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Temperature Behaviour of Human Serum Albumin

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Structural alterations of albumin, their dependence on concentration and the role of free –SH groups at thermal denaturation, as well as the reversibility of thermally induced structural changes, were studied. Application of various physical methods provides information on a series of structural parameters in a major concentration range. Apart from changes of the helix content, heat treatment gives rise to β structures which are amplified on cooling and which are correlated with the aggregation of albumin. With rising temperature and concentration the proportion of β structures and aggregates increases.

At degrees of denaturation of up to 20% complete renaturation is possible in every case. The structure content is concentration-dependent even at room temperature. It may be that intermolecular interactions induce additional α-helix structures which are less stable, however, than the ones stabilized by intramolecular interactions. Unfolding of the pocket containing the free –SH group of cysteine-34 enables disulphide bridges to be formed leading to stable aggregates and irreversible structural alterations. Through binding of *N*-ethylmaleimide to free –SH groups, which blocks the formation of disulphide bridges, it is possible to prevent aggregation and irreversible conformational changes. At temperatures below 65–70 °C, oligomers are formed mainly via intermolecular β structures.

In the preparation of human serum albumin for clinical purposes its behaviour at different temperatures is of importance. The albumin is treated at 60 °C for about 10 h to inactivate the hepatitis virus. The structure should be largely retained of course in this treatment. Sodium octanoate or sodium octanoate + acetyltryptophan may be used as stabilizer. We distinguish in general two stages in the heat treatment of albumin. The first stage includes reversible structural alterations, the second one includes irreversible structural alterations, which may not necessarily result in a complete destruction of the ordered structure [1–3]. Although a number of investigations are available on the problem of the thermal exposure of albumin, there are still open questions to be answered concerning in particular the nature of structural alterations, the limits of reversibility, the influence of concentration and environmental conditions and the molecular mechanism of the action of the stabilizers and their relative effectiveness under various conditions. Experiments performed on horse serum albumin by Zimmermann and Dittmar [4] showed that higher molecular weight components will be increasingly formed after heat treatment of 15 min at 100 °C. If the time of exposure is 60 min or more, the aggregation products will decompose into low-molecular-weight fragments which are serologically inactive. They may still cause, however, severe shock reactions in anaphylaxia experiments. The authors suggest that species despecifications and desantigenization may only be obtained by a specific splitting of peptide bonds and not by changing the secondary structure. Aoki et al. [5] studied the termal denaturation of bovine serum albumin in the alkali pH range. The results obtained by gel electrophoresis show various components after the heat treatment at 65 °C. Brand and Anderson [6] described the influence of heating and fatty acids on the aggregate formation.

The changes in the physicochemical properties of albumin, which have already been described in the literature, are partly contradictory. The reason is that on the one hand the results obtained imply method-specific information, for instance on the aggregational behaviour and the development of β structure [2, 6–10], and on the other hand the con-

Abbreviations. H→^{2}H exchange, hydrogen–deuterium exchange; ESR, electron spin resonance; CD, circular dichroism; MalNEt, *N*-ethylmaleimide.

centration dependence of the effects have been largely neglected.

We have, therefore, attempted to obtain more profound information on the changes in the physicochemical properties of albumin during thermal treatment, in particular on the influence of the concentration and of the free −SH groups of albumin by the use of various physicochemical methods which also permit the carrying out of investigations in a larger concentration range. A later communication will report on possibilities of influencing structural alterations in the thermal treatment of albumin.

MATERIALS AND METHODS

Human serum albumin (commercial product, lyophilized) from the Institut für Impfstoffe (Dessau, G.D.R.) was used for the investigations. If not indicated otherwise, 0.1 M phosphate buffer, pH 6, containing 0.2 M NaCl, served as solvent. The determination of concentration was performed by spectral photometry with $\lambda = 280$ nm on the basis of an absorption coefficient of $A^{1\%}_{1\,cm} = 5.8$ [10]. The samples used for electron spin resonance (ESR) measurements had been filtered through gel (Sephadex G-150). Mercaptalbumin was chromatographically prepared by the separation of albumin molecules by DEAE-Sephadex A-50 [11]. The electrophoretic analysis showed, in agreement with sedimentation measurement, that the mercaptalbumin contained monomers and only a low content of dimers. The labels 2,2,4,4-tetramethyl - 1,2,3,4, - tetrahydro - 5,6 - benzo - γ - carboline-3-oxyl(I) and 3-maleimido-2,2,5,5-tetramethylpyrrolidine-1-oxyl(II) have been used for the ESR investigations. Label I, being non-covalently bound to albumin, was added to the albumin solutions 10 min before the beginning of measurements. A 0.02 M ethanol solution of the label was prepared for that purpose in advance. The conditions of titration were chosen so that the ethanol concentration was 1% or less.

