Journal of Fluorescence, Vol. 16, No. 2, March 2006 (© 2006)
DOI: 10.1007/s10895-005-0051-y

Fibrinogen-β-Estradiol Binding Studied by Fluorescence Spectroscopy: Denaturation and pH Effects

Sónia Gonçalves,[1,2] Nuno C. Santos,[1] J. Martins-Silva,[1] and Carlota Saldanha[1]

Published online: February 14, 2006

Fibrinogen is a blood plasma protein that plays a crucial role in hemostasis. It is known that erythrocyte aggregation increases in the presence of fibrinogen, and that β-estradiol decreases erythrocyte aggregation with a constant fibrinogen concentration. In this work, we have used intrinsic tryptophan fluorescence to obtain information on the conformational changes of fibrinogen upon the recently proposed interaction with β-estradiol. To evaluate the effect on the conformational changes during fibrinogen-β-estradiol binding, fluorescence experiments were performed using guanidine hydrochloride (0–6 M) as denaturant, at different pH values. The results obtained for pH 6.5 and 8.0 showed no effect during the binding. The main differences were observed between pH 4.2 and 7.4, in the absence and in the presence of two different denaturant concentrations (1 and 5 M). A red shift of the fluorescence emission from 344 to 354 nm is observed when denaturant concentration is above 3 M for all studied pH values. This phenomenon may be explained by the loss of compact structure of the protein in the presence of denaturant, with tryptophan residues exposure to the aqueous environment and alteration of fibrinogen-β-estradiol binding. These results demonstrate that the binding sites of fibrinogen are strongly dependent on the conformational state of the protein.

KEY WORDS: Fibrinogen; β-estradiol; denaturation; binding; fluorescence.

INTRODUCTION

Fibrinogen is a 340 kDa water soluble protein, consisting of two sets of three nonidentical polypeptide: the Aα-, Bβ-, and γ-chains [1,2] linked to each other by disulfide bonds. The primary structures of the fibrinogen chains contain a total of 72 intrinsically fluorescent tryptophan residues distributed along the molecule ($\alpha = 11$; $\beta = 14$; $\gamma = 11)_2$ [3]. Several cardiovascular pathologies, such as acute myocardial infarction and cerebrovascular diseases, are associated with the increased fibrinogen concentration, leading to alterations on erythrocyte aggregation and deformability [4]. Fibrinogen also acts as a cofactor in platelet aggregation (through a binding site on the γ-chains) [1]. It has been established that fibrinogen is a major determinant of plasma viscosity, and that increased plasma viscosity is a risk marker for atherosclerotic disease [5]. These hemorheological proprieties depend on erythrocytes intrinsic characteristics and on the physicochemical environment. *In vivo* and *in vitro* studies showed that high concentrations of fibrinogen increase the aggregation and decrease the deformability of erythrocytes [4,6–8]. Currently, the mechanism of fibrinogen-induced erythrocyte aggregation is not known. However, when some ligands are present, such as β-estradiol, the erythrocyte aggregation index decreases [7]. Fibrinogen has the ability to bind a variety of ligands, and much of the interest in this protein function derives from physiological implications, e.g., on the acute phase of an inflammatory process and improved viscosity syndromes [5].

The function of a protein depends on the correct folding of its native structure. Changes in the protein structure due to a denaturation process are widely used to obtain

[1] Instituto de Biopatologia Química and Unidade de Biopatologia Vascular, Instituto de Medicina Molecular, Faculdade de Medicina de Lisboa, 1649-028, Lisboa, Portugal.
[2] To whom correspondence should be addressed. E-mail: sabreu@fm.ul.pt

information about conformational transitions in proteins, association of subunits in oligomeric proteins, protein unfolding or ligand binding to proteins, following the information given by the fluorescence characteristics of the trypthophan residues. The importance of obtaining a precise knowledge regarding the conformation of fibrinogen in physiological conditions facilitates the formulation of plausible mechanisms of interaction.

Ligand-binding studies are interesting probes to monitor conformational alterations in the fibrinogen domains [4]. The study of the β-estradiol binding to fibrinogen can provide information about the functionality of these molecules during erythrocyte aggregation. Previous fluorescence spectroscopy studies [9] indicate a direct interaction of β-estradiol with more than one site of the fibrinogen macromolecule. Those results strongly suggest that the interaction of β-estradiol with fibrinogen triggers a conformational change on the protein, preventing its interaction with the erythrocyte membrane, and consequently avoiding the pernicious increase on erythrocyte aggregation and decrease on erythrocyte deformability.

