Probing Dynamic Conformations of the High-Molecular-Weight αB-Crystallin Heat Shock Protein Ensemble by NMR Spectroscopy

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Supporting Information

ABSTRACT: Solution- and solid-state nuclear magnetic resonance (NMR) spectroscopy are highly complementary techniques for studying supra-molecular structure. Here they are employed for investigating the molecular chaperone αB-crystallin, a polydisperse ensemble of between 10 and 40 identical subunits with an average molecular mass of approximately 600 kDa. An IxI motif in the C-terminal region of each of the subunits is thought to play a critical role in regulating the size distribution of oligomers and in controlling the kinetics of subunit exchange between them. Previously published solid-state NMR and X-ray results are consistent with a bound IxI conformation, while solution NMR studies provide strong support for a highly dynamic state. Here we demonstrate through FROSTY (freezing rotational diffusion of protein solutions at low temperature and high viscosity) MAS (magic angle spinning) NMR that both populations are present at low temperatures (<0 °C), while at higher temperatures only the mobile state is observed. Solution NMR relaxation dispersion experiments performed under physiologically relevant conditions establish that the motif interchanges between flexible (highly populated) and bound (sparsely populated) states. This work emphasizes the importance of using multiple methods in studies of supra-molecules, especially for highly dynamic ensembles where sample conditions can potentially affect the conformational properties observed.

INTRODUCTION

The native states of proteins are in many cases inherently unstable, and the assistance of “molecular chaperones” is required to ensure that these important biomolecules attain and maintain their functional forms. The molecular chaperone αB-crystallin is a human small heat shock protein (sHSP) that plays an important role in proteostasis in vivo and can prevent amyloid fibril formation in vitro. It is up-regulated in neuronal cells of patients suffering from neurodegenerative disorders including Alzheimer’s and Parkinson’s diseases, where it is found bound to amyloid plaques. An understanding of how this chaperone inhibits protein aggregation has proven elusive. Structural studies are challenged by the fact that αB-crystallin populates a heterogeneous ensemble of inter-converting oligomers at equilibrium, with 95% of the oligomers sized between 20- and 40-mers. Despite this complexity, αB-crystallin oligomers possess several remarkably simplifying features. Both solid- and solution-state nuclear magnetic resonance (NMR) studies have established that the overall structure adopted by the constituent monomers is essentially independent of oligomer size. In addition, we have previously shown that the equilibrium size distribution can be quantitatively explained assuming that both the dimer interface and the interface holding neighboring dimers together are similar in all oligomers. These observations have allowed us to construct structural models of the principally populated oligomers in solution (Figure 1A). Alternative models of αB-crystallin have also been recently put forward.

The primary sequence of αB-crystallin and of sHSPs in general can be divided into three parts, the N- and C-termini and a core domain (Figure 1A-i). The termini are involved in holding the oligomers together, and the motional properties of a highly conserved IxI motif in the C-terminus are thought to play a critical role in regulating the kinetics and thermodynamics of monomer exchange between oligomers. The end of the C-terminus, a region termed the “extension” (green in Figure 1A-i), is intrinsically disordered. For example, residues 164–175 were observed in solution 1H NMR spectra using experiments and samples that were not optimized for...
large proteins, which is only possible if this region is highly mobile.\(^{17-19}\) Of interest, mutations in the C-terminus modulate chaperone activity\(^{20,21}\) and have been linked to disease.\(^{22-24}\)

