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The structure and oxidation of the eye lens chaperone α A-crystallin

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3

4 **The structure and oxidation of the eye lens chaperone α A-**
5 **crystallin**

6

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29

30

31 **Abstract**

32

33 The small heat shock protein (sHsp) α A-crystallin is a molecular chaperone important for the
34 optical properties of the vertebrate eye lens. It forms heterogeneous oligomeric ensembles.
35 We determined the structures of human α A-crystallin oligomers combining cryo-electron
36 microscopy, cross-linking/mass spectrometry, nuclear magnetic resonance spectroscopy and
37 molecular modeling. The different oligomers interconvert by the exchange of tetramers
38 leading to mainly 12-, 16- and 20-meric assemblies in which interactions between N-terminal
39 regions are important. Cross-dimer domain-swapping of the C-terminal region is a
40 determinant of α A-crystallin heterogeneity. Human α A-crystallin contains two cysteines which
41 can form an intramolecular disulfide *in vivo*. Oxidation *in vitro* requires conformational
42 changes and oligomer dissociation. The oxidized oligomers, which are larger than reduced
43 α A-crystallin and destabilized against unfolding, are active chaperones and can transfer the
44 disulfide to destabilized substrate proteins. The insight into the structure and function of α A-
45 crystallin provides a basis for understanding its role in the eye lens.

46 Introduction

47

48 The small heat shock proteins (sHsps) α A- and α B-crystallin are major constituents of the
49 vertebrate eye lens¹. They ensure lens transparency^{1,2} and prevent lens proteins from
50 aggregation^{3,4}. Mutations in both α -crystallins result in cataract and in a variety of eye
51 disorders, emphasizing their importance for the lens^{2,5}. Besides the commonality of the
52 processes in which α A- and α B-crystallin are involved, differences in their expression
53 patterns and distribution in the lens exist⁶⁻⁹. Specifically, α A-crystallin is predominantly
54 expressed in the eye lens. *In vitro* studies point towards mechanistic differences between
55 α A- and α B-crystallin in suppressing the aggregation of model substrates^{10,11} and mutations
56 of conserved residues have different impact on the two crystallins¹².

57 Human α A-crystallin, a 19.9 kDa protein with 173 residues, consists of three structurally
58 distinct regions: the conserved α -crystallin domain (ACD, residues 61-145) flanked by the N-
59 terminal region (NTR, residues 1-60) and the short, flexible C-terminal region (CTR,
60 residues 146-173)^{13,14}. The ACD adopts a β -sandwich fold composed of two anti-parallel
61 sheets of three and four β -strands, respectively. It dimerizes through the interaction of the
62 β 6+7-strands of two adjacent protomers (" β 7-interface dimer")^{15,16}. α A-crystallin assembles
63 into polydisperse oligomers with extensive size heterogeneity and a constant exchange of
64 subunits between oligomers¹⁷⁻¹⁹. There is yet no structural information available either for full-
65 length α A-crystallin or for α A-crystallin in any oligomeric form. Consequently, the structural
66 elements critical for assembly and those conferring plasticity to the oligomeric assembly are
67 poorly understood. The involvement of the NTR in oligomer formation is indicated by the shift
68 of the average oligomer ensemble to smaller species, dimers and/or tetramers, upon its
69 truncation²⁰⁻²³. Studies on C-terminal truncation mutants of α A-crystallin from different
70 organisms display significant disparities leaving the role of the CTR in oligomer formation still
71 ill defined^{18,21,24-27}. The CTR of α A-crystallin exhibits greater overall flexibility than that of α B-
72 crystallin²⁸ including the segment containing the conserved IXI motif, which promotes
73 oligomer formation by binding into the β 4/ β 8 groove within the ACD of a neighboring
74 protomer^{29,30}.

75 A characteristic of human α A-crystallin is the presence of two cysteines in its ACD, the
76 invariant C131 found in most species and an additional cysteine at position 142 also found in
77 α A-crystallin from primates and zebrafish (*Danio rerio*)³¹. C131 was predicted to be buried,
78 whereas C142 was suggested to be fully solvent-exposed^{32,33}. In the crystal structure of the
79 zebrafish α A-crystallin ACD, the CTR covers C132 while leaving C143 accessible¹⁶. Notably,
80 already in young human lenses and during the first ~30 years of age, ~45 % of α A-crystallin
81 exhibit an intramolecular disulfide bond (henceforth denoted as oxidized α A-crystallin, α A_{ox}),
82 in the remaining fraction the cysteines are in the free sulfhydryl form (reduced α A-crystallin,

83 αA_{red})³⁴⁻³⁷. In young lenses, a subpopulation might form intermolecular disulfides as well³⁸.
84 With ageing, the amount of αA_{ox} increases up to 90 %³⁶ and it becomes a major constituent
85 of high molecular weight aggregates³⁹⁻⁴¹, concomitant with an age-dependent loss of the
86 chaperone activity of α -crystallin⁴². αA_{red} is undetectable in cataractous lenses³⁵. Despite
87 their importance, the structural and functional consequences of αA -crystallin oxidation are
88 yet unknown.

89 Here we present the architecture and plasticity of human αA -crystallin oligomers as well as
90 the structural and functional consequences of its oxidation. The structures of human αA -
91 crystallin assemblies and pseudo-atomic models of a 16-meric assembly reveal the domain-
92 swapping of the CTR to be a key determinant of αA -crystallin heterogeneity. Formation of the
93 intramolecular disulfide bond leads to distinct oligomers that are chaperone active and can
94 transfer its intramolecular disulfide to destabilized substrate proteins.

95 **Results**

96

97 **Oligomer architecture and conformational heterogeneity of reduced α A-crystallin**

98 To determine the oligomer architecture of reduced human α A-crystallin, we employed single-
99 particle cryo-EM (**Extended Data Fig. 1**). The initial analysis of ~74,000 projection images
100 revealed two distinct populations, one containing round particles with 3-, 4- and 5-fold
101 symmetries and diameters varying between 6 and 16 nm (**Extended Data Fig. 1b**), the other
102 one comprising elongated structures with 2-fold symmetry and a nearly uniform long axis of
103 13-14 nm (**Extended Data Fig. 1c**). The two populations seemed to represent end- and side-
104 on projections of a barrel-like architecture with varying subunit stoichiometries. On this basis,
105 we established a 3D-reconstruction procedure which allowed us to assign ~ 80 % of the
106 particles to 12-, 16- and 20-meric assemblies with abundances of approximately 36 %, 27 %
107 and 19 % and calculate the corresponding 3D models (**Fig. 1**) at resolutions of 9.2 Å, 9.8 Å
108 and 9.0 Å, respectively (**Extended Data Fig. 1h, Table 1**).

109 According to the reconstructed EM volumes (**Fig. 1**), all three assemblies form hollow, barrel-
110 like structures with a recurring unit resembling a tilted „Z“ comprising two substructures that
111 are connected in the mid-plane of the barrel. Each of these substructures is large enough to
112 accommodate an α A-crystallin dimer implying that the Z-shaped structures represent
113 tetramers (dimers of dimers) which serve as building blocks of the oligomers. Notably, in the
114 average structures of the 12- and 16-mers, adjacent tetramers are not connected in the
115 equatorial plane of the barrel (**Fig. 1a,b**) whereas there is a well-resolved density bridging
116 neighboring tetramers in the 20-mer (**Fig. 1c**).

117 To elucidate the structural variability of α A_{red} oligomers, each oligomer population was
118 subjected to 3D sampling and classification which revealed that the density corresponding to
119 an α A-crystallin dimer remains almost invariant within all three populations (**Extended Data**
120 **Fig. 2**). In contrast, significant heterogeneity exists in areas where adjacent tetramers come
121 together in apical and equatorial regions which most likely harbor the N- and/or C-termini.
122 The oligomers differ in the density connecting the tetramers in the equatorial plane: this
123 density is lacking in all sub-ensembles of the 12-mer population, while it is present in
124 approximately 30 % of the 16-mer population and in all sub-ensembles of the 20-mer
125 population (**Extended Data Fig. 2 and Supplementary Table 1**). The observed
126 heterogeneity is suggestive of dynamic inter-subunit interactions involving N- and/or C-
127 terminal regions. This conformational heterogeneity together with very similar projection
128 views of different oligomers presumably limits the resolution of the reconstructions.

129 **Pseudo-atomic model of the α A-crystallin 16-mer**

130 To obtain pseudo-atomic models of the αA_{red} 16-mer, we subjected full-length protein to
131 cross-linking and mass spectrometry. Using the cross-linker bis(sulfosuccinimidyl)suberate
132 (BS3), we identified numerous intra- and intermolecular cross-links (**Extended Data Figs.**
133 **3,4 and Supplementary Table 2**). The data demonstrated the structural similarity of human
134 α A-crystallin in its ACD and CTR to zebrafish and bovine α A-crystallin, as all distances
135 between corresponding residue pairs resolved in the respective crystal structures were below
136 30 Å - approximately the upper distance limit dictated by the utilized cross-linker⁴³ (**Extended**
137 **Data Fig. 4f**).

