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POLY-S-CARBOBENZOXYMETHYL-L-CYSTEINE: A MODEL SYSTEM FOR THE DENATURATION OF NON-HELICAL PROTEINS

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SUMMARY

The optical-rotatory dispersion and viscosity properties of poly-S-carbobenzoxymethyl-L-cysteine have been studied in mixtures of dichloroacetic acid and ethylene dichloride. As the proportion of the more polar solvent increases there is a large increase in the intrinsic viscosity $[\eta]$ and large decreases in the specific optical rotation $[\alpha]_\lambda$ and the parameter a_0 calculated from the MOFFITT–YANG equation. The changes in these properties occur over such a narrow range of solvent composition as to suggest that a cooperative transition is occurring between two molecular forms. However, there is no change in the optical-rotatory constant b_0, indicating that the transition is not one between a helical and a random-coil conformation. The changes observed with this polypeptide are typical of those which occur during the denaturation of such globular proteins as β-lactoglobulin and γ-globulin which are also characterised by the absence of appreciable helical contents in their native states.

INTRODUCTION

Several years ago the pioneering work of DOTY, BLOUT, BAMFORD and co-workers (*cf.* URNES AND DOTY[1]) established that, according to the solvent environment, many polyamino acids can exist in either of two conformational states. These states may be characterised by the value of the parameter b_0 in the MOFFITT–YANG[2] equation relating specific optical rotation $[\alpha]$ with wavelength (λ)

$$m' = \frac{3}{n^2+2}\cdot\frac{M}{100}\cdot[\alpha] = a_0\left(\frac{\lambda_0^2}{\lambda^2-\lambda_0^2}\right) + b_0\left(\frac{\lambda_0^2}{\lambda^2-\lambda_0^2}\right)^2$$

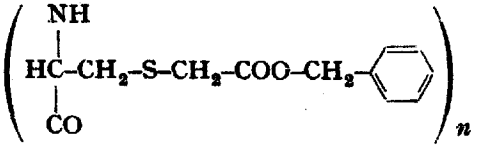
In this equation m' is the reduced residue rotation in which $[\alpha]$ is corrected for the refractive index of the solvent n and the mean residue weight M, and a_0, b_0, and λ_0 (taken as 212 mμ) are constants.

Under one set of solvent conditions the constant b_0 had a characteristic value in the vicinity of —640 and the polyamino acid showed solution properties expected for a rod-like particle with a mass per unit length consistent with the α-helical conformation. By suitably altering the conditions of its environment (*e.g.* by a change in temperature, solvent, pH etc.) the polyamino acid could be transformed to the random-coil conformation and under these conditions the value of b_0 was zero.

The changes in properties which occur during the helix–coil transition for such polyamino acids are very similar to those which occur on denaturation of many globular proteins and denaturation was therefore often interpreted in terms of a helix–coil transition. Thus, the denaturation of bovine serum albumin in 8 M urea solutions was correlated with the concomitant decrease in the fraction of the molecule in the helical form f_H as calculated from the optical-rotatory-dispersion constant b_0 ($f_H = -b_0/640$) (see ref. 3).

On the other hand, it has become apparent during recent years that the denaturation of native proteins cannot always be interpreted in terms of this type of transition. For example proteins such as β-lactoglobulin and γ-globulin are apparently almost devoid of helix in the native state as indicated by a value of b_0 close to zero[4–7]; on denaturation these proteins show no change in b_0 but both a_0 and $[\alpha]_\lambda$ become much more negative. It has been suggested[5] that the changes in a_0 and $[\alpha]_\lambda$ are due to alterations in the environment of the peptide bonds as a result of changes in the tertiary structure of the protein on denaturation. TANFORD, DE AND TAGGART[5] consider that in the native state the peptide groups of these proteins are mainly buried in a non-polar environment due to the compact folding of the molecule; on denaturation the protein unfolds so that the peptide groups are in a more polar aqueous environment. TANFORD[8] has recently measured the optical-rotatory dispersion properties in dioxane–water mixtures of a model compound *N*-acetylglutamic acid which contains the –CONH– grouping. He showed that, as the fraction of the less polar dioxane in the mixture was increased, both $[\alpha]_\lambda$ and a_0 gradually became more positive. This demonstrated the effect the solvent environment of the amide group could have on the optical-rotatory properties of a protein molecule, even in the absence of any change in its secondary or tertiary structure.

