α-Crystallin Regions Affected by Adenosine 5′-Triphosphate Identified by Hydrogen−Deuterium Exchange†

Azeem Hasan, David L. Smith, and Jean B. Smith*

Department of Chemistry, University of Nebraska, Lincoln, Nebraska 68588-0304

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ABSTRACT: ATP interaction with lens α-crystallins leading to enhanced chaperone activity is not yet well understood. One model for chaperone activity of small heat shock proteins proposes that ATP causes small heat shock proteins to release substrates, which are then renatured by other larger heat shock proteins. A similar role has been proposed for ATP in α-crystallin chaperone activity. To evaluate this model, ATP-induced structural changes of native human α-crystallin assemblies were determined by hydrogen−deuterium exchange. In these experiments, hydrogen−deuterium exchange, measured by mass spectrometry, gave direct evidence that ATP decreases the accessibility of amide hydrogens in multiple regions of both αA and αB. The surface encompassed by these regions is much larger than would be shielded by a single ATP, implying that multiple ATP molecules bind to each subunit and/or ATP causes a more compact α-crystallin structure. Such a conformational change could release a bound substrate. The regions most affected by ATP are near putative substrate binding regions of αA and αB and in the C-terminal extension of αB. The widespread decrease in hydrogen−deuterium exchange with particularly large decreases near substrate binding regions suggests that ATP releases substrates via both direct displacement and a global conformational change.

α-Crystallin, a major structural protein of the human lens, is composed of two subunits, αA and αB, each about 20 kDa, which form oligomers with a molecular mass of approximately 800 kDa. The α-crystallins have regions of homology with other small heat shock proteins (sHsp) (*1*, *2*) and exhibit similar chaperone-like activity in their ability to prevent aggregation of partially denatured proteins (*3*, *4*). The presence of α-crystallins in tissues other than the lens (*1*, *5*) suggests a physiological significance for this chaperone activity beyond preventing insoluble aggregates associated with cataract. In a current model for sHsp chaperone activity, denatured substrates bind to hydrophobic sites forming soluble sHsp/substrate complexes that maintain the substrate in a soluble state until it can be delivered to other larger Hsps for proper refolding (*6*). The chaperone activity of lens α-crystallins appears to be similar to other sHsps with ATP assisting in the delivery of the denatured substrate to another Hsp (*7*). Such a role for ATP is consistent with its high concentration in the lens (3−7 mM) (*8*) and its binding constant to α-crystallin of 7.84×10^3 M^{-1} (*9*, *10*). Whether ATP binding to αA and αB differs has not been determined. Previous evidence for a role for ATP in α-crystallin activity includes ATP enhancement of α-crystallin antiaggregatory effects (*11*) and a requirement for ATP plus other heat shock proteins for α-crystallin restoration of activity to partially denatured enzymes (*7*). ATP hydrolysis is not required for these effects (*12*).

Previous data have not shown whether ATP interaction with α-crystallin leading to enhanced chaperone activity involves both αB and αA, whether the interaction is at specific binding sites, or if the ATP interaction is a more general phenomenon. Enzymatic digestion of recombinant αB-crystallin was severely limited by ATP with the greatest inhibition in a region including residues 60−123 (*13*). These data were interpreted as evidence that ATP binds within this core region. However, since enzymatic digestion of α-crystallin, with or without ATP present, proceeds first with cleavages in the N- and C-termini and the core region of αB is the last to be digested, it may be that prior cleavage in the termini is required before cleavage can proceed in the core region. If this is true, the observed inhibition within αB 60−123 may have been due not to specific binding to sites within this region but to ATP causing a conformational change that produced an α-crystallin resistant to digestion.

To obtain more detailed information about ATP interaction with human α-crystallins, we have used hydrogen−deuterium (H/D)[1] exchange detected by mass spectrometry (*14*) to measure the effect of ATP on the accessibility of amide hydrogens. This technique yielded structural information specific to both αA and αB in their native assemblies under nearly physiological conditions. These H/D exchange data gave direct evidence that ATP induced global structural changes in both αA and aB and indicated specific regions of interaction adjacent to proposed areas critical to chaperone activity.

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* Address correspondence to this author. Phone: 970-264-6647. Fax: 402-472-9402. E-mail: jsmith8@unl.edu.

[1] Abbreviations: H/D, hydrogen−deuterium; EDTA, ethylenediaminetetracetic acid; DTT, dithiothreitol; HPLC, high-pressure liquid chromatography; TFA, trifluoroacetic acid; ESIMS, electrospray ionization mass spectrometry, MS/MS, tandem mass spectrometry.

