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THE CONFORMATION OF EYE-LENS PROTEINS STUDIED BY MEANS OF OPTICAL ROTATORY DISPERSION

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SUMMARY

1. The conformation in solution of eye-lens proteins obtained from cortical extracts of adult bovine lenses, was studied by means of the optical rotatory dispersion method.

2. The rotatory data were analysed according to the modified two-term Drude equation, the MOFFITT–YANG equation and the one-term Drude equation.

3. The lens proteins appeared to consist of a mixture of α-helix, random coil and some other structure, which disappeared upon denaturation by acid, alkali or 8 M urea, and also in 2-chloroethanol.

4. From a comparison with other proteins it seems probable that this other structure has a β conformation.

INTRODUCTION

The ORD method has proved to be very important for the study of protein conformation. Most previous workers in this field have treated their visible and near-ultraviolet ORD data according to the MOFFITT–YANG equation[1] or the one-term Drude equation. These equations provide information about the conformation of the protein molecules in solution and where no structures other than α-helical and random or a mixture of these are present, also give the helix content of the molecules. BLOUT, SCHMIER AND SIMMONS[2] found that ORD measurements in the far ultraviolet give the same information, making use of the conformation-dependent Cotton effects in the 190–240-mμ region. The recent analysis of SHECHTER and co-workers[3–5] of these Cotton effects led to the introduction of a new equation: the modified two-term Drude equation which gives a very good fit of visible and near-ultraviolet rotatory dispersion. The advantage of this method is that it not only allows the determination of α-helix content of proteins in solution, but also permits the detection of other ordered structures.

The present work describes the ORD of some eye-lens proteins in water and

Abbreviation: ORD, optical rotatory dispersion.

other solvents over a wavelength range of 220 to 550 mμ. The rotatory properties of poly-L-glutamic acid, bovine serum albumin, β-lactoglobulin and γ-globulin were measured for comparison. The results suggest that the lens proteins do not consist of either α-helices, random conformations or mixtures of these two conformations, but that other conformations are involved, which disappear upon denaturation by urea, acid or alkali. In the light of recent publications[6–9] it seems probable that these protein molecules consist of a mixture of α-helices, random conformations and β structures.

EXPERIMENTAL PROCEDURE

Materials

Poly-L-glutamic acid, crystalline bovine serum albumin, β-lactoglobulin and bovine γ-globulin were commercial preparations. The lens proteins were obtained by one of us (A.F.V.D.) from cortical extracts of adult bovine lenses[10,11].

α-Crystallin was prepared by repeated gel filtration on Sephadex G-200 and was shown by immunoelectrophoresis to be essentially free from the other lens proteins.

Some other fractions, designated by the symbols B, C, D and E, were obtained in the same way. Agar-gel electrophoresis revealed that B, C and D represented most of the β-crystallins.

Fraction E contained the lens proteins of low molecular weight and was found to consist of the γ-crystallins, β_S- and some other β-crystallins and pre-α-crystallin.

β_S-Crystallin was obtained in homogeneous form from Fraction E by repeated chromatography on DEAE-Sephadex A-50.

ORD measurements

The measurements were made with an automatic recording spectropolarimeter, Polarmatic 62 (Bendix Electronics Ltd.) at room temperature in the wavelength range of 220 to 550 mμ with an Osram 150-W xenon lamp. The entrance slit and the exit slit were set, respectively, at 0.5 and 0.4 mm in the near ultraviolet and at 1.0 and 0.8 mm in the far ultraviolet. The cells had a pathlength of 10 mm and the instrument was calibrated at 546 mμ with high-grade sucrose, dissolved in water. All solutions were filtered through sintered glass or centrifuged before use to remove dust and fibers.

