Subunit composition of rabbit lens beta crystallins

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DEAE-purified rabbit beta crystallins have been studied by sedimentation on sucrose gradients and their subunits characterized by co-electrophoresis in SDS-polyacrylamide gels. The experiments indicate that the beta crystallins isolated by DEAE-cellulose are a large family of related proteins with sedimentation velocities of about 4S. Deaggregation and denaturation of these proteins to polypeptides and subsequent electrophoretic analysis yields three groups of subunits with calculated molecular weights of 21,000, 23,000, and 29,000. The minimum total number of polypeptide chains in the three groups is five. Each of the beta crystallins is made up of two or three such chains. Aggregation and loss of solubility occur readily during concentration procedures. In the course of these experiments, it became evident that alpha crystallin is composed of at least two different sizes of polypeptide chains.

Polyacrylamide gel electrophoresis in the presence of the anionic detergent sodium dodecyl sulfate (SDS) has recently been employed to separate and identify the subunits obtained by vigorous deaggregation, denaturation, and reduction of rabbit lens proteins previously purified by DEAE-cellulose chromatography.[1] The electrophoretic method employed effectively minimized native charge differences of polypeptides and separation is based mainly on molecular weight. In that report, six major subunit groups were obtained from rabbit soluble whole lens proteins. Three groups of chains with calculated molecular weights of 21,000, 23,000, and 29,000 were identified in the beta crystallin region of the DEAE-cellulose chromatogram (0.015M and 0.03M eluates). Further experience with this method in which tritiated heavy and light chains of gamma globulin were co-electrophoresed in double-labeled gels with ^{14}C-labeled lens proteins, yielded the same molecular weights based on assumed values of 55,000[2] and 23,000[2] for the heavy and light chains, respectively. In addition, studies with enzymes of known sizes suggest that the technique can discriminate molecular weight differences as small as 5 per cent between polypeptides.

Previous investigators have described physical heterogeneity in bovine lens beta crystallins prepared in several ways. Thus, Spector[3] reported three major peaks with sedimentation velocities of 5S, 9.2S, and 14.5S. Björk,[4] using gel filtration and column electrophoresis as preparatory procedures, found four fractions which sedimentated at 4.2S, 4.9S, 9.6S, and 13.6S. In high concentrations of urea, deaggregation of beta crystallin to subunits of 1.8S has been reported.[5]

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It is the purpose of this communication to describe the results of studies performed on the four major rabbit lens beta crystallin peaks isolated from DEAE-cellulose and to clarify their subunit compositions.

Materials and methods

Whole lenses from adult rabbits were incubated with radioactive amino acids in amino acid deficient Eagle's medium as previously described.[1] Lens proteins were labeled with either ^{14}C-mixed amino acids (NEC-445), 10 μc per milliliter, or a mixture of ^{3}H-leucine, arginine, lysine, threonine, and valine, 10 μc of each amino acid per milliliter of incubation medium. In all cases, the period of incubation with isotope was 24 hours. The lenses were washed and homogenized, and the soluble proteins prepared as previously described.[1]

The ^{14}C-labeled soluble proteins were then applied to a 30 × 2.5 cm. DEAE-cellulose column (250 mg. total sample) and eluted by stepwise applications of discontinuous buffers.[6] The 0.015M and the 0.03M peaks were lyophilized after extensive dialysis against 0.0005M phosphate, pH 6.85.

Aliquot parts of 250 μg of tritiated soluble whole lens protein were mixed with sufficient amounts of each ^{14}C-labeled, DEAE-purified beta crystallin to give an ^{3}H/^{14}C ratio of 10 to 1. The double-labeled samples were prepared for electrophoresis in sodium dodecyl sulfate-polyacrylamide gels and electrophoresis was carried out for 16 hours at 5 v. per centimeter.[1] In some experiments, electrophoresis was extended to 22 hours at 5.5 v. per centimeter to increase the separation between the beta subunits. After fractionation,[1] samples were collected in 10 ml. of Bray's solution[7] and counted in a Packard Liquid Scintillation Spectrometer. The channel overlap was 1 per cent tritium into carbon and 10 per cent carbon into tritium. Following background subtraction, suitable corrections for channel overlap were made.