When both of the termini are removed, the core domain forms \(\beta\)-sheet-rich dimers (Figure 1A-ii) that serve as the “building blocks” for the oligomeric structures.\(^{25,26}\) Deletion of the N terminus and the C-terminus past the IxI motif (residues 159–161 in \(\alpha\B-crystallin, Figure 1A-i\) leads to predominantly monomers and dimers in solution, although a significant population of oligomers remains.\(^{27}\) When crystallized, the IxI residues in this deletion construct form contacts between dimers,\(^{27}\) mimicking an interaction observed in crystal structures of homologous sHSP oligomers.\(^{18,20}\) Notably, solid-state NMR studies of \(\alpha\B-crystallin comprising full-length protein establish that each dimeric unit adopts a structure similar to that in Figure 1A-ii, with the IxI residues tightly bound to adjacent dimers.\(^{10}\) In contrast, a different picture has emerged from solution-state NMR, where spin relaxation studies of the IxI residues show that they are highly mobile.\(^{9}\) Indeed, the 13C chemical shifts of the Ile \(\delta_1\) carbons in the IxI motif, 12.5 and 12.3 ppm, are consistent with the values observed for a random coil.\(^{30}\) Although it is clear that in solution the IxI motif is disordered and unbound, paramagnetic relaxation enhancement NMR measurements establish that these residues, nevertheless, are localized to a region proximal to a binding pocket\(^{9}\) that has been elucidated by solid-state NMR and X-ray diffraction studies.\(^{10,27}\)

In order to resolve this apparent discrepancy and to further characterize the dynamics of the IxI residues, we have employed a combination of both solution- and solid-state NMR using U-[\(\text{H}\)], Ile-[\(\text{13CH}_3\) \(\delta_1\)], Leu,Val-[\(\text{13CH}_3,12\text{CD}_3\)] samples of \(\alpha\B-crystallin. The solution NMR experiments exploit a methyl-TROSY effect that is critical in studies of very high molecular weight proteins (average molecular mass of 580 kDa).\(^{31,32}\) Following the elegant work of Reif, Oshkinat, and co-workers on the \(\alpha\B-crystallin system\(^{33}\) we have used MAS NMR of samples sedimented by ultracentrifugation\(^{34}\) and prepared according to the FROSTY protocol (referred to as FROSTY-MAS below) to examine both the “liquid-like” and “solid-like” properties of the IxI motif of \(\alpha\B-crystallin. By using the same sample labeling for both sets of experiments, the solution- and solid-state NMR results can be compared directly. Moreover, the extensive protein deuteration that benefits the solution NMR studies is also advantageous in removing unwanted dipolar interactions in the solid-state-based experiments. We show here, through a combination of both solution- and solid-state NMR techniques, that at temperatures below 0 \(\degree\)C the IxI moiety exists in at least two states, corresponding to bound and highly mobile conformations, while above 0 \(\degree\)C only the mobile state is observed in NMR spectra. Solution NMR relaxation dispersion (RD) studies establish further that the mobile IxI conformation interchanges with a sparsely populated state that, remarkably, has structural features that are similar to those of the bound state that has been characterized previously by both solid-state NMR\(^{30}\) and X-ray diffraction.\(^{37}\)

### MATERIALS AND METHODS

#### Protein Production and Purification

\(\alpha\B-crystallin was prepared by overnight expression in E. coli BL21(DE3) cells at 37 \(\degree\)C. Cells were harvested and lysed in 20 mM Tris pH 8 buffer in the presence of a protease inhibitor cocktail (Roche). After purification on a Q-column, with protein eluting at 100 mM NaCl,\(^{35}\) the fractions containing \(\alpha\B-crystallin were pooled, concentrated, and further
purified using an S200 gel filtration column in 150 mM NaCl, 50 mM Tris, pH 8.0. U-13C,15N-[U-13CH3]-αIle-[1-13CH3]-βIle protein was prepared by growing in D2O and M9 media with [13C,15N]-glucose as the sole carbon source and the precursor sodium α-ketobutyrate [1-13CH3,15N]-CD3COONa (60 mg/L) added 1 h prior to induction. 40 U-13C,15N-[U-13CH3]-βIle-[1-13CH3]-δIle, Leu,Val-[1-13CH3]-15N]-CD3 αβ-crystallin was generated as for Ile-(αIle-[1-13CH3],δIle), βIle-[1-13CH3]-15N]-CD3 αβ-crystallin was described previously. 36 NMR samples in the concentration range 200 μM to 1.5 mM were transferred into a buffer containing 2 mM EDTA, 2 mM NaN3, and 30 mM sodium phosphate in 100% D2O, adjusted to the desired pH with DCl and NaOD. Samples for solid-state NMR were then mixed with glycerol and further concentrated, as described below.