Concentration

The protein concentration in the solutions was about 0.5 % for measurements in the visible and near-ultraviolet region and 0.01 % for far-ultraviolet measurements and was determined from the absorbance at 280 mμ. The extinction coefficients ($E^{1\%}_{1\,cm}$ at 280 mμ) were based on dry weight determination after evaporating and drying a known amount of solution of the protein in water at 110° and cooling in a desiccator over P_2O_5. In some experiments (*e.g.* with poly-L-glutamic acid) the solution was made by dilution of a standard solution, the concentration of which was found by dry weight determination. The uncertainty in the protein concentration was estimated at about 2 % and was of the same order or somewhat greater than the uncertainty in the rotation, except in the far-ultraviolet region (below 250 mμ).

Analysis of ORD data

The observed rotations α_λ were expressed in terms of the reduced mean residual specific rotation, $[R']_\lambda$, *i.e.* the specific rotation $[\alpha]_\lambda$ corrected for the mean residual molecular weight M_0 and the refractive index n of the solvent

$$[R']_\lambda = [\alpha]_\lambda \frac{3\,M_0}{100(n^2+2)} \tag{1}$$

where $[\alpha]_\lambda = 100\ \alpha_\lambda/lc$, l is the path length (dm) and c is the concentration of the protein (g/100 ml). For the lens proteins M_0 was assumed to be 116 (except for β_s-crystallin, for which $M_0 = 117$) for β-lactoglobulin and γ-globulin 112, for bovine serum albumin 118 and for poly-L-glutamic acid 169, according to the observation that poly-L-glutamic acid after drying retains 1 mole of hydration water per amino acid residue[12,13]. The refractive indices were taken from FOSS AND SCHELLMAN[14]. The ORD data, measured in the 300–550-mμ region were analyzed according to the one-term Drude equation

$$[\alpha]_\lambda = \frac{K}{\lambda^2 - \lambda_c^2} \tag{2}$$

the MOFFITT–YANG equation

$$[R']_\lambda = \frac{a_0\lambda_0^2}{\lambda^2 - \lambda_0^2} + \frac{b_0\lambda_0^4}{(\lambda^2 - \lambda_0^2)^2} \tag{3}$$

and the modified two-term Drude equation

$$[R']_\lambda = \frac{A_{193}193^2}{\lambda^2 - 193^2} + \frac{A_{225}225^2}{\lambda^2 - 225^2} \tag{4}$$

RESULTS

To test the experimental procedure a SHECHTER AND BLOUT plot of helical poly-L-glutamic acid in water was made. The coefficients A_{225} and A_{193}, found from this plot, are in excellent agreement with those of SHECHTER AND BLOUT ($A_{225} = -2050$, $A_{193} = +2900$). Fig. 1 shows the common Drude plots of α-crystallin in Tris buffer (0.1 M; pH 8.0) and in water. Fig. 2 represents the MOFFITT–YANG plot of α-crystallin in water and Fig. 3 the SHECHTER AND BLOUT plot of α-crystallin in Tris

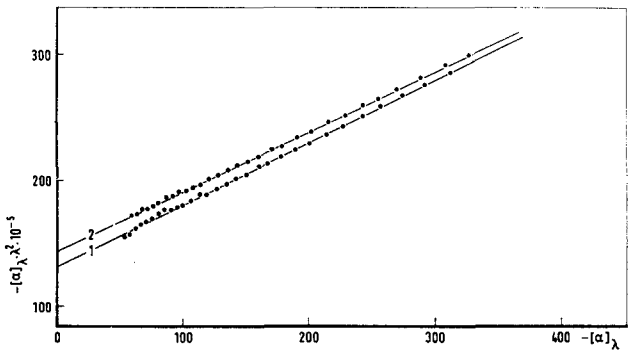

Fig. 1. ORD data of α-crystallin, plotted according to the one-term Drude equation. 1, α-crystallin in water; 2, α-crystallin in 0.1 M Tris buffer (pH 8.0).

buffer. For the other lens protein fractions the rotatory parameters were determined using only the ORD data down to 333 mμ. These measurements were not extended down to 300 mμ, because of the appearance of a Cotton effect around 280 mμ, associated with the aromatic absorption bands of tryptophan and tyrosine. This Cotton effect