For the covalent labelling of Cys-34 with label II the protein solution was incubated with a 50-times label excess at 15 °C for 30 min. Then the unbound label molecules were separated on Sephadex G-25. The number of −SH groups labelled could be determined by double integration of the ESR spectra obtained after labelling. Disc electrophoresis with polyacrylamide gel was used for gel electrophoretic investigations.

The circular dichroism (CD) measurements were performed on a dichrograph CD 185 (Roussel-Jouan). The measurement of temperature dependence was carried out in a hermetically sealed cell after achieving equilibrium, i.e. when a change in CD could no longer be found (this was the case after heating for 1 h).

The accuracy of temperature measurements was ± 0.2 °C. The structure contents (α-helices, β structures and residual structures ϱ) were calculated according to the curve-fitting method [12]. The CD basis spectra of Chen et al. [13] were used with the calculations. The −SH groups were determined by means of Ellman's reagent [14]. The ESR spectra were recorded with a Varian E3 spectrometer. An on-line computer (KRS 4200) served for the spectral analysis [15]. The maximum sucrose concentration was 37.5 w/v in the determination of the rotational correlation time.

The viscosity was measured by a rotating cartesian-diver viscometer and an Ubbelode viscometer.

The infrared spectra of the solutions were measured by the Perkin-Elmer 180 infrared spectrometer 180. The albumin samples dissolved in 0.05 M ^2H_2O phosphate buffer (pH 7.4) were heated in a thermostated cell and kept at the specified temperature until no further spectral change could be demonstrated in the range of amide I band (about 1 h). Infrared spectroscopic measurements on films were performed in connexion with the H → ^{2}H exchange measurements.

H → ^{2}H exchange kinetics were observed on thin films at a temperature of 25 °C and a relative humidity of 97% from the decrease in intensity of the amide A band. The intensities of the amide A band were subsequently normalized to the amide I band. The instrumentation used for the measurements has been described elsewhere [16]. Films of heated albumin solutions (0.5 − 1 h at 80 °C) and untreated albumin solutions were studied. For the film preparation salt-free solutions were used.

The sedimentation behaviour of the albumin samples was studied in an analytical ultracentrifuge Spinco E with ultraviolet absorption optics, monochromator, and photo-electric scanner in the speed range of 17000 to 40000 rev./min at 20 °C. The albumin solutions of different concentrations were first heated to the specified temperature for 1 h and brought to a concentration of 1.4 mg/ml, if required, by dilution with phosphate buffer after cooling down. The concentration gradients were recorded using monochromatic light at $\lambda = 280$ nm. The sedimentation coefficients were calculated from the time shift of the gradients and corrected to standard conditions (water, 20 °C). The concentration portions of monomers and oligomers were calculated from the absorbance ratios of the gradients.

RESULTS

THERMAL DENATURATION

CD Measurements

There are four peaks presents in the CD spectra: at $\lambda = 262$ nm and 268 nm (optically active transitions

