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Thermal-induced Unfolding of β-Crystallin and Disassembly of its Oligomers Revealed by Temperature-Jump Time-Resolved Infrared Spectroscopy

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β-Crystallins are the major structural proteins existing in the vertebrate lens, and their conformational stability is critical in maintaining the life-long transparency and refraction index of the lens. Seven subunits of β-crystallins naturally assemble into various heterogeneous oligomers with different sizes. Here, we systematically investigated the thermal stability of the different secondary structures present in β-crystallins and then the dynamic process for the thermal-induced unfolding of β-crystallins by Fourier transform infrared spectroscopy-monitored thermal titration and temperature-jump nanosecond time-resolved IR difference absorbance spectra. Our results show that the N-terminal anti-parallel β-sheets in β-crystallin are the most unstable with a transition midpoint temperature at 36.0±2.1 °C, leading to the formation of an intermediate consisting vastly of random coil structures. This intermediate structure is temporally assigned to that of the monomer generated by the thermal-induced disassembly of β-crystallin oligomers with a transition midpoint temperature of 40.4±0.7 °C. The global unfolding of β-crystallins that leads to denaturation and aggregation indicated by the formation of intermolecular anti-parallel β-sheets has a transition midpoint temperature determined as 72.4±0.2 °C. Temperature-jump time-resolved IR absorbance difference spectroscopy analysis further reveals that thermal-induced unfolding of β-crystallins occurs firstly in the anti-parallel β-sheets in the N-terminal domains with a time constant of 50 ns.

\textbf{Key words:} β-Crystallin, Protein dynamical structure, Temperature-jump, Time-resolved IR spectrum

I. INTRODUCTION

α-Crystallin and the superfamily of βγ-crystallins are major proteins existing in the vertebrate lens [1−3], whose short-range order at high concentration ensures both the transparency and the high refractive index of lens for image formation on the retina [3−5]. In order to fulfill their optical function in the whole life span of the organism, crystallins have to be soluble and stable [6]. Unfortunately, with aging, crystallins gradually lose solubility and stability owing to misfolding and aggregation in the presence of environmental stresses such as heat, ultraviolet radiation, etc. [4, 7]. The accumulation of insoluble aggregates obstructs the passage of light through the lens, and finally leads to the light scattering, which is generally recognized as the main cause of cataract [4, 7]. The oligomeric β-crystallin and the monomeric γ-crystallin share a common polypeptide chain fold named as βγ-crystallin domain comprising two Greek motifs [8]. Greek motif is a common secondary structural motif comprising four β-strands in protein structure (Fig.1(a)). The two consecutive Greek key motifs intercalate to form a β-sandwich of two anti-parallel β-sheets, having a complex topology in that each 4-stranded motif exchanges its third β-strand to a β-sheet belonging to its partner motif (Fig.1(a) and (b)). Although β-sandwich domains are common in proteins, in βγ-crystallins they are characterized by their high internal conformational symmetry (Fig.1(c)).

In the lens, β-crystallin is the most widely distributed structural protein family including basic β-crystallins (βB1−βB3) and acidic β-crystallins (βA1−βA4) [9, 10], and their stability is critical in maintaining the transparency and refraction index of the lens. These β-crystallins naturally assemble into various heterogene-
ous oligomers with their size in the range of 50–200 kDa. β-Crystallin has been proposed to have high stability due to its oligomeric states [11]. However, some monomeric γ-crystallins are found to be much more stable than the β-crystallin homodimers according to thermodynamic calculation [6], suggesting that the oligomeric states may not be a key determinant in crystallin stability. Therefore, the understanding of conformation stability and unfolding/folding pathway of the separate domains is of great importance to reveal the mechanism of cataract, on which few studies have been reported to the authors’ knowledge.