138 The volume of the equatorial inter-tetramer density present in some 16-mer sub-populations
139 is just large enough to accommodate the CTR (**Extended Data Fig. 2**). Together with its
140 positioning, we concluded that the variability within this area stems from 3D domain
141 swapping of the CTR: in structural classes containing the equatorial inter-tetramer density,
142 the IPV motif binds intermolecularly into the $\beta 4/\beta 8$ pocket of an adjacent protomer (3D
143 domain-swapped configuration), while in classes lacking the density, it binds intramolecularly
144 into the $\beta 4/\beta 8$ pocket of the same polypeptide chain (non-3D domain-swapped
145 configuration). This view is supported by the occurrence of the CTRs in swapped and non-
146 swapped configurations in bovine and zebrafish α A-crystallin ACD crystal structures,
147 respectively^{15,16}. Thus, we generated pseudo-atomic models of the αA_{red} 16-mer with the
148 CTRs in both configurations using (i) shape and symmetry constraints from the cryo-EM
149 envelopes differing in the equatorial inter-tetramer density, (ii) the crystal structures of
150 truncated versions of bovine and zebrafish α A-crystallins as templates, and (iii) intra- and
151 intermolecular distance restraints from cross-linking. During modeling using Molecular
152 Dynamics Flexible Fitting, a homology-modeled structure for the NTR (residues 1-60) was
153 used which contained 3 short helices connected by flexible loops (**Extended Data Fig. 5a,b**).
154 The structures of the central ACD (residues 61-145) and part of the CTR (residues 146-166)
155 were derived from homology modeling based on the above-mentioned crystal structures. The
156 residues 167-173 were not included into the model due to their flexibility²⁸. The fitting
157 procedure resulted in an ensemble of solutions with the NTRs of both apical (M_{ap}) and
158 equatorial protomers (M_{eq}) adopting a variety of possible conformations (**Extended Data Fig.**
159 **5c,d**) consistent with their flexibility. Although no consensus structure could be derived for
160 the NTR, its integration during the fitting process was crucial because it restricted the
161 positioning of the central ACD and CTR. The best structures were selected based on RMSD,
162 stereochemistry and cross correlation with respect to the cryo-EM density and further energy
163 minimized.

164 In the final pseudo-atomic models of the αA_{red} 16-mer (**Fig. 2**) which fit best into the EM-map
165 from all possible models and fulfill cross-linking restrains, all parts of the polypeptide chain

166 are accommodated within the electron density. The models reveal that two protomers form a
167 β 7-interface dimer. Interactions between N-termini mediate the association of two dimers
168 across the equator to form a tetramer (equatorial N-terminal interface, eq-NI) (Fig. 2a,d),
169 which is the recurring unit of the oligomer. Further N-terminal interactions between apical
170 protomers of the tetramers (apical N-terminal interface, ap-NI) serve to form the 16-mer (Fig.
171 2a). The close proximity of the N-terminal segments is corroborated by intermolecular cross-
172 links involving residues M1, K11, and T13 (Supplementary Table 2) which are all satisfied in
173 our models. In contrast to the prevailing contribution of the NTR to oligomer formation, the
174 CTR is barely involved in inter-subunit interactions. In both 16-mer models (Fig. 2a,b), the
175 CTRs of M_{ap} are in a non-3D domain-swapped configuration as the distance between apical
176 protomers is too large compared to the length of the CTR to permit an intermolecular IXI-
177 β 4/ β 8 interaction. On the other hand, although the distance between M_{eq} of neighboring
178 tetramers supports this interaction in both directionalities of the palindromic sequence, the
179 CTR contributes to the assembly by 3D domain swapping in only ~30 % of the 16-mer
180 population (Fig. 2b,f). 3D domain swapping creates an interface (equatorial C-terminal
181 interface, eq-CI) in which the CTRs of M_{eq} from neighboring tetrameric units are in close
182 proximity, consistent with the observed intermolecular cross-link K166-K166 (not used as a
183 modeling constraint), and interact through electrostatic interactions involving residues
184 downstream of the IXI motif (Fig. 2b,f).

185

186 **3D domain-swapping of the C-terminal region in the α A-crystallin ensemble**

187 12- and 20-meric αA_{red} assemblies share the modular architecture of the 16-mer (Fig. 3a,b).
188 In all three cases, the tetramers have the same curvature. The ACD positions within the
189 tetramers are identical. In the apical regions of 12- and 20-mers, the CTRs don't swap
190 domains due to the large distance between adjacent protomers as for the 16-mer. However,
191 the CTRs of all M_{eq} are in the non-3D domain-swapped state in the 12-mer whereas those of
192 the 20-mer connect neighboring tetramers by domain swapping (Fig. 3). As estimated from
193 the relative abundances of all sub-populations, ~20 % of all CTRs are in the 3D domain-
194 swapped state in the ensemble subset studied (Supplementary Table 1). Domain swapping
195 of the CTR as suggested by cryo-EM would require large amplitude motions of the
196 polypeptide chain also in the hinge region (I146-E156) adjacent to the ACD. The CTR of α A-
197 crystallin displays significant flexibility and can be detected by solution-state NMR (residues
198 G149-S173, Fig. 3c)²⁸. To test the prevalence of 3D domain-swapping of the CTR in the
199 reduced α A-crystallin ensemble, we performed paramagnetic relaxation enhancement (PRE)
200 experiments on αA_{red} labelled with the 3-(2-iodoacetamido)-proxyl spin label (IPSL) at the
201 cysteine residues within the ACD (most likely C142). The spectra of the spin-labelled ¹⁵N-
202 αA_{red} sample (¹⁵N- αA_{red} -IPSL) showed a substantial decrease (~40-50 %) in the peak

203 intensity ratios between the paramagnetic (oxidized) and diamagnetic (reduced) states
204 ($I_{\text{para}}/I_{\text{dia}}$) for residues in the IPV region, such as A158, I159 and V161 (Fig. 3e).

205 If the observed PREs were a consequence of random-coil like structural fluctuations of the
206 CTR, PRE effects would localize around C142 (± 10 amino acids)⁴⁴. We observe, however, a
207 flat PRE profile with minimum intensity around A158, suggesting that CTD is either partially
208 structured or exchanges between a bound and a free form. We can exclude the former
209 interpretation as the CTD chemical shifts of oxidized and reduced spin-labeled α A-crystallin
210 are rather similar (Extended Data Fig. 6).

211 Due to the high molecular weight of the α A-crystallin oligomers, no direct PREs can be
212 measured for the CTR bound state. However, chemical exchange between bound and
213 unbound CTRs allows to indirectly access the proximity of the CTD to the β 4/ β 8 groove⁴⁵.
214 The measured transfer-PREs thus allow to probe intra- and intermolecular 3D domain-
215 swapping.

216 To distinguish whether the unbound CTRs are in proximity of the ACD of the same protomer
217 (non-3D domain-swapped) or an adjacent one (3D domain-swapped), we incubated spin-
218 labelled $^{14}\text{N-}\alpha\text{A}_{\text{red}}$ ($^{14}\text{N-}\alpha\text{A}_{\text{red}}$ -IPSL) with $^{15}\text{N-}\alpha\text{A}_{\text{red}}$ in a 1:1 molar ratio ($^{15}\text{N-}\alpha\text{A}_{\text{red}}$ + $^{14}\text{N-}\alpha\text{A}_{\text{red}}$ -
219 IPSL). In case all CTR interactions would involve 3D domain-swapping, an attenuation of the
220 signal intensity by 25 % would be expected. The fact, that only a decrease by 10-15 % in the
221 peak intensity is observed (Fig. 3f) is in agreement with the cryo-EM results that indicate ~20
222 % of the CTRs to be in a 3D domain-swapped state (Supplementary Table 1). Both
223 experiments thus imply that the 3D domain-swapped state is not dominantly populated in the
224 oligomer ensemble of $\alpha\text{A}_{\text{red}}$.

225

226 ***In vitro* formation of an intramolecular disulfide bond in human α A-crystallin**

227 In agreement with the literature^{32,33}, we detected in an Ellman's assay of $\alpha\text{A}_{\text{red}}$ 0.93 ± 0.008
228 mol (SH) / mol (protein) corresponding to one accessible cysteine residue *in vitro*. The
229 presence of only one reactive cysteine is puzzling at first glance, considering that the
230 cysteines C131 and C142 of human α A-crystallin form an intramolecular disulfide bridge *in*
231 *vivo*³⁴⁻³⁷. However, when performed in the presence of urea, the Ellman's assay detected
232 1.92 ± 0.070 mol (SH) / mol (protein) for $\alpha\text{A}_{\text{red}}$. Thus the second cysteine is not readily
233 accessible under native conditions.

