BIOCHEMISTRY
including biophysical chemistry & molecular biology
Article

Biochemistry **2009,** *48,* 9313–9320 9313
DOI: 10.1021/bi900451j

The Chaperone-like Protein α-Crystallin Dissociates Insulin Dimers and Hexamers[†]

Tue Rasmussen,*[,‡,§] Marina R. Kasimova,[‡] Wim Jiskoot,[§] and Marco van de Weert[‡]

[‡]*Faculty of Pharmaceutical Sciences, University of Copenhagen, Copenhagen, Denmark, and* [§]*Division of Drug Delivery Technology, Leiden/Amsterdam Center for Drug Research (LACDR), Leiden University, Leiden, The Netherlands*

Received March 16, 2009; Revised Manuscript Received August 28, 2009

ABSTRACT: The protein α-crystallin, a member of the small heat shock protein family, has the ability to prevent aggregation of partially denatured proteins, an effect demonstrated both in vivo and in vitro. In this work, we have probed the apparent thermal destabilization of bovine insulin by α-crystallin, using differential scanning calorimetry, near-UV circular dichroism, and intrinsic fluorescence spectroscopy. The thermal denaturation of insulin, followed by differential scanning calorimetry, is greatly affected by the presence of α-crystallin. Even at a ratio of α-crystallin subunit to insulin monomers as low as 1:10, a significant decrease in the transition temperature and a change in the shape of the transition are evident. These changes are detected for both zinc-free (mainly dimeric) and zinc-containing (predominantly hexameric) insulin. The transition temperatures measured by near-UV circular dichroism are consistent with the calorimetry results; however, no changes in the spectra of insulin occur below the transition temperature in the presence of α-crystallin. The intrinsic fluorescence of α-crystallin indicates association with insulin above 40 °C. On the basis of this, we conclude that α-crystallin promotes the dissociation of insulin oligomers to a lower-association state species with a lower thermal stability. Furthermore, we propose that the dissociation of insulin is caused by the ability of α-crystallin to bind to the insulin self-association surfaces and thus stabilize insulin dimers and monomers.

Small heat shock proteins (sHsps)[1] make up a family of proteins with chaperone-like activity. While classic chaperones help proteins acquire their native tertiary structure, sHsps associate with partially denatured or misfolded states and prevent their aggregation and subsequent precipitation. The sHsps are a diverse group ranging in size from 12 to 43 kDa with a conserved sequence called the α-crystallin domain. Most members of this group form large oligomeric structures with a molecular mass as high as 1000 kDa (*1*).

The protein α-crystallin, native to the vertebrate eye lens, is the most studied member of the sHsps because of its availability. It exists as an oligomer in the size range of 500−800 kDa and consists of two homologues, αA- and αB-crystallin, in a 3:1 molar ratio. Both homologues have a molecular mass of ∼20 kDa and ∼60% sequence homology (*2*, *3*). While αA-crystallin is primarily expressed in the lens, αB-crystallin has also been found in almost all studied tissue types, and its expression is linked to cellular stress, such as heat and hypertonic stress (*4*, *5*).

The ability of α-crystallin to prevent protein aggregation has been demonstrated in several in vitro settings. This includes the lens proteins γ- and β-crystallin (*6*, *7*), as well as numerous other model proteins (*6*, *8*, *9*). The proposed mechanism for the chaperone-like effect of α-crystallin is an interaction with the exposed hydrophobic surfaces of partially denatured proteins (*10*, *11*), thereby preventing their self-association and irreversible aggregation. This interaction is specific to partially denatured proteins, whereas neither fully denatured nor native proteins are expected to be recognized (*10*). The chaperone-like activity of α-crystallin has been shown to increase with temperature (*12*, *13*). Although α-crystallin undergoes a thermal transition between 55 and 75 °C (*14*, *15*), above its transition midpoint it retains significant structure and even exhibits chaperoning ability (*8*, *16*). It has been proposed that further exposure of α-crystallin hydrophobic residues during heating promotes association with other partially denatured proteins (*13*, *17*), though no clear correlation between the hydrophobicity of α-crystallin and its chaperone-like activity has been established (*18*).

A less well-studied property of α-crystallin is the apparent ability to thermally destabilize multimeric proteins, as recently reported by Khanova et al. in a study of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using differential scanning calorimetry (DSC) (*19*). The suggested mechanism is the dissociation of tetrameric GAPDH to dimers, which have a lower thermal stability than the tetramer. This property is intriguing, as it could have implications for protein association in the eye lens and for the wider understanding of the mechanism of chaperoning by heat shock proteins.

To further elucidate this side of the chaperone-like effect, we have studied the interaction between lens α-crystallin and bovine insulin, a protein capable of existing in three well-defined quaternary structures at physiological pH and temperatures, a monomer at concentrations below 0.1 μM, a dimer at concentrations up to 1 mM, and a hexamer at higher concentrations (*20*, *21*). The dimers also associate to hexamers at concentrations above 0.01 mM, when at least two zinc ions are coordinated per hexamer (*20*). Both zinc ions coordinate three His^{B10} residues,

[†]We acknowledge Novo Nordisk for a grant for the VP-DSC and the Drug Research Academy for a research grant to T.R.

*To whom correspondence should be addressed: Faculty of Pharmaceutical Sciences, Universitetsparken 2, 2100 Copenhagen, Denmark. Phone: (+45) 35336544. Fax: (+45) 35336030. E-mail: tr@farma.ku.dk.

[1]Abbreviations: sHsp, small heat shock protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DSC, differential scanning calorimetry; T_{max}, transition maximum; EDTA, sodium diaminoethanetetraacetic acid; $\Delta H_{cal,app}$, apparent calorimetric enthalpy; T_m, transition midpoint; NATA, *N*-acetyltryptophanamide; NUV CD, near-ultraviolet circular dichroism.

Published on Web 08/28/2009

each originating from one of the six insulin monomers, thus stabilizing the structure (*22*).

The thermal stability of human insulin was studied by Huus et al. (*23*) and found to be dependent on the association state of the protein. At a concentration of 0.6 mM, the reported transition maxima (T_{max}), measured for the zinc-bound and zinc-free insulin, are approximately 87 and 70 °C, respectively. The heat-induced denaturation of the zinc-bound insulin is coupled to dissociation, so that the folded hexamer dissociates into denatured monomers. However, the thermal behavior of the dimeric insulin is not yet well established (*23*).

The effect of chemical denaturants on the denaturation pathway of zinc-bound insulin is different from the one seen for the thermal denaturation. At 0.5 M urea, the insulin hexamer is dissociated to dimers, while the dimer starts to dissociate to monomers above 5.5 M urea (*24*). The monomers retain some tertiary structure even at urea concentrations of > 7 M. A similar result is obtained in denaturation experiments with guanidine hydrochloride; i.e., dissociation of the oligomers precedes the process of denaturation (*25*).

The aim of this study is to probe the apparent thermal destabilization of oligomeric proteins by chaperone-like proteins using bovine insulin and bovine α-crystallin as a model system.

