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Heterogeneity of δ-Crystallins of the Embryonic Mallard Lens. Correlation between Subunit Compositions and Isoelectric Points†

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ABSTRACT: δ-Crystallins from the lenses of embryonic mallards (*Anas platyrhynchos*) were analyzed with respect to native and subunit molecular weight, subunit composition, and isoelectric point. $NaDodSO_4$–urea–polyacrylamide gel electrophoresis showed that unfractionated mallard δ-crystallins are composed of approximately equal amounts of subunits with molecular weights near 47 000 and 48 000. Agarose gel chromatography showed that the embryonic mallard δ-crystallins have native molecular weights slightly less than 200 000. Thus, embryonic mallard δ-crystallins appear to be tetramers. Five major and nine minor δ-crystallins were resolved by isoelectric focusing. The five predominant δ-crystallins each cross-reacted with antichick δ-crystallin antiserum, and each had a different proportion of the larger and smaller subunits, indicating a direct relationship between the isoelectric point and the subunit composition. The presence of numerous, minor species of native δ-crystallins with different isoelectric points suggested that the subunits possess charge heterogeneity as well as size heterogeneity.

δ-Crystallin, also called F.I.S.C. (first important soluble crystallin), is one of the major structural proteins of avian and reptilian lenses [see Clayton (1974) for a review]. Most of our knowledge of δ-crystallin comes from investigations on the chicken lens, where it was first discovered (Rabaey, 1962). Embryonic chick lenses are a particularly good source of δ-crystallin, since they are easily obtained and δ-crystallin represents 60–80% of the soluble protein present in the lens (Rabaey, 1962; Genis-Galvez et al., 1968; Yoshida & Katoh, 1971; Piatigorsky et al., 1972).

One important, unexplained finding concerning δ-crystallins of chickens is that the native proteins can be resolved into at least seven species by isoelectric focusing in a polyacrylamide gel (Bours & van Doorenmaalen, 1970; Brahma & van der Starre, 1975) or in free solution (Bours, 1976). The isoelectric points of the components are clustered between a pH of approximately 5.05 and 5.34, with the different forms being separated from each other by only 0.02–0.06 of a pH unit (Bours, 1974, 1976). It is not known if the difference in the isoelectric points of the different δ-crystallins of the chicken lens involves differences in conformation and/or differences in subunit composition. The second possibility is supported

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by the fact that δ-crystallins are composed of four subunits (Piatigorsky et al., 1974) which appear heterogeneous with respect to size (Reszelbach et al., 1977) and charge (Clayton, 1969, 1970; Truman et al., 1971, 1972; Thomson et al., 1978).

Recently, we have provided evidence that there is a relationship between the isoelectric point and the subunit composition of the δ-crystallins from the embryonic chick lens (Piatigorsky, 1978). It was shown that, in cultured lenses, newly synthesized δ-crystallins with higher isoelectric points had a greater proportion of higher molecular weight subunits (about 50 000) than of lower molecular weight subunits (about 48 000). This demonstration depended upon our ability to change the ratio of synthesis of the polypeptides comprising the two sizes of the δ-crystallin subunits (Piatigorsky & Shinohara, 1977) and to produce a group of δ-crystallins with atypically high isoelectric points (Piatigorsky, 1978). It is much more difficult to demonstrate a direct relationship between the isoelectric point and the subunit composition of native δ-crystallins in the naturally occurring pool of δ-crystallins in the chicken lens. Many of the δ-crystallins are present in very small amounts, their isoelectric points are tightly clustered within a narrow range of pH (Bours, 1974, 1976), and the unfractionated proteins have considerably fewer of the larger subunits than of the smaller subunits (Reszelbach et al., 1977). The unequal amounts of the two size classes of the δ-crystallin subunits increase the difficulty of quantitating differences in their mass ratio within different proteins.