234 In our pseudo-atomic model of $\alpha\text{A}_{\text{red}}$, the cysteines are located on adjacent antiparallel β 8-
235 and β 9-strands and point in opposite directions (Fig. 3d). Their C_α atoms are ~6 Å apart,
236 which is within the C_α - C_α distance range of 3.8 Å – 6.8 Å usually observed for disulfide bond
237 conformations in proteins, but farther than the mean C_α - C_α distance of ~4.6 Å found in cross-
238 strand disulfides⁴⁶. The formation of a cross-strand disulfide bond between C131 and C142

239 would require the rotation of the cysteine side chains towards each other, resulting in
240 significant conformational changes upon oxidation of α A-crystallin.

241 To study the formation of an intramolecular disulfide bond in α A-crystallin, we performed
242 redox titrations with glutathione (Fig. 4). At -149 mV, roughly 50 % of the protein was
243 oxidized to the intramolecularly cross-linked species (α A_{ox}), the remaining fraction consisted
244 of intermolecularly disulfide-linked dimers (~20%) and trimers (~14%) (Fig. 4a). Upon
245 quantification of the ratio of α A_{red} and α A_{ox} monomer bands, the equilibrium constant of the
246 redox reaction for intramolecular disulfide bond formation K_{eq} was determined to be 0.434
247 mM corresponding to a redox potential of the intramolecular disulfide of -135 mV (Fig. 4c).
248 We also performed the titration reactions with α A_{ox}. (Fig. 4b,e). The results indicated that
249 after 20 h, the forward and reverse reactions were indistinguishable and the thermodynamic
250 equilibrium attained (Fig, 4c,f).

251 For comparison, the above value is between the redox potentials of the catalytic disulfides in
252 bacterial DsbA and DsbC, oxido-reductases with a strained conformation in the oxidized
253 state^{47,48}. The low reaction rate and the formation of a substantial amount of intermolecularly
254 disulfide-bridged species suggest a thermodynamically unfavorable conformational state for
255 intramolecular disulfide formation also in the case of α A-crystallin. Thus, a denaturant should
256 facilitate oxidation. Indeed, in the presence of urea, the disulfide-linked oligomers were
257 abolished and half-maximal oxidation was achieved at -222 mV (Fig. 4d,f), again implying
258 that destabilization leads to the accessibility of both cysteines. As a consequence, we
259 prepared α A_{ox} by incubation with GSSG in the presence of urea. According to Ellman's
260 assays this preparation contains no free sulfhydryls (-0.03 ± 0.037 mol (SH) / mol (protein)).

261

262 **Structural impact of the intramolecular disulfide bond on human α A-crystallin**

263 Circular dichroism (CD) spectroscopy showed that oxidation does not lead to pronounced
264 changes in the secondary structure (Extended Data Fig. 7a). However, differences became
265 apparent in the environment of phenylalanine/tyrosine and tryptophan residues (Extended
266 Data Fig. 7b). Since ~60% of the Phe and Tyr residues as well as the single Trp are located
267 within the NTR, altered tertiary interactions within the NTR upon oxidation appear likely.

268 Electron micrographs of negatively-stained α A_{ox} revealed that the protein assembles into
269 oligomers that are more polydisperse and larger than observed for α A_{red} (Fig. 5a). The
270 average oligomer size shifted from ~13.5 nm for α A_{red} to ~17.7 nm for α A_{ox} (Fig. 5b). In
271 agreement, SEC experiments indicated an increase in molecular mass from 380 kDa for
272 α A_{red} to 770 kDa for α A_{ox} (Extended Data Fig. 7c) and sedimentation velocity aUC
273 experiments showed an increase in the sedimentation coefficient ($\langle s_{20,w} \rangle$)¹⁹ from 14 S to 25
274 S (Extended Data Fig. 7d).

275 The projections seen in electron micrographs of negatively-stained αA_{ox} particles were either
276 round or slightly elongated (Fig. 5a) and resembled projections seen in αA_{red} samples. A
277 preliminary 3D-reconstruction of a 32-mer calculated from few class averages including
278 1,500 single particle images (Extended Data Fig. 7e) without employing any starting model
279 revealed a hollow, slightly elongated assembly with D2 symmetry (Fig. 5c and Extended
280 Data Fig. 7f). Notably, the assembly contains the characteristic Z-shaped tetramers as seen
281 in the oligomers of αA_{red} (Fig. 1), but it is expanded through the insertion of further building
282 blocks apparently composed of dimers (Fig. 5c).

283 The overall architecture of the αA_{ox} 32-mer implies altered residue and/or subunit proximities
284 and consequently an altered cross-linking behavior compared to αA_{red} . Due to the lack of
285 quantitative cross-linking data, we only tentatively compared both patterns in their most
286 striking features. The comparison revealed a higher number of interactions in αA_{ox}
287 (Supplementary Table 3) which included those observed for αA_{red} but also indicated
288 differences between the two redox states. As such many cross-links observed only in αA_{ox}
289 involved residues located within the NTR as well as within $\beta 4$ -, $\beta 6+7$ - and $\beta 9$ -strands
290 (including K145) suggesting alteration of their relative positions and/or enhanced sidechain
291 accessibilities in αA_{ox} (Extended Data Fig. 4b).

292

293 **The intramolecular disulfide affects local structural dynamics of αA -crystallin**

294 To further test how intramolecular disulfide formation affects the structure and dynamics of
295 αA -crystallin, we performed hydrogen-deuterium exchange coupled to mass spectrometry
296 (H/DX-MS) (Fig. 6a and Supplementary Fig. 1). In agreement with previous studies⁴⁹,
297 peptides from the NTR were characterized by a moderate protection at short D_2O exposure
298 times, but became increasingly deuterated at longer exposure, consistent with the dynamic
299 nature of this region sampling different conformations. The peptides from the ACD showed in
300 general lower exchange. The $\beta 5$ - and $\beta 6+7$ -strands (F93-E102 and Y109-R119, respectively)
301 were most strongly protected, while the CTR exchanged readily consistent with high
302 exposure/flexibility. Thus, the degree of exchange in αA_{ox} was similar to that observed for
303 αA_{red} , but specific differences existed. In αA_{ox} , the N-terminal stretch comprising residues D2-
304 F10 exhibited increased protection. The $\beta 6+7$ -strand (Y109-R119), the C-terminal region of
305 the $\beta 8$ -strand (L133 and S134) and the $\beta 9$ -strand (L139-G143) as well as the loop
306 connecting the latter became deprotected upon disulfide formation with F141 ($\beta 9$ -strand,
307 neighboring C142) showing the strongest deprotection. These results suggest that the
308 introduction of the intramolecular disulfide affects dynamics, solvent exposure and the
309 hydrogen bonding network around the sites of disulfide formation (Fig. 6b).

310 To test oligomer stability, we performed aUC experiments in the presence of urea. With
311 increasing urea concentrations, both αA_{red} and αA_{ox} oligomers dissociated successively. A

312 dissociated species with a sedimentation coefficient of 2 S was observed at 4.5 M urea for
313 αA_{red} , and at 3.5 M urea for αA_{ox} (**Extended Data Fig. 8a**). Similarly, urea-induced unfolding
314 transitions monitored by intrinsic fluorescence revealed cooperative unfolding with midpoints
315 at 3.8 M and 2.7 M urea for αA_{red} and αA_{ox} , respectively (**Extended Data Fig. 8b**).

316

317 **Chaperone activity of oxidized αA -crystallin**

318 To compare αA_{red} and αA_{ox} functionally, we performed *in vitro* aggregation assays using p53
319 and MDH as model substrates and assessed the redox states of αA -crystallin and the
320 substrate. For comparison, we performed the same experiments in the presence of GSSG
321 and the reduced and oxidized forms of DsbA ($DsbA_{red}$ and $DsbA_{ox}$, respectively). The heat-
322 induced aggregation of p53 was efficiently suppressed only in the presence of αA_{ox} (**Fig. 7a**).
323 Remarkably, when αA_{ox} was present, disulfide-linked large oligomers of p53 were formed
324 early and concomitantly αA_{red} appeared (**Fig. 7b**) indicating that the disulfide in αA_{ox} was
325 transferred to p53. Interestingly, when we added $DsbA_{ox}$, only a slight aggregation
326 suppression activity was detected (**Fig. 7a**). However, also in this case, high molecular
327 weight disulfide-bonded p53 aggregates were formed (**Fig. 7b**) at a rate similar to that
328 observed for αA_{ox} (**Fig. 7c**). These results indicate that αA_{ox} and $DsbA_{ox}$ share the ability to
329 transfer their disulfide bond to destabilized p53. The addition of GSSG neither suppressed
330 aggregation, nor did it lead to the early formation of disulfide-linked species (**Fig. 7a,b**). Such
331 species were also absent when αA_{red} or $DsbA_{red}$ were present (**Extended Data Fig. 9a**).

