Insights into the domains required for dimerization and assembly of human α B crystallin

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Abstract

Protein pin array technology was used to identify subunit–subunit interaction sites in the small heat shock protein (sHSP) α B crystallin. Subunit–subunit interaction sites were defined as consensus sequences that interacted with both human α A crystallin and α B crystallin. The human α B crystallin protein pin array consisted of contiguous and overlapping peptides, eight amino acids in length, immobilized on pins that were in a 96-well ELISA plate format. The interaction of α B crystallin peptides with physiological partner proteins, α A crystallin and α B crystallin, was detected using antibodies and recorded using spectrophotometric absorbance. Five peptide sequences including ₃₇LFPTSTSLSPFYLRPPSF₅₄ in the N terminus, ₇₅FSVNLDVK₈₂ (β 3), ₁₃₁LTITSSLS₁₃₈ (β 8) and ₁₄₁GVLTVNGP₁₄₈ (β 9) that form β strands in the conserved α crystallin core domain, and ₁₅₅PERTIPITREEK₁₆₆ in the C-terminal extension were identified as subunit–subunit interaction sites in human α B crystallin using the novel protein pin array assay. The subunit–subunit interaction sites were mapped to a three-dimensional (3D) homology model of wild-type human α B crystallin that was based on the crystal structure of wheat sHSP16.9 and *Methanococcus jannaschi* sHSP16.5 (Mj sHSP16.5). The subunit–subunit interaction sites identified and mapped onto the homology model were solvent-exposed and had variable secondary structures ranging from β strands to random coils and short α helices. The subunit–subunit interaction sites formed a pattern of hydrophobic patches on the 3D surface of human α B crystallin.

Keywords: α B crystallin; lens; chaperone; small heat shock protein; assembly; molecular modeling

Small heat shock proteins (sHSPs) are a superfamily of proteins with a molecular weight <40 kDa ubiquitously found in a variety of organisms (Wistow 1985). Increased expression of sHSPs occurs in cells in response to abnormal heat, oxidation, osmotic stress, radiation, and other environmental factors. Under conditions of stress, sHSPs can function as molecular chaperones in cells, interacting with unfolded or unfolding target substrate proteins to confer stability on exposed, hydrophobic surfaces and protect against aggregation (de Jong et al. 1989; Piatigorsky 1989; Horwitz 1992; Haslbeck and Buchner 2002). α B crystallin is the

archetype of the sHSP family that is characterized by a conserved “ α -crystallin core” domain consisting of ~80–100 residues organized as a β sandwich similar to an immunoglobulin fold (Kim et al. 1998; van Montfort et al. 2001; Narberhaus 2002). In humans, α B crystallin has been implicated in protein aggregation-related pathologies including cataract, desmin-related myopathies, cardiomyopathies, and neurodegenerative diseases such as Alzheimer’s disease and Alexander’s disease (Iwaki et al. 1989, 1993; Stege et al. 1999; Clark and Muchowski 2000; McLean et al. 2002; Jacob et al. 2003; Wytenbach 2004). In normal cells, α B crystallin is believed to function in the maintenance of lens-specific intermediate filaments that contribute to lens cell transparency (Nicholl and Quinlan 1994; Tardieu 1998; Quinlan and Van Den Ijssel 1999; Alizadeh et al. 2003, 2004) and in the stabilization of partially unfolded proteins that are intermediates in mechanisms of aggrega-

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tion (Horwitz et al. 1992; Muchowski et al. 1997; Derham and Harding 1999; van Boekel et al. 1999; Horwitz 2003). Although the assembly of α B crystallin subunits is associated with chaperone activity (Aquilina et al. 2004), the interactive domains responsible for assembly of homo- and heteromeric α crystallin complexes remain to be characterized. The absence of an X-ray or NMR structure for full-length α B crystallin is limiting progress in understanding the relationships between primary, secondary, and tertiary structure and the interactions necessary for subunit assembly of α B crystallin. The X-ray crystal structures of *Methanococcus jannaschii* sHSP16.5 (Mj sHSP16.5) and wheat sHSP16.9 suggested that sHSPs have common structural features and that the α crystallin core domain is an immunoglobulin-like fold consisting of 7–9 β strands organized in the tertiary structure as a β sandwich (Kim et al. 1998; van Montfort et al. 2001; Studer et al. 2002). In the crystal structure of Mj sHSP16.5, two categories of interactions contributed to the quaternary structure: (1) subunit-subunit interactions that resulted in the formation of a dimeric building block, and (2) dimer-dimer interactions that resulted in the formation of larger oligomeric assemblies. The crystal structures identified three hydrophobic subunit-subunit interaction sites, namely an amphipathic helix in the N terminus, a groove in the α crystallin core domain, and an I-X-I/V motif (where I is isoleucine, X is variable, and V is valine) in the C-terminal extension that were involved in formation of dimers, and was subsequently the smallest structural unit for assembly of dodecamers in Mj sHSP16.5 and 24-mers in wheat sHSP16.9 (van Montfort et al. 2001; Studer et al. 2002). Spectroscopic data suggested that the secondary and tertiary structures of α B crystallin were similar to those of Mj sHSP16.5 and wheat sHSP16.9 (McHaourab et al. 1997; Koteiche and McHaourab 2002).