Denaturation of a protein is a process in which the 3-dimensional structure of a molecule is changed from its native state without rupture of peptide bonds. It is sometimes taken to include disulfide bond rupture on chemical modification of certain groups in the protein if these processes are also accompanied by changes in its overall 3-dimensional structure [1]. Denaturation is frequently irreversible and accompanied by loss of solubility (especially at the isoelectric point) and/or of biological activity [1]. Highly concentrated (6–8 M) guanidine hydrochloride (Gdn–HCl) solutions are used to denature native globular proteins. It apparently disrupts hydrogen bonds which hold the protein in its unique structure. However, there is also evidence suggesting that guanidine hydrochloride may disrupt hydrophobic interactions by promoting the solubility of hydrophobic residues in aqueous solution [10].

β-estradiol is a steroid hormone, unionized and lipophilic at physiological pH. About 15–35% of the total amount of steroids in the bloodstream is transported by erythrocytes, including β-estradiol [7]. Its importance has been lately pointed out, namely on the cardiovascular risk reduction in postmenopausal women, although the mechanism of this cardio protective effect remains unclear. A previous *in vitro* study has shown an impaired erythrocyte aggregation in the presence of β-estradiol [7].

Thus, the aim of this study was to use the intrinsic fluorescence of fibrinogen in denaturant conditions and changes in the pH of the media to obtain information on the possible conformational changes induced by fibrinogen-β-estradiol interaction, potentially relevant for the understanding of the previously verified impaired erythrocyte aggregation in the presence of β-estradiol.

MATERIALS AND METHODS

Chemicals

All reagents were of analytical grade and were purchased from Sigma–Aldrich, Co. (St. Louis, MO, USA). Fibrinogen (fraction I, type I from human plasma) stock solutions were prepared by dissolving the protein in NaCl 0.9% (w/v) to obtain a final concentration of 1.16 g dL^{-1}. In all experiments, fibrinogen concentration was maintained constant. β-estradiol (water soluble) solution 3.38 μM was prepared by dissolving the ligand in NaCl 0.9%. Protein and ligand solutions were stored at -20°C.

Denaturation Experiments

Guanidine hydrochloride (Gdn–HCl) solutions ranging from 0 to 6 M were used as denaturant. Denaturation of the protein was carried out in different buffer solutions. The stock buffer solutions were comprised of acetate buffer (pH 4.2, 10 mM), phosphate buffers (pH 6.5 and 8.0, 40 mM), and Tris–HCl buffer (pH 7.4, 100 mM) with different denaturant concentrations (0–6 M). The protein denaturant solutions were incubated for 20 min before fluorescence analysis.

Absorbance Measurements

Absorbance spectra measurements were carried out in a Genesys 10 UV ThermoSpectronic Spectrophotometer (Rochester, NY, USA) during the fibrinogen-β-estradiol binding experiments. There were no changes in fibrinogen absorbance spectrum (absorption maximum at 280 nm) upon interaction with β-estradiol.

Fluorescence Measurements

Fluorescence measurements were carried out in a Varian Carry Eclipse Fluorescence Spectrophotometer (Mulgrave, Victoria, Australia). The fluorescence spectra were measured with 1 cm path length cells. The excitation and the emission slits were set at 5 nm. Intrinsic fluorescence of the protein was measured by exciting the protein solution at 280 nm and the emission spectra were recorded in the 300–450 nm range. For fluorescence binding measurements, spectra of protein solutions in the absence and presence of varying amounts of β-estradiol were made.

After each addition of small quantities of ligand, the sample was incubated for 5 min before recording the emission spectrum. Fluorescence intensities at the maximum of emission (peak heights) were corrected for inner filter and dilution effects before analysis of the binding data.