In order to obtain the assignments of I159δ/I161δ, I161δ/I161δ, and V169γ/I169γ methyl groups, with the addition of α-ketoisovalerate [1-13CH3,15N]-CD3COONa (80 mg/L) as described previously. 50 NMR samples in the concentration range 200 μM to 1.5 mM were transferred into a buffer containing 2 mM EDTA, 2 mM NaN3, and 30 mM sodium phosphate in 100% D2O, adjusted to the desired pH with DCl and NaOD. Samples for solid-state NMR were then mixed with glycerol and further concentrated, as described below.

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SOLUTION-STATE NMR MEASUREMENTS. 13C-1H correlation spectra were acquired using Varian NMR spectrometers operating at field strengths of 11.7 T (room temperature probe), 14.0 T (cryogenically cooled probe), and 18.8 T (room temperature probe) over a range of pHs (5–9) and temperatures (5–50 °C) using experiments that exploit a methyl-TROSY effect, described in detail previously. 30–32 Relaxation data were recorded on samples at pH 5 where the effects of chemical exchange are maximal. Data showing the same exchange process were obtained at pH 7, though the effects were significantly more challenging to quantify because the population of the excited state is considerably lower (Figure S3). All data were processed using the NMRpipe program. 30

1H transverse relaxation rates, with selection for the slowly relaxing methyl proton component, were measured as described previously 40 over a range of temperatures extending from 5 to 50 °C, pH 5.40, 14.0 T. Similarly, 1H and 13C single-quantum RD experiments were performed over the same temperature range, pH 5, 14.0 T and 18.8 T. A constant time Carr–Purcell–Meiboom–Gill (CPMG) relaxation delay of 40 ms was employed with 17 CPMG frequencies in the range 50–2000 Hz, including several repeat values for error analysis. 43 R2,eff (νCPMG) values were obtained via the relation R2,eff (νCPMG) = 1/T2relax ln(I(νCPMG)/I0), where I(νCPMG) and I0 are peak intensities with and without the 40 ms constant-time CPMG delay, respectively, and νCPMG is the inverse of twice the delay between successive 180° pulses. Values of exchange parameters were extracted from fits of R2,eff (νCPMG) profiles to a two-site exchange model, as described previously 43 using the in-house written program CATIA (http://abragam.med.utoronto.ca/software.html). 44,45

Spin-state selective methyl 13C RD experiments were performed at 50 °C, pH 5 following a procedure described in detail previously. 40 A constant time CPMG element of 40 ms was used, along with 21 CPMG frequencies in the range 50–2000 Hz. Errors in R2,eff were estimated on the basis of repeat νCPMG values. 43 Dispersion data were fitted to a two-site exchange process using a relaxation model that has been described. 44,45 Central to the work here is that residue-specific values for S02 and γ are obtained, where S0 is the square of an order parameter quantifying the amplitude of motion of the methyl rotation axis and γ is the correlation time for the assumed isotropic molecular tumbling. Thus, it is possible to establish the relative mobilities of corresponding methyl groups in the ground and excited states, as discussed below.

SOLID-STATE NMR MEASUREMENTS. αβ-crystallin protein samples were prepared for solid-state NMR analyses according to the “pHUSTY” procedure of Maini et al. 33 In brief, labeled αβ-crystallin was concentrated in a 100 kDa ultrafiltration membrane to a volume of 36 μL. An equal volume of 40% glycerol was added once a concentration of approximately 50 mg/mL was obtained. The sample volume was then reduced by half, yielding a solution of 50 mg/mL protein in 20% v/v glycerol, in the NMR buffer described above. The sample (final volume 36 μL) was subsequently packed into a 3.2 mm thin walled MAS rotor using centrifugation.

Solid-state NMR spectra were acquired on a narrow-bore Varian VNMRS spectrometer operating at a field strength of 11.7 T. All experiments were performed using a triple-resonance T3 MAS probe operated in two-channel mode. In all cases the MAS frequency was 12 kHz, and 1H decoupling during 13C evolution, detection, and mixing periods was achieved with a 120 kHz 1H two-pulse phase modulated (TPPM) scheme. 46 The sample temperature under MAS conditions was calibrated using the 39Pb chemical shift of solid Pb(NO3)2. At −20 and +20 °C, respectively, 1 and 3 °C of sample heating was observed.

