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Identification of a region in alcohol dehydrogenase that binds to α-crystallin during chaperone action

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Abstract

α-Crystallin, the major eye lens protein and a member of the small heat-shock protein family, has been shown to protect the aggregation of several proteins and enzymes under denaturing conditions. The region(s) in the denaturing proteins that interact with α-crystallin during chaperone action has not been identified. Determination of these sites would explain the wide chaperoning action (promiscuity) of α-crystallin. In the present study, using two different methods, we have identified a sequence in yeast alcohol dehydrogenase (ADH) that binds to α-crystallin during chaperone-like action. The first method involved the incubation of α-crystallin with ADH peptides at 48 °C for 1 h followed by separation and analysis of bound peptides. In the second method, α-crystallin was first derivatized with a photoactive trifunctional cross-linker, sulfosuccinimidyl-2[6-(biotinamido)-2-(*p*-azidobenzamido)-hexanoamido]ethyl-1,3di-thiopropionate (sulfo-SBED), and then complexed with ADH at 48 °C for 1 h in the dark. The complex was photolyzed and digested with protease, and the biotinylated peptide fragments were isolated using an avidin column and then analyzed. The amino acid sequencing and mass spectral analysis revealed the sequence YSGVCHTDLHAWHGDWPLPVK (yeast ADH_{40-60}) as the α-crystallin binding site in ADH. The interaction was further confirmed by demonstrating complex formation between α-crystallin and a synthetic peptide representing the binding site of ADH. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: α-Crystallin; Alcohol dehydrogenase; Binding site; Chaperone; Cross-linker

1. Introduction

The failure of proteins to fold correctly leads to aggregation and severe functional deficit [1–3]. Chaperones either assist protein folding or maintain proteins in an unfolded state and provide considerable protection against diseases that are caused by protein aggregation [2–6]. Chaperone action involves interaction and protein–macromolecule complex formation [7–9]. α-Crystallin, the major eye lens protein, is known to suppress the aggregation of β-, γ-crystallins and a wide variety of enzymes under denaturing conditions [10–14]. It has been suggested that α-crystallin acts like a molecular chaperone in vivo and helps to maintain lens clarity [10,15]. The decrease in chaperone activity in cataractous lenses supports this hypothesis [16,17]. Surface hydrophobicity plays an important role in protein–protein interaction and chaperone action [18–21]. Decrease in hydrophobicity has been shown to reduce the chaperone activity of α-crystallin [22,23]. Recently, using a hydrophobic probe, we were able to map the chaperone site in αA-crystallin [24]. We have also confirmed the chaperone site as the functional unit of αA-crystallin by demonstrating chaperone-like activity in a synthetic peptide [25].

Another approach to identifying the domains of protein interactions include site-directed or deletion mutagenesis [26–28]. However, mutagenesis studies can provide only indirect information about the nature of biomolecular interaction and cannot precisely define the complementary domain in the target protein because mutations often result in changes in the tertiary structure of protein. Currently, studies on binding site identification mainly involve the proteolysis of the protein complex [29–31] and the use of

Abbreviations: ADH, alcohol dehydrogenase; GuHCl, guanidine hydrochloride; MALDI, matrix-assisted laser desorption mass spectroscopy; sulfo-SBED, sulfosuccinimidyl-2[6-(biotinamido)-2-(*p*-azidobenzamido)-hexanoamido]ethyl-1,3di-thiopropionate; TMAE, trimethylaminoethyl; TFE, trifluoroacetic acid

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photoactive cross-linkers [32–34]. Photoactive cross-linkers have an advantage over chemical cross-linkers in that they are not chemically reactive without exposure to the proper wavelength of light [35]. Using this technique, one can identify the points of contact in interacting proteins. Earlier, using one such photochemical cross-linker, we identified the mellitin binding sites in αA-crystallin [23] and the alcohol dehydrogenase (ADH) binding site in αB-crystallin [34].

Several studies have been directed toward identifying the chaperone site in the αA and αB subunits of α-crystallin [23,26,27,36]. However, the site[s] in target proteins important for chaperone action has not been mapped. Recently, we have shown that the chaperone-like action in α-crystallin varies among substrate proteins [14]. Therefore, identifying the α-crystallin interacting sites in substrate proteins would explain the wide chaperoning ability of α-crystallin. In the present study, we have used yeast ADH as the target protein to identify the α-crystallin interacting site during chaperoning.

