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# On the Equilibrium between Monomeric α-Lactalbumin and the Chaperoning Complex of α-Crystallin

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**In chaperoning dithiothreitol-denatured α-lactabumin, α-crystallin forms a chaperoning complex. In order to study the kinetics of such chaperoning it needs to be established whether the formation of the chaperoning complex is a reversible or irreversible process. The chaperoning reaction was studied by dynamic light scattering as a function of concentration and weight ratio of α-lactalbumin/α-crystallin. HPLC and subsequent SDS-PAGE gel electrophoresis experiments established that the chaperoning complex formed contains both α-crystallin and α-lactalbumin. Upon rechromatographing the chaperoning complex, the presence of monomeric α-lactalbumin has been demonstrated in addition to the chaperoning complex itself. This and equilibrium dialysis experiments demonstrated conclusively the existence of an equilibrium between monomeric partially denatured α-lactalbumin and the chaperoning complex made of α-lactalbumin and α-crystallin.** 

***Key Words:*** **α-crystallin; α-lactalbumin; chaperone; equilibrium.**

α-Crystallin forms complexes with denaturing proteins thereby preventing their uncontrolled aggregation. This chaperoning process has been shown to follow a saturation type of complexing, i.e., when the capacity of the available α-crystallin to bind the target protein is exceeded, the excess target protein aggregates and eventually precipitates out of solution (1). In modeling the kinetics of such a process using DTT-denatured α-lactalbumin as the target protein, an equilibrium was assumed between monomeric α-lactalbumin and the chaperoning complex (2). In other chaperone systems (e.g., GroEl) the existence of such equilibrium has been demonstrated (3, 4). In the small heat shock family of chaperones (e.g., Hsp 25), to which α-crystallin belongs, the assumption is that they do not release proteins once complexed (5). Other results suggest that they may do so *in vitro* via secondary interaction of the high molecular weight complex with Hsp70 (6). With α-crystallin there is a lack of experimental evidence to indicate whether the association of partially denatured α-lactalbumin with α-crystallin is a reversible or irreversible process. Lindner *et al.* (7) studied the interaction of α-crystallin with α-lactalbumin by proton NMR spectroscopy. They have shown that α-crystallin interacts only with the molten globule state of α-lactalbumin along the denaturation pathway. Such molten globule states are in equilibrium with the folded protein. According to the authors, α-crystallin interacts with the large hydrophobic surface of the molten globule form of α-lactalbumin and stabilizes it when the latter is on its way to irreversible aggregation. Whether such a complex can shed unfolded α-lactalbumin was not addressed. Recently in a similar system, DTT denatured lysozyme chaperoned by α-crystallin (8), it was found that α-crystallin interacts only with the irreversibly destabilized form of lysozyme. However, whether this interaction is reversible remains unclear.

## MATERIALS AND METHODS

Bovine eyes were obtained from a local slaughterhouse. The lenses were removed and homogenized in 7 volumes of 0.05 M Tris buffer, pH 7.4 which also contained 0.1 M KCl, 1 mM EDTA, 10 mM 2-mercaptoethanol and 0.02% $NaN_3$. After centrifugation at 10,000*g* for 30 min at 4°C, the supernatant was loaded on a Sepharose-CL6B or Sephacryl 300 column (2.5 × 75 cm), which was pre-equilibrated with the same buffer and five mL fractions were collected (9). The α-crystallin peak was identified by SDS-PAGE gel electrophoresis (10) and fractions were pooled, concentrated by ultrafiltration, and rechromatographed on the same column (9). The purified α-crystallin was desalted, lyophilized and stored at −20°C. Calcium depleted, Type III, α-lactalbumin was purchased from Sigma. Dithiothreitol (DTT) was purchased from Schwarz Mann Biochem. For the chaperoning experiments, phosphate buffer at pH 6.82 was used containing 50 mM $Na_3PO_4$, 0.1 M NaCl, 2 mM EDTA. α-Crystallin and α-lactalbumin stock solutions in phosphate buffer were mixed first to obtain the desired concentration and weight ratio. The experiments

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were performed at room temperature (23°C) and initiated by adding DTT dissolved in phosphate buffer. The final concentration of DTT was 50 mM.

