Physicochemical Investigations on a Complex Protein: the Soluble Protein of the Eye Lens, α-Crystallin*

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Exact data of molecular weight, form, and shape can be obtained only from proteins which have been prepared in a pure and homogeneous state (see Waugh (1), Lundgren and Williams (2), and others). However, it is possible to obtain useful information about proteins that show complexity by comparing carefully the results of several physicochemical measurements. The investigation of the soluble lens protein α-crystallin gives an example of this comparative method.

α-Crystallin is highly organ-specific and can thus be used with advantage in immunobiological investigations of problems of induction and differentiation (see Woerdeman et al. (3, 4) and de Vincentiis (6)). It is probable that the investigation of the chemical structure and the physical behavior of the lens proteins would provide a better understanding of some problems of proteinogenesis in correlation with the morphogenesis of the organism (6).

EXPERIMENTAL PROCEDURE

The preparation of fairly pure α-crystallin is rather easy and can be effected without the specific equipment of a specialized laboratory. Extracts of the eye lens of vertebrates with diluted buffer solutions at a pH above 7.0 are opalescent solutions which contain soluble and insoluble proteins. The periphery of the eye lens disperses easily, whereas the nucleus of the adult lens has to be crushed in a mortar. The nucleus contains insoluble protein aggregates of the peripheral soluble proteins (7). To obtain the soluble proteins, only the periphery has to be extracted. This can be done simply, by stirring slowly for a few minutes with an aqueous solvent, approximately pH 8, at a temperature between 0 and 4°.

The main soluble proteins of the peripheral lens extract can be separated by making use of the different solubility of the protein components in concentrated salt solutions, by using the great differences in electrophoretic mobility, by ultracentrifugal sedimentation, and by isoelectric precipitation.

RESULTS

Analysis of Peripheral Extract—Analysis of the electrophoretic and sedimentation patterns of peripheral extracts of adult lens
does not at first glance give corresponding results, as is shown in Figs. 1A and 1B. Electrophoresis according to Tiselius or in solid carriers (e.g. paper, agar, starch) displays two clearly visible boundaries.

From these observations one might conclude that the peripheral extract contains, for the most part, two protein components (in Fig. 1A marked α and β). Sedimentation in a high centrifugal field, however, results in the formation of three clearly visible boundaries (in Fig. 1B designated by A, B, and C). From this one might conclude that the peripheral extract contains three main protein components.

Acidifying the extract with acetic acid to pH 5.0 precipitates a whitish substance which, after washing and redissolving in weak alkaline buffers, gives a clear solution. Electrophoresis of this solution shows only one boundary (Fig. 2A) at different pH values, varying from pH 3.0 to pH 10.0, with isoelectric point pH 5.0, whereas sedimentation produces two boundaries (Fig. 2B). The sedimentation constants are of the order of 15 and 7.5 S. The remaining solution, after precipitation at pH 5.0, forms only one complex when examined by electrophoresis (Fig. 3) and by sedimentation in the ultracentrifuge. The sedimentation constant of this protein complex is of the order of 3 S (boundary C in Fig. 1B).

α- and β-Crystallin—It thus appears that the isoelectric precipitate formed from the extract of the eye lens at pH 5.0 comprises two components (A and B) which give rise to the formation of two boundaries in the ultracentrifuge and only one boundary with electrophoresis. Furthermore, the eye lens extract contains a complex component (C) which does not precipitate at pH 5.0 and which gives only one boundary with both methods. This component has an isoelectric point of pH 6.1 (estimated with electrophoresis at different pH, buffer solution 0.1 M NaOAc and 0.2 M Glycin).

The precipitate at pH 5.0 is called α-crystallin; the protein complex which remains in solution is called “β-crystallin.” The amino acid composition of α- and β-crystallin has been estimated by several investigators. The N-terminal amino acids of α-crystallin were determined by means of the dinitrofluorobenzene (DNFB) method, and the total amino acid content after hydrolyses by their dinitrophenyl-derivatives (Bloemendal (8)). The absorption spectrum in the ultraviolet of α-crystallin has been measured by the present author (9). According to these analytical data, the composition of α-crystallin may be considered practically independent of the method of preparation.