1D 13C spectra were obtained using either INEPT (insensitive nuclei enhanced by polarization transfer) or CP transfer from 1H, with each spectrum taking approximately 2 h to acquire. For CP experiments, 13C and 1H field strengths of approximately 50 and 62 kHz respectively were used, with contact times of 1.2–1.4 ms and a small linear ramp on the 1H channel. 48 2D 13C-1H correlation spectra were recorded using a finite-pulse radio frequency driven recoupling (RFDR) 49 sequence for 13C recoupling, with a mixing period of 8 ms and 25 kHz 13C fields during RFDR. The transmitter was centered within the aliphatic region for RFDR experiments, and 40 t1 points (sweep width of 3770 Hz) were acquired in the indirect dimension, with a total experiment time of 2 s. 2D 1H-13C Heteronuclear correlation (HETCOR) spectra were obtained using either CP or INEPT transfer following the t1 (1H evolution) period. 1H homonuclear decoupling was employed during the t1 period of the CP-based experiment using a frequency-switched Lee–Goldberg (FSLG) sequence 50 at a field strength of 80 kHz. A total of 64 t1 points were acquired, with a sweep width of 9600 Hz in the indirect dimension and a total experiment time of 18 h. A scaling factor of 1/√3 was applied to the 1H chemical shift dimension of the FSLG-CP HETCOR, as previously described. 51

RESULTS AND DISCUSSION

As described in the Introduction, the IxI motif in the C-terminal region of αβ-crystallin, Figure 1A, plays a critical role in controlling the kinetics and thermodynamics of subunit interchange between the ensemble of oligomeric structures that this protein populates in solution 9 (see below). It is important, therefore, to reconcile the differences between solution-state NMR results establishing that the Ile residues are highly mobile and solid-state NMR data showing that the IxI motif is in a bound conformation, and hence (relatively) rigid. To this end we have recorded both solution- and solid-state NMR spectra (Figure 1B) using samples prepared with identical labeling to serve as a starting point for further analysis of the dynamics of this important region 19–24 (see below). FROSTY-MAS HETCOR experiments using cross-polarization (CP) for magnetization transfer have been recorded previously on samples of αβ-crystallin at low temperatures. 5,3 We show here that a multiple-temperature analysis of such HETCOR spectra, measured with CP and INEPT magnetization transfers, and solution heteronuclear multiple quantum coherence (HMQC) data sets gives insight into how the structures observed by solid-state NMR relate to those populated in solution, which are the more physiologically relevant.

In a previous publication we noted that there are four very intense correlations in the Ile, Leu, Val 13C-1H solution HMQC spectrum of αβ-crystallin, 9 derived from I159δ/I161δ, I161δ/I161δ, and V169γ/I169γ methyl groups localized to the C-terminus of the protein (Figure 1B-i) (see also Figure S1). Interestingly, none of the N-terminal Ile residues (I3δ/I3δ, I5δ/I5δ, I10δ/I10δ) give rise to cross peaks of comparable intensities, suggesting that they are much less dynamic. The proton and carbon spins from each of I159δ, I161δ, and V169γ/I169γ methyl groups have relaxation...
rates and chemical shifts that are consistent with a disordered region.\textsuperscript{5} The intensities of the V169\textgamma resonances increase with temperature in a manner consistent with increased tumbling rates. In contrast, I159\textgamma and I161\textgamma peak intensities increase much less significantly and in fact decrease at higher temperatures, consistent with a conformational exchange process that becomes more significant with increasing temperature.\textsuperscript{9} The observed intensity vs temperature profile for these Ile and Val residues does not reflect different temperature dependencies for $^1H$ $T_1$ values, as measurements establish essentially identical relative values at 25 and 45 °C, $R_2^{\text{Ile}}/R_2^{\text{Val}} = 2.0 \pm 0.1$. In fact, as we show below, both the $^1H$ and $^{13}C$ line widths for I159\textgamma and I161\textgamma are significantly larger than for V169\textgamma at 50 °C, with the differences becoming pronounced already at 30 °C.