RESULTS AND DISCUSSION

Protein structure is now known as an ensemble of many fluctuating micro-states [11]. A protein is considered to be denatured when it has lost its characteristic native structure. The structural changes will depend on the type of protein examined, the kind of denaturing conditions the protein has been subjected to, as well as on the intensity and duration of exposure. The denaturation may affect general properties of the protein, such as the solubility, and additionally, more specific properties related to the function of the protein [12]. Fibrinogen is easily denatured by a vast number of compounds or treatments [12]. The distinction between denatured and nondenatured molecules of fibrinogen has been based on one of the functions of fibrinogen; namely, the polymerization and formation of an insoluble fibrin gel after the cleavage of peptide bonds by thrombin [12]. In the present work, we used fluorescence spectroscopy to monitor the changes observed during fibrinogen-β-estradiol binding by varying the media pH and in the presence of different denaturant concentrations.

Fibrinogen, as most of the proteins, may be affected by the pH of the medium. In buffered solutions, fibrinogen has an absorbance maximum at 280 nm typical of Trp residues. When excited at this wavelength, fibrinogen has an emission spectrum in the 300–450 nm wavelength range, with an emission maximum at 344 nm. In order to obtain information about pH influence on the fibrinogen response to β-estradiol binding, fluorescence emission spectra were measured at different pH buffer solutions. Figure 1 shows normalized spectra of fibrinogen at different pH values. In all cases, the emission wavelength was 344 nm (Figure inset, right axis). No emission spectra shifts were observed upon the changes in the solution pH. The fluorescence intensities obtained for pH 8.0 are only slightly higher than those of other pH values. An insignificant decrease is observed when the pH solution is changed from 8.0 to 4.2. The fluorescence intensities decrease 3.8% in relation with the maximum value at pH 7.4 and 4.2.

To provide an insight into the molecular aspects of fibrinogen-Gdn–HCl interaction and the effects during the denaturation processes, spectra of the protein with different denaturant concentrations and varying the media pH were obtained in the 300–450 nm range.

Figure 2 shows fibrinogen response as a function of denaturant concentration for all pH buffer solutions. For denaturant concentrations until 1 M, the emission wavelengths of the maxima do not change. In this region no appreciable effect was observed due to the influence of denaturant concentration or solution pH. From 1 to 3 M, a shift of the emission wavelength from 344 nm to 350–355 nm is observed, maintaining a constant value for higher concentrations. This red shift due to the presence of denaturant was reported before for several other Trp containing proteins (e.g., [13]). It is due to the Gdn–HCl induced protein complete unfolding, with the exposure of all the Trp residues to the aqueous environment and the consequent shift of their fluorescence emission to higher wavelengths. The reason for the phenomenon observed at low Gdn–HCl concentrations is because there is a folding and refolding process caused by the denaturation process. Above 4 M of Gdn–HCl the emission wavelength of the protein is kept constant. The inset in Fig. 2 shows the spectra obtained when the solution pH is equal to 6.5. As it can be seen, a 60% decrease in the fluorescence intensity is observed when Gdn–HCl concentration changes from 1 to 2 M. This behavior is also explained by the exposure to the aqueous solution of the Trp residues previously buried in the fibrinogen tertiary and quaternary structures, with a consequent decrease in their fluorescence quantum yields. The same spectral variation trends were obtained for the other pH values (data not shown). The denaturation induced decrease on the fluorescence quantum yield, tending to the same plateau resulting form the full exposure to the aqueous environment, can clearly be noticed in Fig. 3. This figure shows the variation of the fluorescence emission intensity as a function of Gdn–HCl concentration at different pH values. As previously described, a gradual decrease in fluorescence intensity is obtained by increasing the denaturant concentration (0 to 2 M range). After this point the intensity appears to maintain constant. Below 2 M of Gdn–HCl a noticeable difference was encountered for pH 4.2 where the fluorescence intensity decreases more rapidly than for the other buffered solutions. This result is probably caused by a more extensive protein unfolding enhanced by the lower solution pH. This behavior has been reported before for other proteins that shows an unfolding state in the vicinity of the pH 3–4 [14,15].