During the course of a comparative study to be reported elsewhere (Williams & Piatigorsky, unpublished experiments), we found that unfractionated δ-crystallins from the embryonic mallard lens, in contrast to those from the chick lens, are resolved by $NaDodSO_4$–urea–polyacrylamide gel electrophoresis into two bands containing approximately equal amounts of protein. We thus thought that the mallard might be an advantageous organism for investigating the possibility that the different isoelectric points of δ-crystallins reflect differences in their subunit composition. In the present report we show that embryonic mallard δ-crystallins, like chicken δ-crystallins, are an array of proteins with different isoelectric points, that the different mallard δ-crystallins are more highly resolved than the chicken δ-crystallins by isoelectric focusing, and that there is indeed a direct relationship between the isoelectric point and the subunit composition of the different δ-crystallins.

Materials and Methods

Fertile eggs of mallards (*Anas platyrhynchos*) were obtained from the National Zoo (Washington, DC) and were incubated for 15–19 days in a forced draft, humidifed incubator at 37 °C. Lenses were removed with jeweler's forceps and placed into ground-glass homogenizing tubes containing Ham's F-10 medium (Ham, 1963). The lenses were homogenized in 0.01 M sodium phosphate, pH 6.8, and centrifuged at 10 000 rpm at 4 °C for 10 min. The supernatant fraction was adjusted to pH 5.0 with acetic acid, supplemented with ethanol to a final concentration of 10%, set at 4 °C for 20 min, and centrifuged at 10 000 rpm at 4 °C for 10 min. The resulting supernatant fraction (the pH 5 soluble fraction) contained the δ-crystallin.

The pH 5 soluble fraction was isoelectrically focused for 3 days at 4 °C in a sucrose density gradient between pH 3 and 10 (LKB ampholytes) in an LKB 110-mL column according to the manufacturer's instructions. The pH and optical density at 280 nm were determined in 1-mL fractions, which were collected with a peristaltic pump. The δ-crystallin fractions were dialyzed against deionized water at 4 °C before being analyzed further. Isoelectric focusing was also performed in a 10% polyacrylamide gel between pH 4 and 6 as described elsewhere (Piatigorsky, 1976). Ampholines were removed by soaking the gel overnight in 2% (w/v) sulfosalicylic acid, 11% (w/v) trichloroacetic acid, and 27% (v/v) methanol in water (Söderholm et al., 1972). The gel was stained in 0.1% Coomassie brilliant blue R (Schwarz/Mann) in 50% methanol and 7% acetic acid and destained in 8.5% acetic acid and 27% ethanol.

Discontinuous electrophoresis was conducted in 10% polyacrylamide gel slabs containing 0.1% $NaDodSO_4$ and 8 M urea (Schwarz/Mann) as given previously (Reszelbach et al., 1977). The δ-crystallin samples were dissolved in 1% $NaDodSO_4$, 4 M urea, 1% 2-mercaptoethanol, 0.6% Tris-HCl, pH 6-8, and 10% glycerol and heated to 100 °C for 2–3 min before electrophoresis in order to eliminate any possible disulfide bonds.

Double immunodiffusion was performed on Ouchterlony plates (Hyland Division Travenol Laboratories, Inc.). Anti-δ-crystallin sheep antiserum was prepared against δ-crystallin which was purified by isoelectric focusing from 15-day-old embryonic chick lenses (Piatigorsky et al., 1974). Immunodiffusion took place at room temperature for 24 h. The Ouchterlony plates were washed with 0.15 M NaCl at room temperature for 1 week, stained with 0.1% Coomassie brilliant blue R in 50% ethanol and 7% acetic acid for 4 h at room temperature, and destained with 7% acetic acid and 5% methanol for 1–2 days.

Agarose gel chromatography was performed with Bio-Gel A-1.5 M, 200–400 mesh (Bio-Rad Laboratories), in a glass column (1 × 45 cm) at room temperature. The elution buffer was 0.15 M NaCl and 0.05 M Tris-HCl, pH 7.5. Twenty-drop fractions (0.55 mL) were collected every 5–6 min by gravity, diluted to 2 mL with water, and assayed for fluorescence. Excitation was at 280 nm and emission was at 350 nm. [^{35}S]Methionine-labeled δ-crystallin (1 μg) purified by isoelectric focusing (Piatigorsky et al., 1974) from 6-day-old embryonic chick lenses was added to the sample before chromatography as an internal marker; its location within the fractions was determined by assaying 50-μL aliquots by scintillation counting.