2. Materials and methods

2.1. Materials

Yeast ADH was obtained from Sigma Chemical Co. Sulfo-SBED and Immunopure® immobilized monomeric avidin were procured from Pierce Chemical Co., and lysyl endopeptidase from Wako Chemicals USA, Inc. All other chemicals were of the highest grade and commercially available.

2.2. Isolation of α-crystallin

α-Crystallin was isolated from bovine lens cortex by gel filtration on Sephadex G-200 and purified by passing through a trimethylaminoethyl (TMAE)-fractogel column [34]. The α-crystallin obtained by this method was > 99% pure, as judged by SDS-PAGE. The concentration of α-crystallin was determined based on an absorption coefficient of 0.83 for 1 mg/ml protein [36].

2.3. Chaperone assay

Native ADH or its peptides were heated in 50 mM phosphate buffer containing 0.1 M NaCl (pH 7.4) (buffer A) in the presence or absence of α-crystallin in a final volume of 1 ml at 48 °C. The aggregation of protein was followed at 360 nm for 1 h using a temperature-regulated Shimadzu UV–Vis spectrophotometer.

2.4. Identification of binding sites

2.4.1. Proteolysis method

ADH (5 mg) was taken in 1 ml of buffer A and digested with lysyl endopeptidase (200:1, mol/mol) for 6 h at 30 °C. The peptides generated were separated from the protease by filtering the sample through a 10 kDa filter. The peptides were then incubated with 1 mg α-crystallin in buffer A at 48 °C for 1 h. The ADH peptide(s) bound to α-crystallin were separated from the free peptides by passing the incubation mixture through an agarose (A-0.5 M) (3 × 45 cm) column equilibrated with buffer A. Fractions containing the α-crystallin-ADH peptide complex were pooled and concentrated to 1 ml. The bound peptide(s) were dissociated from the crystallin by treating the complex with 2 M guanidine hydrochloride (GuHCl) and filtering through a 10 kDa filter. The filtrate was analyzed on a HPLC fitted with a C18 column (Vydac C18, 218TP1010 from The Separation Group, Hesparia, CA), using a linear gradient (0–70%) of water and acetonitrile containing 0.1% TFA. The peptide peak was concentrated by vacuum centrifugation and sequenced at the protein core facility (University of Missouri, Columbia, MO). The apparent molecular weight of the peptide was also determined by matrix-assisted laser desorption mass spectroscopy (MALDI) at the Biomedical Research core facility of the University of Michigan, Ann Arbor, MI.

2.4.2. Cross-linking method

α-Crystallin was derivatized with Sulfo-SBED using the procedure described by the supplier. In brief, α-crystallin (5 mg) in buffer A was reacted in the dark with 0.5 mg sulfo-SBED in dimethylsulfoxide. After 15 min, the unreacted sulfo-SBED was removed by passing the mixture through a buffer A-equilibrated Sephadex G-25 column in the dark. The SBED-conjugated α-crystallin (2 mg) was mixed with an equal amount of ADH and incubated at 48 °C in the dark for 1 h. The ADH–α-crystallin–SBED complex was isolated by passing the mixture through an agarose (0.5 M) column. The fractions containing the complex were photolyzed on an ice bath using a UV lamp (365 nm) for 20 min, and then digested with lysyl endopeptidase. After 6 h, the protease was inactivated by the addition of 1 mM diisopropylfluorophosphate. The digested mixture was treated with 2-mercaptoethanol to cleave the disulfide bond of the cross-linker. The ADH peptide, now containing the biotin group of the cross-linker, was isolated using an immunopure monomeric avidin kit as per the protocol given by Pierce [37]. In brief, the sample mixture was passed through an avidin column and the unbound peptides were washed off using buffer A. The bound material was eluted using 0.1 M glycine (pH 2.8). The eluate was analyzed by HPLC and sequenced as described earlier.

2.5. Further confirmation of binding region

After identification of a binding site, a peptide having a sequence corresponding to the α-crystallin interaction region in ADH was synthesized (Research Genetics Inc., AL). The ability of this ADH peptide (200 μg) to bind and form a complex with α-crystallin (0.5 mg) was tested at

room temperature and at 48 °C. After 1 h incubation in phosphate buffer, the samples were centrifuged to pellet any aggregate formed and the supernatant was injected into an HPLC fitted with a Synchropack G100 gel filtration column. Individual peaks were further analyzed on a HPLC (fitted with a reverse-phase column) over water/acetonitrile gradient containing 0.1% TFA.