In vitro the size of the sHSP complexes may depend on the length and nature of the N terminus and C terminus extensions that flank the α crystallin core domain (Feil et al. 2001). Cryo-electron microscopy indicated that complexes of recombinant human α B crystallin existed as a broad distribution of sizes from 20 to 80 subunits. The quaternary structure observed was a hollow sphere with an internal diameter of ~10 nm (Haley et al. 2000). Human α B crystallin has slightly longer N and C termini compared to Mj sHSP16.5 and wheat sHSP16.9, which form helical and flexible structures of 40 and 20 residues, respectively. Assembly depends on solvent conditions as well as posttranslational modifications, including phosphorylation of serine residues (MacCoss et al. 2002; Horwitz 2003). Abraham and coworkers reported that truncated and phosphorylated lens α B crystallin formed assemblies that were different from those formed by full-length unmodified wild-type α B crystallin (Cherian and Abraham 1995; Thampi et al. 2002). Experiments involving *C. elegans* sHSPs 12.2, 12.3, and 12.6 indicated that sHSPs with short N and C termini form

small assemblies and have diminished chaperone function (Leroux et al. 1997; Kokke et al. 1998). Similarly in *P. furiosus* sHSP, the C terminus is believed to play an important role in oligomer formation (Laksanalamai and Robb 2004). Characterization of the interactions between α crystallin dimers is expected to provide new information on the structural basis of sHSP function.

The identification and characterization of residues belonging to the subunit-subunit interaction sites in human α B crystallin, the archetype of the small heat shock protein family, are the objectives of the current report. In the absence of a crystal structure for human α B crystallin, a peptide scanning method called a protein pin array was used to identify peptide sequences in human α B crystallin that interacted with α A crystallin and α B crystallin subunits. Protein pin arrays employ peptides that represent interactive domains of proteins. Interactive domains are usually comprised of multiple sequences that are in close proximity and form a three-dimensional (3D) interactive surface. Though individual peptides only form part of the entire interactive surface, pin arrays have been successful in mapping the discrete sequences that form interactive domains in proteins. Protein pin arrays were used to map antigen epitopes in antigen-antibody interactions and several receptor-ligand interactive sites that depend on the 3D structure of the interacting partner proteins (Slootstra et al. 1996, 1997; Meleen et al. 2000, 2001; Bernard et al. 2004; Timmerman et al. 2004). While it cannot be ruled out that subunit-subunit interactions in α B crystallin require higher-order structure that cannot be detected using protein pin array assays, it is likely that the pin arrays can identify sequences important for the subunit-subunit interactions of α B crystallin. Recently a similar strategy was employed to identify oligomerization and substrate interactions sites in a protein homologous to α B crystallin, *Bradyrhizobium japonicum* sHSPB (Lentze and Narberhaus 2004). In the absence of a crystal or NMR structure of α B crystallin, a homology model of α B crystallin was constructed using Molecular Operating Environment (MOE) software and compared with the crystal structures of Mj sHSP16.5 and wheat sHSP16.9. The results of the protein pin arrays were mapped onto the 3D-computed model for human α B crystallin. Five interactive sequences, an N terminus helix motif, three β strands in the α crystallin core domain, and a sequence containing the I-X-I/V motif in the C terminus were identified as subunit-subunit interactive sites in α B crystallin using the pin array and 3D model of α B crystallin.

Results

Sequential peptide fragments corresponding to the primary sequence of human α B crystallin were synthesized, immobilized on the pin arrays, and tested for interaction with myoglobin, α A crystallin, and α B crystallin (Fig. 1). The

Figure 1. (A, left) Protein pin array and ELISA assay identified interactive sequences of human α B crystallin. Sequential 8-mer peptides were synthesized on the individual tips of derivatized polyethylene pins arranged in a 96-well microtiter plate format. Each pin contained nanomoles of a specific eight-amino-acid in length peptide immobilized on it and consecutive peptides were offset by two amino acids. All peptides were covalently bonded to the surface of the plastic pins. The first peptide was $_1$ MDIAIHHP $_8$, and the last peptide was $_{168}$ PAVTAAPK $_{175}$ for the human α B crystallin. In all, 84 peptides corresponding to the 175-amino-acid primary sequence of human α B crystallin were synthesized and immobilized on 84 individual pins of the pin array (Table 2). (B, center) ELISA plate result of the negative control for interactions between human myoglobin and the human α B crystallin peptide library. Blue coloration indicates a positive interaction, and a clear solution indicates no interaction. (C, right) ELISA plate result for the interactions between human α B crystallin and the peptides of the human α B crystallin protein pin array. Blue coloration indicates a positive interaction; a clear solution indicates no interaction.

ELISA-based method used primary antibodies for myoglobin, α A crystallin, or α B crystallin and horseradish peroxidase (HRP)-conjugated secondary antibodies that turned 3,3',5,5'-Tetramethylbenzidine (TMB) substrate from colorless to blue when binding occurred. When the wells of the ELISA plate contained $0.06 \mu\text{M}$ myoglobin, no blue color was observed, indicating that no interaction occurred between the immobilized α B crystallin peptides and human myoglobin (Fig. 1B). When each well of the ELISA plate contained $0.05 \mu\text{M}$ human α B crystallin, 18 wells were dark blue, 11 wells were light blue, and 55 wells were colorless (Fig. 1C). The last three peptides of the α B crystallin pin array were the epitope for the antibody to human α B crystallin, were dark blue, and served as the positive control for the pin array experiment.

A plot of the absorbance measured (Y -axis) in each well of the ELISA plate against the 8-mer peptide sequence (X -axis) of the α B crystallin pin array resulted in a pattern of high and low absorbance that permitted the identification of interactive sequences (Fig. 2). The protein pin array identified 18 interactive sequences when human α B crystallin was the target protein (Table 1, column 2) and 11 interactive sequences when human α A crystallin was the target protein (Table 1, column 3). The well corresponding to peptide $_{57}$ RTIPITRE $_{64}$ was the most intense blue color when α A crystallin was the ligand, and the well corresponding to peptide $_{75}$ FSVNLDVK $_{82}$ was the most intense blue color when α B crystallin was the ligand. For α A crystallin, five consecutive overlapping peptides in the N terminus, three discrete nonoverlapping peptides in the α crystallin core domain, and three consecutive overlapping peptides in the C

terminus region were identified by intense blue color. When α B crystallin was the ligand, more peptides had intense blue color and the absorbance pattern was similar to the pattern observed for α A crystallin. As stated above, the last three peptides of the human α B crystallin protein pin array were the epitopes of the α B crystallin antibody used and were not considered in the data analysis. Several interactive sequences that overlapped significantly in their sequence ($_{37}$ LFPTSTSL $_{44}$, $_{41}$ STLSPPFY $_{48}$, $_{43}$ SLSPFYLR $_{50}$, $_{45}$ SPFYLRPP $_{52}$, $_{47}$ FYLRPPSF $_{54}$) and ($_{155}$ PERTIPIT $_{162}$, $_{157}$ RTIPITRE $_{164}$, $_{159}$ IPITREEK $_{166}$) were consolidated into longer contiguous sequences $_{37}$ LFPTSTLSPPFYLRPPSF $_{54}$ and $_{155}$ PERTIPITREEK $_{166}$, respectively.

