Lens α-crystallin is a 600–800-kDa heterogeneous oligomer protein consisting of two subunits, αA and αB. The homogeneous oligomers (αA- and αB-crystallins) have been prepared by recombinant DNA technology and shown to differ in the following biophysical/biochemical properties: hydrophobicity, chaperone-like activity, subunit exchange rate, and thermal stability. In this study, we studied their thermodynamic stability by unfolding in guanidine hydrochloride. The unfolding was probed by three spectroscopic parameters: absorbance at 235 nm, Trp fluorescence intensity at 320 nm, and far-UV circular dichroism at 223 nm. Global analysis indicated that a three-state model better describes the unfolding behavior than a two-state model, an indication that there are stable intermediates for both αA- and αB-crystallins. In terms of standard free energy \( \Delta G^{\text{UNF}}_{\text{H}2\text{O}} \), αA-crystallin is slightly more stable than αB-crystallin. The significance of the intermediates may be related to the functioning of α-crystallins as chaperone-like molecules.

Human lens proteins become progressively less soluble with age and cataract formation. The accumulation of high molecular mass aggregated and insoluble proteins was thought to be the main cause of lens opacity (1, 2). The major crystallin in high molecular mass and insoluble proteins, α-crystallin, has been thought to play a major role in the maintenance of lens transparency (3), as manifested by the recent finding that α-crystallin acts like a chaperone in \textit{in vitro} experiments (4). However, the mechanisms of high molecular mass aggregation and insolubilization have not been well established. A reasonable assumption is that these events are related to proteins being in an unstable or not fully folded state, and thus tending to aggregate when their concentrations are high. A study on thermodynamic and kinetic stability may provide a reliable clue.

An earlier report indicated that the native α-crystallin is thermally very stable (5). It does not denature even at 100 °C, but undergoes a thermal transition at 60–65 °C. Later, it was reported that α-crystallin becomes partially unfolded at this temperature (6). The refolding is irreversible after exposure to the high temperature (6, 7). On the other hand, the reversibility of denaturation unfolding of α-crystallin has been reported (8, 9). The thermodynamic and kinetic stability, however, has not been studied in detail, presumably because of the heterogeneity and oligomeric nature of α-crystallin. In contrast, the monomeric γ-crystallin has been extensively studied (10–13). Recently, we reported a thermodynamic and kinetic study on γF-crystallin (14), which is the most stable γ-crystallin gene product (15, 16).

To obtain pure homogeneous α-crystallin, we have cloned human lens αA- and αB-crystallins (17), which are good models for studying the unfolding and refolding properties of α-crystallin. In addition to the unfolding intermediate, we also need to consider whether α-crystallin has a dissociation intermediate (18). In this work, we used the chemical denaturant GdnHCl\(^1\) to compare thermodynamic stability between αA- and αB-crystallins and global analysis to obtain the standard free energy, \( \Delta G^{\text{H}2\text{O}} \). Global analysis uses multiple data sets, such as results from CD and fluorescence measurements, and involves a simultaneous nonlinear analysis to obtain a single set of parameters that satisfy all sets of data (19–21).

**MATERIALS AND METHODS**

**Preparation of Recombinant α-Crystallin Samples**

Preparation of the recombinant αA- and αB-crystallins was as described in a previous report (17). They are present in solution as 600–800-kDa homo-oligomers. We have also reported some of their biochemical and biophysical properties (17, 22, 23). Protein concentrations were determined by absorption based on aromatic amino acid composition (24).

**Spectroscopic Measurements**

Absorption was measured with a Perkin-Elmer Model Lambda 11 spectrophotometer. The absorption readings at 235 nm were plotted against GdnHCl concentrations.

CD spectra were measured with an Aviv Model 60 DS circular dichroism spectrometer. Five scans were recorded, and the averaged spectrum was smoothed by a polynomial-fitting program. The readings at 223 nm were compiled with GdnHCl concentrations. Appropriate base lines were always subtracted from spectra obtained with the corresponding GdnHCl concentrations.

Fluorescence was measured with a Shimadzu Model RF-5301PC spectrofluorometer. Trp emission spectra were scanned with an excitation wavelength at 290–295 nm. To avoid the effect of a red shift in the emission maximum by unfolding, emission intensities at 320 or 350 nm were obtained.