Figure 4 shows the fluorescence intensity as a function of added β-estradiol concentration for fibrinogen, in the absence and in the presence of denaturant, in acetate buffer pH 4.2. In the absence of denaturant, the addition of β-estradiol to the protein solution caused a decrease in the fluorescence intensity. In the presence of Gdn–HCl 1 M, no significant changes were

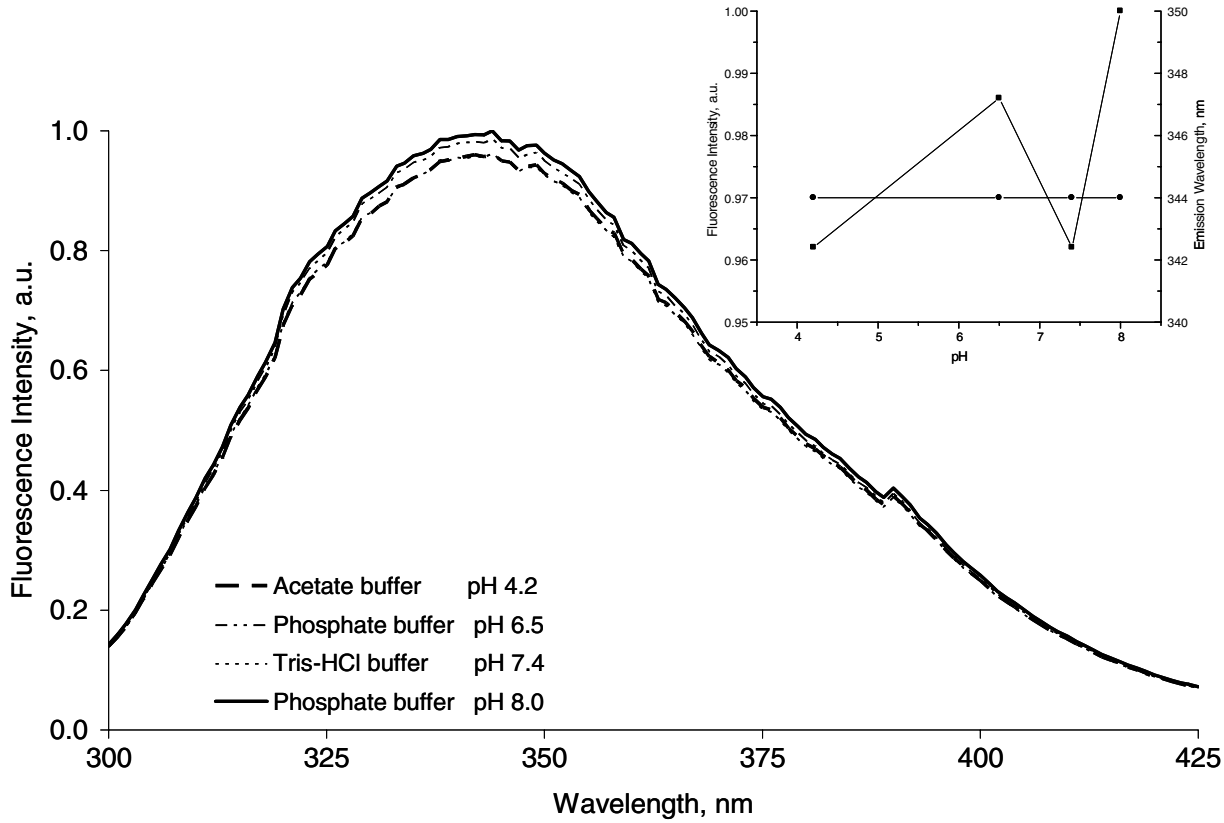

Fig. 1. Emission spectra of fibrinogen at different buffers solutions: acetate buffer (pH 4.2; 10 mM); phosphate buffers (pH 6.5 and pH 8.0; 40 mM); Tris–HCl buffer (pH 7.4; 100 mM). Excitation wavelength: 280 nm. Fibrinogen concentration is kept constant (202 mg L^{-1}). Inset: variation of the maxima fluorescence intensity (left) and emission wavelength (right) of fibrinogen at different pH values (●—emission wavelength; ■—fluorescence intensity). The fluorescence intensities (peak heights) were normalized by dividing by the maximum fluorescence intensity of the spectra obtained at pH 8.0.

observed in relation to denaturant free fibrinogen. In both situations, the fibrinogen-β-estradiol binding seems to induce a conformational change resulting in a decrease of the Trp residues fluorescence. However, when the Gdn–HCl concentration is increased to 5 M, a substantial change in the response of the system is observed: the fluorescence intensity is almost maintained constant as β-estradiol concentration increases. This effect may be explained by the complete protein denaturation at this Gdn–HCl concentration, impairing the binding of β-estradiol and/or the conformational change induced by it.