332 Both αA_{red} and αA_{ox} suppressed the heat-induced aggregation of MDH (**Extended Data Fig.**
333 **9b**). As in the case of p53, αA_{ox} transferred its intramolecular disulfide almost quantitatively
334 to MDH resulting in intermolecularly cross-linked MDH species (**Extended Data Fig. 9c,d**).
335 In the presence of $DsbA_{ox}$, the MDH monomer band disappeared (**Extended Data Fig. 9c**),
336 but disulfide-bonded oligomers were not detected possibly due to their large size (**Extended**
337 **Data Fig. 9d**). In the presence of GSSG or $DsbA_{red}$, the aggregation kinetics of MDH was
338 similar to the control and no cross-linked MDH species were observed (**Extended Data Fig.**
339 **9b,d,e**).

340 Taken together, the above results demonstrate that αA_{red} and αA_{ox} differ in their *in vitro*
341 chaperone activities towards model substrates and αA_{ox} , similar to DsbA, is capable of
342 transferring its disulfide bond to destabilized model substrates.

343 Discussion

344

345 Human α A-crystallin exists in heterogeneous ensembles of oligomers of varying subunit
346 stoichiometries. The atomic models determined by combining data from cryo-EM, X-ray
347 crystallography, NMR and molecular modeling reveal the roles of the NTR and CTR in
348 oligomerization and C-terminal domain swapping as a determinant of ensemble
349 heterogeneity. The recurring unit of α A_{red} oligomers is a tetramer in which two β 7-interface
350 dimers associate at the equator of the barrel-shaped assembly through N-terminal
351 interactions. Further N-terminal interactions at the poles mediate the formation of higher-
352 order assemblies by linking tetrameric units. A tetrameric building block is consistent with
353 previous studies²⁰⁻²³.

354 The homology-modeled structure of the NTR contains 3 short helices connected by flexible
355 loops consistent with the propensity of NTRs to adopt secondary structure elements^{29,50}.
356 These regions are highly dynamic and exist as ensembles of heterogeneous
357 conformations^{29,51,52}. In α A-crystallin, even the protomers in the apical and equatorial regions
358 of the same oligomer possess different conformations. In our model, representing one of
359 several possibilities, the N-terminal interactions occur mainly between the loops connecting
360 helices α 2 and α 3. The amphipathic helix α 2 (residues 20-27) covering the conserved
361 phenylalanine-rich sequence RLFDQXFG¹⁴ dictates the position of the interacting loop
362 regions in equatorial protomers. This motif was implicated to contribute to the higher order
363 subunit assembly, oligomer stability and dynamics⁵³.

364 The CTR of α A_{red} occurs in non-3D and 3D domain-swapped configurations as previously
365 captured in crystals of truncated forms of zebrafish¹⁶ and bovine α A-crystallin¹⁵, respectively.
366 We show that both configurations coexist in solution. The transition between the two states
367 requires the dissociation of the IPV motif from the β 4/ β 8 groove. However, NMR studies on
368 human α B-crystallin⁵⁴ and Hsp27⁵⁵ show that the IXI motif is highly dynamic in solution and
369 not rigidly bound to the protein scaffold. In α A-crystallin, the enhanced dynamics of the CTR
370 is likely to facilitate domain swapping.

371 In human α A-crystallin, the interplay between the geometric constraints imposed by the
372 assembly architecture and the hinge loop connecting the CTR to the ACD is likely to dictate
373 the propensity for domain swapping. In all three assemblies, distance constraints preclude
374 intermolecular binding of the CTR in apical protomers. Our reconstructions are of similar
375 dimensions but differ in their number of subunits leading to closer packing of protomers, i.e.
376 equatorial inter-protomer distances decrease gradually from 12-mer to 20-mer.
377 Consequently, in equatorial protomers of the 12-mer, the non-3D domain-swapped
378 configuration is favored as a flexible chain of a given length is less likely to span large
379 distances relative to its own length, resulting in folding back of the chain on itself (non-3D

380 domain-swapped configuration). Shorter distances promote domain swapping in all
381 equatorial protomers of the 20-mer population. In the 16-mer population, both configurations
382 coexist, nevertheless, the domain-swapped state might impose more strain on the hinge
383 region, and is thus less favored.

384 Despite the high similarity at the sequence level and virtually the same monomer length, α A-
385 and α B-crystallin form different geometric bodies utilizing the same type of interactions: the
386 β 7-interface mediates dimerization and oligomerization is supported by N-terminal
387 interactions, as well as by IXI-binding to the neighboring protomer. While the CTR is decisive
388 in the formation of hexameric species of human α B-crystallin^{56,57}, the CTR of human α A-
389 crystallin contributes to the formation of higher-order oligomers only in the 3D domain-
390 swapped form. N-terminal interactions are key to oligomer formation for all assembly types:
391 the 12-meric species, the most abundant oligomer population, assembles without
392 participation of the CTR in intermolecular interactions indicating that there is no stringent
393 contribution of the IXI motif to oligomer formation. Consistent with this, both human⁵⁸ and
394 bovine⁵⁹ α A-crystallin retain their ability to oligomerize upon mutations of the IXI motif or its
395 deletion²⁴.

396 α A_{ox} is highly abundant in young lenses without interfering with lens transparency³⁶. The
397 oxidation of α A-crystallin *in vitro* requires the presence of destabilizing agents which leads to
398 the dissociation of oligomers. This suggests that local conformational changes and/or partial
399 unfolding occur, putting the two cysteines in the β 8- and β 9-strands in an appropriate spatial
400 proximity. It has been suggested that partial unfolding of monomers upon dissociation may
401 be a common property of human sHsps and partly unfolded monomers may exist within
402 larger oligomers^{60,61}. Upon removal of urea, α A_{ox} reassembles into oligomers that are distinct
403 from those of α A_{red} harboring subunits which are locally more dynamic in their β 6+7-, β 8- and
404 β 9-strands.

405 The redox potential of the intramolecular disulfide bridge in human α A-crystallin is
406 comparable to that determined for thiol-disulfide oxido-reductases^{47,62}. In the presence of
407 urea, the intramolecular disulfide is formed at -220 mV, which is even below the estimated
408 redox potentials of approximately -204 mV and -217 mV at the nuclear and cortical regions,
409 respectively, of the young lens^{63,64}, thus enabling the formation of the intramolecular disulfide
410 bridge *in vivo*. Although it is delicate to deduce the redox potential of a disulfide bond *in vivo*
411 from the redox potential determined *in vitro* under equilibrium conditions in dilute solutions,
412 the mere existence of the intramolecular disulfide in α A-crystallin *in vivo* hints at certain
413 similarities of its redox properties *in vitro* and *in vivo*.

414 The intramolecular disulfide bridge in human α A-crystallin is a cross-strand disulfide. Such
415 disulfides are often reactive redox-based conformational switches due to their strained
416 conformation⁶⁵. Although the edge strand β 8 might tolerate the conformational

417 changes/distortions caused by the disulfide bond to a certain extent, the diminished stability
418 of αA_{ox} against urea-induced dissociation and unfolding compared to αA_{red} supports a
419 strained structure.

420 Interestingly, the two cysteines in human αA -crystallin are conserved among primates. *In*
421 *vivo*, introduction of additional cysteine residues may be detrimental, as naturally occurring
422 arginine to cysteine mutations of human αA -crystallin are all associated with cataract⁵.
423 Together with the general evolutionary selection against cysteines, this suggests that the
424 cysteines of human αA -crystallin must serve a function in the eye lens. This notion appears
425 contradictory to the increase of intramolecular disulfides concomitant with a decrease in
426 chaperone activity of αA -crystallin during ageing and cataractogenesis⁴². It should, however,
427 be noted that mere coincidence of these processes has yet been demonstrated, but not a
428 direct causality. The precise relationship between cysteine oxidation and cataractogenesis
429 needs to be further clarified.

430 αA_{ox} is able to transfer its intramolecular disulfide to destabilized substrates, i.e. it has redox
431 properties intermediate between disulfide oxidases DsbA and DsbC. Given that it constitutes
432 roughly ~15-20 % of the eye lens protein, this corresponds to an intracellular concentration of
433 3-4 mM. The lenticular glutathione concentration is in the order of ~3.7 mM in the outer
434 cortical regions and ~2.8 mM in the nuclear regions of young lenses^{64,66}. It is therefore likely
435 that the redox state of the eye lens is not solely dictated by the glutathione system, but αA -
436 crystallin itself will be an integral co-determinant of the lenticular redox system and a yet
437 unknown player in lenticular redox homeostasis. It could well be that the preferential
438 oxidation of αA -crystallin prevents the formation of nonnative disulfide bonds in other
439 crystallins and thus their aggregation in the eye lens. Further *in vivo* studies are required to
440 address this issue.

