Classification of Rat Lens Crystallins and Identification of Proteins Encoded by Rat Lens mRNA

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Endogenous rat lens crystallins have been separated by gel filtration into four fractions, α, β′, β, and γ-crystallin. Elution patterns of soluble lens proteins from animals of different ages show a relative decrease of β′ and γ-crystallin during aging. Conversely, the relative amounts of α and β-crystallin are enhanced. The rat crystallin subunits from the four fractions were characterized by one-dimensional and two-dimensional gel electrophoretic techniques. From the results a classification could be derived and a nomenclature for the soluble rat lens proteins is proposed. The products synthesized by rat lens mRNAs in a heterologous cell-free system have also been characterized. Co-electrophoresis of the radioactive products synthesized de novo together with the isolated unlabeled protein fractions on two-dimensional gels shows the relation between primary gene products and their posttranslationally modified derivatives.

Investigations on the total population of rat lens crystallin mRNAs and their translation products urged us to develop a system for the identification of the individual polypeptides. In particular we felt the need for a nomenclature of rat lens crystallins in order to facilitate discussions in recombinant DNA studies. In previous studies on rat lens crystallins [1–8] combinations of gel filtration, sodium dodecyl sulphate/polyacrylamide gel electrophoresis and/or isoelectric focusing have been used for the identification of the soluble rat lens proteins. In this paper we present a classification based upon size fractionation by gel filtration of the four native protein classes, and one-dimensional and two-dimensional gel electrophoresis of their respective subunits or components. We describe the identification of the polypeptides that arise by posttranslational modifications of the primary gene products. The nomenclature that we propose for the rat lens crystallin polypeptides is in accord, at least as far as possible, with the nomenclature introduced for the calf lens crystallins by Kibbelaar and Bloemendal [9,10].

MATERIALS AND METHODS

Animals, Materials and Reagents

Wistar rats of different ages, as specified below, were used and obtained from MDL-TNO (Zeist, The Netherlands). Ultrogel ACA 34 and Sephadex G-200 (fine grade) were purchased from L.K.B. (Bromma, Sweden) and Pharmacia (Uppsala, Sweden), respectively. Oligo(dT)-cellulose (T2) was from Collaborative Research (Waltham, MA, USA) and l-[35S]methionine (spec. act. 500 Ci/mmol) from the Radiochemical Centre (Amersham, UK). Methylmercury hydroxide was bought as a 1 M stock solution from the Ventron Division (Danvers, MA, USA). All manipulations involving polyribosomes and total cytoplasmic RNA were performed under RNase-free conditions. Plastic and glassware were autoclaved or heated for several hours at 220°C. All solutions except detergents, which were directly dissolved in RNase-free water, were treated with 0.05% (v/v) diethylpyrocarbonate (Fluka AG, Buchs, Switzerland) by rigorous shaking at room temperature followed by extensive boiling.

Isolation and Fractionation of Rat Lens Proteins

Animals, between 30 and 180 days in age, were used. Lenses were dissected from rat eyes, decapsulated and stirred for 3 h at 4°C in a buffer containing 50 mM Tris/HCl at pH 7.5, 50 mM NaCl and 1 mM EDTA. The 5000 x g supernatant of this suspension contained the soluble lens proteins. Fractionation of these proteins into four fractions was achieved by gel filtration either on Sephadex G-200 or Ultrogel ACA34 at room temperature with the isolation medium as running buffer. Peak fractions were extensively dialyzed at 4°C against twice-distilled water and lyophilized. One part of each fraction was dissolved in gel application buffer containing 60 mM Tris/HCl at pH 6.8, 2.3% dodecyl sulphate, 5% 2-mercaptoethanol, 10% glycerol and Bromophenol blue as a marker dye. Samples were stored at –20°C.

