Aust. J. Biol. Sci., 1976, **29**, 481–5

Sedimentation Equilibrium Studies on Protein from Kookaburra Beak

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Abstract

Fractionated samples of the soluble *S*-carboxymethyl proteins from kookaburra beak (Frenkel and Gillespie 1976) were examined by equilibrium sedimentation. The molecular weight was found to be 11 300 when the photoelectric scanning absorption optical system was employed and 13 700 when Rayleigh interference optics were used. Possible explanations for this difference are considered and it is concluded that it must arise from heterogeneity of the protein. Optical rotatory dispersion measurements indicate that the proteins probably exist as random coils in dilute aqueous buffer.

Introduction

The preceding paper (Frenkel and Gillespie 1976) described the preparation of soluble proteins from beaks and fractionation of the proteins from kookaburra beak. In this paper the heterogeneity and molecular weight of the kookaburra beak proteins are examined by equilibrium sedimentation.

Materials and Methods

Proteins

The zinc-precipitable fraction of kookaburra beak and a more highly fractionated sample (fraction 4b) prepared by Frenkel and Gillespie (1976) were used in this study.

Apparent Partial Specific Volumes

These were determined from the densities of dialysed solutions and their diffusates. The densities were measured with a precision density meter DMA-02 (Anton Paar, Graz). The zinc-precipitable protein was used for the density measurements because of a shortage of fractionated material. The protein was dialysed against 0·01 M sodium tetraborate–0·1 M NaCl (pH 9·1) and the concentration was determined by measuring the refractive index increment with a Brice–Phoenix differential refractometer using the value for dn/dc of 0·186 ml/g previously found for solubilized feather keratin in the same buffer (Harrap and Woods 1964). The absorbance of the solution was then measured with a Beckman DB spectrophotometer at 276 nm after dilution with 6 M guanidine hydrochloride and a value of 19·7 was found for $E^{1\%}_{1\,cm}$. This value was then used to determine the concentration of the protein after dialysis against 6 M guanidine hydrochloride.

Sedimentation Equilibrium

Experiments were carried out with a Beckman Model E ultracentrifuge equipped with Rayleigh interference optics and the photoelectric scanning absorption optical system. The more highly purified fraction 4b was used for this study. Molecular weights were measured by the low speed equilibrium method (Chervenka 1970) and the meniscus depletion method (Yphantis 1964). Column heights of 3 mm were employed. Base lines for the absorption optical system were obtained by scans at 430 nm where the protein did not absorb (Eisenberg *et al.* 1972). The computer program of Roark and Yphantis (1969) was used to calculate the point-average molecular weights in the meniscus depletion runs.

Optical Rotatory Dispersion

Measurements were made at 20°C with a Perkin Elmer spectropolarimeter Model 141 in the wavelength range 365–578 nm. Values of b_0 and a_0 from the Moffitt equation were determined graphically. The value of λ_0 was taken as 212 nm and the mean residue weight as 99·5, calculated from the amino acid composition of the crude protein (Frenkel and Gillespie 1976).

Results and Discussion

Partial Specific Volume

A value of 0·710 ml/g was obtained in 0·01 M sodium tetraborate–0·1 M NaCl and two separate dialyses gave values of 0·701 and 0·698 ml/g in 6 M guanidine hydrochloride–0·01 M sodium tetraborate (pH 8·3). For calculation of molecular weights in both urea and guanidine hydrochloride solutions the mean value of 0·700 ml/g was used. This is the correct value for use in guanidine hydrochloride solutions since it has been determined on dialysed solutions according to the Casassa and Eisenberg (1964) definition. Its use for experiments carried out in 8 M urea assumes that the selective solvation is the same in both solvents.