By means of comparison we have also recorded $^{13}C$ detected FROSTY-MAS\textsuperscript{33} based HETCOR spectra where the $^1H$-$^{13}C$ polarization transfer step is carried out using an INEPT scheme\textsuperscript{52} (MAS INEPT HETCOR, Figure 1B-ii). The INEPT transfer will preferentially “select” flexible regions so that the solid-state data set recorded in this manner is very similar to the solution spectrum, with intense correlations observed for I159\textgamma, I161\textgamma, and Val169\textgamma methyl groups. All correlations increase significantly with temperature from −22 to 0 °C. Interestingly, both Ile peaks decrease in intensity from 0 to 30 °C, consistent with an exchange process, as observed in the solution state, although in solution the increased line broadening from exchange is compensated to some extent by more rapid overall tumbling as the temperature increases. FROSTY-MAS HETCOR spectra have also been obtained with $^1H$-$^{13}C$ cross-polarization\textsuperscript{48,51} (MAS FSLG-CP HETCOR, Figure 1B-iii) that are very similar to those previously recorded by Reif and co-workers.\textsuperscript{33} Notably, at −22 °C a resonance from I159\textgamma is observed at 9.5 ppm, in contrast to 12.3 ppm for the $^1H_2$-$^{13}C$ chemical shift difference in the excited state. This information is critical to our model of subunit exchange that is described below.

In order to probe the conformational exchange process involving the Ile residues (see above) in more detail and to understand how the inter-converting states may be related structurally, we have carried out solution-state CPMG\textsuperscript{53,54} RD experiments (Figure 2). The single-quantum $^{13}C$ RD profiles\textsuperscript{42} obtained for both I159\textgamma and I161\textgamma show the characteristic signature of exchange with effective transverse relaxation rates, $R_2^{\text{eff}}$, decreasing as a function of increasing pulse rate ($\nu_{\text{CPMG}}$). In contrast, the RD curves for the two methyl resonances from V169 are independent of $\nu_{\text{CPMG}}$. Consistent with the absence of chemical exchange or with a scenario where there is little chemical shift difference between exchanging states. A detailed analysis of the RD data presented previously indicates that the major conformation, corresponding to the dynamic unbound IxI state, exchanges with a second conformer which has a maximum fractional population of 2% at 50 °C, pH 7, increasing with decreasing pH.\textsuperscript{9} The effects of exchange as a function of both pH and temperature as quantified by both NMR and mass spectrometry have been described previously,\textsuperscript{8,9} establishing that the same exchange process manifests over the complete pH and temperature range examined. For completeness here, Figure S3 shows $^1H$ 1D, $^{13}C$-edited NMR spectra recorded at a number of temperatures and pH values, with exchange contributions increasing at the lower pH values and at higher temperatures. As described in Materials and Methods and below, we have carried out further detailed RD experiments at pH 5 to maximize the observed exchange effects.

At the time of our initial relaxation experiments,\textsuperscript{9} we were not able to determine the Ile $^{13}C\delta_1$ chemical shifts in the sparsely populated state since only the absolute values of the differences in shifts between exchanging conformers are available from RD data sets. The structural features of the IxI motif in the bound conformation were thus not established. Additionally, no information was available concerning the motional properties of IxI in the excited state. This information is critical to our model of subunit exchange that is described below.

With the recent development of $R_{xy}$ experiments that provide signs of chemical shift differences between exchanging states even in cases where large differences in intrinsic relaxation rates are present (see below), we can now establish that the $^{13}C$ chemical shifts of I159\textgamma and I161\textgamma in the excited state are 10.5 and 14.1 ppm, respectively. These shifts are similar to those for the bound state from solid-state NMR at −22 °C (9.5 and 15 ppm respectively, Figure 1B-iii), indicating that the sparsely populated state may resemble the bound conformation observed by solid-state NMR.\textsuperscript{10}