Dynamic light scattering (DLS) measurements (11) were performed in an apparatus (12, 13) which uses a fiber optic DLS probe with a semiconductor laser (670 nm) as a light source, tightly focused to produce a 20-$\mu$m-diameter spot. The scattered light was collected at 154°C. The time correlation function (TCF) was computed using a digital correlator (BI9000). The TCF data were analyzed using commercial data inversion routines supplied by the Brookhaven Instrument Corporation (Holtsville, NY). We used the exponential sampling method (14) for all runs. The instrument performance was checked by measuring the dynamic light scattering of standard suspensions of polystyrene beads having 80 nm diameter.

High performance liquid chromatography (HPLC) was performed using a Superose HR-6 sizing column attached to a Pharmacia LKB Biotechnology Inc. HPLC system. Samples of 0.5 ml were taken from the DLS experiments at different times. They were filtered and 0.2 ml was loaded onto the column. The elution was at 0.5 ml/min at room temperature (23°C) and 0.5 ml fractions were collected.

SDS-PAGE electrophoresis was run on 14% acrylamide gels according to Laemmli (15) using pre-cast gels (Novex) in a Novex apparatus. The gels were stained by Coomassie Blue R-250 and silver.

Equilibrium dialysis was performed using both 25 and 50 kDa membranes in a dialysis chamber (Amika). An aliquot of 0.5 ml of the pooled fractions of the chaperoning complex was placed in the chamber and dialyzed overnight at 4°C against the phosphate buffer used in the chaperoning experiments. The UV spectrum of the dialysate was obtained. The dialysate was concentrated and SDS-PAGE gel electrophoresis was performed using silver staining.

## RESULTS

Chaperoning by $\alpha$-crystallin was investigated as a function of concentration (using 3–9 mg/ml protein concentrations) and of weight ratios of $\alpha$-crystallin/$\alpha$-lactalbumin varying from 1:3 to 3:1. The progress of chaperoning was followed by DLS. The initial distribution pattern, obtained immediately after the addition of DTT, was centered around 17 nm (the size of the $\alpha$-crystallin), the average size of the chaperoning. Ten $\mu$s first delay time was used to focus the attention on the chaperoning complex. With this delay time the low molecular weight (14 kD) $\alpha$-lactalbumin does not appear in the size distribution pattern (1). The size of the chaperoning complex seemed to grow slightly for the first few minutes, followed by a slow decline reaching a steady state after 15 min. Eventually both the overall scattering intensity as well as the size parameters began to rise (Figs. 1a and 1b). Samples for analysis by HPLC were taken usually when high molecular weight aggregates began to appear (45–80 min). Figure 2 shows such an example where particles with the range of 60–100 nm are already present. In that particular run $\alpha$-crystallin and $\alpha$-lactalbumin were present in weight 1:1 ratio and the sample was taken 60 min after the addition of DTT.

A sample, such as shown in Fig. 2, was run on HPLC and produced three peaks: (1) a sharp peak of high molecular species at fractions 13–16; (2) a broad peak with fractions 19–27; and (3) a strong peak of fractions 33–38 (Fig. 3). The ratio of intensities of peak (1)/peak (3) were roughly proportional to the initial $\alpha$-crystallin/$\alpha$-lactalbumin weight ratio of a particular experiment.

