Molecular Biology Reports **18**: 209–215, 1993.

Comparison of the homologous carboxy-terminal domain and tail of α-crystallin and small heat shock protein

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Received 15 April 1993; accepted 19 May 1993

Key words: eye lens; molecular chaperone; mouse HSP25; α-crystallin; stress proteins

Abstract

The C-terminal domain and tail, which is the most conserved region of the α-crystallin/small heat shock protein (HSP) family, was obtained from rat αA-crystallin, bovine αB-crystallin and mouse HSP25. All three domains have primarily β-sheet conformation and less than 10% of α-helix, like the proteins from which they are derived. Whereas the C-terminal part of αA-crystallin forms dimers or tetramers, the corresponding regions of αB-crystallin and HSP25 form larger aggregates. The heat-protective activity, recently described for the α-crystallin/small HSP family, is not retained in the C-terminal domain and tail. In the course of this study some differences with the previously published sequence of HSP25 were observed, and a revision is proposed.

Abbreviations: αA2Dt, residues 64–173 of rat α-crystallin; αB2Dt, residues 70–175 of bovine αB-crystallin; bp, base pair; HSP2Dt, residues 92–209 of HSP25; HSP(s), heat shock protein(s); HSP25, mouse small HSP; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl chloride; SDS, sodium dodecyl sulfate; polyacrylamide; WSF, water-soluble fraction

Introduction

The α-crystallins are evolutionarily, structurally and functionally related with the ubiquitous small heat shock proteins (HSPs) [1, 2]. Both α-crystallin and the small HSPs have recently been shown to have the properties of a molecular chaperone [3–5]. Both proteins form large aggregates, which is obviously related to their common functional features. However, no conclusive data about their quaternary structure is yet available. Different models for the arrangement of subunits in α-crystallin have been proposed, ranging from a multi-layer to a micellar arrangement of subunits [6–9]. A way to get more information about the quaternary structure is to find the regions that are involved in intersubunit interactions. The α-crystallin and small HSP subunits are probably composed of two compact structural domains

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and an extending C-terminal tail [10]. It has been proposed that the C-terminal domain, which is the best conserved sequence within this protein family, is the 'aggregation domain' [1]. We previously showed that the C-terminal domain and tail of αA-crystallin forms dimers or tetramers, whereas the N-terminal domain aggregates into less defined large complexes [11]. Based on this finding, a new model for the quaternary structure of α-crystallin and the small HSPs, has been proposed, in which a rhombic dodecahedric arrangement of tetrameric building blocks occurs [12].

We now address the question as to whether also the corresponding C-terminal sequences of αB-crystallin and a small HSP form tetramers. Therefore we produced the C-terminal sequences of bovine αB-crystallin and mouse HSP25, and analyzed their quaternary structures by gel permeation chromatography and crosslinking. Far-UV circular dichroism spectroscopy was used to check the presence of regularly folded structure, and to find out whether the secondary structure of the C-terminal domains and tails is conserved. We further investigated whether the heat-protective capacity of the α-crystallin subunits and HSP25 is associated with the homologous C-terminal sequences.

Materials and methods

Bacterial strains and plasmids

Escherichia coli strains HB101 [13] and TG1 [14] were used as recipients for the constructs pAK3038p25 [15] and pET3bHSP2Dt, respectively. For overproduction of HSP2Dt *E. coli* strain B BL21(DE3)pLysS [16] was used. The construction of pAK3038p25 has been described earlier [15], and pET3bαA2Dt has previously been prepared in our laboratory [11].

Cloning procedures

A DNA fragment encoding the C-terminal domain and tail of HSP25 was produced by the polymerase chain reaction and inserted into the expression plasmid pET3b. Plasmid DNA (pET3bHSP2Dt) was sequenced by the dideoxynucleotide method [17] and transformed in BL21(DE3)pLysS cells.