The fluorescent probe 1-anilino-8-naphthalenesulfonic acid (ANS) was used to study the hydrophobicity of the protein molecule (25). ANS solution (50 \( \mu \text{M} \)) was added to α-crystallin solutions with increasing GdnHCl concentrations, which had been incubated overnight to attain equilibrium. The samples were incubated for 30 min before fluorescence measurements. Emission spectra were scanned with an excitation wavelength at 395 nm.

**FPLC Gel Filtration and Light Scattering**

Dissociation of the α-crystallin aggregate was studied with FPLC gel filtration and light scattering at 400 nm. α-Crystallin samples incu-
bated for 24 h with increasing GdnHCl concentrations were applied to a Superose-6 column (Amersham Pharmacia Biotech FPLC system) and eluted with the same GdnHCl buffer. The column was pre-equilibrated with the appropriate GdnHCl solution.

Light scattering was measured at a 90° angle with the Shimadzu spectrofluorometer set at 400 nm for both excitation and emission wavelengths. Scattering intensity of the samples with increasing GdnHCl concentrations was plotted against GdnHCl concentration.

Unfolding and Refolding Studies

The folding reactions have been described in several previous publications (26–28). For oligomeric a-crystallin, the investigation of unfolding needs to consider whether there is a dissociation intermediate in addition to an unfolding intermediate (18). The presence of a dissociation intermediate can be determined by investigating the molecular mass as a function of the denaturant concentration by FPLC gel filtration. In many systems, dissociation of the subunit aggregate usually precedes subunit unfolding.

Equilibrium Studies

The unfolding transition was monitored by three probes: Trp fluorescence and absorption (local or tertiary structural change) and far-UV CD (secondary structural change). The data sets can be analyzed either individually or simultaneously by global analysis. The simplest model is a two-state transition.

Two-state Model N ⇄ U—This is the simplest case of denaturant-induced unfolding in which the transition is two-state, with rate constants $k_1$ and $k_2$, between a native state N and an unfolded state U and an insignificantly low population of intermediate. Equations 1–4 describe the thermodynamics of this model.

$$K_U = [U]/[N]$$  \hspace{1cm} (Eq. 1)

$$Q = 1 + K_U$$  \hspace{1cm} (Eq. 2)

$$X_N = 1/Q; X_U = K_U/Q = 1 - X_N$$  \hspace{1cm} (Eq. 3)

$$\Delta G_U = \Delta G_U^{H_2O} - mD = -RT \ln K_U$$  \hspace{1cm} (Eq. 4)

$K_U$ is the unfolding equilibrium constant; $Q$ is the partition function; and $X_N$ and $X_U$ are mole fractions of the proteins in the native and unfolded states, respectively. $\Delta G_U$ and $\Delta G_U^{H_2O}$ are the standard free energy changes for the unfolding transition in the presence and absence of denaturant D, and m is the denaturant susceptibility parameter that describes the dependence of $\Delta G_U$ on denaturant concentration. Substituting Equation 4 for Equations 2 and 3, we get Equations 5 and 6.

$$Q = 1 + \exp(mD - \Delta G_U^{H_2O}/RT)$$  \hspace{1cm} (Eq. 5)

$$X_U = (\exp(mD - \Delta G_U^{H_2O}/RT))/Q$$  \hspace{1cm} (Eq. 6)

The observed parameter $F[D]$ at any denaturant concentration [D] is given by Equation 7.

$$F[D] = X_NF_N + X_UF_U$$  \hspace{1cm} (Eq. 7)

where $F_N$ and $F_U$ are the parameters of the native and unfolded states, respectively. A nonlinear least-squares fitting program will yield the free energy difference in the absence of denaturant ($\Delta G_U^{H_2O}$) and m.

Three-state Model N ⇄ I ⇄ U—If a detectable intermediate I is involved, then a three-state model with rate constants $k_1$, $k_1'$, $k_2$, and $k_2'$ should be applied. The treatment of data and the equations used (Equations 8–13) are similar to those of the two-state model.
Global Analysis

Global analysis considers all measurement parameters simultaneously; the thermodynamic model is acceptable only if it fits all three spectral signal changes (20, 21). A nonlinear least-squares fitting program was written in association with SigmaPlot (SPSS, Chicago, IL). The initial parameters were provided, and regression was run until the R or R² values were minimal.