Results

Subunit and Native Molecular Weights of δ-Crystallin. The subunits of δ-crystallin from embryonic mallard lenses were compared with those from embryonic chick lenses by electrophoresis in a polyacrylamide gel slab containing $NaDodSO_4$ and urea. As expected under these conditions (Reszelbach et al., 1977), embryonic chick δ-crystallin gave two bands with molecular weights of approximately 48 000 and 50 000 in a ratio of about 1 to 3 in favor of the smaller species (Figure 1A). Embryonic mallard δ-crystallin also gave two bands in this gel (Figure 1B). In contrast to the chick δ-crystallin polypeptides, the mallard δ-crystallin polypeptides had molecular weights of approximately 47 000 and 48 000 and were present in nearly equal amounts. The same results were obtained whether or not the δ-crystallins were reduced at 100 °C in 1% 2-mercaptoethanol before electrophoresis.

The native molecular weight of δ-crystallin from the embryonic mallard lens was also compared with that from the chick lens by gel filtration on agarose beads. An internal marker of [^{35}S]methionine-labeled δ-crystallin from the embryonic chick lens was cochromatographed with the mallard δ-crystallin, since it is known that chick δ-crystallin has a native molecular weight of approximately 200 000 (Piatigorsky et al., 1974). The peak of the mallard δ-crystallin was very ho-

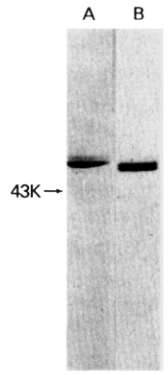

FIGURE 1: $NaDodSO_4$–urea–polyacrylamide gel electrophoresis of δ-crystallin from embryonic chick (A) and mallard (B) lenses. Approximately 5 μg of protein from the pH 5 soluble fraction of each preparation was examined. Ovalbumin was subjected to electrophoresis in a parallel slot and served as the 43 000 molecular weight marker. The two bands of chick δ-crystallin are about 50 000 and 48 000 (Reszelbach et al., 1977).

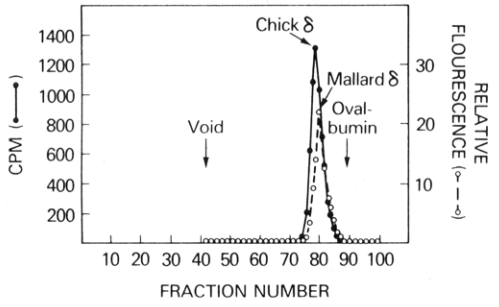

FIGURE 2: Agarose gel chromatography of δ-crystallins from embryonic chick and mallard lenses. Approximately 1 μg of [^{35}S]-methionine-labeled δ-crystallin from 6-day-old chick embryos was cochromatographed with the mallard δ-crystallin. Mallard δ-crystallins were pooled from fractions 1 to 5 shown in Figure 3. The labeled chick δ-crystallin was not present in enough quantity to give detectable fluorescence. Ovalbumin was chromatographed separately. The void volume was determined with Dextran blue.

mogeneous and eluted just one fraction after the peak of the chick δ-crystallins, indicating that the δ-crystallins from the embryonic mallard lenses are polymeric proteins (Figure 2). No mallard protein eluted with a molecular weight comparable to ovalbumin which would have represented monomeric δ-crystallins. Although the resolution of this column was limited, the gel filtration pattern is consistent with the possibility that the mallard δ-crystallins, like the chick δ-crystallins (Piatigorsky et al., 1974), are composed of four polypeptides. The fact that the δ-crystallins from the embryonic mallard lens are slightly smaller than those from the embryonic chick lens is also consistent with the finding that δ-crystallin subunits of the mallard are slightly smaller than those of the chick.