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MATERIALS AND METHODS

Isolation of α-Crystallins. α-Crystallins were isolated from two clear lenses obtained from a 19-year-old organ donor (Lions Eye Bank, Omaha, NE). The lenses had no visible opacities, and the donor history indicated no diseases known to affect lens clarity. The lens was homogenized using a stirring bar for 4 h in a buffer (5 mM sodium phosphate, 0.1 mM EDTA, 0.1 mM DTT, and 0.02% NaN_3, pH 6.9). The lens homogenate was centrifuged at 15000*g* and 4 °C for 1 h. The supernatant (water-soluble crystallins) was separated from the pellet and stored at −80 °C until analysis. α-Crystallins were isolated from other water-soluble crystallins by size exclusion chromatography (2.5 × 95 cm, Toyo-pearl HW-55sf, Toso HAAS, Montgomeryville, PA). The proteins were eluted by gravity at a flow rate of 12 mL/h using the same buffer that was used for the protein extraction. Protein elution was monitored at 280 nm. Fractions corresponding to α-crystallins were pooled and stored at −80 °C until analysis (*15*).

Construction of Peptide Maps. Preliminary to determining the effect of ATP on H/D exchange of α-crystallins, it was necessary to establish the fragmentation patterns of αA- and αB-crystallins digested by pepsin. For these identifications, αA- and αB-crystallins were prepared from α-crystallins (2 nmol) by reversed-phase HPLC (Vydac C4 column, 4.6 mm × 150 mm, 5 μm) using a binary gradient system (Rainin, Woburn, MA). Solvent A was water and solvent B was acetonitrile, both with 0.1% trifluoroacetic acid (TFA). The proteins were eluted using a gradient of 30−50% solvent B in 20 min and detected by their absorbances at 280 nm. The HPLC fractions corresponding to αA and αB were pooled separately and then concentrated and resuspended in a buffer (5 mM sodium phosphate, pH 2.5).

Enzymatic digestion of the α-crystallins, αA and αB separately, was performed with the same conditions that would be used to digest the deuterated crystallins in the H/D exchange experiments. These conditions, 0 °C and pH 2.5, were chosen to minimize back-exchange of deuterium. A high pepsin:crystallin ratio of 2:1 (w:w) with 1 nmol of protein permitted use of a short digestion time (5 min), which also minimized back-exchange. Following digestion, the peptides were separated and analyzed by reversed-phase HPLC directly coupled to a mass spectrometer. The HPLC consisted of a microbore column (Vydac C18, 1 mm × 50 mm, 300 Å, 5 μm) and a binary gradient system (Shimadzu, Columbia, MD). Much of the HPLC system was maintained at 0 °C to avoid back-exchange of deuterium. Solvent A was water and solvent B was acetonitrile, both with 0.05% TFA. Peptides were eluted with a gradient of 2−60% solvent B in 60 min and a flow of 50 μL/min. The eluate (5 μL min^{-1}) was directed on-line to an ion trap mass spectrometer equipped with an electrospray ionization source (LCQ, Finnigan MAT, San Jose, CA) and the other eluate (45 μL) was monitored by a UV detector and collected for further analysis. The instrument was calibrated with a mixture of Ultramark, caffeine, and the peptide MRFA over a mass range of 50−2000 Da with a mass uncertainty of ±0.1 Da.

Peptides were identified by matching their molecular masses with the calculated masses of peptides derived from typical cleavage sites of pepsin (L, I, F, Y, and W), based on the published sequences of human lens αA- and αB-crystallins. Each peptide identification was confirmed by collision-induced tandem mass spectrometry (MS/MS) performed as part of the peptide analysis on the ion trap mass spectrometer. The isolation width was 1.5 Da. Peptides were fragmented by helium at a collision energy of 25−30%.