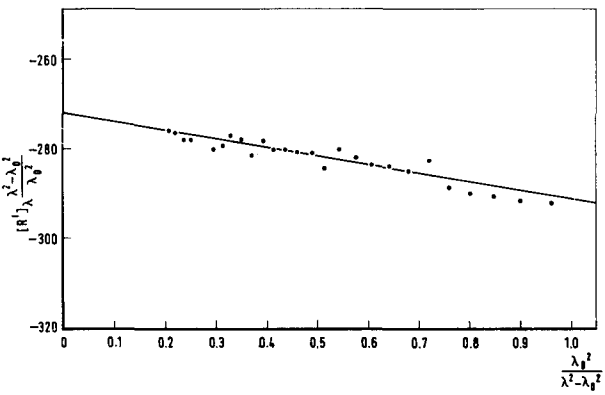

Fig. 2. MOFFITT–YANG plot of α-crystallin in water. $\lambda_0 = 212$ mμ.

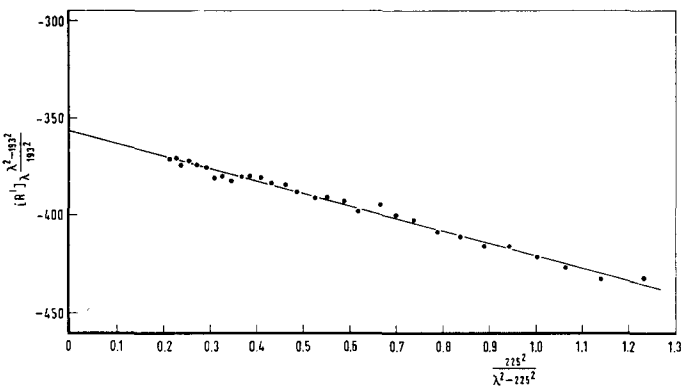

Fig. 3. SHECHTER AND BLOUT plot of α-crystallin in 0.1 M Tris buffer (pH 8.0).

has also been reported for some other proteins, *e.g.* for ribonuclease (*cf.* GLAZER AND SIMMONS[15]). The ORD curves of the lens proteins between 220 and 300 mμ are shown in Fig. 4. The proteins had to be dissolved in water instead of in 0.1 M Tris buffer because of the high absorption of the buffer in the far ultraviolet. Around 280 mμ the anomalous behaviour, corresponding to the aromatic absorption bands, can be seen. All proteins showed a slight trough with the minimum value lying between 233 and 236 mμ. Since polypeptides and proteins of the α-helix–random coil type always show a trough at 233 mμ and the magnitude of the rotation at this wavelength permits the estimation of α-helix content of the molecules[2] we derived also from Fig. 4 the value of $[R']_{233\,m\mu}$ for the various lens proteins. The optical rotation measurements below 220 mμ became very questionable due to the high absorbance of the proteins and are therefore not reported here.

The rotatory parameters of the lens proteins and the three other proteins in

some solvents and in various states of denaturation are collected in Table I. The coefficients A_{193} and A_{225} from the first and the second column of this table are plotted according to the method of SHECHTER AND BLOUT in Fig. 5. The results suggest that the lens proteins do not show great differences in conformation among themselves and are not made up of a mixture of α-helix and random coil alone, because they do not fall on the line determined by SHECHTER AND BLOUT for α-helical proteins,

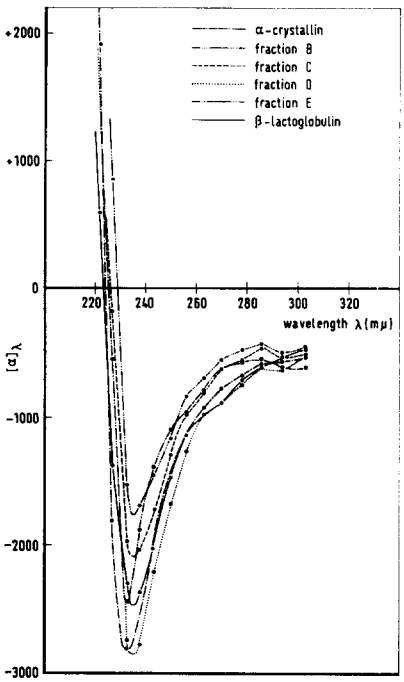

Fig. 4. ORD curves of the lens proteins and β-lactoglobulin in the far ultraviolet. The proteins are dissolved in water.