Gradient density centrifugation[8] was employed to approximate the sedimentation velocities of each of the four ^{14}C-labeled beta crystallin peaks eluted from DEAE. These samples were not lyophilized or concentrated in any manner. Five-tenths milliliter (about 100 μg) samples, eluted at the height of each peak, were layered on 15 ml. linear 5 to 20 per cent (w/v) sucrose gradients[8] prepared in 0.14M NaCl with 0.01M Tris pH 7.4. The gradients were centrifuged at 24,000 r.p.m. for 39 hours at 4° C. in the SW 25.3 rotor in a Spinco L-2 65 Preparative Ultracentrifuge with diffusion pump. DEAE-purified rabbit gamma globulin[9] and human hemoglobin were run in the same and/or parallel gradients as markers with assumed sedimentation velocities of 6.6S and 4.3S. No protein interaction was detected. After centrifugation, the gradients were tapped

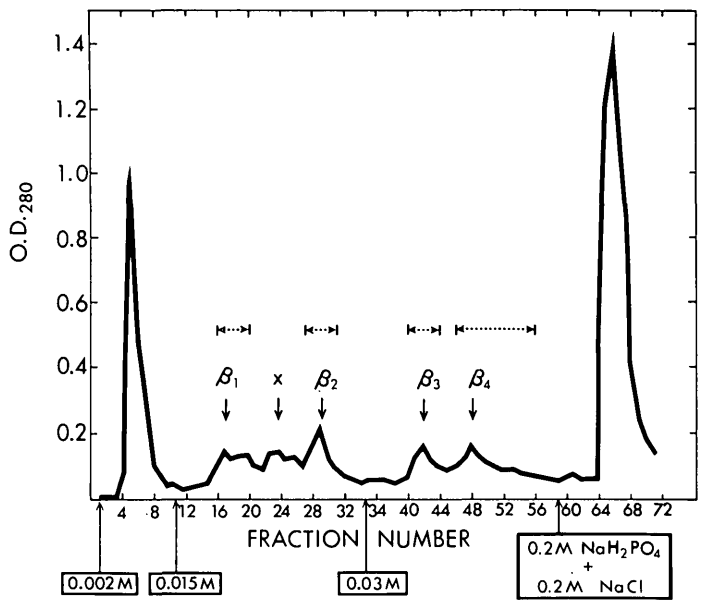

Fig. 1. DEAE-cellulose chromatogram of rabbit lens protein. The column was 30 × 2.5 cm. and the total sample applied was 250 mg. The beta crystallin fractions pooled and lyophilized for subunit analysis are indicated (| < . . . . . > |).

from above, the gamma globulin band detected by absorption at 280 mμ, and hemoglobin by absorption at 410 mμ. Aliquot parts of each fraction were added to 10 ml. of Bray's solution and counted.

Results

A typical DEAE-cellulose chromatogram for rabbit lens protein is shown in Fig. 1. In the experiment illustrated here, alpha crystallin was eluted with 0.2M NaH_2PO_4 + 0.2M NaCl. Usually, two peaks (labeled β_1 and β_2) were eluted by 0.015M phosphate. In some experiments, however, an additional peak (labeled *X*) could be seen midway between them. The 0.03M eluate always yielded two peaks (labeled β_3 and β_4). Gamma and beta crystallin represented 18 and 38 per cent, respectively, of the total optical density recovered from the column. The remainder (44 per cent) was eluted with 0.2M NaH_2PO_4 + 0.2M NaCl.

The approximate sedimentation velocities of each of the β peaks (relative to that of hemoglobin and gamma globulin as markers) are shown in Table I. All of the beta crystallins migrated in their gradients as reasonably narrow, symmetrical bands. Each band, however, was slightly broader

