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EFFECT OF GUANIDINE HYDROCHLORIDE ON THE STATE OF TRYPTOPHAN-CONTAINING SITES OF HUMAN, BOVINE AND RABBIT PLASMINOGENS

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One of the features of plasminogen (Pg) activation into plasmin, initiated by streptokinase (Sk), is a obvious manifestation of species specificity. Thus, Sk activates human, canine, rabbit Pgs relatively easily, but sheep, bovine, murine zymogens — very slowly [1—3]. It was considered to be predetermined by the zymogen structure peculiarities [4], which are still obscure. Indeed, molecules of human, rabbit and bovine zymogens differ in the state of tryptophan and tyrosine residues, as well as in secondary and tertiary structures [5,6]. But these data do not account for the different rates of Pg activation by Sk. They only suggest that the reason of the formation of stable Sk-human Pg complexes (but not rabbit or bovine Pgs) occurs due to a more flexible structure of the human zymogen. The studies on the state of tryptophan-containing sites of human, rabbit and bovine Pgs depending upon pH value testify actually to less flexible structure of the bovine zymogen [7]. But these findings are not sufficient to make a conclusion on zymogen structural mobility.

In the present study, the state of tryptophan-containing protein sites under the influence of guanidine hydrochloride (GuHCl), another factor perturbing protein structure, is investigated by means of intrinsic tryptophan fluorescence method.

MATERIALS AND METHODS

Investigations were made with purified human, rabbit and bovine Pgs samples, isolated by affinity chromatography from blood plasma (rabbit and bovine Pgs) or from β-globulin enriched blood fraction (human Pg). Methods of purification, evalution of homogeneity, specific activity of the samples and their characteristics were described in our previous paper [7]. Tryptophan fluorescence spectra were measured on a Fica—55 or a Hitachi—840 spectrofluorometers with excitation wavelength $\lambda = 296$ nm. 0.06M phosphate buffer (pH 7.4), containing 0.1M NaCl, was used as a solvent. The spectra were analysed according to the fluorescence intensity ($I_{relative}$) in maximum, the position of spectrum maximum (λ_{max}), its half-widths ($\Delta\lambda$) and I_{355}/I_{328} ratio, that characterizes the contribution of different groups of tryptophan residues to fluorescence. All measurements were repeated not less than four times.

The following reagents and chemicals were used: Sk preparation «Streptase» (Behringwerke AG, Germany), urokinase (Iskra-Industry, Japan), L-Lysine, polyacrylamide gel reactants (Reanal, Hungary), disodium dodecyl sulfate (Serva, Germany), CNBr-Sepharose (Pharmacia, Sweden). Other reagents were analytical grade. L-Lysine-Sepharose was synthesized from L-Lysine and CNBr-Sepharose in 0.1M phosphate buffer pH 9.0 [8].

RESULTS AND DISCUSSION

Human Pg. GuHCl within a 2—4 M concentration range caused a slight (18%) quenching of tryptophan fluorescence, which was weakened with 5M denaturant concentration (Fig.1). As seen from fluorescence spectra parameters, the state of tryptophan-containing sites is not changed

greatly by the denaturant at a concentration less than 3 M. Thus, changes in λ_{max} and $\Delta\lambda$ did not exceed 2 nm, and changes in I_{355}/I_{328} ratio constitute 17% (Fig. 2). This supposes some disordering in tryptophan residues surrounding.

At 3—4 M GuHCl, stages could be traced in the changes of tryptophan-containinig sites. During the first 5 min a remarkable batochromic shift of λ_{max}, depending upon denaturant concentration, was observed. At 3 M GuHCl this shift increased considerably by 30 min without further changes. When GuHCl concentration was 4 M, gradual (but less considerable) increase in batochromic shift of λ_{max} was observed during 120 min. $\Delta\lambda$ value changes identically and stages can be also seen, depending upon the effect of the two above mentioned GuHCl concentrations: considerable increase of this parameter value starts already at 5 min., and then increased evenly, but less obviously till 60 min. Fluorescence quenching, induced by GuHCl within this range, did not increase as compared to the 2 M denaturant effect. Probably, the increase in contacts of tryptophan residues with solvent took place at the above mentioned concentrations. Moreover, strong spatial «uncoupling» («dispersal») of tryptophan-containing sites was observed. In any case, practically similar $\Delta\lambda$ dynamics with differing changes in λ_{max} leads us to such an assumption. The value of $I_{355}\backslash I_{328}$ in the presence of 3 M GuHCl increased sharply in 30 min, whereas at 4 M the changes in this parameter became maximum already at 5 min (Fig.2). It appears that in the last case the denaturant caused rapid quenching of mainly short-wave («blue») tryptophan residues.

