COMPARISON OF HUMAN HAEMOGLOBIN AND GLOBIN IN DENATURATION

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The paper compares the behaviour of human methaemoglobin cyanide and globin under denaturing conditions. Changes of dichroic spectra, produced by isothermal denaturation with urea and/or guanidine hydrochloride, and heat precipitation in the presence of the two denaturants have been investigated. The courses of the denaturation curves, structures of the denatured forms and the effects of the denaturants on heat-stability of the proteins are discussed.

Globin is known to have fewer α-helical segments than haemoglobin; we have found it to be less resistant to denaturation in all cases. This indicates that those peptide parts of haemoglobin whose helicity has been upset by the splitting-off of the haem group do not, in globin, form a structure comparable in stability to the α-helix. In view of the low thermal stability of globin we believe that the groups, which interact with haem in haemoglobin, are on the surface of the globin molecule and that their hydrophobicity increases its tendency to precipitate.

Human haemoglobin and its apoprotein, globin, were compared from various points of view in our laboratory[1,2] and elsewhere[3-5]. The purpose of these studies was to elucidate the structure and function of haemoglobin, as well as the effect of prosthetic groups on the spatial arrangement and other properties of protein generally.

The behaviour of haemoglobin under various denaturing conditions has been studied extensively[6-8], but comparable data for globin are still lacking. Being interested in the conformational features of globin we have attempted to compare its denaturation with that of haemoglobin. Differences in stability of the native structures and in denaturation reactions may contribute to information on the effect of haem on the conformation of the peptide part of the protein molecule.

First we investigated isothermal denaturation of haemoglobin and globin by urea and/or guanidine hydrochloride (GuHCl). Either denaturant is known to weaken a native protein conformation to such an extent that highly ordered globular structures collapse into random coils[9]. With α-helix-rich proteins, such as haemoglobin[10,11] and globin[2], this breakdown is accompanied by a decrease in the negative dichroic band at 222 nm, characteristic of α-helical peptide chains[12].

Another process paid attention to in this study was heat-induced precipitation of the proteins. Since intermolecular hydrophobic interactions play the decisive role in this process[13] the location of hydrophobic groups in the protein molecule,

particularly their exposure to the solvent and their possible intermolecular reactions, can be assessed from the precipitation curves.

EXPERIMENTAL

Materials. A solution of haemoglobin was prepared by lysis of washed human red cells. The haemolysate was purified with a fresh gel of aluminium hydroxide and centrifuged 30 min at 3000*g* (ref.[13]). The haemoglobin was then converted into methaemoglobin cyanide (MetHbCN) by oxidation with potassium ferricyanide in the presence of potassium cyanide and dialysed against buffer. All experiments were carried out with this form of haemoglobin as the most stable one. In addition, it has approximately the same maximum absorptivity at 540 nm as the corresponding hemichromogen[14], so that even in denaturation the protein concentration can be determined spectrophotometrically.

Globin was prepared according to the procedure described by Rossi-Fanelli and coworkers[15]; the denatured portions were removed by four-fold precipitation at pH 7 (ref.[16]). After dialysis against buffer the solution was centrifuged 30 min at 30000*g*. The solutions used in the experiments were not older than five days, being stored at 4°C.

All measurements were performed in 0·05M sodium phosphate buffer, pH 7·0. All chemicals (Lachema, Brno) were A.G. Guanidine hydrochloride was prepared from twice crystallized guanidine carbonate.

Methods. Circular dichroic (CD) curves were measured in an apparatus JASCO ORD/UV-5 with a CD attachment in a 1 mm cell at 23–25°C, the protein concentration being 0·1%. The curves were registered in several 24-h intervals, until the differences between a curve and the preceding one might be due to experimental error only. The mean residual weight was 108.

