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## INTERACTION OF NEWLY SYNTHESIZED $\alpha$ -CRYSTALLIN WITH ISOLATED LENS PLASMA MEMBRANES

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### Summary

Translation of lens polyribosomes in a reticulocyte cell-free system results mainly in the synthesis of the water-soluble crystallins. After incubation of the translation products with isolated lens fiber plasma membranes, the newly synthesized  $\alpha$ -crystallin interacts with this fraction and becomes water-insoluble. Urea extraction of the reisolated plasma membranes shows that part of the polymeric  $\alpha$ -crystallin, in particular the  $\alpha A$  chains, becomes urea-insoluble. When the membranes were isolated under conditions that stabilize complex formation with the cytoskeleton, only  $\alpha A_2$  seems to interact with this complex. In contrast, interaction with  $\beta$ - and  $\gamma$ -crystallin could not be observed.

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### Introduction

Although  $\alpha$ -crystallin is mainly concentrated in the water-soluble part of bovine lens fibers, part of this protein seems to exist in close association with the fiber plasma membranes [1].

The latter  $\alpha$ -crystallin fraction cannot be removed from the membranes by extraction with a low-ionic strength buffer and even resists mild proteolytic digestion applied to the plasma membranes [2]. Moreover, when the isolated membranes are treated with 6 M urea,  $\alpha$ -crystallin is only partially solubilized. Therefore this  $\alpha$ -crystallin has been regarded as an integral plasma membrane constituent [3]. Earlier investigations [4] demonstrated that  $\alpha$ -crystallin may also exist in close association with microfilaments, which can be obtained

together with plasma membranes, provided proper ionic conditions are applied [5]. Bracchi et al. [6] suggested, on basis of absorption experiments, that only aged  $\alpha$ -crystallin can be made water insoluble by interaction with lens membrane fragments. They furthermore discussed the possibility that age-related changes in conformation and/or the aggregation of  $\alpha$ -crystallin as shown by several workers [7–9] would be necessary for this interaction.

Albeit it might well be that aging will promote the interaction, the results presented in this paper provide evidence that also newly synthesized  $\alpha$ -crystallin interacts with lens fiber plasma membranes, indicating that no age-related modifications per se are necessary for association with lens plasma membranes.

## Materials and Methods

*Isolation of lens fiber plasma membranes.* Fresh calf lenses were decapsulated and homogenized by continuous stirring for 2 h in either bicarbonate buffer (1 mM  $\text{NaHCO}_3$ , 1 mM  $\text{CaCl}_2$ ) or in cytoskeleton-stabilizing buffer (50 mM Tris-HCl, pH 7.4/25 mM KCl/5 mM magnesium acetate).

Plasma membranes were isolated by discontinuous sucrose gradient centrifugation as described earlier [2] either in bicarbonate or cytoskeleton-stabilizing buffer. The plasma membrane fractions obtained at the sucrose densities 1.20–1.22 g/cm<sup>3</sup> and 1.18–1.20 g/cm<sup>3</sup> were washed three times in the respective buffers and centrifuged in a Ti 50 rotor. In the isolation procedure, using cytoskeleton-stabilizing buffer, membranes are found only at the density 1.20–1.22 g/cm<sup>3</sup>. Protein concentration was determined as described by Lowry et al. [10].

*De novo synthesis of lens proteins.* Calf lens polyribosomes were isolated as described by Bloemendal et al. [11]. Rabbit reticulocytes were prepared as described by Evans and Lingrel and lysed by addition of water [12]. A 30 000  $\times g$  supernatant fraction of the lysed cells was used as cell-free system, and incubations were performed at 30°C for 1 h. The reaction mixture contained per ml: 0.6 ml of reticulocyte cell-free extract, 1  $\mu\text{mol}$  ATP, 0.2  $\mu\text{mol}$  GTP, 1  $\mu\text{mol}$  2-mercaptoethanol, 10  $\mu\text{mol}$  creatine phosphate, 50  $\mu\text{g}$  creatine phosphokinase, 20  $\mu\text{mol}$  Tris-HCl, pH 7.4, 100  $\mu\text{mol}$  KCl, 1  $\mu\text{mol}$  magnesium acetate, and 0.1  $\mu\text{mol}$  of 19 amino acids, 40  $\mu\text{Ci}$  of [<sup>35</sup>S]methionine was added as only labeled amino acid. The lysate was made mRNA dependent by preincubation with 10  $\mu\text{g/ml}$  micrococcal nuclease in the presence of 1 mM  $\text{CaCl}_2$  for 15 min at 20°C as described by Pelham and Jackson [13]. After the incubation 2 mM ethylene glycol bis( $\beta$ -aminoethyl ether)-N,N'-tetraacetic acid (EGTA) was added to chelate the  $\text{Ca}^{2+}$ . Polyribosomes were added in a concentration of 400  $\mu\text{g/ml}$  (determined spectrophotometrically, assuming an extinction coefficient of 13 at 260 nm).