3. Results and discussion

We used ADH as a substrate protein for the identification of α-crystallin binding site(s) since at 48 °C ADH destabilizes and aggregates, whereas α-crystallin retains significant native structure at this temperature [38]. At 48 °C in the presence of α-crystallin, ADH forms a complex and the reaction mixture remains clear. In the present study, prior to incubating with α-crystallin, we digested ADH with lysyl endopeptidase to generate relatively large fragments compared to the peptides that will be generated by digestion with trypsin and chymotrypsin. The digestion was carried out for 6 h, based on the specificity data supplied by the manufacturer of the enzyme. The peptide cocktail derived from the digestion of ADH was filtered through a 10 kDa filter, and an aliquot of the filtrate was injected into an HPLC fitted with a reverse-phase column to obtain a profile for the digested peptides (Fig. 1). It appears that a number of peptides with varying affinity to the C18 column were generated during digestion of ADH.

The light-scattering property of the digested ADH mixture was measured at 48 °C. Digestion of ADH significantly reduced its light-scattering property during thermal denaturation (Fig. 2). In the presence of α-crystallin, the ADH peptides did not show any light scattering and the solution remained clear, indicating an interaction between α-crystallin and the aggregation-prone peptides.

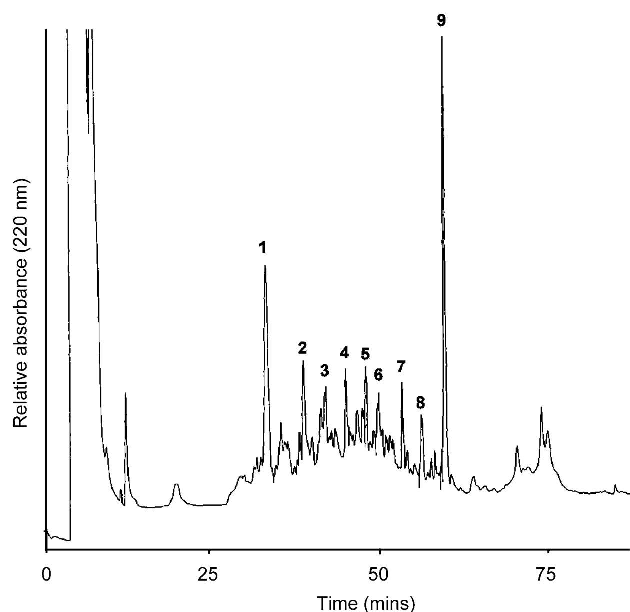

Fig. 1. Reverse-phase HPLC profile of ADH peptides. ADH was digested with lysyl endopeptidase for 6 h. The mixture was filtered on a 10 kDa filter and the filtrate was injected into an HPLC fitted with a C18 column. Major peaks are identified by numbers 1–9.

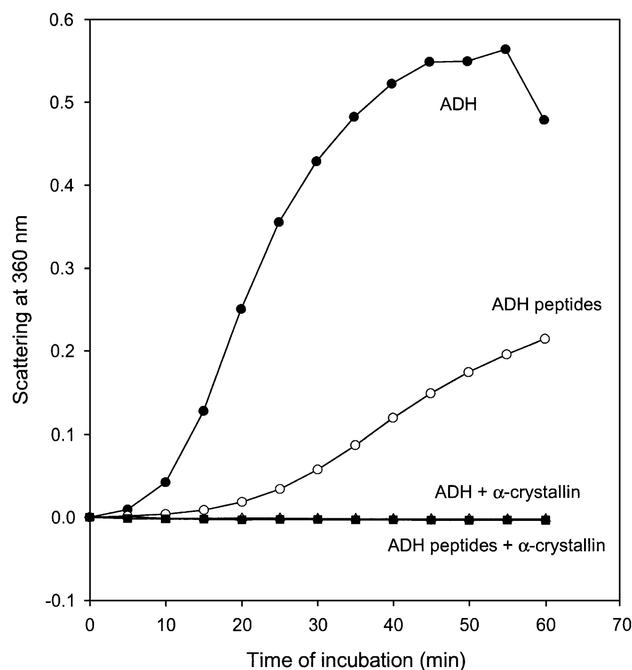

Fig. 2. Thermal aggregation kinetics of ADH (250 μg) and its peptide (500 μg) in the presence and absence of α-crystallin (100 μg). Assay was done using phosphate buffer pH 7.4 at 48 °C for 1 h.