The temperature-jump (T-jump) method can initiate a thermal-induced protein unfolding/folding process as fast as 70 ps [12] while the time-resolved IR difference spectroscopy can probe the dynamical structural changes of protein secondary structures in amide I band [13–17]. T-jump coupled to time-resolved IR difference spectroscopy has proven to be a powerful tool to trace the protein dynamical structures. In the current work, the unfolding process of β-crystallins has been examined, in terms of both the thermodynamics and the dynamics. Thermal titration curves were performed using FTIR spectroscopy to reveal the thermal stability of the major secondary structural components in the β-crystallins, then T-jump time-resolved IR difference spectroscopy was applied to probe the thermal-induced unfolding dynamical process for these secondary structural components.

II. MATERIALS AND METHODS

A. Chemicals

Bovine lenses were purchased from Beijing Zhuo Chen Livestock Company Limited. Sepacryl S-300HR was obtained from GE Healthcare. β-crystallin antibody was purchased from Abcam. Na₂HPO₄ and K₂HPO₄ were purchased from Avantor Performance Materials and Duksan Pure Chemicals, respectively. NaCl and KCl were obtained from Yi Ren Da Chemicals.

B. β-Crystallin isolation and purification

β-crystallin was isolated from the lenses of freshly excised bovine eyes obtained from a local slaughterhouse. The lenses were removed and separated into nucleus and cortex. The cortical material was homogenized in phosphate buffered saline (NaCl 137 mmol/L, KCl 2.7 mmol/L, Na₂HPO₄ 10 mmol/L, K₂HPO₄ 2 mmol/L, pH=7.4). A water-soluble fraction was obtained by centrifuging the homogenate at 10⁴ r/min for 30 min at 4 °C. The supernatant containing the soluble fraction was fractionated by gel filtration on a FPLC (fast protein liquid chromatography, AKTA purifier plus, GE Healthcare, SE) system using a Sepacryl S-300HR (26 mm×600 mm) (Fig.2(a)). We collected the soluble purified protein in each peak. The purified protein was identified by SDS-polyacrylamide gel electrophoresis and Western blot (Fig.2 (b) and (c)). The proteins of the sample were separated using SDS polyacrylamide gel electrophoresis. SDS polyacrylamide gel electrophoresis maintained polypeptides in a denatured state once they had been treated with strong reducing agents to remove secondary and tertiary structures and thus allowed separation of proteins by their molec-

FIG. 1 Cartoon representation of the crystal structure of β-Crystallin. (a) and (b) The βγ-crystallin domain is made from two linear sequence-related Greek motifs. (c) The crystal structure of a subunit of β-crystallins, i.e., dimeric βB2-crystallin (PDB ID: 2BB2). (b) and (c) were drawn using pymol program.

FIG. 2 β-Crystallin isolation and identification. (a) β-crystallin was isolated by gel filtration on a FPLC at 280 nm. The soluble purified protein in each peak was collected. The purified β-crystallin was identified by (b) SDS-polyacrylamide gel electrophoresis and (c) Western blot analysis with antibody to β-crystallin.
cular weight (the molecular weight of \( \beta \)-crystallin was predicted 23 kDa). During the process of Western blot, \( \beta \)-crystallin antibodies which were linked to a reporter enzyme were specific detection tools that bound \( \beta \)-crystallin directly. When exposed to an appropriate substrate this enzyme drove a colourimetric reaction and produced a color. The sample which produced a color was referred to \( \beta \)-crystallin. It has been reported that the presence of salt in protein solution could cause complexity in amide I' IR absorption [18, 19], the buffer of the purified \( \beta \)-crystallin for all the IR experiments was replaced by D\( _2 \)O (pD=7.4) and the concentration of \( \beta \)-crystallin was kept at about 25 mg/mL.