441 Taken together, our structural analysis of αA -crystallin revealing the assembly principles of
442 its oligomer ensembles together with properties αA_{red} and αA_{ox} provides a framework for
443 understanding its role in the normal lens and in cataractogenesis.

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454

455 **Author Contributions**

456 C.J.O.K., J.B. and S.W. designed and conceived the research plan. C.P., B.Ro. and C.J.O.K.
457 performed electron microscopy experiments and processed the data. C.J.O.K. carried out,
458 with contributions from P.W.N.S., E.V.M. and M.H., the experiments for the biochemical and
459 biophysical characterization. V.D. provided full-length human recombinant p53. M.S. and
460 S.A. performed NMR experiments. M.S. and B.Re. analyzed the NMR data. J.Z. conducted
461 cross-linking/mass spectrometry experiments. J.Z. and J.R. analyzed the cross-linking data.
462 M.Z. performed molecular dynamics simulations and model building. C.J.O.K., J.B. and S.W.
463 wrote the manuscript with input from all authors.

464

465 **Competing Interests**

466 The authors declare no competing interests.

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633

634 **Figure legends**

635

636 **Figure 1: Cryo-EM 3D-reconstructions of human α A-crystallin (reduced) oligomers.**

637 **a)** 12-mer (D3 symmetry) viewed along a 3-fold (left) (top view) and 2-fold symmetry axes
638 (middle, right) (side views). The volume corresponding to an α A-crystallin dimer is indicated
639 by a black ellipse. Apical (ap) and equatorial (eq) regions of the barrel-shaped 12-mer are
640 marked by dashed ellipses. Top and side views of the 16-mer (D4 symmetry) (**b**) and of the
641 20-mer (D5 symmetry) (**c**). For clarity, three tetramers of the 16-mer are outlined. The empty
642 arrowheads in (**a**) and (**b**) indicate the missing density between two adjacent tetramers in the
643 equatorial plane of the barrel, the filled arrowhead in (**c**) the inter-tetramer density. Scale bar:
644 10 nm. The isosurface thresholds were set to render a volume corresponding to a protein
645 mass of 239 kDa for the 12-mer, 318 kDa for the 16-mer, and 398 kDa for the 20-mer.

646

647 **Figure 2: Pseudo-atomic models of human α A-crystallin (reduced) 16-mer.**

648 **a)** Top and side views of the cryo-EM map of α A-crystallin 16-mer (reduced) superimposed
649 with the atomic model (ribbon representation) containing the CTRs of apical (M_{ap}) and
650 equatorial (M_{eq}) protomers in a non-3D domain-swapped conformation. ap-NI: apical N-
651 terminal interface; eq-NI: equatorial N-terminal interface (black dashed ellipse). **b)** 16-mer
652 containing the CTRs of M_{eq} in a 3D domain-swapped conformation. eq-CI: equatorial C-
653 terminal interface (black solid ellipse). **c)** The domain organisation of human α A-crystallin.
654 NTR (residues 1–60, sienna), ACD (residues 61–145, gray), CTR (residues 146–173, green).
655 **d)** Close-up view of the eq-NI with intermolecular cross-links involving the residues M1 and
656 K11. **e)** Close-up view of two neighboring equatorial protomers with their CTRs in non-3D
657 domain-swapped configuration. The IPV motifs are shown in orange, β 4- and β 8-strands in
658 light blue. **f)** Close-up view of eq-CI. Negatively and positively charged residues located
659 within the CTRs are shown in red and blue, respectively.

660

661 **Figure 3: CTR interactions in the reduced α A-crystallin oligomer ensemble.**

662 **a)** αA_{red} 12-mer superimposed with the pseudo-atomic model containing the CTRs of both
663 M_{ap} and M_{eq} in a non-3D domain-swapped conformation. **b)** αA_{red} 20-mer superimposed with
664 the pseudo-atomic model containing the CTRs of M_{eq} in a 3D domain-swapped conformation.
665 Domain color coding is as in Fig. 2. Dashed lines indicate the inter-protomer distances
666 (measured as the distance between I146 and P160 of neighboring protomers) to be spanned
667 by the linker between β 9 and β 10 for 3D domain-swap. **c)** 1H , ^{15}N HSQC solution-state NMR
668 spectrum of αA_{red} . In the inset, assigned backbone resonances involving the residues G149-
669 S173 are shown in black, non-detectable residues in grey. **d)** Alignment of an equatorial,
670 non-3D domain-swapped protomer (M_{eq-nds} , gray) with an equatorial, 3D domain swapped
671 protomer (M_{eq-ds} , blue). The positions of C131, C142, G149 and of the IPV motif are
672 indicated. **e)** PRE intensity ratios of a ^{15}N - and spin-labelled sample (^{15}N αA_{red} -IPSL) as a
673 function of the residue number. As the spin label is attached to ^{15}N -labelled protein, intra-
674 and intermolecular PREs are not distinguishable. **f)** PRE intensity ratios of a mixed sample
675 containing ^{15}N -labelled αA_{red} (^{15}N - αA_{red}) and unlabeled protein bearing the spin label (^{14}N -
676 αA_{red} -IPSL) in a 1:1 ratio (^{15}N - αA_{red} + ^{14}N - αA_{red} -IPSL). As the spin label is attached to ^{14}N - αA ,
677 only intermolecular contacts lead to signal quenching. For e-f, the experimental error was
678 determined from the signal-to-noise ratios of the individual cross peaks (details are outlined
679 in the Supplementary Note 1).

680

681 **Figure 4: Intramolecular disulfide cross-linking in human α A-crystallin.**

682 **a)** Denaturing, non-reducing PAGE of αA_{red} incubated at 43 °C for 20 h in the presence of
683 different GSH:GSSG ratios from fully oxidizing (2.5 mM GSSG) to fully reducing (5 mM GSH)
684 conditions. ox: oxidized α A-crystallin; red: reduced α A-crystallin; 2-mer, 3-mer: disulfide-

685 linked dimers and trimers. Note that even in the presence of 2.5 mM GSH minute amounts of
686 intermolecular disulfide-bonded dimers form, likely as a result from GSSG-impurities present
687 in the commercial GSSG-preparation. **b)** The same titration as in **(a)** using αA_{ox} at reaction
688 start. **c)** Relative intensities of the αA_{red} and αA_{ox} monomer bands of the gels shown in **(a)**
689 and **(b)** as a function of the GSH²:GSSG ratio. Half-maximal oxidation (dashed line) at a
690 redox potential of -135 mV (K_{eq} : 0.434 mM) for αA_{red} and at -145 mV (K_{eq} : 0.92 mM) for αA_{ox} .
691 **d)** Denaturing, non-reducing PAGE of αA_{red} incubated for 20 h at 43 °C in the presence of
692 4.5 M urea and at varying GSH²:GSSG ratios. Note that even under fully reducing conditions
693 (5 mM GSH), approximately 50 % of αA -crystallin is oxidized, likely due to GSSG impurities.
694 **e)** The same titration as in **(d)** using αA_{ox} at reaction start. **f)** Relative intensities of αA_{red} and
695 αA_{ox} monomer bands of the gels shown in **(d)** and **(e)** as a function of the GSH²:GSSG ratio.
696 Half-maximal oxidation (dashed line) at a redox potential of -222 mV (K_{eq} : 257 mM) for αA_{red}
697 and -224 mV (K_{ox} 306 mM) for αA_{ox} . In **a-f**, the shaded areas (in green) indicate the lenticular
698 GSH²:GSSG redox potential range. In **c** and **f**, values plotted are mean and s.d. of n=3
699 replicate determinations of one titration.

700

701 **Figure 5: Oligomer architecture of oxidized human αA -crystallin.**

702 **a)** Electron micrographs of αA_{red} (left) and αA_{ox} (right) oligomers negatively stained with 2%
703 uranyl acetate. Scale bar: 50 nm. Note the increased oligomer size and polydispersity in
704 αA_{ox} . **b)** Size distributions of the oligomers of αA_{red} (black bars) and αA_{ox} (gray bars). The
705 average oligomer size is shifted from ~13.5 nm in αA_{red} to ~17.7 nm in αA_{ox} . **c)** Different
706 views of the 3D-reconstruction of a 32-meric assembly of αA_{ox} . Scale bar: 10 nm. Dimeric
707 building blocks are indicated by ellipses.

