ELSEVIER

Biophysical Chemistry 116 (2005) 159 – 165

Biophysical Chemistry

http://www.elsevier.com/locate/biophyschem

Acid-induced denaturation and refolding of prothrombin

Dilip Kumar Debnath[1], Kasturi Mukhopadhyay[2], Soumen Basak*

Chemical Sciences Division, Saha Institute of Nuclear Physics, 1/AF Bidhannagar, Calcutta 700064, India

Received 13 January 2005; accepted 3 February 2005
Available online 29 April 2005

Abstract

Structural transitions of the blood coagulation factor prothrombin (extracted from goat blood) in response to reduction of pH were investigated by fluorescence, circular dichroism and light scattering measurements. The study revealed the presence of a partially unfolded state at around pH 3.5, characterized by marked enhancement of fluorescence from ANS bound to the protein, increase of bimolecular rate constant for tryptophan fluorescence quenching and a sharp peak in the light scattering intensity. Further lowering of the pH caused reversal of the trend of variation of these parameters, suggesting that prothrombin folds back to a compact state containing native-like secondary structural elements. The refolded state at low pH (<pH 3) fits the description of the A-state, the end-point of acid-induced denaturation process of several other monomeric proteins, and is a possible candidate for the class of folding intermediates known as molten globules.

Keywords: Prothrombin; Structural transitions; Acid; Partial unfolding; Molten globule; ANS fluorescence; Circular dichroism; Light scattering

1. Introduction

Electrostatic interactions between charged residues on the surface of a protein play an important role in conferring stability to its folded structure. Change of pH alters the ionisation state of these residues, causing intramolecular charge repulsion and possible disruption of salt bridges that can lead to destabilization of the native protein conformation [1]. pH is an important factor determining protein structure and function. Most proteins are stable and active at physiological pH and show varying degrees of denaturation in acid medium. However, as the acid concentration increases, a number of these proteins revert back to a compact conformation containing substantial secondary structure that resemble the folding intermediates known as molten globules [2,3]. Study of the structural stability of a protein as a function of pH thus helps understand the thermodynamic or kinetic intermediates in its folding pathway and identifies the electrostatic interactions important for the stability of its folded state [4,5].

Prothrombin, a vitamin K-dependent plasma glycoprotein, is the inactive zymogen form of thrombin, the final regulatory enzyme generated during blood coagulation [6]. It is activated to thrombin most readily in presence of the fully assembled enzyme complex prothrombinase, consisting of blood coagulation factors X_a (a serine protease) and V_a (a cofactor), Ca^{2+} ions and a negatively charged membrane surface [7]. Prothrombin is a single chain, multi-domain protein (M.W. ~70 kD) consisting of three-Gla, kringle and catalytic-domains. The N-terminal Gla domain contains ten highly conserved γ-carboxyglutamate (Gla) residues that exist in the deprotonated (negatively charged) form at neutral pH and mediate the binding of prothrombin to phospholipid membranes through Ca^{2+} bridges formed between the charged carboxyl groups on the Gla residues and the lipid head groups [8,9]. The calcium ions induce a conformational change of prothrombin to ensure tight membrane binding, while two of its peptide bonds are cleaved by factor X_a to generate thrombin. Thrombin, in turn, cleaves fibrinogen to form fibrin, which then forms a net-like framework to produce the blood clot.

* Corresponding author. Fax: +91 33 2337 4637.
E-mail address: soumen.basak@saha.ac.in (S. Basak).
[1] Present Address: Raiganj College, Raiganj 733134, West Bengal, India.
[2] Present Address: Harbor–UCLA Research and Education Institute, CA 90502, USA.