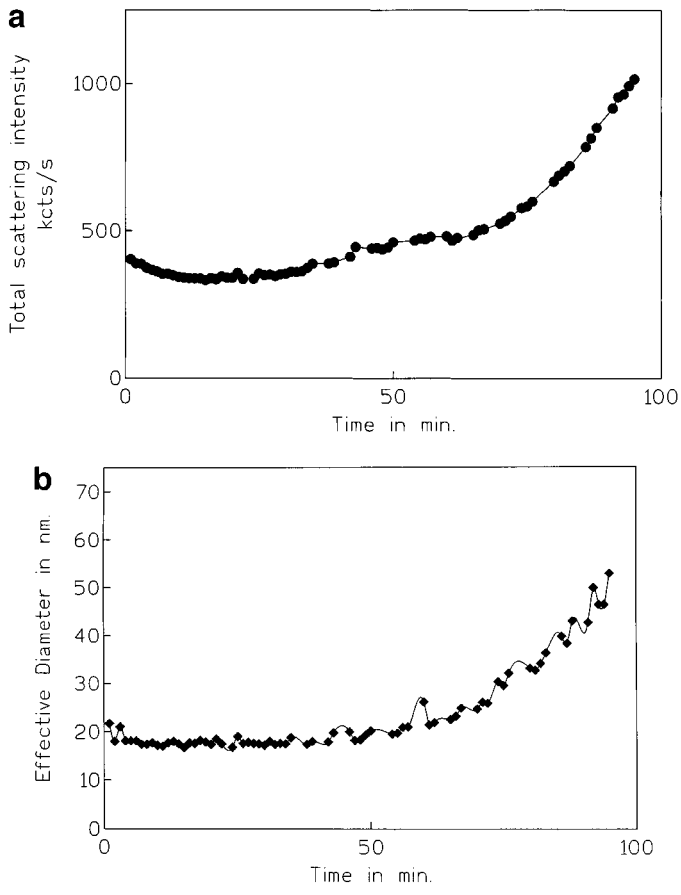


**FIG. 1.** Change in the DLS parameters as a function of chaperoning time (in minutes). (a) Overall scattering intensity (kcts/s). (b) Average effective particle diameter.

Identification of the composition of the peaks in the HPLC pattern was established by SDS-PAGE gel electrophoresis (Fig. 4). Peak (1) contained mostly $\alpha$-lactalbumin; peak (2) represents the chaperoning complex containing both $\alpha$-crystallin as well as $\alpha$-lactalbumin; peak (3) has only $\alpha$-lactalbumin. Upon rechromatographing the chaperoning complex (peak 2 in Fig. 3) one obtains an HPLC pattern consisting of a broad peak containing fractions 19–27 and two smaller peaks made of fractions 35–37 and 38–41 (Fig. 5). SDS-PAGE gel electrophoresis identified the 19–27 fractions as the chaperoning complex containing both $\alpha$-crystallin and $\alpha$-lactalbumin while the peaks containing fractions 35–38 and 38–41 showed the presence of $\alpha$-lactalbumin only (Fig. 6). The peak of fractions 42–45 contained DTT only. A similar pattern was found for all concentrations and weight ratios investigated. The appearance of monomeric $\alpha$-lactalbumin in the rechromatographed chaperoning complex indicates the existence of equilibrium

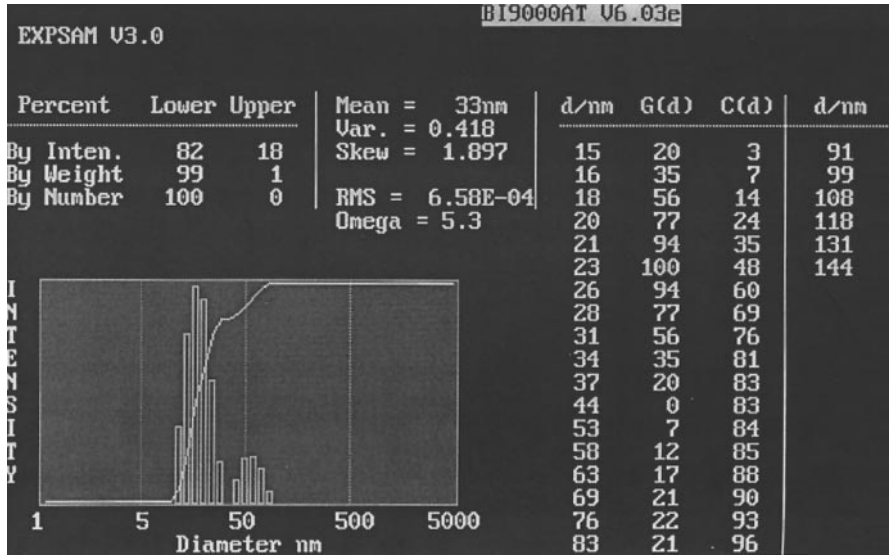


**FIG. 2.** Distribution pattern of particles 100 min. after the addition of DTT. The analysis of the DLS intensity was done with exponential sampling. The d/nm column gives the diameter of the particles in nm. The G(d) column lists the relative abundance of the particles with the most abundant diameter labeled as 100. The C(d) column depicts the cumulative distribution of particles.

between chaperoning complex and monomeric $\alpha$-lactalbumin. $\alpha$-Lactalbumin appears in higher fractions in the rechromatographed HPLC pattern (34–41) than in the original HPLC pattern (33–38). This may indicate that the $\alpha$-lactalbumin coming off the chaperoning complex interacts with the column.