One-Dimensional and Two-Dimensional Gel Electrophoresis

One-dimensional gel electrophoresis in dodecyl sulphate-containing polyacrylamide gels was performed according to Laemmli [11] with the modification that slab gels instead of gel rods were used. The gels were 12 cm long and contained 13% acrylamide, 0.4% bisacrylamide and 0.1% sodium dodecyl sulphate. In this method a stacking gel was applied, while staining with Coomassie brilliant blue and destaining were performed as described by Weber and Osborn [12]. Two-dimensional gel electrophoresis, using isoelectric focusing in 9 M urea in the first dimension and dodecyl sulphate slab gels as second dimension, was carried out as described by O’Farrell [13]. Radioactive polypeptide bands or spots were visualized by fluorography [14] in combination with the drying procedure described by Berns and Bloemendal [15].
Isolation of Rat Lens Polyribosomes

Decapsulated lenses from three-month-old rats were homogenized at 4 °C in a buffer containing 50 mM Tris/HCl at pH 7.4, 25 mM KCl and 5 mM MgCl₂. The 5000 x g supernatant from this homogenate was brought to a final concentration of 0.25% (v/v) sodium deoxycholate and 0.25% (v/v) Nonidet P40 and centrifuged through a cushion of 2 M sucrose in the homogenization buffer at 4 °C and 78000 x g for 16 h. The polyribosome pellet was resuspended in water and stored at −80 °C.

Isolation, Fractionation and Characterization
of Rat Lens mRNAs

Total cytoplasmic RNA from three-day-old rat lenses was prepared essentially by the method of Palmiter [16]. Briefly, whole lenses were homogenized with a loose-fitting Dounce apparatus in a tenfold excess of ice-cold hypotonic buffer containing 25 mM Tris/HCl at pH 7.5, 25 mM NaCl, 5 mM MgCl₂ and 2% (v/v) Triton X-100. After lysis at 0 °C for 10 min the homogenate was centrifuged at 0 °C, 12000 x g for 10 min. The supernatant was stored on ice and the pellet was thoroughly washed with the hypotonic buffer by syringing through small-gauge needles and centrifuged as above. After a second wash of the pellet the combined supernatants were brought to a final concentration of 1 mg/ml heparin and 100 mM MgCl₂ and kept on ice for 60 min. The precipitate was collected through a cushion of 0.2 M sucrose in hypotonic buffer without Triton X-100 by centrifugation at 0 °C, 20000 x g for 30 min. Magnesium ions were removed by dissolving the pellet in 50 mM Tris/HCl at pH 7.5, 50 mM 1,2-cyclohexylenedinitrilotetraacetic acid monohydrate, 0.25% dodecyl sulphate, followed by ethanol precipitation overnight at −20 °C. The nucleic acid pellet was recovered by centrifugation and directly applied to oligo(dT) cellulose affinity chromatography [17]. The rat lens poly(A)-containing RNA thus obtained was fractionated on a 5–29% (w/w) isokinetic sucrose density gradient after denaturation with 10 mM methylmercury hydroxide as described by Dodemont et al. [18]. The sucrose gradient was divided into 0.4-ml fractions and protein-synthesizing activity in each fraction was monitored in two different ways in a nuclelease-treated rabbit reticulocyte lysate [19]. Either a 1-μl aliquot from each fraction was directly assayed in the cell-free system or equal amounts of mRNA throughout the gradient were subjected to translation in vitro. In the latter case variable volumetric amounts of mRNA were ethanol precipitated in the presence of 10 μg tRNA from Escherichia coli. The pellets were washed twice with 75% ethanol, dried, resuspended into 1 μl water and finally assayed. Translation products were analyzed on dodecyl sulphate/polyacrylamide gels as described above.

