Complete Protection by α-Crystallin of Lens Sorbitol Dehydrogenase Undergoing Thermal Stress*

Received for publication, July 12, 2000
Published, JBC Papers in Press, August 4, 2000, DOI 10.1074/jbc.M006133200

Isabella Marini, Roberta Moschini, Antonella Del Corso, and Umberto Mura‡
From the Università di Pisa, Dipartimento di Fisiologia e Biochimica, Laboratorio di Biochimica, via S. Maria 55, 56100 Pisa, Italy

Sorbitol dehydrogenase (L-iditol:NAD+ 2-oxidoreductase, E.C. 1.1.1.14) (SDH) was significantly protected from thermally induced inactivation and aggregation by bovine lens α-crystallin. An α-crystallin/SDH ratio as low as 1:2 in weight was sufficient to preserve the transparency of the enzyme solution kept for at least 2 h at 55 °C. Moreover, an α-crystallin/SDH ratio of 5:1 (w/w) was sufficient to preserve the enzyme activity fully at 55 °C for at least 40 min. The protection by α-crystallin of SDH activity was essentially unaffected by high ionic strength (i.e. 0.5 M NaCl). On the other hand, the transparency of the protein solution was lost at a high salt concentration because of the precipitation of the α-crystallin/SDH adduct. Magnesium and calcium ions present at millimolar concentrations antagonized the protective action exerted by α-crystallin against the thermally induced inactivation and aggregation of SDH. The lack of protection of α-crystallin against the inactivation of SDH induced at 55 °C by thiol blocking agents or EDTA together with the additive effect of NADH in stabilizing the enzyme in the presence of α-crystallin suggest that functional groups involved in catalysis are freely accessible in SDH while interacting with α-crystallin. Two different adducts between α-crystallin and SDH were isolated by gel filtration chromatography. One adduct was characterized by a high Mr of approximately 800,000 and carried exclusively inactive SDH. A second adduct, carrying active SDH, had a size consistent with an interaction of the enzyme with monomers or low Mr aggregates of α-crystallin. Even though it had a reduced efficiency with respect to α-crystallin, bovine serum albumin was shown to mimic the chaperone-like activity of α-crystallin in protecting SDH from thermal denaturation. These findings suggest that the multimeric structural organization of α-crystallin may not be a necessary requirement for the stabilization of the enzyme activity.

α-Crystallin, which represents approximately 35% of lens soluble proteins, is a multiaggregate of two different 20-KDa subunits, αA and αB, which have a high degree of sequence homology. Believed to be strictly a lens-specific protein, α-crystallin has now been found in several cells and non-lenticular tissues (1, 2). Both αA and αB chains have a conserved domain, called the “α-crystallin domain,” which is peculiar to the SHSP family (3, 4). Like SHSP, α-crystallin subunits can be induced by heat and other stress conditions (5) and appear to be involved in the pathogenesis of various degenerative diseases (6, 7) and in apoptosis (8).

The chaperone-like action of α-crystallin, which was assumed from the observed sequence homology between α-crystallin subunits and the SHSP gene of Drosophila (9), was then confirmed by the strong in vitro anti-aggregation effect of α-crystallin on denaturating thermally stressed proteins (10). In fact, the effectiveness of α-crystallin as an anti-aggregator is often used as a simple assay for in vitro assessments of molecular chaperone power (11). The thermally induced precipitation of β- and γ-crystallin (12, 13) and several enzymes (14, 15) was efficiently prevented by α-crystallin present at variable ratios with respect to the target protein. Enzymes undergoing thermal stress were protected by α-crystallin from aggregation but generally not from inactivation, except for the restriction enzyme NdeI (16) and for bovine liver catalase in which activity was only slightly protected by α-crystallin (17). The situation was different when the enzyme was subjected to different post-translational modifications, such as glycation, carbamylation, and steroid-induced inactivation where α-crystallin was shown to protect the enzyme activity (17).

In this work, we studied the chaperone-like activity of α-crystallin on sorbitol dehydrogenase (L-iditol:NAD+ 2-oxidoreductase, E.C. 1.1.1.14) (SDH), purified from bovine lens. SDH is a heat labile tetrameric enzyme that requires Zn2+ and NADH as cofactors for its activity (18) and is the only known mechanism responsible for the removal of sorbitol in the cell. SDH was protected by bovine lens α-crystallin not only from thermally induced aggregation but also from inactivation.

EXPERIMENTAL PROCEDURES

Materials—Calf eyes were obtained from a local abattoir soon after slaughtering; the lenses were removed and frozen at −20 °C until used. Molecular weight markers for SDS-PAGE and gel filtration, dithiothreitol, NAD+, β-D-(−)-fructose, and D-sorbitol were from Sigma-Aldrich. NADH was supplied by Roche Molecular Biochemicals, EDTA by Serva Feinbiochemica, and YM30 ultrafiltration membrane by Amicon. The electrophoretic equipment was from Bio-Rad. Sepharose CL-4B and Sepharose 6B, were from Amersham Pharmacia Biotech. Spectra/Por molecular porous membrane tubing (cut off 60KDa) was from Spectrum. All other chemicals were of reagent grade.