Expression and purification of proteins

αB-Crystallin and βL-crystallin were obtained from calf lenses as described [18]. Recombinant bovine αA-crystallin and mouse HSP25 were isolated according to Merck *et al.* [4] and Gaestel *et al.* [19], respectively. To be able to distinguish between the C-terminal domain with and without the extending tail, we will further refer to the former as 2Dt and to the latter as 2D. αA2Dt (previously αA2D [11]) was expressed in *E. coli*. A water-soluble fraction of the *E. coli* lysate was obtained as described [11]. αA2Dt was partially purified from the WSF by Bio-Gel hydroxylapatite chromatography (Bio-Rad). Proteins of the WSF were bound to hydroxylapatite in 1 mM of sodium phosphate (pH 6.8) and eluted with a linear gradient of sodium phosphate (1.4 mM/min). αA2Dt was further purified by reversed phase HPLC, using an RP-4 column (Selectosil, 5C4, 300 Å in a gradient of acetonitrile in 0.1% TFA, at a flow rate of 0.8 ml/min. HSP2Dt was directly purified from the WSF of the bacterial strain overexpressing HSP2Dt by RP-4 chromatography. αB2Dt was obtained by cyanogen bromide cleavage of αB-crystallin according to standard procedures [18] and isolated by RP-4 chromatography. Up to 10 mg of protein could be loaded on the column and a maximum of 3 mg of pure protein (αB2Dt) was obtained per run. A maximum of 10 mg of pure αA2Dt and HSP2Dt could be obtained from a 500 ml *E. coli* culture. All solutions and buffers, including dialysis water, contained protease inhibitors (20 μM PMSF, 100 μM EDTA, 40 μg/l bacitracin and 30 μg/l benzamidin).

Far-UV CD-spectroscopy

Far-UV CD-measurements were carried out as described before [4]. The samples were dissolved

in 50 mM sodium phosphate buffer containing 0.1 M NaCl, pH 7.0. Protein concentrations were determined by amino acid analysis.

Miscellaneous methods

Before gel permeation chromatography, cross-linking and far-UV CD- and heat-protection measurements, protein samples were denatured and refolded, as described before [4]. The N-terminal amino acid sequence of HSP2Dt was determined on an Applied Biosystems Model 470A Protein Sequencer, equipped on-line with a Model 120A PTH Analyser. Gel permeation analysis was carried out on a Superose 6 HR 10/30 (Pharmacia-LKB) column [11]. Crosslinking was performed with 25 mM of dimethyl suberimidate, as described earlier [11]. All samples used for the heat protection experiments were dissolved in 50 mM sodium phosphate buffer, pH 7.0 containing 0.1 M NaCl. They were then centrifuged at 20000 rpm for 20 min. Heat protection was measured as described before [3].

Results

To compare the properties of the C-terminal domain with extending tail (abbreviated as 2Dt) of αA-crystallin, αB-crystallin and mouse HSP25 we could use previously described procedures to produce αA2Dt and αB2Dt, but had to construct first an expression plasmid for HSP2Dt. The respective sequence of the three proteins are aligned in Fig. 1.

Fig. 2 summarizes the strategy chosen for the creation of a clone capable of directing the synthesis of the C-terminal domain and tail of HSP25. The corresponding DNA fragment, obtained by PCR, was cloned into the pET3b expression vector and transformed. DNA-sequencing revealed differences with the expected sequence of HSP2Dt [15]. The inferred differences corresponded with 3 amino acid substitutions and the insertion of one amino acid (see Fig. 1). Protein sequencing confirmed that the amino acid sequence corresponded with the nucleotide sequence. The corrected amino acid sequence of HSP25 better corresponds with that

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                             *
                * 10        20        30     *  40
HSP2Dt    MIRQTADRWRVSLDVNHFAPEELTVKTKEGVVEITGKHEERQ
 αA2Dt    MVRSDRDKFVIFLDVKHFSPEDLTVKVLEDFVEIHGKHNERQ
 αB2Dt      RLEKDRFSVNLDVKHFSPEELKVKVLGDVIEVHGKHEERQ
                *            *               *