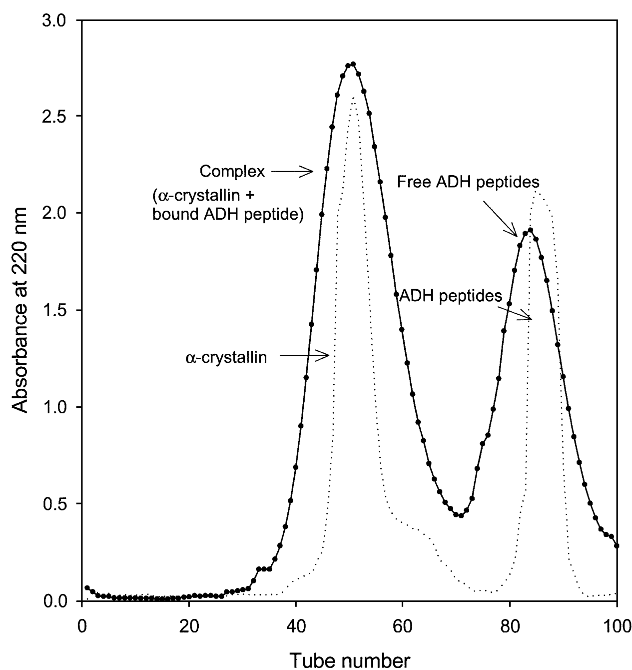

Fig. 3. Elution profile of α-crystallin and ADH–peptide mixture (incubated at 48 °C) on an agarose (A-0.5 M) column. The profile of the unheated α-crystallin and peptide mixture is shown as a dotted line.

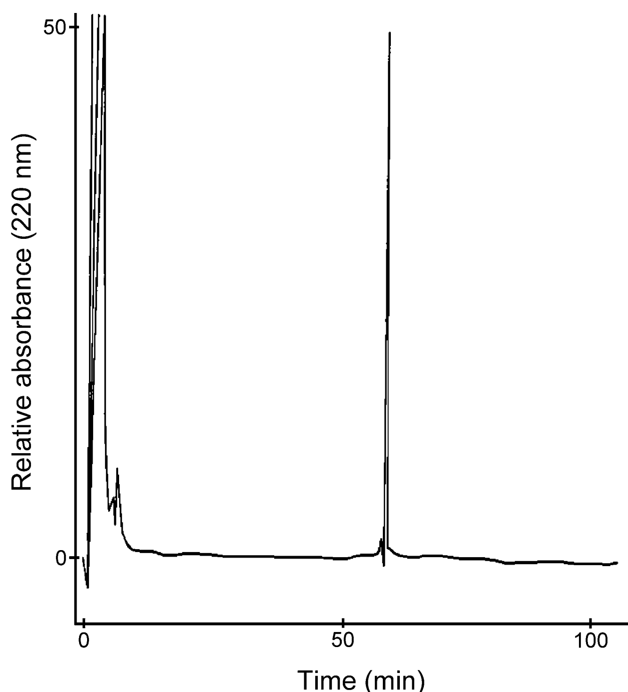

Fig. 4. HPLC profile of ADH peptide bound to α-crystallin. The complex from the agarose column was dissociated by GuHCl treatment and filtered through a 10 kDa filter. The filtrate was then injected for HPLC analysis.

When the incubation mixture containing α-crystallin–ADH peptides was passed through an agarose [0.5 M] column, two peaks were observed (Fig. 3). The first peak represented α-crystallin + bound ADH peptide(s), whereas the second peak was that of free ADH peptides. The complex was disrupted with GuHCl and filtered through a 10 kDa filter. Both the filtrate and the retentate were analyzed by HPLC. While the retentate showed two peaks, corresponding to the elution points of αB- and αA-crystallin, the filtrate showed a single peak of peptide eluting at 60 min (Fig. 4). The elution time of the ADH peptide was same as that of peak 9 in Fig. 1. HPLC analysis of the free ADH peptide fractions (tube no. 80–90) from the agarose separation did not show this peak (profile not shown). Furthermore, HPLC analysis of the α-crystallin peak recovered when a mixture of ADH peptides and α-crystallin was subjected to gel filtration prior to 48 °C incubation also did not show the presence of the ADH peptide eluting at 60 min (data not shown). To ensure that the peptide peak seen in Fig. 4 was a result of binding and not due to co-elution of aggregated peptide with α-crystallin, we heated the ADH peptide cocktail at 48 °C in phosphate buffer for 1 h. The sample was then centrifuged to pellet the aggregate and the supernatant was loaded onto an agarose gel filtration column. When the column was developed, we did not see any peak in the α-crystallin eluting region, but a peak was observed in the peptide-eluting region (figure not shown). These data suggest that only in the presence of α-crystallin the aggregation prone ADH peptides elute with α-crystallin, due to their binding with the chaperone protein.