Enzyme Purification and Assay—Sorbitol dehydrogenase was purified to electrophoretic homogeneity as described previously (19). The final enzyme preparation (approximately 1 mg/ml) with a specific activity of 51 units/mg was stored at 4 °C in 10 mM sodium phosphate, pH 7 (S-buffer) supplemented with 2 mM dithioretilt and 0.1 mM NADH. The purified enzyme stored in the above preservative mixture was dialyzed against S-buffer just before use.

The assay of enzyme activity was performed at 37 °C as described previously (19) by following the decrease in absorbance at 340 nm in a sorbitol dehydrogenase/BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis.
reaction mixture (0.5 ml final volume) containing 0.24 mM NADH and 0.4 mM d-fructose in a 100 mM Tris-HCl buffer, pH 7.4. The rate of NADH oxidation measured in a parallel assay in which the substrate was omitted was subtracted as a blank. One unit of enzyme activity is the amount of SDH that catalyzes the oxidation of 1 μmol/min of NADH.

α-Crystallin Isolation—All procedures were carried out at 4 °C unless otherwise stated. Frozen lenses were suspended (1.5 g/ml in 0.4 ml of S-buffer) and then stored at −20 °C in an S-buffer until use at a final protein concentration of approximately 2 mg/ml.

Protein Aggregation Assay—The thermal denaturation of SDH both in the absence and presence of α-crystallin was followed by monitoring, as an index of turbidity, the absorbance at 360 nm in a Beckman DU-6 spectrophotometer. Incubations of SDH (0.1–0.3 mg/ml) were performed at 55 °C both in S-buffer and in 50 mM Tris-HCl buffer pH 7.4, using a water bath at a controlled temperature.

Gel Filtration Chromatography—Gel filtration analyses of SDH/α-crystallin mixtures were carried out at 25 and 55 °C by Sepharose CL-4B on a XK16/70 column (Amersham Pharmacia Biotech) equipped with a thermostatic jacket. Elution was performed with S-buffer at a flow rate of 60 ml/h, and fractions of 1 ml were collected. The following standards were used for apparent molecular weight calibration: apoferritin, M<sub>r</sub> 44,000; β-amylase, M<sub>r</sub> 200,000; alcohol dehydrogenase, M<sub>r</sub> 150,000. The relative elution volume (R<sub>e</sub>) is expressed as R<sub>e</sub> = (V<sub>e</sub> - V<sub>c</sub>) / V<sub>c</sub> where V<sub>c</sub> and V<sub>e</sub> refer to the sample elution volume, the column exclusion volume (as determined by the elution of a dextran blue standard), and the resin bed volume, respectively.

Analysis of the Kinetic Data—SDH initial velocity measurements were performed spectrophotometrically as described above. For each set of measurements, data were analyzed by double reciprocal plots. Rate measurements in each set of assays were in duplicate with a reproducibility of <5%, and each plot was repeated at least twice. K<sub>m</sub> and V<sub>max</sub> values measured for fructose and NADH were determined by non-linear regression with a relative standard deviation of 5%.

Other Methods—Protein concentration was determined by the Coomassie Blue binding assay (20), using bovine serum albumin as a standard. SDH and α-crystallin localizations in the elution profiles of chromatographic analyses were carried out by SDS-PAGE according to Laemmli (21) using 12% acrylamide slab gels, 0.75-mm thick. The following standards were used for apparent molecular weight calibration: bovine serum albumin, M<sub>r</sub> 66,000; ovalbumin, M<sub>r</sub> 45,000; glycerol-

RESULTS

Thermal Inactivation of Sorbitol Dehydrogenase—A progressive inactivation of SDH was observed by incubating the enzyme at increasing temperatures. The pseudo-first order inactivation rate constant increased from 3.51 × 10<sup>−4</sup> ± 0.29 × 10<sup>−4</sup> s<sup>−1</sup> to 1.40 × 10<sup>−3</sup> ± 0.07 × 10<sup>−3</sup> s<sup>−1</sup> when the incubation temperature of SDH (0.01 mg/ml) was raised from 25 °C to 60 °C. The inactivation process was affected by the protein concentration, nature, and concentration of the buffer and by the presence of the pyridine cofactor.

NADH, up to 0.1 mM, partially protected the enzyme against thermal inactivation. However, for all the temperatures tested the NADH-dependent protection led to a SDH activity that was never more than 20% higher than the control values. The time course of SDH inactivation at 55 °C with different final enzyme concentrations, ranging from 0.01 to 3 mg/ml, showed that after 15 min of incubation the residual activity was increased from approximately 25 to 70%, respectively. However, incubations of SDH in the concentration range of 0.1–0.3 mg/ml performed at 55 °C for more than 15 min led to protein precipitation (see below). The precipitation phenomena at long incubation times were affected by the nature and concentration of the buffer. Although the 10 mM phosphate buffer, pH 7, appeared to protect the protein from precipitation, the Tris-HCl buffer used in this study (50 mM, pH 7.4) appeared to magnify the susceptibility of SDH to thermal aggregation. Moreover, the concentration of the Tris buffer affected the enzyme’s stability. At 55 °C, the residual activity of SDH (0.01 mg/ml) after 40 min of incubation rose from approximately 20 to 40% when the buffer concentration was increased from 10 to 50 mM (data not shown). The changes in the nominal pH values occurring in the buffer in the temperature range from 25 to 55 °C (pH from 7.4 to 6.3) did not affect the enzyme inactivation (data not shown).