As described above, previous solution NMR studies have established that the IxI ground state is highly dynamic.\textsuperscript{9} Further evidence is derived from the relative intensities of the $^{13}C$ methyl multiplet components recorded in an $^1H_1$-coupled HSQC data set. In such a spectrum four individual components...
of the \(^{13}\)CH\(_3\) group are observed corresponding to the \(^{13}\)C spin coupled to protons either all “up” (\(\alpha\alpha\alpha\)) or “down” (\(\beta\beta\beta\)), outer multiplet components, or to protons in the “2-up/1-down” (\(\alpha\alpha\beta\)) or “2-down/1-up” (\(\beta\beta\alpha\)) states, inner components. For a highly dynamic methyl group there is little difference in transverse relaxation rates of each of the carbon lines, so a 3:1:1:3 multiplet structure is predicted.\(^{32}\) In contrast, in the slow tumbling limit the net \(^{13}\)C–\(^{1}\)H dipolar interaction is 3-fold larger for the outer lines, leading to a decay rate that is approximately 9 times more rapid than for the inner components.\(^{36}\) Similar ratios of outer to inner components, 3:1:1 to 2:9:1, are found for both I159\(_1\) and I161\(_1\), confirming the highly dynamic nature of these residues in the ground state (Figure 3A). To obtain insight into the motional properties of these isoleucines in the excited state, we recorded the decay of methyl \(^{1}\)H transverse magnetization,\(^{44}\) \(R_{2,1\text{H}}\), as a function of temperature (Figure 3B). As a control we also measured \(R_{2,1\text{H}}\) values for the methyl protons of V169. These were found to decrease with temperature, as expected on the basis of decreasing solution viscosity and enhanced molecular tumbling. Between 0 and 20 °C, proton relaxation rates for I159\(_1\) and I161\(_1\) methyl groups also followed this trend. Above 20 °C, however, \(R_{2,1\text{H}}\) values were found to increase significantly, and at 50 °C, \(^{1}\)H transverse relaxation rates for the \(\delta\) methyls of I159/I161 were approximately 3-fold larger than for the protons from methyls of V169. The increase in relaxation rates (minimum in \(R_2\) vs temperature profile, Figure 3B) coincides precisely with the point at which the excited state, and so the magnitude of the difference in relaxation between the outer and inner lines, is maximal.

