A SPECTROPHOTOMETRIC STUDY OF REVERSIBILITY OF HUMAN MERCAPTOALBUMIN DENATURATION BY UREA

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The renaturation of the human mercaptoalbumin denatured by urea has been studied spectrophotometrically. The denaturation is reversible up to 15 min treatment with 8 mol dm^{-3} urea, whereas a longer treatment brings about irreversible changes of the mercaptoalbumin molecule. Samples have been prepared of the renatured mercaptoalbumin pre-denatured by 8 mol dm^{-3} urea for a period of either 1 min or 200 min. The temperature perturbation differential and derivation spectrophotometries were used to investigate the localization of tyrosyl residues in native mercaptoalbumin and in the renatured forms. From the results it follows that the localization of tyrosyls in the renatured mercaptoalbumin after 1 min denaturation by 8 mol dm^{-3} urea is the same as that in the native protein. On the other hand, the renaturated mercaptoalbumin after 200 min denaturation contains a greater number of exposed tyrosyls than is the number in the native protein.

In our earlier studies we found considerable differences in the investigations of renaturation of human mercaptoalbumin adopting different methods. Whereas in the case of polarographic monitoring of the renaturation kinetics we found[1] a stepwise lowering of the Brdička current within 15 min, in the case of polarimetry[2] a large change in optical rotation took place before the first measurement was possible (up to 1 min renaturation) followed by a smaller change (about 20% of total change) which ended within 5 min of renaturation. Another observable phenomenon was the dependence of the denaturation reversibility on the time of action of urea. It was found that in the case of 1 min denaturation of mercaptoalbumin with 8 mol dm^{-3} urea this process is reversible, whereas the chromatographic, electrophoretic[3], and polarographic[1] properties of the protein obtained by the renaturation of mercaptoalbumin after 200 min denaturation by 8 mol dm^{-3} urea distinctly differ from the properties of native mercaptoalbumin.

Therefore, we tried to elucidate the differences observed by means of another method describing the conformational changes during denaturation and renaturation from a viewpoint different from that of the above-given methods. For this purpose, we

chose the UV spectrophotometry which characterizes the localization of aromatic chromophores in peptides[4].

The human serumalbumin (HSA) contains 1 tryptophyl, 31 phenylalanyls, and 18 tyrosyls[5]. In studies of HSA structure attention was mostly paid to the tyrosyls out of which about one half are localized on the surface of the molecule. Malan and Edeldoch[4] state that 8.7 tyrosyls are accessible to the nitration agent. Morávek et al.[6] found that 8 residues were strongly nitrated, 4 residues were nitrated to a medium extent, and 6 residues did not undergo nitration. The denaturation makes all the tyrosyls accessible, which is accompanied by a hypsochromic shift of the absorption band[3,7,8], and during renaturation they are again buried.

Beside the problem of denaturation reversibility of mercaptoalbumin by urea in the present paper also dealt with is the localization of tyrosyls and its changes during the denaturation and renaturation processes. For the characterization of arrangement of the tyrosyl residues in the molecules of both native and renatured mercaptoalbumin we adopted the temperature perturbation differential and derivation spectrophotometries[9]. For these measurements we prepared solid samples of renatured mercaptoalbumin, because even low concentrations of urea very much interfere with these measurements.

EXPERIMENTAL

Reagents

Mercaptoalbumin was prepared from human serumalbumin (Imuna, Šarišské Michaľany) by a known procedure[3]. The renatured mercaptoalbumin was prepared in the following way: 100 ml of $5.5 \cdot 10^{-5}$ mol dm^{-3} mercaptoalbumin solution was treated with 800 ml of 9 mol dm^{-3} urea solution, and the denaturation action of urea was allowed to take place either 1 min or 200 min, whereafter the solution was poured into 2 700 ml distilled water to decrese the urea concentration to 2 mol dm^{-3}. Then the mixture was dialyzed in a dialyzation tube (Kalle, Wiesbaden) of 40 mm diameter for 5 × 8 h using a 15 times greater volume of distilled water which was intensively stirred. The solutions of mercaptoalbumin rid of urea were concentrated to a half of original volume using an air flow. The solid samples of renatured mercaptoalbumin R_{SH}^{1} and R_{SH}^{200} were obtained by lyophilization.

The measurements were carried out with an SP8 400 spectrophotometer (Pye Unicam) in 10 mm quartz cells placed in a thermostated holder. The concentrations of mercaptoalbumin were determined correctly with application of the extinction coefficient[10] and checked by means of polarography[3]. The measurements of temperature perturbation differential and derivation spectra are described in detail elsewhere[9].