RESULTS

Gel Filtration

In order to fractionate the individual crystallin sub-classes we used gel filtration on Sephadex G-200 or Ultrogel ACA 34 columns. The resulting separation patterns are shown in Fig. 1. In the four panels the soluble native crystallins are presented from one, two, three and six-month-old rat lenses respectively. The most striking feature of these distribution patterns is the decrease of both β₂ and γ-crystallin with increasing age and concomitantly the drastic increase in β₁-crystallin, which becomes the major fraction. We did not attempt to achieve complete separation between the high-molecular-weight α-crystallin (αHM) and the α-crystallin fractions, which emerge together with the void volume. From previous experiments it was known that qualitatively both fractions comprise only α-crystallin subunits [4]. A one-dimensional electropherogram of the polypeptides present in each of the four native crystallin fractions is shown in Fig. 2. In lane b the three types of rat α-crystallin subunits αA, αB and αAM, present in the combined α and αM-crystallin peaks, are indicated. Polypeptides of the β and/or γ-crystallin family do not occur here. The main differences between β₁ and β₂-crystallin (lanes c and d) are represented by a β₁-specific band in the 30-kDa region and some quantitative differences, especially obvious for βBp, which is the major band in the β₁ pattern. The γ-crystallin fraction (lane e) results, when analyzed on a dodecyl sulphate/polyacrylamide gel, in a rather diffuse pattern with some more or less distinct polypeptide bands, which are all located in the 17–22-kDa range. A comparison of the rat lens crystallin fractions with the complete mixture of calf lens crystallins (lane f) and bovine α-crystallin in the marker slot (lane a) shows that, although the rat α chain is equal in size to the calf α chain [20], the apparent molecular weight of αA-crystallin from rat lens as deduced from this gel is markedly lower (see also [4–6]). Also the β₁-specific component from rat lens (βB₁; asterisk) has a molecular weight which is apparently lower than the corresponding β₂-specific bands from the calf (named βB₁ and βB₂; arrow heads; see [21,22]). This does, however, not necessarily exclude the possibility that the actual lengths of the β₁-specific polypeptides from calf and rat are similar. It is known that the differences in gel-electro-
phoretic behavior of the αA chains from rat and calf can be explained by minor differences between the amino acid sequences [20]. This may also be true for calf and rat βB1 sequences.

Two-Dimensional Gel Electrophoresis

Fig. 3A—D shows two-dimensional gel-electrophoretic analyses of the α, β1, β1, and γ-crystallin fractions, respectively, from three-month-old rat lenses. On the basis of these patterns we propose a nomenclature for the rat lens crystallins, based upon the following criteria:

a) The polypeptide chains (or groups of chains) are designated α, β, or γ after their occurrence under the respective gel chromatographic peaks, αLM, α, β(High) and β(Low), and γ.

b) The α and β-crystallin chains are subsequently named A (acidic) or B (basic) according to their isoelectric points. The division between acidic and basic polypeptides is fixed between βB1 and αA. For the γ-crystallins such a subdivision is not feasible and therefore not introduced, since all γs have basic isoelectric points.

c) The polypeptides are further identified according to their isoelectric points, the lowest number representing the most basic component. When more components have identical isoelectric points the one with the highest molecular weight has been given the lowest number. One exception has to be made in this respect in order to avoid confusion in the literature. Since the α-crystallin subunits from rat have extensively been described [4—7], we adapt the nomenclature for these polypeptides as suggested by Cohen et al. [5]. The components will, therefore, be named αA, αB and αA

d) Posttranslationally modified polypeptides of known identity will be named after the primary gene product from which they are derived. The primary βB1 product for example will be marked a, while its derivative is marked b. Also here we must make an exception for the α-crystallin constituents. Primary gene products are called αA, αA and αB, while their well-established deamidation products are named αA and αB.

For the nomenclature based on these criteria we refer to Fig. 3A—D. Earlier studies in our laboratory [6] revealed the occurrence of a genetically highly interesting α-type chain in rodent α-crystallin, named αA. This latter component has been shown to be identical to αA except for an insertion of 22 amino acids in internal position between residues 63 and 64 of the αA chain. The localization of this additional chain relative to αA and αB could easily be indicated on the electropherogram shown in Fig. 3A since the electrophoretic
behavior of this polypeptide both on isoelectric focusing and dodecyl sulphate-containing gels was known [4]. There are also a few weaker spots, which have not been identified hitherto. They may well represent shortened and/or deamidated polypeptides, similar to such chains detected in bovine lens [9, 23]. The fact that the latter components are not found among newly synthesized α-crystallin (see below) supports the assumption that they are posttranslationally modified. The electrophoretic pattern of the β-crystallins is much more complicated. About ten components can be observed, both in the βH (Fig. 3 B) and in the βL-crystallin class (Fig. 3 C). Qualitatively the differences between the two patterns are not very pronounced. As in many mammals [21] the rat lens βH fraction is characterized by two highly basic chains designated β1A and β1B1, respectively (cf. [9, 10, 18, 22, 24]). With regard to these polypeptides the βH pattern differs remarkably from the βL pattern in that the β1A polypeptide is only present in a trace amount in βL-crystallin. Furthermore, βB2 (which may be comparable to βBP in the calf lens system) is obviously the main component in the βL-crystallin fraction. Similar to the calf β-crystallin distribution pattern, acidic chains are found which have been named rat lens βA1, βA2 and βA3, respectively.