Chemically the two fractions with isoelectric points pH 5.0 and pH 6.1 behave differently toward sodium nitroprussiate:

1 With a trace of a slower moving third component.
physicochemical investigations on a complex protein

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Fig. 1A. Electrophoresis diagram of peripheral eye lens extract of the cow. Ascending boundary. Conditions: 0.1 M NaOAc, 0.05 M Glycin, pH 7.8 (NaOH), 5 volts per cm, 4°. B: Sedimentation diagram of peripheral eye lens extract of the cow. Conditions: 50, 100 r.p.m., 3180 seconds, 15° angle, 2.5% concentration, same buffer.

α-crystallin gives a negative reaction, β-crystallin a strongly positive one. This means that β-crystallin contains free SH groups, whereas α-crystallin does not.

Subunits of α-Crystallin—According to the sedimentation analysis, α-crystallin seems to be binary (αA and αB), but it behaves (quasi-) homogeneously in electrophoresis. Sedimentation measurements prove that the concentration ratio in which the two subunits occur in the α-crystallin fraction is far from being constant. On the contrary, this ratio differs considerably in different preparations. α-Crystallin might consist of two different proteins with equal mobility and the same isoelectric point, but in this case it is improbable that the coupled components could be easily separated by a centrifugal field but not by an electric field, nor by dissolving the isoelectric precipitates by making use of the very radical influence of ions in the buffer solution which force the precipitated particles to dissolve. Assuming, however, that the two subunits are derived from the same protein, many experimental facts can easily be explained.

In my earlier publications on this subject (10) native α-crystallin was assumed to have a molecular form which gives the fast moving boundary with a sedimentation constant of the order of $s_{20} = 15$. The extraction of this native protein, as it occurs in the lens periphery, should cause partial transmutation into particles with a sedimentation constant of the order of $s_{20} = 7.5$. This must be considered as the first step towards denaturation (Fig. 2B). An argument for this assumption is the following. The precipitate at pH 5.0 can be dissolved by acidifying to pH 2.5. A clear solution results which gives in the ultracentrifuge a diagram with one boundary with a low sedimentation constant (about 1 S). The same occurs by acidifying an alkaline solution of α-crystallin. This means that both the subunits dissociate into only one component. This component shows typical protein properties and serologically appears to be identical with α-crystallin. It is thus proved that the antigenically active group must be present in both subunits, and therefore, strong support is lent to the assumption that the two components, αA and αB, are subunits of the same protein. By continuing the sedimentation until the αA component is fully precipitated and decanting the supernatant liquid, one can separate, more or less quantitatively, the two components. A successful result is obtained only if the diffusion rate is very low; therefore, the sedimentation must be carried out at a low temperature (0°C).

Method: 100 mg sodium nitroprussiate is dissolved in 0.5 ml water; one drop of 25% ammonia is added. On a porcelain disk one drop of this solution is brought in contact with a drop of the protein solution. β-crystallin gives a reddish color; α-crystallin does not.

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Fig. 2A. Electrophoresis diagram of isoelectrically precipitated α-crystallin. Conditions: 5 volts per cm, 40 minutes, 8.0°, 0.1 M NaOAc, 0.05 M Glycin, pH 7.8 (NaOH). B: Sedimentation diagram of the same preparation. Conditions: 50,000 r.p.m., 1500 seconds, 17°, 15° angle, 1.1% concentration.

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It is also possible, however, that already in the lens periphery, the α-crystallin occurs in the two subunits.

Positive precipitin reaction with rabbit antisera against bovine α-crystallin; for technical details see ten Cate, et al. (4).
FIG. 3. Paper-electrophoresis of a 3% solution of "β-crystallin" of the bovine eye lens. Conditions: pH 7.8, 0.1 M NaOAc, 0.06 M Glycin, Whatman No. 1 filter paper, 5 hours, 5 volts per cm, 6250 A-diagram, log 1/T distance from origin.

FIG. 4. Sedimentation diagrams of α-crystallin after sedimentation of the fast moving component. A: Supernatant fluid, containing only the slow moving fraction of the protein. B: Diagram of the dissolved sediment. Both 47760 r.p.m., 900 seconds, 30° angle, about 20°. Buffer: 0.1 M NaOAc, 0.05 M Glycin, pH 7.8.