Figure 5 presents the fibrinogen-β-estradiol binding curve in the absence and in the presence of Gdn–HCl, at pH 7.4. At this pH value, similar results were obtained for fibrinogen in the absence of denaturant and with 1 M of Gdn–HCl. When denaturant concentration arises to 5 M, the curve shows a change in the response to the increase in β-estradiol concentration. By comparing Figs. 4 and 5 it can be noticed that when denaturant concentration is equal to 1 M the behavior of the fibrinogen during the binding process is quite similar for both buffer solutions. However, there is a major difference between the results obtained with Gdn–HCl 5 M at pH 7.4 and 4.2. While at pH 4.2 the denaturation leads to a total inhibition of the decrease in fluorescence intensity indicative of fibrinogen-β-estradiol binding, at pH 7.4 the binding is less extensive at Gdn–HCl 5 M than in its absence, but the fibrinogen-β-estradiol binding still occurs. This effect can be due to a stabilization of the fibrinogen conformation at pH 7.4, enabling the β-estradiol binding even in the presence of higher concentrations of denaturant. The decrease on the extent of the binding can be explained by a preservation of some of the binding sites of fibrinogen while other sites are unfolded by the denaturation process. The decrease could also be explained by an impairment of the binding in some of the proteins while another sub-population keeps their β-estradiol binding properties.

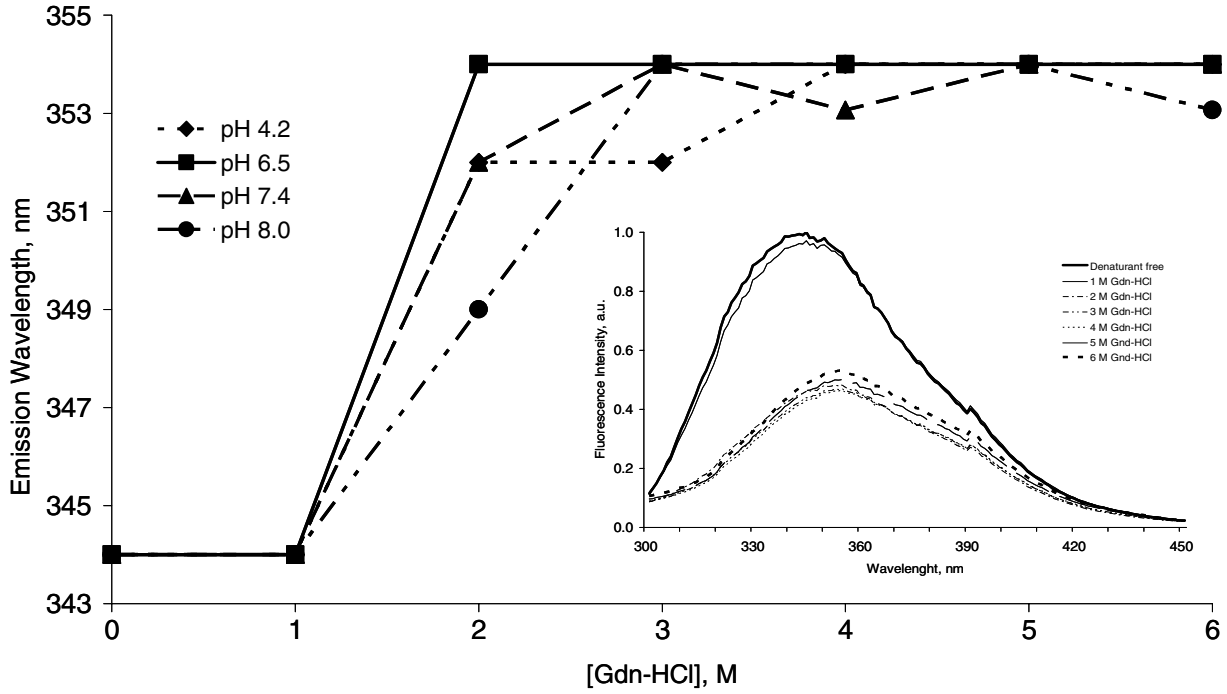

Fig. 2. Emission wavelength values as a function of Gdn–HCl concentration for fibrinogen at different buffers: pH 4.2, 10 mM (acetate buffer); pH 6.5 and 8.0, 40 mM (phosphate buffer); pH 7.4, 100 mM (Tris–HCl buffer). Excitation wavelength: 280 nm. Fibrinogen concentration is kept constant (202 mg L^{-1}). Inset: Fluorescence spectrum of fibrinogen in the absence and in the presence of different denaturant concentrations when pH is equal to 6.5. The fluorescence intensities (peak heights) of the inset were normalized by dividing by the maximum fluorescence intensity of the spectra obtained in the absence of denaturant.