EXPERIMENTAL PROCEDURES

Materials. All proteins were purchased from Sigma-Aldrich. Bovine insulin was dissolved in a minimal amount of 0.05 M hydrochloric acid, then diluted in a large excess of 7 mM phosphate buffer, and adjusted to pH 7.4 with sodium hydroxide. The insulin concentration was set using an extinction coefficient of 6190 M^{-1} cm^{-1} at 276 nm (*26*). Two samples were prepared, both with a final concentration of 0.6 mM insulin: zinc-bound insulin, containing 0.25 mM zinc ions (2.5 zinc ions per hexamer), and zinc-free insulin where 1 mM disodium diaminoethanetetraacetic acid (EDTA) was used to chelate zinc ions. Bovine eye lens α-crystallin was dissolved directly in 7 mM phosphate buffer (pH 7.4), and the concentration was measured using extinction coefficients of 13300 M^{-1} cm^{-1} at 280 nm. The extinction coefficient is derived from the amino acid sequence as described in ref *27*, and a 3:1 ratio of αA to αB subunits is assumed (*2*). Where mentioned, zinc ions were added as $ZnCl_2$.

Differential Scanning Calorimetry. Experiments were conducted on a VP-DSC microcalorimeter (MicroCal, Northampton, MA) (*28*). The temperature scans were conducted at a scan rate of 1 °C/min. The reference chamber was filled with 7 mM phosphate buffer, and a reference scan (buffer−buffer) was subtracted before the concentration was normalized. The thermograms were analyzed for the apparent calorimetric enthalpy ($\Delta H_{cal,app}$) of the transition and the T_{max}. The T_{max} is defined as the temperature at which the highest heat flow is recorded, i.e., the peak maximum, which is not necessarily the midpoint of the transition. We tested the reversibility of the unfolding reactions by scanning to a temperature slightly above the T_{max} and then rescanning the sample again through the full temperature range. The analysis of the obtained thermograms was performed using the DSC module of Origin version 7.0 (OriginLab, Northampton, MA).

Circular Dichroism Spectroscopy. The experiments were conducted on a JASCO J-815 circular dichroism spectrometer (Jasco, Tokyo, Japan). The insulin samples were scanned in a 2 mm quartz sample cell from 300 to 250 nm using a bandwidth of 2 nm, a response time of 2 s, and a scan rate of 100 nm/min. Each spectrum was an average of three scans, and a reference scan of the relevant buffer was subtracted. The temperature was controlled with a Peltier element and increased at a rate of 0.25 °C/min over the whole temperature range. The insulin transition was followed at 276 nm, and the data were fitted according to the method of Pace et al. (*29*) to obtain the transition midpoint (T_m) value, which is defined as the midpoint between the pre- and post-transition baseline on the sigmoidal denaturation curve.

Fluorescence Spectroscopy. Steady state fluorescence emission scans were conducted on an FLS 920 spectrometer (Edinburgh instruments Ltd., Livingston, U.K.). The measurements were conducted in front face mode, because of the high sample concentration. The excitation wavelength was set at 295 nm to selectively excite the tryptophans present in α-crystallin. Technical emission scans for the steady state were acquired between 315 and 450 nm, and a reference scan was subtracted to remove background signals. For the measurements, the excitation and emission slits were set at 4.5 nm, the steps were set at 0.5 nm, and the dwell time was set at 0.2 s, and the plotted data are an accumulation of two scans.

The exact determination of the peak maximum and intensity was conducted between 340 and 365 nm. For these measurements, the excitation and emission slits were set at 4.5 nm, the steps were set at 0.5 nm, and the dwell time was set at 0.5 s. The measurement was an accumulation of three scans, and a background scan was subtracted. *N*-Acetyltryptophanamide (NATA) was analyzed to obtain the peak maximum and intensity of a fully solvent-exposed tryptophan. NATA was dissolved in the experimental buffer at a concentration of 0.025 mg/mL, thus having a fluorescence intensity comparable to that of the protein samples. The cell holder temperature was controlled by a Peltier element, and temperature equilibration was conducted for at least 5 min after the measurement temperature had been reached to ensure equilibration of the sample. The peak wavelength data were smoothed with a window of 5 (~2.5 nm), and the maximum value was subsequently determined. Furthermore, the peaks were manually inspected to prevent local maxima from displacing the global maximum of the peak.

RESULTS

Differential Scanning Calorimetry. The thermal denaturation of bovine insulin with addition of various concentrations of the chaperone-like protein α-crystallin was investigated by DSC. The thermal transition of zinc-bound insulin has a sharp peak with a T_{max} of 85.6 °C (Figure 1A), equivalent to the coupled dissociation and denaturation of the insulin hexamer. The transition is slightly biphasic, with a smaller peak around 70 °C corresponding to the transition of dimeric insulin (*23*). Overall, the unfolding was 95% reversible, equivalent to what was reported by Huus et al. for human insulin (*23*). Zinc-free insulin, which is predominantly dimeric, undergoes a transition with a single peak with a T_{max} of 67.1 °C (Figure 1C) with the unfolding being 95% reversible. The results, summarized in Table 1, show that the $\Delta H_{cal,app}$ values of zinc-bound insulin and zinc-free insulin are approximately 137 and 84.7 kJ/mol, respectively.

Addition of 0.06 mM α-crystallin to zinc-bound insulin (0.6 mM, 0.25 mM zinc ions) causes the T_{max} to decrease, while the transition peak becomes wider. At the same time, $\Delta H_{cal,app}$ decreases to 122 kJ/mol and the transition is less reversible (~70%).