Isoelectric Points of Native δ-Crystallins. Native δ-crystallin of the embryonic mallard lenses was isoelectrically focused in a sucrose density gradient. A broad distribution of partially resolved proteins was observed between pH 5 and 5.8 (Figure 3). These results indicate that embryonic mallard δ-crystallins have a broader range of isoelectric points than do embryonic chick δ-crystallins, which show a much more uniform peak of absorption at 280 nm with an isoelectric point near pH 5.1 (Craig & Piatigorsky, 1973). Five fractions, as indicated in Figure 3, were selected for further analysis (see below).

Embryonic mallard δ-crystallins were also examined by isoelectric focusing in a polyacrylamide gel containing ampholines with a pH distribution between 4 and 6 (Figure 4, total). Five major bands (denoted by the arrows) were found

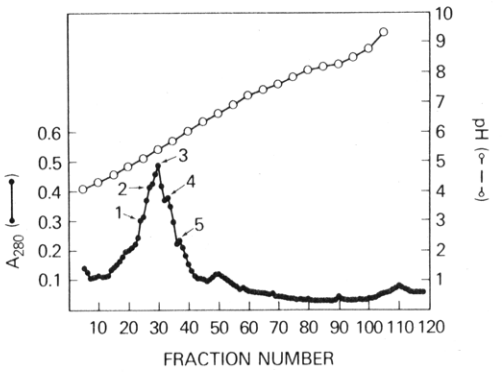

FIGURE 3: Isoelectric focusing of embryonic mallard δ-crystallins in a sucrose density gradient. Approximately 5 A_{280} units of the dialyzed pH 5 soluble fraction was examined.

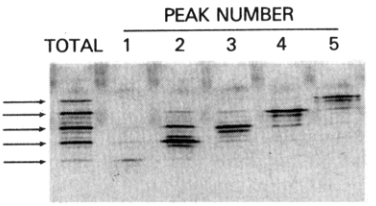

FIGURE 4: Isoelectric focusing of embryonic mallard δ-crystallins in a polyacrylamide gel slab containing ampholines in a pH range of 4–6. Approximately 9.4 μg of protein from the pH 5 soluble fraction was examined in the total (unfractionated) preparation. Protein (2–5 μg) from fractions 1 to 5 of Figure 3 was examined in the fractionated δ-crystallins. The arrows point to the five major species of δ-crystallins. The top represents the less acidic and the bottom represents the more acidic region of the gel. The bottom two-thirds of the gel did not contain protein and was cut off the photograph.

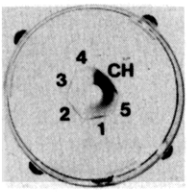

FIGURE 5: Immunodiffusion of the protein in the regions designated 1–5 in Figure 3. Sheep antiserum was prepared against 15-day-old embryonic chick δ-crystallin purified by isoelectric focusing. CH is 15-day-old embryonic chick δ-crystallin. Note the similarity between the precipitin lines of the mallard and the chick δ-crystallins. Thirty microliters of the following preparations was placed in the wells: chick, A_{280} = 0.25; mallard 1, A_{280} = 0.12; mallard 2, A_{280} = 0.168; mallard 3, A_{280} = 0.194; mallard 4, A_{280} = 0.152; mallard 5, A_{280} = 0.094.

in the top third of the gel, indicating that their isoelectric points were comparable to those observed in the sucrose density gradient. In addition to the predominant bands of protein, there were at least nine minor bands of protein visible in the stained gel. The mallard δ-crystallins thus have numerous isoelectric forms in a polyacrylamide gel, as do the chick δ-crystallins (Bours & van Doorenmaalen, 1970; Bours, 1976), except that the resolution of the former is greater than that of the latter.

The five fractions of protein isolated by isoelectric focusing in a sucrose density gradient (Figure 3) were refocused in a polyacrylamide gel in order to confirm their differences in isoelectric point and to obtain a measure of their purity. The stained polyacrylamide gel presented in Figure 4 shows that the five fractions of mallard δ-crystallins represent the five, partially purified, major bands of protein with different isoelectric points.