0301-4622/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.bpc.2005.02.003

A previous study had examined the pH- and ionic strength-dependence of the equilibrium between fast- and slow-folding forms of bovine prothrombin fragment 1 (N-terminal residues 1–156) and proposed the existence of a pH-dependent self-association to explain the data [10]. We report here a study of the effect of acidic pH on the conformational properties of intact prothrombin isolated from goat blood. As a starting point, we assume, on the basis of species similarity between cow and goat, that the sequence and structural features of goat prothrombin are similar to those of bovine prothrombin, which contains 14 tryptophan (trp) residues and 12 disulphide bonds [11]. Structural alterations were monitored by both steady-state and time-resolved measurements of trp and ANS fluorescence, acrylamide quenching of trp emission, circular dichroism and light scattering measurements. Our results show that above pH 4, prothrombin exists in a compact native state characterized by pronounced secondary structure and a low degree of ANS fluorescence. With decrease in pH, it undergoes a sharp transition to a partially unfolded conformation at ~pH 3.5, characterized by marked enhancements in ANS fluorescence, trp fluorescence quenching efficiency and light scattering signal. However, in even more acidic medium, these properties revert back to their values existing in the native state at higher pH, suggesting refolding of the protein into a compact state with secondary structure and restored tertiary packing.

2. Materials and methods

Ultrapure acrylamide and Tris were procured from Sigma (St. Louis, MO, USA). 1,8-anilino naphthalene sulphonate (ANS) was purchased from Molecular Probes. All other chemicals were of reagent grade or better. The following buffers were used for pH-dependent studies: phosphate for pH 6–7, acetate–acetic acid for pH 4–5 and glycine–HCl for lower pH down to pH 1 (all of strength 25 mM).

2.1. Isolation of prothrombin

Prothrombin was isolated from goat (*Cipra Hirca*) blood plasma by the barium citrate adsorption technique following published protocols for bovine prothrombin [6]. Briefly, fresh goat blood collected from the slaughterhouse was mixed with 2.85% sodium citrate solution (anticoagulant) in the ratio 8:1 (v/v) and centrifuged at 3000 *g* for 15 min. Serum from the top layer was collected, made into a barium citrate suspension (by adding 80 ml 1M $BaCl_2$ per litre of plasma) and subjected to three cycles of centrifugation (3600 *g* for 30 min) and resuspension of the precipitate in a citrate–saline solution containing 0.9% NaCl and 20 mM sodium citrate. The resulting barium citrate complex was suspended in 20 mM EDTA solution, pH 7.4 (120 ml/l of plasma) and dialyzed extensively against a citrate–saline solution (20 mM citrate, 0.09% NaCl) containing 20 mM EDTA, pH 7.4. Prothrombin was precipitated from the dialysate by treatment with ammonium sulfate, dissolved in 25 mM sodium citrate buffer, pH 6, containing 0.1% PMSF and dialyzed against the citrate buffer. Insoluble protein, which normally precipitates after dialysis, was removed by centrifugation at 3600 *g* for 20 min. The supernatant was applied to a DEAE Sephadex column (2 cm × 15 cm), washed with the same citrate buffer and eluted with citrate–saline containing 25 mM sodium citrate, pH 6 and 0.1 M NaCl. The purity of the preparation was checked by 8% SDS–polyacrylamide gel electrophoresis under reducing condition. A single band characteristic of prothrombin (M.W. ~70 kD) appeared after Coomassie blue staining. Prothrombin concentration was determined using the extinction coefficient of the bovine protein ($\varepsilon = 1.44$ mg^{-1} ml cm^{-1} at 280 nm) and verified by Lowry's method using BSA as standard [12,13]. A correction factor due to Rayleigh scattering was applied to the spectrophotometric analysis using the relation [12]

$$A_{280}(\text{corr}) = A_{280}(\text{obs}) - 1.7065 \times A_{345}(\text{obs}). \quad (1)$$

2.2. Spectroscopic measurements

Absorbance spectra were recorded on a Shimadzu UV-2101 spectrophotometer. A Hitachi F-4010 spectrofluorometer was used for steady-state fluorescence measurements. Tryptophan fluorescence was observed with excitation at 295 nm, bandwidths of 5 nm in both excitation and emission channels and typical prothrombin concentrations of 0.25 μM. Fluorescence emission from ANS was measured using excitation at 350 nm, bandwidths of 5/10 nm in excitation/emission channels and ANS:prothrombin ratio of 25:1. The concentration of ANS stock in water was determined using an extinction coefficient of 4950 $M^{-1}cm^{-1}$ at 350 nm [14]. All measurements were made at 25 °C.