We have recently been studying the influence of the sulphur atom in the side-chain substituents of polyamino acids on their conformational properties (*cf.* ref. 9). This has led to the discovery of an even more striking demonstration of the effect of the polarity of the solvent on optical-rotatory-dispersion properties. Moreover, the changes observed are more akin to those occurring on protein denaturation since not only does the molecule poly-*S*-carbobenzoxymethyl-L-cysteine



contain a polypeptide backbone but it also undergoes a conformational transition over a very narrow range of solvent composition.

EXPERIMENTAL

Materials

Methylene chloride was distilled from solid potassium hydroxide pellets immediately before use. Dioxane was purified and dried according to the method of VOGEL[10]. Light petroleum of boiling range 55–70° was dried over sodium. Benzene was dried over sodium and redistilled. Tri-*n*-butylamine (A. R. grade) was stored over solid potassium hydroxide and used without further purification. Dichloroacetic acid was

redistilled at 20 mm pressure and ethylene dichloride at normal pressure. Benzyl chloroacetate was prepared from chloroacetyl chloride and benzyl alcohol according to the method of REMIZOV AND KROMOV-BORISOV[11]. The fraction of b.p. 103° at 0.8 mm was collected $n_D^{24} = 1.5236$.

Melting points are uncorrected. Microanalyses were performed by the C.S.I.R.O. and University of Melbourne microanalytical laboratory.

S-Carbobenzoxymethyl-L-cysteine: Cysteine (L. Light and Co. Ltd.) (12.1 g) was dissolved in water (100 ml) and filtered through a Celite filter pad. The solution was stirred vigorously and sodium bicarbonate (13 g) and a solution of benzyl chloroacetate (22 g) in ethanol (20 ml) were added all at once. Stirring was continued for 24 h, the crystalline solid filtered and washed successively with water, alcohol, and ether and dried in air. Yield 22.0 g (82 %). The solid recrystallized as needles from hot water–ethanol. m.p. 160–161°; $[\alpha]_D^{21} -7.6°$ in 1 N HCl (c = 1.12). (Found: C, 53.17; H, 5.84; O, 23.5; S, 11.88. $C_{12}H_{15}NO_4S$ requires, C, 53.51; H, 5.61; O, 23.76; S, 11.90 %.)

S-Carbobenzoxymethyl-N-carboxy-L-cysteine anhydride: S-carbobenzoxymethyl-L-cysteine (2.0 g) was suspended in dry dioxane (50 ml) at 50° and phosgene passed in under stirring for 30 min, after which time the gas inlet was disconnected and the stirring continued for a further 60 min. Dry nitrogen was then passed in for 2 h to remove excess phosgene and the filtered solution taken to dryness *in vacuo* at 40°. The crystalline residue was recrystallized twice from boiling dry benzene, yield 1.75 g (80 %). m.p. 109° (decomp.); $[\alpha]_D^{21} -48.7°$ in ethylacetate (c = 1.25). (Found: C, 52.97; H, 4.91; O, 26.9; S, 10.74. $C_{13}H_{13}NO_5S$ requires C, 52.87; H, 4.44; O, 27.09; S, 10.86 %.)

Poly-S-carbobenzoxymethyl-L-cysteine: S-carbobenzoxymethyl-N-carboxy-L-cysteine anhydride (10.2 g) was dissolved in methylene chloride (400 ml) and tri-*n*-butylamine (2.13 ml of a 1 % (w/v) solution in benzene) added. After standing for 5 days at room temperature, the gelatinous suspension was poured with stirring into light petroleum (2 volumes) and the polymer collected and dried in air, yield 8.5 g (96 %). $\eta_{sp}/c = 0.42$ dl/g in dichloroacetic acid (c = 0.5). (Found: C, 56.66; H, 5.31; O, 20.0; S, 13.07. $(C_{12}H_{13}NO_3S)_n$ requires C, 57.35; H, 5.21; O, 19.10; S, 12.76 %.) Soluble in dichloroacetic acid, trifluoroacetic acid, and acetophenone.