Renaturation

The renaturation was accomplished by diluting the denaturation mixture (concentrations: 8 mol dm^{-3} urea, $5 \cdot 10^{-5}$ mol dm^{-3} mercaptoalbumin) with a 3× larger volume of distilled water. The renaturation kinetics was monitored directly in the renaturation mixture. For determination of the dependence of denaturation reversibility on the time of action of urea, samples were taken from the denaturation mixture at definite time intervals, and after 100 min renaturation initiated by lowering the urea concentration from 8 to 2 mol dm^{-3} the differential spectra were recorded with a solution of 1.25 mol dm^{-3} mercaptoalbumin and 2 mol dm^{-3} urea in the reference cell.

RESULTS AND DISCUSSION

When studying the renaturation of the mercaptoalbumin denatured for 1 min, D^1_{SH}, by decreasing the urea concentration from 8 to 2 mol dm^{-3}, we found that the absorbance changed from the value corresponding to the denatured mercaptoalbumin[3] before the first measurement was possible (1 min renaturation) to the value of renatured mercaptoalbumin, R^1_{SH}, which corresponds to the absorbance of mercaptoalbumin in 2 mol dm^{-3} urea. With increasing renaturation time no further changes were observed.

The time course of renaturation of the mercaptoalbumin denatured by 8 mol dm^{-3} urea for 200 min, D^{200}_{SH}, was the same as that in the previous cases, but the final absorbance of the renatured mercaptoalbumin, R^{200}_{SH}, differed from the value obtained in the denaturation of mercaptoalbumin by 2 mol dm^{-3} urea. The absorbance difference between the renatured and native samples (as measured in the differential arrangement) was a measure of denaturation reversibility in the study of its dependence on the time of action of the denaturation agent. The absorbance values were measured after 100 min renaturation (brought about by the urea concentration decrease from 8 to 2 mol dm^{-3}) at the wavelengths of 278 nm (characteristic of tyrosyls) and 292 nm (where the effect of tryptophyls is more distinct). The results of measurements of the dependence of denaturation reversibility on the time of action of 8 mol dm^{-3} urea are given in Fig. 1. From the spectrophotometric viewpoint, the denaturation of mercaptoalbumin with 8 mol dm^{-3} ceases to be reversible after roughly 15 min of the action of urea.

A characteristic temperature perturbation differential spectrum (TPDS) of native mercaptoalbumin is presented in Fig. 2 (heavy line). The main differential peak found at 292 nm is caused by the perturbation of both tyrosyls and tryptophyls. Several well defined characteristic positive peaks in the region of 255 – 270 nm belong to the perturbation of phenylalanyls[11,12].

The absorbance differences $\Delta\varepsilon$ for the wavelengths λ_1 = 292 nm and λ_2 = 300 nm were plotted as the functions of temperature difference ΔT. The mean values of the slope $(\Delta\varepsilon/\Delta T)^{\lambda}$ obtained from five measurements each are 3.338 m^2 mol^{-1} K^{-1} (292 nm) and 0.927 m^2 mol^{-1} K^{-1} (300 nm).

The numbers of exposed tyrosyls and tryptophyls were calculated by the equation system[11] (*1*),

$$x\left(\frac{\Delta\varepsilon}{\Delta T}\right)^{\lambda_1}_{\mathrm{TYR}} + y\left(\frac{\Delta\varepsilon}{\Delta T}\right)^{\lambda_1}_{\mathrm{TRP}} = \left(\frac{\Delta\varepsilon}{\Delta T}\right)^{\lambda_1}_{\mathrm{PROTEIN}}$$

$$x\left(\frac{\Delta\varepsilon}{\Delta T}\right)^{\lambda_2}_{\mathrm{TYR}} + y\left(\frac{\Delta\varepsilon}{\Delta T}\right)^{\lambda_2}_{\mathrm{TRP}} = \left(\frac{\Delta\varepsilon}{\Delta T}\right)^{\lambda_2}_{\mathrm{PROTEIN}} \qquad (1)$$

where λ_1 and λ_2 are the wavelengths of the radiation used, x and y are the numbers of exposed tyrosyls and tryptophyls, respectively. The slope values of the temperature dependences of the absorption coefficients of tyrosyl and tryptophyl were taken from ref.[9].

The solution of the equation system (*1*) gives x = 10.2 and y = 0.93 for native mercaptoalbumin. Therefrom it follows that in the native mercaptoalbumin ten tyrosyls and one tryptophyl are exposed into the solution.

The temperature perturbation differential spectra were measured for both the samples of the renatured mercaptoalbumin. The spectrum of R_{SH}^1 (Fig. 2, dotted line) is practically identical with that of the native mercaptoalbumin in Fig. 2. Also very close is the value of slopes at the wavelengths λ_1 = 292 nm and λ_2 = 300 nm. Hence, according to these measurements the mercaptoalbumin renaturated after 1 min denaturation has the same numbers of exposed tyrosyls and tryptophyls as has the native protein.