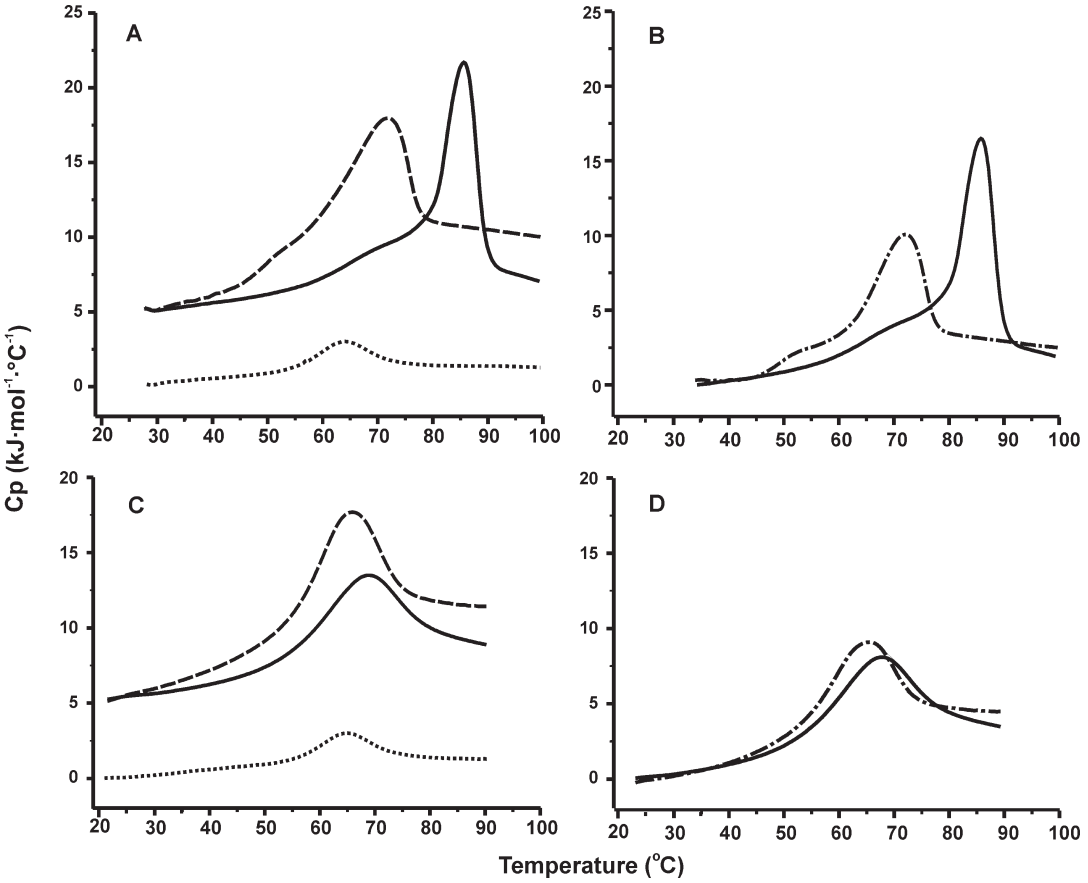

FIGURE 1: DSC of insulin in the presence of α-crystallin. Effect of α-crystallin on the thermal transition of zinc-bound insulin (A and B) and zinc-free insulin (C and D). The solid line depicts data for 0.6 mM insulin without α-crystallin. The dashed line depicts data for 0.6 mM insulin with 0.06 mM α-crystallin present. The dotted line is the thermogram of 0.06 mM α-crystallin. The dashed−dotted line is the thermogram of 0.6 mM insulin with 0.06 mM α-crystallin, but the thermogram of 0.06 mM α-crystallin was subtracted instead of a buffer scan. All thermograms were normalized to the insulin concentration.

Table 1: Transition Parameters for Insulin with the Addition of α-Crystallin[a]

		$\Delta H_{cal,app}$ (kJ/mol)[b]		
	T_{max} (°C)[b]	total[d]	corrected[d]	T_m (°C)[c]
Zinc-Bound Insulin				
no addition	85.6	137	137	86.5
0.012 mM α-crystallin	83.4	130	126	−
0.024 mM α-crystallin	81.8	130	122	−
0.04 mM α-crystallin	73.2	128	118	75.2
0.06 mM α-crystallin	71.3	122	102	73.8
0.12 mM α-crystallin	66.8	131	91.3	67.2
Zinc-Free Insulin				
no addition	67.1	84.8	84.8	71.7
0.06 mM α-crystallin	64.5	107	87.0	67.9
0.12 mM α-crystallin	−	−	−	64.6

[a]The calorimetric data are an average of analysis of two sets of thermograms. The T_{max} values varied by less than 0.8 °C between experiments, while the apparent calorimetric enthalpy varied by less than 5%. The T_m values were calculated from NUV CD. [b]Obtained via DSC. [c]Obtained via NUV CD. [d]Total = total $\Delta H_{cal,app}$. Corrected = total $\Delta H_{cal,app}$ with the $\Delta H_{cal,app}$ of α-crystallin subtracted.

The denaturation of α-crystallin is independent of concentration and is characterized by a T_{max} of 63.8 °C and a $\Delta H_{cal,app}$ of 201 kJ/mol (Figure 1), and the transition is ~85% reversible. Thus, the endotherm of α-crystallin itself would have an only slight influence on the thermograms of insulin with α-crystallin and could not account for the observed change in the thermal transition. Examples of subtracted thermograms can be seen in Figure 1B,D, showing that no significant change in T_{max} is observed after the subtraction.

Figure 2A shows the effect of an increasing α-crystallin concentration while the zinc-bound insulin concentration was kept constant (0.6 mM, 0.25 mM zinc ions). It is apparent from these data that increasing the α-crystallin concentration causes a lowering of the T_{max} (Figure 2A). The shape of the thermograms changes as well, first acquiring a more biphasic character, followed by the disappearance of the high-temperature peak (compare curves 1−6 in Figure 2A). The thermodynamic parameters for these experiments are summarized in Table 1. The overall $\Delta H_{cal,app}$ associated with the transitions remains stable in the 120−140 kJ/mol range, even with the increasing α-crystallin concentrations. When the estimated contribution of α-crystallin to the $\Delta H_{cal,app}$ is subtracted, the remaining $\Delta H_{cal,app}$ for insulin shows a steady decrease from 137 to 91.3 kJ/mol with increasing α-crystallin concentrations. It is important to notice that this treatment of data does not take into account the enthalpic contributions that could occur due to direct interaction between the two proteins.

Addition of 0.06 mM α-crystallin to zinc-free insulin causes the T_{max} to decrease slightly to 64.5 °C as seen in Figure 2B and summarized in Table 1. The $\Delta H_{cal,app}$ corrected for the α-crystallin contribution increases slightly to 87.0 kJ/mol.

Circular Dichroism. The DSC scans represent the combined heat flows from both insulin and α-crystallin. Although the contribution of α-crystallin is relatively small, as discussed above, it is difficult to fully distinguish the effect of α-crystallin on insulin from the transition $\Delta H_{cal,app}$ of the chaperone-like protein. Therefore, it is important to supplement the thermodynamic data with other techniques.

Near-ultraviolet circular dichroism (NUV CD) was used to probe the structural changes. The NUV CD signal between 250 and 300 nm of 0.6 mM insulin greatly outweighs the signal of 0.06 mM α-crystallin (Figure 3). This means that the NUV CD spectra of insulin in the presence of α-crystallin can be assigned specifically to the structural features of the insulin.

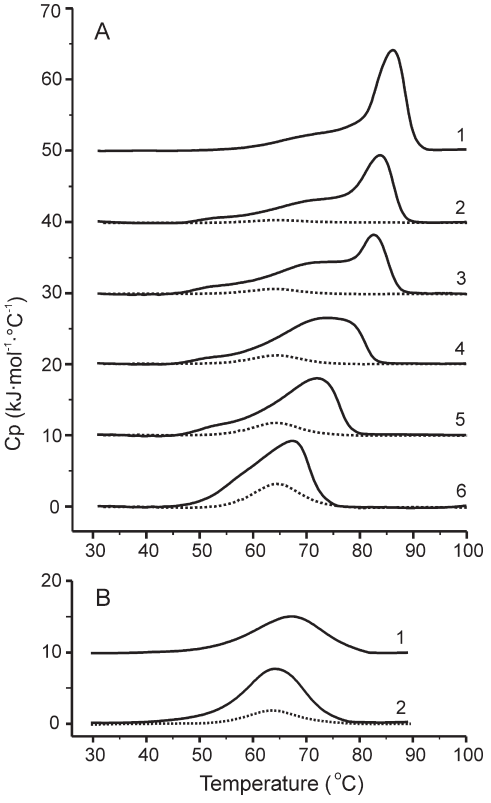

FIGURE 2: Thermal transition of insulin in the presence of different concentrations of α-crystallin. DSC scans of 0.6 mM insulin. Panel A shows data for zinc-bound insulin with the additions of the following concentrations of α-crystallin: 0 (1), 0.012 (2), 0.024 (3), 0.04 (4), 0.06 (5), and 0.12 mM (6). Panel B shows data for zinc-free insulin without (1) and with 0.06 mM (2) α-crystallin. The solid lines represent the measured thermograms, while the dotted lines are the expected thermograms of the α-crystallin transition. All thermograms were normalized to the insulin concentration of 0.6 mM. Furthermore, a baseline constructed in Origin was subtracted from each scan.