Fluorescence quenching experiments were performed by successive addition of 2 μl aliquots of a 2 M stock solution of acrylamide to a solution of prothrombin and monitoring the change of trp emission intensity at 340 nm. The measured intensity was corrected due to acrylamide absorption (at 295 nm) by multiplying with a factor of antilog(ΔOD/2), where ΔOD is the increase in optical density at the tryptophan excitation wavelength as acrylamide is added and 0.5 cm is the effective path length of the cell [15]. The Stern–Volmer quenching constant (K_{SV}) was calculated as the slope of the linear region of the F_0/F_{corr} vs. $[Q]$ plots near the origin, where F_0 and F_{corr} are, respectively, the corrected fluorescence intensities in absence and in presence of a concentration $[Q]$ of quencher molecules. The bimolecular quenching rate constant k_q was calculated using the relation $k_q = K_{SV}/\langle\tau\rangle$, where $\langle\tau\rangle$ is the mean fluorescence lifetime determined as described below.

Fluorescence lifetimes of trp and ANS were determined from measurement of their total emission intensity decays, using a nanosecond time-domain fluorometer assembled in

our laboratory and operated in the time-correlated single-photon-counting mode [16]. Excitation was provided by a pulsed high-pressure (1.5 atm) N_2-lamp operating at 25 kHz, the pulse profile having a FWHM of 1.3 ns. The 295-nm and 358-nm N_2 lines were used to excite trp and ANS, respectively, while their emissions were monitored at 340 nm and 470 nm. Slits of bandwidth 16 nm were used in both excitation and emission channels. Intensity decay profiles were fitted to the sum-of-exponentials series

$$I(t) = \sum_i A_i \exp(-t/\tau_i) \quad (2)$$

where A_i is a factor representing the fractional contribution to the time-resolved decay of the component with a lifetime of τ_i. Best-fit parameters were estimated using software supplied by Edinburgh Instruments (UK), implementing a non-linear least-squares iterative fitting procedure. Mean lifetimes were calculated using the relation

$$\langle \tau \rangle = \sum_i A_i \tau_i^2 / \sum_i A_i \tau_i. \quad (3)$$

Circular dichroic (CD) spectra in the far-UV region were recorded on a JASCO J-720 spectropolarimeter using a cylindrical quartz cell of path length 1 mm. Spectra shown are averages of five consecutive scans performed at a scan speed of 20 nm/min, corrected by subtracting corresponding blanks and subjected to noise reduction. Results are presented as mean residue molar ellipticity (Θ):

$$\Theta = \theta_{obs}/(10nlc) \quad (4)$$

where θ_{obs} is the measured ellipticity in millidegrees, n the number of residues in the protein, l the path length of the cell expressed in cm and c the molar concentration of protein. The relative fractions of three different types of secondary structure (α-helix, β-sheet and random coil) present in the CD spectra were calculated using an in-house computer program that minimises the mean-squared deviation (i.e., Chi-square) between each measured spectrum and a linear combination of standard spectra representing the three different secondary structures [17,18].

Static light scattering measurements were performed on the Hitachi F-4010 spectrofluorometer by setting both its excitation and emission channels at the same wavelength (450 nm) and using a bandwidth of 1.5 nm in both channels.