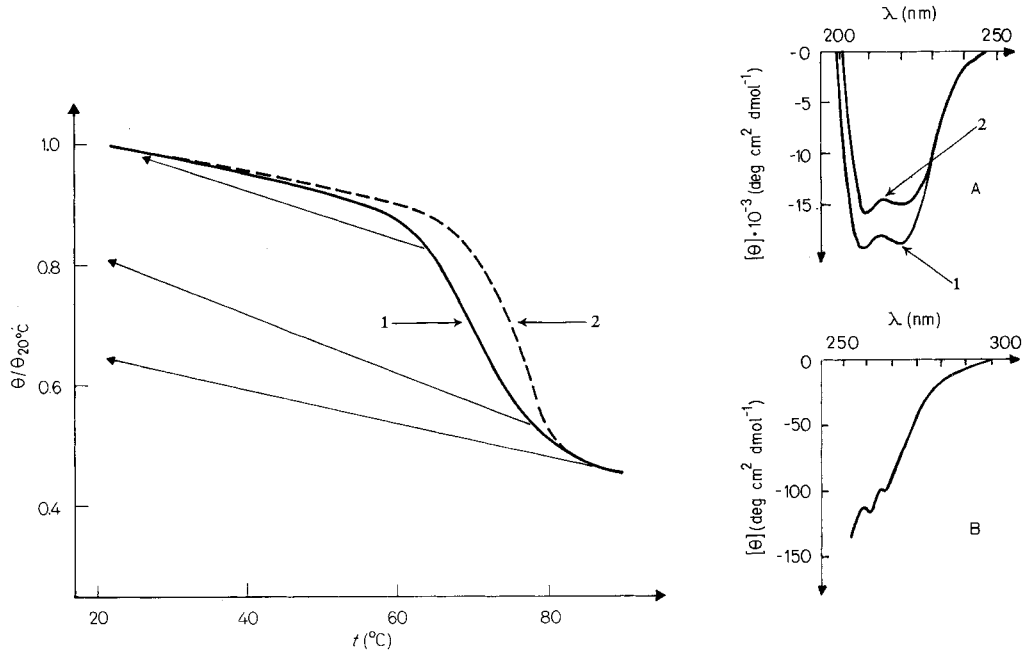

Fig. 1. *Normalized CD melting curves of albumin using* $[\Theta]_{220}$. (1) $c = 0.5$ mg/ml; (2) $c = 0.05$ mg/ml. The degree of renaturation after heating to $t = 65\,^{\circ}C$; $t = 75\,^{\circ}C$; $t = 90\,^{\circ}C$ and recooling to 20 °C is indicated by arrows. Insert (A): CD spectrum of albumin in the peptide chromophore region. (1) $c = 0.5$ mg/ml; (2) $c = 0.05$ mg/ml; path lengths = 1 mm and 10 mm respectively. Insert (B): CD spectrum of albumin in the region of aromatic chromophores. $c = 2$ mg/ml; path length = 10 mm

of the aromatic amino acids and disulphide bonds) and at $\lambda = 208$ nm and 220 nm (peptide absorption range) (Fig. 1).

The amplitude of these peaks do not show any major difference in their dependence on temperature. Below 60 °C the melting curves measured at $\lambda = 262$ nm and 268 nm have a somewhat more pronounced decrease than those measured at $\lambda = 208$ nm and 220 nm, where the measurements in the temperature range are reflecting those changes on the albumin molecule which do not yet have any influence on the helix content and are reversible. The CD peak at 220 nm has been analyzed mainly for the present investigaton as the amplitude of this band is correlated with the helix content of the protein.

We could find only a slight decrease of the CD amplitude up to 60 °C; then a further more pronounced reduction follows between 65 °C and 80 °C. There is a degree of denaturation of about 50% at 80 °C (Fig. 1). While the changes in the CD spectrum which may be obtained up to 65 °C are completely reversible, the samples heated to 80 °C will be subject to an irreversible denaturation of about 40% (Table 1).

The structure contents of the renaturated samples heated to $t_D = 65\,^{\circ}C$ correspond approximately to those of the original values. The denaturation at 75 °C results in an increase in the residual structure contents at the cost of the α-helix content. The β structure contents will remain nearly constant, but increase, however, during cooling down at the cost of the α-helices (Table 1).

ESR Measurements

The spin labels I (non-covalently bound) and II (covalently bound on the cysteine-34) were used to characterize the structural changes. The advantage of label I and label II as compared to other labels is that I [2] as well as II [17] has only one binding site on the albumin. When labelling with II one obtains an ESR spectrum consisting of two components (A and B in Fig. 2) ($c_{albumin} = 5$ mg/ml). There is 98% of the bound spin labels in a structured environment (in Fig. 2 marked with A) at 20 °C. The remaining 2% (B) correspond to an unfolded environment of the spin labels. From the temperature dependence of the ratio between the components A and B in the ESR spectrum the beginning of a more pronounced unfolding of the albumin molecule from 55 °C can be seen (Fig. 3). This unfolding will be still reversible up to a temperature of 68 °C.