Protective Effect of α-Crystallin against the Thermal Aggregation and Inactivation of SDH—When SDH was thermally stressed in the presence of α-crystallin, both enzyme inactivation and precipitation were prevented. α-Crystallin present in the incubation at a ratio as low as 1.2 (w/w) with SDH prevented protein aggregation when the enzyme (0.3 mg/ml, final concentration) was incubated at 55 °C (Fig. 1). High ionic strength interfered with the anti-aggregation capability of α-crystallin; this is shown by the increase in turbidity observed when transient α-crystallin/SDH mixtures, but not α-crystallin alone, were supplemented at 55 °C with NaCl ranging from 0.2 to 0.5 M. The effect exerted by 0.5 M NaCl is shown in Fig. 1. Under these conditions, the rate of the increase in absorbance at 360 nm was inversely proportional to the α-crystallin/SDH ratio in the mixture.

To determine whether the precipitation induced by high ionic strength in α-crystallin/SDH mixtures involved only SDH or both target and protector proteins, the precipitate formed after NaCl addition was collected by centrifugation, washed three times by warmed (i.e. 55 °C) 0.5 M NaCl in Tris-HCl buffer, and then analyzed by SDS-PAGE. The results, shown in the inset of...
Fig. 1, indicate that both SDH and α-crysin are present in the precipitate in a proportion comparable with the original composition of the mixture (compare lanes a and b of Fig. 1, inset).

The ability of α-crysin to protect enzyme activity was evaluated at various temperatures. The residual SDH activity was measured during incubation in the absence or presence of α-crysin at a ratio with the enzyme of 5:1 (w/w) (Fig. 2). In all cases up to 55 °C, the presence of α-crysin fully protected the enzyme for at least 40 min of incubation. Prolonged incubations at 55 °C led to a gradual decline in SDH activity. The remaining enzyme activity after 3 h of incubation at 55 °C of 0.15 mg/ml SDH with 1:5 (w/w) α-crysin was 75–80% of the initial activity (data not shown). At a higher temperature (i.e., 60 °C), α-crysin was only partially active. In fact, as shown in Fig. 2, after 40 min of incubation at 60 °C, although SDH alone was completely inactive, 50% of the initial activity was still detectable when α-crysin was present with the enzyme.

When under the above conditions (5:1, w/w α-crysin/SDH mixtures at 0.1 mg/ml SDH, 55 °C), the ionic strength of α-crysin/SDH mixtures was increased by the addition of 0.5 mM NaCl, a rather modest effect on the stabilizing ability of α-crysin toward SDH activity was observed. A recovery of approximately 75% of the control value was detected 45 min after the addition of the salt.

Effect of Ca2+ and Mg2+ on the Chaperone-like Activity of α-Crysin—Calcium and magnesium ions interfere with the protective action of α-crysin against both thermal inactivation and aggregation of SDH. Both metal ions at millimolar concentrations induced protein aggregation when added to clear mixtures of SDH and α-crysin at 55 °C. After 40 min of incubation at 55 °C, the SDH present in a transparent mixture (0.1 mg/ml) containing α-crysin 1:1 (w/w) was readily precipitated following the addition of 2 mM Ca2+ or 5 mM Mg2+ (Fig. 3A). No precipitation was observed when both metal ions were added at 55 °C to a solution containing α-crysin alone (data not shown).

A concentration-dependent action of Ca2+ and Mg2+ in antagonizing the stabilization exerted by α-crysin on SDH activity is reported in Fig. 3B. The strongest effect of Ca2+ was observed at concentrations between 0.1 and 1 mM, whereas the strongest effect of Mg2+ was between 0.5 and 3 mM. The increase in the α-crysin/SDH ratio resulted in a reduced efficiency for both of the metal ions to induce enzyme inactivation. In particular, when the ratio of α-crysin/SDH was increased from 1:1 to 5:1, the residual activity measured after 20 min at 55 °C from the addition of the metal ion (either 2 mM Ca2+ or 5 mM Mg2+) increased from approximately 50 to 80% (data not shown).

ATP, Mg2+, and KCl were necessary for the GroE system to exert its chaperone action (23). Moreover, ATP, in the presence of Mg2+, KCl, and α-crysin, elicited the partial recovery of citrate synthase activity after the enzyme had been previously denatured by guanidinium chloride (24). In contrast, in our study, the presence of 5–10 mM ATP and 2 mM KCl neither...
improved the stabilization exerted by α-crystallin on SDH nor interfered with the antagonism to the stabilization process exerted by Mg$^{2+}$.