L-[<sup>35</sup>S]Methionine (specific activity 200 Ci/mmol) was purchased from the Radiochemical Center, Amersham (U.K.). Radioactivity was determined in a Packard liquid Scintillation counter type 2450.

*Incubation and reisolation of lens plasma membranes.* The reticulocyte cell-free incubation was supplemented with purified lens plasma membranes 1 h after the polyribosomes had been added and the total mixture was incubated for an additional hour at 30°C, under occasional stirring. In the incuba-

tion with plasma membranes isolated under cytoskeleton-stabilizing conditions, ionic conditions were adjusted to these concentrations. The membranes were reisolated by centrifugation at  $10\,000 \times g$  and three subsequent washings in bicarbonate or cytoskeleton-stabilizing buffer, respectively.

The plasma membranes isolated in bicarbonate were extracted twice with 6 M urea in bicarbonate buffer. The remaining membrane pellets were dissolved for gel electrophoretic analysis.

**Polyacrylamide gel electrophoresis.** Analyses were performed by sodium dodecyl sulfate polyacrylamide gel electrophoresis according to Laemmli [14] with the modification that a slab gel instead of gel rods was used. The gel was 12 cm long and contained a 7–18% acrylamide gradient, 0.4% methylene-bisacrylamide and 0.1% sodium dodecylsulfate. In this method a stacking gel was applied. Staining and destaining were performed as described by Weber and Osborn [15]. Gels were processed for autoradiography. For the detection of the labeled proteins the procedure of Bonner and Laskey [16] was used in combination with the drying procedure described by Berns and Bloemendal [17]. Two-dimensional gel electrophoresis, using isoelectric focussing in the first dimension was performed according to the method of O'Farrell [18].

## Results

Isolated polyribosomes from lens fiber cells direct preferentially the synthesis of the so-called water-soluble crystallins when added to a reticulocyte cell-free system. Fig. 1A (lane 1) shows the one-dimensional pattern of newly synthesized crystallin polypeptides after electrophoresis in an SDS-polyacrylamide gel. The major components are the  $\alpha$ -crystallin subunits in the 20 000 molecular weight region and two  $\beta$ -crystallin subunits with apparent molecular weight in the 25 000 and 32 000 region. The protein distribution after lens polysome translation has further been verified on two-dimensional gels, using isoelectric focussing in the first dimension (Fig. 2a).

Comparison of the two-dimensional autoradiograph with the stained two-dimensional gel pattern, showing the complete set of soluble lens proteins (Fig. 2c), reveals that  $\alpha A_2$  and  $\alpha B_2$  are newly synthesized in a ratio comparable to the amounts occurring in the native state. Since it was already known that an appreciable amount of  $\alpha$ -crystallin is consistently found in the water-soluble lens fraction (compare Fig. 1B, lanes a–f) we wondered whether the newly synthesized crystallin would be able to interact with the lens fiber membranes. If so, aging of  $\alpha$ -crystallins would not necessarily be a prerequisite for interaction of  $\alpha$ -crystallins with membranous structures (cf. Ref. 6).

Therefore the incubation mixtures comprising lens polysomes and the cell-free lysate were supplemented with purified lens fiber plasma membranes and subjected to prolonged incubation. Thereafter, the membranes were reisolated in two different ways. First under ionic conditions that stabilize the interaction of the cytoskeleton with the plasma membranes. It can be seen from the autoradiograph in Fig. 1A (lane a) that two major radioactive polypeptides occur. Further analysis by two-dimensional electrophoresis shows that the two bands correspond to  $\alpha A_2$  and actin. No  $\alpha B_2$  chain can be observed (Fig. 2b). From the autoradiograph of the control incubation in which no lens polysomes were