3. Results and discussion

Fig. 1 shows the tryptophan fluorescence emission spectra of prothrombin incubated in buffers of varying pH (from 1 to 7). Between pH 7 and pH 4, the spectra show emission maxima at ~336 nm, characteristic of trp residues located in regions of low effective dielectric constant, such as inside a compact folded protein. Between pH 4 and pH 2, the emission maximum undergoes a rapid red shift of 7 nm to 343 nm, pointing to a reduced hydrophobicity of the trp

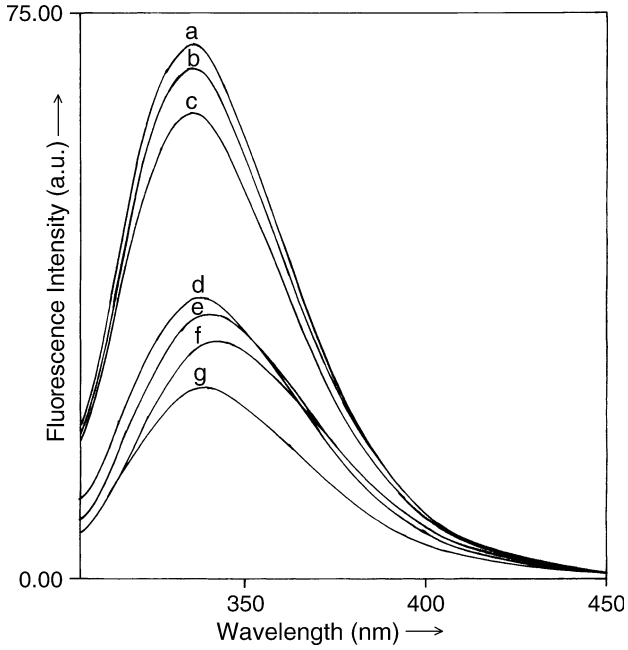

Fig. 1. Tryptophan emission spectra of prothrombin at (a) pH 6, (b) pH 7, (c) pH 4.5, (d) pH 4, (e) pH 3, (f) pH 2 and (g) pH 1.

microenvironments caused by lowering of pH (Fig. 2(a)). In the same pH interval, the intensity of the emission maximum decreases by ~50% (Fig. 2(b)).

Fig. 3 shows the trp emission intensity decay profiles at several pH values. The mean excited state lifetime, as determined from these measurements, decreases from 3.8 ns at pH 4.5 to 3.0 ns at pH 2 (Table 1). The diminished quantum yield indicates activation of non-radiative pathways of fluorescence decay, such as those caused by exposure to the aqueous environment or to neighbouring residues that can quench emission from trp.

The hydrophobic dye molecule ANS (1-anilinonaphthalene-8-sulfonic acid) prefers to occupy hydrophobic sites in a protein and can thus be used as an extrinsic fluorophore to report on the degree of compactness of protein structures. When excited at 337 nm, ANS in aqueous medium shows a broad emission spectrum at ~530 nm with very low quantum yield, but upon binding to hydrophobic regions in proteins it exhibits a pronounced blue shift of the emission maximum and a marked increase of fluorescence intensity [19]. Changes in ANS fluorescence are frequently used to detect non-native conformations of globular proteins, especially those that are characterized by the presence of solvent exposed hydrophobic clusters or patches [20].

Fig. 4 shows the steady-state emission spectra of ANS pre-incubated with prothrombin in buffers of varying pH, while Fig. 5 shows the corresponding time-resolved intensity decay profiles at some of these pH values. Fig. 6 summarizes the dependence of the fluorescence parameters of ANS on pH. Between pH 7 and pH 4.5, the position and intensity of the emission peak remain unaltered at the values