The protein pin array provided information only on the primary sequence of the interactive regions in human α B crystallin that included intramolecular interactions as well as intermolecular interactions. To distinguish between intramolecular interactions and intermolecular interactions, side-chain contacts were calculated using the 3D homology model for α B crystallin (Table 1, column 4). Protein side-chain contacts included three types of interactions: hydrogen bonds, hydrophobic interactions, and salt bridges. Human α B crystallin does not contain cysteines, and disulfide bonds are not involved in intramolecular interactions. Column 5 lists exposed residues in the interactive sequences that are capable of forming intermolecular interactions with either α A crystallin or α B crystallin. Of the 18 peptides that interacted strongly with α B crystallin, residues belonging to peptides 5, 10, 26, 38, 44–45, 48, 54–57, and 71–74 were involved in intramolecular interactions and had no exposed residues capable of intermolecular interactions. Interestingly, peptides 5, 10, 26, 38, 44–45, 48, 54–57, and 71–74 that did not have any exposed residues did not show interaction with α A crystallin (column 3). These peptides were excluded from the set of sequences identified as domains for subunit–subunit interactions in α B crystallin. Based on these data, consensus sequences were identified in α B crystallin that interacted with both α A crystallin and α B crystallin (Table 1, column 6). Consensus sequences were defined as sequences that were capable of intermolecular interactions with both α A crystallin subunits and α B crystallin subunits. All 11 peptides that interacted strongly with α A crystallin were identified as α B crystallin subunit–subunit interaction sequences. Human α A crystallin and human α B crystallin are <57% homologous, and form mixed oligomers. Consensus sequences identified by the protein pin arrays are expected to be important for both α A crystallin $\leftrightarrow$ α B crystallin and α B crystallin $\leftrightarrow$ α B crystallin interactions.

The alignment of the primary sequence for human α B crystallin with the primary sequences for wheat sHSP16.9 and Mj sHSP16.5 resulted in good alignment of the predicted secondary structure of α B crystallin with the observed secondary of wheat sHSP16.9 and Mj sHSP16.5 (Fig. 3). Although there was variability in the primary se-

Table 1. Protein contacts for wt human α B crystallin peptides that interacted with either human α A crystallin or human α B crystallin

Pin #	Peptides that bind α B crystallin	Peptides that bind α A crystallin	Internal protein contacts 4Å - hydrophobic 3Å - ionic cutoff	Exposed residues, i.e., residues capable of intermolecular interactions	Consensus subunit-subunit interaction sequences (α B $\leftrightarrow$ α B, α B $\leftrightarrow$ α A)
5	¹¹ RRPFFPFH ₁₈	—	F17→I10, H18→F54	—	—
10	²¹ SRLFDQFF ₂₈	—	R22→L55, D25→R123	—	—
18	³⁷ LFPTSTSL ₄₄	³⁷ LFPTSTSL ₄₄	F38→T40, F38→T42 P39→S43	L37, S41, L44	—
20	—	⁴¹ STSLSPFY ₄₈	—	S41, L44, S45, P46, F47, Y48	—
21	—	⁴³ SLSPFYLR ₅₀	—	L44, S45, P46, F47, Y48, L49, R50	³⁷ LFPTSTSLSPFYLRPPSF ₅₄
22	—	⁴⁵ SPFYLRPP ₅₂	—	S45, P46, F47, Y48, L49, R50, P51, P52	—
23	—	⁴⁷ FYLRPPSF ₅₄	—	F47, Y48, L49, R50, P51, P52, S53	—
26	⁵³ SFLRAPSW ₆₀	—	F54→H18, L55→R22, A57→S19, W60→F118	—	—
37	⁷⁵ FSVNLDVK ₈₂	⁷⁵ FSVNLDVK ₈₂	F75→N146, S76→R69, V77→I98	N78, L79, D80, V81, K82	⁷⁵ FSVNLDVK ₈₂
38	⁷⁷ VNLDVKHF ₈₄	—	F84→L89	—	—
44	⁸⁹ LKVKVLGD ₉₆	—	L89→F84	—	—
45	⁹¹ VKVLGDVI ₉₈	—	I98→V77	—	—
48	⁹⁷ VIEVHGKH ₁₀₄	—	I98→V7, E99→K121, V100→L89	—	—
54	¹⁰⁹ DEHGFISR ₁₁₆	—	—	—	—
55	¹¹¹ FISREFHR ₁₁₈	—	F118→W60	—	—
56	¹¹³ SREFHRKY ₁₂₀	—	F118→W60	—	—
57	¹¹⁵ EFHRKYRI ₁₂₂	—	F118→W60, K121→E99	—	—
65	—	¹³¹ LTITSSLS ₁₃₈	I133→V128	L131, T132, T134, S135, S136, L137, S138	¹³¹ LTITSSLS ₁₃₈
70	¹⁴¹ GVLTVNGP ₁₄₈	¹⁴¹ GVLTVNGP ₁₄₈	L143→L89, N146→F75	G141, V142, T144, V145, G247, P148	¹⁴¹ GVLTVNGP ₁₄₈
71	¹⁴³ LTVNGPRK ₁₅₀	—	K150→D127, K150→D129	—	—
73	¹⁴⁷ GPRKQVSG ₁₅₂	—	K150→D127, K150→D129	—	—
74	¹⁴⁹ RKQVSGPE ₁₅₄	—	K150→D127, K150→D129	—	—
77	—	¹⁵⁵ PERTIPIT ₁₆₂	—	P155, E156, R157, T158, I159, P160, I161, T162	—
78	¹⁵⁷ RTIPITRE ₁₆₄	¹⁵⁷ RTIPITRE ₁₆₄	—	R157, T158, I159, P160, I161, T162, R163, E164	¹⁵⁵ PERTIPITREEK ₁₆₆
79	—	¹⁵⁹ IPITREEK ₁₆₆	—	I159, P160, I161, T162, R163, E164, E165, K166	—