The structural transition was followed at 276 nm, the absorption maximum of insulin (Figures 3 and 4). The thermal transition of insulin produces a sigmoidal curve (Figure 4) where the molar ellipticity is an indicator of the association state of insulin (*23*, *25*, *30*). Zinc-free insulin is measured to have an approximate ellipticity of -230 deg cm^2 dmol^{-1} residue^{-1} at 25 °C. The molar ellipticity of zinc-bound insulin is ca. -370 deg cm^2 dmol^{-1} residue^{-1} at 25 °C, consistent with more structuring in the insulin hexamer compared to the dimeric insulin. These data are consistent with the fact that the self-association of the dimers into hexamers results in shielding of several surface-exposed aromatic residues from water (*30*).

The NUV CD data were analyzed with regard to T_m (Figure 4; see also Experimental Procedures). A good fit was obtained for the zinc-free insulin with a T_m of 71.7 °C. The fit of zinc-bound insulin is worse, because the transition is biphasic, as also observed in the DSC experiments. The T_m of zinc-bound insulin was determined to be 86.5 °C. The presence of increasing concentrations of α-crystallin to zinc-bound insulin (0.6 mM) causes a downshift in T_m (Table 1). Notably, the intensity of the pretransitional CD signal remains the same as for the zinc-bound insulin without the chaperone-like protein (Figure 4A).

In the DSC scans, the addition of 0.06 mM α-crystallin to zinc-free insulin causes only a minor depression of the T_{max}, but the circular dichroism data confirm that the insulin structural transition occurs at lower temperatures upon addition of α-crystallin (Figure 4B). The T_m values are calculated to be 67.9 and 64.6 °C with addition of 0.06 and 0.12 mM α-crystallin, respectively (Table 1).

Fluorescence Spectroscopy. To probe if insulin directly interacts with α-crystallin, we used steady state fluorescence to determine the solvent exposure of the tryptophan residues in α-crystallin in the presence of insulin. Insulin lacks tryptophan residues, whereas the αA-crystallin subunit contains one and the αB-crystallin subunit two. This enables selective measurement of α-crystallin at an excitation wavelength of 295 nm, where only tryptophan is excited (Figure 5).

The increase in temperature has two main effects on the tryptophan emission of 0.06 mM α-crystallin (Figures 5 and 6). The intensity is lowered as the temperature increases, an effect due to dynamic quenching by the solvent or neighboring amino

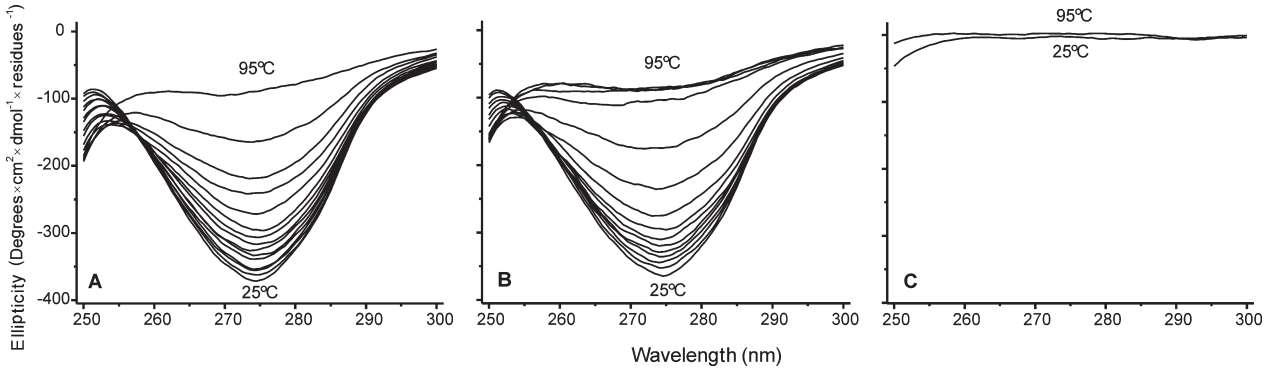

FIGURE 3: Temperature dependence of the NUV CD spectra of insulin and α-crystallin. Spectra of 0.6 mM zinc-bound insulin are shown in panel A. Spectra of 0.6 mM zinc-bound insulin with the addition of 0.06 mM α-crystallin are shown in panel B. Spectra of 0.06 mM α-crystallin are shown in panel C. The spectra were collected in the temperature range from 25 to 95 °C, with 5 °C intervals, except for panel C, which shows only the two spectra at the temperature extremes.

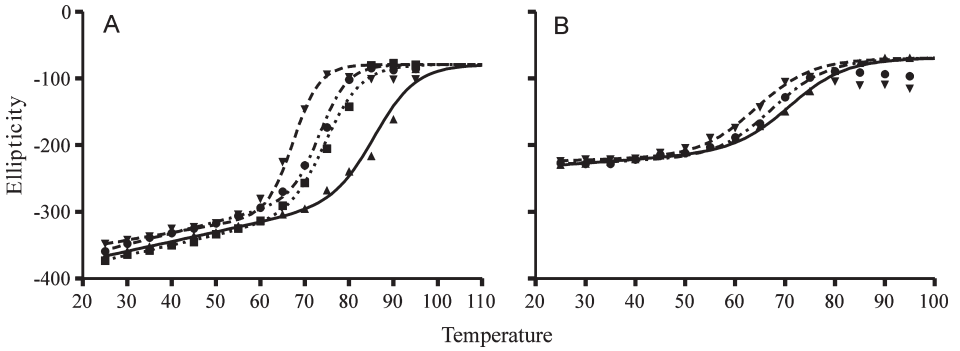

FIGURE 4: Thermal denaturation of insulin followed by NUV CD at 276 nm in the presence of α-crystallin. Molar ellipticity of 0.6 mM zinc-bound insulin (A) and zinc-free insulin (B) in the temperature range of 25−95 °C. α-Crystallin was present at the following concentrations: 0 (▲, solid line), 0.12 (▼, dashed line), 0.06 (●, dash-dotted line), and 0.04 mM (■, dotted line). All the data points are corrected for the relevant buffer contribution. The lines show the fit of the transition used to determine T_m. The posttransitional baseline of zinc-free insulin was used for all the data sets under the assumption that denatured species could be considered the same.