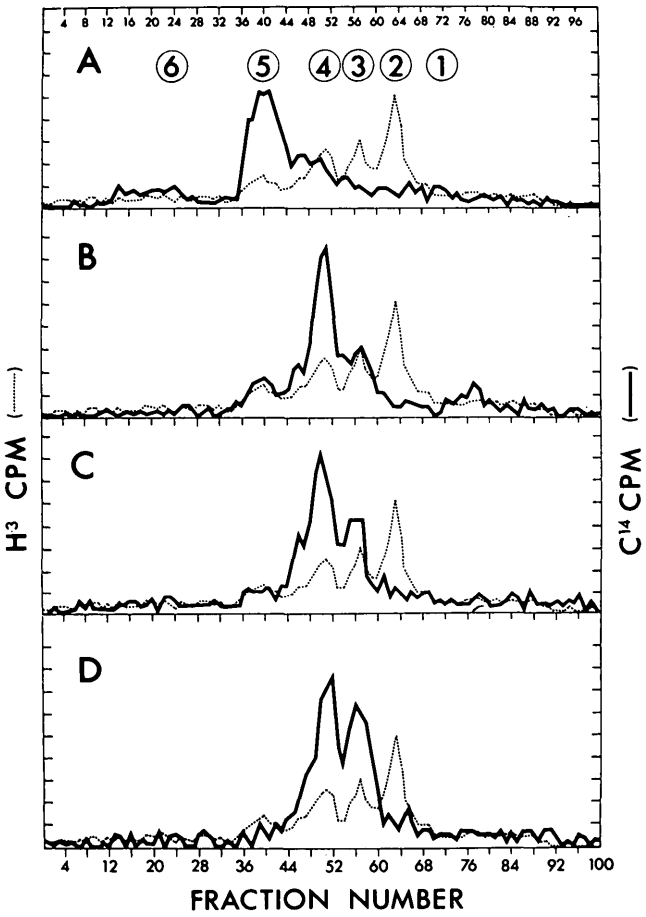

Fig. 2. Subunit composition of the four major DEAE-purified beta crystallins. Tritiated soluble whole lens proteins (. . . . .) as reference markers were added to ^{14}C-labeled beta crystallins (———), the mixture denatured to polypeptide chains, and co-electrophoresed at 5 v. per centimeter for 16 hours. Panels *A*, *B*, *C*, and *D* are samples of β_1, β_2, β_3, and β_4 crystallins, respectively. The circled numbers indicate the positions of the six major subunit groups obtained from soluble whole lens proteins.[1]

than that of the purified marker proteins. The center of no band sedimentated at velocities greater than 4.5S, and no counts were found in the pellet.

The subunit composition of the four major beta crystallin components isolated from DEAE is shown in Fig. 2. The dotted pattern in each panel represents tritiated whole lens protein prepared and co-electrophoresed with each of the ^{14}C-labeled beta crystallins to show the six major groups of polypeptides[1] obtained from whole lens proteins. As shown previously,[1] subunit groups 3, 4, and 5 correspond to the subunits of beta crystallin and have calculated molecular weights of 21,000, 23,000, and 29,000, respectively.

In the experiments illustrated in Fig. 2, all samples were denatured by sodium dodecyl sulfate, reduced by mercaptoethanol, and alkylated with iodoacetamide prior to electrophoresis. In an attempt to determine if any of the four beta crystallins isolated from DEAE-cellulose are disulfide linked, duplicate samples were treated with SDS alone and alkylated with 0.15M iodoacetamide before electrophoresis to block sulfhydryls exposed by SDS. In the absence of reduction before electrophoresis, the subunits of β_1, β_3, and β_4 crystallins were considerably diminished but not completely absent. In each case material of higher molecular weight was found near the origin of the gel, indicating disulfide bonding. β_2, however, was completely dissociated by SDS alone.

β_1 consists predominantly of polypeptide chains with an approximate molecular weight of 29,000. β_2 and β_3 appear to be similar in subunit composition and consist mainly of chains of molecular weight 23,000. The β_4 material consists of chains of molecular weight 23,000 and 21,000. The subunit composition of the minor peak (X) is identical with β_1. It is evident from this figure that there is considerable heterogeneity and overlap in subunits obtained from each of the beta crystallins. This is in large part a function of the difficulty in achieving sharp resolution of the individual beta crystallins by DEAE-chromatography. Indeed, if fractions eluted at the height of each peak (rather than pooled, lyophilized materials) are examined, much of the heterogeneity is eliminated.

Prolonged electrophoresis (22 hours)

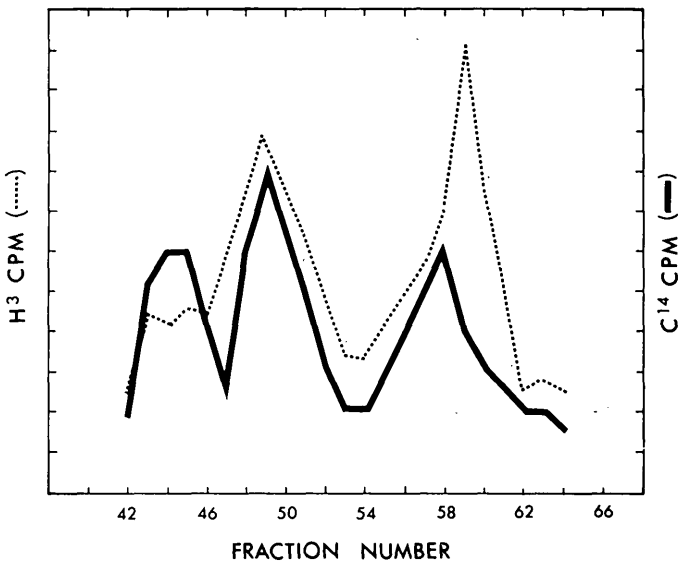

Fig. 3. Subunits of a sample of pooled 0.03M eluate co-electrophoresed with subunits of soluble whole lens proteins at 5.5 v. per centimeter for 22 hours to increase the separation between subunits. Only fractions 42 to 64 are plotted in this figure.