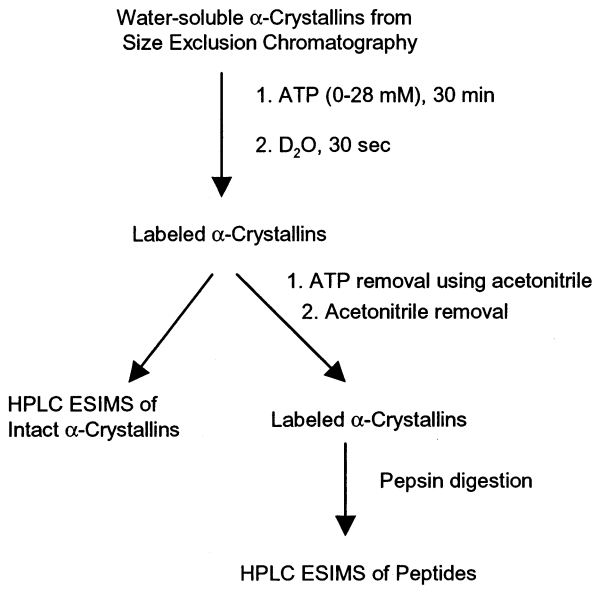

FIGURE 1: Procedure for hydrogen−deuterium (H/D) exchange of α-crystallins in the presence of ATP. The isolated α-crystallins were incubated with ATP and then deuterated in D_2O. The deuterium incorporated into α-crystallins was determined from the molecular masses of the intact deuterated crystallins analyzed by ESIMS. After removal of ATP, which inhibits enzymatic digestion, the α-crystallins were digested with pepsin under conditions that permitted little back-exchange of deuterium. The peptides were analyzed by ESIMS to determine which regions of α-crystallin were affected by ATP.

Reaction of α-Crystallins with ATP Followed by Hydrogen−Deuterium Exchange. A scheme illustrating the experimental procedure for the H/D exchange of α-crystallins and ATP is shown in Figure 1. Aliquots of α-crystallins (300 pmol/10 μL) were incubated with equal volumes of ATP in 50 mM phosphate buffer to give final concentrations of 0, 7, 14, and 28 mM, pH 7.0 at 37 °C, for 30 min. After incubation, each sample was diluted 20-fold with D_2O phosphate buffer (50 mM, pD 7.0) containing the same concentration of ATP and incubated again for 30 s. H/D exchange was quenched by lowering the pD to 2.5 and immediately placing the samples in dry ice. Each sample was analyzed as intact proteins and as peptides formed by digestion of the proteins.

To determine the extent of deuterium back-exchange occurring during analysis, fully deuterated αA and αB were prepared by heating the proteins in D_2O at 80 °C for 8 h. These samples were analyzed by the same procedures as the other deuterated α-crystallins.

Analysis of Intact α-Crystallins after Reaction with ATP and Hydrogen−Deuterium Exchange. The deuterated α-crystallins were trapped in a protein microtrap cartridge (5 μL; Michrom Resources, Auburn, CA) and desalted with 400 μL of a solution of 2% acetonitrile in water with 0.1% TFA. The proteins were then eluted and separated into αA- and αB-crystallins by reversed-phase HPLC (Vydac C4 microbore column, 1 × 50 mm, 5 μm, Micro-Tech Scientific) using a gradient of 35−50% acetonitrile in 5 min. The

effluent from the HPLC flowed on-line into a quadrupole ESIMS (Micromass Platform II, Manchester, U.K.). All scans of fractions containing protein were summed to obtain the spectra and assign molecular masses. For protein analysis, the mass spectrometer was calibrated with myoglobin (M_r 16951) with a typical mass accuracy of ±2 Da for a 20 kDa protein. Data were processed with Mass Lynx 3.3 software.

ATP Removal. The presence of ATP inhibited pepsin digestion of the crystallins. Several procedures for removing the ATP were tried and dismissed either because they were not effective or because too much of the incorporated deuterium was lost during ATP removal. We found ATP could be removed with minimal loss in deuterium by loading the sample onto a protein trap cartridge, washing it with 15% acetonitrile containing 0.05% TFA to remove the ATP, and then eluting the protein with 50% acetonitrile. Because acetonitrile interfered with the subsequent reversed-phase HPLC, the sample was collected in chilled ether, which facilitated extraction of the acetonitrile. The ether layer was removed, and a stream of nitrogen was blown over the water-soluble sample to remove any remaining ether. This procedure could be performed in 90 s.

Analysis of α-Crystallins after Hydrogen−Deuterium Exchange and ATP Removal. The α-crystallin samples incubated in 7 mM ATP were chosen for further examination after ATP removal. These proteins were analyzed intact as described above and also as peptides after digestion by pepsin. Following digestion, the peptides were concentrated and desalted using a peptide cartridge (5 μL; Michrom Resources), fractionated by reversed-phase HPLC, and analyzed by a procedure similar to the one described above for construction of peptide maps, but employing a hybrid quadrupole, time-of-flight mass spectrometer (Micromass Q-Tof 1). This mass spectrometer was calibrated with [Glu]-fibrinopeptide B over a mass range of 100−2000 Da. Masses of peptides from deuterated α-crystallins reflected the number of deuteriums incorporated into the intact protein during the D_2O incubation at pD 7.0. Because H/D exchange occurred before the pH was lowered and the protein was digested, incorporation of deuterium indicated exchange in the native assemblies of αA and αB. Comparison of the sample incubated in 7 mM ATP with one taken through the same procedure but with 0 mM ATP showed the effect of ATP on H/D exchange. Uncertainty of the average mass determinations was typically ±0.05 Da.