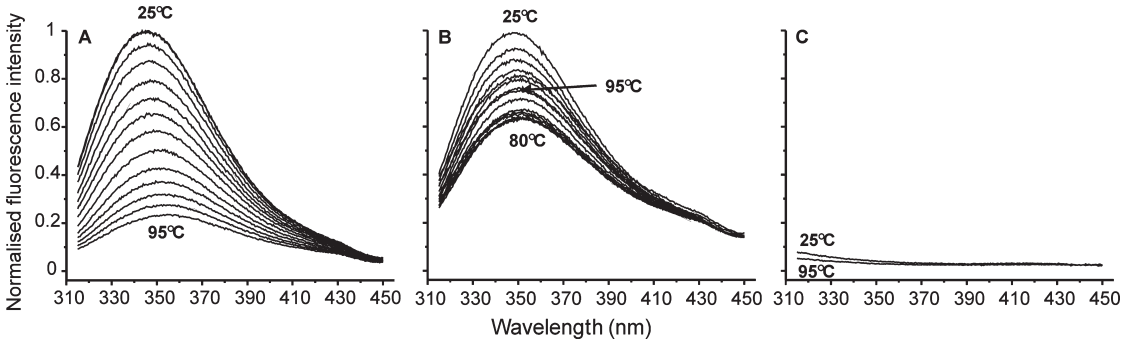

FIGURE 5: Temperature dependence of the intrinsic fluorescence spectra of α-crystallin excited at 295 nm in the presence of insulin: (A) 0.06 mM α-crystallin, (B) 0.06 mM α-crystallin with 0.6 mM zinc-bound insulin, and (C) 0.6 mM zinc-bound insulin. The spectra shown are in the temperature range from 25 to 95 °C, with 5 °C intervals, except for panel C which shows only the two spectra at the temperature extremes.

acid side chains (*31*). The second effect is a red shift in the peak maximum from 347 nm at 25 °C to 358 nm at 95 °C. For the sake of comparison, the fully solvent exposed tryptophan analogue NATA was measured to have an emission maximum of 362 nm, which is constant over the full temperature range used in the experiment. The red shift in the peak maximum can thus be interpreted as a shift toward a higher degree of solvent exposure, typical for loosening of the protein structure (*31*). Neither the intensity value nor the peak maximum shows the well-defined thermal transition of α-crystallin at 65 °C as observed by DSC.

The presence of zinc-bound insulin (0.6 mM) affects both the emission intensity and the red shift of the peak maximum of α-crystallin at increased temperatures (Figure 6). In the range between 30 and 70 °C, both fluorescence parameters of the mixture become less dependent on temperature as compared to those of the α-crystallin alone. The fluorescence intensity seems to level off between 70 and 85 °C, while increasing again at higher temperatures (Figure 6A). The emission maximum is only slightly increased with temperature, returning back toward the value at room temperature above 80 °C (Figure 6B). To ensure that these changes are not caused by the interaction of α-crystallin with zinc ions, we repeated the measurements without insulin, using an α-crystallin solution with 0.25 mM zinc ions. The obtained results were equivalent to those for α-crystallin in the absence of zinc ions (data not shown).

With addition of zinc-free insulin, the difference between the temperature dependence of pure α-crystallin fluorescence and α-crystallin with insulin present is less apparent than for zinc-bound insulin. In the range of 25−80 °C, the two fluorescence intensity curves lie within experimental error, although they deviate from each other above 80 °C (Figure 6). The wavelength maximum of the fluorescence spectrum is more affected than the intensity and deviates at 70 °C, with the largest deviation observed above 80 °C.

DISCUSSION

This work was initiated to probe the apparent thermal destabilization of oligomeric proteins by the chaperone-like protein α-crystallin recently reported by Khanova et al. (*19*). Whereas the primary focus of the paper by Khanova et al. was on prevention of aggregation of the protein GAPDH by α-crystallin (*19*), this work focuses on the characterization of the destabilizing action of α-crystallin using another oligomeric protein, namely insulin.

Although α-crystallin is normally recognized as a stabilizing entity which prevents protein aggregation (for a review, see ref *32*), our experiments are consistent with a concentration-dependent destabilizing influence of α-crystallin on the thermal stability of insulin. When 0.12 mM α-crystallin is added to 0.6 mM zinc-bound insulin, the thermal transition maximum is lowered from 85.6 to 66.8 °C, whereas the $\Delta H_{cal,app}$ decreases from 137 to 91.3 kJ/mol, if corrected for the α-crystallin contribution. These new values are close to those observed for the zinc-free, dimeric, insulin, with a T_{max} of 67.1 °C and a $\Delta H_{cal,app}$ of 84.8 kJ/mol (Table 1). The addition of α-crystallin to zinc-free insulin causes a further decrease in T_{max}, indicating that both hexameric and dimeric forms of insulin are affected by the

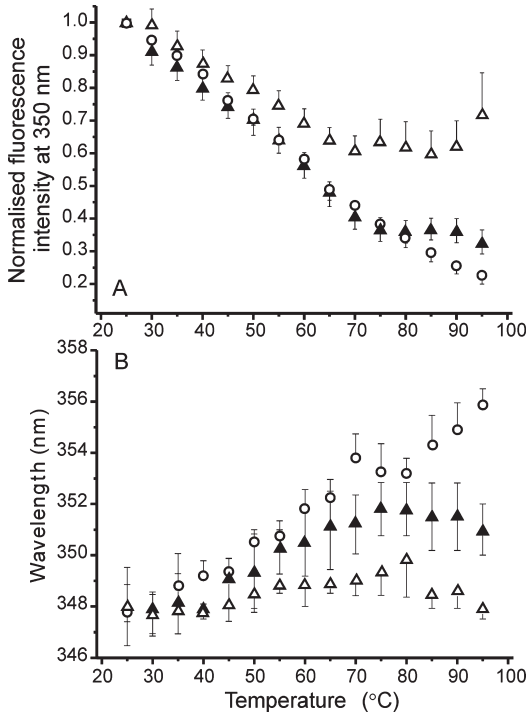

FIGURE 6: Temperature dependence of the tryptophan fluorescence of α-crystallin in the presence of insulin. Panel A shows the temperature-dependent intensity of the emission at 350 nm as a fraction of the maximum emission at 25 °C. Panel B shows the temperature-dependent fluorescence emission maximum. The graphs show data for 0.06 mM α-crystallin (○), 0.06 mM α-crystallin with 0.6 mM zinc-bound insulin (△), and 0.06 mM α-crystallin with 0.6 mM zinc-free insulin (▲). The intensity of the emission at 350 nm has been normalized to the emission at 25 °C. Error bars represent the standard deviation of three separate measurements.

presence of the chaperone-like protein. As higher-order oligomers are more stable because of the stabilizing contribution from formation of the intermolecular interface (*33*), the decrease in T_{max} and ΔH_{cal} favors the conclusion that α-crystallin promotes the dissociation of insulin hexamers and dimers.