Preparation of solutions

Although dissolution is slow, requiring several hours stirring, poly-S-carbobenzoxymethyl-L-cysteine is very soluble in dichloroacetic acid and 5 % solutions could be prepared without difficulty. On the other hand the polymer could not be directly dissolved in ethylene dichloride and solutions in the mixed solvent system were prepared by adding appropriate volumes of ethylene dichloride to solutions in dichloroacetic acid. The solutions thus prepared were stable and showed no tendency to precipitate or gel on standing. Ethylene dichloride does not seem to be a non-solvent for this polymer as in one instance a solution in ethylene dichloride – dichloroacetic acid (9:1, v/v) was dialysed using Visking 18/32 "Cellophane" tubing against several changes of ethylene dichloride. Although a large proportion of the polymer diffused through the membrane so that the resulting solution was too dilute for physical measurements, the polymer retained by the membrane remained in solution even

though the dichloroacetic acid content of the solution was probably in the vicinity of 0.01 %.

The polymer concentrations of the solutions on which optical-rotatory-dispersion measurements were made were in the range 0.3–0.4 %.

Methods

Optical-rotatory-dispersion measurements were made using a photoelectric polarimeter (W. F. Stanley and Son) in conjunction with a quartz-prism monochromator (Carl Zeiss). A mercury arc light source was employed, measurements being made at 578, 546, 486, 436, 405, 365 and 334 mμ. Solutions were contained in jacketed 1-dm cells through which water at 25° was circulated.

Viscosity measurements were made at 25° using simple Ostwald viscometers.

RESULTS AND DISCUSSION

The optical-rotatory dispersion data obtained in ethylene dichloride–dichloroacetic acid mixtures of different compositions (v/v) are shown in Fig. 1 plotted in terms of the MOFFITT–YANG equation taking $\lambda_0 = 212$ mμ. There is very little change in the slope of these plots ($b_0 = -70 \pm 10$) as the composition of solvent is varied, suggesting that the solvent composition has no influence on the secondary structure of this polypeptide. The small magnitude of the value for b_0 makes it unlikely that there is any periodic arrangement of the polypeptide backbone even when the proportion of helix-favouring solvent (ethylene dichloride) is as high as 95 %.

In contrast to the lack of any change in b_0, Fig. 1 demonstrates the striking effect on a_0 (*i.e.* the intercept of the curve with the ordinate) as the proportion of the less polar ethylene dichloride is increased. These changes were shown to be reversible by adding dichloroacetic acid to a 93 % ethylene dichloride solution ($a_0 = +350$)

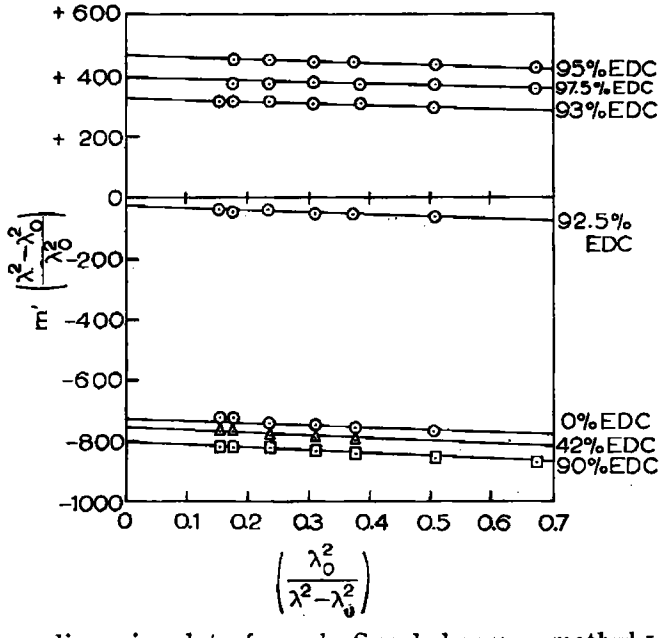


Fig. 1. Optical-rotatory-dispersion data for poly-*S*-carbobenzoxy-methyl-L-cysteine in dichloroacetic acid–ethylene dichloride mixtures plotted in terms of the MOFFITT–YANG equation taking $\lambda_0 = 212$ mμ. The slope of these plots gives the parameter b_0 and their intercept with the ordinate the parameter a_0. EDC, ethylene dichloride.