To establish whether the decreased H/D exchange of α-crystallins was specific to ATP, similar experiments were performed with 7−28 mM KCl and 7−28 mM ADP. Neither KCl nor ADP affected deuterium incorporation.

RESULTS

Analysis of Intact αA- and αB-Crystallins after Incubation with ATP and H/D Exchange. The α-crystallins examined by H/D exchange were native assemblies of αA and αB isolated from total water-soluble crystallins by size exclusion chromatography. H/D exchange of the α-crystallins, indicated by the increase in molecular mass of the intact proteins, showed a gain of 82 deuteriums for αA-crystallin and 88 deuteriums for αB after a 30 s incubation in D_2O (Table 1). When ATP was present, the uptake of deuterium was less and depended on the concentration of ATP (Figure 2). The decrease in the number of deuteriums in αA and αB ranged from 5 Da in both αA and αB in 3.5 mM ATP to 11 Da in αA and 13 Da in αB in 14 mM ATP.

Table 1: Molecular Masses of α-Crystallins

	αA	ΔD	αB	ΔD
native	19951		20201	
30 s D_2O labeling	20233	82	20289	88
3.5 mM ATP plus 30 s labeling	20028	77	20284	83
7.0 mM ATP plus 30 s labeling	20026	75	20079	78
14.0 mM ATP plus 30 s labeling	20022	71	20076	75
28.0 mM ATP plus 30 s labeling	20023	72	20076	75

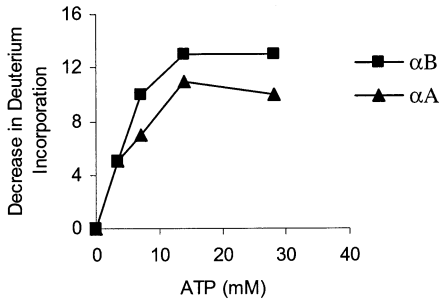

FIGURE 2: Relationship between the decrease in deuteriums incorporated into αA and αB and the concentration of ATP present during H/D exchange.

The extent of deuterium back-exchange occurring during desalting and analysis was determined by comparing the number of deuteriums incorporated into heat-denatured samples incubated for 8 h with the maximum possible number of incorporated deuteriums calculated from the amide hydrogens in each sequence. Deuterium recovery was 81.4% for αA and 81.1% for αB. When the procedure for extracting ATP was included, recovery was 72.7% for αA and 71.7% for αB. Therefore, the ATP extraction step was included in the analysis of samples used to determine the effect of ATP, even if no ATP was present.

H/D exchange in 7 mM ATP, the concentration chosen for the pepsin digestion experiments, showed 7 fewer deuteriums in αA and 10 fewer in αB than at 0 mM ATP. Because the additional time required to remove ATP before pepsin digestion allowed more deuterium loss, protein samples analyzed after ATP removal had only 73 deuteriums in αA and 76 in αB (Figure 3, middle). However, H/D exchange of proteins incubated in 0 mM ATP (Figure 3, middle) and 7 mM ATP (Figure 3, bottom), both taken through the ATP removal procedure, again showed that 7 mM ATP decreased deuterium by 7 for αA and 9 for αB. The additional small peaks in the spectra (Figure 3) are due to loss of the C-terminal serine (−87 Da) of αA, and/or potassium adducts (+39 Da), acetylation (+42 Da), and phosphorylation (+80 Da) of αA and αB.

Analysis of Peptides of αA- and αB-Crystallins. Before deuterated α-crystallins could be analyzed as peptides, each peptide produced by pepsin digestion had to be unambiguously identified. Pepsin digestion of α-crystallins is reproducible but not totally predictable. Typical cleavage sites are at either side of Leu, Ile, Phe, Tyr, and Trp. The peptides, identified from their molecular masses and confirmed by the masses of fragments in MS/MS analysis, represented 97% of αA and 99% of αB (column 1, Table 2).