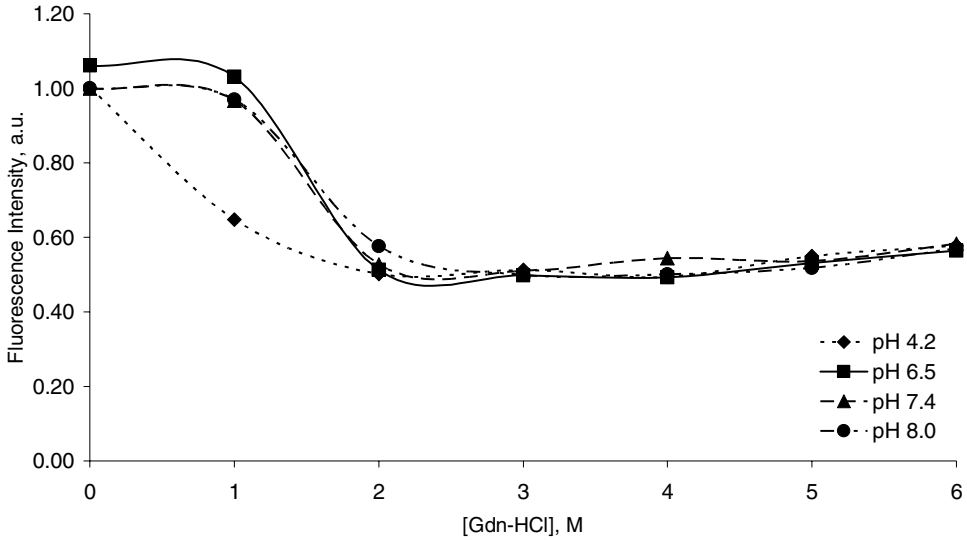

Fig. 3. Fluorescence intensity of fibrinogen as a function of Gdn–HCl concentration at different buffers: pH 4.2, 10 mM (acetate buffer); pH 6.5 and 8.0, 40 mM (phosphate buffer); pH 7.4, 100 mM (Tris–HCl buffer). The fluorescence intensities at the maximum of emission (peak heights) were normalized by dividing by the maximum fluorescence intensity of the spectra obtained in the absence of denaturant.