When redissolved, the sediment yields a clear solution, which gives two boundaries in the ultracentrifuge with about the same relative concentrations as the original solution. The supernatant solution, however, shows a sedimentation diagram in which the aA-component has almost entirely disappeared (Figs. 4A and 4B). One might expect that the concentration of the fast sedimenting aA-component would be very much greater than that of the slow moving aB-component. This is not the case, however, and consequently, this experiment provides an argument for the assumption that the aA-component changes into the aB-component during re-solution. Continuation of the sedimentation brings to light yet another fact: the aB-component aggregates very easily. This points to a remarkable unfolding of the αB-particles. The same phenomenon occurs after all prolonged operations, such as dialysis, etc. In those cases in which one main boundary appears, it is practically always the aA-component, the aB-boundary having disappeared in a multitude of aggregation peaks6 (Fig. 5). The fact that the nucleus of the lens of adult mammals (cow, older than 5 years) contains α-crystallin, which is practically insoluble in neutral and weak alkaline solvents, shows that the deterioration starts in the living lens, leading to denaturation and aggregation.

6 By adding urea, etc.; however, the native component (the aA-unit) seems to be attacked.

FIG. 5. Sedimentation diagram of α-crystallin with aggregated aB-unit. Twice precipitated isoelectrically, redissolved in 0.1 M NaOAc, 0.05 M Glycin, pH 7.1; 1.4%, 47760 r.p.m., 2520 seconds, 12.1°, 45° angle.

FIG. 6. Sedimentation diagram of once (A) and twice (B) precipitated α-crystallin. Conditions: 4200 r.p.m., 1500 seconds, 45° angle, 1.2% concentration. A, 21.1°; B, 20.0°. Buffer: 0.1 M NaOAc, 0.05 M Glycin, pH 7.8.

After reprecipitating the dissolved precipitate of peripheral α-crystallin by the repeated addition of acetic acid until pH 5.0 is reached and redissolving this precipitate at pH 7.8, the sedimentation pattern changes remarkably: the concentration of the fast component is lower, that of the other component is increased (Figs. 6A and 6B). This is another argument for our assumption.

DISCUSSION

Boundaries in Peripheral Extract—It is possible that the existence of two boundaries in the ultracentrifuge is due to the fact that the two components differ in shape but not in molecular weight. The fast moving boundary corresponds, in this case, to the globular α-crystallin particles, the slow moving boundary to the stretched particles which are formed as a result of the breaking of noncovalent bonds which keep the tertiary structure of the globular particle intact. The very low opalescence of freshly prepared peripheral α-crystallin solutions, the solubility of the protein, and the "Gaussian" form of the Philpot curve of the aA-subunit during sedimentation make it probable that the aA-particle is the native form of α-crystallin. This could be proved only if it were possible to investigate solutions which contain only the aA-unit in a very pure state. Also the critical tests on the frictional coefficient could be achieved only if it were possible to separate the two units quantitatively.

**Diffusion Constants of Particles**—According to the formula

$$M = \frac{RTS}{D(1 - V_p)}$$
in which \( S = \) sedimentation constant, \( D = \) diffusion constant, \( V = \) the partial specific volume of the solute, and \( \rho = \) the specific weight of the solvent, the molecular weight being constant, the sedimentation constant \( S \) is directly proportional to the diffusion constant \( D \). Corey and Pauling (11) claim that the stretching of globular proteins may produce an axial ratio of 20, which means that a globular particle with a radius \( r \) obtains a length of about \( 12r \) to a diameter of about \( 0.6r \). According to Herszog, Illig, and Kudar (12), the diffusion constant of such a stretched particle is about half that of the corresponding globular particle. Therefore, a solution which contains globular and stretched particles of the same molecular weight may give in the ultracentrifuge two boundaries with sedimentation constants in the ratio of 1 to 2. This is indeed the case, as we have seen before. The dependence of shape of the electrophoretic mobility is much less pronounced, so it seems quite possible that the globular and the stretched units have essentially the same mobility (see also (13)). Bloemendal claimed that he succeeded in separating the subunits quantitatively by starch electrophoresis. In that case it should be possible to measure the diffusion constants exactly. There exists in each case a pronounced difference in diffusion which can be observed during the sedimentation in the ultracentrifuge.