The masses of peptides derived from deuterated proteins showed the extent of H/D exchange in the specific regions

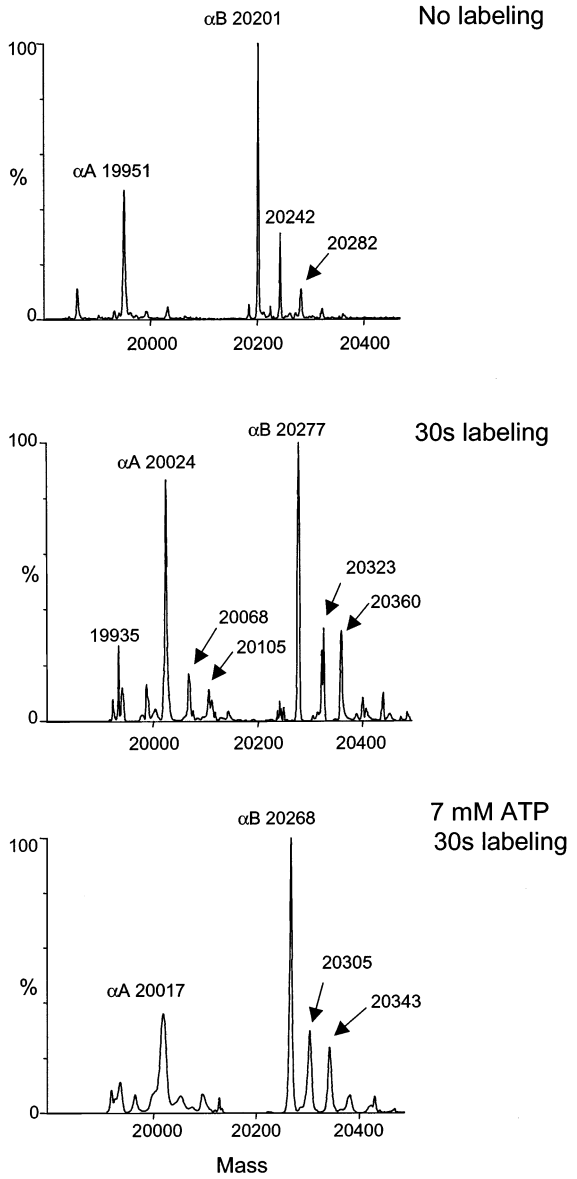

FIGURE 3: Mass spectra of intact α-crystallins. The top spectrum shows undeuterated αA (M_r 19951) and αB (M_r 20201). Additional peaks are due to αA minus the C-terminal serine, acetylated and phosphorylated forms of αA and αB, and/or adducts of potassium. The middle spectrum shows increases of 73 Da for αA and 76 Da for αB due to deuterium uptake during incubation in D_2O for 30 s. In the bottom spectrum, α-crystallins deuterated after incubation in 7 mM ATP show less deuterium uptake, 7 fewer deuteriums in αA and 9 fewer in αB. To obtain proper controls, deuterated samples, even those not incubated in ATP, were taken through the ATP removal procedure.

from which the peptides were derived. Peptides from most regions of deuterated αA and αB, both with and without ATP incubation, were found in the pepsin digests. These regions are underlined in Figure 4. For many peptides, the mass of the peptide from α-crystallins incubated with 7 mM ATP was within 1 Da of the same peptide from α-crystallin not exposed to ATP (dotted lines in Figure 4). Four regions, αA 59−93, αA 106−127, αB 64−99, and αB 144−175, showed decreases of more than 1 Da for the proteins incubated in ATP (solid lines in Figure 4). The increase in mass of each peptide due to H/D exchange (columns 2 and 3), the difference in deuterium uptake when ATP was present (column 4), and the average difference per exchangeable amide hydrogen for each peptide (column 5) are given in Table 2. Residue numbers in italics indicate overlapping peptides that are part of a larger peptide. With the exception of αA 106−127, the peptides with greater than 0.1 Da decreases per exchangeable residue were the same peptides that had greater than 1 Da decrease for the whole peptide.

Table 2: Change in Peptide Masses of Deuterated α-Crystallins (Da)