RESULTS

Dissociation Study by FPLC and Light Scattering—We sought to establish whether the α-crystallin aggregate (600–800 kDa) dissociates gradually or abruptly with increasing GdnHCl concentrations. Fig. 1 shows the FPLC elution profiles with increasing GdnHCl concentrations. Both αA- and αB-crystallins displayed a sharp peak at 20–22-ml fractions and started dissociating at around 1.0 M GdnHCl. A dissociated intermediate appeared and was in equilibrium with the undissociated multimer and the completely unfolded monomer at 1.0–1.5 M GdnHCl for both αA- and αB-crystallins. The peak position for the undissociated multimer, however, shifted to a position of slightly higher molecular mass. The transition range was narrow and completed at 2.0 M GdnHCl. With heating (6), no dissociation intermediate was observed, and the fate of the partially unfolded protein is to undergo high molecular mass aggregation.

The scattering data also reflect on the size of aggregates (Fig. 2). The transition is apparently two phases involving a stable intermediate.

ANS Study—ANS intensity initially increased with increasing GdnHCl concentration, reached a maximum intensity at 0.4 M GdnHCl, and then decreased (Fig. 3). The total loss of binding was achieved at a lower GdnHCl concentration for αB-crystallin than for αA-crystallin.

Equilibrium Study—α-Crystallin samples with increasing GdnHCl concentrations were left overnight at room temperature to reach equilibrium. Three spectroscopic measurements were used to probe the unfolding transition: far-UV CD for secondary structure and Trp fluorescence and absorption for tertiary structure. Fig. 4 (A and B) shows plots of absorbance at 235 nm, Trp fluorescence intensity at 320 nm, and CD intensity at 223 nm versus GdnHCl concentration. An initial global analysis with the two-state model gave an exceptionally poor fit, and the data were abandoned. A three-state fit is presented in Fig. 4 (A and B) (solid lines), and the resulting ΔG\text{H2O}\text{H2O} and m values are listed in Table I. Thermodynamically, αA-crystallin (ΔG\text{H2O}\text{H2O} = 6.38 kcal/mol) is more stable than αB-crystallin (6.92 kcal/mol).

![FIG. 4: Unfolding curves of αA-crystallin (A) and αB-crystallin (B) with GdnHCl probed by various spectroscopic measurements: absorption at 235 nm, Trp fluorescence at 320 nm, and far-UV CD at 223 nm. The solid lines are lines fit by global analysis with the three-state model.]

### Table I

| Crystallin | ΔG\text{H2O}\text{H2O} | ΔG\text{H2O}\text{H2O} | ΔG\text{H2O}\text{H2O} | m\text{H} | m\text{H} | m\text{H} |
|-----------|-----------------|-----------------|-----------------|--------|--------|--------|
| αA        | 2.90 ± 0.50     | 3.48 ± 0.83     | 6.38 ± 0.67     | 2.89 ± 0.18 | 3.91 ± 0.45 | 9.92 ± 0.32 |
| αB        | 1.69 ± 0.11     | 3.35 ± 0.33     | 5.04 ± 0.22     | 4.76 ± 0.39 | 2.53 ± 0.20 | 7.29 ± 0.30 |
(5.04 kcal/mol), although the difference (1.34 ± 0.705 kcal/mol) is borderline significant. The small difference in $\Delta H$ (9.92 versus 7.29 kcal/mol/mol) between $\alpha$A- and $\alpha$B-crystallins apparently indicates that their conformations are similar. The small difference in $\Delta C_P$ (9.92 versus 7.29 kcal/mol/mol) between $\alpha$A- and $\alpha$B-crystallins apparently indicates that their conformations are similar. Fig. 5 (A and B) displays the residuals of the fits, which should be randomly distributed about zero. Apparently, the three-state model is not a perfect fit, but the four-state model is too complicated to analyze, with too many parameters involved.

**DISCUSSION**

$\alpha$-Crystallin subunits $\alpha$A and $\alpha$B previously were separated under denaturation in urea. The reconstituted homopolymeric $\alpha$A- and $\alpha$B-crystallins were either smaller than the native protein or not in the native conformation (29–31), which posed a serious problem in studying their physical/chemical properties, especially comparing their conformational stability. The use of recombinant technology to clone $\alpha$A- and $\alpha$B-crystallins has overcome this difficulty and facilitated many kinds of studies (17, 32–37). Recently, interest in $\alpha$-crystallin has been intensified following many findings: $\alpha$-crystallin possesses a chaperone-like property (4, 38); $\alpha$B-crystallin is a small heat-shock protein expressed in non-lenticular tissues, and its expression increases in some neurological diseases (39–41).