Column 1: the pin peptide number; column 2: the peptides that interacted with α B crystallin; column 3: the peptide sequences that interacted with α A crystallin; column 4: the intramolecular interactions identified using the protein contact algorithm (Materials and Methods); column 5: exposed residues containing side chains that are capable of forming intermolecular interactions with either α A crystallin or α B crystallin; column 6: the consensus sequences for subunit-subunit interactions involving either one α A crystallin subunit and one α B crystallin subunit or two α B crystallin subunits.

quence of the N terminus, the secondary structure was largely helical in all three proteins. In the conserved α crystallin core domain, both the primary and secondary structure were similar and consisted of 7, 8, and 9 β strands in α B crystallin, sHSP16.9, and sHSP16.5, respectively. In contrast to the N terminus and the core α crystallin domain, helices and β strands were absent in the C terminus. While the I-X-I/V motif was conserved, the C termini of all three proteins were variable in sequence and length. The secondary structure of the α crystallin core domains was similar for

human α B crystallin, wheat sHSP16.9, and Mj sHSP16.5, except that the β 6 contained in the loop region of both wheat sHSP16.9 and Mj sHSP16.5 was absent in α B crystallin. Subunit-subunit interaction sites (Fig. 3, orange boxes) identified using the human α B crystallin protein pin arrays aligned well with the interactive sequences identified in the crystal structures of wheat sHSP16.9 (Fig. 3, green boxes) and Mj sHSP16.5 (Fig. 3, cyan boxes). The N-terminal sequence ³⁷LFPTSTSLPFYLRPPSF₅₄ corresponded to the sequence ²⁰PFDTFRSI₂₇ that formed a helix in the

Figure 2. A plot of the α B crystallin peptide vs. absorbance when human α A crystallin or human α B crystallin was used as ligand in the human α B crystallin protein pin array. Patterns of absorbance indicates the interactive sequences in human α B crystallin. Absorbance maxima were observed at three sequences in the α crystallin core domain and one in each of the extensions of the C and N termini. The X-axis lists the 8-mer peptides in order on the 84 pins of the protein pin array. The Y-axis is the absorbance measured at 405 nm in each well of the ELISA plate when α A crystallin and α B crystallin were screened using an ELISA-based colorimetric method. Interactions between human α A crystallin and the peptides of the human α B crystallin protein pin array were detected in a similar way. The height of the vertical bars represents the absorbance for each peptide and is proportional to the interaction of that peptide with the ligand protein (human α A crystallin or human α B crystallin). Absorbance readings in the range of 0.00–0.05 were observed in the absence of any ligand and were considered baseline noise. Solid black bars represent the absorbance for human α B crystallin at room temperature; diagonal striped bars represent the absorbance recorded when human α A crystallin was used as the ligand. At room temperature, interactions were not observed at every peptide, and there was a distinct pattern to the interactions. Wells corresponding to interactive peptides were blue and are the taller bars in the plot. Wells without color are short bars or no bars. Peptides that had tall bars for both the human α A crystallin and human α B crystallin experiments were selected as the sequences involved in subunit–subunit interactions, and are identified with arrows and brackets. The predicted secondary structure of human α B crystallin (–, helix; bars, β strands; black lines, unstructured stretch or loop) is at the top of the figure. The figure is divided into three structural regions. Sequences in the left (pink) were in the N-terminal extension. Sequences in the C-terminal extension are to the right (light blue), and sequences in the α crystallin core domain are in the center (dark blue).

crystal structure of wheat sHSP16.9. The sequences 75 FSVNLDVK 82 (β 3), 131 LTITSSLS 138 (β 8), and 141 GVLT/NGP 148 (β 9) that formed strands β 3, β 8, and β 9 in the conserved “ α crystallin core domain” corresponded to the sequences EAHVFKAD (β 3), VEEVKAGL (β 8), and GVLTVTVP (β 9) in the wheat sHSP16.9. The sequence 155 PERTIPTREEK 166 contained the conserved I-X-I/V motif that corresponded to the sequence EVKAIQIS in the flexible C-terminal extension of wheat sHSP16.9 crystal structure. Peptides in the loop region of α B crystallin were not identified as subunit–subunit interaction sites by the pin array.

The homology model of human α B crystallin (red) contained three structural domains: the N terminus, the α crystallin core domain, and the C terminus (Fig. 4). The 65-residue N-terminal region contained three helix-turn-helix motifs in the homology model. The 89-residue α crystallin core domain formed a β sandwich that resembled an immunoglobulin fold. The last 21 residues formed the flexible C terminus that extended from the α crystallin core domain. When the 3D homology model of α B crystallin was superimposed with the crystal structures of wheat sHSP16.9 and Mj sHSP16.5, the C α root mean square deviation (RMSD) of the fit was 3.25 Å. Superimposition of the conserved α