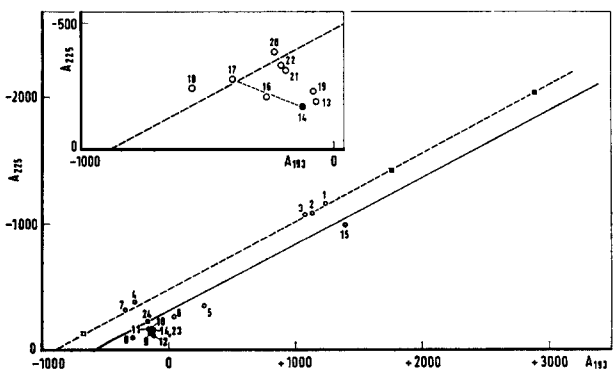

Fig. 5. Plot of A_{225} *versus* A_{193} for poly-L-glutamic acid, the lens proteins and some other proteins. The numbers correspond to the entries in Table I. Poly-L-glutamic acid in aqueous solution, pH 4.66 (■), pH 5.34 (⊠), pH 8.14 (□). α-Helical proteins in aqueous solution and in organic solvents are represented respectively by the broken line and the dashed line according to SHECHTER AND BLOUT. The inset graph represents α-crystallin in various solvents.

TABLE I

ROTATORY PARAMETERS OF EYE-LENS PROTEINS AND SOME OTHER PROTEINS

The parameters were calculated from Eqns. 2–4. The duration of action of the denaturing agent is placed in parentheses when it is longer than about 2 h. Tris means 0.1 M Tris buffer (pH 8.0). The values of $[R']_{233\ m\mu}$ are determined for solutions of the proteins in water.

Protein	*Solvent*	*Modified two-term Drude equation*		*Moffitt–Yang equation*		*One-term Drude equation*	$[R']_{233\ m\mu}$
		A_{193}	A_{225}	b_0	a_0	λ_c	
1 bovine serum albumin	Water	+1236	−1158	−344	−298	(263)	
2 bovine serum albumin	Tris	+1128	−1083	−320	−304	(258)	
3 bovine serum albumin	0.1 M NaCl	+1075	−1067	−313	−320	(257)	
4 bovine serum albumin	Tris + 8 M urea (30 h)	− 267	− 383	− 36	−654	219	
5 β-lactoglobulin	Water	+ 280	− 344	− 75	−166	247	−2000
6 β-lactoglobulin	Tris	+ 51	− 270	− 45	−265	232	
7 β-lactoglobulin	Tris + 8 M urea (20 h)	− 338	− 319	− 15	−640	216	
8 γ-globulin	Tris	− 282	− 88	+ 19	−334	208	
9 crystallin, Fraction B	Tris	− 151	− 129	− 5	−271	215	−1350
10 crystallin, Fraction C	Tris	− 126	− 169	− 12	−295	219	−1750
11 crystallin, Fraction D	Tris	− 147	− 161	− 14	−300	220	−2450
12 crystallin, Fraction E	Tris	− 122	− 102	− 7	−226	218	−2150
23 β_s-crystallin	Tris	− 121	− 161	− 11	−281	220	
24 β_s-crystallin	Tris + 8 M urea	− 162	− 217	− 25	−375	222	
13 α-crystallin	Water	− 70	− 188	− 19	−272	223	−2500
14 α-crystallin	Tris	− 125	− 169	− 13	−295	218	
15 α-crystallin	2-Chloroethanol	+1395	− 985	−336	+ 30	(303)	
16 α-crystallin	Tris + 8 M urea	− 266	− 209	− 2	−457	215	
17 α-crystallin	Tris + 8 M urea (24 h)	− 400	− 278	− 6	−639	216	
18 α-crystallin	Tris + 8 M urea (65 h)	− 560	− 245	+ 3	−731	212	
19 α-crystallin	Tris (urea removed by dialysis)	− 79	− 228	− 32	−323	220	
20 α-crystallin	HCl (pH 2.7)	− 234	− 386	− 47	−625	223	
21 α-crystallin	NaOH (pH 11.0)	− 191	− 311	− 24	−514	219	
22 α-crystallin	NaOH (pH 12.2, 24 h)	− 209	− 327	− 24	−548	218	