                             *
             *     50        60        70    *   80
HSP2Dt       DEHGYISRCFTRKYTLPPGVDPTLVSSSLSPEGTLTVEA
 αA2Dt       DDHGYISREFHRRYRLPSNVDQSALSCSLSADGMLTFSG
 αB2Dt       DEHGFISREFHRKYRIPADVDPLAITSSLSSDGVLTVNG
             *               *               *

                 90        100       110       120
HSP2Dt    PLPKAVTQS--AEITIPVTFEARAQIGGPEAGKSEQSGAK
 αA2Dt    PKVQSGLDAGHSERAIPVSREEKPSSAPSS
 αB2Dt    PRKQASG----PERTIPITREEKPAVTAAPKK
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Fig. 1. Alignment of the homologous C-terminal domains and tails of the mouse small heat shock protein, rat αA-crystallin and bovine αB-crystallin. The aligned sequences are displayed in three blocks, the first two corresponding with the two putative motifs in the C-terminal domain of α-crystallin, and the last one representing the C-terminal extension, as proposed by Wistow [10]. Lysine residues are printed in bold-type. The conserved aspartyl, prolyl and glycyl residues are indicated by asterisks. The very flexible C-terminal tail of αA- and αB-crystallin [25] is represented by the wavy line. The original sequence of HSP2Dt [15] was corrected as follows: 8(del→R), 18(V→F), 19(I→A) 93(T→A), and are underlined. The same corrections of the murine HSP25 sequence have independently been deduced from the genomic sequence [26].

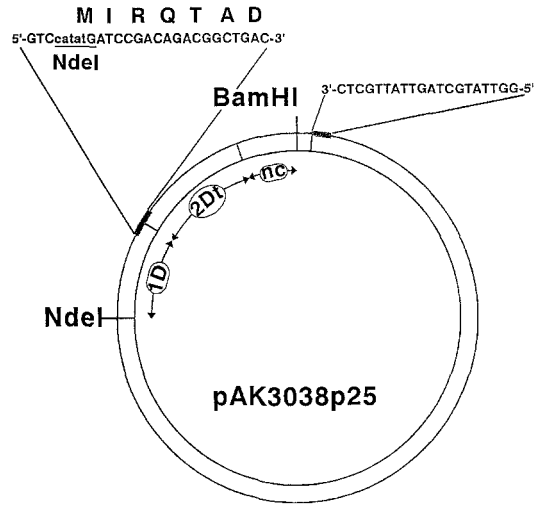

Fig. 2. Construction of the HSP2Dt expression vector. The clone pAK3038p25, containing the full-length cDNA of mouse HSP25 [15] was used to amplify by PCR the DNA fragment encoding HSP2Dt. This DNA fragment starts at nucleotide 364 of the cDNA p25, corresponding with Ile-92 of mouse HSP25 [15], and comprises the 3′ sequence of the p25 cDNA, including the entire 3′ noncoding region as well as 69 nucleotides downstream from the BamHI cloning site of pAK3038p25 [15]. The used primers are shown. The 5′-primer contained mismatches, indicated with lower case letters, to introduce an NdeI restriction site and a translation initiation codon. The resulting DNA fragment of 604 bp was cleaved with NdeI and BamHI and cloned in the NdeI/BamHI-cleaved pET3b vector to yield the pET3bHSP2Dt construct. 1D, 2Dt and nc are the nucleotide sequences corresponding with the N-terminal domain, C-terminal domain and tail, and the 3′ non-coding region, respectively.

of chinese hamster HSP27 [20] and human HSP27 [21, 22].

αA2Dt and HSP2Dt were expressed in *E. coli* and purified (Materials and methods (Fig. 3A). By virtue of the fact that a single internal methionine residue is present at position 67 in bovine αB-crystallin, precisely preceding the C-terminal domain, αB2Dt could simply be obtained by cleavage with cyanogen bromide (Fig. 3A, lane 8) and purification over an RP-4 column.