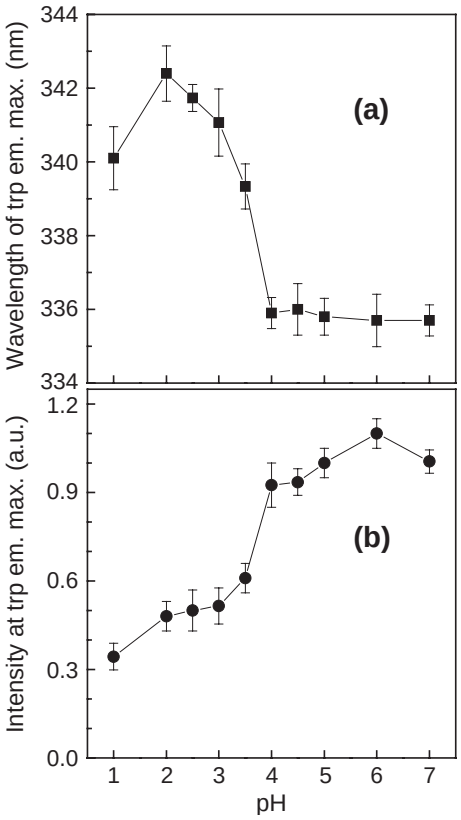

Fig. 2. Variation of (a) wavelength and (b) intensity of the tryptophan emission maximum of prothrombin with pH.

characteristic of fluorescence from ANS alone in water. Below pH 4.5, however, the peak position undergoes a marked blue shift to ~475 nm while the peak intensity increases dramatically by a factor of nearly 30 within the small interval of pH 4.5 to pH 3. The increase in intensity is mirrored by a simultaneous increase of the mean excited

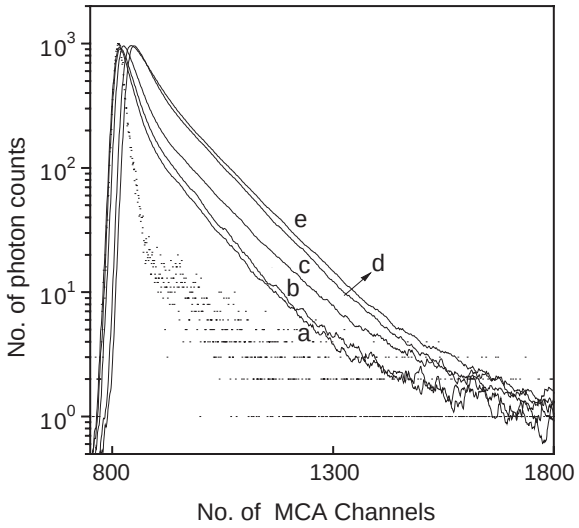

Fig. 3. Continuous curves showing the results of biexponential fits to tryptophan fluorescence intensity decay profiles at (a) pH 1, (b) pH 3, (c) pH 3.5 and (d) pH 6. The dotted curve represents the instrumental profile of the lifetime spectrometer.

Table 1
Fluorescence lifetimes ($\langle\tau\rangle$), Stern–Volmer quenching constants (K_{SV}) and bimolecular quenching rate constants (k_q) for acrylamide quenching of tryptophan emission from prothrombin at different pH

pH of solution	τ_1 (ns)	α_1 (%)	τ_2 (ns)	α_2 (%)	$\langle\tau\rangle$ (ns)	K_{SV} (M^{-1})	$k_q \times 10^{-9}$ (M^{-1} s^{-1})
1.0	0.99	57.6	4.61	42.4	2.52	–	–
2.0	1.04	47.5	4.77	52.5	3.00	–	–
2.5	1.09	46.7	4.72	53.3	3.02	8.22	2.72
3.0	1.05	48.2	4.85	51.8	3.02	8.69	2.88
3.5	0.90	48.4	4.88	51.6	2.95	15.5	5.25
4.0	1.00	39.6	4.82	60.4	3.31	13.54	4.09
5.0	1.20	26.8	4.78	73.3	3.83	11.7	3.05
6.0	1.33	24.5	4.93	75.5	4.08	10.97	2.69

Excitation: 297 nm, emission: 340 nm.

state lifetime of ANS in presence of prothrombin, from 8 ns at pH 6 to 14 ns at pH 3. These results suggest that between pH 4 and pH 3, prothrombin undergoes a structural transition that exposes hydrophobic patches in the interior of the protein to the solvent, facilitating the binding of ANS molecules to their preferred hydrophobic sites. On further decrease of pH below 3, both the emission intensity and the lifetime decrease considerably down to pH 1, which can be interpreted to mean that the protein reverts back to a (relatively) more compact folded structure below pH 3.