A different situation is encountered in the case of the mercaptoalbumin renatured after 200 min denaturation, R_{SH}^{200}. Its temperature perturbation differential spectrum is given in Fig. 2 (dashed line), from which it can be seen that the overall shape of spectrum has changed. The main differential peak is shifted towards shorter wavelengths (291 nm) and its shape is changed as well. Changes are encountered also in the region characteristic of the perturbation of phenylalanyls. Summarily it can be stated that with a number of residues there obviously takes place a change in exposition; particularly the hypsochromic shift of the main differential peak indicates a polarity increase in the neighbourhood of some tyrosyls.

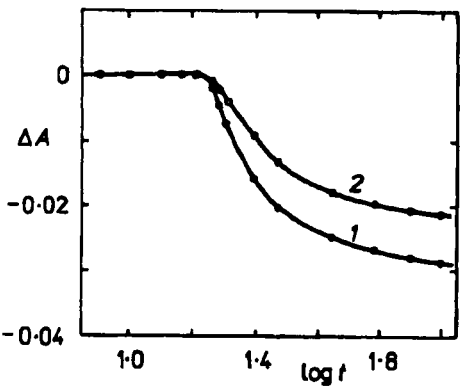

FIG. 1

The dependence of the absorbance differences ΔA of the renatured and native mercaptoalbumin on the denaturation time t (in min) with 8 mol dm^{-3} urea. Temperature 22 °C, pH 6.8. *1* 278 nm, *2* 292 nm

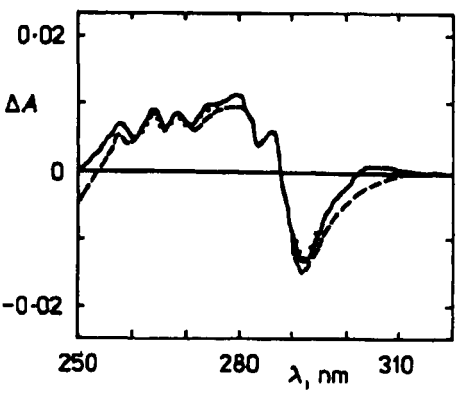

FIG. 2

TPDS of mercaptoalbumin, concentration 2.4 . 10^{-5} mol dm^{-3}; ΔT = -15 K; pH 6.8; curves: —— native peptide, ····· renatured R^1, - - - - renatured R^{200}

The $\Delta\varepsilon$ values at the wavelengths $\lambda_1 = 291$ nm and $\lambda_2 = 300$ nm were plotted as a function of temperature difference, and slopes of these dependences were determined: their mean values obtained from five measurements each are 4.277 $m^2\ mol^{-1}\ K^{-1}$ (291 nm) and 1.141 $m^2\ mol^{-1}\ K^{-1}$ (300 nm). In this case the solution of the equation system (*1*) is $x = 13.6$ and $y = 0.96$. For the mercaptoalbumin renatured after 200 min denaturation with 8 mol dm^{-3} urea it was found that the numbers of exposed tyrosyls and tryptophyls are 14 and 1, respectively.

The derivation spectrum of native mercaptoalbumin is given in Fig. 3. The dotted line expresses the originally obtained derivation spectrum, the heavy line represents the derivation spectrum from which the contribution of one tryptophyl (contained therein) has been subtracted. It is obvious that subtraction of this contribution made the main negative band (which – before – was a product of superposition of both the chromophores) more smooth. A distinct change was also observed in the region of the tyrosyl band at 280 nm whose shape was affected by the superposition of the tryptophyl contribution. No substantial change was observed in the region characteristic of phenylalanyl. The parameters of heterogeneity[13] for the native mercaptoalbumin were determined on the basis of three independent measurements: $R = 6$, $\lambda_{1/2} = 6.4$, $H = 38.4$. Hence, according to the method by Brandts and Kaplan[13], the degree of heterogeneity of tyrosyls is very high. This indicates that there are two groups of tyrosyls of roughly the same magnitude in two different states.

In contrast to the derivation spectrum of R_{SH}^{200} given in Fig. 4, the derivation spectrum of R_{SH}^{1} is completely identical with the spectrum of native mercaptoalbumin. Once again the heavy line represents the derivation spectrum with separated contribution of tryptophyl. In comparison with the derivation spectrum of native mercaptoalbumin, in this case the relative heights of the bands at 280 and 290 nm are interchanged, which is most important from the standpoint of determination of heterogeneity of tyrosyls. Besides, in the region characteristic of phenylalanyls there occurred very distinct changes both in magnitude and number of the peaks.