Figure 3. (A) Trace through a \(^{1}\)H–\(^{13}\)C HSQC spectrum of U−[\(^{1}\)H\]Ile−[\(^{13}\)CH\(_3\), 61], Leu−[\(^{13}\)CH\(_3\),\(^{15}\)CD\(_3\)], \(\alpha\beta\)-crystallin, 45 °C, 14.0T, pH 5, recorded without proton decoupling during acquisition of carbon chemical shift. The resonances from I159\(_1\) and I161\(_1\) are approximately in the ratio 3:1:1:3. (B) \(^{1}\)H \(R_2\) values for the methyl protons of V169 as a function of temperature for I159\(_1\) and I161\(_1\), and V169\(_1\)/\(\gamma\2) (stereospecific assignments of V169 methyl groups are not available; hence V169 methyls are denoted “A” and “B”). Rates are measured as described previously.\(^{9}\) \(R_{2,1\text{H}}\) values for V169 decrease in a manner consistent with decreasing solution viscosity. Similarly, \(R_{2,1\text{H}}\) rates for I159\(_1\) and I161\(_1\) decrease with increasing temperature in the range 0–20 °C, while at higher temperatures the rates increase rapidly. Solid lines in the figure are meant to guide the eye. (C) 2D \(^{13}\)C–\(^{1}\)H correlation map, corresponding to the first plane of a spin-state-selective CPMG RD experiment,\(^{45,57}\) with the four spin-state-selective multiplet components observed. The \(^{1}\)H spin states associated with each multiplet line are as indicated with \(\alpha\), \(\beta\), or \(\gamma\), corresponding to \(^{1}\)H spin-up and down. (D) Fits of RD curves of the four separated \(^{13}\)C methyl multiplet components can establish the dynamic properties of the sparsely populated (‘invisible’) state, as described in detail previously.\(^{45}\) The sizes of dispersion profiles (arrows) for the outer lines (only the most upfield line is shown) are smaller than for the inner lines (only one of the two is indicated) in the case where the methyl probe in the excited state is less dynamic than in the ground conformation. (E) RD curves of the four separated components for I159\(_1\) (i) and I161\(_1\) (ii) pH 5, 40 °C, 18.8 T, analyzed and fit (solid lines) as described previously.\(^{45}\) The sizes of dispersion profiles derived from the inner lines are larger than those from the outer lines for both residues, corresponding to \(R_{2,1\text{H}}\) increase. Data were recorded at pH 5, where the population of the excited state, and so the magnitude of the difference in relaxation between the outer and inner lines, is maximal.
superimposed, as illustrated schematically in Figure 3D. The main features in this figure can be understood most simply by considering a two-state exchanging spin system in moderately slow exchange. In the limit that the CPMG pulsing rate is very slow, $\nu_{\text{CPMG}} \to 0$, $R_{2,\text{eff}}$ rates for each of the four lines are given by $R_{2,G}^{\text{int}}(0) = k_{G} + R_{2,G}^{\text{int}}j \in \{\alpha\alpha\alpha\beta\beta\beta\beta\beta\beta\}$, where $R_{2,G}^{\text{int}}j$ is the intrinsic $^1$C methyl $j$ spin-state transverse relaxation rate for the ground-state conformer and $k_{G}$ is the rate of exchange from state $m$ to $n$. In the case where pulsing is rapid, $\nu_{\text{CPMG}} \to \infty$, the value of $R_{2,\text{eff}}$ becomes the population-weighted average of relaxation rates in each of the inter-converting states (assuming that exchange is faster than $k_{G} + R_{2,G}^{\text{int}}$), $R_{2,G}^{\text{int}}(\infty) = p_{G}R_{2,G}^{\text{int}} + p_{E}R_{2,E}^{\text{int}}$, where $p_{E}$ and $p_{G}$ are the fractional populations of the ground and excited states, respectively. The "size" of the dispersion profile (arrow in Figure 3D) is thus given by $R_{2,G}^{\text{int}}(0) - R_{2,G}^{\text{int}}(\infty) = k_{G} + p_{E}(R_{2,E}^{\text{int}} - R_{2,G}^{\text{int}})$. Noting that in the macromolecule limit (such as for proteins) the outer multiplet components relax more rapidly than the inner lines, $R_{2,G}^{\text{int}} > R_{2,E}^{\text{int}}$ (see above) and assuming that $R_{2,G}^{\text{int}} > R_{2,G}^{\text{int}}$ the schematic shown in Figure 3D is obtained where dispersion profiles from the inner lines are larger than those from the outer components. In contrast, when $R_{2,G}^{\text{int}} < R_{2,G}^{\text{int}}$ the situation is reversed. Figure 3E-i,ii shows RD profiles for each of the four lines for I159° (i) and I161° (ii) (18.8 T, 50 °C), and it is clear that substantial differences in the curves are obtained, exactly as predicted in Figure 3D. Even without a detailed analysis of the data, it follows simply by inspection that the excited-state conformation is one in which the Ile residues are very much less mobile than in the populated ground state. Fits (solid lines) to the experimental data (squares), measured at fields of 11.7, 14.0, and 18.8 T, using a two-site model of exchange were performed as described in detail previously, assuming that both Ile residues report on the same exchange process (same populations and rates). An important parameter obtained from the fit is $\Delta(S(\tau_{c})) = (S^{2}\tau_{c})_{E} - (S^{2}\tau_{c})_{G}$, where $S^{2}$ is a methyl axis order parameter and $\tau_{c}$ is the (assumed isotropic) correlation time in either the excited (E) or the ground (G) state. Values of $\Delta(S^{2}\tau_{c})$ of $+180$ and $+100$ ns were obtained for I159° and I161°, respectively, confirming that the excited state is indeed bound.