SDH and α-Crystallin Form Two Sizes of Complexes—The elution profiles of a size exclusion chromatography of a mixture of α-crystallin and SDH (5:1, w/w) after incubation and separation at 25 and 55 °C are shown in Fig. 4, panels A and B, respectively. In Fig. 4C, the profile of a chromatographic analysis at 25 °C of SDH in the absence of α-crystallin is also reported. The elution profile at 280 nm reported in Fig. 4A revealed two peaks, one of which at a $R_f$ of 0.71 (low $M_r$ peak) was associated with SDH activity. SDS-PAGE analysis (inset) revealed that whereas SDH was indeed exclusively in the low $M_r$ peak, α-crystallin, mainly present at a $R_f$ of 0.45 (high $M_r$ peak), was distributed in a wide range of elution volumes overlapping the peak of SDH activity. The staining intensity of the α-crystallin bands in the fractions containing active SDH was higher when compared with fractions eluting immediately before the SDH activity peak (compare lanes c and b in Fig. 4A, inset). Moreover, no α-crystallin bands were detectable by SDS-PAGE in fractions with elution volumes corresponding to low $M_r$ peak of chromatographic runs performed at 25 °C (data not shown) in which α-crystallin was analyzed in the absence of SDH (compare lanes c and d in Fig. 4A, inset). The $R_f$ values of standard proteins are indicated by numbered arrows as follows: 1, apoferritin; 2, β-amylase; 3, alcohol dehydrogenase.

![Fig. 4](image-url)  
Size exclusion chromatography on Sepharose CL-4B of the SDH/α-crystallin mixtures at 25 and 55 °C. Panel A, purified SDH (0.17 mg/ml) was incubated in Tris-HCl, pH 7.4, at 25 °C for 15 min in the presence of α-crystallin (0.85 mg/ml) and then chromatographed at the same temperature. Panel B, the same as described in panel A, except the temperature of pre-incubation and separation was 55 instead of 25 °C. Panel C, purified SDH (0.17 mg/ml) was chromatographed as described above at 25 °C in the absence of α-crystallin. In each of the panels, the continuous line and the closed circles refer to the absorbance at 280 nm and to SDH activity (units/ml), respectively. Insets for each panel show the SDS-PAGE analysis of the eluted fractions as follows: lanes a, b, and c refer to the fractions marked by arrows a, b, and c, respectively; lane d refers to the SDS-PAGE analysis of the fractions, equivalent to arrow c coming from a chromatographic analysis (not shown) performed at 25 °C (A) and 55 °C (B) in the absence of SDH. O and F in the insets refer to the origin and the front of the electrophoretic migration, respectively. The $R_f$ values of standard proteins are indicated by numbered arrows as follows: 1, apoferritin; 2, β-amylase; 3, alcohol dehydrogenase.
the high $M_r$ region ($R_p = 0.47$).

The elution volume of active SDH in the chromatographic analysis performed at 25 °C on an α-crystallin/SDH mixture (Fig. 4A) is slightly lower than that observed for SDH analyzed in the absence of α-crystallin ($R_p = 0.73$, Fig. 4C). However, such a decrease (approximately 3 ml, corresponding to an increase of 30 KDa) is too small to attempt any specific evaluation of the $M_r$ of the α-crystallin/SDH complex. A further decrease in the elution volume of active SDH was observed when the α-crystallin/SDH mixture was analyzed at 55 °C. However, in such a condition, even though high $M_r$ α-crystallin aggregate and active SDH were separated (see inset, Fig. 4B), a slight delay in α-crystallin elution was also observed (differential $R_p = 0.02$). This finding would seem to indicate a modest but still detectable change in the resolution power of the column at 55 °C. Because of the difficulties in finding suitable standard proteins, no calibration of the column could be done at 55 °C.

The possibility that low $M_r$ aggregates of α-crystallin subunits might protect SDH from thermal inactivation was tested by measuring at different times the enzyme activity of SDH kept at 50 °C in the presence of a dialysis bag containing α-crystallin (see "Experimental Procedures"). Under those conditions, it was possible to measure a residual activity of approximately 90% after 1 h of incubation. When α-crystallin were not present in the dialysis bag, a residual activity of approximately 70% was measured.

**Accessibility of the Active Site of SDH while Interacting with α-Crystallin**—The ability of α-crystallin to protect SDH activity against thiol blocking agents and metal chelators was tested at 55 °C in mixtures containing α-crystallin and SDH in a ratio of 5:1 (w/w).