C. FTIR spectroscopy

Temperature-dependent FTIR spectra were collected on a spectrometer (VERTEX 70 V, Bruker Optics, DE) using a temperature controlled IR cell as described previously [20]. The two-compartment CaF\(_2\) sample cell with a 50-\( \mu \)m thick Teflon spacer was used for both the protein solution and reference D\( _2 \)O, respectively. Temperature regulation was controlled by water circulation at an accuracy of \( \pm 0.1 \) °C. To eliminate spectral interference from atmospheric water vapor, the measurements were performed in a home-built vacuum chamber. We recorded each spectrum in the amide I' band at a resolution of 4 cm\(^{-1}\) and 40 scans were signal averaged.

D. \( T \)-jump time-resolved IR difference absorbance spectra

The details of \( T \)-jump time-resolved IR absorbance difference spectrometer have been described previously [20–22]. Briefly, a 1.9-\( \mu \)m pulsed laser, which was generated via Raman shifting the fundamental output of a Nd:YAG laser (Lab 170, 10 Hz, 8–12 ns; Spectra Physics, Mountain View, CA) at 1064 nm, was used to heat the solution rapidly, known as temperature jump. The induced transient IR absorbance changes were probed by a CO mid-IR laser at a spectral spacing about 4 cm\(^{-1}\) (Dalian University of Technology, Dalian, CN) in conjunction with a MCT detector (Kolmar, Newburyport, MA) and a digital oscilloscope (Tektronix, TDS 520D, Wilsonville, OR). The temperature-dependent FTIR spectra of D\( _2 \)O were taken as an internal standard for \( T \)-jump calibration [20, 23, 24]. The temporal resolution of this system was about 30 ns after the deconvolution of the instrumental response [20].

It is important that the protein should undergo a complete reversal process when the temperature of the sample relaxes back to its starting temperature after the heating laser pulse (the relaxation time is less than 10 ms, much shorter than the interval between two successive heating laser pulses, \( i.e. \), 100 ms), which would guarantee the reliability of the \( T \)-jump time-resolved IR difference absorbance measurement in a multi-shot measuring mode. Thus, we collected heating-and-cooling cycled FTIR spectra to determine the highest accessible temperature at which the reversibility of the protein unfolding/folding can still remain [20], which is about 46 °C for \( \beta \)-crystallins in D\( _2 \)O solution.

III. RESULTS AND DISCUSSION

As shown in Fig.1, \( \beta \)-crystallin contains two major secondary structures, \( i.e. \), anti-parallel \( \beta \)-sheets, random coils. The temperature-dependent FTIR spectra together with the corresponding second derivative spectra are displayed in Fig.3. Apparently all the temperature-dependent spectra can be roughly categorized into two groups, \( i.e. \), those below and above 70 °C. At low temperatures, the IR spectra have a slight increase of IR intensity around 1648 cm\(^{-1}\) (Fig.3(a)), while at high temperatures above 70 °C, they are dominated by a loss of IR intensity in the whole amide I' band except those around 1617 cm\(^{-1}\). Second derivatives of the absorption spectra with an enhanced spectral sensitivity are used to find the component absorption peaks, displayed in Fig.3(b), where the peak at 1631 and 1684 cm\(^{-1}\) assigned to the low and high vibration frequency respectively for the intramolecular

![Fig. 3 FTIR absorption spectra and corresponding second derivative spectra for \( \beta \)-crystallin. (a) Absorption spectra of \( \beta \)-crystallin at 25 mg/mL, in D\( _2 \)O (pD=7.4). (b) The corresponding second derivative spectra. Experiments were carried out at the temperatures as indicated.](image-url)
anti-parallel β-sheets, 1645 cm\(^{-1}\) to the random coils, 1654 cm\(^{-1}\) to the α-helices and loops, and 1669 cm\(^{-1}\) to the β-turns. At high temperatures above 70 °C, a distinct new feature is the simultaneous appearance of 1617 and 1687 cm\(^{-1}\), which is a clear spectral sign of the formation of intermolecular anti-parallel β-sheets observed in the aggregate of denatured proteins [25]. The IR bands at high wavenumbers (1684, 1687 cm\(^{-1}\)) come from the vibration of transition dipole moments (TDM) parallel to the chain direction [26], while the ones at low wavenumbers (1617, 1631 cm\(^{-1}\)) come from the vibration of TDM perpendicular to the chain direction [26]. The number of chains of the intermolecular anti-parallel β-sheets from the aggregate of denatured proteins would be larger than that of the intramolecular anti-parallel β-sheets. Thus, the band splitting of intermolecular anti-parallel β-sheets would be larger than that of intramolecular anti-parallel β-sheets owing to the stronger coupling between the neighboring chains in the intermolecular aggregates.