residue no.	30 s D_2O	7 mM ATP 30 s D_2O	change due to ATP	av change/ exchangeable residue
αA				
3−22	5.0	4.3	−0.7	−0.04
23−27	0.8	0.6	−0.2	−0.03
28−33	1.7	1.4	−0.3	−0.06
32−37	1.6	1.3	−0.3	−0.05
38−52	6.1	5.9	−0.2	−0.02
53−57	1.7	1.4	−0.3	−0.01
59−93	15.2	11.2	−4.0	−0.12
63−76	*5.0*	*3.6*	*−1.4*	*−0.10*
72−75	*0.3*	*0.2*	*−0.1*	*−0.03*
75−85	*2.3*	*1.2*	*−1.1*	*−0.12*
94−108	1.1	0.9	−0.2	−0.02
106−127	9.7	8.1	−1.6	−0.07
130−133	0.7	0.7	−0.0	−0.00
134−139	0.9	0.7	−0.2	−0.04
140−173				
			sum αA:[a] −6.6	
αB				
3−24	6.2	5.2	−1.0	−0.05
24−27	0.8	0.6	−0.2	−0.05
28−37	2.9	2.2	−0.7	−0.08
38−54	7.3	5.7	−1.6	−0.11
55−67	5.1	4.3	−0.8	−0.08
64−99	15.8	11.7	−4.1	−0.12
68−75	*2.8*	*2.4*	*−0.4*	*−0.06*
76−79	*0.8*	*0.6*	*−0.2*	*−0.06*
80−89	*1.8*	*0.4*	*−1.4*	*−0.18*
90−112	5.3			
113−137				
138−143	0.7	0.6	−0.1	−0.02
144−175	13.0	10.1	−2.9	−0.11
			sum αB:[a] −9.3	

[a] The sums do not include overlapping peptides.

Overlapping peptides in some regions allowed more precise location of the amide hydrogens most affected by ATP. Within αA 59−93, where deuterium was decreased by 4.0, peptide 63−76 contained 1.4 of the reduction and peptide 75−85 included 1.1. Since peptide 72−75 showed only 0.1 Da reduction, a major portion of the reduction shown by 59−93 was in residues 59−62 and/or 86−93, two peptides not observed individually. These results suggest the reduction in deuterium uptake is widespread throughout the region of 59−93 except for 72−75. Similarly, of the 4.1 Da reduction in αB peptide 64−99, 0.4 Da was in peptide 68−75, 0.2 Da in peptide 76−79, and 1.4 Da in peptide 80−89, indicating considerable decrease in H/D exchange in peptides 64−67 and/or 90−99 and again suggesting widespread decrease in deuterium uptake throughout the region of 64−99 except for 68−79. It should be noted that summing H/D exchange of component peptides to give the H/D exchange of the parent peptide is only approximate because the amide linkage that is cleaved to form peptides is not included.

Spectra demonstrating H/D exchange in αA peptide 75−85, a region exhibiting a large reduction per residue in the presence of ATP, are shown in Figure 5. The average mass

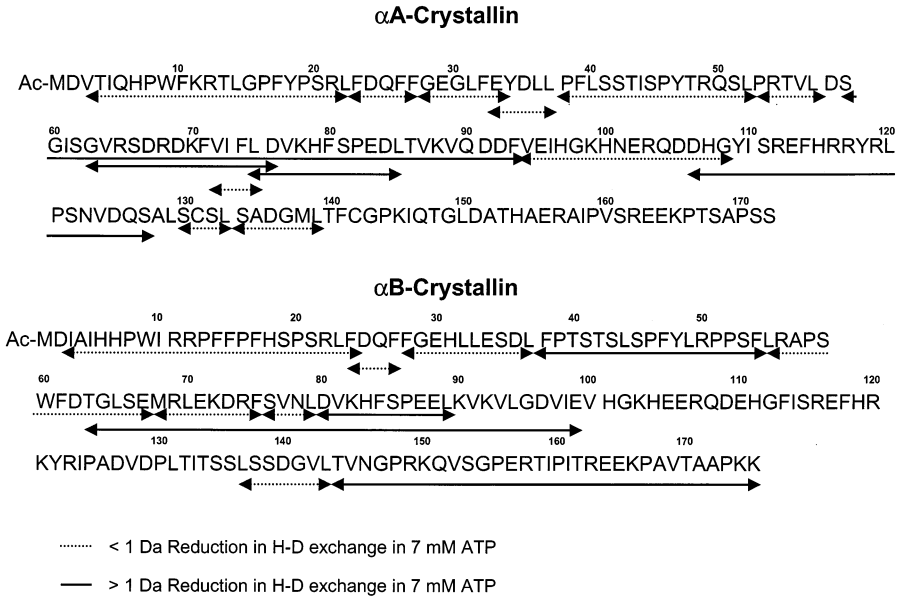

FIGURE 4: Sequences of αA and αB showing peptides produced by pepsin digestion. The masses of peptides from α-crystallins in which H/D exchange had taken place were determined and compared with masses from α-crystallins incubated in 7 mM ATP before the H/D exchange. For those underlined with a solid line, ATP caused greater than 1 Da decrease in deuterium uptake, while for those underlined with a dotted line, ATP caused less than 1 Da decrease. Regions not underlined did not yield mass spectra from which H/D uptake in the presence of ATP could be calculated.