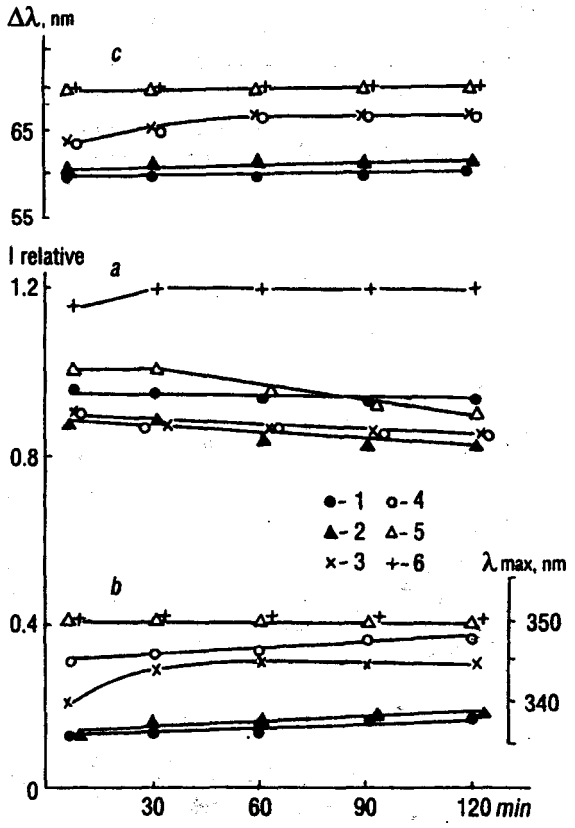

Fig. 1. Changes of fluorescence relative intensity ($I_{relative}$, a), position of the maximum (λ_{max}, b), and the half-widths ($\Delta\lambda$, c) of the tryptophan fluorescence spectra of human plasminogen after guanidine hydrochloride addition at final 1 (1), 2 (2), 3 (3), 4 (4), 5 (5) and 6 (6) M concentrations. The solvent is 0.06 M phosphate buffer pH 7.4, containing 0.1 M NaCl; 25.0 °C

At 5—6 M denaturant changes of λ_{max}, $\Delta\lambda$ and I_{355}/I_{328} ratio reached their maximum at once (Fig.1,2). The addition of 5 M guanidine salt noticeably weakened fluorescence quenching, but 6 M denaturant increased its intensity by 15—20%. Under these conditions, local disordering of zymogen structure seemed to result in the elimination of quenching effect of neighbouring groups on fluorescence of long-wave («red») tryptophan residues. It allows us to suggest that the latter are oriented in the protein molecule with participation of comparatively strong bonds, which could not be broken at lower denaturant concentrations.

Thus, GuHCl effect on the human zymogen molecule is characterized by comparatively small (±20%) changes of fluorescence intensity with considerable changes in the state of tryptophan residues only at ≥3 M denaturant and very weak dynamics of parameters (except the narrow range of 3—4 M concentrations).

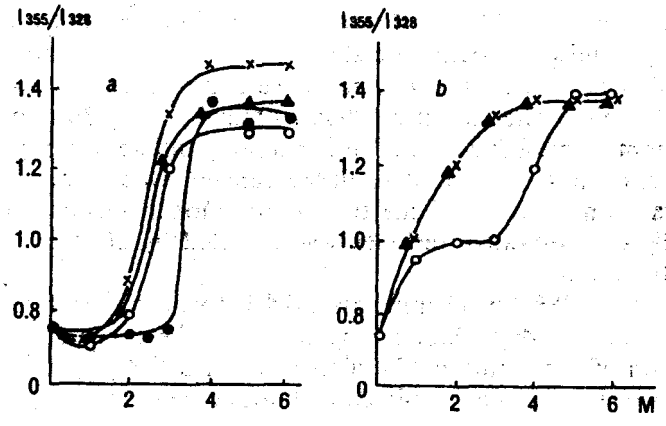

Fig. 2. $I_{355}\backslash I_{328}$ values (ratio «fluorescence intensity at 355 nm band: fluorescence intensity at 328 nm band») for human (a) or bovine (b) plasminogens after guanidine hydrochloride additions in 5 (●-●-●), 30 (×-×-×), 60 (o-o-o) and 120 (▲-▲-▲) min