Figure 3. Sequence alignment, secondary structure elements, and interactive sequences for human α B crystallin, sHSP16.5, and sHSP16.9. The observed secondary structure of wheat sHSP16.9 and *Methanococcus jannaschi* HSP16.5 along with the predicted secondary structure of human α B crystallin ($\sim$, α helix; $\sim$, β strand) are shown. The interactive sequences in the dodecameric quaternary structure of wheat HSP16.9 are in green boxes; the interactive sequences for the Mj sHSP16.5 24-mer structure are in blue boxes. The interactive domains identified in human α B crystallin using protein pin arrays are in orange boxes and corresponded with the interactive sequences observed in the crystal structures of Mj sHSP16.5 and wheat sHSP16.9. In the crystal structure of wheat sHSP16.9, the α helix in the N terminus interacted with β 4 and β 7 in the α crystallin core domain.

crystallin core domains of the three structures resulted in a $C\alpha$ RMSD of 2.06 Å. The α crystallin core domain of α B crystallin consisted of seven β strands organized in two β sheets forming a compact β sandwich. The first sheet was formed from β 2, β 3, β 8, and β 9, and the second sheet was formed from β 4, β 5, and β 7 (Figs. 3,4). The β sandwich was formed primarily by the packing of hydrophobic resi-

Figure 4. Superposition of the monomeric subunits of Mj sHSP16.5 (blue), wheat sHSP16.9 (green), and human α B crystallin (red). The 3D coordinates of the three structures were superimposed yielding a final RMSD of 3.25 Å. All three structures have the same basic topology. The hydrophobic N terminus is largely helical (except in Mj sHSP16.5, where the N terminus is unresolved in the X-ray structure). An immunoglobulin-like α crystallin core domain consists of β strands (11 in Mj sHSP16.5, eight in wheat sHSP16.9, and seven in α B crystallin) that form two antiparallel β sheets connected by a flexible loop. The C-terminal extension is highly charged and unstructured. Note that the C-terminal extension is unresolved in the X-ray structure of Mj sHSP16.5.

dues F75→N146, V77→I98, F84→L89, I133→V128, L143→L89, and N146→F75, and three ionic bonds, S76→R69, K150→D127, and K150→D129 (Table 1). The N-terminal residues were largely helical and formed three helix-turn-helix motifs. The interactions between F17→I10, H18→F54, S19→A57, R22→L55, F38→T40, F38→T42, and P39→S43 were either intrahelical or interhelical (Table 1, column 4). The interactions between D25 and W60 of the N terminus with the α crystallin core domain residues R123 and F118, respectively, may stabilize the flexible N terminus through an interaction with the α crystallin core domain (Table 1, column 4). The C-terminal extension was flexible and solvent-exposed and was not involved in any intramolecular interactions with either the N terminus or the α crystallin core domain.

The subunit-subunit interaction sequences LFPTSTSLSPFYLRPPSF, FSVNLDVK, LTITSSLS, GVLTVNGP, and PERTIPTREEK (green) identified by the α B crystallin protein pin array were mapped onto the 3D homology model of α B crystallin (gray) (Fig. 5A). Subunit-subunit interaction sites were found in the N-terminal domain, the α crystallin core domain, and the C terminus. Interactive sequences in α B crystallin did not map to the loop region connecting the two β sheets in the α crystallin core domain. In each β strand, the surface-exposed residues (ball and stick) contributed to an interface for interaction with subunits in assembly of the α crystallin complex. The N-terminal sequence LFPTSTSLSPFYLRPPSF formed a helix and was solvent-exposed. The α crystallin core domain sequences FSVNLDVK, LTITSSLS, and GVLTVNGP

Table 2. Sequential 8-mer peptides of human α B crystallin that were synthesized in a protein pin array format

#	Peptide	Hydrophobicity	Mol. wt. (Da)	#	Peptide	Hydrophobicity	Mol. wt. (Da)
1	MDIAIHHP	0.67	915.09	43	SPEELKVK	0.04	911.07
2	IAIHHPWI	1.12	968.17	44	EELKVKVL	0.32	939.16
3	IHHPWIRR	0.6	1096.31	45	LKVKVLGD	0.39	853.06
4	HPWIRRP	0.67	1090.31	46	VKVLGDVI	0.68	824.02
5	WIRRPFFP	0.88	1100.35	47	VLGDVIEV	0.72	824.97
6	RRPFFPFH	0.62	1085.3	48	GDVIEVHG	0.37	806.87
7	PFPPFHSP	0.95	957.12	49	VIEVHGKH	0.36	900.04
8	FPFHSPSR	0.51	956.09	50	EVHGKHEE	-0.18	945.99
9	FHSPSRLF	0.63	972.13	51	HGKHEERQ	-0.41	1002.06
10	SPSRLFDQ	0.27	931.03	52	KHEERQDE	-0.6	1052.08
11	SRLFDQFF	0.63	1041.19	53	EERQDEHG	-0.47	980.96
12	LFDQFFGE	0.68	984.09	54	RQDEHGFI	0.14	983.06
13	DQFFGEHL	0.47	974.05	55	DEHGFISR	0.16	942.01
14	FFGEHLLE	0.73	973.11	56	HGFISREF	0.48	974.1
15	GEHLLESD	0.18	880.92	57	FISREFHR	0.35	1073.24
16	HLLESDLF	0.7	955.09	58	SREFHRKY	-0.1	1104.25
17	LESDFLPT	0.59	903.02	59	EFHRKYRI	0.13	1130.33
18	SDLFPTST	0.49	848.93	60	HRKYRIPA	0.11	1022.23
19	LFPTSTSL	0.79	847	61	KYRIPADV	0.28	943.12
20	PTSTSLSP	0.44	770.86	62	RIPADVDP	0.28	863.98
21	STSLSPFY	0.66	882.99	63	PADVDPDLT	0.42	808.9
22	SLSPFYLR	0.72	964.15	64	DVDPLTIT	0.55	854.97
23	SPFYLRPP	0.7	958.15	65	DPLTITSS	0.49	814.91
24	FYLRPPSF	0.83	1008.21	66	LTITSSLS	0.7	802.94
25	LRPPSFRL	0.57	967.2	67	ITSSLSSD	0.35	790.84
26	PPSFRLAP	0.61	866.05	68	SSLSSDGV	0.25	732.75
27	SFLRAPSW	0.71	945.1	69	LSSDGVLT	0.5	772.86
28	LRAPSWFD	0.62	973.11	70	SDGVLTVN	0.37	785.85
29	APSWFDTG	0.56	861.92	71	GVLTVNNGP	0.57	737.85
30	SWFDTGSL	0.64	893.96	72	LTVNNGPRK	0.16	866.03
31	FDTGLSEM	0.44	880.99	73	VNGPRKQV	0.04	879.02
32	TGLSEMRL	0.4	888.07	74	GPRKQVSG	-0.04	809.92
33	LSEMRLEK	0.16	987.2	75	RKQVSGPE	-0.12	881.99
34	EMRLEKDR	-0.27	1058.24	76	QVSGPERT	0.04	854.93
35	RLEKDRFS	-0.12	1032.18	77	SGPERTIP	0.23	837.95
36	EKDRFSVN	-0.13	976.06	78	PERTIPIT	0.49	908.09
37	DRFSVNLD	0.19	947.02	79	RTIPITRE	0.27	967.16
38	FSVNLDVK	0.44	903.04	80	IPITREEK	0.16	967.15
39	VNLDVKHF	0.46	953.1	81	ITREEKPA	-0.02	925.07
40	LDVKHFSP	0.47	924.07	82	REEKPAVT	-0.1	911.04
41	VKHFSPEE	0.19	954.06	83	EKPAVTAA	0.19	767.89
42	HFSPEELK	0.25	968.09	84	PAVTAAPK	0.36	735.89