The isothermal denaturation curves were fitted on a computer EAI 640, the computation being based on following the diminishing root-mean-square deviation of experimental values from the theoretical curve. The constants were calculated for two valid numerals. Since the values of the constants served only to compare the protein stabilities, no corrections had to be taken for the refractive indices and the activity coefficients. The programme in FORTRAN IV EAI language was worked out in collaboration with Dr I. Kalousek of our Institute.

Heat-denaturation was followed by measuring the decreasing absorbance of the protein solution. The solution (1% MetHbCN, 0·33% globin) was kept in a thermostat for 1 h at a given temperature, then centrifuged. The absorbance of the supernatant, measured at room temperature in a spectrophotometer Unicam SP 700 (540 nm for MetHbCN, 280 nm for globin) and related to the absorbance of the non-heated solution at the same wave length (A_{rel}) was plotted *vs* the given temperature.

RESULTS

Isothermal Denaturation

Globin. Ellipticity of globin at 222 nm, Θ, in relation to concentration of GuHCl or urea is shown in Fig. 1. The two curves obey the frequently used equation[7,9]

$$K = A \,.\, c^{\nu}, \qquad (1)$$

where A and v are constants and c is the molar concentration of a denaturant. The symbol K designates the equilibrium constant of the denaturation, which can be expressed as

$$K = [\mathrm{D}]/[\mathrm{N}] = (\Theta_0 - \Theta)/(\Theta - \Theta_\infty)\,, \qquad (2)$$

where Θ_0 denotes ellipticity at $c = 0$, Θ_∞ the limit ellipticity at high c values and Θ the measured ellipticity; [N] and [D] designate the native and the denaturated portions, respectively. Combination of (*1*) and (*2*) gives

$$\Theta = \frac{\Theta_0 + \Theta_\infty \,.\, Ac^v}{1 + Ac^v}\,. \qquad (3)$$

This equation describes the curves for globin in Fig. 1. The constants A and v were determined by the curve-fitting method; the parameters Θ_0 and Θ_∞ were read out from the curves.

The mid-transition denaturant concentration ($c_{1/2}$) is a parameter expressing the efficiency of a denaturant on a given protein; its value can be taken directly from the curve as a denaturant concentration corresponding to the ellipticity $(\Theta_0 + \Theta_\infty)/2$. If K is set equal to 1 and $c_{1/2}$ is substituted for c in equation (*1*) the quantity A can be expressed

$$A = 1/(c_{1/2})^v\,. \qquad (4)$$

The values of A from Eq. (*4*), designated as A_{cal}, along with $c_{1/2}$, A, v, Θ_0 and Θ_∞ are given in Table I; A_{cal}'s agree with the A values obtained by the curve-fitting method.

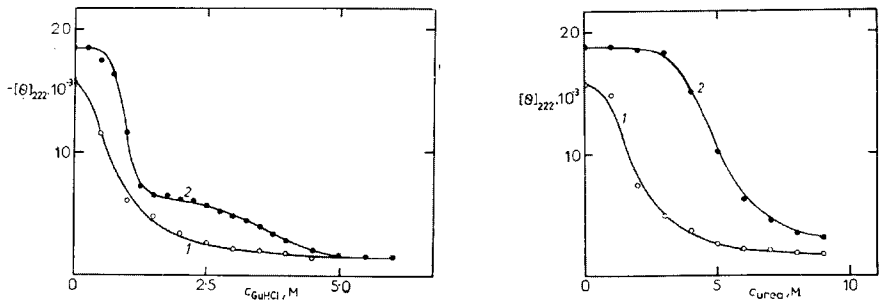

FIG. 1

Mean Residual Ellipticity of Proteins at 222 nm in Relation to Concentration of Denaturants
a) Guanidine hydrochloride, *b*) urea; *1* human globin, *2* human methaemoglobin cyanide.

Methaemoglobin cyanide. The denaturation curve of MetHbCN in urea (Fig. 1*b*) is similar to that of globin, so that it could be described by equations (*3*) and (*4*). In this case, however, the denaturant concentration could not be raised to such a degree as to allow us to read out the Θ_∞ value directly from the curve. For this reason it was determined tentatively, until the experimental points agreed with the calculated curve throughout the concentration range of the denaturant. The value of Θ_∞ is higher than that for globin (Table I).