To investigate whether the conserved C-terminal sequence of αB-crystallin and HSP25 forms small oligomers, like αA2Dt [11], we subjected the purified and reaggregated polypeptides to gel permeation chromatography (Fig. 4). In contrast to α2Dt [11] both αB2Dt and HSP2Dt form large aggregates, of approximately 235 kDa (18–20 subunits) and 170 kDa (12–13 subunits), respectively. However, the elution profile of αB2Dt is very asymmetric, indicating that αB2Dt is a heterogeneous population of aggregates, with the largest aggregate consisting of 18–20 subunits. To further analyze the quaternary structure, the polypeptides were crosslinked by 25 mM dimethyl suberimidate. In the case of αB2Dt more than 7 crosslinking products can be distinguished (Fig. 3B). However, in the case of HSP2Dt, predominantly dimers are formed, and only a faint tetramer band can be observed.

All three domains are perfectly water-soluble, indicating that no improper interactions between secondary structure elements, leading to aspecific aggregation, take place. Far-UV CD-measurements were performed, to provide additional evidence that the domains are properly folded, and that they contain the same secondary structure elements as α-crystallin and HSP25, which are known to have predominantly β-sheet structure and at most 10% of α-helix [4]. In Fig. 5, the normalized far-UV CD-spectra are shown. The curves of all three domains are shaped like the proteins from which they are derived. All proteins and protein fragments can be estimated to contain approximately 10% of α-helix. This convincingly shows that, although the domains have different tendencies for oligomerization, their secondary structures are similar and intact.

It has recently been discovered that both α-crystallin and HSP25 can function as molecular chaperones [3–5]. To investigate whether this function can be assigned to the conserved C-terminal domain and tail of these proteins, a heat protection experiment was carried out. βL-crystallin was heated in the presence of the C-terminal sequences of αA, αB and HSP25. Heat denaturation of βL-crystallin was monitored spectrophotometrically. It turned out that none of the domains is capable of protecting βL-crystallin against heat denaturation (results

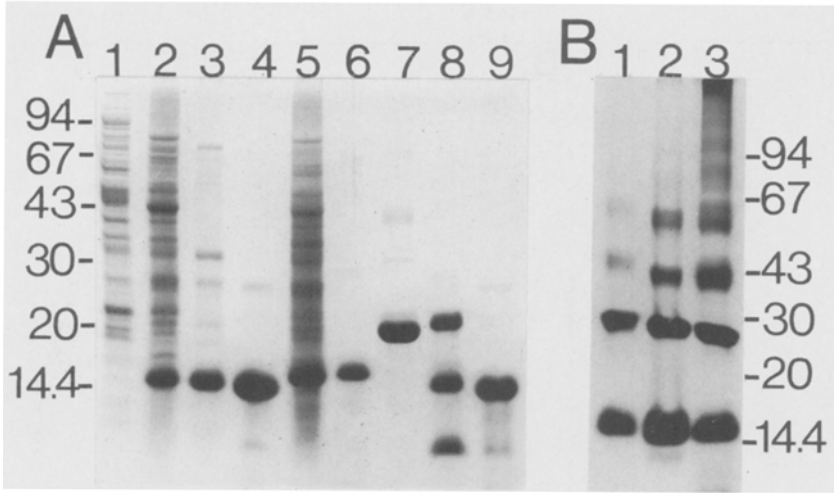

Fig. 3. Purification and crosslinking of αA2Dt, αB2Dt and HSP2Dt. Panel A: Tricine SDS-PAGE pattern of samples taken during the purification procedure of αA2Dt, αB2Dt and HSP2Dt. Lane 1, total protein of *E. coli* BL21(DE3).pLysS, containing plasmid pET3bαA2Dt, without induction. Lane 2, WSF of this strain after induction. Lane 3, αA2Dt after hydroxylapatite chromatography of this WSF. Lane 4, αA2Dt after subsequent RP-4 HPLC. Lane 5, WSF of induced *E. coli* BL21(DE3).pLysS, containing plasmid pET3bHSP2Dt. Lane 6, HSP2Dt after RP-4 HPLC of the WSF of this strain. Lane 7, calf αB-crystallin. Lane 8, αB-crystallin after cleavage with cyanogen bromide, resulting in a mixture of three polypeptides: uncleaved αB-crystallin, residues 2–67 and residues 68–175 (αB2Dt). Lane 9, pure αB2Dt after RP-4 chromatography of cyanogen bromide cleaved αB-crystallin. Panel B: Tricine SDS-PAGE pattern of crosslinked samples of HSP2Dt (lane 1), αA2Dt (lane 2) and αB2Dt (lane 3). SDS-PAGE molecular mass markers are indicated in kDa.

not shown), despite the fact that they are apparently properly folded.