Immunodiffusion of δ-Crystallins with Different Isoelectric Points. In order to establish that the fractions of mallard lens protein examined above were indeed δ-crystallins, they were

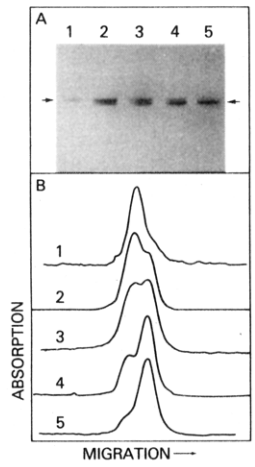

FIGURE 6: $NaDodSO_4$–urea–polyacrylamide gel electrophoresis of isoelectrically focused δ-crystallins from embryonic mallard lenses. (A) Staining pattern of 1–4 μg of δ-crystallins from the regions designated 1–5 in Figure 3. The arrow on the left points to the higher molecular weight band and the arrow on the right points to the lower molecular weight band. (B) Scans of the stained polyacrylamide gel.

analyzed by immunodiffusion against anti-δ-crystallin sheep antiserum prepared from purified δ-crystallin isolated from the embryonic chick lens. The results are presented in Figure 5. Each of the five fractions of mallard protein obtained from the sucrose density gradient showed a precipitin band at the same relative position on the Ouchterlony plate as did the purified δ-crystallin from the embryonic chick lens. The precipitin band obtained from the embryonic chick δ-crystallin is darker than those obtained from the mallard δ-crystallins because more protein was placed in the well.

Subunit Composition of δ-Crystallins with Different Isoelectric Points. The five fractions of mallard lens protein obtained by isoelectric focusing in a sucrose density gradient were examined by $NaDodSO_4$–polyacrylamide gel electrophoresis. The stained gel slab showed that fraction 1 was composed almost entirely of the higher molecular weight polypeptides and fraction 5 was composed almost entirely of the lower molecular weight polypeptides (Figure 6A). By contrast, fractions 2, 3, and 4 consisted of both bands of protein. In order to estimate the relative amounts of protein in the two bands of δ-crystallin, the stained gels were scanned (Figure 6B). The ratios of protein in the higher molecular weight band to that in the lower molecular weight band in fractions 2, 3, and 4 were obtained by integrating the areas under the peaks of the scans. This calculation was performed by an integrator built into the scanner. The ratios were 2.5:1, 1:1, and 1:3 for fractions 2, 3, and 4, respectively. This result is clearly an approximation in view of the overlap of the curves of the two bands obtained in the scans.

Discussion

Comparative studies have shown that δ-crystallin is present among many different orders of birds (see Clayton, 1974), including mallards (Rabaey, 1962, 1965). The δ-crystallins of different species of birds cross-react with antiserum directed against δ-crystallin of the embryonic chick lens and thus share antigenic determinants. In the present experiments we have demonstrated that embryonic δ-crystallin of mallards (which belong to the order Anseriformes) has many similarities with embryonic δ-crystallin of chicks (which belong to the order Galliformes). Both δ-crystallins appear to be tetrameric proteins with generally similar, although not identical, native and subunit molecular weights, both are resolved into two polypeptide bands by $NaDodSO_4$–urea–polyacrylamide gel electrophoresis, both consist of a spectrum of proteins with slightly different isoelectric points between pH 5 and 6, and both are soluble at their isoelectric point.

The present results show that the different isoelectric points of the mallard δ-crystallins are directly related to their subunit composition. The five major forms of δ-crystallin with different isoelectric points each had a different ratio of the higher to the lower molecular weight polypeptides. All possible combinations of the larger and smaller subunits of δ-crystallin were found in a ratio which suggests that there are no preferences for assembly. In vitro experiments have also indicated that chick δ-crystallin subunits can assemble in different combinations (Clayton & Truman, 1967) and even that chick and duck δ-crystallins can reassociate into hybrid aggregates (Clayton, 1969). The ability of different δ-crystallin polypeptides to associate with each other in different combinations is consistent with their similarity in amino acid sequence, as judged by analysis of their peptides (Piatigorsky, 1976; Reszelbach et al., 1977).

