method of Studier [29]. For this purpose, bacteria were grown in 3-fold Luria-Bertani (LB) media for 8 h at 37 °C. Afterwards temperature was decreased to 20 °C and culturing was continued for 16–18 h. Recombinant wild-type HspB5 and its mutants were purified by ammonium sulfate fractionation (0–40% saturation), followed by ion-exchange chromatography on High Trap Q and size-exclusion chromatography on Superdex 200 [28]. Recombinant human HspB6 and the so-called 3D phosphomimicking mutant of HspB1 with residues Ser15, Ser78 and Ser82 replaced by Asp were expressed and purified as described earlier [30]. According to the SDS gel electrophoresis [31] the purity of all proteins exceeded 95%. Bovine lens β_L- and β_H-crystallin were obtained according to Horwitz et al. [32], dialyzed against water and freeze-dried.

2.2. Formation of heterooligomeric complexes

The wild-type HspB5 and its point mutants were mixed with either HspB6 or 3D mutant of HspB1 in buffer B (20 mM Tris/Acetate pH 7.6, 10 mM NaCl, 0.1 mM EDTA, 0.1 mM PMSF, 15 mM β-mercaptoethanol). The total final concentration of sHsp monomers was 210 μM and the molar ratio of HspB5 to two other small heat shock proteins was varied in the range between 1/5 and 5/1. This mixture was incubated either at 4° C (low rate of subunit exchange) or at 42° C (high rate of subunit exchange) for 1 h. Formation of heterooligomeric complexes was analyzed by means of size-exclusion chromatography or native gel electrophoresis.

The wild-type HspB5 and its point mutants were mixed with the wild-type human HspB4 in buffer C (20 mM PIPES/NaOH pH 6.8, 15 mM β-mercaptoethanol, 0.1 mM PMSF) at the final concentration of each protein equal to 50 μM. After incubation for 1 h either at 4 or 42 °C formation of heterooligomeric complexes was analyzed by ion-exchange chromatography.

2.3. Size-exclusion chromatography

Oligomeric structure of homo- and heterooligomers of HspB5 and its mutants was analyzed by size-exclusion chromatography on Superdex 200 HR 10/30 column equilibrated with buffer S (20 mM Tris/Acetate pH 7.6, 150 mM NaCl, 0.1 mM EDTA, 0.1 mM PMSF, 15 mM β-mercaptoethanol). The protein sample (150 μl) was loaded on the column and eluted with the rate of 0.5 ml/min. The following standards were used for calibration: thyroglobulin (669 kDa), ferritin (440 kDa), rabbit skeletal muscle glyceraldehyde phosphate dehydrogenase (144 kDa), bovine serum albumin (68 kDa) and ovalbumin (43 kDa).

2.4. Native gel electrophoresis

Gradient (5–15%) polyacrylamide gel electrophoresis performed by the method of Davis [33] was used for the analysis of different mixtures of sHsps. Isolated proteins or their mixtures preincubated at low (4 °C) or high (42 °C) temperatures were run on a gradient gel as described earlier [34]. The running buffer had a pH of 8.8. Under these conditions, all sHsps studied were migrating towards the positive pole.

2.5. Ion-exchange chromatography of HspB5 and its heterooligomeric complexes with HspB4

Formation of heterooligomeric complexes of HspB5 and its mutants with HspB4 was analyzed by means of ion-exchange chromatography [35]. Isolated HspB4, HspB5 or their mixtures (100 μl) preincubated either at 4 or 42° C were loaded on 1 ml DEAE-HiTrap column equilibrated by buffer C (20 mM PIPES/NaOH pH 6.8, 15 mM β-mercaptoethanol, 0.1 mM PMSF). The column was washed by 5 column volumes of buffer C and eluted by 20 column volumes of linear gradient of buffer C containing 0.5 M NaCl. Under the conditions used, the isolated HspB5 (theoretical pI = 6.76) was not binding to the column, whereas HspB4 (theoretical pI = 5.77) was retained on the column and could be eluted by increasing salt concentration. Heterooligomeric complexes formed by HspB4 and HspB5 are expected to have intermediate pI values and therefore their chromatographic properties should differ from the corresponding properties of isolated proteins. Peak fractions (400 μl each) were collected, combined, dialyzed against water and freeze-dried. Their protein composition was analyzed by urea-gel electrophoresis performed in Davis [33] or Perrie and Perry [36] system on 10% polyacrylamide gel containing 8 M urea in separation gels.