Structural changes in proteins can be detected by monitoring their circular dichroic (CD) spectra, especially in the far-UV region where the main contribution to the spectra comes from the secondary structure of the peptide backbone. CD spectra of prothrombin at various pH values are shown in Fig. 7. Using a least-squares procedure for spectral data fitting (as described in the Materials and Methods section), we calculated the following relative

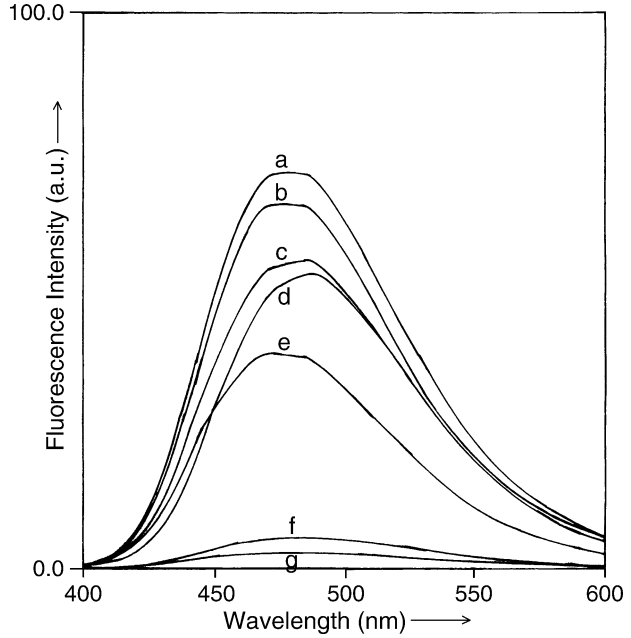

Fig. 4. Fluorescence spectra of ANS pre-incubated in prothrombin at (a) pH 3, (b) pH 3.5, (c) pH 2, (d) pH 2.5, (e) pH 4, (f) pH 4.5 and (g) pH 7.

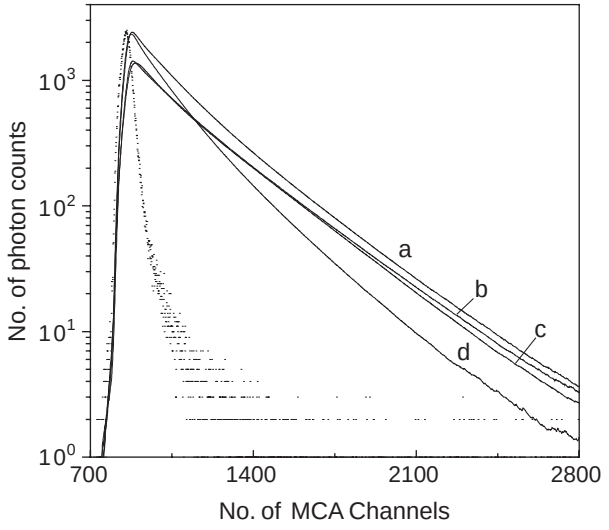

Fig. 5. Biexponential fits to fluorescence intensity decay profiles of ANS pre-incubated in prothrombin at (a) pH 4.5, (b) pH 3, (c) pH 2 and (d) pH 6. The sharp decay curve at left (dotted line) represents the instrumental profile.