To better elucidate the temperature effect on the secondary structures of β-crystallin, the thermal titration curves are derived from the global multiple Gaussian-peak fitting method of all the FTIR spectra acquired at different temperatures with fixed peak positions found in the second derivative spectra (Fig.3(b)). The FTIR absorbance spectrum observed at 25 °C and its fitted spectrum by the global multiple Gaussian-peak fitting method are displayed in Fig.4(a). The ratio of the area for a given fitting peak to that of the total area is taken as the relative composition for the corresponding secondary structure component, which is used to construct the thermal titration curve. Thermal titration curves conducted at 1631 and 1684 cm\(^{-1}\) (Fig.4(b)) show that they have the approximate first transition midpoint temperatures (\(T_m\)) of 36.0±2.1 and 36.9±2.2 °C respectively, indicating that the anti-parallel β-sheets within the βγ-crystallin domains are unstable, which undergo thermal-induced disruption at around the physiological temperature. Figure 4(c) shows that the thermal titra-

FIG. 4 (a) The FTIR absorbance spectrum observed at 25 °C and its fitted spectrum by the global multiple Gaussian-peak fitting method. Baselines are subtract from the FTIR absorbance spectra before the global multiple Gaussian-peak fitting analysis. (b) The area percent thermal titration curves at 1631 and 1684 cm\(^{-1}\). The data at 1684 cm\(^{-1}\) were vertically shifted by adding a value of 5%. \(T_{m1}\) and \(T_{m2}\) are 36.0±2.1 and 72.1±0.1 °C for 1631 cm\(^{-1}\), and \(T_{m1}\) and \(T_{m2}\) are 36.9±2.2 and 73.4±1.0 °C for 1684 cm\(^{-1}\). (c) The area percent thermal titration curves at 1645 and 1654 cm\(^{-1}\). The data at 1654 cm\(^{-1}\) were vertically shifted by adding a value of 22%. \(T_{m1}\) and \(T_{m2}\) are 40.8±0.9 and 74.3±0.6 °C for 1645 cm\(^{-1}\). (d) The area percent thermal titration curves at 1617 and 1687 cm\(^{-1}\). \(T_m\) are 72.8±0.4 and 73.9±0.5 °C for 1617 and 1687 cm\(^{-1}\).
FIG. 5 The results of the SVD analysis on the temperature-dependent FTIR spectra in amide I′ band. (a) The SVD components and the corresponding singular values. (b) The resolved three species-associated spectra. (c)–(e) The corresponding second derivative spectra of the three major species-associated spectra, together with their counterparts found in the experiment. (f) The population thermal titration curves of the three major components retrieved by SVD. T_{m1} and T_{m2} are 40.4 ± 0.7 and 72.9 ± 0.3 °C for intermediate, and T_m is 72.4 ± 0.2 °C for denatured.

As shown in Fig.4, the thermal titration curve of β-crystallins at 1645 cm\(^{-1}\) exhibits a three-state transition process with the first T_m at 40.8 ± 0.9 °C and the second at 74.3 ± 0.6 °C. The first transition process can be assigned to the formation of random coil structures after the disruption of the intramolecular anti-parallel β-sheets, which is further supported by the folding/unfolding kinetics after T-jump in the later section. The second transition process at high temperatures is assigned to the global unfolding of β-crystallins which leads to protein denaturation and final aggregation, which correlates well with the transition process of the peaks centered at 1617 and 1687 cm\(^{-1}\) (Fig.4(d)) whose appearance at high temperatures is a spectral fingerprint for the formation of intermolecular anti-parallel β-sheet in the denatured protein aggregates [25]. It’s known that the absorption peaks of random coil and α-helix are well separated in most of the cases in D_2O [26, 27]. However, the trend of β-sheets unfolding into random coils has been found in our previous work [22]. Moreover, it’s noticed that there are a smaller account of α-helices than random coils, thus we assigned the IR band at 1654 cm\(^{-1}\) to the absorption of α-helices, because the titration curve at 1654 cm\(^{-1}\) shows a weak IR intensity and a little change with temperature increasing.