It may be concluded from the expansion of the (+1) component of the spectral content A and the increasing distance of the components of A ($2A'_{zz}$ in Fig. 2) by 0.5 G as compared to the spectrum at 20 °C that particles of different molecular weight will be formed (increase of the rotational correlation time by the formation of larger particles). The t_m value of 73 °C (50% content in an immobilized environment, Fig. 3) is in relatively good agreement with the values obtained from CD data. That means that the increase in label mobility by unfolding of the pocket containing the free −SH group of cysteine-34 [18] and the con-

Table 1. *Degree of denaturation* D_t *(at the temperature of denaturation* t_D*), degree of irreversible denaturation* I_t *(measured after heating to the temperature of denaturation* t_D *and subsequent cooling down to 20 °C), and structure contents (α, β, ϱ) at different temperatures* t_D *with the mean standard deviations of the curve-fitting results*
D = denatured; R = renatured

Substance	t_D	D_t	I_t	α_D	β_D	ϱ_D	α_R	β_R	ϱ_R
	°C	%							
Albumin	20	–	–	61.1 ± 0.4	22.2 ± 1.5	16.7 ± 1.4	–	–	–
Albumin + MalNEt	20	–	–	69.9 ± 0.5	24.9 ± 1.9	5.3 ± 1.9	–	–	–
Albumin	65	12	0	54.9 ± 0.5	16.3 ± 2.0	28.8 ± 1.9	59.6 ± 0.3	24.7 ± 1.2	15.8 ± 1.2
Albumin + MalNEt	65	10	0	60.0 ± 0.6	18.0 ± 2.2	22.5 ± 2.1	66.7 ± 0.5	21.5 ± 2.0	11.8 ± 2.0
Albumin	75	45	30	33.6 ± 0.4	22.6 ± 1.5	43.8 ± 1.4	40.8 ± 0.4	28.1 ± 1.7	31.0 ± 1.6
Albumin + MalNEt	75	30	15	50.8 ± 0.5	21.8 ± 2.1	27.4 ± 2.0	61.8 ± 0.5	29.4 ± 2.0	8.7 ± 2.0
Mercaptalbumin	20	–	–	66.3 ± 0.5	11.0 ± 2.4	22.7 ± 2.3	–	–	–
Mercaptalbumin + MalNEt	20	–	–	68.3 ± 0.6	7.0 ± 2.4	24.7 ± 2.4	–	–	–
Mercaptalbumin	65	12	0	60.0 ± 0.8	7.2 ± 2.9	32.8 ± 2.8	68.5 ± 0.9	3.3 ± 3.6	28.1 ± 3.5
Mercaptalbumin + MalNEt	65	10	0	62.8 ± 0.8	7.0 ± 3.2	30.3 ± 3.1	68.1 ± 0.6	11.9 ± 2.2	20.2 ± 2.1
Mercaptalbumin	75	38	22	43.6 ± 0.7	10.4 ± 2.9	46.0 ± 2.8	53.4 ± 0.6	18.8 ± 2.2	27.8 ± 2.2
Mercaptalbumin + MalNEt	75	20	0	57.0 ± 0.8	6.5 ± 3.2	36.5 ± 3.1	68.6 ± 0.6	10.9 ± 2.4	20.5 ± 2.3