The structural changes of insulin upon its thermal denaturation in the presence of α-crystallin were followed by NUV CD. Although the transition temperatures measured correspond well to the T_{max} values obtained by DSC, a close examination of the NUV CD data leads to the important observation that addition of α-crystallin does not affect the ellipticity of the native insulin species at ambient temperature. Dissociation of insulin hexamers into dimers by the removal of zinc ions results in a change in molar ellipticity from approximately −360 to −230 deg cm^2 dmol^{-1} residue^{-1} at 25 °C (cf. Figure 4A,B). The NUV CD signal of insulin in the presence of α-crystallin does not change significantly, suggesting that no dissociation is taking place below the thermal transition temperature.

Involvement of Zinc Ions. The lowering of the thermal transition temperature of zinc-bound insulin might, at least in part, be explained by a competitive binding of zinc ions by α-crystallin. Indeed, several studies indicate that α-crystallin is capable of binding a range of divalent cations, including zinc (*34*, *35*). Human recombinant α-crystallin binds zinc ions with an association constant of 5×10^3 M^{-1} (at 25 °C and pH 7) (*35*). In contrast, the association constants for porcine and bovine insulin are reported to be 1.9×10^6 M^{-1} (pH 7 and 20 °C) and 1.85×10^5 M^{-1} (pH 8 and 30 °C), respectively (*36*, *37*). The binding of zinc ions to α-crystallin is thus presumably several orders of magnitude weaker than the binding of zinc ions to the main insulin binding site at ambient temperatures, which makes the competition for zinc ions minimal. Although at higher temperatures the affinity of α-crystallin for zinc ions could ultimately exceed that of insulin, zinc ion competition seems an unlikely explanation, as α-crystallin also causes the lowering of the thermal denaturation temperature of zinc-free insulin. Still, competition for zinc ions would take place and could play a minor role in the dissociation of the zinc-bound insulin hexamer. The fluorescence data collected on α-crystallin suggest that the tryptophan fluorescence is affected more when the zinc-bound insulin, rather than zinc-free insulin, is used as a substrate. This effect could be due to a reported improvement in the chaperone-like ability of α-crystallin in the presence of zinc ions (*34*, *35*).

Stabilization of the Denatured State of Insulin. The lowering of the thermal transition temperature could be due to preferential binding of α-crystallin to the denatured insulin species. This would cause a lowering of the thermal stability by displacement of the equilibrium from the native states of proteins through the displacement of the folding reaction toward the denatured state. For this model to be valid, α-crystallin should also destabilize monomeric proteins. We tested the two monomeric proteins equine myoglobin and bovine ribonuclease A by DSC under conditions (buffer, concentration range, and DSC conditions) similar to those of the insulin experiments. No lowering of the T_{max} of these proteins was evident in the presence of α-crystallin (data not shown). Our experiments correlate with earlier results of other groups showing that α-crystallin does not recognize fully denatured proteins (*10*, *11*, *38*), but more likely partly denatured proteins with extended hydrophobic patches. This suggests that the lowering of T_{max} is not a simple displacement of the equilibrium between the native and denatured state of proteins, and a more expanded model should be considered to explain the lowering of the transition temperature.

Stabilization of Intermediate States of Insulin. Protein−protein interaction in the native state is considered to be stabilizing, which is also the case in the insulin system. This stability increase from the monomer to hexamer is reflected in the consequent increase in the T_{max} and $\Delta H_{cal,app}$ values of insulin with the increase in the degree of association. Therefore, it is surprising that in the presence of α-crystallin insulin becomes less thermostable. We propose that α-crystallin actually stabilizes a lower association state and thus dissociates the higher association state. Our calorimetric data for zinc-bound insulin strongly suggest a change from a hexameric transition to a dimeric transition upon the addition of α-crystallin as both T_{max} and $\Delta H_{cal,app}$ (corrected for the contribution from α-crystallin) decrease to a level equivalent to the zinc-free insulin transition. The same argument can be applied in concluding that the insulin dimers dissociate further into monomers. Unfortunately, the thermodynamic parameters for the denaturation of the insulin monomer are not easy to obtain experimentally due to the fact that the monomeric state becomes significantly populated only at very low concentrations, a condition that is not suitable for the DSC or NUV CD experiments.

A Mechanism of Interaction of Insulin with α-Crystallin. The assembly of the insulin hexamer at neutral pH is well-described in the literature. The dimer is stabilized by hydrophobic interactions between primarily aromatic residues in the B chain of two insulin monomers. As observed from the X-ray crystallography data for porcine insulin (*22*), the side chains of Val^{B12}, Tyr^{B16}, Tyr^{B26}, Phe^{B24}, and Phe^{B25} from the insulin B chain are

present at the dimerization interface. Formation of the hexamer is supported by both hydrophobic interactions and hydrogen bonding among the three constituent dimers. Several hydrophobic and aromatic residues, Leu^{A13}, Tyr^{A14}, Phe^{B1}, Val^{B2}, and Leu^{B17}, are found to participate in hexamer formation (*22*, *39*). The dimer−dimer interaction is considerably weaker than the monomer−monomer interaction (*22*, *40*), but the hexamer is further stabilized by the coordination of two zinc ions to the six His^{B10} residues.

The structural information lays the foundation for our model of stabilization of intermediate states of insulin, as both the dimerization and hexamerization interfaces on insulin are hydrophobic as well as rich in aromatic residues. We propose that α-crystallin is able to bind to these hydrophobic patches of insulin in a manner resembling its binding to partially denatured proteins, thus preventing insulin self-association. This in turn would lead to the lower association states being populated and subsequently made available for thermal denaturation. This simplified model is depicted in Figure 7, where I_6, I_2, and I_1 are insulin hexamers, dimers, and monomers, respectively, and α is α-crystallin. The thermal denaturation temperatures of these species can be ranked as follows: $I_6 > I_2\alpha \geq I_2 > I_1\alpha \geq I_1$; the lower insulin association states have lower thermal stability, and the α-crystallin-associated monomers and dimers would be expected to have a slightly higher thermal stability than non-associated monomers and dimers due to the stabilizing interaction with α-crystallin. Though no rigorous analysis of the DSC thermograms is possible, the shape of the denaturation curve of zinc-bound insulin becomes increasingly asymmetric with increasing α-crystallin concentrations and could appear to consist of several underlying denaturation peaks (Figure 2A) consistent with different protein species (*41*). We suggest these species are insulin dimers and monomer bound to α-crystallin (Figure 7).