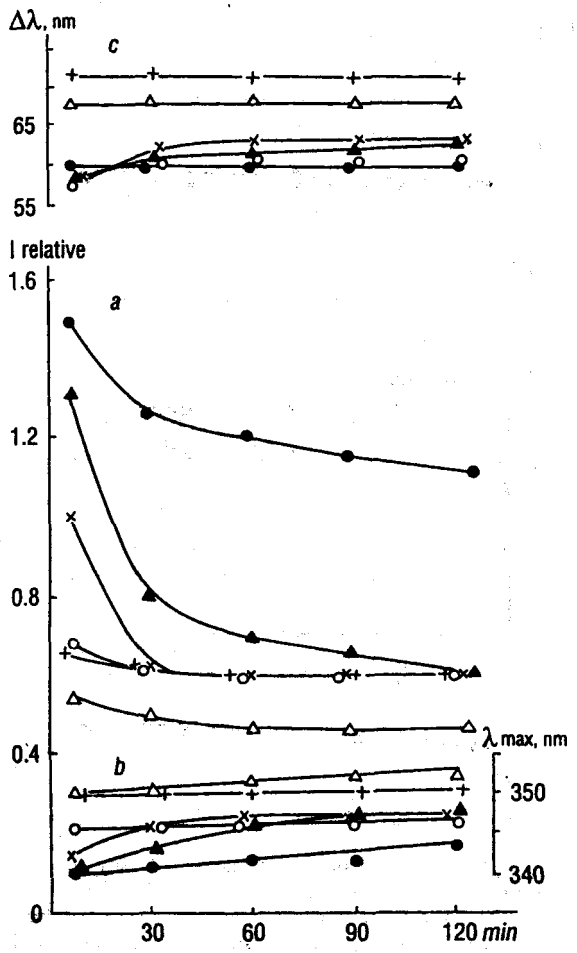

Fig. 3. Dependence of fluorescence intensity ($I_{relative}$, a), position of the maximum (λ_{max}, b) and the half-width ($\Delta\lambda$, c) of the tryptophan fluorescence spectra of bovine plasminogen on guanidine hydrochloride concentration. The conditions and designations are the same as in Fig. 1

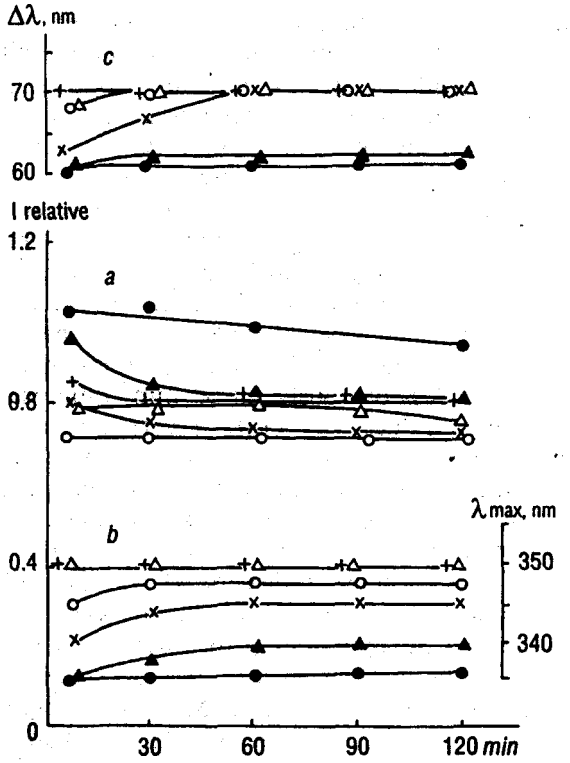

Fig. 4. Guanidine hydrochloride additions influence the fluorescence intensity ($I_{relative}$, a), position of the maximum (λ_{max}, b) and the half-widths ($\Delta\lambda$, c) of the tryptophan fluorescence spectra of rabbit plasminogen. The conditions and designation are the same as in Fig. 1

Bovine Pg. At final 1 M denaturant concentration, essential increase of fluorescence intensity occurred during the first 30 min (Fig. 3). This effect, though weaker, was also observed in 120 min. This weakening was combined with slow and small batochromic shift of λ_{max} (by 3 nm). Besides, at 5 min after denaturant addition, I_{355}/I_{328} ratio increased sharply (Fig.2). Perhaps, the break of the limited number of relatively weak bonds, stabilizing the surrounding of tryptophan residues, eliminated at first the effect of quenching groups on tryptophan long-wave residues. Further loosening of sites contributed to the changes in the surrounding of these residues in such a way that it made impossible the increase of fluorescence and short-wave residues fluorescence was quenched.

