



## THE STATE OF TRYPTOPHAN-CONTAINING SITES OF HUMAN, BOVINE AND RABBIT PLASMINOGENS WITH CHANGING SOLUTION pHs

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**Abstract**—1. The state of tryptophan-containing sites is proved to be stable by intrinsic tryptophan fluorescence with pH 5–8, 7–9, and 6–9 in human, rabbit and bovine plasminogen molecules, respectively.

2. With pH < 5.0 tryptophan-containing sites of human zymogen (in contrast to rabbit and bovine ones) undergo conformational transitions.

3. With the shift of solution pH from 9 to 12 tryptophan-containing sites of human and rabbit plasminogens are partially disorganized, while tryptophan residues become more available for solvent.

4. Tryptophan-containing sites of bovine plasminogen molecules are less mobile in structure during changes of solution pH.

### INTRODUCTION

Fibrinolysis plays a very important role in several physiologic and pathologic processes, such as histogenesis and tissue differentiation, ovulation and embryo implantation, malignancy and metastatic spread of tumors, inflammation etc. (Kristensen *et al.*, 1984; Laiho and Keski-Oja, 1989; Niedbala and Sartorelli, 1989). The key reaction in fibrinolysis is plasminogen (Pg) transformation into plasmin, an active serine proteinase, by so-called "proteinase" or "non-proteinase" pathways (Jackson *et al.*, 1980).

The mechanism of Pg activation by a non-proteinase pathway remains a subject of many years of research, but is still far from clear. One of the results of this activation is action on Pg by streptokinase (SK), a protein factor, synthesized by  $\beta$ -haemolytic streptococci, of ~50 kDa molecular weight and devoid of any hydrolase activity (Reddy, 1988). The SK activating function is characterized by species specificity: it activates human, canine, and rabbit Pgs but not sheep, bovine or mouse Pgs (Ablondi and Hagan, 1958; Wulf and Mertz, 1969; Castellino, 1979). SK also forms a comparatively stable complex with human Pg or

human plasmin, which is considered a carrier of activating function, and the formation of this complex is considered the major condition for activation to occur (Castellino, 1979; Reddy, 1988). Accordingly, SK does not form such complexes with sheep and bovine Pgs (Summaria *et al.*, 1974; Paoni and Castellino, 1979). However, there is one exception from this general rule: SK activates native rabbit Pg, but does not form a stable complex with it (Schick and Castellino, 1973).

The reasons for the species specificity of SK action were sought after by looking at peculiarities of zymogen structures (Ablondi and Hagan, 1957). However, until the present time differences in structural organization between human and animal Pg molecules have not been clearly identified. In previous studies we determined states of tyrosine and tryptophan residues, secondary and tertiary structures between human, bovine and rabbit Pg molecules. We also put forward suppositions about more structural flexibility of the human zymogen molecule (Nikandrov *et al.*, 1989, 1992).

This problem is still far from being solved. In fact, there are only a few articles on acidic transformations of human and swine Pg using intrinsic tryptophan fluorescence (Rodier, 1976;

Zyma *et al.*, 1981). In the present study, we used an intrinsic tryptophan fluorescence method and examined tryptophan-containing sites of human, rabbit and bovine Pg molecules in a wide range of solvent pH. In some experiments the method of selective quenching of fluorescence was used.

## MATERIALS AND METHODS

The human Pg samples were prepared from a  $\beta$ -globulin enriched blood fraction by affinity chromatography on lysine-sepharose (Brockway and Castellino, 1971). Rabbit and bovine Pg samples were purified from blood plasma by affinity chromatography on lysine-polymer-silochrome (Demidchik, 1985). Specific activity of Pg samples, determined by a caseinolytic method (Taylor and Tomar, 1970), after activation of human plasminogen and rabbit Pgs by SK and bovine Pg by urokinase, comprised 25 caseinolytic units per mg of protein. Protein concentration in purified Pg samples was measured spectrophotometrically on the value of absorption of water solutions with 280 nm, taking  $A_{1\text{cm}}^{1\%}$  17.0; 18.1, 15.3 for human, rabbit and bovine Pgs, respectively (Wohl *et al.*, 1977; Summaria *et al.*, 1973).

Homogeneity of the obtained samples of purified proteins was proved by electrophoresis in 12.5% polyacrylamide gel in the presence of sodium dodecyl sulfate (Weber and Osborn, 1969).