e.g. bovine serum albumin, in water. The same phenomenon is observed for β-lactoglobulin and γ-globulin. All of these proteins must therefore contain some other structures, the ORD of which is different from that for the helix or the random conformation. As can be seen in Fig. 5, this structure disappeared for the greater part, or completely, upon denaturation of α-crystallin by 8 M urea, acid or alkali, since the data for the denatured protein fall on or near the line for α-helix and random-coil proteins. The denaturation by urea showed a time dependence; after 24 h α-crystallin seemed to become a mixture of α-helix and random conformation only, with a very low α-helix content. The denaturation by urea seemed also to be reversible, since the original structure largely reappeared after dialysis against Tris buffer. For β-lactoglobulin, the other structure also disappeared upon denaturation by urea. It is known that α-crystallin splits up into subunits when it becomes denatured, because the sedimentation coefficient $s_{20,w}$ is 1.8 S in 7 M urea (BLOEMENDAL *et al.*[16]) in contrast with the value 19.4 S in water at pH 8.1 (SPECTOR AND KATZ[17]). Since the dissociation into subunits paralleled the disappearance in 8 M urea of the non-helical ordered structure, it seems very probable that this structure keeps the subunits together in the native protein. The subunits themselves can further consist of a mixture of α-helix and random coil.

The same phenomenon was observed for the action of acid and alkali on α-crystallin, which was very similar but did not show a time dependence. In these solvents too, the protein consisted of a mixture of helix and random conformation only, with a higher helix content than in 8 M urea. SPECTOR AND KATZ[17] found for α-crystallin at pH 2.1 two components with $s_{20,w}$ values of 1.3 S and 5.9 S, so under these circumstances the protein also dissociates into subunits.

The data for α-crystallin in 2-chloroethanol fall near the line, determined by SHECHTER AND BLOUT, for α-helical proteins in organic solvents. The deviation from the straight line is not more than observed by SHECHTER AND BLOUT for various proteins in organic solvents. This means that in chloroethanol also the original conformation which was not helical or random, disappeared and was presumably transformed into a helix, since the helical content was much higher in chloroethanol than in water or Tris buffer solution. The disappearance of this other structure was again accompanied by a dissociation into subunits, because we found that $s_{20,w}$ was 1.2 S in 2-chloroethanol.

In conclusion we think that α-crystallin in the native state consists of a mixture of α-helix, random conformation and another structure, which keeps the subunits together in the native protein. In 8 M urea, acid, alkali or 2-chloroethanol this structure disappears and the protein dissociates into subunits. The subunits are composed of both helices and random conformations. The helix content of the individual subunits decreases by the denaturing action of urea, but increases by the helix-promoting solvent 2-chloroethanol.

As can be seen from Fig. 5 and Table I, there was some difference between the ORD measurements for bovine serum albumin, β-lactoglobulin and α-crystallin in pure water and in Tris buffer solutions. The results for bovine serum albumin lead to the conclusion that the helix content in water was somewhat higher than in the buffer solutions. We think this was an effect of ionic strength rather than a slight denaturing action of the Tris buffer, since the same effect was found in 0.1 M NaCl. The smallest effect was shown by α-crystallin and the greatest by β-lactoglobulin.

Our values for β-lactoglobulin in water also differ a little from those of SHECHTER AND BLOUT. This may have been caused by a small difference of the protein samples.