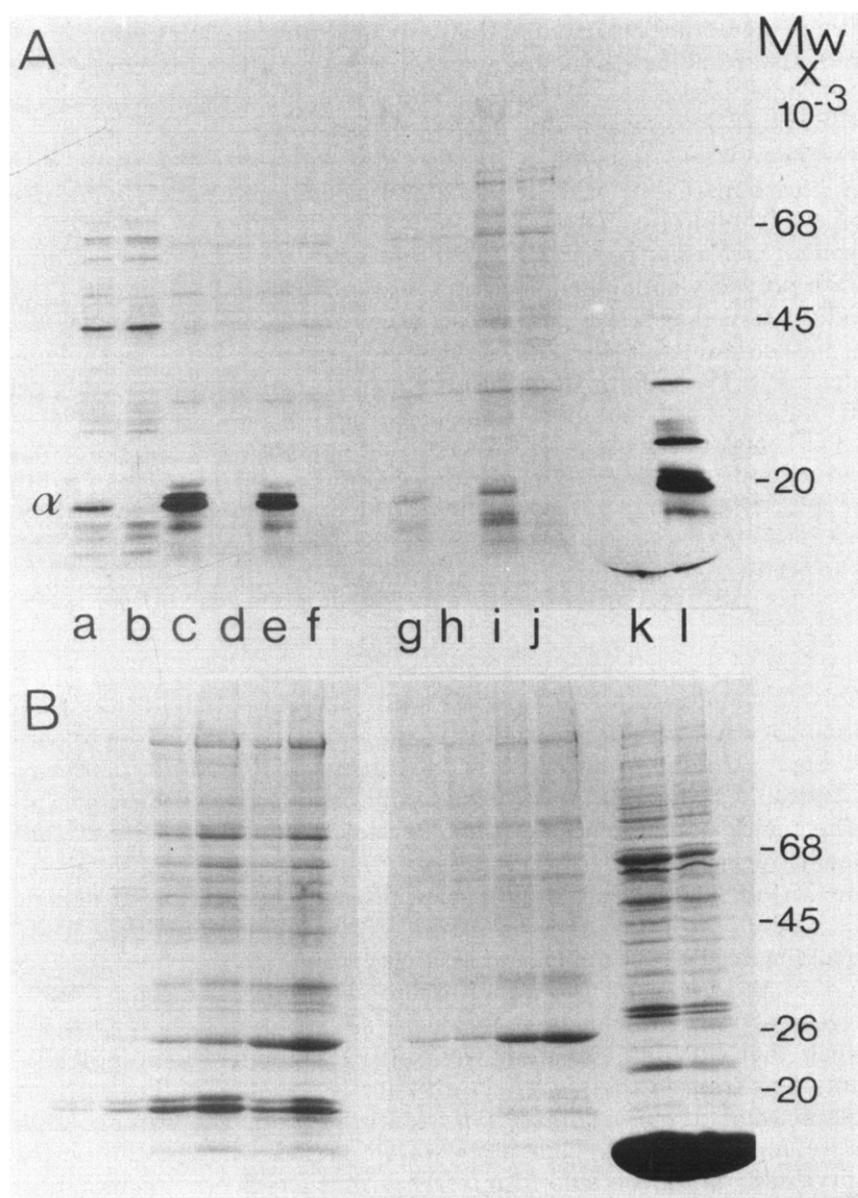


Fig. 1. (A) Autoradiograph of the one-dimensional SDS gel electrophoretic patterns of lens plasma membrane fractions, reisolated after incubation in a reticulocyte lysate in the presence or absence of lens fiber polyribosomes. a, the membranes were isolated under cytoskeleton-stabilizing conditions and collected from discontinuous sucrose gradients at a density of  $1.22\text{--}1.20\text{ g/cm}^3$ ; incubation plus lens fiber polyribosomes. b, membranes isolated as described under a; incubation without lens polyribosomes. c, the membranes were isolated in bicarbonate buffer and collected from a discontinuous sucrose gradient at density  $1.22\text{--}1.20\text{ g/cm}^3$ ; incubation plus lens polyribosomes. d, membranes isolated as described under c; incubation without lens polyribosomes. e, the membranes were isolated in bicarbonate buffer and collected from a discontinuous sucrose gradient at density  $1.20\text{--}1.18\text{ g/cm}^3$ ; incubation plus lens polyribosomes. f, membranes isolated as described under e; incubation without polyribosomes. g–i, pellets remaining after 6 M urea extraction of the membrane fractions in c–f, respectively. k, autoradiograph of the incubation system without addition of lens polyribosomes (control). l, autoradiograph of the newly synthesized crystallins in the reticulocyte lysate under the direction of lens polyribosomes. (B) Stained SDS gel electrophoretic pattern of various reisolated lens fiber plasma membrane fractions. The different lanes correspond to those in (A). Molecular weight markers used in this experiment were:  $\alpha$ -crystallin (20 000); ovalbumin (45 000); bovine serum albumin (68 000);  $\alpha$ ,  $\alpha$ -crystallin.