The dissociation of insulin oligomers can be induced by chaotropic denaturants, such as urea and guanidine hydrochloride (*24*, *25*). This is also the case with addition of cyclodextrins, which have been shown to increase the dissociation constant of the insulin dimer by up to 2 orders of magnitude at pH 2.5 and 7.4 in an isothermal titration calorimetry study (*42*). Cyclodextrins are known to improve the stability of proteins by reducing their tendency toward aggregation and precipitation, probably by interacting with the aromatic side chains on the protein surface. In the case of insulin, where the majority of aromatic residues participate in the formation of dimers or hexamers, the binding to cyclodextrins competes with insulin self-association. Convincing evidence of this mechanism was presented by a nuclear magnetic resonance spectroscopy study showing interaction at pH 6.8 between *β*-cyclodextrins and several insulin residues, including Tyr^{B16}, which participates in the formation of the dimer, and Tyr^{A14} and Phe^{B1}, which contribute to hexamer formation (*22*, *43*). It is clear that hydrophobicity of the inner cavity of cyclodextrins is the origin of their interaction with insulin and thus resembles the mechanism of interaction between insulin and α-crystallin.

Our model also explains why the NUV CD signal of insulin in the presence of α-crystallin does not change significantly at ambient temperatures (Figure 4) even though the fluorescence data, particularly the blue shift of the emission maximum of α-crystallin in the presence of insulin, suggest that binding between the two proteins occurs at lower temperatures (~40 °C). The association state of insulin can be assessed from the level of the NUV CD signal as mentioned previously (*23*, *30*).

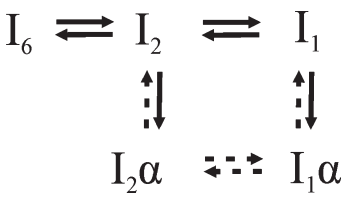

FIGURE 7: Simplified model for the dissociation of insulin during heating in the presence of α-crystallin. The model shows insulin (I) and its association state (1, 2, or 6) and α-crystallin (α). The dashed arrows indicate reactions for which we have no evidence of whether they take place.

The signal is a consequence of the solvent-exposed aromatic residues changing from being in an ordered environment, within the hexamer, to a disordered state when exposed to water, in the dimer and more so in the monomer. However, when the dissociation of the oligomer is coupled to binding to α-crystallin, the interpretation of the NUV CD data is more difficult. We propose that α-crystallin binds to the insulin self-association interfaces. Therefore, the environment around the insulin aromatic side chains, which are mostly located at these interfaces (*22*, *30*), would not change considerably when switching from being ordered in the self-association surface of insulin to binding to α-crystallin. This could explain why the NUV CD signal of insulin in the presence of α-crystallin does not change significantly at temperatures below the thermal transition even though the fluorescence data indicate binding between both proteins. The intrinsic fluorescence data do not show substantial changes below 40 °C in the α-crystallin fluorescence when insulin is present. This could be due to a thermal activation step of α-crystallin needed to provide sufficient hydrophobic surface to accommodate the insulin self-association surfaces (*10*, *12*, *16*).

A puzzling feature of the fluorescence data is that the emission maximum of the α-crystallin in the presence of insulin is considerably blue-shifted even at the temperatures above the thermal transition temperature of insulin (Figure 6), suggesting that no dissociation of insulin and α-crystallin occurs. At this molar ratio of 1:10 (α-crystallin subunit to insulin monomer), the dimeric transition is predominant (Figure 2), with no remnants of the hexamer transition. This could suggest that α-crystallin is able to accommodate binding of as many as five dimers per α-crystallin subunit, a feat which is unlikely solely on the basis of the size of the protein molecules involved. Another possible explanation is that α-crystallin is dissociated during insulin denaturation, and at temperatures above the thermal transition of both proteins, they irreversibly co-aggregate. However, our results do not allow us to draw any definitive conclusions about this.

CONCLUDING REMARKS

We have presented strong evidence, both thermodynamic and structural, that α-crystallin promotes the dissociation of insulin oligomers to lower-association state species. Literature data (*24*, *25*, *42*, *43*) show that insulin oligomers can be dissociated in a way that can be compared to the interaction of insulin with α-crystallin, and our intrinsic fluorescence results show that a direct interaction between α-crystallin and insulin in fact occurs. On the basis of this, we conclude that the dissociation of insulin is caused by the ability of α-crystallin to associate with the insulin self-association interfaces and stabilize the dimers and monomers. The interaction between α-crystallin and insulin occurs

only at temperature above 40 °C, which could be due to a thermal activation step of α-crystallin (*10*, *12*, *16*).

Our results do not imply that α-crystallin has a general destabilizing function at physiological temperatures, but that heat-activated α-crystallin and possible other sHsps can dissociate native oligomeric proteins and thereby mediate their thermal denaturation. The ability to dissociate native oligomeric structures closely resembles the ability of sHsp to prevent or dissociate aggregates by interaction with exposed patches of hydrophobic surfaces.

ACKNOWLEDGMENT

We thank Dr. Andrea Hawe for advice on the fluorescence studies.