The heterogeneity parameters found from three independent measurements for R_{SH}^{200} are: $R = 3.7$, $\lambda_{1/2} = 7.2$, $H = 26.6$. Thus it is obvious that the heterogeneity of tyrosyls in R_{SH}^{200} is lower than that in the native mercaptoalbumin.

On the basis of the measurements of temperature perturbation differential spectra it can be concluded that the molecule of native mercaptoalbumin has about 10 tyrosyls and 1 tryptophyl exposed into the solution (out of the total numbers of 18 tyrosyls and 1 tryptophyl). This number of tyrosyls localized at the surface of the molecule is also indicated by the results of the derivation spectrophotometry. The heterogeneity parameter H was found to be 38.4, which corresponds to a very high heterogeneity of tyrosyls in the scale by Brandts and Kaplan[13]. Hence it can be stated that in the molecule there are two groups (of roughly the same magnitude) of tyrosyls in optically different states; with simplification we can denote them as free and buried tyrosyls. With regard

to the total number of tyrosyls in the molecule of mercaptoalbumin it is possible to deduce from the derivation spectra that the number of tyrosyls in each group is near to 9. Thus both the spectrophotometric methods give very close pictures of the state of tyrosyls in the molecule of native mercaptoalbumin. These results agree well with literature data[4,6].

When applying the methods of TPDS and derivation spectrophotometry to R_{SH}^{1} we arrive at identical conclusions as in the case of the native mercaptoalbumin. R_{SH}^{1} has roughly 10 tyrosyls and 1 tryptophyl exposed into the solution, and in this respect it is identical with the native protein.

Different values are obtained for R_{SH}^{200}. According to TPDS in this case there are 14 tyrosyls and 1 tryptophyl localized at the surface of the molecule. Thus the number of exposed tyrosyls in R_{SH}^{200} is markedly increased as compared with the native mercaptoalbumin. The heterogeneity parameter H is much lower in the case of R_{SH}^{200} than in the case of the native mercaptoalbumin. The value of $H = 26.6$ corresponds to a medium degree of heterogeneity of tyrosyls, which means that in the molecule of R_{SH}^{200} one group of tyrosyls predominated over the other; from the derivation spectra alone it cannot be decided whether the number of free tyrosyls or that of the buried ones has increased as compared with the native mercaptoalbumin. However, the comparison with the results of TPDS clearly shows that the significant lowering of heterogeneity of tyrosyls in R_{SH}^{200} – as compared with the native mercaptoalbumin – is due to the higher number of exposed tyrosyls.

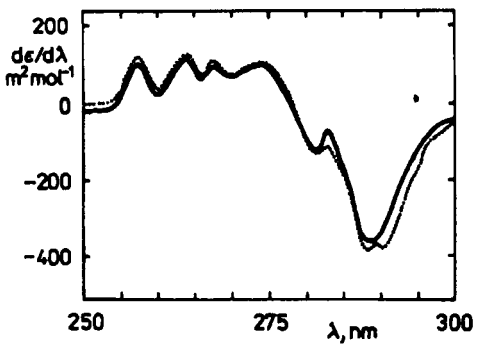

FIG. 3
Derivation spectrum of native mercaptoalbumin (· · · · ·), derivation spectrum of HSA after subtraction of contribution of tryptophyl (——). Temperature 22 °C, pH 6.8

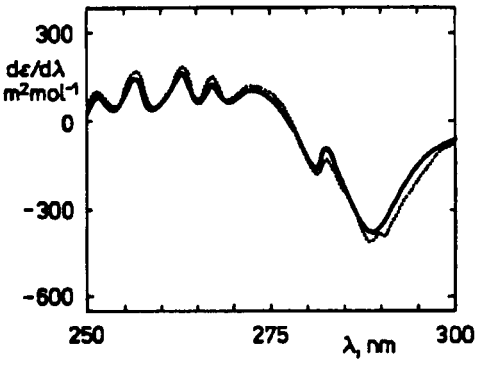

FIG. 4
Derivation spectrum of R^{200}. The original derivation spectrum (· · · · ·), the derivation spectrum after subtraction of contribution of tryptophyl (——). Temperature 22 °C, pH 6.8

On the basis of the values of exposition of tyrosyls in the three samples mentioned it can be stated that the conformation of R_{SH}^{1} is probably identical with the native conformation, whereas R_{SH}^{200} markedly differs from the native molecule.

The results of investigation of denaturation reversibility of mercaptoalbumin with 8 mol dm^{-3} urea at the conditions used by us show that the longest denaturation time after which the molecule can be reversibly renatured is roughly 15 min (see Fig. 1). With longer denaturation times the denaturation is irreversible, and the renaturation products have a greater number of tyrosyls exposed into the solution as compared with the native mercaptoalbumin.

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