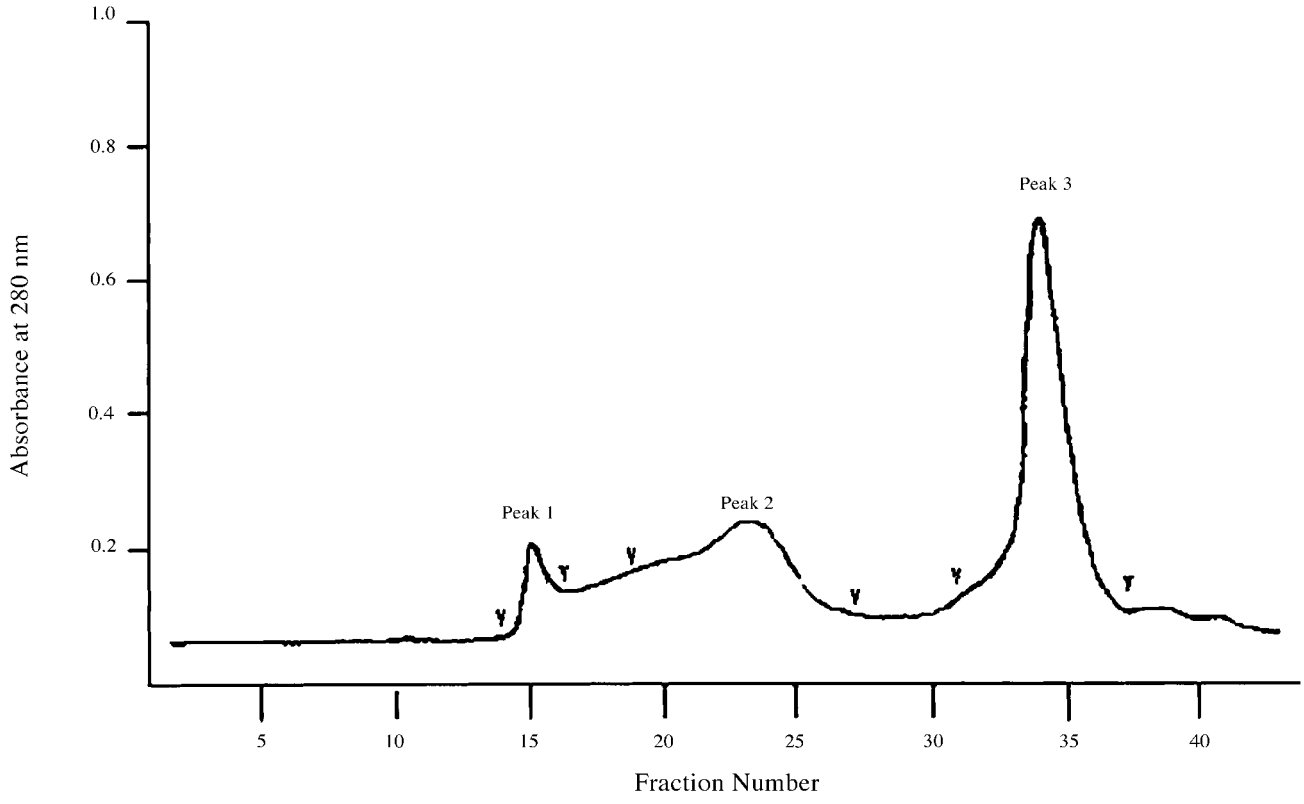


**FIG. 3.** HPLC pattern of a chaperoning mixture run taken after 60 min. The concentrations of both $\alpha$-crystallin and $\alpha$-lactalbumin was 2.4 mg/ml. Peak 1, fractions 13–16; peak 2, fractions 18–27; peak 3, 33–38.