REFERENCES

1. Sun, Y., and Macrae, T. H. (2005) The small heat shock proteins and their role in human disease. *FEBS J. 272*, 2613–2627.
2. Bloemendal, H. (1981) Molecular and Cellular Biology of the Eye Lens , John Wiley, New York.
3. Bloemendal, H., and de Jong, W. W. (1991) Lens proteins and their genes. *Prog. Nucleic Acid Res. Mol. Biol. 41*, 259–281.
4. Klemenz, R., Frohli, E., Steiger, R. H., Schafer, R., and Aoyama, A. (1991) αB-Crystallin is a small heat-shock protein. *Proc. Natl. Acad. Sci. U.S.A. 88*, 3652–3656.
5. Dasgupta, S., Hohman, T. C., and Carper, D. (1992) Hypertonic stress induces αB-crystallin expression. *Exp. Eye Res. 54*, 461–470.
6. Horwitz, J. (1992) α-Crystallin can function as a molecular chaperone. *Proc. Natl. Acad. Sci. U.S.A. 89*, 10449–10453.
7. Wang, K., and Spector, A. (1994) The chaperone activity of bovine α-crystallin. Interaction with other lens crystallins in native and denatured states. *J. Biol. Chem. 269*, 13601–13608.
8. Lee, J. S., Satoh, T., Shinoda, H., Samejima, T., Wu, S. H., and Chiou, S. H. (1997) Effect of heat-induced structural perturbation of secondary and tertiary structures on the chaperone activity of α-crystallin. *Biochem. Biophys. Res. Commun. 237*, 277–282.
9. Rajaraman, K., Raman, B., and Rao, C. M. (1996) Molten-globule state of carbonic anhydrase binds to the chaperone-like α-crystallin. *J. Biol. Chem. 271*, 27595–27600.
10. Mchaourab, H. S., Dodson, E. K., and Koteiche, H. A. (2002) Mechanism of chaperone function in small heat shock proteins. Two-mode binding of the excited states of T4 lysozyme mutants by αA-crystallin. *J. Biol. Chem. 277*, 40557–40566.
11. Rajaraman, K., Raman, B., Ramakrishna, T., and Rao, C. M. (1998) The chaperone-like α-crystallin forms a complex only with the aggregation-prone molten globule state of α-lactalbumin. *Biochem. Biophys. Res. Commun. 249*, 917–921.
12. Raman, B., and Rao, C. M. (1994) Chaperone-like activity and quaternary structure of α-crystallin. *J. Biol. Chem. 269*, 27264–27268.
13. Das, K. P., and Surewicz, W. K. (1995) Temperature-induced exposure of hydrophobic surfaces and its effect on the chaperone activity of α-crystallin. *FEBS Lett. 369*, 321–325.
14. Gesierich, U., and Pfeil, W. (1996) The conformational stability of α-crystallin is rather low: Calorimetric results. *FEBS Lett. 393*, 151–154.
15. Surewicz, W. K., and Olesen, P. R. (1995) On the thermal-stability of α-crystallin: A new insight from infrared-spectroscopy. *Biochemistry 34*, 9655–9660.
16. Raman, B., and Rao, C. M. (1997) Chaperone-like activity and temperature structural changes of α-crystallin. *J. Biol. Chem. 272*, 23559–23564.
17. Rao, C. M., Raman, B., Ramakrishna, T., Rajaraman, K., Ghosh, D., Datta, S., Trivedi, V. D., and Sukhaswami, M. B. (1998) Structural perturbation of α-crystallin and its chaperone-like activity. *Int. J. Biol. Macromol. 22*, 271–281.
18. Reddy, G. B., Kumar, P. A., and Kumar, M. S. (2006) Chaperone-like activity and hydrophobicity of α-crystallin. *IUBMB Life 58*, 632–641.
19. Khanova, H. A., Markossian, K. A., Kleimenov, S. Y., Levitsky, D. I., Chebotareva, N. A., Golub, N. V., Asryants, R. A., Muronetz, V. I., Saso, L., Yudin, I. K., Muranov, K. O., Ostrovsky, M. A., and Kurganov, B. I. (2007) Effect of α-crystallin on thermal denaturation and aggregation of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase. *Biophys. Chem. 125*, 521–531.
20. Hansen, J. F. (1991) The self-association of zinc-free human insulin and insulin analog B13-glutamine. *Biophys. Chem. 39*, 107–110.
21. Brange, J., and Langkjoer, L. (1993) Insulin structure and stability. *Pharm. Biotechnol. 5*, 315–350.
22. Baker, E. N., Blundell, T. L., Cutfield, J. F., Cutfield, S. M., Dodson, E. J., Dodson, G. G., Hodgkin, D. M. C., Hubbard, R. E., Isaacs, N. W., Reynolds, C. D., Sakabe, K., Sakabe, N., and Vijayan, N. M. (1988) The structure of 2Zn pig insulin crystals at 1.5-Å resolution. *Philos. Trans. R. Soc. London, Ser. B 319*, 369–456.
23. Huus, K., Havelund, S., Olsen, H. B., van de Weert, M., and Frokjaer, S. (2005) Thermal dissociation and unfolding of insulin. *Biochemistry 44*, 11171–11177.
24. Ahmad, A., Millett, I. S., Doniach, S., Uversky, V. N., and Fink, A. L. (2004) Stimulation of insulin fibrillation by urea-induced intermediates. *J. Biol. Chem. 279*, 14999–15013.
25. Ahmad, A., Millett, I. S., Doniach, S., Uversky, V. N., and Fink, A. L. (2003) Partially folded intermediates in insulin fibrillation. *Biochemistry 42*, 11404–11416.
26. Nielsen, L., Frokjaer, S., Brange, J., Uversky, V. N., and Fink, A. L. (2001) Probing the mechanism of insulin fibril formation with insulin mutants. *Biochemistry 40*, 8397–8409.
27. Pace, C. N., Vajdos, F., Fee, L., Grimsley, G., and Gray, T. (1995) How to measure and predict the molar absorption coefficient of a protein. *Protein Sci. 4*, 2411–2423.
28. Plotnikov, V. V., Brandts, J. M., Lin, L. N., and Brandts, J. F. (1997) A new ultrasensitive scanning calorimeter. *Anal. Biochem. 250*, 237–244.
29. Pace, C. N., Shirley, B. A., and Thomson, J. A. (1989) Measuring the conformational stability of a protein. In Protein Structure: A practical approach (Creighton, T. E., Ed.) pp 311−330, IRL Press Ltd., Oxford, U.K.
30. Strickland, E. H., and Mercola, D. (1976) Near-ultraviolet tyrosyl circular-dichroism of pig insulin monomers, dimers, and hexamers: Dipole-dipole coupling calculations in monopole approximation. *Biochemistry 15*, 3875–3884.
31. Lakowicz, J. R. (1999) Principles of fluorescence spectroscopy, Kluwer Academic, New York.
32. Van, M. R., Slingsby, C., and Vierling, E. (2001) Structure and function of the small heat shock protein/α-crystallin family of molecular chaperones. *Adv. Protein Chem. 59*, 105–156.
33. Johnson, C. R., Morin, P. E., Arrowsmith, C. H., and Freire, E. (1995) Thermodynamic analysis of the structural stability of the tetrameric oligomerization domain of P53 tumor-suppressor. *Biochemistry 34*, 5309–5316.
34. Ganadu, M. L., Aru, M., Mura, G. M., Coi, A., Mlynarz, P., and Kozlowski, H. (2004) Effects of divalent metal ions on the αβ-crystallin chaperone-like activity: Spectroscopic evidence for a complex between copper(II) and protein. *J. Inorg. Biochem. 98*, 1103–1109.
35. Biswas, A., and Das, K. P. (2008) Zn^{2+} enhances the molecular chaperone function and stability of α-crystallin. *Biochemistry 47*, 804–816.
36. Grant, P. T., Frank, B. H., and Coombs, T. L. (1972) Differences in Nature of Interaction of Insulin and Proinsulin with Zinc. *Biochem. J. 126*, 433–440.
37. Goldman, J., and Carpenter, F. H. (1974) Zinc binding, circular dichroism, and equilibrium sedimentation studies on insulin (bovine) and several of its derivatives. *Biochemistry 13*, 4566–4574.
38. Carver, J. A., Guerreiro, N., Nicholls, K. A., and Truscott, R. J. W. (1995) On the Interaction of α-crystallin with unfolded proteins. *Biochim. Biophys. Acta 1252*, 251–260.
39. Blundell, T. L., Cutfield, J. F., Cutfield, S. M., Dodson, E. J., Dodson, G. G., Hodgkin, D. C., Mercola, D. A., and Vijayan, M. (1971) Atomic positions in rhombohedral 2-zinc insulin crystals. *Nature 231*, 506–511.
40. Pocker, Y., and Biswas, S. B. (1981) Self-association of insulin and the role of hydrophobic bonding: A thermodynamic model of insulin dimerization. *Biochemistry 20*, 4354–4361.
41. Privalov, P. L. (1989) Thermodynamic problems of protein structure. *Annu. Rev. Biophys. Biophys. Chem. 18*, 47–69.
42. Lovatt, A., Cooper, A., and Camilleri, P. (1996) Energetics of cyclodextrin-induced dissociation of insulin. *Eur. Biophys. J. 24*, 354–357.
43. Aachmann, F. L., Otzen, D. E., Larsen, K. L., and Wimmer, R. (2003) Structural background of cyclodextrin-protein interactions. *Protein Eng. 16*, 905–912.