to give an ethylene dichloride concentration of 83% whereupon a_0 decreased to −830. The effect of adding ethylene dichloride to dichloroacetic acid solutions of polymer is in the same direction as that observed by TANFORD[8] for *N*-acetylglutamic acid in the system dioxane–water in that a_0 becomes more positive as the proportion of less polar solvent is increased. However, whereas the change in a_0 was continuous over the whole range of solvent composition for the small molecule, for the polypeptide a_0 changed from approx. −700 to approx. +400 over the range 92–94% ethylene dichloride. Changes in $[\alpha]_{546}$ of the same magnitude and in the same direction have also been observed by FASMAN AND BLOUT[12] for poly-*O*-acetyl-L-serine in the solvent system dichloroacetic acid–chloroform. These changes occurred over a somewhat wider range of solvent composition than that encountered in the present study and at high chloroform contents the solution gelled on standing. These results were interpreted in terms of formation of a β-conformation on addition of chloroform. The sharp change in physical properties over such a narrow range of solvent composition reported here is characteristic of a cooperative transition between two different forms of a molecule. As has been pointed out before (*e.g.* TANFORD, DE AND TAGGART[5]) although a value of b_0 close to zero is indicative of a lack of regularity in the arrangements of the polypeptide backbone it does not preclude other types of folding in the molecule.

TABLE I

VALUES FOR $[\eta]$, a_0, AND $[\alpha]_{546}$ FOR POLY-*S*-CARBOBENZOXYMETHYL-L-CYSTEINE IN DICHLOROACETIC ACID–ETHYLENE DICHLORIDE MIXTURES

Solvent (% ethylene dichloride (v/v))	$[\eta]$ *(dl/g)*	a_0	$[\alpha]_{546}$
0	0.43	−730	−76
88	0.45	−800	−80
95	0.075	+400	+37

In order to test whether in fact the transition observed in optical-rotatory properties was associated with two forms of the molecule of different shape, viscosities of solutions of the polyamino acid in dichloroacetic acid, 88% ethylene dichloride, and 95% ethylene dichloride were determined, the values of intrinsic viscosity $[\eta]$ being shown in Table I, together with the corresponding values of a_0 and also $[\alpha]_{546}$. The values of $[\eta]$ in the solvent mixtures are subject to fairly large errors (±10%) due to the volatility of ethylene dichloride and the sensitivity of the solvent viscosity to small changes in solvent composition. Nevertheless, it is clear that, over the same range of solvent compositions in which a_0 becomes considerably more positive, the molecule changes to a form which is either much more compactly folded or considerable less solvated.

There is a striking similarity in the transitions reported above to those which occur on denaturation of a group of globular proteins of which γ-globulin[6] and β-lactoglobulin[13] are typical examples. This group is characterised by values of b_0 close to zero for the protein in its native state, indicative of the absence of any α-helical structure. On denaturation by, *e.g.*, urea or heating there is scarcely any change in b_0 but both a_0 and $[\alpha]\lambda$ become considerably more negative. Moreover the

transition occurring on denaturation[6] appears to be of a cooperative nature similar to that encountered with the polyamino acid. At the same time there is a considerable increase in viscosity on denaturation. As mentioned in the introduction TANFORD, DE AND TAGGART[5] have suggested that in the native state the molecule is compactly folded and the peptide groups are in a relatively non-polar environment. On denaturation the molecule unfolds leading to the observed increase in intrinsic viscosity and at the same time the peptide groups are exposed to a more polar (aqueous) environment. A similar interpretation can be placed on the results for the polyamino acids reported above. At concentrations of ethylene dichloride above 93 % the molecule is tightly folded so that the peptide groups are not accessible to the dichloroacetic acid. With the high proportion of benzyl groups the environment is likely to be much more non-polar than that in a protein and thus correspondingly more positive values for a_0 and $[\alpha]\lambda$ are found. When the proportion of dichloroacetic acid reaches a critical value the molecule apparently unfolds so that the peptide groups become accessible to the polar solvent, whereupon there is an increase in $[\eta]$, and a_0 and $[\alpha]\lambda$ decrease to values typical of denatured proteins.

Thus, in the same way as polyamino acids such as poly-L-glutamic acid, poly-L-lysine etc. serve as models for proteins which are at least partially helical in the native state, so the polyamino acid described in this paper can serve as a model for proteins which are folded but not helical in the native state.

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