Optical Rotation

The values of b_0 and a_0 in the borate–NaCl buffer for the zinc-precipitable fraction were $+59°$ and $-556°$ respectively; in 6 M guanidine hydrochloride the values were $+66°$ and $-470°$. These values indicate lack of α-helical structure. The slightly positive value of b_0 is outside the range ($-10° \pm 20°$) expected for a random coil and may be due to the presence of some β-structure. The value of a_0, however, is more negative than that usually associated with a β-structure in proteins (Imahori and Nicola 1973). The difference in a_0 between the borate–NaCl buffer and guanidine hydrochloride may be a solvent effect since a_0 values are very solvent dependent. If any significant amount of β-structure was present it should be abolished in guanidine hydrochloride, reducing the b_0 value to near zero and the a_0 to a more negative value. Thus although the presence of some β-structure is not excluded, it may be reasonably concluded that the extracted beak proteins exist as random coils in dilute aqueous buffers. This does not imply that bird's beak keratin is structureless *in situ*. Rudall (1947) has shown that the hard portions of beak give a feather-type X-ray pattern. In feather the proteins are folded into an antiparallel pleated sheet conformation (Fraser *et al.* 1971) whilst the soluble *S*-carboxymethyl proteins show random coil behaviour in solution (Harrap and Woods 1964).

Molecular Weight

(i) *Absorption optics*

Two low-speed runs which were carried out at 16000 and 18000 rev/min in 8 M urea–0·05 M sodium phosphate, pH 7·0, gave linear plots of log (optical density) against r^2 (r is the distance from the centre of rotation). Scans were made at 276 and 286 nm. Values of 10800 and 11400 were calculated for the molecular weight. A meniscus depletion experiment (52000 rev/min) gave a linear plot of log (optical density) against r^2 and a value of 11600 for the molecular weight. The average molecular weights from the computer analysis showed a slight increase down the column, and they were in the order $M_n < M_w < M_z$ although the differences were probably within experimental error. The concentration at the highest optical density

was approximately 0·45 mg/ml so non-ideality has been virtually eliminated. There was a small region near the base of the cell of optical density >1 which was not counted in the analysis. The mean of the values obtained with the use of the absorption optical system was 11 300.

(ii) *Interference optics*

Experiments were carried out by the meniscus depletion method at concentrations between 0·02 and 0·05% in 8 M urea–0·05 M sodium phosphate, pH 7·0, and in 6 M guanidine hydrochloride unbuffered at pH 6·3 and also in guanidine hydrochloride–0·01 M sodium tetraborate, pH 8·3, at speeds of 44 000 and 52 000 rev/min. There were no obvious differences between the results in urea and guanidine hydrochloride. In all cases plots of log (fringe displacement) against r^2 were linear. However, the point-average molecular weights from the computer analysis indicated a slight decrease at first and then an increase down the column, i.e. the distribution of molecular weights down the column was sigmoidal. The computer program (Roark and Yphantis 1969) also calculates the 'midpoint moments' which are generated by fitting the entire data simultaneously and taking the slope at the midpoint. The value of M_w calculated in this way (mean from six experiments) was 13 500, M_z was also 13 500 and the average of M_n over the cell contents was 13 800. Extrapolation of the average molecular weights for each experiment to zero concentration, placing most weight on points between 200 and 500 μm displacement, gave 13 800. From all the data the molecular weight is estimated to be 13 700. Although these results indicate a high degree of homogeneity, some individual experiments suggested heterogeneity. One experiment at 0·5 mg/ml and 52 000 rev/min gave a significantly lower value of 12 400 although in this case there was incomplete resolution to the base of the column. The non-ideality present in 6 M guanidine hydrochloride would tend to mask heterogeneity. The concentration at the base of the cell in most experiments was of the order 1–2 mg/ml and a decrease in molecular weight of 5–10% over the solution would be expected in the absence of heterogeneity. Thus the slightly sigmoidal nature of the molecular weight distribution probably arises from a combination of non-ideality and heterogeneity.