The components found in the low-molecular-weight crystallin fraction have been numbered consecutively γ1-γ7 (Fig. 3 D). Much work has still to be done in order to improve the characterization of the individual polypeptides of this group. Sequencing of cloned γ-crystallin-specific cDNAs derived from the corresponding mRNAs [18] has revealed already that one γ-crystallin polypeptide corresponds, to a large extent, to the γ1 fraction from the calf [25, 26], whereas another chain is similar in sequence, but by no means identical [35]. This means that the two proteins represent different γ-gene products.

Polypeptides Encoded by Rat Lens mRNA

The classification described was used for the identification of the products of cell-free translation directed by isolated rat lens polyribosomes and mRNAs. From these experiments we could, to a certain extent, also distinguish between primary products of translation and their posttranslational derivatives. In initial experiments rat lens polyribosomes were assayed for cell-free translation and the resulting translation products analysed on one-dimensional gels. These studies did, however, not give conclusive data on the primary gene products in quantitative as well as in qualitative respect because of overcrowding of products in similar molecular weight ranges (data not shown). Therefore, total rat lens mRNA was fractionated on an isokinetic sucrose density gradient after denaturation with dimethylmercury hydroxide [18]. Fig. 4 shows a typical example of the gradient profile obtained. Fractionated mRNA was translated in two different ways. Either a 1-μl aliquot of each individual gradient fraction was directly assayed for translation in vitro, irrespective of the amount of mRNA present, or in the other approach equal amounts of mRNA, recovered by ethanol precipitation from the gradient fractions, were analyzed. The first method is merely a confirmation of the high mRNA resolution (cf. Fig. 2 in [18]). We observed that the 10-S peak contains α2m mRNA, whereas the mRNA coding for the α2m subunits is present in the 14-S peak. In the 16-18-S range of the gradient mRNAs sediment that code for proteins which comigrate with the established cytoskeletal proteins derived from calf lens, namely actin, tubulin and vimentin [27-29] as well as some other yet unidentified proteins.

The second method, based upon translation of equal amounts of mRNA, may provide a better insight into the relative abundance of all possible in vitro translation products actually encoded by the mRNAs in each gradient fraction (Fig. 4 B). As a result γ-crystallin-synthesizing capacity is detected in the 16-18-S peak region. This is obscured when mRNAs were assayed for translation in vitro according to the first procedure. An explanation for this phenomenon may be found in the fact that the γ-crystallin mRNAs which actually appear in the 10-S region of the gradient [18] are
so abundant that they trail in a shallow curve towards higher sedimentation values. Moreover they have proven to be extremely active templates in protein biosynthesis (Dodemont, unpublished) as compared to the cytoskeletal mRNAs, which are present in the 16–18 S range [30, 31].

The final identification of the newly synthesized rat lens crystallin polypeptides was achieved by two-dimensional gel electrophoresis. By co-electrophoresis of the radioactive products with the unlabeled proteins present in the native $z$, $\beta_h$, $\gamma_h$ and $\gamma$-crystallin classes we were able to identify those crystallin constituents which are primary gene products. Fig.5 shows a typical two-dimensional gel pattern of the total mixture of newly synthesized rat lens polypeptides under the direction of polyribosomes from three-month-old rat lenses in a reticulocyte lysate. Certain chains are absent or almost below detection level as compared to control crystallin gel patterns. Therefore, it is assumed that all $\gamma$-crystallin polypeptides except $\gamma_4$ crystallin are primary gene products. Also $\beta_{1b}$, $\beta_{4a}$, $\beta_{5}$ and $\beta_{A2}$ as well as $\beta_{B2}$, $\beta_{A2}$ and $\alpha_{A1}$ are clearly newly synthesized. This result was confirmed upon translation of partially purified poly(A)$^+$ RNA from rat lens (not shown).