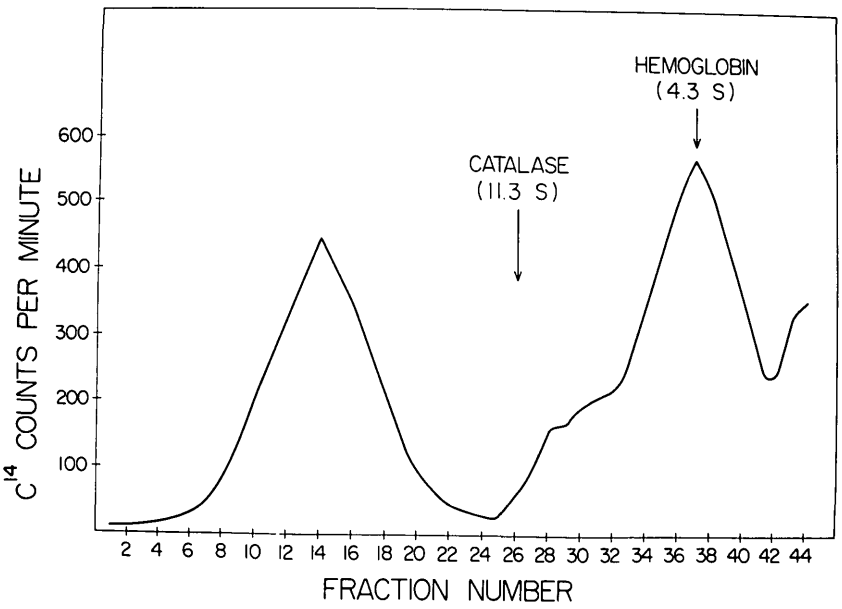

Fig. 4. Sucrose gradient of labeled rabbit lens proteins. The soluble proteins, following 24 hours incubation with ^{14}C amino acids, were centrifuged at 24,000 r.p.m. in the SW 25.3 rotor for 31 hours. The positions of catalase and hemoglobin as reference markers are indicated by vertical arrows.

successfully increased the resolving power of the technique. In those studies it became apparent that the 29,000 subunit really consists of a group of 2 to 3 chains of similar molecular weights. Similarly, it became evident that the shoulders at fractions 46 and 56 of peaks 4 and 3, respectively, in Fig. 2 (dotted curves) were distinct and separate chains. Fig. 3 illustrates the effect of increased time of electrophoresis upon a sample of pooled 0.03M eluate. Here, peak 2 (alpha and some gamma crystallin) has been run completely off the gel into the anodal buffer chamber, and there is greater separation of the slower components. It is clear that peak 4 consists of at least two chains with similar molecular weights. It can also be seen that peak 3 must consist of more than beta crystallin materials, since the ^{14}C and ^{3}H peaks do not precisely coincide (see Discussion).

Fig. 4 illustrates the sedimentation pattern of newly synthesized rabbit whole lens proteins. Two major peaks are seen, one at 18.5S and one at 4S, with an incompletely resolved shoulder at 9S.

Discussion

Because of the ability of this analytic method to discriminate between subunits of slightly different molecular weights, it has been possible to show that the beta crystallins consist of three groups of subunits. The *minimum* total number of chains of different sizes in these groups is five (two each of molecular weight 29,000 and 23,000, and one of molecular weight 21,000). This suggests that the beta crystallins are likely a large family of closely related molecules. The sucrose gradient data presented in Table I as well as the elution pattern from DEAE-cellulose support this impression.

A rough approximation of the molecular weights of proteins can be derived from density gradient data by application of the formula[8]:

$$\frac{S_1}{S_2} = \frac{(MW_1)^{2/3}}{(MW_2)}$$

Table I. Sedimentation velocity of DEAE-purified beta crystallins on 5 to 20 per cent linear sucrose gradients

Peak designation	*Average S (four determinations)*
β_1	4.2
X	3.8
β_2	4.3
β_3	4.0
β_4	4.0

Approximately 100 μg of ^{14}C-labeled protein eluted at the C. height of each peak were centrifuged at 4° C. for 39 hours in the SW 25.3 rotor in a Spinco L-2 65 Preparative Ultracentrifuge.