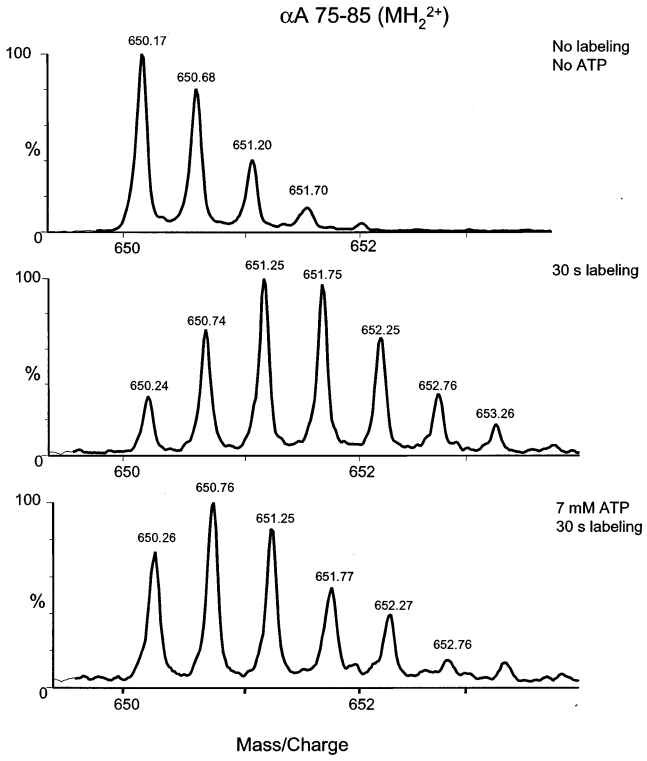

FIGURE 5: Mass spectra showing the doubly charged form of peptide αA 75−85. The top spectrum shows the isotope pattern due to natural abundance of ^{13}C. The shift in the masses in the middle spectrum is due to incorporation of deuterium during a 30s incubation in D_2O. The bottom spectrum shows inhibition of deuterium uptake in this peptide when the protein was incubated in 7 mM ATP before H/D exchange.

of the doubly charged ion for αA 75−85 increased from 650.69 to 651.85 Da due to H/D exchange. When the peptide came from αA incubated in 7 mM ATP, the mass of the doubly charged ion was 651.30 Da, a decrease of 0.55 Da. This difference corresponded to 1.10 fewer deuteriums in this part of the protein in the presence of 7 mM ATP.

Summation of the decreases in H/D exchange for the individual peptides in the presence of ATP yielded 6.6 for αA and 9.3 αB (Table 2), approximating the decreases (7 Da for αA and 10 Da for αB) measured for the intact proteins (Figure 3). The similarity in these approximate numbers suggests that H/D exchange of regions that could not be determined was affected little by ATP, since reduction in exchange was accounted for in regions that provided data.

DISCUSSION

The role of ATP in chaperone activity of small heat shock proteins, including α-crystallins, appears to be that of an assistant. α-Crystallins, without ATP, can sequester partially denatured proteins, preventing their aggregation and precipitation (*3*), but they are more effective when ATP is present (*11*). Even though ATP hydrolysis is not required (*12*), the phenomenon is specific to ATP. Wang and Spector noted that α-crystallins alone prevent irreversible aggregation of enzymes, but restoration of enzyme activity requires other heat shock proteins and ATP (*7*). These data for α-crystallin are consistent with a model proposed for other sHsps, in which the sHsps bind partially denatured proteins and then act cooperatively with other heat shock proteins, in the presence of ATP, to restore activity (*6*). Wang and Spector proposed that ATP releases the substrate by destabilizing the α-crystallin−denatured protein complex (*16*). Specifics about this interaction of ATP with α-crystallin and how it induces release of substrates were not determined. Destabilization may be due to ATP displacing the substrate, and/or ATP may induce structural changes that contribute to release of the substrate. The structural information obtained from the present H/D exchange experiments allows comparison of the regions of α-crystallin affected by ATP binding with proposed substrate binding sites. Such detailed structural information is required for creating and testing a model explaining ATP enhancement of chaperone activity.

Several studies have addressed the question of where substrates bind to α-crystallin. Because exposure of hydrophobic regions correlates with increased chaperone activity (*17*, *18*), some putative binding sites have been proposed on the basis of binding of hydrophobic probes. The hydrophobic probe, bis-ANS, binds to the N-terminal domain of αB-crystallin (*19*) as well as to peptides αA 79−88, αB 73−82, and αB 93−103 (*20*). These regions are near the areas with the largest ATP-induced reductions in H/D exchange (see Figure 4). As indicated in Table 2, ATP reduced H/D exchange by 1.6 Da for αB 38−54 in the N-terminus and 1.1 and 1.4 Da in peptides αA 75−85 and αB 80−89, respectively.