Assembly of polypeptides in different combinations is not unique to δ-crystallin and has been observed in many other proteins. One well-known example is lactate dehydrogenase, where two different subunits assemble in five combinations (Markert, 1963, 1968). Differential assembly of polypeptides also occurs in other lens crystallins. For example, bovine α-crystallin subunits can associate in different combinations in vivo (Delcour & Papaconstantinou, 1970, 1974) and in vitro (Li & Spector, 1972, 1973), and α- and β-crystallin subunits may even form hybrid aggregates in vitro (Bloemendal et al., 1975). β-Crystallin polypeptides also associate in different combinations in mammalian (Herbrink & Bloemendal, 1974; Zigler & Sidbury, 1976a) and submammalian (Truman & Clayton, 1974; Zigler & Sidbury, 1976b) species. One source of heterogeneity among lens crystallins, therefore, appears to be due to different combinations of aggregated polypeptides.

Finally, the existence of numerous, minor forms of native δ-crystallin having different isoelectric points from the principal forms suggests that the larger and smaller molecular weight polypeptides also possess charge heterogeneity. Electrophoretic tests of duck δ-crystallin in the presence of urea have provided evidence for charge heterogeneity among the subunits (Clayton, 1969, 1970). Further experiments are required in order to establish the number of different subunits composing mallard δ-crystallin, to understand the biochemical basis for their differences, and to determine whether each minor species of δ-crystallin with a different isoelectric point also has a different subunit composition.

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Accessibility of Deoxyribonucleic Acid in Chromatin to the Covalent Binding of the Chemical Carcinogen Benzo[*a*]pyrene†

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ABSTRACT: A model system utilizing rat liver microsomes to activate [^{3}H]benzo[*a*]pyrene (BP) in the presence of calf thymus nuclei was used to examine the ability of BP to bind regions of DNA which differ in their accessibility in chromatin. [^{3}H]BP-modified nuclei were digested with staphylococcal nuclease and DNase I, and the specific activity (cpm of [^{3}H]BP/A_{260} of DNA) of the DNA remaining undigested was determined. Both enzymes resulted in characteristic changes in specific activity as a function of digestion. No changes occurred during digestion of isolated [^{3}H]BP-DNA, and BP had no effect on the kinetics of digestion of DNA or nuclei, indicating that the specific activity changes seen in nuclear digests were due to preferential binding to DNA in regions of chromatin differing in enzyme susceptibility. The nucleosomal sites of [^{3}H]BP binding were determined by electrophoretic analysis of the resistant DNA and by examining the specific activity as a function of digestion of (1) nucleosome multimers isolated by sucrose gradient sedimentation of [^{3}H]BP-modified nuclei partially digested with staphylococcal nuclease and of (2) monomer subfractions obtained by KCl precipitation of H1-containing monomers. In addition, the distribution of [^{3}H]BP in fragments obtained from a DNase I digest of nuclei was compared to that of an isolated monomer fraction. These data led to the conclusion that BP binds to the spacer region and the outermost portions of the nucleosome core.

The polycyclic aromatic hydrocarbon benzo[*a*]pyrene (BP)[1] is ubiquitous as an environmental pollutant and is carcinogenic in animal test systems. Like most chemical carcinogens, it undergoes metabolic activation to form highly reactive intermediates capable of covalent binding to macromolecules. The 7,8-dihydrodiol 9,10-epoxide of BP has been identified as the major metabolite bound to DNA (Sims et al., 1974; King et al., 1976; Weinstein et al., 1976). By comparison to other metabolites, its increased reactivity with DNA has been correlated with its greater potency as a carcinogen (Jerina et al., 1976; Slaga et al., 1976; Levin et al., 1976), mutagen (Huberman et al., 1976; Jerina et al., 1976; Newbold & Brookes, 1976; Levin et al., 1977; Malaveille et al., 1977; Marquardt et al., 1977), and transforming agent (Marquardt et al., 1976, 1977). The in vivo covalent binding of BP has been shown to occur by derivitization of the 2-amino group

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[1] Abbreviations used: BP, benzo[*a*]pyrene; EDTA, ethylenediaminetetraacetate.

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