At 2 M denaturant, fluorescence intensity increase was not so considerable and in 30 min it was replaced with quenching by 20%, which reached 40% by 120 min. It was just quenching but not the fluorescence increase that occurred on the background of batochromic shift of λ_{max} that grew within 30—90 min, accompanying $\Delta\lambda$ growth as well. Changes in I_{355}/I_{328} ratio were presented by two stages: at 5 min this value increase was 1/3 of the initial one, and it reached its maximum by 30 min. It enables us to suggest that during the first 5 min fluorescence contribution among different groups of tryptophan residues was redistributed: the part of long-wave residues fluorescence grew simultaneously with the decrease in the part of short-wave ones (may be, due to conformational freedom of globule structure). Only fluorescence quenching of short-wave residues was revealed by 30 min in consequence of certain labilization of protein structure.

3 M GuHCl induced fluorescence quenching by 38% in 30 min, essential batochromic shift of λ_{max}, coinciding with I_{355}/I_{328} ratio growth and only a slight broadening of the fluorescence spectrum. We believe that just the same redistribution of contribution of fluorescence of different groups of tryptophan residues, as it was described above, took place in this case. Further denaturant con-

centration growth (4 M) only increased the appearance of the mentioned shifts. The actual absence of changes in Δ(is rather surprising. Perhaps, when concentration was ≤4 M, GuHCl only loosened tryptophan-containing sites in the protein molecule without considerable disorganization of their structure. Besides, during the first 5 min of its effect, fluorescence of tryptophan «red» residues seemed to increase.

At 5 M denaturant concentration the value of λ_{max} shift reached actually its maximum already in 5 min (Fig.3). It was combined with fluorescence quenching by 45% (54% — by 60 min), $\Delta\lambda$ changes were considerable (8 nm), but they reached their maximum only at 6 M GuHCl. Different tryptophan-containing sites of bovine Pg molecule seemed to be retained by means of strong bonds. Fluorescence quenching decreased a little in the presence of 5—6 M denaturant, and proceeding from I_{355}/I_{328} ratio value, quenching of «blue» tryptophan residues was firstly (5 M) observed, and then some increase in long-wave chromophore fluorescence. As compared with human Pg, GuHCl effect on bovine zymogen fluorescence intensity was more considerable, ±50%. At low denaturant concentrations fluorescence intensity increased at the initial stage of the effect, but then it might be replaced by quenching. λ_{max} changes were more prominent, than in experiments with human Pg, and within the whole range of concentrations the denaturant led to manifestation of dynamics of tryptophan fluorescence parameters. During the first 5 min at 1 M denaturant effect on zymogen, redistribution of fluorescence intensity a part of different groups of tryptophan residues was already detected.

In contrast to human zymogen, the dependence, approaching the exponent (Fig. 2), but not S-like dependence of I_{355}/I_{328} ratio from denaturant concentration (that presupposes the cooperative character of changes in tryptophan-containing sites) was observed. The character of this dependence suggests two stages of rearrangement of tryptophan-containing sites during the first 5 min of denaturant effect.

Rabbit Pg. 1 M GuHCl addition actually caused only $\Delta\lambda$ increase by 2 nm (Fig.4). 2 M denaturant determined a slight fluorescence quenching, beginning from 30 min that was combined with batochromic shift of λ_{max} and growth in $\Delta\lambda$. Probably it indicates partial loosening of tryptophan-containing sites in the protein.

3 M denaturant effect resulted in fluorescence quenching by 20—28%. This level of quenching was maximal when denaturant affected the given protein. Besides, even in the first 5 min a noticeable batochromic shift of λ_{max} and an increase in $\Delta\lambda$ were observed, and they grew by 60 min. Under these conditions $\Delta\lambda$ reached its maximum and a further increase in GuHCl concentration did not lead to growth of this parameter. The shift of λ_{max} was considerable, but did not reach its limit. That is why it is supposed that the increase in heterogeneity of tryptophan-containing sites (for example, their spatial «uncoupling» («dispersal»), increase of differences in their surrounding), but not disordering of these sites, is most probable. Spatial «uncoupling» («dispersal») can be caused by the break in some bonds, determining mutual domain orientation.