Both iodoacetamide and iodoacetic acid led to a complete inactivation of SDH. When 0.01 mg/ml bovine lens SDH was incubated in 50 mM Tris-HCl, pH 7.4, buffer at 55 °C with 0.5 mM of either iodoacetamide or iodoacetic acid, an inactivation occurred at the same rate (with a pseudo-first order rate constant of $2.5 \times 10^{-3} \pm 0.6 \times 10^{-3}$ s$^{-1}$) irrespective of the presence of α-crystallin. The presence of 0.1 mM NADH significantly delayed ($k = 6.5 \times 10^{-4} \pm 1 \times 10^{-4}$ s$^{-1}$) but did not prevent the loss of enzyme activity. Bovine lens SDH, which contains Zn$^{2+}$ as a prosthetic group (18), is inactivated by EDTA and recovers its activity upon treatment with ZnSO$_4$ (19). When 0.01 mg/ml SDH was treated with 100 µM EDTA at 55 °C, no reactivation occurred upon addition of the Zn$^{2+}$. α-Crystallin, 5:1 (w/w) with the enzyme, did not protect SDH at 55 °C from the rapid and complete loss of activity induced by EDTA. However, the inactivation was completely reversed when the inactive protein mixture, following thermal treatment, was incubated at 25 °C in the presence of 100 µM ZnSO$_4$ (data not shown). NADH, which has been shown to protect SDH at 37 °C from EDTA-induced inactivation (19), was also able at 55 °C to protect SDH from EDTA in the presence of α-crystallin.

**Kinetic Features of SDH Following Thermal Stress**—The effectiveness of SDH as a catalyst of the NADH-dependent reduction of fructose was tested after the thermal treatment of the enzyme. Reaction rates were measured at 37 °C at different substrate concentrations by using 0.01 mg/ml SDH pre-incubated at 55 °C for different times (0–40 min) both in the absence and in the presence of α-crystallin in a ratio of 5:1 (w/w) with respect to the enzyme. No changes in $K_m$ values with respect to the untreated SDH for both fructose (200 ± 3 mM) and NADH (0.049 ± 0.001 mM) were observed, either for the thermally stressed enzyme stabilized by α-crystallin (205 ± 4 and 0.046 ± 0.005 mM for fructose and NADH, respectively) or for the enzyme undergoing inactivation in the absence of α-crystallin (200 ± 3 and 0.049 ± 0.001 mM for fructose and NADH, respectively). Moreover, $V_{max}$ measured for the enzyme previously heated at 55 °C in the presence of α-crystallin (0.068 ± 0.008 µmol/min/mg of protein) was essentially identical to the one measured under the same conditions on the native unstressed enzyme (0.064 ± 0.005 µmol/min/mg of protein).

**BSA Mimics the Chaperone-like Activity of α-Crystallin**—To assess the specificity of action of α-crystallin, we made use of BSA, a thermally stable protein, which shows a completely different structural organization with respect to α-crystallin (25). The molecular chaperone-like activities of BSA and α-crystallin were compared by evaluating their ability to stabilize SDH activity (0.01 mg/ml of enzyme) at protector/target ratios ranging from 0 to 1 (data not shown). The results indicated that at low protector/target protein ratios, α-crystallin was the most effective protective agent. 50% of initial enzyme activity was still detectable after 40 min of incubation at 55 °C at an α-crystallin/SDH ratio of 0.1. To achieve the same protection, a ratio of BSA/SDH of at least 0.4 was required. However, at high protector/target ratios (higher than 0.7), α-crystallin and BSA were equivalent as protective agents, both being able to almost completely preserve SDH activity. When the anti-aggregation action was evaluated, no protection against SDH precipitation at 55 °C was observed when α-crystallin was substituted by an equivalent concentration (mg/ml) of BSA under the conditions adopted in Fig. 1. However, a significant delay before precipitation (approximately 50 min) and a 50% reduction in the maximal extent of protein precipitation were observed when the BSA/SDH ratio was increased from 0.5 to 1.0 (data not shown).

**DISCUSSION**

After incubation at 55 °C, SDH purified from bovine lens aggregated and α-crystallin, present at a ratio of 2:1 in weight with the enzyme, completely suppressed precipitation phenomena, subsequently keeping the solution transparent for at least two h. A new target protein, SDH, thus confirms the ability of α-crystallin to prevent thermally induced protein aggregation and insolubilization. Moreover, unlike what was observed for other target proteins (10), besides being protected from thermal aggregation, SDH activity was completely stabilized by α-crystallin even in terms of enzyme activity. Indeed, at an α-crystallin/SDH ratio of 5:1, a full retention of SDH activity for at least a 40-min incubation at 55 °C was observed (Fig. 2). Because it is a multimeric protein, in which activity is linked to a pyridine cofactor and to a bound metal ion (i.e. Zn$^{2+}$) acting as a prosthetic group, SDH may undergo inactivation by a variety of triggering events, which probably occur through different unfolding pathways. It is, therefore, not surprising that SDH is quite sensitive to heat and appeared to lose activity at relatively low temperatures (Fig. 2). The protein concentration considerably affects the rate of inactivation. A significant increase in the residual activity (from 25 to 70%) was observed following incubation of the enzyme for 15 min at 55 °C when the protein concentration was raised from 0.01 to 0.3 mg/ml (data not shown). This fact suggests that protein-protein interaction is an important factor for SDH stability. The pyridine cofactor too is able to partially protect the enzyme from thermally induced inactivation at all of the temperatures tested. However, neither the high protein concentration nor the pyridine cofactor was able to completely counteract thermal stress. On the other hand, the protection by α-crystallin was complete in the entire range of temperatures up to 55 °C. At higher temperatures, the protective effectiveness of α-crystallin decreased considerably. At 60 °C, for instance, despite the presence of α-crystallin, the residual activity after 40 min of incu-
bation dropped from 100 to approximately 50% of initial activity (Fig. 2).