As shown in Fig.4, the thermal titration curve of β-crystallins at each wavenumber exhibits a three-state transition curve, revealing that there exist three states of β-crystallins in the whole temperature range of 25–85 °C. To extract intermediate species-associated population conversion process, we applied singular value decomposition (SVD) analysis on a series of FTIR spectra acquired at different temperatures. SVD analysis has proven to be a powerful method in multicomponent spectral analysis with an external variable such as time, temperature, etc. [28, 29]. By using SVD analysis method, species-associated spectra with their corresponding population change as a function of the external variable can be resolved from the integrated raw data [28, 29]. The temperature-dependent raw spectra between 1600–1700 cm\(^{-1}\) were taken for SVD analysis, and the result of the SVD analysis is displayed in Fig.5(a), which shows the first three singular values are significant, since the smallest one of the first three is larger than the maximum of the remaining values by an order in magnitude. The resolved three species-associated spectra are shown in Fig.5(b), while the corresponding second derivative spectra of these three species are shown in Fig.5 (c)–(e). Interestingly, the second derivative spectrum for the first component is almost the same as that for the natural protein acquired at room temperature (25 °C) (Fig.5(c)), thus the
SVD-resolved first component can be unambiguously assigned to the natural protein. The second derivative spectrum of the second component matches well with that at 64 °C (Fig. 5(d)), this would come from an intermediate species formed by thermal-induced unfolding of the natural protein. The spectral feature reveals that in addition to the existing β-sheet structures, the intermediate species also contains plenty of random coil structures centered at 1645 cm\(^{-1}\). Furthermore the second derivative spectrum of the third SVD-resolved component is almost the same as that for the denatured protein at high temperature (85 °C) (Fig. 5(e)), therefore the third component can be safely assigned to the denatured protein dominated by the two absorbing peaks for intermolecular anti-parallel β-sheets at 1617 and 1687 cm\(^{-1}\) respectively and a broad peak of random coils centered at 1645 cm\(^{-1}\). The successive conversion processes from the natural protein to the intermolecular aggregates are well reflected in the corresponding population titration curves retrieved by SVD as shown in Fig. 5(f). Figure 5(f) reveals that content of the natural protein keeps decreasing with the increasing temperature, in contrast, that of the denatured protein has a zero population at low temperatures and arises drastically above 64 °C, clearly shows that the denaturation of β-crystallins occurs at high temperatures with a \(T_m\) of 72.4±0.2 °C. Figure 5(f) also shows the intermediate species presents a three-state transition with the first \(T_m\) at 40.4±0.7 °C, indicating formation of an intermediate prior to the denaturation of β-crystallins. Apparently the \(T_m\) for the formation of the intermediate species is consistent with that for the formation of the random coil structures and unfolding of the β-sheet structures but with a better accuracy in determination of the \(T_m\) value.