For the data presented in Table I, the molecular weights of proteins with S values of 3.8 to 4.3 would be about 55,000 to 65,000. Since the estimated molecular weights of the beta crystallin subunits are 21,000 to 29,000, each of the beta crystallins, as eluted from DEAE-cellulose, contains two or three such chains.

Recent data derived from sequence studies of mouse light chains[10] indicate that the molecular weight of the light chain may be 10 to 15 per cent higher than the assumed value of 23,000 used in these experiments. Thus, it is quite likely that each of the beta crystallins consists of two polypeptide chains.

β_1 is a dimer of the largest subunits (29,000). β_2 is an aggregate of chains of molecular weight 23,000, and it appears that β_3 and β_2 have similar compositions. β_2 and β_3 may elute at different molarities because of differences in configuration of the aggregates and in resulting exposed charges. Alternatively, they may be composed of closely related but not identical subunits.

Electrophoresis of unreduced beta crystallins in SDS suggests that β_1, β_3, and β_4 may be composed of disulfide-linked chains, while β_2 is composed of subunits joined by noncovalent bonds. This interpretation is, however, dependent on adequate alkylation of sulfhydryls exposed by SDS to prevent sulfhydryls on two separate chains from interacting to form an interchain disulfide bond. The presence of small amounts of subunits of β_1, β_3, and β_4 after treatment with SDS alone again indicates heterogeneity within each lyophilized peak eluted from DEAE-cellulose.

As shown previously,[1] after 24 hours of incubation with labeled amino acids, about 20 per cent of the total incorporated counts in whole lens proteins are found in the subunit group of 21,000 molecular weight (peak 3), while 17 per cent are in the 23,000 subunit (peak 4). The 0.015M and 0.03M eluates can be shown to account for the latter group quantitatively, but much of the group of smaller chains cannot be accounted for in these eluant fractions. DEAE-isolated alpha crystallin sedimented on 5 to 20 per cent sucrose gradients in the manner described yields a major 18.5S peak and a minor 4S component. Double label studies of the subunits of the 18.5 material have demonstrated that about 30 per cent of the 0.4M eluate (alpha crystallin) is an alpha crystallin chain of calculated molecular weight 20,500.[11] It must be emphasized that our data indicate that the electrophoretic method employed separates proteins or subunits on the basis of size only and cannot, therefore, cleanly resolve components that are very similar in molecular weight. The alpha crystallin subunit of calculated molecular weight 20,500 makes up a considerable percentage of the leading edge of subunit group 3 from whole lens and explains the lack of total and precise superimposition of ^{14}C and ^{3}H at peak 3 in Figs. 2 and 3, since the ^{3}H counts of peak 3 are derived from whole lens and represent some alpha as well as beta crystallin subunits.

Our recent studies have demonstrated the existence of at least three different sizes of alpha crystallin subunits (calculated molecular weights 18,500, 20,500 and 19,500) and will be reported in a subsequent communication in more detail.

In the sucrose gradient determinations reported here, no concentration procedure was employed and the concentration of protein in the samples applied to the gradients was about 0.02 per cent. In preliminary

attempts to obtain sedimentation velocities performed with lyophilized beta crystallins or with eluates concentrated by dialysis against 60 per cent sucrose in buffered saline, some visible aggregation occurred. When these samples were centrifuged on sucrose gradients, all of the soluble protein had an S value of about 4S, while the aggregated material was found at the bottom of the centrifuge tube. This finding suggests that the degree of aggregation of beta crystallins and their sedimentation velocities may be concentration dependent and that denaturation and aggregation can occur readily during manipulation. Indeed, centrifugation of labeled, freshly prepared rabbit soluble whole lens proteins on sucrose gradients yields only two distinct peaks, 18.5S (alpha) and 4S (beta), although a shoulder is detectable at 9S (Fig. 4). Similar findings have been reported for bovine whole lens proteins.[5]

The demonstrated complexity of the beta crystallins and their subunit compositions are formidable obstacles to studies designed to trace their synthesis and assembly. It is conceivable, however, that the synthesis of different beta crystallins may occur in different layers of the lens (epithelium and fibers) and this possibility is now being studied.

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