Judging from the growth of batochromic shift of λ_{max}, the absense of considerable changes in $\Delta\lambda$ and fluorescense intensity, 4 M GuHCl promoted further exposure of tryptophan residues in the solvent. This exposure was likely to reach its limits at 5 M denaturant concentrations, when λ_{max} shift was maximal. Further increase in GuHCl concentration did not increase changes in $\Delta\lambda$ and λ_{max}, and fluorescence quenching weaked. We believe that profound disorganization of tryptophan-containing sites causes weakening of quenching effect of the groups in the neighbourhood of tryptophan-containing sites. Thus, denaturant effect on the rabbit Pg wascharacterized by prominent, but not exceeding 30% quenching of tryptophan fluorescence at 3—4 M agent. GuHCl effect was not accompanied by fluorescence increase in any case. Considerable changes in the state of tryptophan-containing sites of the protein began at 2 M denaturant and reached their maximum at 4—5 M.

CONCLUSIONS

Thus, GuHCl produced strong disorganization effect on Pgs tryptophan-containing sites, that is much stronger than that of alkaline medium (see [7]).

The general character of the changes in the parameters of tryptophan fluorescence spectra is rather complex. First of all it is accounted for by the complexity zymogen structure: for example, human Pg is a multidomain protein [9,10], which contains ~ 20 tryptophan residues (see [6]). Denaturant effect on protein molecule is manifold: GuHCl is considered not only to compete for hydrogen bonds and to modify hydrophobic interactions, but it also produces the effect, peculiar to neutral salts (ex. [11]). Moreover, the registration of parameters of tryptophan fluorescence in the dynamics of the denaturant effect allows to detect the stages of unfolding of tryptophan-containing sites.

GuHCl causes the changes in fluorescence intensity in all three Pgs. The degree of changes becomes lower, in the order of decreasing, in bovine, rabbit and human zymogens. On the whole,

similar values of shifts in the position of spectrum maximum and its half-width are observed when all zymogens are affected. Nevertheless, the dynamics of these parameters and their dependence upon denaturant concentrations are different in the zymogens. It leads us to some conclusions on peculiarities of zymogen structure lability inspite of the fact that due to the potent (in comparison with alkaline medium) GuHCl effect, these molecular structures are subjected to more generalized rearrangements and the data obtained show the maximum changes in the state of tryptophan residues.

So, considerable changes in the structure of tryptophan-containing sites in human and rabbit Pgs are realized within the narrower range of denaturant concentrations than in bovine zymogen. Structure rearrangements of these sites in the rabbit protein reach maximum at lower guanidine salt concentration. S-like character of plot shows changes in I_{355}/I_{328} ratio in human Pg (unlike bovine zymogen) and it enables us to assume that in human Pg, a considerable role in orientation of tryptophan-containing sites and (or) in their organization, is played by some «critical» number of intramolecular interactions resistant to low denaturant concentrations. It was of special interest that tertiary structure of human zymogen is better ordered judging from circular dichroism spectra [5,6]. Perhaps, this factor determines cooperative character of tryptophan-containing sites rearrangement. In rabbit zymogen intramolecular interactions, which are sensitive to GuHCl, are less significant for orientation of groups, quenching tryptophan fluorescence.

In general it seems that rabbit zymogen is more sensitive to GuHCl effect: considerable changes in the state of tryptophan-containing sites start to manifest themselves and reach maximum at lower denaturant concentrations than those in the other zymogens. From this viewpoint tryptophan-containing sites in bovine zymogen are more stable. It goes without saying that our findings do not reveal reasons of different conformation lability of zymogens. But it is quite clear that these differences may be very significant. And it stimulates further investigations in comparative physical biochemistry of plasminogen.

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EFFECT OF GUANIDINE HYDROCHLORIDE ON THE STATE OF TRYPTOPHAN-CONTAINING SITES OF HUMAN, BOVINE, AND RABBIT PLASMINOGENS

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Summary

Considerable changes in the state of tryptophan-containing sites occured under the effect of 3M (5M), 2M (4M) and 2M (6M) guanidine hydrochloride in human, rabbit and bovine plasminogen molecules, respectively (and reached a maximum at 5M, 4M and 6M denaturant). In human plasminogen, but not in bovine, guanidine hydrochloride effect was able to cause rearrangements of tryptophan-containing sites of cooperative character. In bovine zymogen the denaturant induced gradual unfolding of such sites. In human and bovine plasminogens, but not in rabbit, the orientation of groups (neighboured with tryptophan residues), quenching tryptophan fluorescence, seemed to be realized with the participation of bonds broken by guanidine hydrochloride. On the whole, the molecule of rabbit plasminogen was more sensitive to denaturant effect, however the strongest changes of fluorescence intensity of tryptophan residues were observed under the effect of guanidine salt on bovine plasminogen.