The spectra of tryptophan fluorescence were taken on a Fica 55 spectrofluorometer with excitation of  $\lambda = 296$  nm. Fluorescence quenching was measured with addition of 3 M solution of CsCl,  $\text{NaNO}_3$  or acrylamide to 2 ml of Pg solution ( $1.6\text{--}2.1 \times 10^{-6}$  M) immediately before measurement (aliquots of quencher solutions were 5–10  $\mu\text{l}$ ). The data on quenching were processed according to the Stern-Volmer equation, applying corrections for reabsorption for  $\text{NaNO}_3$  (Burstein, 1977).

Solution pH was determined with the use of the following buffering systems: pH 1–2, 0.05 M KCl–HCl; pH 3–8, 0.05 M citric acid– $\text{Na}_2\text{HPO}_4$ ; pH 9, 0.05 M  $\text{KH}_2\text{PO}_4$ – $\text{Na}_2\text{B}_4\text{O}_7$ ; pH 10–11, 0.05 M  $\text{Na}_2\text{CO}_3$ –HCl; pH 12, 0.1 M  $\text{Na}_2\text{HPO}_4$ –NaOH. All the buffering systems contained in addition 0.1 M NaCl.

All measurements were repeated not less than four times.

The following materials were used: SK preparation "Streptase" (Behringwerke AG,

Germany), urokinase (Iskra-Industry, Japan), acrylamide, L-lysine, polyacrylamide gel reactants (Reanal, Hungary), disodium dodecyl sulfate (Serva, Germany), CNBr-Sephacryl (Pharmacia, Sweden). Other reagents were Russian production (analytical grade). L-lysine–CNBr-Sephacryl was synthesized from L-lysine and CNBr-Sephacryl in 0.1 M phosphate buffer pH 9.0 (Brockway and Castellino, 1971).

## RESULTS AND DISCUSSION

Proceedings from the obtained data, the tryptophan-containing sites of molecules are in a stable state with pH 5–8 for human Pg (Fig. 1), pH 7–9 for rabbit Pg (Fig. 2), and pH 6–9 for bovine Pg (Fig. 3). Beyond these pH ranges tryptophan fluorescence quenches.

Thus, the value of "q" for these three zymogens decreased by 42–50% with pH 1.0. Taking into account changes of value of quantum yield (q) of zymogen fluorescence with pH from 7.0 to 1.0 shift, we determined the value of  $pK_a$  (according to the general rule of ionization constant evaluation for spectrophotometric measurements, Albert and Sargent, 1964), which comprised 3.0, 4.37, and 3.92 for human, rabbit and bovine Pgs, respectively. It enables us to conclude that tryptophan fluorescence quenching in acid medium for rabbit Pg is caused by interaction with protonated carboxyl of Glu or Asp residues. At the same time,  $pK_a$  of human and bovine zymogens is considerably less than that of Glu and Asp side groups in proteins,

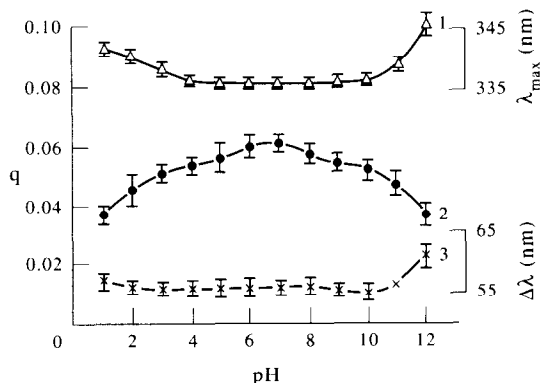


Fig. 1. The changes of quantum yield (q, 1), position of the maximum ( $\lambda_{\text{max}}$ , 2) and the half-widths ( $\Delta\lambda$ , 3) of the tryptophan fluorescence spectra of human Pg at different solution pHs. The solvents are 0.05 M KCl–HCl (for pH 1–2), 0.05 M citric acid– $\text{Na}_2\text{HPO}_4$  (for pH 3–8), 0.05 M  $\text{KH}_2\text{PO}_4$ – $\text{Na}_2\text{B}_4\text{O}_7$  (for pH 9), 0.05 M  $\text{Na}_2\text{CO}_3$ –HCl (for pH 10–11) and 0.1 M  $\text{Na}_2\text{HPO}_4$ –NaOH (for pH 12.0). 0.1 M NaCl was added to all buffer systems.