The values of b_0 from the MOFFITT–YANG equation, collected in Table I, are near zero for the crystallins, suggesting that these proteins have a very low helix content. In 2-chloroethanol the helix content was much higher and almost the same as that of bovine serum albumin in water, because b_0 was strongly negative and nearly the same for both. However, when the protein contains another structure than α-helix and random coil only, it is not possible to calculate the helix content from b_0, unless the contribution of the other structure to b_0 is known.

λ_c was also very low for the lens proteins. This is typical for a denatured protein, *e.g.* bovine serum albumin or β-lactoglobulin in 8 M urea. For bovine serum albumin in aqueous solutions, and for α-crystallin in 2-chloroethanol, λ_c could not be determined with any accuracy because the plot of $[\alpha]_\lambda\lambda^2$ *versus* $[\alpha]_\lambda$ did not give a straight line. This means that the dispersion was not simple and that the proteins presumably contained a rather high α-helix content. For this reason, the λ_c values for these samples in Table I are placed in parentheses. For Fraction B, λ_c was somewhat lower than for the other lens proteins, although the difference was in the order of magnitude of the accuracy (about 2 mμ).

The published $[R']_{233m\mu}$ values for the helical and the random conformation are very uncertain (YANG AND MCCABE[12]) and vary from −12000 to −18000 for a complete α-helix and from −1800 to −2000 for a random coil. In spite of this uncertainty, the values for the crystallins in Table I suggest that these proteins did not contain α-helix at all, or only a very small fraction. This conclusion, however, would be justified only if no structures other than helical and random were present, since these structures presumably give a contribution to the rotation at 233 mμ. Moreover, the value for Fraction B was so low that it seemed not to be a random protein only.

DISCUSSION

The most interesting question now is, what other structure or structures may be present in the lens proteins and particularly in α-crystallin. Comparing the ORD properties of the crystallins with those of other proteins and polypeptides, for example β-lactoglobulin, γ-globulin, ovalbumin, lysozyme and poly-L-serine, the conformation of which has been studied previously by means of the ORD method and other techniques, we found it very likely, that β structures are also present.

To correlate the rather low levorotation and the low b_0 of β-lactoglobulin, URNES AND DOTY[18] supposed that in these protein helices, random conformations and β structures were present. A different explanation is given by TANFORD, DE AND TAGGART[19] who proposed the occurrence of hydrophobic-bonded areas in β-lactoglobulin. TOMIMATSU AND GAFFIELD[6] studied the ORD properties of ovalbumin, conalbumin and lysozyme in 0.05 M NaCl and found that the data for these proteins, analyzed by the method of SHECHTER AND BLOUT, do not fall on the line for helical proteins in aqueous solution. According to these authors it is most likely that the three proteins also contain hydrophobic-bonded segments. The occurrence of β conformations seems not very probable to these authors, since if these conformations were present, they would have to be present as aggregates of denatured protein which is not very likely in this case. On the other hand, it is reasonable to conceive the native

α-crystallin molecules as aggregates build up of a rather large number of subunits, so that the presence of β structures is quite possible.

Concerning the structure of lysozyme, PHILLIPS[9] reported that in this protein β conformations are most likely present. Thus we think that the structure, found by TOMIMATSU AND GAFFIELD in aqueous solutions of lysozyme, which is not helical or random, can at least partly be a β conformation.

Recently TIMASHEFF AND TOWNEND[7] reported that it is very probable that in β-lactoglobulin β structures are present, since the infrared spectrum of an aqueous solution of this protein in the native form displays an amide I band with a maximum at 1632 cm^{-1}, a frequency normally associated with the β conformation, while upon denaturation a shift to 1643 cm^{-1} is observed, pointing to a transformation into random-coil protein. Preliminary studies of the infrared spectrum of α-crystallin in KBr discs showed an amide I band at 1629 cm^{-1} supporting the presence of a β conformation. For the α-helix this band lies at about 1650 cm^{-1}.