It is currently accepted that the chaperone-like action of α-crystallin is because of its ability to bind hydrophobic regions of the target protein with the generation of soluble adducts (26, 27). Nevertheless, the increase in ionic strength (from 0.2 to 0.5 M NaCl), although having no effect on the solubility of α-crystallin present alone at 55 °C, induced protein precipitation in mixtures of α-crystallin/SDH. Thus, the protective action of α-crystallin against protein precipitation failed either because of the release of SDH from the complex or because of the precipitation of the complex itself. Indeed, SDS-PAGE analysis of the precipitate, obtained upon the addition of 0.5 M NaCl (Fig. 1), revealed the presence of both SDH and α-crystallin in the same ratio as in the mixture before the addition of the salt (Fig. 1, inset). In this regard, the reduced rate of precipitation observed at higher α-crystallin/SDH ratios could be explained by a stabilization effect exerted by α-crystallin on the adduct. This interpretation is consistent with the fact that in conditions unfavorable to protein precipitation (i.e. high α-crystallin/SDH ratios at absolute low protein concentrations), the increase in ionic strength had only a modest effect on the protection exerted by α-crystallin on SDH activity.

Calcium and magnesium ions were able to antagonize the protective action of α-crystallin against the thermally induced aggregation of SDH as shown previously for aldose reductase and γ-crystallin (28, 29). In fact, both metal ions added at a millimolar level at 55 °C to a transparent mixture of SDH and α-crystallin led to protein precipitation (Fig. 3A). Furthermore, Ca2+ and Mg2+ impaired the protective action exerted by α-crystallin on SDH activity in conditions of thermal stress. The effects of different concentrations of both Ca2+ and Mg2+ on the protective ability of α-crystallin meant that an optimal level of effectiveness could be defined for both metal ions (Fig. 3B, inset). It is worth noting that all our attempts to modify the effectiveness of α-crystallin to act as chaperone-like protein by the addition of ATP failed both in the presence and absence of Mg2+ ion (data not shown). If the occurrence of an ATP-driven process can be ruled out, the chaperone-like action of α-crystallin should be explained exclusively in terms of the intrinsic properties of its structure. In other words, the interaction alone between protector and target protein should be sufficient to provide protection. In this regard, the effectiveness of α-crystallin to prevent SDH precipitation was compared with that of BSA, a hydrophobic protein (25) that, as occurs with α-crystallin, is resistant to thermally induced precipitation. When BSA was present at a ratio of 1:2 in weight with SDH, which in the case of α-crystallin led to full protection (Fig. 1), protein precipitation occurred to the same extent and with the same kinetics as control incubations performed in the absence of the protector protein (data not shown). However, as the BSA/SDH ratio was increased to 1:1, protection against precipitation, even though not complete, was observed. Thus, BSA may intervene in the SDH aggregation process. The potential of a chaperone-like action of BSA appeared to be more evident in the protection exerted by the protein toward thermally induced SDH inactivation. Therefore, the chaperone-like action did not appear to be a specific feature of α-crystallin, although α-crystallin was more effective than BSA in protecting SDH. This result differs from those previously reported for the restriction enzyme NdeI and catalase (16, 17), whose limited protection by α-crystallin against thermally induced inactivation did not find a comparable protection by BSA. The protective action exerted on SDH by BSA, which is not as structurally organized as α-crystallin, raises the question of the relevance of the multimeric structure of α-crystallin in determining its chaperone-like activity. The apparent specificity of action of α-crystallin, at least in the case of SDH, appears to be confined to rather low protector/target protein ratios.

The ability of α-crystallin to protect the catalytic activity of SDH provides an additional tool in the study of its interaction with the target protein. We thus attempted to take advantage of this behavior to gain a better understanding of the features of the protein-protein interaction. A comparison of the kinetic parameters of the enzyme thermally stressed in the presence of α-crystallin with those of the native SDH and with those of the enzyme undergoing thermal inactivation in the absence of the protector protein revealed no differences. This fact ruled out the possibility of using the measurement of the enzyme activity to follow the complex formation. Nevertheless, this result suggests the occurrence of two possible situations. (i) The interaction between α-crystallin and the target protein ceases at temperatures lower than those inducing stress. In this case, a decrease from 55 to 37 °C (the temperature at which the enzyme assay was performed) would have been sufficient for the release of an active SDH, which would at that point be indistinguishable from the native enzyme. (ii) The interaction between α-crystallin and SDH is kept at a low temperature, but it does not involve functional groups at the active site. In this case, the enzyme could freely express catalysis while it is bound to α-crystallin.