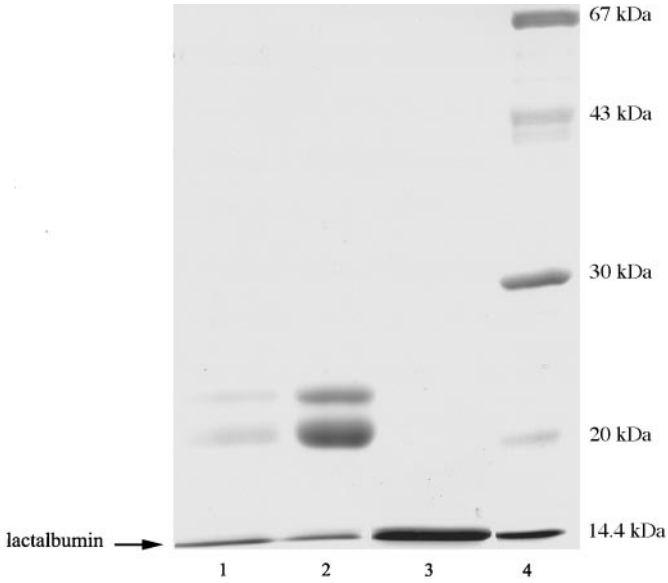


**FIG. 4.** SDS-PAGE gel electrophoresis pattern of the peaks from Fig. 3. Lane 1, peak 1 containing mainly $\alpha$-lactalbumin with small amount of heavy molecular weight $\alpha$-crystallin; lane 2, peak 2 consisting of $\alpha$-crystallin and $\alpha$-lactalbumin; lane 3, peak 3 containing $\alpha$-lactalbumin only. Lane 4, molecular weight standards.

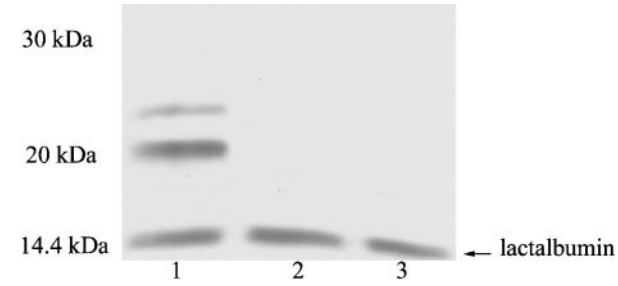


**FIG. 6.** Silver-stained SDS-PAGE electrophoresis pattern of the peaks of the rechromatographed chaperoning complex from Fig. 5. Lane 1, (peak 1) containing fractions 19–27 showing the presence of $\alpha$-crystallin and $\alpha$-lactalbumin; lane 2 (peak 2) made of fractions 35–37 indicating the presence of $\alpha$-lactalbumin; and lane 3 (peak 3) made of fractions 38–41 showing the presence of $\alpha$-lactalbumin.

Equilibrium dialysis of the chaperoning complex band from the HPLC chromatography (peak 2) demonstrated the presence of a protein in the dialysate. The SDS-PAGE gel electrophoresis of the dialysate (Fig. 7) identifies the presence of $\alpha$-lactalbumin.

## DISCUSSION

The existence of an equilibrium between the chaperoning complex and monomeric lactalbumin was established using HPLC chromatography and equilibrium dialysis experiments combined with SDS-PAGE gel electrophoresis. Mixtures of $\alpha$-crystallin and $\alpha$-lactalbumin in the presence of DTT showed a time course of chaperoning resembling a flattened parabolic curve. One can observe in detail what we originally (1) labeled as a fluffy chaperoning ball, that is, we see a slight decline in the average size parameters (Diameter and Effective diameter) and especially in the average light scattering intensity (kcts/s) for the first 15 min after which a steady size is obtained followed by an increase in all these parameters (Figs. 1a and 1b). Within these parabolic curves, however, there are certain temporal fluctuations, but nothing that could be perceived as being periodic. These fluctuations may be the signature of the dynamic reorganization of interparticle exchange of $\alpha$-A and $\alpha$-B subunits of $\alpha$-crystallin and denatured $\alpha$-lactalbumin that has been reported in the