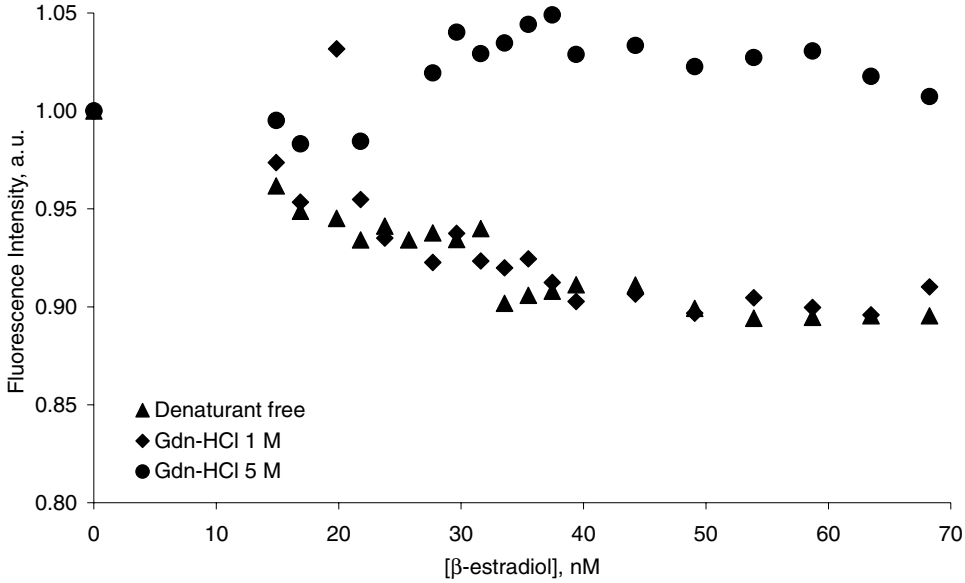

Fig. 4. Normalized fluorescence intensity peak height values obtained for fibrinogen-β-estradiol binding in acetate buffer pH 4.2, 10 mM (excitation wavelength 280 nm; emission wavelength 344 nm for denaturant free ([Gdn–HCl] = 0 M) and [Gdn–HCl] = 1 M; 354 nm for [Gdn–HCl] = 5 M). Fibrinogen concentration is kept constant (202 mg L^{-1}).

It has been suggested that certain ordered protein conformations, commonly called molten globular states, can represent protein folding intermediates [16,17]. The emerging consensus is that these partially ordered forms exhibit a high content of secondary structure, considerable compactness, nonspecific tertiary structure, and significant structural flexibility. These characteristics may be used to define a general state of the protein,

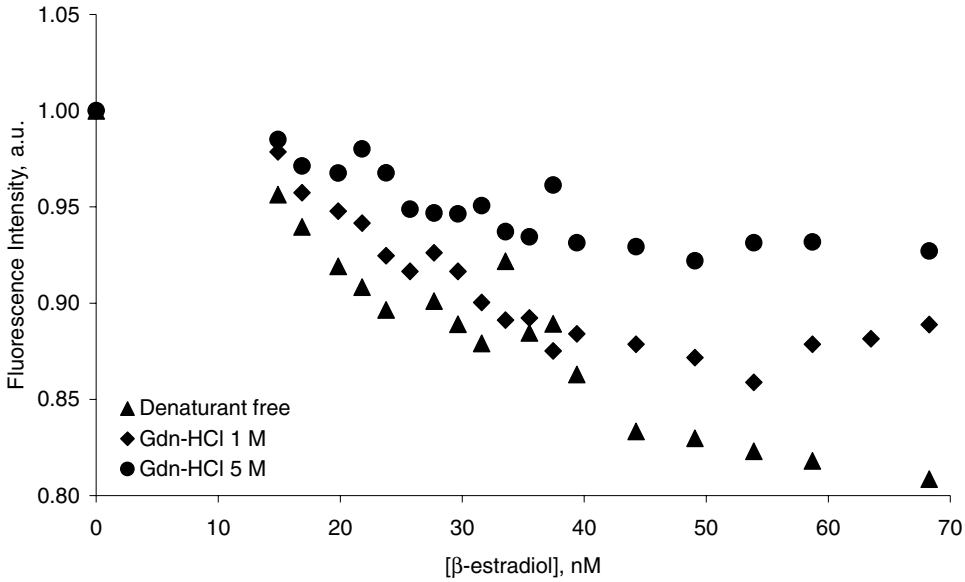

Fig. 5. Normalized fluorescence intensity peak height values obtained for fibrinogen-β-estradiol binding in Tris–HCl buffer pH 7.4, 10 mM (excitation wavelength 280 nm; emission wavelength 344 nm for denaturant free ([Gdn–HCl] = 0 M) and [Gdn–HCl] = 1 M; 354 nm for [Gdn–HCl] = 5 M). Fibrinogen concentration is kept constant (202 mg L^{-1}).

called the molten globule state, which is structurally and thermodynamically distinct from both the native state and the denatured state [18]. In our work, this phenomenon can be reflected in the maintenance by fibrinogen of the β-estradiol binding properties at low Gdn–HCl concentration (at both pH values) and/or at high Gdn–HCl concentration at pH 7.4. It may be speculated that these results reinforce the hypothesis that some of the erythrocyte hypoaggregation effect mediated by β-estradiol comes from the fibrinogen-β-estradiol binding ability. However, it should be stressed that further experiments and different methodological approaches must be conducted in order to further explain these aspects.

In conclusion, changes in the protein structure due to the denaturation process were observed during fibrinogen-β-estradiol binding. At high concentration of denaturant, when it is supposed that the protein is completely unfolded, the pH of the solution can create a new hydrophobic environment in the folding-unfolding system that enables fibrinogen-β-estradiol binding properties following a new binding process not so distinctive from the observed for the protein in the native conformation.