MALDI analysis of the α-crystallin bound peptide showed a relative mass of 2418 Da. N-terminal amino acid sequencing gave the sequence YSGVCH. This peptide probably has the sequence YSGVCHTDLHAWHGDWPLPVK, corresponding to the residues 40–60 of yeast ADH (Fig. 5), since we used lysyl endopeptidase to digest native ADH. These results indicate one binding site in ADH for α-crystallin.

The use of protease prior to complex formation between two proteins can destroy some binding sites that harbor the cleavage site for the enzyme. Therefore, to verify our results, we used a photoactive trifunctional cross-linker, sulfo-SBED, to determine the binding site in ADH. Photoactive chemicals have been used to map the interacting sites of proteins, hormones and enzymes [34,39,40]. Sulfo-SBED has an *n*-hydroxysuccinamide group for coupling with the amino groups of α-crystallin, a photoactive aryl azide that can react with the proximal amino acid of the interacting target protein, and a central disulfide linker to release the labeled protein after cleavage. The advantage of sulfo-SBED is the presence of a biotin for easy isolation and purification of the conjugated protein or peptide using avidin affinity chromatography [25,37].

α-Crystallin was first treated with sulfo-SBED in the dark, and the free cross-linker was removed by Sephadex G-25 chromatography. Coupling of SBED to α-crystallin did not alter its chaperone activity (data not shown), consistent with our earlier findings that derivatizing α-crystallin with a similar cross-linker does not diminish its chaperone-like activity [34]. SBED-derivatized α-crystallin was then incubated with ADH in buffer A at 48 °C in the dark to form a complex. The complex was photolyzed for the activation

1 MSIPETQKGV IFYESHGKLE HKDIPVPKPK ANELLINVK**Y SGVCHTDLHA WHGDWPLPVK**

61 LPLVGGHEGA GVVVGMGENV KGWKIGDYAG IKWLNGSCMA CEYCELGNES NCPHADLSGY

121 THDGSFQQYA TADAVQAAHI PQGTDLAQVA PILCAGITVY KALKSANLMA GHWVAISGAA

181 GGLGSLAVQY AKAMGYRVLG IDGGEGKEEL FRSIGGEVFI DFTKEKDIVG AVLKATDGGA

241 HGVINVSVSE AAIEASTRYV RANGTTVLVG MPAGAKCCSD VFNQVVKSIS IVGSYVGNRA

301 DTREALDFFA RGLVKSPIKV VGLSTLPEIY EKMEKGQIVG RYVVDTSK

Fig. 5. Amino acid sequence of yeast alcohol dehydrogenase showing the α-crystallin binding site (bold).

and insertion of the aryl azide group onto ADH at the interacting site. The complex was then dissociated with 2 M GuHCl and digested with lysyl endopeptidase. The ADH peptide containing the biotin group was isolated using an immunopure monomeric avidin kit. HPLC analysis of the purified sample showed one peptide peak eluting at 58 min (peak 3 in Fig. 6A) and two other peaks. The first peak (23 min) in Fig. 6A was identified as biotin and the second peak (32 min) could be due to some cross-linker leaching out of the chromatography [41], since we were unable to identify any amino acid during sequencing. The slight difference in the elution time of the peptide (peak 3 in Fig. 6A) compared to the previous method (Fig. 4) is likely due to the modification of the peptide by the cross-linker. Additionally, the ADH peptides that did not bind to the biotin column did not show any peak eluting in this region (58–60 min) on reverse-phase HPLC (Fig. 6B). The N-terminal sequencing of the peptide (peak 3 in Fig. 6A) gave a sequence YSGVC corresponding to the residues 40–44 of ADH. This peptide is also likely to have a