The formation of the excited state has previously been linked with the process of subunit exchange in $\alpha\beta$-crystallin. The finding that it is bound leads to a straightforward model that can explain why subunit exchange occurs in terms of the formation and breaking of specific interactions within oligomers. Our previous studies have established that monomers of $\alpha\beta$-crystallin (half-ellipses in Figure 4) are held in place within a given oligomer by two types of interactions. The first is intra-dimer, depicted in our model in Figure 4 by a red ring, while the second class is inter-dimer, denoted by wavy lines (black or gray). A model that assumes only these two classes of interactions, which are essentially independent of oligomer size, quantitatively accounts for the oligomeric distributions observed over a wide range of solution conditions, as measured by mass spectrometry. While the structural basis of the intra-dimer interactions is well understood, the atomic contacts that constitute the inter-dimer interface are presently less well known. Previous work using NMR paramagnetic relaxation enhancement measurements has shown that the intrinsically disordered "ground state" of the C-terminus (yellow) makes transient contacts with a specific hydrophobic groove on adjacent monomers, contributing to the stabilization of the inter-dimer interface (denoted by gray wavy lines in Figure 4). Other interactions between monomers or involving the N-terminus have also been identified as contributing toward the stability of this interface and are denoted by black wavy lines.

Using results from a combination of mass spectrometry, X-ray diffraction, and NMR analyses, we concluded previously that (1) all monomers must be in equivalent environments, (2) oligomers are comprised of dimeric building blocks, (3) each monomer is connected to an oligomer via a pair of C-terminal "cross-linking" interactions that must be broken prior to monomer release, and (4) models of oligomers could be constructed based on polyhedral scaffolds. Taken together, in concert with experimental data from ion-mobility...
mass spectrometry and electron microscopy, the structure of a 24-mer can be described by an octahedron, shown schematically in Figure 4B ("oligomer i"). Focusing on a single polyhedral vertex (they are all the same), it is clear that four dimers come together with each specific monomer interacting with its two immediate neighbors (black and gray wavy lines). In order for a monomer to dissociate from the oligomer, therefore, both of the “immediate neighbor” stabilizing interactions must be broken. Supporting this notion, NMR RD and mass spectrometry kinetic data can be reconciled assuming that the dissociation process involves the simultaneous reannexation of a pair of C-termini (including IxI motifs), as depicted in the model described by Figure 4. Here, disordered C-termini, corresponding to ground states (yellow), simultaneously form ordered low-populated conformers, denoted here as either inter-dimer (green, left side) or intra-dimer (blue, right side), leading to the release of the C-terminal interactions. In this model, formation of either two inter- or two intra-dimer C-terminal interactions, or a mixture of both, corresponding to an ordering of the IxI region, results in a distortion of the oligomer, leading to the breaking of interactions that hold a specific monomer in place and thereby facilitating its departure. Subsequently the structure rearranges so that all monomers occupy an identical environment. The model thus provides a link between the macroscopic process of subunit exchange and microscopic fluctuations of the C-terminal IxI.

**CONCLUDING REMARKS**

The present study establishes that at least two distinct structural environments are adopted by the C-terminal IxI residues of aB-crystallin, one free and one bound, with relative populations varying substantially with temperature. While both disordered and bound states are seen at low temperatures, above 0 °C only the mobile conformation is appreciably populated. Interestingly, this “ground-state conformer exchange with a second sparsely populated state where the IxI moiety is more rigid bound” and where the 13C Ile δ1 methyl chemical shifts are similar to those reported from a solid-state NMR study of aB-crystallin at low temperature. The excited state has been shown previously to play an important role in controlling the kinetics and thermodynamics of subunit interchange between different oligomers and the resulting particle size distribution. Intriguingly, the present study has established that the excited-state conformation of the IxI motif shares several important features with the “bound”-state structure reported from both solid-state NMR and X-ray methods, leading to a proposed mechanism for the subunit exchange process. The complementary information derived here from both solid- and solution-state NMR experiments is important for elucidating the detailed mechanism by which dynamics are able to regulate the function of this important molecule.

**ASSOCIATED CONTENT**

Supporting Information

![Figures showing assignment of 1159/161 δ1 methyl groups, solid-state NMR spectra, effects of pH and temperature on 1159/161 and V169 1H NMR spectra, and 1H/13C CPMG RD profiles of aB-crystallin. This material is available free of charge via the Internet at http://pubs.acs.org.](http://pubs.acs.org)

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**Notes**

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