The first hypothesis is consistent with the previously reported effect of temperature on the α-crystallin structure (30, 31). In fact, a structural change in α-crystallin that occurs at around 55 °C has been proposed as necessary for α-crystallin to be active as a protein stabilizer. However, in studies on the anti-aggregation activity of α-crystallin, it has also been shown that once the complex between α-crystallin and the target protein had been formed, it remained stable at lower temperatures. In particular, this was shown when the anti-aggregation activity of α-crystallin was tested toward ALR2 (28) and carbonic anhydrase (14), which were protected by α-crystallin against aggregation but not against inactivation. In these cases, the gel filtration analysis of the α-crystallin/target mixtures previously subjected to thermal stress revealed that both ALR2 (Mr 34,000) and carbonic anhydrase (Mr 31,000) coeluted as inactive enzymes with α-crystallin in the high molecular weight region (~Mr 300,000). If this was also the case for SDH, one would expect to find a high Mr complex between α-crystallin and the enzyme in which SDH could express its catalytic activity. Such an event would be consistent with the second hypothesis presented above, which assumes that the active site on the α-crystallin-bound SDH is freely accessible. The occurrence of an active α-crystallin-SDH adduct is supported by the results of SDH inactivation induced by thiol blocking agents or EDTA. In particular, while at 25 °C the inactivation of SDH induced by EDTA was reversed by the addition of ZnSO4, at 55 °C the loss of enzyme activity could be recovered by ZnSO4 only when α-crystallin was present with SDH during treatment with EDTA. This fact is a further indication that the microenvironmental conditions of SDH undergoing inactivation by EDTA when α-crystallin was present were different from those in which SDH was inactivated in the absence of α-crystallin. This is likely because of the binding between the protector/target enzyme. It is worth noting that in the presence of α-crystallin, NADH protected the SDH from EDTA-induced inactivation in a manner similar to that previously reported when the enzyme was subjected to EDTA treatment at a lower temperature (19). Thus, even if SDH were bound to α-crystallin, its cofactor site would be freely accessible.

The hypothesis that a stable active adduct was generated
between SDH and α-crystallin was tested by gel filtration chromatography performed both at 25 °C and under thermal stress conditions (i.e. 55 °C). The chromatographic separation of a mixture of SDH/α-crystallin performed at 55 °C revealed that some SDH co-migrated with α-crystallin in the range of high Mr fractions but that no activity was associated with that enzyme population (Fig. 4B and lane a of the inset). The occurrence of such an adduct between α-crystallin and the target enzyme may well represent the basis of the potent chaperone-like action of α-crystallin against protein aggregation phenomena. However, the lack of SDH activity in the high Mr complex raises the question of how SDH is protected against thermal inactivation.

The interaction between active SDH and α-crystallin both at 25 and 55 °C is indicated by the evident enrichment in the α-crystallin content of the eluted fractions containing the active enzyme (Fig. 4, A and B, insets). The retention volumes of active SDH separated from α-crystallin both at 25 and 55 °C appeared smaller than the elution volume of SDH analyzed alone at 25 °C in the absence of α-crystallin. The differences, however, were too small to predict any specific Mr for the active-SDH/α-crystallin adduct. Thus, the interaction between the active enzyme and the protector protein should occur with isolated α-crystallin subunits or with oligomeric α-crystallin aggregates whose size would be so small as to not significantly alter the elution volume of SDH upon binding.

Another possibility that would explain the elution profile and electrophoretic analysis of Fig. 4 is that the stabilization of SDH activity occurred through a weak interaction between the enzyme and aggregates of α-crystallin subunits of a size comparable with SDH. In this regard, it is interesting to note the recently proposed structural organization for α-crystallin, which would appear to arise from an aggregation of four decameric substructural units (32). In this case, the formation of the hypothesized oligomeric α-crystallin aggregate must be induced by the presence of SDH. In fact, when the chromatographic analysis was performed both at 25 and at 55 °C on α-crystallin samples in which SDH was absent, the SDS-PAGE fractions of which molecular species of 150–200 KDa should elute, none or only a very modest amount of α-crystallin was revealed at 25 and at 55 °C, respectively (see lane d in the insets of Fig. 4, A and B). The chromatographic evidence of an interaction between low molecular weight aggregates of α-crystallin subunits and SDH was supported by the even partial protection exerted by α-crystallin against SDH inactivation observed while protector and target proteins were kept at 50 °C, segregated from each other by a 60-kDa cut-off dialysis membrane.

It is worth noting that the active-SDH/α-crystallin interaction does not require any thermal stress to occur. In fact, an increase in the relative content of α-crystallin in the fractions containing active SDH is observed in the chromatographic analysis performed at 25 °C (Fig. 4A, inset, lanes a–c). This fact explains the protective action exerted by α-crystallin against SDH inactivation even at low temperatures (Fig. 2). The results presented indicate that the overall stabilizing action exerted by α-crystallin on SDH (i.e. anti-aggregation and enzyme activity preservation) occurs with the generation of at least two different interactive complexes between the protector and target proteins.