We have reported differences in hydrophobicity, chaperone-like activity, thermal stability, and rate of subunit exchange between $\alpha$A- and $\alpha$B-crystallins (17, 22, 23). The thermal stability study indicated that $\alpha$B-crystallin is more susceptible to aggregation than $\alpha$A-crystallin and that addition of $\alpha$A- to $\alpha$B-crystallin reduces aggregation (22). The lower thermal stability for $\alpha$B-crystallin at high temperature may be related to its greater hydrophobicity (17, 42). The present thermodynamic study also indicated that $\alpha$A-crystallin is conformationally more stable than $\alpha$B-crystallin. The difference may contribute to the differential susceptibility to modification-related aggregation and insolubilization. We are currently studying the human lens insoluble fraction to see whether it contains equal or different amounts of $\alpha$A- and $\alpha$B-crystallins by an immunochemical method. The low conformational stability of either $\alpha$A- or $\alpha$B-crystallin may imply that in vivo, $\alpha$-crystallin is more susceptible to aggregation and insolubilization than other crystallins. Low conformational stability of calf lens $\alpha$-crystallin was also reported by calorimetric measurement ($\Delta G_{U,H_2O}^{H_2O} = 6$ kcal/mol) (43). In contrast, we have reported rather high conformational stability for calf lens $\gamma$F-crystallin ($\Delta G_{U,H_2O}^{U,H_2O} = 9$ kcal/mol) (14).

$\alpha$-Crystallin has been suggested to have a stable intermediate (8, 29, 45–47). An ANS study of calf lens $\alpha$-crystallin demonstrated that it undergoes partial unfolding in dilute GdnHCl concentrations, and an intermediate was observed at 0.8–1.0 M GdnHCl with maximum hydrophobicity and chaperone-like activity (9). None of the earlier reports, however, distinguished the unfolding intermediate from the dissociation intermediate. Our FPLC data indicated that $\alpha$A- and $\alpha$B-crystallins started dissociating at 1.0 M GdnHCl. The fractions between 25 and 30 ml contained partially dissociated proteins, whereas the fractions between 35 and 40 ml contained mostly unfolded proteins. The corresponding unfolding curve of the Trp emission maximum shows a gradual shift from 336 to 352 nm (>3.0 M GdnHCl), indicating an initial partial unfolding to complete unfolding (data not shown). The fact that dissociation yields only one oligomer with a defined size indicates that the dissociation is a cooperative reaction. A nonspecific dissociation will yield many oligomers with various sizes, resulting in a very broad FPLC peak. The sequence of events appears to be partially unfolding and loosing of the quaternary structure, followed by dissociation and complete unfolding.

No three-dimensional x-ray crystal structure is available for

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**Fig. 5.** Residuals of three-state fits of $\alpha$A-crystallin (A) and $\alpha$B-crystallin (B). The residuals are the observed minus the calculated signals. Fl, fluorescence.
α-crystallin, and many structural models have been proposed, including a three-layer model (48, 49), a micellar model (50), a chaperone two-stack model (51), a “pitted-flexiball” model (52), and a model of a spherical assembly with a central cavity (53). More recently, a three-dimensional structural model was constructed (54) based on the similarity between the sequences of human αB-crystallin and the sHsp Mj HSP 16.5, whose high resolution structure is known (55). The building block is a dimer in which the monomer folding unit composes a β-sandwich arrangement: eight β-strands in the core “α-crystallin” (Glu67–Ile161) and one β-strand in the N-terminal domain. Further study is needed to establish whether the building block dimer is the intermediate observed in this study.

One analogous system for the unfolding study of α-crystallin is chaperonin-60 (GroEL). GroEL is an oligomer consisting of 14 subunits of 57 kDa each, with a molecular mass close to the 800 kDa of α-crystallin (56). The quaternary structure is two heptameric rings stacked on top of each other to form a cylinder. Each subunit is folded into three functional domains. The process and that a...