The difference of 20% between the values obtained for the molecular weight by the two optical systems is difficult to explain. Several factors should be considered in the interpretation of interference fringe patterns. Generally the quality of interference fringes in concentrated urea or guanidine hydrochloride is inferior to those obtained with dilute buffers. Blank runs carried out with water after the equilibrium experiment may not exactly correspond to the experimental conditions pertaining during the equilibrium run. The use of teflon window liners as recommended by Ansevin *et al.* (1970) substantially reduced window distortion. However, this does not overcome the problem of baseline variability which is discussed in more detail by Horbett and Teller (1972). The results were also computed without the inclusion of the blank run, and the computer analysis was allowed to determine the zero concentration level. The mean value for M_w for the six experiments calculated in this way was no different although individual experiments showed marked variations from the blank corrected data. A more serious error is the existence of uncompensated buffer gradients between the sample and reference solution. Such problems can arise with concentrated urea and guanidine hydrochloride unless care is taken in handling solutions or if there is a mismatch in the column heights of the solution and reference. The distribution of

solvent components also negates the requirement of constant chemical potential which exists after dialysis equilibrium and which should be maintained at sedimentation equilibrium for the Casassa and Eisenberg (1964) treatment to apply. Another source of error is the influence of Wiener skewing on the results (Yphantis 1964). For a 12-mm cell and with 6 M guanidine hydrochloride at 52 000 rev/min this would make the molecular weights 3–4% too high when the camera is focused on the mid-plane of the cell as in these experiments.

In the photoelectric scanning optical system problems also arise with the baseline. This was overcome by scanning at 430 nm (Eisenberg *et al.* 1972). The agreement between the low-speed and meniscus depletion runs suggests that baseline error is insignificant. Moreover the meniscus depletion experiment gave a baseline at the top of the column which was coincident with the 430-nm scan, indicating the absence of very low-molecular-weight material which absorbs in the u.v. region. Low-speed sedimentation equilibrium experiments were performed on bovine serum albumin, egg albumin and β-lactoglobulin dissolved in 0·1 M NaCl to check the operation of the absorption optical system. In all cases molecular weights were within 7% of the expected values. More extensive measurements were made on reduced and carboxymethylated lysozyme in 8 M urea at the same speeds used for the meniscus depletion experiments on the beak protein. Both optical systems gave molecular weights which were within 4% of the sequence molecular weight. The values found with the absorption optical system were generally a few per cent lower than those determined with Rayleigh interference optics. Inconsistency of molecular weights determined by the two optical systems appears to be a characteristic of solubilized keratins. This was found previously for a high-tyrosine component from wool (Frenkel *et al.* 1973) and is also found with the low-sulphur components (E. F. Woods, unpublished data).

Unless all the possible errors discussed above are cumulative the only way to explain the molecular weight data is on the basis of heterogeneity of the solution. Proteins vary little in their refractive index increments but their u.v. absorbances vary by a factor of ten or more. Thus in mixtures, a component of very low u.v. absorbance will contribute little to the heterogeneity analysis when using the photoelectric scanning absorption optical system even though it is present in an appreciable amount. In methods of analysis based on the refractive index increment of the solute, such as the Rayleigh interference method, such solutions will show heterogeneity. Conversely a component (protein or nucleic acid) of high u.v. absorbance will contribute to the u.v. scan on the basis of its absorption and not its weight fraction in solution, and thus its amount is overestimated when the scanner is used. The differences in the beak protein molecular weight found with the two optical systems may be explained by the presence of a higher-molecular-weight component which has a low u.v. absorbance. Electrophoresis of the zinc-precipitable fraction in SDS–polyacrylamide gels (Fig. 3*c* of Frenkel and Gillespie 1976) shows only one main band with just a trace of higher-molecular-weight material. Cellulose acetate electrophoresis of fraction 4b (Fig. 6*b* of Frenkel and Gillespie 1976) shows only a trace of contaminating protein which corresponds in electrophoretic mobility to the other major protein found in the zinc-precipitable fraction. However, as discussed by Frenkel and Gillespie (1976), the amino acid composition does indicate chemical heterogeneity since many amino acids are not present in integral amounts per mole. Nevertheless, it appears justified to conclude that the molecular weight of the main component would have

a value between that obtained by the two optical systems, i.e. between 11 300 and 13 700.

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Manuscript received 26 July 1976