The dependence of Θ for MetHbCN on concentration of GuHCl (Fig. 1*a*) exhibits a long plateau in the region of 2M-GuHCl. Therefore we assume that there is a relatively stable intermediate[9] between the native and the denatured forms. The two equilibrium constants can then be defined by

$$K_1 = [\mathrm{I}]/[\mathrm{N}] = A_1 c^{\nu_1}, \tag{5}$$

$$K_2 = [\mathrm{D}]/[\mathrm{I}] = A_2 c^{\nu_2}, \tag{6}$$

where [I] is the portion of the intermediate of ellipticity Θ_I, the other symbols being the same as in equations (*1*) and (*2*). Combination of equations (*5*) and (*6*) with the assumptions

$$[\mathrm{N}] + [\mathrm{I}] + [\mathrm{D}] = 1$$

and

$$\Theta = \Theta_0[\mathrm{N}] + \Theta_\mathrm{I}[\mathrm{I}] + \Theta_\infty[\mathrm{D}]$$

yields

$$\Theta = \frac{\Theta_0 + \Theta_\mathrm{I} A_1 c^{\nu_1} + \Theta_\infty A_1 A_2 c^{(\nu_1+\nu_2)}}{1 + A_1 c^{\nu_1} + A_1 A_2 c^{(\nu_1+\nu_2)}}, \tag{7}$$

TABLE I

Parameters of Equations (*3*) and (*7*)

The respective curves are given in Figs 1 and 2.

Protein	Denaturant	$c_{1/2}$ mol/l	ν	A l/mol	A_{cal} l/mol	θ_0 deg. cm^2	θ decimol^{-1}
Globin	urea	1·9	2·6	0·18	0·19	15 600	1 500
	GuHCl	0·75	2·0	1·7	1·8	15 600	1 300
Methaemoglobin cyanide	urea	4·9	5·7	$1{\cdot}0 \cdot 10^{-4}$	$1{\cdot}2 \cdot 10^{-4}$	18 600	2 600
		0·95	6·7	1·4	1·4	18 600	5 900[a]
	GuHCl	3·65	7·1	$1{\cdot}0 \cdot 10^{-4}$	$1{\cdot}0 \cdot 10^{-4}$	5 900[a]	1·300

[a] The θ_I value.

which equation we fitted on the curve for MetHbCN, the quantities A_1, A_2, ν_1, ν_2 and Θ_1 being variables (Table I).

Heat Denaturation

Precipitation of globin and MetHbCN in relation to temperature is shown in Fig. 2. The curves for GuHCl (Fig. 2*a*) are monotonic; the globin curves take a simple sigmoidal course, whereas the MetHbCN curves have plateaus around 75°C.