Columns 2 and 6: The peptide sequence. Columns 3 and 7: Hydrophobicity (arbitrary units) as provided by manufacturer (Mimotopes). Columns 4 and 8: The calculated molecular weight as provided by the manufacturer (Mimotopes).

formed strands β 3, β 8, and β 9, and buried residues from these three β strands interacted to form the compact hydrophobic core of the β sandwich. The C-terminal extension sequence PERTIPTREEK was in the unstructured region of the C terminus and was solvent-exposed.

A space-filled model of α B crystallin was generated to view the structural topology of the α B crystallin subunit-subunit interactions sites (Fig. 5B). The hydrophobic surface is presented as green, hydrophilic surface is orange, and charged surface is red or blue for positive or negative charges, respectively. The surfaces not involved in the subunit-subunit interaction are white. The N-terminal helical

sequence LFPTSTSLSPFYLRPPSF was solvent-exposed, with an exposed hydrophobic surface calculated to be 79%. The hydrophobic surface for the sequences FSVNLDVK, LTITSSLS, and GVLTVNNGP that formed β 3, β 8, and β 9 was calculated to be 65%, 68%, and 71%, respectively, and an average of 68% for the interface formed by all three β strands. The calculated hydrophobic surface for the C-terminal extension PERTIPTREEK was 58% hydrophobic. The high proportion of hydrophobic surface in the interactive sequences was consistent with the importance of hydrophobic side chains in interactions between human α B crystallin subunits.

Figure 5. (A, top) Mapping of the interactive sequences to the 3D computer model of human α B crystallin. All five binding regions (one in the N terminus, one in the C terminus, and three in the α crystallin core domain) are solvent-exposed in the monomeric subunit. Interactive sites identified by the protein pin arrays are in color and coded as follows: blue, acidic; red, basic; green, hydrophobic; orange, hydrophilic. (B, bottom) A space-filling representation of human α B crystallin using the same colors as in A.

Discussion

The results obtained using the α B crystallin protein pin array identified five consensus sequences involved in the interactions between subunits of α B crystallin and α A crystallin: $_{37}\text{LFPTSTSLSPFYLRPPSF}_{54}$, $_{75}\text{FSVNLDVK}_{82}$, $_{131}\text{LTITSSLS}_{138}$, $_{141}\text{GVLTVNGP}_{148}$, and $_{155}\text{PERTIPITREEK}_{166}$. Although several groups have identified individual residues that might be important for structure and/or function of α B crystallin, the results reported in this paper are the first to identify specific interactive sequences in α B crystallin that might be important in both structure and function. The sequences identified using protein pin arrays were consistent with interactive sequences identified using the X-ray crystal structures of Mj sHSP16.5 and wheat sHSP16.9 (Kim et al. 1998; van Montfort et al. 2001). In spite of the remarkable overlap in the interacting sequences of α B crystallin, wheat sHSP16.9, and Mj sHSP16.5, it is intriguing that α B crystallin does not form mono-disperse assemblies as observed in wheat sHSP16.9 and Mj sHSP16.5. Although the α crystallin core domain is highly conserved, the N- and C-termini of α B crystallin are not only longer but vary significantly in sequence compared to those of wheat sHSP16.9 and Mj sHSP16.5. These differ-

ences in the primary sequence can account for the variability in the quaternary structure and the observed polydispersity of α B crystallin assemblies in solution. The 3D structural model of human α B crystallin constructed using the 2.8 Å crystal structure of wheat sHSP16.9 contained an “ α crystallin core” domain composed of seven β strands that formed a compact β sandwich characteristic of sHSPs, and agrees remarkably well with previous homology models (Muchowski et al. 1999b; Feil et al. 2001; van Montfort et al. 2001; Guruprasad and Kumari 2003). Superposition of the human α B crystallin homology model with the wheat sHSP16.9 and Mj sHSP16.5 crystal structures resulted in remarkable overlap that agreed with the secondary structure predicted by McHaourab using electron spin resonance (ESR) data (Koteiche et al. 1998; Koteiche and McHaourab 1999). Point mutations of conserved residues in α B crystallin that had little or no effect on complex assembly or chaperone function were outside the interactive sequences identified using protein pin arrays (Plater et al. 1996; Hepburne-Scott and Crabbe 1999; Muchowski et al. 1999a; Derham et al. 2001). When mutations were made in *B. japonicum* sHSPH (small heat shock protein H, found in mammals) within sequences that were homologous to those identified by the α B crystallin pin arrays (F118, I124, S133), smaller assemblies were observed, whereas mutations made outside of the sequences identified by the pin array (H83, E87, G102, D109, E110) did not change the oligomeric assembly of sHSPH (Lentze et al. 2003). N78D and N146D mutations in $_{75}\text{FSVNLDVK}_{83}$ and $_{141}\text{GVLTVNGP}_{149}$ resulted in larger oligomeric assemblies (Gupta and Srivastava 2004) that were consistent with the pin array results that identified these sites as subunit-subunit interaction sites. Together, these data reinforce the importance of the α crystallin core domain in dimerization and the role of the N and C termini of α B crystallin in higher-order assembly.