The success of resolving the intermediate species-associated IR spectra appearing in the heating process by using SVD seems surprising, since the secondary structures undergo a continuous change while SVD is a linear operation in mathematics. In principle, a continuous structure change cannot be treated mathematically with a linear combination of a number of limited components as representative structures. However, a successful example can be found in linear decomposition of the temperature-dependent absorption spectra of liquid water. It has been observed that the IR absorption of water in a large temperature region exists isosbestic points, therefore the corresponding spectra can be delineated by a linear combination of a high density water spectrum and a low density spectrum, despite of the fact that water molecules are in a continuous breaking of hydrogen-bonding network of the liquid water [30]. In the current case, the thermal-induced structure change of β-crystallin is also a continuous process, and an isosbestic point at 1626 cm\(^{-1}\) exits in the temperature-dependent FTIR spectra (Fig. 3(a)), this may accounts for the justice of suing SVD method in parallel to the linear combination method used in water spectra. However, the physics underneath the success of such linear mathematical treatments needs further exploration.

SVD analysis clearly suggests that, heating β-crystallins solution at low temperatures not only brings about the change in the secondary structures, but also creates an intermediate species with distinct IR absorption spectroscopic feature the same as that of the protein heated to 64 °C, this species has a \(T_m\) of 40.4±0.7 °C for formation and 72.4±0.2 °C for full denaturation and aggregation. Wiegmann et al. proposed a three-state model for unfolding of dimeric βB2-crystallin via the urea-induced denaturation experiments, wherein unfolding of the N-terminal domain is the first step, this leads to the formation of the monomeric intermediate with unfolded N-terminal domain [5]. With the fact that the highest temperature for maintaining the reversibility of the protein subjected to thermal-induced unfolding/folding being 46 °C, we then propose tentatively that this intermediate species is the partially unfolded monomeric form of β-crystallin oligomers, which is produced by the thermal-induced disassembly of β-crystallin oligomers as represented in the following scheme:

\[
M_m + \ldots + M_n \xrightarrow{\Delta} (m + \ldots + n)M_{\text{partially unfolded}}
\] (1)

Where \(M_m\) and \(M_n\) represent the β-crystallin oligomers of varied monomeric units denoted by \(m\) and \(n\), and \(M\) represents the monomer. To trace the thermal-induced dynamical structural changes in β-crystallins, we acquired \(T\)-jump time-resolved IR absorbance difference spectrum in amide I’ region recorded at 2 µs after the \(T\)-jump at 34 °C with a \(\Delta T\) of 9 °C for β-crystallins in D\(_2\)O solution (Fig. 6), which shows a broad bleaching peak at 1685 cm\(^{-1}\) (the high vibration frequency of the intramolecular anti-parallel (β-sheet) and a broad absorption peak at 1645 cm\(^{-1}\) (random coil). The steady-state difference IR spectrum between two

FIG. 6 The transient IR absorbance difference spectrum recorded at 2 µs after the \(T\)-jump from 34 °C to 43 °C. The steady-state FTIR absorbance difference spectrum between 43 and 34 °C is also included for comparison.
typical temperatures 43 and 34 °C is also plotted for comparison. Obviously, most part of the T-jump time-resolved IR absorbance difference spectrum follows that of the steady-state difference spectrum. The bleaching peak indicates that T-jump induces the unfolding of the anti-parallel β-sheets, whereas the absorption peak indicates the formation of the random coil structures. All the observed IR absorption peaks in FTIR and T-jump time resolved IR difference spectra together with their corresponding assignments of the secondary structural components are listed in Table I.

Figure 7 presents the bleaching kinetics at 1685 cm⁻¹ with a time constant of 48±10 ns, and the absorption kinetics at 1645 cm⁻¹ with a time constant of 57±10 ns, which indicates that anti-parallel β-sheets unfolds slightly faster than the random coils fold roughly by 10 ns in magnitude. The fact suggests that the disruption of anti-parallel β-sheets within the βγ-crystallin domains leads to the formation of the random coil structures, revealing the occurrence of a thermal-induced intermediate. The urea-induced equilibrium denaturation experiments revealed that the unfolding of dimeric βB2-crystallin begins at the unfolding of the N-terminal domain, which leads to forming the monomeric intermediate with unfolded N-terminal domain [5]. Considering the highly-conserved conformation of β-crystallins, each N-terminal domain should have the same structural change in our experiments. Therefore, we proposed a model for the thermal-induced unfolding of a β-crystallin domain as illustrated in Fig.8, wherein the anti-parallel β-sheet in N-terminal unfolds firstly in around 50 ns under heat pulse. It was reported that all β-crystallins, except for βB2-crystallin, form heteromers via exchanging subunits [31], the structure of β-crystallins are in a dynamic equilibrium between monomers and dimers [32]. Thus, unfolding of N-terminal domain accompanied by the formation of flexible random coil structures might facilitate the subunit exchange of β-crystallins.