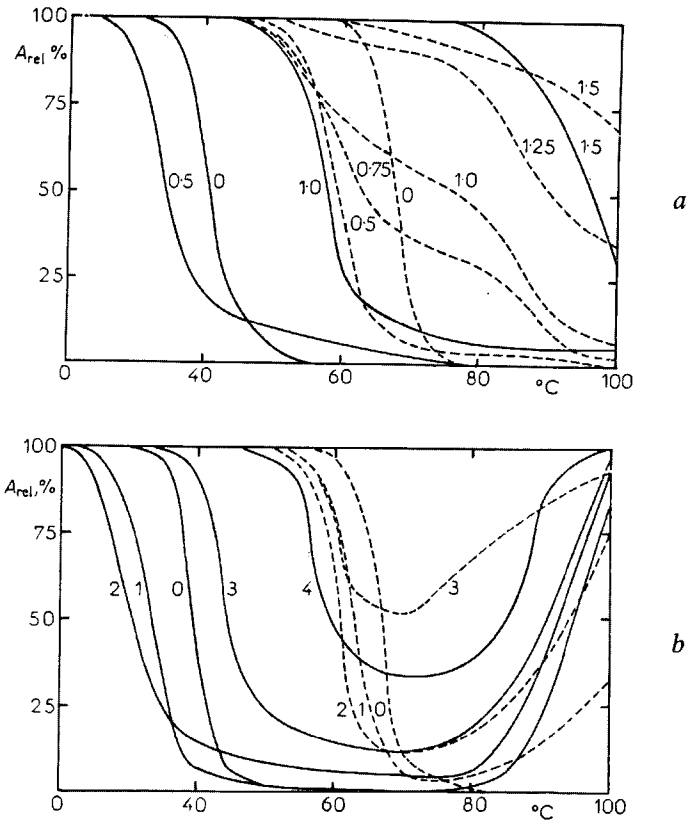

FIG. 2

Temperature Dependence of the Relative Absorbance of Protein Solutions after Heating in the Presence of Denaturants

a Guanidine hydrochloride, *b* urea; solid line: human globin, dashed line: human methaemoglobin cyanide. The starting solutions (0·33% globin and 1% methaemoglobin cyanide) were heated for 1 h; absorbance was measured at 280 and 540 nm, respectively. The molar concentrations of the denaturants are given at the curves. The experimental points are not presented for better survey.

The presence of urea makes the precipitation curves more complex (Fig. 2*b*). With increasing temperature A_{rel} decreases to a minimum, corresponding to about 70°C, then increases. Since at elevated temperatures urea is converted into cyanate[17] we examined the effect of potassium cyanate on the two proteins. Globin boiled in 0·1M-KCNO for 1 h had the same electrophoretic mobility in a polyacrylamide gel as globin boiled in 1M or 4M urea. Further we found that after heating to 90°C in 0·1M-KCNO for 1 h 100% of globin and 50% of MetHbCN remained in the solution. From these results we conclude that the increased solubility of the two proteins in neutral concentrated solutions of urea at high temperatures is due to a decrease in the number of free NH_3^+ groups, as a result of carbamylation[18]. This reaction entails a decrease of the isoelectric point of a protein. In neutral media this decrease is accompanied by an increase in the net-charge and, thus, in solubility.

DISCUSSION

The mechanism of the denaturing action of GuHCl and urea has been widely discussed[9], but not yet satisfactorily explained. In many experiments GuHCl appeared to be a stronger denaturant than urea. This experience accords with our results, both in the isothermal denaturation (see the values of $c_{1/2}$) and in the heat denaturation. Studying the first denaturation step of CO-haemoglobin (dissociation to subunits), Kawahara and coworkers[8] also demonstrated a stronger effect of GuHCl compared to that of urea; the unfolding of the molecule, following the dissociation, was not studied by these authors.

The usual product of an isothermal denaturation by urea or GuHCl is a random coil[9]. This structure was demonstrated by Tanford and coworkers for haemoglobin in 6M-GuHCl by hydrodynamic methods[8,19] and by gel chromatography[20]. The determined value of Θ_∞ in this medium suggests that globin forms a random coil too. In the maximum possible concentration of urea (9M) the structures of the two proteins are not so certain. The value of Θ_∞ for globin was more negative than in Gu. .HCl, but this slight difference might be due to the impossibility of further increasing the urea concentration in order to reach a limit state; correction for the refraction index might also be of some importance. The value of Θ_∞ for MetHbCN in urea was about twice that in GuHCl. This increase can be interpreted as with globin or can be attributed to the existence of a stable intermediate of $\Theta_I \approx -2500$ deg . . $cm^2\ dmol^{-1}$. Denaturation of haemoglobin in solutions of urea and various substituted amides has been described by Elbau and coworkers[7]. The values of the constants in equation (*1*) rather differed from ours; the authors measured the ORD curves too soon (a few hours after the denaturation had started) when, in our experience, no equilibrium could have established.