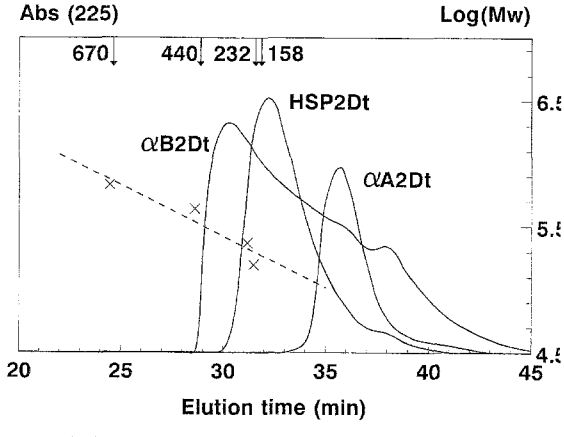

Fig. 4. Gel permeation chromatography of αA2Dt, αB2Dt and HSP2Dt. Elution profiles of 25 μg of αB2Dt and HSP2Dt after Superose 6B chromatography, compared with that of αA2Dt. Elution positions of gel filtration markers are indicated at the top of the figure. Molecular masses are given in kDa. The broken line indicates the calibration line (elution time vs. log(Mw)).

Discussion

By far-UV CD-analysis of αA2Dt, αB2Dt and HSP2Dt, we have demonstrated that the three C-terminal regions are structurally well organized, being predominantly in β-sheet conformation. At most 10% of α-helix is present. The same distribution of secondary structure elements is found in the proteins from which these C-terminal fragments are derived. This is in agreement with the proposal that the α/HSP subunits are composed of two domains with similar structural motifs [10]. The CD-spectra thus indicate that the C-terminal domains and tails are properly folded and can exist as independent structural units. These units are likely to correspond with genuine domains, since smaller fragments of β-sheet proteins are relatively devoid of secondary structure [23]. The fact that the CD-spectra of all protein fragments resemble each other suggests that the secondary structure, as could be expected, has evolutionarily been conserved.