708

709 **Figure 6: Dynamics of oxidized human αA -crystallin**

710 **a)** Relative fractional deuterium uptake (Rel frac D uptake) of all peptides detected in H/DX-
711 MS experiments. The deuteration behavior for early timepoints (10 s and 60 s) of the
712 exchange-reaction is shown. Peptides ordered by their midpoint and the peptide start and
713 end amino acid positions are indicated at the abscissa. Note that the uptake pattern is overall
714 well conserved among αA_{red} and αA_{ox} . Values plotted are mean and s.d. of n=3 technical
715 replicates is plotted. The error bars reflect the corresponding s.d. **b)** Differences in amide
716 hydrogen protection in αA_{red} and αA_{ox} mapped onto the model of a non-3D domain-swapped
717 monomer of αA -crystallin. Differences in deuterium uptake were obtained by the difference in
718 local relative deuterium uptake (ΔD uptake αA_{ox} - αA_{red}). The difference data were averaged
719 using the algorithm DynamX 3.0 (Waters). Regions in αA_{ox} with unchanged protection from
720 deuteration are colored white, with decreased protection red, and with increased protection
721 blue.

722

723 **Figure 7: α A-crystallin is capable of transferring disulfide bonds to human p53.**

724 **a)** Heat-induced aggregation of recombinant p53 (2 μ M) in the presence of a two-fold molar
 725 excess of GSSG, α A_{red}, α A_{ox} and reduced (DsbA_{red}) or oxidized (DsbA_{ox}) recombinant *E.*
 726 *coli* DsbA. Note that the aggregation of p53 is only suppressed in the presence of α A_{ox}. **b)**
 727 Non-reducing PAGE of samples withdrawn at the indicated timepoints (red arrows) from the
 728 aggregation assays in the presence of GSSG, α A_{ox} and DsbA_{ox} shown in **(a)**. Note that
 729 disulfide-bridged species of p53 are formed both in the presence of α A_{ox} and DsbA_{ox}. **c)**
 730 Relative intensity of the p53 monomer band as a fraction of the intensity (amount of
 731 monomer) at the beginning of each aggregation kinetics experiment (t = 0 min). Values
 732 plotted are mean and s.d. of n=2 independent experiments.

733

734 **Table 1: Cryo-EM data collection and validation statistics for α A-crystallin oligomer**
 735 **reconstructions**

	12-mer (D3) (EMD-4895)	16-mer (D4) (EMD-4894,PDB 6T1R)	20-mer (D5) (EMD-4896)
Data collection and processing			
Molecular mass (kDa)	238.8	318.4	398
Magnification	37000	37000	37000
Voltage (kV)	300	300	300
Electron exposure (e ⁻ /Å ²)	30	30	30
Defocus range (μ m)	1.2 – 2.5	1.2 – 2.5	1.2 – 2.5
Pixel size (Å)	1.35	1.35	1.35
Symmetry imposed	D3	D4	D5
Initial particle images (no.)	74068	74068	74068
Final particle images (no.)	26596	19783	14336
Relative abundance (%)*	35.9	26.7	19.4
Map resolution (Å)	9.2	9.8	9.0
FSC threshold	0.143	0.143	0.143
Dimensions (width x height in Å)	10.8 x 13.6	10.9 x 13.8	12.0 x 13.7
Validation			
MolProbity score	-	2.23	-
Clashscore	-	17	-
Poor rotamers (%)	-	0	-
Ramachandran plot	-	-	-
Favored (%)	-	92	-
Allowed (%)	-	8	-
Disallowed (%)	-	1	-

736 * Relative abundance with respect to the total number of images in the initial cryo-EM dataset.

737

738 **Online Methods**

739

740 **Cloning and protein purification**

741 Wild-type human α A-crystallin was recombinantly produced in *Escherichia coli* at 20°C. The
 742 cells were harvested by centrifugation and disrupted in the presence of protease inhibitor mix

743 G (Serva, Heidelberg, Germany). The first purification step was anion exchange
744 chromatography (Q-Sepharose FF). After fraction pooling, urea was added to 4.5 M final
745 concentration, then cation exchange (SP-Sepharose FF) and gel filtration chromatography
746 (Superdex 75) were performed. After an additional high-resolution anion exchange
747 chromatography step, urea was removed by dialysis against PBS (137 mM NaCl, 2.7 mM
748 KCl, 8.1 mM Na₂HPO₄, 1.76 mM KH₂PO₄, pH 7.4), 1 mM EDTA, 1 mM DTT. As a final
749 polishing step, a gel filtration run (Superdex 200; PBS, 1 mM EDTA, 1 mM DTT) was
750 performed, yielding αA_{red} preparations of > 95% homogeneity as judged by SDS-PAGE.
751 Aliquots were stored at -80 °C in gel filtration buffer. Prior to all experiments, protein aliquots
752 were thawed and incubated for 3 h at 37 °C to ensure proper thermal equilibration. If not
753 stated otherwise, all experiments were performed in PBS buffer, 1mM EDTA, with either 1
754 mM DTT or 1 mM trans-4,5-dihydroxy-1,2-dithiane (DTT_{ox}) present. For experiments in which
755 reductants or oxidants were likely to interfere, the buffer was exchanged to thoroughly
756 degassed and N₂-purged PBS, 1 mM EDTA using 7K MWCO polyacrylamide desalting
757 columns (Thermo Scientific, Waltham, MA, USA) to remove residual DTT. Further
758 experimental details are described in Supplementary Note 1.

759

760 **Redox titration, preparative protein oxidation and Ellman's assay**

761 Redox titrations were performed according to Wunderlich & Glockshuber⁴⁷. To rule out a
762 potential interference of O₂ from air during redox titrations, the water used for the preparation
763 of buffer was thoroughly degassed and purged with N₂ over night. Pipetting steps and
764 incubation for equilibration were carried out in an anaerobic chamber (Coy laboratory
765 products, Grass Lake, MI, USA) under N₂/H₂ (98%/2%) atmosphere. All solutions were
766 transferred into the anaerobic chamber immediately after preparation in N₂-purged water. All
767 reagents and tubes were stored in the anaerobic chamber at least 24 h before the start of the
768 experiment. Initially, the storage buffer of αA_{red} was exchanged to PBS, 1 mM EDTA using
769 gel filtration spin columns, the protein was transferred to the anaerobic chamber and diluted
770 (10 μM final concentration) into buffers of a specified redox-potential as defined by a mixture
771 of oxidized and reduced glutathione, GSSG and GSH, respectively, in PBS, 1 mM EDTA.
772 The total concentration of glutathione monomers was kept at 5 mM for all reactions. After
773 equilibration for 20 h at 43 °C, disulfide exchange was quenched by the addition of 25 mM N-
774 ethyl-maleimide (NEM, dissolved in dry ethanol) and incubation for 20 min at 20°C. For
775 redox-titrations in the presence of urea, all redox buffers and the buffer for initial DTT
776 removal contained 4.5 M urea. The quenched reactions were analyzed by loading 1 μg of
777 total protein per lane onto gradient gels (TG Prime, 8-16%, Serva, Heidelberg, Germany)
778 using non-reducing sample buffer. The relative amount of remnant reduced and oxidized
779 monomeric αA-crystallin (*R*) was quantified by densitometry using ImageJ. The equilibrium

780 constant for the formation of the intramolecular disulfide K_{eq} was determined through
781 nonlinear regression of the data using the function $R = ([GSH]^2/[GSSG]) / (K_{eq} +$
782 $([GSH]^2)/[GSSG])$. The K_{eq} obtained (4.34×10^{-4} M for the reaction of αA_{red} under native
783 conditions and 0.257 M for the reaction of αA_{red} in the presence of urea and 0.306 M for the
784 reaction of αA_{ox} in the presence of urea) were used to determine the corresponding redox
785 potential at 43 °C and pH 7.4 from the Nernst equation $E_{0 \alpha A} = E_{0 GSH/GSSG} - (RT/nF) \times$
786 $\ln K_{eq}$ with $E_{0 GSH/GSSG} = -240$ mV, which is the standard potential for the glutathione redox
787 pair at 40 °C and pH 7.4⁶⁷. To assess the reversibility of the reaction, the titrations were
788 equivalently performed using αA_{ox} . The data for αA_{ox} were processed as for αA_{red} .

789 To produce preparative amounts of αA_{ox} , protein at a concentration of 50 μ M was subjected
790 to buffer exchange using a HiPrep 26/10 desalting column (GE Healthcare) equilibrated in
791 PBS, 1 mM EDTA, 4.5 M urea. Subsequently, the eluting protein was brought to 37 °C,
792 supplemented with 2.5 mM GSSG and 5 μ M GSH, and incubated for 6 h at 37 °C. After
793 incubation, the redox system was removed by exchanging the buffer to PBS, 1 mM EDTA,
794 4.5 M urea. The eluate was then dialyzed twice against 5 L of PBS, 1 mM EDTA. The
795 oxidation state of the protein was validated after quenching with NEM by non-reducing SDS-
796 PAGE.

797 The Ellman's assay was performed according to Simpson, 2008⁶⁸. The reaction was scaled
798 to 100 μ L volume and the final protein concentrations in the reaction mixture were 26 μ M for
799 αA_{red} and 22 μ M for αA_{ox} .