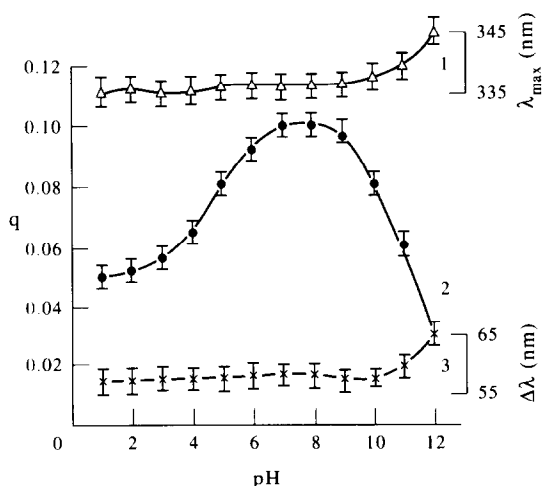


Fig. 2. The dependence of quantum yield ( $q$ , 1), position of the maximum ( $\lambda_{max}$ , 2) and the half-widths ( $\Delta\lambda$ , 3) of the tryptophan fluorescence spectra of rabbit Pg on the pH solution. The conditions are the same as in Fig. 1.

namely 4.4–4.6 (Cantor and Schimmel, 1984). It seems, that in molecules of these zymogens tryptophan fluorescence quenching manifests itself in different ways: for example, as a result of interaction between  $\alpha$ -carboxyl of the C-terminal residue of the molecule, or of protein conformation transition. The latter phenomenon possibly conforms with human Pg action,

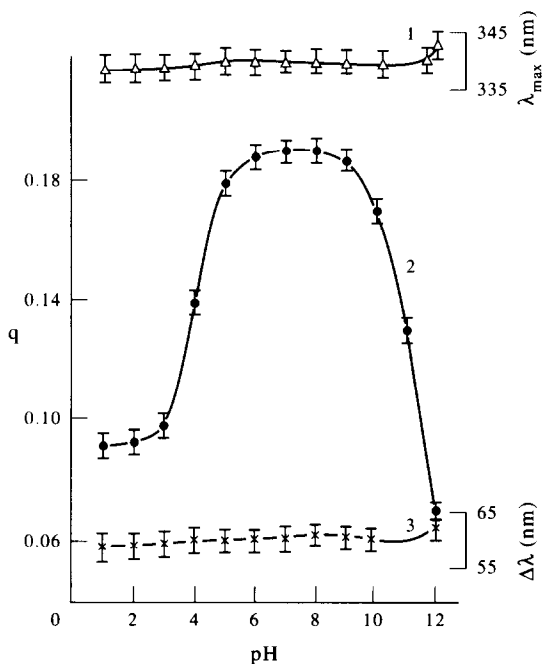


Fig. 3. The influence of solution pH upon the changes of quantum yield (1), position of the maximum (2) and the half-widths (3) of the tryptophan fluorescence spectra of bovine Pg. The conditions are the same as in Fig. 1.

because fluorescence quenching is followed, starting from pH 4.0, by a significant bathochromic shift of  $\lambda_{max}$  (Fig. 1). This enables us to suggest increased availability of tryptophan residues for solvent. It conforms with more effective fluorescence quenching, especially by the  $\text{NO}_3^-$  anion with pH 3.0, compared with pH 7.0 (see Table 1), and with a significant increase of  $I_{355}/I_{328}$  ratio (Fig. 4). Taking into consideration rabbit Pg, the Stern–Volmer constant is somewhat lower with pH 3.0, than that with pH 7.0. In general, the degree of fluorescence quenching (%) is slightly higher in an acid medium than with pH 7.0, this is especially characteristic of bovine Pg. However, the absence of changes of  $\lambda_{max}$  and fluorescence spectrum half-widths ( $\Delta\lambda$ ) in acid medium in rabbit and bovine Pgs (Figs 2 and 3) enables us to presuppose that there are no significant structural transformations of tryptophan-containing sites in the molecules of these Pgs.

Tryptophan fluorescence is quenched in all three zymogens when the solution pH is higher than 9.0. But with pH 12.0 the degree of quenching differs among these proteins: for human Pg quenching reaches 10% (Fig. 1), while for rabbit and bovine Pgs this value decreases by 72 and 68%, respectively (Figs 2 and 3).

The  $pK_a$  values of quenching groups, calculated from “q” alterations in pH range 8–12, comprise 9.86, 10.9 and 11.0 for human, rabbit and bovine Pgs, accordingly. For rabbit and bovine Pgs these values practically equal the  $pK_a$  of tyrosine residue ionization in Pg molecules, i.e. 11.2 (Nikandrov *et al.*, 1989, 1992). This enables us to conclude that tryptophan fluorescence quenching in these zymogens is caused by tyrosyl ionization. For human Pg, the  $pK_a$  value, derived from tryptophan fluorescence quenching, is close to the  $pK_a$  of ionization of six strongly ionized tyrosine residues, i.e. 10.2 (Nikandrov *et al.*, 1989, 1992). Consequently, these three Pgs share a basic mechanism of tryptophan fluorescence quenching in an alkaline medium.

