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Research paper

Physico-chemical properties of R140G and K141Q mutants of human small heat shock protein HspB1 associated with hereditary peripheral neuropathies

Victoria V. Nefedova [a], Petr N. Datskevich [a], Maria V. Sudnitsyna [a], Sergei V. Strelkov [b], Nikolai B. Gusev [a,*]

[a] Department of Biochemistry, School of Biology, Moscow State University, Moscow 119991, Russian Federation
[b] Laboratory for Biocrystallography, Department of Pharmaceutical and Pharmacological Sciences, KU Leuven, Belgium

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ABSTRACT

Some physico-chemical properties of R140G and K141Q mutants of human small heat shock protein HspB1 associated with hereditary peripheral neuropathy were analyzed. Mutation K141Q did not affect intrinsic Trp fluorescence and interaction with hydrophobic probe bis-ANS, whereas mutation R140G decreased both intrinsic fluorescence and fluorescence of bis-ANS bound to HspB1. Both mutations decreased thermal stability of HspB1. Mutation R140G increased, whereas mutation K141Q decreased the rate of trypsinolysis of the central part (residues 5–188) of HspB1. Both the wild type HspB1 and its K141Q mutant formed large oligomers with apparent molecular weight ~560 kDa. The R140G mutant formed two types of oligomers, i.e. large oligomers tending to aggregate and small oligomers with apparent molecular weight ~70 kDa. The wild type HspB1 formed mixed homooligomers with R140G mutant with apparent molecular weight ~610 kDa. The R140G mutant was unable to form high molecular weight heterooligomers with HspB6, whereas the K141Q mutant formed two types of heterooligomers with HspB6. *In vitro* measured chaperone-like activity of the wild type HspB1 was comparable with that of K141Q mutant and was much higher than that of R140G mutant. Mutations of homologous hot-spot Arg (R140G of HspB1 and R120G of αB-crystallin) induced similar changes in the properties of two small heat shock proteins, whereas mutations of two neighboring residues (R140 and K141) induced different changes in the properties of HspB1.

1. Introduction

Small heat shock proteins (sHsp) comprise a large family of proteins ubiquitously expressed in different human tissues. Ten members of sHsp family (HspB1–HspB10) are encoded in human genome [1,2]. All these proteins have small molecular weight of monomers (17.0–28.4 kDa), contain conservative α-crystallin domain of ~90 residues, variable N- and C-terminal domains, and tend to form homo- and heterooligomers with highly dynamic quaternary structure [3,4]. sHsp play important house-keeping role protecting the cell against different unfavorable conditions [5]. For instance, sHsp are involved in regulation of redox state [6] and protection of cytoskeleton [7]. sHsp possess chaperone-like activity and protect the cell against accumulation of protein aggregates [8] and participate in regulation of apoptosis [9]. Since sHsp are involved in many intracellular processes, their mutations correlate with development of different congenital diseases such as cataract, dilated cardiomyopathy, different forms of motor neuropathies and myofibrillar myopathy [8,10–12].

At present about twenty mutations of HspB1 are described in the literature [8,13]. However, only few of these mutations are thoroughly investigated [14–16]. In order to understand the molecular mechanisms of congenital diseases associated with HspB1 mutations it is desirable to compare the structure and properties of mutated and wild type proteins. This paper deals with investigation of some physico-chemical properties of two mutants of HspB1 (R140G and K141Q) associated with distal hereditary motor neuropathy and/or Charcot–Marie–Tooth disease type 2 [17,18].

Abbreviations: ACD, α-crystallin domain; AUC, analytical ultracentrifugation; DLS, dynamic light scattering; DTT, dithiothreitol; ME, β-mercaptoethanol; PMSF, phenylmethanesulfonyl fluoride; SEC, size-exclusion chromatography; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone; WT, wild type.

* Corresponding author. Tel./fax: +7 495 939 2747.
E-mail address: NBGusev@mail.ru (N.B. Gusev).

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2. Materials and methods

2.1. Cloning and expression of human small heat shock proteins

cDNA of the wild type HspB1 and HspB6 in pET23b was obtained as described earlier [19]. The following forward (fw) (5′-tctcccgg tgcttcacgggcaaa −3′) and reverse (rev) (5′- atttgcccgtgaagcaccggga-3′) mutant primers were used for obtaining R140G mutant of HspB1. K141Q mutant of HspB1 was obtained by using 5′-cccggtgcttcac gcggcaatac-3′ forward and 5′-tgtattgccgcgtgaagcaccgg-3′ reverse primers (triplets corresponding to mutated residues are underlined). In the first round of PCR we obtained two fragments by using two pairs of primers, for instance wild type HspB1 fw and mutant HspB1-K141Q rev and mutant HspB1-K141Q fw and wild type HspB1 rev primers. In the second round of PCR two beforehand purified fragments were annealed by using wild type HspB1 fw and wild type HspB1 rev primers. The construct obtained was cloned into pET23b vector at NdeI and XhoI sites and integrity of sHsp coding sequence was confirmed by DNA sequencing. cDNA of the so-called Cys-mutant of HspB6 with double mutation C46S/E116C was obtained as described earlier [20].