800

801 **Quaternary structure analysis**

802 The quaternary structure of αA -crystallin samples was determined by analytical gel filtration
803 (SEC), analytical ultracentrifugation (aUC) and negative stain electron microscopy (NS-EM).
804 SEC runs were performed on a Superose 6 10/300 GL (GE Healthcare, Chalfont St Giles,
805 UK) column using fluorescence detection. For aUC, sedimentation velocity experiments were
806 carried out on a ProteomeLab XL-I analytical ultracentrifuge (Beckman Coulter, Brea, CA,
807 USA) at 20 °C. Protein was detected by UV absorbance. For urea titration aUC experiments,
808 αA -crystallin stock solution was diluted to 20 μ M in buffer containing the indicated
809 concentrations of urea. The solution was allowed to equilibrate at 20 °C for 5 h. The
810 sedimentation velocity profiles were analyzed with the dc/dt method⁶⁹ and normalized to
811 $s(20,w)$. Negative staining experiments were conducted as described previously¹⁹. Oligomer
812 sizes were determined as diameters of circumscribing circles of the class averages using
813 ImageJ (version 1.47t). Further experimental details are described in Supplementary Note 1.

814

815 **Extrinsic and intrinsic fluorescence and circular dichroism (CD) spectroscopy**

816 To assess protein stability, urea-induced unfolding equilibria were determined in dependence
817 of the urea concentration by intrinsic fluorescence measurements. Unfolding mixtures
818 contained 4 μM of protein and the indicated amount of urea dissolved in degassed, N_2 -
819 purged PBS, 1 mM EDTA with 1 mM DTT or DTT_{ox} . The concentration of urea was verified
820 by refractive index determination. Samples were incubated for 16 h. Spectra were measured
821 in triplicates using a Jasco FP-6500 spectrofluorimeter (Jasco, Tokyo, Japan) connected to a
822 thermostat. Hellma QS 10mm x 2 mm fluorescence ultra-micro cuvettes (Hellma, Müllheim,
823 Germany) were used for fluorescence measurements. The excitation wavelength was set to
824 295 nm and emission spectra were recorded from 305 nm to 450 nm. During incubation and
825 measurement, the samples were kept at 20 °C. The acquired spectra were corrected for the
826 corresponding buffer signal. For each spectrum, at a given concentration of urea (denoted as
827 $[\text{urea}]$), the intensity averaged emission wavelength $\langle\lambda\rangle_{[\text{urea}]}$ was calculated⁷². The fraction of
828 natively folded protein (f_{folded}) was calculated for the measurement range between 0 M urea
829 and 7.5 M urea as $f_{\text{folded}} = \langle\lambda\rangle_{(7.5\text{M urea})} - \langle\lambda\rangle_{[\text{urea}]} / \langle\lambda\rangle_{(7.5\text{M urea})} - \langle\lambda\rangle_{(0\text{M urea})}$ and plotted versus
830 denaturant concentration.

831 Circular dichroism (CD) spectra in the near (250-320 nm) and far (200-260 nm) UV-range
832 were measured using a Jasco J-710 (Jasco, Tokyo, Japan) or a Chirascan (Applied
833 Phostophysics, Leatherhead, United Kingdom) circular dichroism spectrophotometers
834 equipped with a thermostated cuvette holder set to 20 °C. Near-UV CD spectra were
835 recorded at a protein concentration of 100 μM , far-UV CD spectra were measured at 40 μM
836 in 20 mM $\text{KH}_2\text{PO}_4/\text{KOH}$, pH 7.4, 1 mM EDTA. To record near-UV spectra, a QS 1 cm cuvette
837 was used, for far-UV spectra a detachable window QS 0.2 mm cuvette (both Hellma,
838 Müllheim, Germany).

839

840 **Hydrogen/Deuterium exchange–mass spectrometry (H/DX-MS)**

841 H/DX-MS experiments were performed using an ACQUITY UPLC M-class system with H/DX
842 technology (Waters, Milford, MA, USA). H/DX kinetics were determined by measuring data
843 points at 0, 10, 60, 600 and 1800 s exposure to deuterated buffer at 20°C. At each data
844 point, 4 μl of a solution of 30 μM protein were diluted automatically 1:20 into PBS, 1 mM
845 EDTA, 1 mM DTT or 1mM trans-4,5-dihydroxy-1,2-dithiane, pD 7.5, prepared with 99.9 %
846 D_2O or H_2O as reference buffer. The reaction mixture was quenched by the addition of 200
847 mM KH_2PO_4 , 200 mM Na_2HPO_4 , 4 M GdnHCl, 300 mM TCEP, pH 2.3 (titrated with HCl) in a
848 ratio of 1:1 at 0 °C. 50 μl of the resulting sample were subjected to on-column peptic digest
849 on an in-line Enzymate BEH pepsin column (2.1 x 30 mm) at 20°C. Peptides were trapped
850 and desalted by reverse phase chromatography at 0° C using an Acquity UPLC C18 BEH
851 VanGuard pre-column (1.7 μm C18 beads, 2.1 x 5 mm, Waters). For separation, an Acquity
852 UPLC BEH C18 (1.7 μm , 1 x 100 mm) analytical column (Waters) at 0° C, and gradients with

853 stepwise increasing acetonitrile (in 0.1 % formic acid) concentration from 5 – 35 % in 6 min,
854 from 35 – 40 % in 1 min and from 40 – 95 % in 1 min were applied. The eluted peptides were
855 analyzed using an in-line Synapt G2-Si QTOF HDMS mass spectrometer (Waters). MS data
856 were collected over an m/z range of 100-2000. Mass accuracy was ensured by calibration
857 with [Glu1]-Fibrinopeptide B (Waters) and peptides were identified by triplicate MS^E ramping
858 the collision energy from 20-50 V. Data were analyzed using ProteinLynx Global Server
859 (PLGS, Version 3.0.3) and DynamX (Version 3.0) software packages (Waters). As all
860 samples were handled under identical conditions, deuterium levels were not corrected for
861 back-exchange and were therefore reported as relative deuterium uptake levels. Briefly, for
862 each peptide relative fractional exchange, is calculated dividing the deuterium level
863 incorporated at a given timepoint (in Da) by the total number of backbone amide hydrogens
864 in the peptide (this equals the number of amino acids, minus proline residues minus 1 for the
865 N-terminal amide⁷³. All experiments were performed with triplicate determination at each time
866 point.

867

868 **Aggregation assays**

869 Chaperone assays and disulfide-transfer reactions were performed in parallel using malate
870 dehydrogenase (MDH) or human p53 (p53) as model substrates in the absence of reductant.
871 MDH was diluted to 4 μ M and p53 was diluted to 2 μ M into PBS containing 1 mM EDTA on
872 ice. Oxidized and reduced α A-crystallin, oxidized and reduced DsbA and GSSG were added
873 to a final concentration of 4 μ M. The reaction mix was split in two aliquots. One of the
874 aliquots was used to follow the aggregation of model substrates at 45 °C by recording the
875 absorbance at 360 nm for 60-120 min using a Cary 50 UV/VIS spectrophotometer (Varian,
876 Palo Alto, CA, USA) equipped with a temperature-adjustable cuvette holder. The other
877 aliquot was used to withdraw samples at the indicated timepoints. One sample was
878 withdrawn at t= 0 min for reference before starting the reaction by placement of the reaction
879 mixtures to 45 °C in the photometer. The disulfide exchange reaction in every sample was
880 quenched immediately after withdrawal by the addition of NEM dissolved in dry ethanol to 25
881 mM final concentration. After 20 min of incubation at 20 °C, non-reducing SDS-PAGE sample
882 buffer was added, the sample was incubated at 95°C for 5 min and analyzed on an 8-
883 16% gradient gel (TG Prime, Serva, Heidelberg, Germany).

884

885 **Cryo-electron microscopy (Cryo-EM), image processing and 3D-reconstruction**

886 **Sample preparation, data collection** – For the preparation of the cryo-EM samples, 4 μ L of
887 α A_{red} solution (0.3 mg/mL in PBS, 1 mM EDTA, 1 mM DTT, pH 7.4) were applied to glow-
888 discharged Quantifoil R 2/1 holey carbon copper grids, incubated for 30 sec, blotted, and
889 plunge-frozen in liquid ethane using a manual plunger. The samples were mounted into

890 autoloader cartridges and transferred into a Titan Krios electron microscope (FEI) equipped
891 with a K2 Summit direct detector (GATAN) and operated in energy-filtered transmission
892 electron microscopy (EFTEM) mode at 300 kV. Automatic data acquisition was performed
893 using the TOM toolbox⁷⁴. 2334 movie images were collected at defocus values ranging from
894 - 1.2 μm to - 2.5 μm and at a nominal magnification of $\times 37,000$ (1.35 \AA per pixel) in “super-
895 resolution mode” (0.675 \AA per pixel). The movies were recorded at dose rates of 7 - 8
896 electrons per pixel per second, with exposure times of 0.37 - 0.27 s per frame and a target
897 total dose of 25 - 36 electrons per square \AA . The frames were aligned, averaged and
898 binned to a final pixel size of 1.35 \AA per pixel. Well-separated particle images were selected
899 manually and extracted into 200 X 200 pixel boxes using „e2boxer“ of the EMAN2 software
900 package⁷⁰. Images were corrected for the contrast transfer function by phase flipping using
901 Bsoft⁷⁵. All subsequent image processing procedures were carried out within the IMAGIC5
902 suite⁷¹.