BOHAK AND KATCHALSKI[20] measured the ORD of poly-L-serine and their results point to the presence of a β conformation. These results were confirmed by the X-ray powder diagram and the infrared data for poly-L-serine in KBr discs. The amide I band was found at 1634 cm^{-1}. Remarkable in this respect is the finding of SHECHTER AND BLOUT that poly-L-serine in water also must contain structures other than α-helix and random coil only.

TROITSKII[8] applied the MOFFITT equation, as extended by WADA, TSUBOI AND KONISHI[21] to the conformational analysis of a number of proteins including β-lactoglobulin, γ-globulin and ovalbumin. He found that in all these proteins, except serum albumin, β structures are present. It is possible to employ the method of WADA, TSUBOI AND KONISHI for calculating the content of β conformation. The results, however, are in our opinion not very certain since the measurements of WADA, TSUBOI AND KONISHI were carried out in organic solvents and the rotatory constants of the β form, determined by these authors, are not in agreement with the results of other investigators. (For a detailed review see URNES AND DOTY[18].) Using the data of WADA, TSUBOI AND KONISHI we calculated the fraction of α-helix (f_H), β conformation (f_β) and random coil (f_R) for α-crystallin in the native form and in some states of denaturation, by means of the following equations proposed by IMAHORI[22].

$$a_0 = a_0^R + f_H a_0^H + f_\beta a_0^\beta \quad (5)$$

$$b_0 = f_H b_0^H + f_\beta b_0^\beta \quad (6)$$

$$f_H + f_R + f_\beta = 1 \quad (7)$$

Here a_0^R means the contribution to a_0 from the completely disordered protein. a_0^H, a_0^β, b_0^H and b_0^β are the values of a_0 and b_0 for the α-helix and the β conformation, respectively. The random coil does not give a contribution to b_0. For these constants we used the following values: $a_0^R = -730$, $a_0^H = +650$, $a_0^\beta = +840$, $b_0^H = -630$ and $b_0^\beta = +420$. The results of the calculation are collected in Table II. The low absolute value of b_0 for the lens proteins can be explained by supposing that the proteins contain both helices and β structures, because the contribution to b_0 is negative for the helix and positive for the β conformation. For both structures the contribution to a_0 is positive but for the random coil strongly negative. This explains why, on denaturation of α-crystallin, b_0 does not change much but a_0 becomes more negative since helices and

β structures may be broken by the denaturing agent. For a_0^R we used the value found for α-crystallin in Tris buffer *plus* 8 M urea after 65 h. However, it is not certain that the protein was completely disordered in this case. Moreover, it is not certain either that in other solvents (*e.g.* in Tris buffer without 8 M urea) a_0^R does not alter. So the data in Table II must be considered with some reserve.

TABLE II

FRACTIONS OF THE HELIX, RANDOM COIL AND β FORM OF α-CRYSTALLIN IN VARIOUS SOLVENTS

Solvent	b_0	a_0	f_H	$f\beta$	f_R
Tris	−13	−295	0.24	0.33	0.43
Tris + 8 M urea	− 2	−457	0.15	0.22	0.63
Tris + 8 M urea (24 h)	− 6	−639	0.05	0.06	0.89
Tris + 8 M urea (65 h)	+ 3	−731	0.00	0.00	1.00
Tris (urea removed by dialysis)	−32	−323	0.25	0.29	0.46
HCl (pH 2.7)	−47	−625	0.10	0.04	0.86

In conclusion we can say that the eye-lens proteins must contain other structure or structures than α-helical or random conformations only. It seems very probable that this other structure (or at least one of them) is the β conformation. We are aware of the fact that the presence of β structures has not been proved and that this conformation is not *per se* the only possibility, but we think it gives a reasonable explanation.

It is known that the eye lens has a layered structure[23] and that β conformations in proteins can lead to the formation of flat structures. When these flat frames indeed give rise to the forming of layers it would be possible for the β conformation in the lens proteins to be responsible for the particular structure of the eye lens. Moreover the transformation of α-crystallin into insoluble albumoid during aging of the eye lens may be ascribed to the known tendency of β structures to aggregate (*cf.* ref. 24).

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