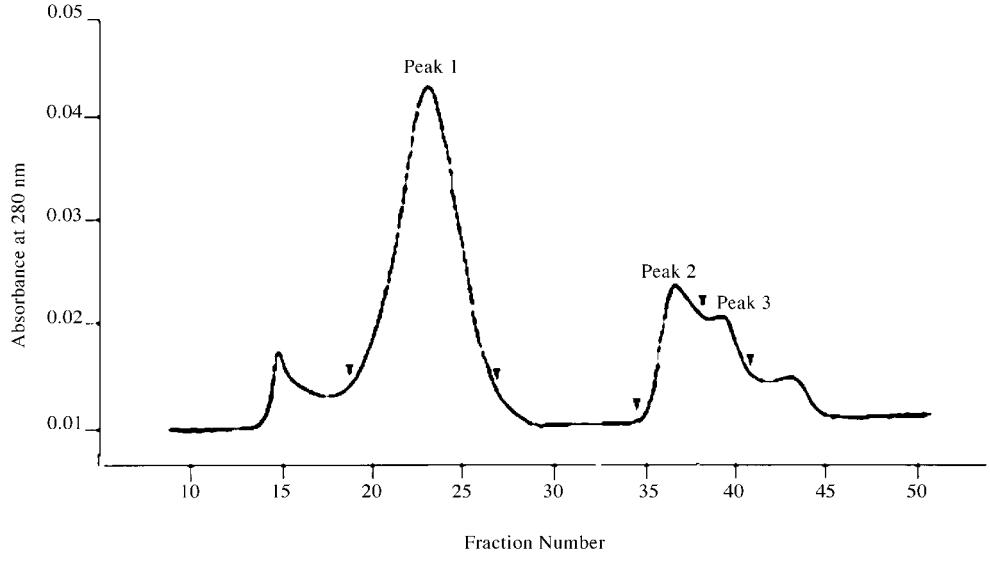


**FIG. 5.** HPLC pattern of the rechromatographed chaperoning complex peak from Fig. 3. Peak 1, fractions 19–27; peak 2, fractions 35–37; peak 3, fractions 38–41. These peaks were identified in Fig. 6. The peak in void volume (13–16) contained aggregated $\alpha$-lactalbumin. The peak of fractions 42–45 contained DTT.

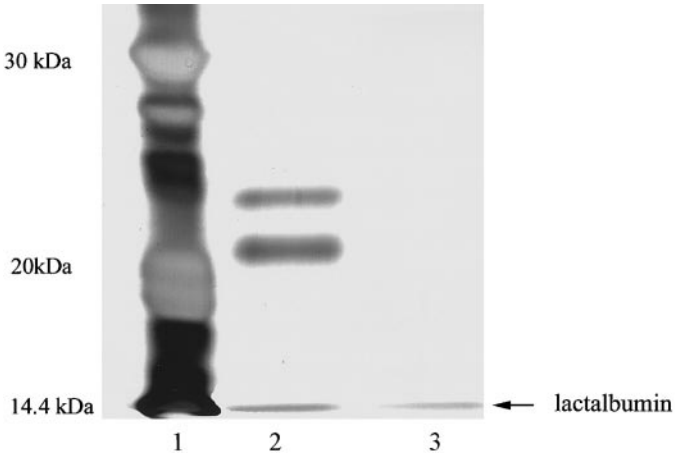


**FIG. 7.** Silver-stained SDS-PAGE electrophoresis pattern of equilibrium dialysate. Lane 1, molecular weight standards; lane 2, chaperoning complex to be dialyzed. Lane 3, equilibrium dialysate of the chaperoning complex showing $\alpha$-lactalbumin only.

literature (16, 17). The general trend that we characterized as fluffy-ball type of chaperoning complex (1) that may decline in size has also been reported in other DTT denatured proteins (insulin, lysozyme and conalbumin) undergoing chaperoning (17). Upon addition of DTT one observes a wide distribution of molecular weights (similar to the first band in Fig. 2). The subsequent decline in size (Figs. 1a and 1b) was accompanied by a decrease in the width of the molecular size distribution of the first band in Fig. 2. This indicates that the chaperoning complex formation occurs in a number of different steps. Therefore, the model proposed in a preliminary report on kinetics (2), namely a one step equilibrium between $\alpha$-crystallin and $\alpha$-lactalbumin as reactants and the chaperoning complex as a product, is an oversimplification.

However, that such an equilibrium does exist is demonstrated by HPLC experiments. The isolation of a chaperoning complex (Figs. 3 and 4) that upon rechromatography yields separate peaks of $\alpha$-lactalbumin plus the original peak of the chaperoning complex (Figs. 5 and 6) is a proof of such an equilibrium. A second proof for the existence of such an equilibrium was the equilibrium dialysis of the isolated chaperoning complex against the buffer. In each case the presence of a protein in the dialysate has been shown by UV absorption spectroscopy and the protein was identified as $\alpha$-lactalbumin (Fig. 7). Therefore, at least in the $\alpha$-lactalbumin/$\alpha$-crystallin model the $\alpha$-crystallin, like other large heat shock proteins (3, 4), is capable of releasing soluble $\alpha$-lactalbumin from the chaperoning complex. Thus its function may be more than simply stabilizing the molten globules of $\alpha$-lactalbumin (5, 7).

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