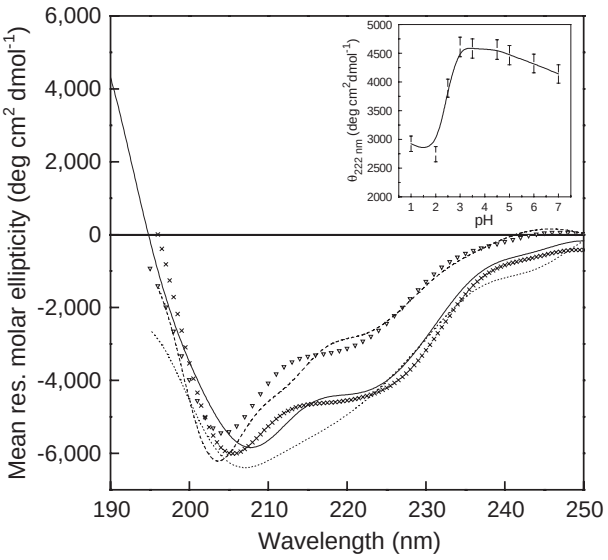

Fig. 7. Far-uv CD spectra of prothrombin at various pH: pH 1 (▽), pH 2 (- - - -), pH 3 (····), pH 5 (x) and pH 6 (—). Inset shows ellipticity at 222 nm as a function of pH. Concentration of protein used was ~0.2 mg/ml (3 μM).

contributions of secondary structure elements to be present in the CD spectrum at pH 6: 26% α-helix, 21% β-sheet and 53% random coil [18]. These numbers are comparable to those obtained from CD spectra of prothrombin from other vertebrate species [11]. The nature of the spectrum changes noticeably below pH 3, leading to a reduction in the depths of the double minima around 208 and 222 nm that are characteristic of the α-helix. The magnitude of the mean residue molar ellipticity at 222 nm decreases abruptly (from ~5000 to ~3000 mdeg cm^2 $dmol^{-1}$) below pH 3 but remains constant thereafter (inset of Fig. 7), indicating presence of a reduced level of secondary structure even in the most acidic state.

Although near-UV CD spectra can reveal the local ordering of aromatic residues, i.e., the tertiary structure in proteins, such spectra of prothrombin at acidic pH could not be measured as the protein was found to precipitate at low pH (≤4) at concentrations above ~4 μM. Instead, we have used quenching studies of fluorescence emission from tryptophan to extract information on the accessibility of tryptophan residue(s) to the quencher molecules, thereby indirectly monitoring the rigidity of tertiary packing in the interior of the protein [21]. Fig. 8(a) shows the Stern–Volmer plots for acrylamide quenching of trp emission from prothrombin incubated at various pH, while Fig. 8(b) shows the quenching constant (K_{SV}) plotted as a function of pH. Table 1 shows the pH-dependence of the bimolecular rate constant k_q ($=K_{SV}/\langle\tau\rangle$), which is a more accurate indicator of the efficiency of quenching. It is observed that the value of k_q increases from 2.7×10^9 M^{-1} s^{-1} at pH 6 to 5.3×10^9 M^{-1} s^{-1} at pH 3.5, suggesting relaxation of tertiary packing of the protein and formation of a comparatively open structure that offers increased access of the quenchers to the trp residues. Below pH 3.5, however, k_q drops sharply to 2.8×10^9 M^{-1} s^{-1}, suggesting that the unfolding caused by the reduction of pH is reversed below pH 3.5, resulting once again in a compact structure.

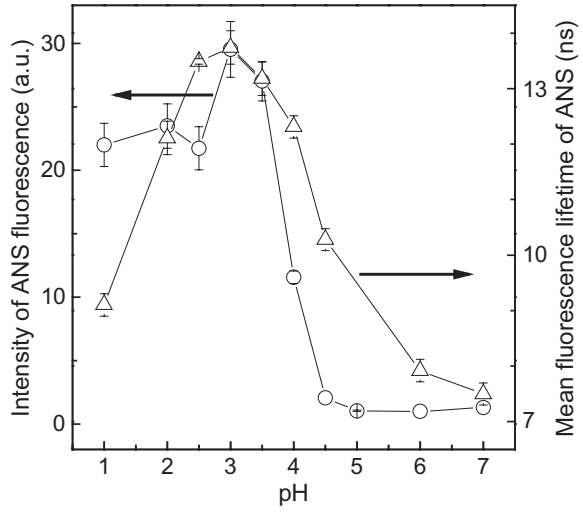

Fig. 6. Fluorescence parameters of ANS incubated with prothrombin at various pH: intensity at emission maximum (○) and mean lifetime (△).