903 ***Image processing and 3D-reconstruction*** – For the processing of the cryo-EM data,
904 reference-free class averages were generated from 74,068 CTF-corrected and band-pass
905 filtered (20 \AA - 160 \AA) single particle images. The class averages revealed almost spherical
906 particles ranging in size between 6 and 16 nm as well as elongated ones with a maximum
907 length of 14 nm. The presence of 2-, 3-, 4- and 5-fold symmetries in projection images
908 together with the variation of particle dimensions and shapes suggested the presence of
909 multiple structures of a similar barrel like architecture but of varying subunit stoichiometries.
910 Based on this, three models each consisting of a bundle of „pillars“ (3, 4 and 5 pillars
911 compatible with 3-, 4- and 5-fold symmetries) were generated as starting references.
912 Reference-free class averages were sorted into initial particle subsets based on particle
913 diameter and symmetry. Within each subset, an initial 3D-reconstruction was calculated by
914 projection matching cycles using the above “pillar-bundle” models as starting models. In a
915 next step of data sorting, the three initial 3D-reconstructions were used as references to align
916 and sort all single particle images of the cryo-EM data set into three final subsets in an
917 iterative procedure. All particles were aligned independently by multi reference alignment
918 (MRA) to each of the three reference structures. Within each of the three particle sets,
919 multivariate statistical analysis (MSA) was applied to generate class averages. The Euler
920 angles of the class averages were assigned by angular reconstitution (AR)⁷⁶. Subsequently,
921 classes that mismatched with the corresponding model reprojections were sorted out. This
922 “purification” step by MSA/AR was repeated until all remaining class averages agreed well
923 with reference reprojections, resulting in three distinct particle subsets.

924 For 3D-reconstruction, the final class averages were refined iteratively by 6 rounds of MRA
925 and AR using starting models generated from the input class averages of the respective set.
926 For the refinement, single particle images of each class were aligned with the corresponding

927 reprojection of the respective preliminary reference, while particles that were rotated by more
928 than 9 degrees as well as the 10% of images with the lowest cross correlation coefficients
929 were ignored. Euler angles of the class averages comprising the remaining particles were
930 refined by AR and a new 3D-reconstruction was calculated, which served as a reference for
931 the subsequent refinement cycle. During refinement, filtering of input and output images as
932 well of the reference models changed in every iteration starting from 20 Å and ending at 7 Å
933 to sequentially allow more details to affect the alignment. Class averages and 3D-
934 reconstructions were masked with an adaptive soft edge mask unless when used for Fourier
935 shell correlation (FSC) calculations.

936 For resolution determination, the “gold standard” 0.143 criterion was used⁷⁷. FSCs were
937 calculated between the final reconstructions of independently processed half sets using the
938 FSC validation server (<http://pdbe.org/fsc>) within the PDBe (Protein Data Bank in Europe)⁷⁸.
939 3D reconstructions and atomic models were rendered using UCSF Chimera⁷⁹.

940 **3D sampling and classification** – To analyze the conformational heterogeneity of human
941 α A-crystallin oligomers, the particles from the final oligomer subsets were subjected to band
942 pass filtering (140 Å - 10 Å), normalisation and 3D sampling⁸⁰ followed by 3D classification.
943 Within each data set, 1,000 random 3D-reconstructions from randomly selected 1,000
944 projection images were generated (3D sampling of structure sub-ensembles). The 3D-
945 reconstructions were analyzed by 3D MSA followed by clustering into 10 distinct sub-
946 populations by 3D-classification using IMAGIC5.

947

948 **NMR spectroscopy**

949 α A_{red} was ¹⁵N/¹³C-labeled upon recombinant expression and purified as described above.
950 Purified protein was dialyzed against 10 mM HEPES/KOH (pH 7.4), 2 mM DTT, 1 mM EDTA.
951 Solution-state NMR experiments were carried out employing a Bruker Biospin Avance III
952 spectrometer operating at a ¹H Larmor frequency of 950 MHz (22.3 T) using a CPTCI triple-
953 resonance cryoprobe. All experiments were performed at 300 K in HEPES/KOH buffer
954 containing 5% D₂O. For spin-labeling experiments 3-(2-iodoacetamido)-proxyl (IPSL) (Sigma-
955 Aldrich, St. Louis, MO, USA; 50 mM stock dissolved in DMSO) was used. As only one
956 cysteine residue is readily surface accessible in native α A-crystallin, the label was most likely
957 reacted with C142^{32,33}. As a control, protein-bound IPSL was reduced with a 10 molar
958 excess of freshly prepared ascorbic acid in HEPES/KOH buffer to yield the diamagnetic
959 species. Paramagnetic relaxation enhancements (PREs) arising from the spin label were
960 determined using the ratio of peak intensities of the ¹H, ¹⁵N-HSQC spectra obtained for the
961 paramagnetic (oxidized) and the diamagnetic (reduced) state (I_{para}/I_{dia}), in the absence and
962 presence of 10 molar equivalents of ascorbic acid. Further experimental details are
963 described in Supplementary Note 1.

964

965 **Cross-linking and mass spectrometry**

966 For cross-linking experiments, bis(sulfosuccinimidyl)suberate (BS3) cross-linker (Thermo
967 Scientific, Waltham, MA, USA) was added to the protein upon continuous vortexing of the
968 protein solution. The reaction mixture was incubated at 20 °C for 1 h and quenched. The
969 samples were loaded on gradient gels, which were run at a constant voltage of 200 V using
970 MOPS-SDS-running buffer. The protein in excised gel bands was alkylated with
971 iodoacetamide (IAA) and digested with trypsin (Thermo Fischer Scientific), following
972 previously established protocols⁸². Peptides were separated by reverse-phase
973 chromatography and analysed by LC-MS/MS on a Orbitrap Fusion Lumos (Thermo Fisher
974 Scientific) with a “high/high” acquisition strategy. The mass spectrometric raw files were
975 processed into peak lists using MaxQuant (version 1.5.3.30)⁸⁵, and cross-linked peptides
976 were matched to spectra using Xi software (version 1.6.745)⁸⁶. FDR was estimated using
977 XiFDR on 5% residue level⁸⁸. Further experimental details are described in Supplementary
978 Note 1.

979

980 **Model building**

981 Structural modeling of the human α A-crystallin 16-mer was based on homology models of
982 either the non-3D domain-swapped structure of truncated α A-crystallin (α A_{60–166}) from
983 zebrafish (PDB 3N3E)¹⁶ or on the 3D domain-swapped structure of bovine truncated α A-
984 crystallin (α A_{59–163}) (PDB 3L1E)¹⁵ using the program Modeller⁸⁹. The N-terminal segment
985 (α A_{1–59}) was modeled using I-Tasser⁹⁰. Homology modeled ACD dimer structures were fitted
986 as rigid bodies into the corresponding cryo-EM densities using the program colores of the
987 Situs package⁹¹. The N-terminal modeled segment (α A_{1–59}) was placed randomly in various
988 positions. The oligomers were energy minimized using the Sander module of the Amber
989 software package (Amber16)⁹². Molecular dynamics (MD) flexible fitting was started from
990 energy-minimized structures using the emap option in Sander⁹³. For each of the initial
991 placements of the N-terminal segments, the final flexibly fitted structure was evaluated based
992 on RMSD, stereochemistry and cross correlation with respect to the cryo-EM density. The
993 non-3D and 3D domain-swapped structures with low force field energy and best cross
994 correlation to the cryo-EM density were selected as best representative solutions. Further
995 experimental details are described in Supplementary Note 1.

996

997 **Reporting Summary**

998 Further information on experimental design is available in the Nature Research Reporting
999 Summary linked to this article.

1000

1001 **Data Availability**

1002 The cryo-EM density maps of α A-crystallin oligomers have been deposited in the EMBD
1003 under accession codes EMD-4895 (12-mer), EMD-4894 (16-mer) and EMD-4896 (20-mer).
1004 The coordinates for the 16-mer model were deposited in the wwPDB under accession
1005 number PDB 6T1R. The mass spectrometry proteomics data have been deposited to the
1006 ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier
1007 PXD013587. The ^1H , ^{15}N , ^{13}C chemical shifts of reduced α A-crystallin are available at the
1008 BioMagResBank (BMRB) with the accession number BMRB-27109. All other data are
1009 available from the corresponding authors upon reasonable request.

1010

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