As has been mentioned, the curves of isothermal denaturation of globin take a simple sigmoidal course, described by equation (*3*). This suggests that the transition

from the native to the denatured state is accompanied by no formation of any stable intermediate. With MetHbCN the denaturation is not so simple, especially in the use of GuHCl. Analysis based on a one-intermediate model gave good results, but considering the other complicating factors of the process (dissociation to subunits[8], non-equivalency of the subunits[3,4], the splitting-off and dimerization of the haem group[21], hemichromogen formation, *etc.*) other reaction mechanisms cannot be ruled out either. A deeper investigation of this problem was outside the scope of the present paper.

Comparison of the isothermal denaturation curves of MetHbCN and globin (the values of $c_{1/2}$) reveals that globin is less resistant to the action of the denaturants than MetHbCN. Similarly, the ν values for MetHbCN are greater than those for globin. It is well known that the greater the extent of change underwent by a protein molecule in the course of denaturation the higher the value of ν (ref.[9]). This indicates that with globin the difference between the native and the denatured structures is smaller than with MetHbCN, or, in other words, a "native" globin molecule, compared to native MetHbCN, contains denatured portions. The parameter ν offers a new approach to assessing the structures of MetHbCN denatured by urea. For assuming that the ν values for GuHCl and urea are approximately equal if the conformation changes of a protein in the two denaturants are the same (which is evidently true with globin), the structure of MetHbCN in 9M urea does not seem fully unfolded, because the ν value is considerably smaller than the sum $(\nu_1 + \nu_2)$ for MetHbCN in GuHCl.

The precipitation of globin and haemoglobin in a non-oxidative medium has been ascribed to association of the protein molecules by interactions of a hydrophobic nature[13]. Although the two proteins precipitate by similar mechanism they differ in their resistance to denaturation. In all experiments globin was more prone to precipitation (precipitated at lower temperatures) than MetHbCN. This phenomenon seems to be due to the exposure of those hydrophobic regions of globin which were in contact with the haem group; regions can reac t intermolecularly, thereby occasioning precipitation. On the other hand the relatively "closed" molecule of MetHbCN, with a majority of hydrophobic groups inaccessible to intermolecular interactions[11], precipitates only at higher temperatures, at which some of the hydrophobic groups are exposed[6].

The denaturants had a strong effect on the heat stability of the proteins: precipitation was enhanced by low concentrations of urea (0–2M) or GuHCl (0–0·75), but suppressed by higher concentrations of the denaturants. Beyond certain concentrations the denaturants prevented the precipitation completely. This phenomenon seems to reflect the dual effect of the denaturants; on the one hand they upset a protein conformation, exposing the hydrophobic parts and inducing precipitation, on the other they function as dispersing agents[20] by weakening hydrophobic

interactions[9]. At low concentrations of the denaturants the exposure of some hydrophobic groups, masked in the native protein, is evidently the dominating factor; at high concentrations the dispersing effect outweighs the former, thus preventing even random coils from precipitation.

In the absence of a denaturant the heat-denaturation curves have the expected sigmoidal form, corresponding to a pronounced cooperative transition[9]. In the presence of a denaturant the curves take a more complex course. With urea they exhibit a minimum near 70°C – we ascribe this phenomenon to shifts of the isoelectric points of the proteins, as a result of carbamylation of NH_3^+ groups. A noteworthy feature of the precipitation curves for GuHCl (especially with MetHbCN) is the plateaus around 75°C. These plateaus indicate that the transition under study is not a simple process (the formation of an intermediate and other complicating factors are possible).

On the basis of our results we arrive at some conclusions on the conformation of a "native" globin molecule. As is known, globin contains fewer α-helical segments than haemoglobin[2,4]. The weaker resistance of globin to chemical denaturants suggests that the conformation stability of those non-periodically built parts which formed the α-helix in haemoglobin is much reduced in globin. These parts appear to be placed on the surface of the globin molecule and, owing to their hydrophobicity, to increase markedly its tendency to precipitate.

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