ACKNOWLEDGMENTS

This work was supported by the Fundação para a Ciência e Tecnologia (FCT) of the Portuguese Ministry of Science, Technology and Higher Education (MCTES).

REFERENCES

1. A. D. Smith (2004). *Oxford Dictionary of Biochemistry and Molecular Biology*, Rev. edn., Oxford University Press, Oxford.
2. Y. Veklich, E. K. Ang, L. Lorand, and J. W. Weisel (1998). The complementary aggregation sites if fibrin investigated through examination of polymers of fibrinogen with fragment E. *Proc. Natl. Acad. Sci. USA* **95**, 1438–1442.
3. NCBI Protein Database; http://www.ncbi.nlm.nih.gov/entrez, accessed on October 2005.
4. D. Lominadze and L. W. Dean (2002). Involvement of fibrinogen specific binding in erythrocyte aggregation. *FEBS Lett.* **517**, 41–44.
5. T. Jensen, S. Halvorsen, H. Godal, P. M. Sandset, and O. Skjønsberg (2004). The viscosity of fibrinogen subfractions and of EDTA denatured fibrinogen do not differ from that of native fibrinogen. *Thromb. Res.* **113**, 51–56.
6. J. M. Montejo, K. R. Naqvi, M. P. Lillo, J. González-Rodriguez, and A. U. Acuña (1992). Conformation of human fibrinogen in solution from polarized triplet spectroscopy. *Biochemistry* **31**, 7580–7586.
7. I. Gonçalves, C. Saldanha, and J. Martins-Silva (2001). β-estradiol effect on erythrocyte aggregation — A controlled *in vitro* study. *Clin. Hemorheol. Microcirc.* **25**, 127–134.
8. M. Prokopowicz, J. Lukasiak, B. Banecki, and A. Przyjazny (2005). *In vitro* measurement of conformational stability of fibrinogen adsorbed on siloxane. *Biomacromolecules* **6**, 39–45.
9. S. Gonçalves, N. C. Santos, J. Martins-Silva, and C. Saldanha (2006). Fluorescence spectroscopy evaluation of fibrinogen-β-estradiol binding. Submitted.
10. http://www.agsci.ubc.ca/courses/fnh/410protein/1_54.htm, accessed on October 2005.
11. Y. Tanaka, K. Tsumo, M. Umetsu, T. Nakanishi, Y. Yasutake, N. Sakai, M. Yao, I. Tanaka, T. Arakawa, and I. Kumagai (2004). Structural evidence for guanidine-protein side chain interactions: Crystal structure of CutA from *Pyrococcus horikoshii* in 3 M guanidine hydrochloride. *Biochem. Biophys. Res. Commun.* **323**, 185–191.
12. U. Haddeland, A. Bennick, and F. Brosstad (1995). Stimulating effect on tissue-type plasminogen activator — A new and sensitive indicator of denatured fibrinogen. *Thromb. Res.* **77**, 329–336.
13. C. H. Ramos (2004). A spectroscopic-based laboratory experiment for protein conformational studies. *Biochem. Mol. Biol. Educ.* **32**, 31–34.
14. A. L. Fink, L. J. Calciano, Y. Goto, T. Kurotsu, and D. R. Palleros (1994). Classification of acid denaturation of proteins: Intermediates and unfolded state. *Biochemistry* **33**, 12504–12511.
15. D. K. Debnath, K. Mukhopadhyay, and S. Basak (2005). Acid-induced denaturation and refolding of prothrombin. *Biophys. Chem.* **116**, 159–165.
16. R. Khodarahmi and R. Yazdanparast (2005). Suppression effect of guanidine hydrochloride on α-cyclodextrin-assisted refolding of denatured α-amylase. *Process Biochem.* **40**, 2973–2979.
17. O. B. Ptitsyn, R. H. Pain, G. V. Semisotnov, E. Zerovnik, and O. I. Razgulyaev (1990). Evidence for a molten globule state as a general intermediate in protein folding. *FEBS Lett.* **262**, 20–24.
18. D. T. Haynie and E. Freire (2004). Structural energetics of the molten globule state. *Proteins* **16**, 115–140.