2.2. Expression and purification of recombinant human sHsp

Recombinant sHsp were expressed in *Escherichia coli* BL21 pLysS. Bacteria were grown on standard Luria–Bertani (LB) media containing ampicillin (0.1 g/l) at 37 °C up to the optical density 0.6 at 600 nm. Expression was induced by addition of 0.5 mM IPTG and lasted for 3 h. The cells were collected by centrifugation and stored at −20 °C. Bacterial cells were sonicated and the soluble fraction obtained after centrifugation was subjected to ammonium sulfate fractionation (0–40% saturation), ion exchange chromatography on High TrapQ column, followed by size-exclusion chromatography on Superdex 200 HiLoad 26/60 column [19]. Thus obtained protein samples were concentrated, dialyzed and stored in buffer B (20 mM Tris–acetate pH 7.6, 10 mM NaCl, 0.1 mM EDTA, 0.1 mM PMSF, 1 mM dithiothreitol (DTT)) at −20 °C. According to the SDS-gel electrophoresis [21] the purity of proteins was nor less than 95%. Four different preparations of the wild type HspB1, three preparations of HspB6 and two preparations of each of R140G and K141Q mutants were used in the course of this investigation. No significant differences were observed between different batches of the same proteins.

2.3. Fluorescence measurements

All measurements were performed on Cary Eclipse spectrofluorometer (Varian). Bis-ANS titration and measurement of intrinsic Trp fluorescence was performed at 25 °C in buffer F containing 50 mM sodium phosphate (pH 7.5), 150 mM NaCl and 15 mM β-mercaptoethanol (ME). Trp fluorescence was exited at 295 nm (slit width 5 nm) and recorded in the range 300–400 nm (slit width 5 nm). Interaction of the wild type HspB1 and its mutants with hydrophobic fluorescent probe bis-ANS was followed by titration of protein solution (0.05 mg/ml) by stock solution of bis-ANS (170–220 μM) in buffer F. Fluorescence was excited at 395 (slit width 5 nm) and recorded at 495 nm (slit width 5 nm).

Thermally induced conformation changes of HspB1 were followed by recording Trp fluorescence at different temperatures. The protein samples (0.05–0.10 mg/ml) in buffer FF (20 mM HEPES/NaOH pH 7.5, 100 mM NaCl, 2 mM DTT) were heated with the constant rate 1 °C/min from 20 up to 80 °C and cooled back with the same rate using automatic Peltier cell holder of Cary Eclipse spectrofluorometer. Fluorescence was excited at 295 nm and recorded at 320 and 360 nm (slits 5 nm). As it was shown earlier [22], before and after thermal transition the temperature dependence of reciprocal fluorescence at fixed wavelength is described by linear equation $1/I_T = a + b^*T/\eta$, where I is fluorescence intensity, T is absolute temperature, η is solution viscosity (in cP) and a and b are constants. In the range of thermal transition $I = (1 - \alpha)^*I_n + \alpha^*I_d$, where I_n and I_d are intensities of fluorescence of native and denatured protein and α is fraction of conversion from native to denatured state. I_n and I_d were determined from the linear portion of $1/I$ against T/η plot. Dependence of α on temperature provides information of thermal stability of HspB1 and its mutants.

2.4. Limited trypsinolysis of HspB1 and its mutants

Limited proteolysis of HspB1 (1 mg/ml) was performed at 20 °C in buffer B at a weight ratio HspB1/TPCK-trypsin varying in the range 3000/1–8000/1. After different time of incubation reaction was stopped by addition of PMSF up to the final concentration 5 mM and thus obtained samples were subjected to SDS-gel electrophoresis [21]. The composition of certain peptides obtained after limited proteolysis was determined by tandem mass-spectroscopy performed on Ultraflex Extreme BRUKER mass spectrometer.

2.5. Size-exclusion chromatography (SEC)

The sample (150 μl) containing different quantities of HspB1 (or its mutants) or the mixture of HspB1 and HspB6 in buffer B, containing 150 mM NaCl was loaded on Superdex 200 HR10/30 column equilibrated with buffer B, containing 150 mM NaCl. The column was run at the flow rate 0.5 ml/min and fractions (400 μl) were collected and analyzed by SDS-gel electrophoresis [21]. The column was calibrated with the following protein markers: thyroglobulin (669 kDa), ferritin (440 kDa), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (144 kDa), bovine serum albumin (68 kDa) and ovalbumin (43 kDa).

2.6. Dynamic light scattering (DLS)

Dynamic light scattering was measured at 25 °C on Zetasizer Nano (Malvern) at two protein concentrations (0.1 and 0.3 mg/ml) in buffer D (20 mM Tris/acetate pH 7.6, 150 mM NaCl, 0.1 mM EDTA, 0,1 PMSF, 15 mM ME). Each measurement lasted for 15 s and was repeated 10 times. Ten cycles of these measurements were repeated and thus 100 measurements were performed for each sample. The data presented were obtained from both intensity and number distributions.

2.7. Analytical ultracentrifugation

Sedimentation velocity experiments were performed in Spinco model E analytical ultracentrifuge at 20 °C in a 6-hole AnJ-Ti rotor and 12 mm double sector cells. Absorbance optics, a photoelectric scanner and computer on-line were used to record absorbance at 280 nm and all cells were scanned simultaneously with 1.5 min interval. The proteins were dialyzed overnight against 50 mM phosphate (pH 7.5) containing 150 mM NaCl and 2 mM DTT. The samples obtained after dialysis were centrifuged for 10 min at 12.000 *g*. Thus obtained protein samples at two different concentrations (0.2 and 0.4 mg/ml) were subjected to ultracentrifugation at 44,000 rpm. The sedimentation coefficients were estimated from differential distribution of sedimentation coefficient [$c(s, f/f_0)$ versus s] using SEDFIT program [23].

2.8. Formation of homo- and heterooligomeric complexes of HspB1 and HspB6

The samples of the wild type HspB1 (or its mutants), wild type HspB6 or Cys-mutant of HspB6 (C46S/E116C) (each 1 mg/ml in

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