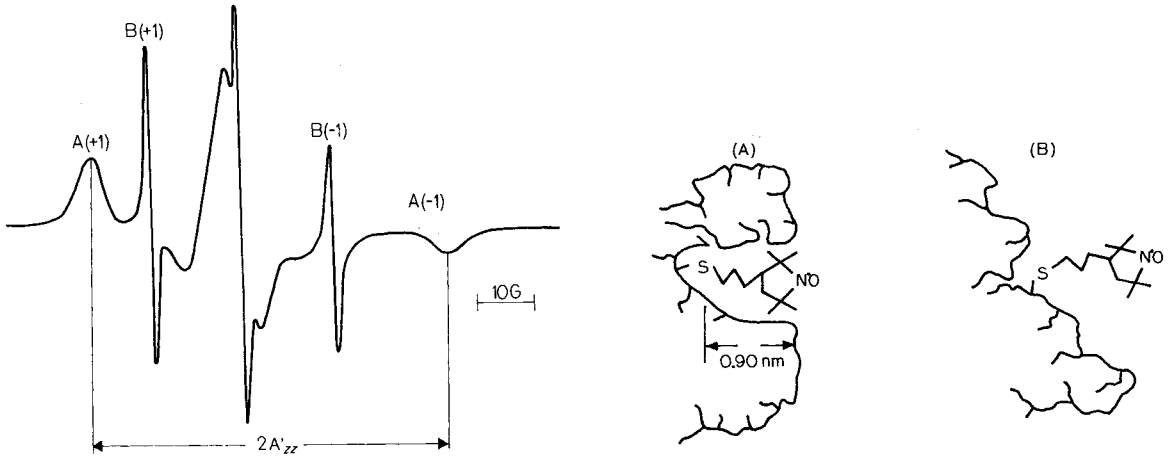

Fig. 2. *ESR spectrum of albumin labelled with label II.* A, strongly immobilized spectral components; B, weakly immobilized spectral components. Insert: schematic drawing of the different environments of label II bound to albumin according to A and B

formation contents measured by CD will take place in parallel, the relative final values obtained, however, being different. A degree of denaturation of the protein of about 45% taken from CD measurements (Table 1) corresponds to an unfolding rate of about 90% near the label. The cooling down of samples to 20 °C after heating to temperatures of 68 °C or more with incubation times of 5 and 20 min will result in different degrees of irreversible structural changes (Fig. 3). In addition the temperature effect was also observed by means of the probe I (Fig. 3). The mobile spectral content of 2% at 20 °C is due in this case to the spin label molecules being free in solution.

The increase in the content of free spin label molecules, which may be found with a temperature rise, can be explained by a decreasing structurization of the binding area of the label on the protein. The same t_m value of 73 °C may be obtained when using the probe I and the label II as well (Fig. 3).

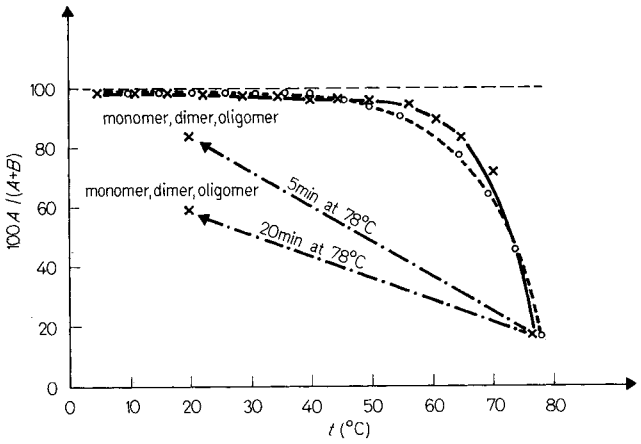

Fig. 3. *Temperature dependence of the ESR spectral component A (%) of label II covalently bound to albumin* (×). The arrows indicate the reversibility after different heating times. Temperature dependence of the ESR spectral component A of label I (%) noncovalently bound to albumin (O)

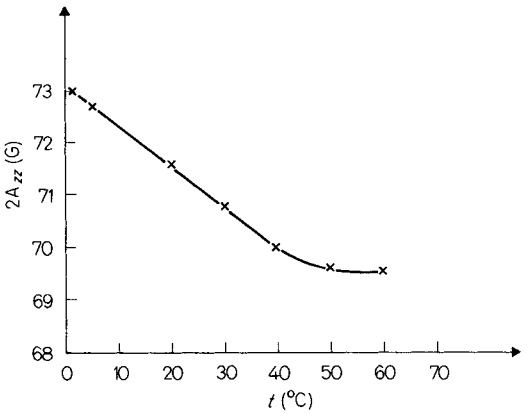

Fig. 4. *Determination of the* A_{zz} *values* ($2A_{zz}$) *at different temperatures, using McConnell's method [19]*