TABLE I Assignment of the observed amide I' band to its corresponding secondary structures.

<table>
<thead>
<tr>
<th>Observed/cm⁻¹</th>
<th>Assignment</th>
<th>Reported/cm⁻¹</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>FTIR</td>
<td>T-jump</td>
<td>Average</td>
<td></td>
</tr>
<tr>
<td>1617</td>
<td>Intermolecular anti-parallel β-sheet from aggregate</td>
<td>1630</td>
<td>[22, 23, 27, 34–37]</td>
</tr>
<tr>
<td>1687</td>
<td>Intermolecular anti-parallel β-sheet from aggregate</td>
<td>1679</td>
<td>[22, 23, 27, 34–37]</td>
</tr>
<tr>
<td>1631 1685</td>
<td>Intramolecular anti-parallel β-sheet</td>
<td>1630</td>
<td>[22, 23, 27, 34–37]</td>
</tr>
<tr>
<td>1684 1685</td>
<td>Intramolecular anti-parallel β-sheet</td>
<td>1679</td>
<td>[22, 23, 27, 34–37]</td>
</tr>
<tr>
<td>1645 1645</td>
<td>Random coil</td>
<td>1645 1640 – 1650</td>
<td>[22, 27, 35, 38]</td>
</tr>
<tr>
<td>1654</td>
<td>α-helix and loop</td>
<td>1652</td>
<td>[15, 20, 27, 38–41]</td>
</tr>
<tr>
<td>1669</td>
<td>β-turn</td>
<td>1671</td>
<td>[22, 27, 42, 43]</td>
</tr>
</tbody>
</table>

FIG. 7 Unfolding and folding kinetic curves for the N-terminal secondary structure components of the β-crystallins acquired by time-resolved IR absorbance difference spectroscopy at 1645 and 1685 cm⁻¹, after a T-jump from 34 °C to 43 °C.

FIG. 8 Model for the first step of thermal-induced unfolding of β-crystallins.

IV. CONCLUSION

In the past years much progress has been made in characterizing the β-crystallins. Yet the details of the structural change during β-crystallin unfolding remain elusive [33]. In an attempt to elucidate the dynamic process for the thermal-induced disassembly of β-crystallin oligomers in relevance to their biological function, we examined the thermal stability of the secondary structure components in β-crystallin oligomers by thermal titration using FTIR spectroscopy, and their unfolding/folding dynamics using time-resolved IR dif-

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ference spectroscopy after T-jump. In this work, thermal titration analyses reveal that the thermal-induced disassembly of the β-crystallin oligomers occur via dissociation into an intermediate species tentatively assigned as the partially-unfolded monomeric form, with a midpoint temperature of 40.4±0.7 °C. Our T-jump nanosecond time-resolved IR difference spectroscopic experimental results demonstrate that the anti-parallel β-sheet in N-terminal domain unfolds firstly in around 50 ns, accompanied by the formation of random coil structures in around 60 ns. These revelations strongly suggest that the β-crystallins unfold in a sequential manner under heat stress, with the N-terminal anti-parallel β-sheet serving as a sensor for the environmental conditions such as temperature change. The fact that the β-crystallin oligomers start to dissociate into monomers even at the room temperature, strongly implicates that this protein exists in a dynamic equilibrium between monomers and different ordered oligomers at the normal physiological temperature of 37 °C. Formation of the random coil structures in N-terminal would increase the structural flexibility of β-crystallins.

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