In an alkaline medium all the Pgs under study produce a bathochromic shift of  $\lambda_{max}$ , highly manifested in human and rabbit zymogens (Figs 1 and 2). But only in rabbit Pg,  $q$  and  $\lambda_{max}$  changes are parallel, while in the other cases  $\lambda_{max}$  shifts only with pH > 11.0. The same pattern applies for  $\Delta\lambda$ . However, it was particularly pronounced with rabbit Pg. These phenomena support the assumption that in an alkaline medium the structure of tryptophan-containing

Table 1. Stern-Volmer constant values ( $\text{mol}^{-1}$ ) of the human, rabbit and bovine Pgs, tryptophan fluorescence quenching under change of solution pH

Pgs	Quenching by $\text{Cs}^+$ at a solution pH of			Quenching by $\text{NO}_3^-$ at a solution pH of			Quenching by acrylamide at a solution pH of		
	3.0	7.0	11.0	3.0	7.0	11.0	3.0	7.0	11.0
Human	1.5	1.6	1.1	10.0	7.5	5.4	2.9	2.5	3.0
Rabbit	—	—	—	3.0	10.4	5.0	5.5	6.0	2.9
Bovine	—	—	—	7.5	6.5	5.1	4.6	3.2	2.2

Pg sites is partially disorganized, while tryptophan residues become more available for solvent. Taking into consideration changes of  $I_{355}/I_{328}$  ratio, it could be applied to human and rabbit Pgs (Fig. 4). Besides with pH 9–12 these ratio changes conform with  $\lambda_{\text{max}}$  shifts. At the same time, for rabbit Pg these shifts do not comply with the decreased effectiveness of fluorescence quenching when pH equals 11.0 (see Table 1). We could presuppose that the latter phenomenon depends on the width of “pockets”, in which tryptophan residues are localized. In fact, the radius of an  $\text{OH}^-$  ion is 0.15 nm, whereas that of a  $\text{NO}_3^-$  ion is not less than 0.19 nm.

### CONCLUSION

Thus, we determined differences in mobility of tryptophan-containing sites of human, bovine and rabbit Pg molecules. When pH shifts from 7.0 to 1.0, human Pg molecules experience a significant conformational transition of these sites, judging by parameters of tryptophan

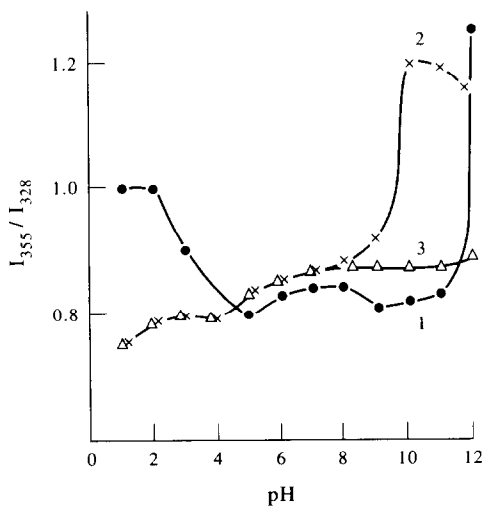


Fig. 4. The values of  $I_{355}/I_{328}$  ratio (ratio “fluorescence intensity at 355 nm band: fluorescence intensity at 328 nm band”) for human (—●—), rabbit (—X—) and bovine (—△—) Pgs under changes in solution pH.

fluorescence spectra and the effectiveness of its selective quenching. These transitions are manifested with  $\text{pH} < 4.0$ . In an alkaline medium tryptophan-containing sites of rabbit Pg are more labile in structure, as their transition begins even with a  $\text{pH} > 9.0$ , while conformational transitions of tryptophan-containing sites in human Pg are manifested only with  $\text{pH} > 11.0$ .

According to previous data (Landmann, 1976), the formation of stoichiometric stable complexes between SK and human Pg is possible in a pH range of 4–10. Consequently, structural reorganization of the human Pg molecule could account for absence of these complexes with  $\text{pH} \leq 3.0$  and  $> 10.5$ .

The bovine Pg molecule, it seems, has the most fixed structure. Considering rabbit Pg, some peculiarities of the state of tryptophan-containing sites make it different from human Pg in an acid medium, and from human and bovine Pgs in an alkaline medium. It should be noted, that data on circular dichroism spectroscopy illustrate its specific interaction with SK, in contrast to human and bovine zymogens (Nikandrov *et al.*, 1992). It proves the necessity of further and more detailed studies on rabbit Pg–SK interaction.

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