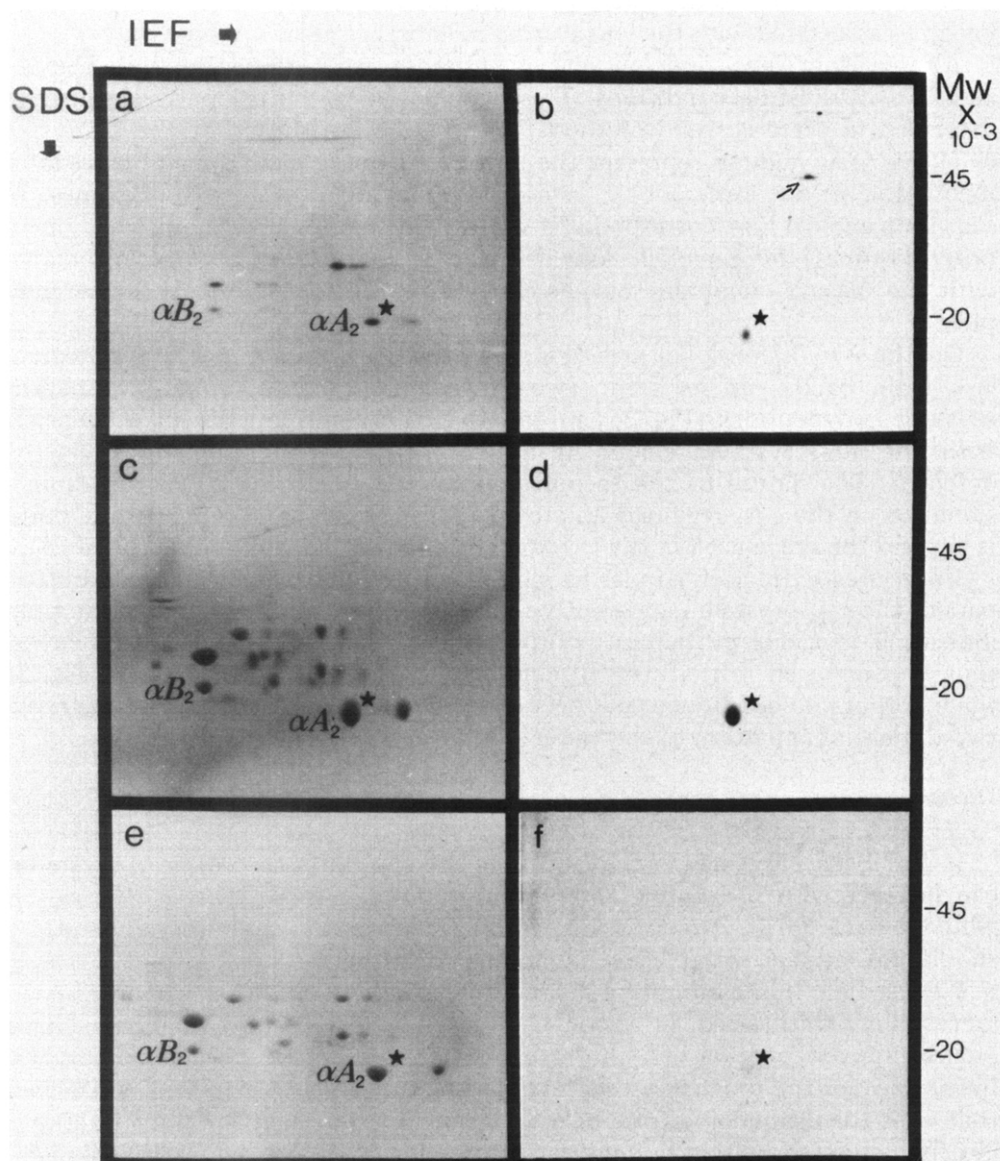


Fig. 2. Two-dimensional gel electrophoretic analysis of soluble lens crystallins and plasma membranes reisolated after incubation in the reticulocyte lysate. a, autoradiograph of newly synthesized polypeptides in the reticulocyte cell-free system under the direction of lens polyribosomes.  $\alpha A_2$  and  $\alpha B_2$  indicate the newly synthesized  $\alpha$ -crystallin chains. (The asterisk in all pictures indicates the  $\alpha A_2$  chain.) b, autoradiograph of plasma membranes incubated in a reticulocyte cell-free system in which lens polyribosomes had been translated. The membranes were reisolated under cytoskeleton-stabilizing conditions. (Arrow indicates actin.) c, stained pattern of native soluble crystallins to which reisolated membranes, isolated in bicarbonate, were added. d, autoradiograph of plasma membranes incubated in the reticulocyte cell-free system in which lens polyribosomes had been translated. The membranes were reisolated in bicarbonate buffer. e, as c, but the membranes were subjected to urea extraction prior to electrophoresis. f, as d, but the membranes were subjected to urea extraction prior to electrophoresis.

added it can be concluded that the reticulocyte lysate synthesizes actin which becomes associated with the lens plasma membranes (Fig. 1A, lane b).

Alternatively lens fiber plasma membranes were reisolated under cytoskeleton-dissociating conditions. This is achieved by bicarbonate washings as described in Materials and Methods. The results are shown in Fig. 1A (lanes c–f). Fig. 1Ac and Ae represent the pattern of the reisolated membranes after incubation in the reticulocyte lysate supplemented with lens polysomes. In Fig. 1Ad and Af the corresponding control incubations (without addition of polyribosomes) are depicted. Also in this case,  $\alpha$ -crystallin becomes associated with the plasma membrane but, as expected, actin is not present in the complex.

On the one-dimensional gels (Fig. 1Ac and Ae), in the  $\alpha$ -crystallin region, two main bands can be seen. Two-dimensional gel electrophoretic analysis of these polypeptides (Fig. 2d) reveals that the main constituent is identical with the  $\alpha A_2$ -crystallin chain. Again, no  $\alpha B_2$ -crystallin polypeptide can be detected. The nature of the second polypeptide, occurring in the one-dimensional gel in the  $\alpha A_2$  region is unknown, but it seems that its isoelectric point is beyond the range used in the two-dimensional gel electrophoresis (pH 5–8).