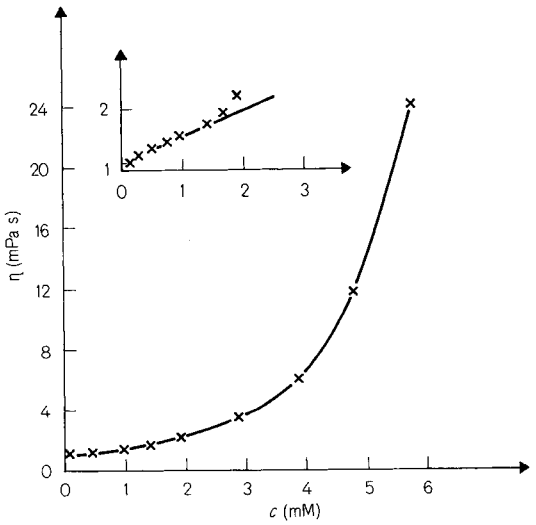

Fig. 5. *Dynamic viscosity* η *of albumin as a function of protein concentration.* Insert: linear region for low protein concentrations

The change in polarity around the NO was observed by means of label I (Fig. 4). With rising temperature the interaction between the spin label and the albumin molecule (according to the van der Waal's interaction) becomes more intensive and the NO group comes into stronger interaction with apolar areas. At 60 °C the line width changes as the rotational movement grows faster so that a further determination of this parameter is no longer possible.

INFLUENCE OF ALBUMIN CONCENTRATION ON THE TEMPERATURE BEHAVIOUR

CD Measurements

The t_m value for the lower concentration (0.05 mg/ml) is higher than that for the higher concentration (0.5 mg/ml) (Fig. 1). The difference (Δt_m) is 5 °C. Albumin concentrations of 0.05 mg/ml and 0.5 mg/ml yield different CD curves (Fig. 1). The structure contents are also different for both the concentrations. We determined the following structure contents at 20 °C and an albumin concentration of 0.05 mg/ml: α-helices = 52%; β structures = 6%; residual structures ϱ = 41%. The α-helix content with $c_{albumin}$ = 0.05 mg/ml is about 15% lower than with $c_{albumin}$ = 0.5 mg/ml.

After heating to 90 °C (we may heat up to 90 °C at $c_{albumin}$ = 0.05 mg/ml without any visible turbidity) and cooling down to 20 °C we have: α = 41%; β = 12% and ϱ = 48%.

It appears to be essential that in addition to the helix contents the β structure contents are also lower than with $c_{albumin}$ = 0.5 mg/ml. This is certainly due to a lower degree of aggregation with less intermolecular β structure contents (see Table 1). While no turbidity of the solution could be found with albumin concentrations $\leq$ 0.5 mg/ml at a temperature of 75 °C, a turbidity and precipitation were observed at the same temperature with $c_{albumin}$ = 5 mg/ml, so that ultraviolet optical investigations in this range of concentrations at such high temperatures are no longer possible. This can be demonstrated by CD measurements at λ = 268 nm when a higher concentration can be used because of the relatively low absorption. With $c_{albumin}$ = 2 mg/ml, after decreasing, the CD values begin to increase at 78 °C, indicating increasing turbidity. At 82 °C an apparent CD value will be a attained which corresponds to the original value at 20 °C.

The degree of irreversible denaturation does not exceed 40% in any case investigated. When stabilizers are used (M. Zinke, M. Becker, R. Wetzel, unpublished results) the same final result will also be obtained. Gel electrophoretic investigation then shows only monomers and higher aggregates.

Oligomers of different sizes could be found at temperatures of 60 °C or more (but less than 80 °C) and c = 0.5 mg/ml.

Mercaptalbumin, however, will precipitate by 85 °C even when 2 mM sodium octanoate is used as stabilizer (M. Zinke, M. Becker, R. Wetzel, unpublished results), as measured by gel electrophoresis.

Without addition of sodium octanoate the samples with $c_{albumin}$ = 5.0 mg/ml and $c_{albumin}$ = 50 mg/ml will precipitate at a temperature of 75 °C.

Viscometric Investigations

We have to distinguish between a linear range of the concentration dependence in the viscometric behaviour up to 65 mg/ml (approximately 1 mM)

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