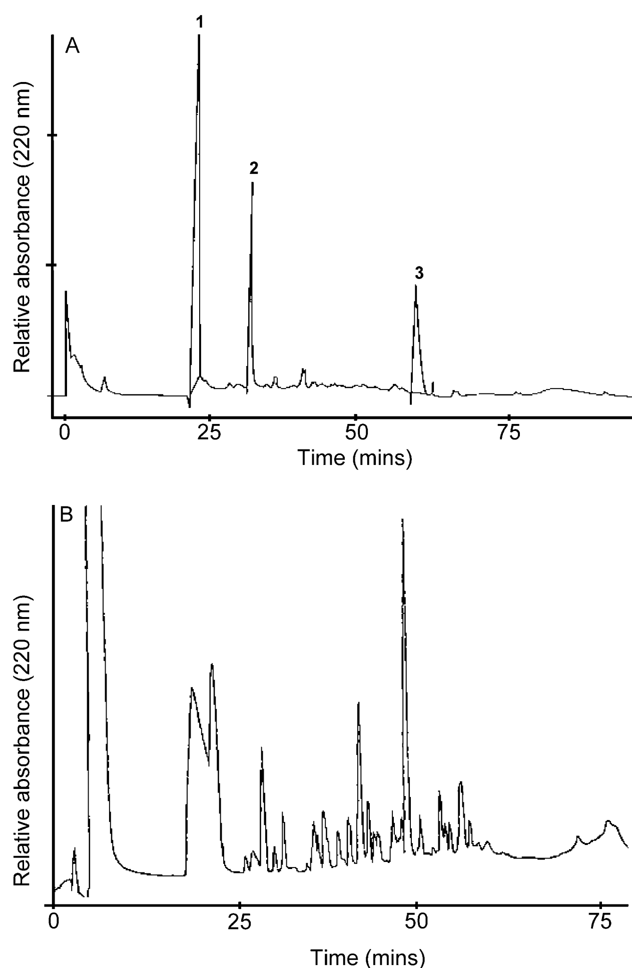

Fig. 6. (A) HPLC profile of biotinylated-ADH peptide (peak 3) purified by avidin chromatography. Peak 1 was found to be free biotin and peak 2 was unidentified. (B) HPLC profile of ADH peptides that did not bind to the avidin column.

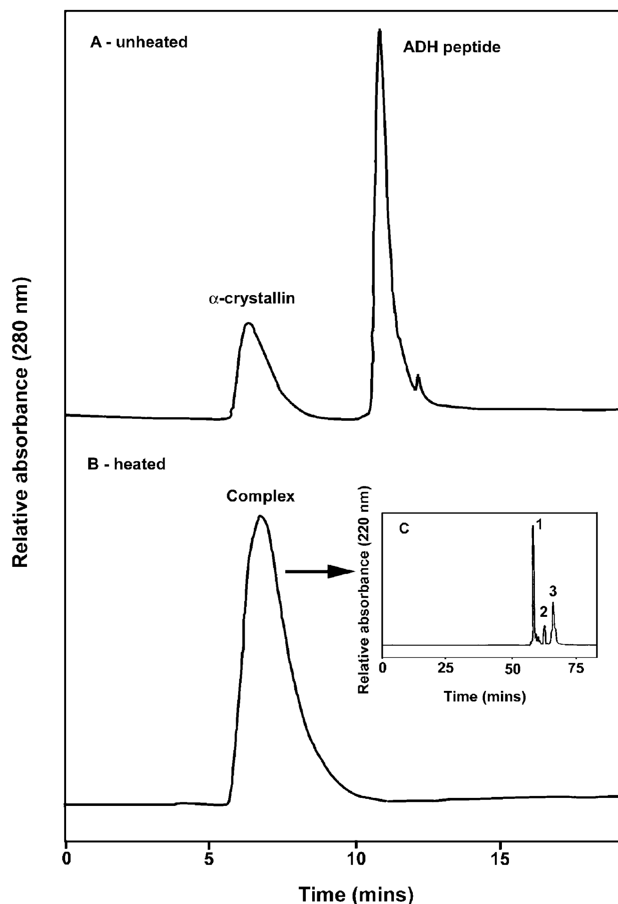

Fig. 7. Gel filtration profile of synthetic ADH peptide and α-crystallin incubations. (A) After incubation at room temperature for 1 h. (B) After incubation at 48 °C for 1 h. (C) HPLC profile of the complex on a reverse-phase column. ADH peptide elutes as peak 1. α-Crystallin dissociates to form αB-crystallin (peak 2) and αA-crystallin (peak 3).

sequence YSGVCHTDLHAWHGDWPLPVK, since we used lysyl endopeptidase to release this fragment from the α-crystallin–ADH complex. The ADH sequence 40–60 is also the sequence of the ADH peptide interacted with α-crystallin earlier. Taken together, the results from two different approaches confirm that residues 40–60 constitute an α-crystallin binding site in yeast ADH.