Deletion of the N-terminal conserved motif SRLFDQFFG in both α A and α B crystallin resulted in altered subunit exchange dynamics but did not change the oligomer size distribution in either protein (Pasta et al. 2003). Although the sequence $_{21}\text{SRLFDQFF}_{28}$ interacted strongly with α B crystallin, there was no significant interaction with α A crystallin. In addition, the 3D model of α B crystallin indicated that the sequence SRLFDQFFG was involved in intramolecular interactions and stabilization of the N terminus. α B crystallin truncation mutants in which the first 56 residues in the N terminus and the last 18 residues in the C terminus and α A crystallin N- and C-terminal truncation mutants in which residues corresponding to the α B crystallin sequences 1–56, 1–65, and 1–85 were deleted resulted in smaller oligomers that are consistent with the pin array data (Bova et al. 2000; Feil et al. 2001). The pin array identified the N-terminal sequence $_{37}\text{LFPTSTSLSPFYLRPPSF}_{54}$ and the C-terminal sequence $_{155}\text{PERTIPITREEK}_{166}$ that appear

in these deletion regions. Cross-linking experiments between α A crystallin and α B crystallin also suggested that C terminus extensions in both α A crystallin and α B crystallin are in close proximity in the oligomer (Swaim et al. 2004). It is likely that the sequences $_{37}\text{LFPTSTSLSPFYLRPPSF}_{54}$ and $_{155}\text{PERTIPITREEK}_{166}$ are involved in higher-order assembly and are not involved in the dimer formation. The α crystallin core domain sequences $_{75}\text{FSVNLDVK}_{82}$, $_{131}\text{LTITSSLS}_{138}$, and $_{141}\text{GVLTVNGP}_{148}$ may contribute to the formation of a dimer interface. This hypothesis is consistent with previous experiments that found the conserved C-terminal motif I-X-I to be critical for assembly but not for dimer formation (Kim et al. 1998; Studer et al. 2002; Thampi and Abraham 2003) and the α crystallin core domain to be the structural determinant of dimerization (Koteiche and McHaourab 2002). Domain swapping of the N and C termini between *C. elegans* sHSP12.2 and α B crystallin showed that the N and C termini of α B crystallin were responsible for higher-order assemblies (Kokke et al. 2001). These independent reports support the hypothesis that the N terminus sequence $_{37}\text{LFPTSTSLSPFYLRPPSF}_{54}$ and the C terminus sequence $_{155}\text{PERTIPITREEK}_{166}$ are important for assembly. The α A crystallin C terminus truncation mutations (1–168 and 1–172) that were outside the homologous α B crystallin C terminus sequence $_{155}\text{PERTIPITREEK}_{166}$ identified by the pin array did not alter the oligomeric assembly (Bova et al. 2000). When the α A crystallin C terminus was truncated (1–157, 1–162, 1–163) to include the homologous α B crystallin C terminus sequence $_{155}\text{PERTIPITREEK}_{166}$, identified by the pin array, a dramatic effect on the oligomeric assembly was observed (Bova et al. 2000).

Recent studies exploring the role of multiple sequences in the chaperone function of α B crystallin suggest that the when α B crystallin acts as a molecular chaperone, sequences identified in this report are important for selective interactions of α B crystallin with a diverse number of target proteins (Putilina et al. 2003). The effectiveness of the protein pin arrays in identifying interactive sequences is due, in part, to the fact that the β strand-rich α crystallin core domain forms a large extended solvent-exposed surface that may not require significant contributions dependent upon 3D molecular organization. Unlike enzymes and antibodies, which have highly specific interactions with partner proteins and have specialized 3D tertiary structures for these interactions, molecular chaperones interact selectively, rather than specifically, with a variety of proteins. Multiple domains of varying affinity are expected to be advantageous in adaptation of molecular chaperones to variable domains exposed on the surfaces of partially unfolded or unfolding proteins. The 3D model of the structure of the α crystallin core domain, conserved in most sHSPs, was remarkably similar to the crystal structure for Mj sHSP16.5 and wheat sHSP16.9, suggesting that structural conservation of the α

crystallin core domain in the absence of primary sequence conservation may be important for the function of sHSPs. Even though the sequence homology in the conserved α crystallin core domain is <40% in the sHSPs studied, the α crystallin core domain interactive sequences identified in this report corresponded to β strands in the structures of all three sHSPs and suggest a preference for that structural motif in sHSPs (Caspers et al. 1995). The α A crystallin mini-chaperone identified by Sharma et al. (2000) using proteolysis and the *Neurospora crassa* sHSP30 functional domains identified by Narberhaus (2002) using two-hybrid analysis overlapped with the interactive sequences identified by the protein pin arrays (Sharma et al. 2000; Plesofsky and Brambl 2002). The current results are consistent with a binding model involving multiple interactive sites on the surface of the sHSPs and suggest a complex multidomain mechanism for the adaptive and selective binding of ligands to sHSPs with a variety of substrates under conditions of cellular stress. There is an intriguing possibility that a conserved set of interactive domains on the surface of the conserved α crystallin core domain in sHSPs can vary their selective affinity dynamically for adaptation to a diverse set of interactive sequences on target proteins. The structure-function relationships established by the protein pin arrays and the 3D model of α B crystallin will enable site direct mutational analyses of these structurally and functionally important sequence motifs in α B crystallin and other homologous small heat shock proteins. The results confirm the utility of protein pin arrays for the identification of functional sequences that are involved in binding filaments and binding and preventing the unfolding and aggregation of chaperone target proteins.