Cross-linking agents have also been used to identify substrate binding regions. With this approach, Sharma et al. showed interaction of αB 57−69 and 93−107 with alcohol dehydrogenase as the substrate (*21*), again near regions with large ATP-induced reductions in H/D exchange.

Another approach used to identify regions critical to chaperone activity is examination of chaperone activity of α-crystallin mutants. Data from mutant α-crystallins, summarized by Derham and Harding (*22*), show that substitutions in the N-terminus at αB 2, 24, and 27 and in the C-terminus at 174 and 175 decreased chaperone activity. The chaperone activity of αA Asp69Ser was also reduced. In contrast, several cysteine substitutions in αA69−88 caused increased chaperone activity (*23*). Mutations in αA at 9 and 37, throughout the α-crystallin domain from 109 to 120, and in the C-terminus had little effect on chaperone activity. In general, the ineffective mutations were in regions where ATP had little effect on H/D exchange. On the other hand, mutants that affected chaperone activity, both favorably and unfavorably, were near regions where ATP caused the largest decreases in H/D exchange.

The peptides with the largest ATP-induced decreases in H/D exchange, αA 75−85 and αB 80−89, are near the beginning of the "α-crystallin" domain (*6*, *24*), a region of high homology with other sHsps. Sharma and co-workers have identified sequences within the α-crystallin domain overlapping with αA 75−85 and αB 80−89 as functionally active chaperones. In αA, peptide 70−88 acts as a "minichaperone" (*25*), and mutation of Phe71 to Gly makes rat αA-crystallin totally inactive in suppressing aggregation of some proteins (*26*). Similarly, αB peptide 73−92 has chaperone activity, which is eliminated by mutation of Phe84 to Gly (K. Sharma, personal communication).

The structure of α-crystallin in this region critical to chaperone activity is of particular interest. Although a crystal structure of α-crystallin has not yet been obtained, the crystal structures of two sHsps with α-crystallin domains, *Methanococcus jannaschii* HSP 16.5 (*27*) and wheat 16.9 (*6*), have been determined. The regions in these proteins that align with αA 75−85 and αB 80−89 are in the third β-sheet. As in α-crystallin, this region in wheat 16.9 has low H/D exchange (P. Wintrode, personal communication).

The large ATP-induced decreases in H/D exchange in peptides near the putative substrate binding sites and included within the minichaperones support the hypothesis that ATP destabilizes the α-crystallin−denatured protein complex by displacing the bound substrate (*16*). In addition to these regions, ATP markedly reduced H/D exchange in αB in peptide 38−54 and the C-terminus and caused a general reduction in H/D exchange throughout both α-crystallins. No regions of increased H/D exchange were detected. These data indicate that ATP causes a general conformational change to a more compact structure, with less solvent exposure and consequently lower H/D exchange. Such a change is consistent with tryptophan fluorescence data indicating that the tryptophans of α-crystallin are less exposed in the presence of ATP (*10*, *11*, *16*). H/D exchange experiments showed relatively small decreases in deuterium in tryptophan-containing peptides αA 3−22 and αB 2−24 but a large decrease for αB 55−67 at higher ATP concentrations.

The large ATP reduction in H/D exchange in αB peptide 80−90 is consistent with results of proteolysis experiments demonstrating low reactivity of residues 60−123 in the presence of ATP (*13*). However, the effect of ATP on H/D exchange in the C-terminus of αB also offers another explanation for inhibition of enzymatic digestion. Slow digestion in the core could be due to ATP blocking the initial cleavage site in the C-terminus (*28*). A third possibility is that ATP interaction at the C-terminus creates a global conformational change that leads to reduced proteolysis.

Incubation of α-crystallins with neither KCl nor ADP had an effect on H/D exchange, indicating that the interaction was specific for ATP. This specificity for ATP has previously been noted in studies of ATP binding to α-crystallin (*10*), in the effect of ATP on proteolytic digestion of αB (*13*), and in experiments illustrating ATP destabilization of α-crystallin−substrate complexes (*16*).

The global conformational changes in αA and αB and the specific interactions of ATP at substrate binding regions indicate that there are two components to ATP destabilization of α-crystallin−substrate complexes. Both formation of a more compact α-crystallin structure and direct displacement of the bound substrates are likely contributors to ATP enhancement of chaperone activity.

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