Polypeptides probably arising by posttranslational modifications comprise $\alpha_{A1}$, $\alpha_{B1}$, $\beta_{B1b}$ (present in a minute amount in the gel in Fig. 5), $\beta_{B3}$, $\beta_{B2}$, $\beta_{A3}$ (also hardly detectable) and $\gamma_4$. We cannot conclude from our data whether or not $\beta_{A3}$ is a product arisen by posttranslational modification since on the 2D-gel this polypeptide comigrates exactly with $\alpha_{A1}$ (compare Fig. 3B and 5).

**DISCUSSION**

The availability of the two-dimensional gel electrophoretic patterns of the water-soluble rat lens crystallins shown in this study is not only a prerequisite for the identification of cloned rat crystallin cDNAs, described recently [18], but is also extremely useful in aging studies of rat lens proteins. Although the established classification of calf lens crystallins [9, 10] is of some help for the identification of spots on rat lens crystallin two-dimensional gel patterns, a number of obvious differences have been demonstrated. In particular the abundance of $\gamma$-crystallin components in young rat lenses is striking. From the gel filtration patterns in Fig.1 it appears that in young rat lenses (3 months old) $\gamma$-crystallin is the major fraction. This is in agreement with our finding that polyribosomes from 3-month-old lenses, as well as mRNAs from newborn rat lenses, direct the synthesis of high amounts of $\gamma$-crystallin polypeptides in a reticulocyte lysate. The closely similar molecular weights of the $\gamma$-crystallins further explain why these polypeptides appear as a smear in the one-dimensional gels.

During aging a decrease in the $\gamma$-crystallin peak is seen on the protein distribution pattern after gel filtration. On the other hand the amount of $\beta_h$-crystallin increases upon aging, apparently at cost of a decrease of the $\beta_{B1b}$-crystallin peak. In calf lens the opposite effect is seen. There, the $\beta_h$ peak decreases upon aging, while a small increase in the $\beta_h$ fraction is observed [32]. Concomitantly, $\beta_{B1b}$ (which in calf lens is a $\beta_{B1b}$-specific component) is gradually modified posttranslationally into $\beta_{B1b}$. Verma et al. [24] have suggested that $\beta_{B1b}$, therefore, may be necessary for the formation of $\beta_h$ aggregates. In the rat lens both $\beta_{B1b}$ and $\beta_{B1b}$ occur in $\beta_h$ (Fig.3B), while $\beta_{B1b}$ also occurs in $\beta_{B1}$ (Fig. 3C). On the basis of this striking result it is tempting to speculate about the role of $\beta_{B1b}$ in the rat lens system in the formation of $\beta_h$ aggregates, but a general model for the assembly of $\beta$-crystallin aggregates and the possible interconversion of $\beta_h$ and $\beta_{B1b}$ cannot be put forward yet.

By comparison of two-dimensional gel patterns of crystallin polypeptides present in the rat lens with their de novo synthesized counterparts, primary gene products could be distinguished from posttranslationally modified derivatives. Those products that occur in vivo in appreciable amounts but are lacking completely, or are present only in minute amounts, upon translation of rat lens polyribosomes are denoted as posttranslational modifications. Since methionine is present in all crystallins we presume that all newly synthesized polypeptides are visualized in our experiments.

The exact nature of posttranslational modifications of the newly synthesized products is still unclear. As can be seen in Fig. 5, rapid modifications also seem to occur in the reticulocyte cell-free system, since minute amounts of $\beta_{B1b}$ and $\beta_{A3}$ can already be detected after 1 h of incubation. Proteolytic activity in the reticulocyte lysate may be responsible for the observed modifications, i.e. changes in size and charge. For calf lens $\beta_{B1b}$ such a modification has been suggested [33]. Next to limited proteolysis, modifications such as deamidation may occur, while acetylation may be incomplete. This will result in the formation of more or less acidic polypeptides respectively.

The nomenclature described above should be considered merely as a guide to enable easy communication between workers in the field of rat lens crystallins, but may not be, a priori, applicable to other species (cf. Berbers et al. [34]). Only a thorough study of crystallin-specific cDNA sequences and their corresponding genes will provide a definite answer about the exact identity of the individual polypeptides described and their interrelationships.

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