It is not clear so far whether the pathways leading to the generation of the two complexes are part of the same interactive mechanism. However, the remarkable sensitivity of both processes to Ca2+ and Mg2+ would at least suggest the existence of specific common features. In fact, the two divalent cations were able to interfere with both antiaggregation and enzyme activity stabilization at concentrations low enough to indicate specific interactions with the molecular components of the SDH/α-crystallin complexes. Whether the generation of the active-SDH/α-crystallin complex represents a preliminary, obligatory step of a general interactive pathway between the protector and the target protein, leading at the end to very stable high Mr adducts, or is occasionally confined to the peculiar features of the target protein used in the present study cannot be assessed at this point, and further investigation will be required.

Acknowledgments—We are indebted to Dr. Nando Benimeo (Industria Alimentare Carni, Castelvetro, Modena, Italy) for the kind supply of bovine lenses and to Dr. Giovanni Sorlini and the veterinary staff of INALCA for their valuable cooperation with the bovine lens collection.

REFERENCES
1. Kato, K., Shinohara, H., Kurube, N., Inaguma, Y., Shimizu, K., and Oshima, K. (1991) Biochim. Biophys. Acta 1074, 201–208
2. Srinivasan, A. N., Nagineni, C. N., and Bhat, S. P. (1992) J. Biol. Chem. 267, 23337–23341
3. Boelens, W. C., and de Jong, W. W. (1995) Mol. Biol. Reports 21, 75–80
4. Merkel, K. B., Groenen, P. T. J. A., Voorter, C. E. M., de Haard-Hoekman, W. A., Horwitz, J., Bloemendal, H., and de Jong, W. W. (1993) J. Biol. Chem. 268, 1046–1052
5. Head, M. W., Horwitz, L., and Goldman, J. E. (1996) J. Cell Sci. 109, 1029–1039
6. Iwaki, T., Wisniewski, T., Iwaki, A., Corbin, E., Tomokane, N., Tateishi, J., and Goldman, J. E. (1992) Am. J. Pathol. 140, 345–356
7. Van Noort, J. M. (1996) J. Mol. Med. 74, 285–296
8. Mehlen, P., Schultz-Osthoff, and Arrigo, A. P. (1996) J. Biol. Chem. 271, 16510–16514
9. Ingolia, T. D., and Craig, E. A. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 2360–2364
10. Horwitz, J. J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10449–10453
11. Derham, B. K., and Harding, J. J. (1997) Biochim. Biophys. Acta 1336, 187–194
12. Boyle, D., and Takemoto, L. (1994) Exp. Eye Res. 58, 9–16
13. Wang, K., and Spector, A. (1994) J. Biol. Chem. 269, 13601–13608
14. Rao, P. V., Horwitz, J., and Zagler, J. S., Jr. (1993) Biochem. Biophys. Res. Commun. 190, 786–793
15. Carver, J. A., Aquilina, J. A., Cooper, P. G., Williams, G. A., and Truscott, R. J. (1995) Biochem. Biophys. Acta 1252, 195–206
16. Hess, J. F., and Fitzgerald, P. G. (1998) Mol. Vis. 15, 4–29
17. Hook, D. W. A., and Harding, J. J. (1998) Int. J. Biol. Macromol. 22, 295–306
18. Jeffery, J., and Jornvall, H. (1998) Adv. Enzymol. 61, 47–106
19. Marini, I., Bucchioni, L., Borella, P., Del Corso, A., and Mura, U. (1997) Arch. Biochem. Biophys. 340, 383–391
20. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
21. Laemmli U. K. (1970) Nature 227, 680–685
22. Wray, W., Boulikas, T., Wray, V. P., and Hancox, R. (1981) Anal. Biochem. 118, 197–203
23. Martin, J., Mayhew, M., Langer, T., and Hartl, F. U. (1993) Nature 366, 228–233
24. Muchowski, P. J., and Clark, J. I. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 1004–1009
25. Peters, T. (1989) Adv. Protein Chem. 37, 161–245
26. Carver, J. A., Guerreiro, N., Nicholas, K. A., and Truscott, R. J. (1995) Biochim. Biophys. Acta 1252, 251–260
27. Das, K. P., and Suresh, W. K. (1995) FEBS Lett. 369, 321–325
28. Marini, I., Bucchioni, L., Vahedi, M., Del Corso, A., and Mura, U. (1995) Biochem. Biophys. Res. Commun. 212, 413–420
29. Korets, J. F., Doss, E. W., and LaButi, J. N. (1998) Int. J. Biol. Macromol. 22, 283–294
30. Raman, B. R., and Rao, C. M. (1994) J. Biol. Chem. 269, 37264–37268
31. Raman, B. R., and Rao, C. M. (1997) J. Biol. Chem. 272, 23559–23564
32. Smuldiers, R. H. P., van Boekel, M. A. M., and de Jong, W. W. (1998) Int. J. Biol. Macromol. 22, 187–196