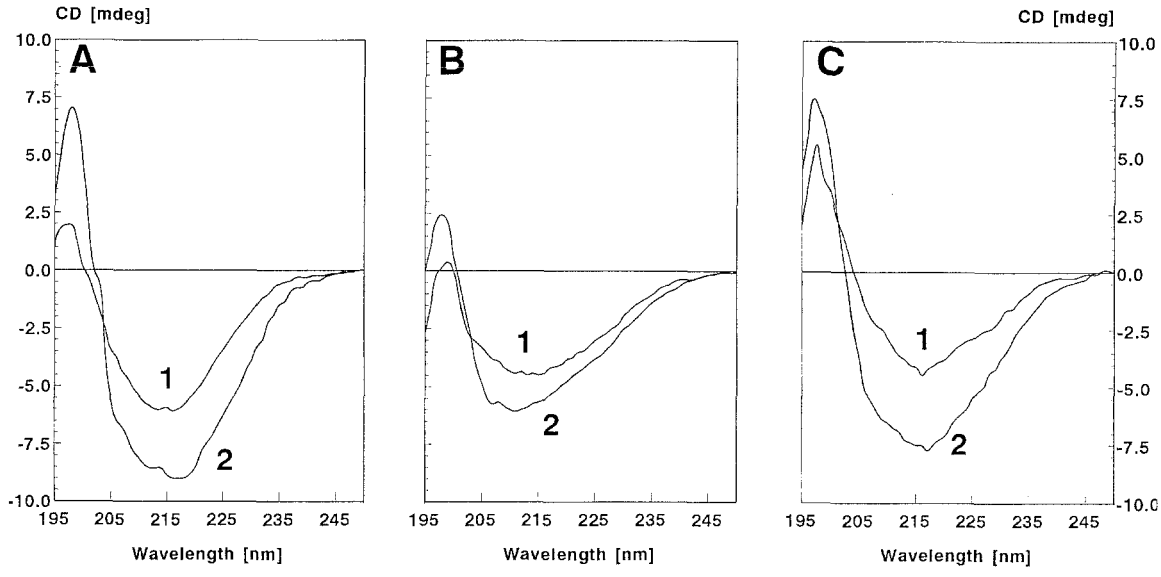

Fig. 5. Far-UV CD-spectra of αA2Dt, αB2Dt and HSP2Dt. Panel A compares the CD-spectra of αA2Dt (curve 1) and recombinant αA-crystallin (curve 2); panel B shows the CD-spectra of αB2Dt (curve 1) and calf αB-crystallin (curve 2); panel C compares the CD-spectra of HSP2Dt (curve 1) and recombinant HSP25 (curve 2). Cuvet path length is 0.5 mm in all cases. The spectra, averages of 8 scans, are normalized to a concentration of 25 μM.

The conservation of the interactions between these protein fragments, however, is not obvious. Earlier, we showed that αA2Dt forms dimers or tetramers [11]. Gel permeation analysis of αB2Dt and HSP2Dt now revealed that these two domains form larger aggregates. αB2Dt is easily crosslinked to high crosslinking products, whereas HSP2Dt forms only dimers or tetramers. The different location of lysines in the three proteins may account for differences in crosslinking (Fig. 1). The fact that lysines are present at positions 26, 37, 55 and 84/85 and at the very C-terminal end in both HSP2Dt and αB2Dt, indicates that the regions, around these lysines are not involved in contacts between tetramers. One could rather expect Lys-6 and -16 to be responsible for the more extensive crosslinking in αB2Dt. The absence of these lysines in HSP2Dt then explains that HSP2Dt forms larger aggregates, but cannot be crosslinked to oligomers larger than dimers or tetramers.

Wherever the positions of the interactive surfaces may be, the present results can be taken to fit the rhombic dodecahedric model [12]. In this model, which proposes that α-crystallin and small HSP aggregates are made up of tetrameric building blocks, the interactions between subunits within a tetramer differ from those between tetramers. The fact that HSP2Dt can be crosslinked to tetramers, while forming *per se* large aggregates, is indeed an indication that at least two types of contact exist and that tetramers might be the basic building block. In this model it is also proposed that α-crystallin and small HSP aggregates may have no single quaternary structure, but at least one stable structure, the rhombic dodecahedron. The fact that αB2Dt apparently forms a heterogeneous population of aggregates (Fig. 4) may in fact reflect that α-crystallin and small HSP aggregates are dynamic structures, with several possibilities for oligomerization.

Recently, it has been discovered that both α-crystallin and HSP25 have *in vitro* chaperone activity [3–5]. Since the C-terminal domain is the essential conserved feature of this family [2], it is likely that the chaperone properties of these proteins are primarily associated with this region. Also the fact that the C-terminal domain with tail can form independent orderly structures, and that this region is probably located at the exterior

of native the aggregates [12], made it relevant to test whether these structures exhibit chaperone activity. Moreover, also in the case of HSP70 the protein-binding activity is associated with a separate domain of the protein [24]. However, it turned out that none of the three protein fragments revealed protective capacity. Apparently, other or additional parts of the proteins are required for heat protection activity. It is yet unknown which molecules are chaperoned by α-crystallin and the small HSPs *in vivo* and which protein regions are involved in interactions between these molecular chaperones and their substrates. A more detailed study in which the α-crystallin subunits and HSP25 are less drastically changed, may give information about the elements necessary for aggregation, substrate binding and chaperone activity.

Acknowledgements

This investigation was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for Scientific Research (NWO). We thank Willeke de Haard-Hoekman for technical assistance. Amino acid sequences were determined at the SON Gas-Phase Sequenator Facility (State University of Leiden).

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