To confirm our results further, we used a purified synthetic peptide fragment, YSGVCHTDLHAWHGDWPLPVK, to demonstrate that ADH sequence 40–60 forms a complex with α-crystallin. Incubations conducted at room temperature showed two peaks on gel filtration (Fig. 7A) that corresponded to the α-crystallin and ADH peptide elution points, suggesting no binding between them at this temperature. The sample incubated at 48 °C showed a single peak eluting at 6.9 min, and the peptide peak was completely missing (Fig. 7B). Further analysis of the 6.9 min peak on a reverse-phase column showed three peaks representing the ADH peptide and αB- and αA-crystallin (Fig. 7C). These results confirm that the binding of the ADH peptide to α-crystallin occurs only on heating the reaction mixture to 48 °C, and that such a complex

remains soluble. We also saw the aggregation of the synthetic ADH peptide at 48 °C in the absence of α-crystallin.

Fig. 5 shows the location of the α-crystallin binding region in ADH. It is interesting to note that the α-crystallin binding region in ADH is rich in beta-sheet forming amino acids and has two tryptophan residues [42]. The β-sheet forming residues 41–45 in native protein also interact with other β-sheet forming residues 68–71 and 369–374. Amino acids from this region also contribute to the active site, pyrophosphate binding site and hydrophobic core. The Cys-44 in this region acts as a metal-binding site [43]. Generally, tryptophan is buried inside the protein core and gets exposed slowly on thermal denaturation [44,45]. Probably, this structural perturbation in ADH triggers the interaction of α-crystallin and the tryptophan and other hydrophobic residues in the binding region of ADH. We speculate that this in turn increases the binding efficiency. In the current study, we report that α-crystallin binds less than 6% of the primary sequence in target protein, unlike the 50% reported earlier with other chaperone systems [29–31].

In the present study, we identified only one site in ADH for the binding of α-crystallin. It is not uncommon to see a binding region corresponding to a continuous short sequence. We reported earlier that the target protein interacting site in αA-crystallin is represented by a single continuous sequence [25]. However, studies with Sec B have shown that this molecular chaperone interacts at multiple points with maltose-binding protein and galactose-binding protein [29,30]. Those studies also report that the entire binding region covers half of the primary sequence of the interacting protein in extended form [29,30,46]. A similar observation was made by Hlodan et al. [31] with regard to Rhodonese binding to chaperonin, GroEL. This discrepancy could be due to the difference in the nature of the methods employed to obtain complexes. While earlier studies used GuHCl to denature the protein, rapid dilution in the presence of the chaperone to form complexes, and the digestion of the complexes by protease [29–31], we digested ADH prior to incubation with α-crystallin and used thermal treatment to denature the ADH fragments. Unlike GuHCl-induced denaturation, thermal treatment induces gradual unfolding of the protein. Furthermore, the initial binding to the chaperone may be blocking extensive unfolding and multiple interactions between chaperone and ADH. Recent study on rhodopsin and transducin interaction investigated by Cai et al. [47] using photoactive cross-linkers showed that only a short stretch of transducin binds to rhodopsin. Additionally, it has been reported that α-crystallin binds to near-native protein [9,48,49] and that there is 20% or less surface area covered in a protein–protein interactions. Therefore, it appears that the stressed target protein ADH does not expose the entire hydrophobic core to the solvent and only a small part of ADH is involved in interaction with α-crystallin. Surprisingly, the binding region of ADH falls within the two cleavage sites of lysyl endopeptidase. This may not be the case with studies involving other proteins; therefore, a careful choice of the protease is required to keep the binding region intact. The experimental approach described in this study can be further applied to probe binding sites in other proteins and enzymes. Mapping of the α-crystallin interacting site(s) in β- and γ-crystallin may explain their roles in high-molecular-weight aggregate formation.

Acknowledgements

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