Materials and methods

Synthesis of pins, binding, and detection of peptides binding to ligand proteins

The α B crystallin protein pin array was designed to measure the binding of multiple peptides to either myoglobin or α A crystallin or α B crystallin in a parallel and simultaneous manner. Peptides corresponding to residues 1–175 of human α B crystallin were synthesized employing a simultaneous peptide synthesis strategy developed by Geysen, called Multipin Peptide Synthesis (Mimotopes) (Geysen 1990; Chin et al. 1997). These peptides were synthesized on derivatized polyethylene pins arranged in a microtiter plate format (Fig. 1A). Each peptide was eight amino acids in length, and consecutive peptides were offset by two amino acids. All peptides were covalently bonded to the surface plastic pins. The first peptide was $_{1}\text{MDIAIHHP}_{8}$, and the last peptide was $_{168}\text{PAVTAAPK}_{175}$ for the human α B crystallin. Eighty-four peptides corresponding to the 175-amino-acid primary sequence of human α B crystallin were synthesized. Human Myoglobin (cat. no. 6036) and mouse anti-human Myoglobin antibody (cat. no. M7773) were purchased from Sigma-Aldrich. Recombinant human α A and α B crystallin were expressed and purified to greater than 98% purity as determined by SDS-PAGE (Muchowski et al.

1999b). Anti-human α A crystallin (cat. no. SPA-221) and anti-human α B crystallin (cat. no. SPA-222) antibodies were purchased from Stressgen. After synthesis, the pins were precoated at room temperature with 2% bovine serum albumin (BSA), 0.1% Tween-20, and 0.1% sodium azide in 10 mM phosphate buffered saline (pH 7.2) (PBS) for 1 h, and washed three times for 10 min each with 10 mM PBS. To screen for binding to the peptides, fixed concentrations of myoglobin, α A crystallin, or α B crystallin were dissolved in 10 mM PBS containing 0.05% Tween-20, added to each well and incubated for 1 h at room temperature. The pin array was washed three times for 10 min each with 10 mM PBS containing 0.05% Tween-20. Monoclonal/polyclonal primary antibodies for myoglobin, α A crystallin, or α B crystallin were diluted into PBS buffer, added to each well and incubated for 1 h at room temperature. Subsequently, the pin array was washed three times for 10 min each with 10 mM PBS containing 0.05% Tween-20. Anti-rabbit horseradish peroxidase conjugate secondary antibodies were diluted into PBS buffer, added to each well, and incubated for 1 h at room temperature. The pin array was washed three times for 10 min each with 10 mM PBS containing 0.05% Tween-20. A chromogenic substrate of horseradish peroxidase 3,3',5,5'-Tetramethylbenzidine (TMB) (Pharmingen) which is colorless was added, and the reaction was carried out for 45 min. A positive reaction resulted in the formation of a blue color. The reaction was stopped by adding 6N sulfuric acid, which changed the color from blue to yellow. The absorption at 450 nm was measured by an ELISA reader (BioTek). The pin array was regenerated by sonicating in a water bath (VWR Aquasonic) with 100 mM PBS, containing 1% sodium dodecyl sulfate (SDS) and 0.1% 2-mercaptoethanol at 60°C for 10 min. Next, the pin array was rinsed three times in deionized water, preheated to 60°C for 30 sec each time, and shaken in water preheated at 60°C for 30 min. The pin array was then washed with methanol at 60°C for 15 sec and air-dried and stored at -20°C for future use. Each ligand was assayed 2–5 times against the human α B crystallin protein pin array peptides. A single pin array was used for all experiments and did not show any significant decrease in efficiency, even after repeated use up to 30 times.

Molecular modeling of human α B crystallin

The homology modeling program Molecular Operating Environment (Chemical Computing Group) was used to construct the 3D homology model of human α B crystallin. MOE employs a number of techniques including and not limited to multiple sequence alignment, structure superposition, contact analyzer, and fold identification to develop homology models based on available high-resolution crystal and/or NMR structures of the template protein molecule (Levitt 1992). It also uses advanced techniques such as analysis of the stereochemical quality of the predicted models which takes into account parameters like planarity, chirality, ϕ/ψ preferences, χ angles, nonbonded contact distances, unsatisfied donors, and acceptors (Fechteler et al. 1995). The overall result agreed well with existing biochemical information for the α B crystallin structure.

The wheat sHSP16.9 crystal structure was chosen as the template for the homology modeling of human α B crystallin because the wheat sHSP16.9 has the highest degree of sequence similarity with human α B crystallin (40% in the α crystallin core domain and 25.4% overall) of all the available crystal structures of sHSPs. In building the homology model of human α B crystallin, the primary sequence of human α B crystallin was first coarsely aligned to that of the template protein wheat sHSP16.9 primary sequence using ClustalX (Jeanmougin et al. 1998; Ayiyar 2000). The predicted

secondary structure of human α B crystallin was then obtained (JPhred) and verified with the available spin labeling information about the structural elements (Koteiche and McHaourab 1999). The verified predicted secondary structure of human α B crystallin (Fig. 4) was then structurally aligned with the observed secondary structure of the wheat sHSP16.9. This alignment was used by MOE to create a series of 10 energy-minimized putative models. Each model was evaluated using the ModelEval module of MOE, and the best model was picked as the final model. This model was further verified by Procheck (Morris et al. 1992).

For calculation of the percent surface that was hydrophobic for the subunit-subunit interaction sequences, an electrostatic potential was applied to the homology model and a script was used to generate the hydrophobic surface area based on the hydrophobic residues. Internal residue interactions were identified using the Protein Contact module of MOE.

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