Upon urea extraction of plasma membranes reisolated in bicarbonate buffer, part of the  $\alpha A_2$ -crystallin apparently remains present in the membrane fraction, indicating that this protein is incorporated into the membrane lipid bilayer, thus becoming an intrinsic membrane protein (Fig. 1A, lanes g–j). In Fig. 2f the incorporated component has been identified as  $\alpha A_2$  by comparison with the two-dimensional pattern of the water-soluble crystallins (Fig. 2e).

## Discussion

It has been suggested previously that the soluble lens protein  $\alpha$ -crystallin can interact with plasma membranes only upon aging [6]. If this observation would reflect the *in vivo* situation, newly synthesized  $\alpha$ -crystallin polypeptides should not interact with plasma membrane constituents.

We verified this assumption by adding isolated lens fiber membranes to  $\alpha$ -crystallin synthesized *in vitro*. Our results clearly show that the major  $\alpha$ -crystallin polypeptide ( $\alpha A_2$ ) interacts preferentially with plasma membranes, irrespective of the procedure used for the isolation of these membranes. The fact that  $\alpha A_1$ , the deamidated form of  $\alpha A_2$ , is not found in the membrane fractions, clearly demonstrates that no maturation or aging process is required. Strikingly, newly synthesized  $\alpha B$ -chains, the other constituents of polymeric native  $\alpha$ -crystallin, are not involved in the complex formation. Similar results have been described for the interaction of  $\alpha$ -crystallin polypeptides with reticulocyte plasma membranes [19].

From the translation patterns of lens polyribosomes in a reticulocyte lysate (Fig. 1A1) it is evident that quantitatively there is a much greater synthesis of  $\alpha$ -crystallin compared to the formation of actin (molecular weight about 45 000). Membranes isolated under cytoskeleton-stabilizing conditions (Fig. 1Aa and Ab) select newly synthesized actin from the incubation mixture. Strikingly, when lens polyribosomes were translated in the incubation,  $\alpha A_2$  becomes incorporated in the membrane-cytoskeletal complex in a concentra-

tion comparable to that of actin. It seems, therefore, that in the interaction between plasma membrane-cytoskeleton and  $\alpha$ -crystallin a stoichiometric relationship does exist, rather than a random distribution of  $\alpha$ -crystallin polypeptides in the complex. Whether these findings have implications for the in situ organization of the 'soluble' lens proteins remains to be established.

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