The slower unit has a much smaller diffusion rate than the fast moving unit. All observations, therefore, demonstrate the possibility that our last assumption is correct.

\( \alpha \)-Crystallin exists in solution (with a pH ranging from 3.5 to 0.5) in two subunits, differing only in shape, a more or less globular unit and a stretched unit. The solubility of the unfolded form is nearly the same as that of the globular form; the serological specificity and the absorption in the ultraviolet do not show remarkable differences. If the aim is to study the covalent chemical structure of \( \alpha \)-crystallin, there is no need to begin by isolating the globular form. For the purpose of investigating the "architecture" of the native globular protein in the sense of Corey and Pauling, however, this has to be done first of all. But at the moment there seems to be no means of obtaining the subunits free from each other in a pure state. The ultracentrifugal analysis shows that a part of the molecules always exists in a stretched and even aggregated state (see also Wood et al. (14)).

**Purification of \( \alpha \)-Crystallin**—At the isoelectric point the solubility of \( \alpha \)-crystallin is low. This simple method of preparing \( \alpha \)-crystallin is by far the best means of purification without causing serious damage to the \( \alpha \)-crystallin molecule.

The precipitation has to be done by acidifying a weak alkaline solution with acetic acid (0.1 n), and the buffer solutions used should consist of univalent salts and should have an ionic strength of about 0.1 \( \mu \). The addition of glycine retards the aggregation of the unfolded particles.

In physicochemical experiments no trace of other components can be found in \( \alpha \)-crystallin prepared in this way. Ultracentrifugal analysis shows the absence of other proteins and other components with molecular weights higher than 10,000, with an accuracy of about 99%. Light scattering analysis shows the absence of insoluble components with still greater accuracy, and free electrophoresis shows the absence of migrating, foreign components with an accuracy of about 98% (microelectrophoresis with Philpot-Svensson optic).

It may thus be established by these criteria that the twice precipitated \( \alpha \)-crystallin from the periphery of the lens of adult mammals has a purity of at least 98%.

In this publication we have considered only \( \alpha \)-crystallin of the eye lens of adult mammals. Investigations of the properties of \( \alpha \)-crystallin prepared from the eye lens of embryos and of post-natal organisms are in progress (6, 15). It would seem that the physicochemical "development" of the protein \( \alpha \)-crystallin correlates with the biological development of the organism. Native \( \alpha \)-crystallin of the calf shows pronounced asymmetry (see also Reznik (16)); that of the adult cow seems to be globular in shape.

**Conclusions**—The probability that the complex soluble eye lens protein \( \alpha \)-crystallin consists of two subunits with equal molecular weight, differing in shape only, has been demonstrated. Concerning the problem of homogeneity or complexity of proteins the following general conclusions can be drawn: *Evidence of more than one boundary and complexity of the boundaries themselves provides no certainty concerning the presence of different proteins in a solution. One single soluble protein may form unfolded units which aggregate easily. In general, these units and aggregations have different diffusion velocities and different sedimentation constants.*

Another proof of homogeneity can be obtained with the serological Oucherterly technique. Here the same problem arises, for the diffusion in agar of different units and aggregates of the same protein results in the formation of several precipitation zones. Hence, one single protein may form quite complex patterns, also with this technique. It is thus very difficult to reach a definite conclusion. In any case, it is dangerous to reach a conclusion about the existence of different proteins in a solution if only one method is applied. Several publications show that this does not receive sufficient consideration. See, for example, François and Rabae (17) in which the authors' deductions concerning the presence of not less than eight different proteins in mamalian eye lens extracts are based merely on some electrophoretic and serological investigations.

**SUMMARY**

The probability that the complex soluble eye lens protein \( \alpha \)-crystallin consists of two subunits with equal molecular weight, differing in shape, is demonstrated by ultracentrifugal and electrophoretic data.

**REFERENCES**


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