Unfolding of proteins is usually reflected in a concomitant increase in their size, typically characterized by the hydrodynamic radius R_H [20]. Fig. 9 shows the result of static light scattering measurements performed as a function of pH to test for the loss of compactness of prothrombin in the partially unfolded state at low pH. A pronounced maximum in the intensity of Rayleigh scattered light is observed at pH 3.5, clearly indicating an increase in R_H at this pH. The data also show that the compact state of the protein is restored again at lower pH, i.e., below pH 3.

The effect of pH on protein structure is not unique, varying from one protein to another. Most proteins unfold in

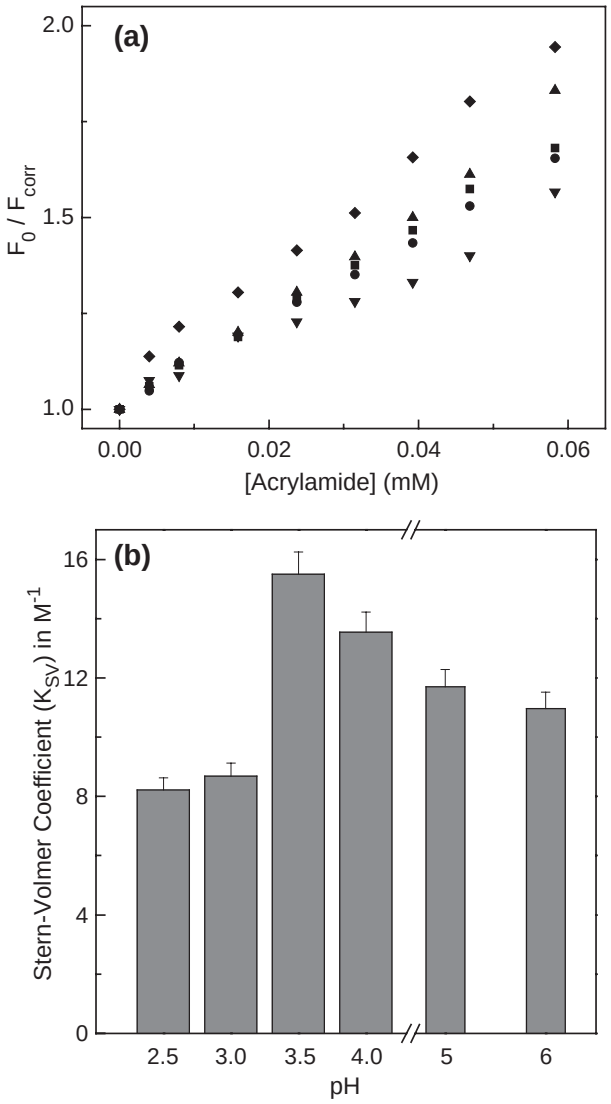

Fig. 8. (a) Stern–Volmer plots for acrylamide quenching of tryptophan fluorescence from prothrombin at pH 6 (●), pH 5 (■), ph 4 (▲), pH 3.5 (♦) and pH 3 (▼). (b) The Stern–Volmer coefficients (K_{SV}) plotted as a function of pH.

acid medium, though a few show no such tendency. Of those that do, several monomeric globular proteins undergo partial unfolding between pH 3 and pH 4, followed by return to a compact folded state at even lower pH (~pH 2) [1–3]. In the present study, we have observed a similar pattern of acid-induced structural transitions, whose origin should lie in changes in electrostatic interactions between charged residues on the surface of the protein molecule arising out of the change in pH of the solvent. The X-ray crystal structure of the N-terminal fragment 1 (Res 1–156) of bovine prothrombin, belonging to the same family of vertebrate prothrombins as goat, reveals the presence of a number of interacting ion pairs that provide stability to the folded structure [22]. These are Lys57-Glu60, Glu99-Arg135, Asp109-Arg116 and Asp109-Arg111, the formation of all of which is favoured at or near the physiological pH. At acidic pH, the negatively charged carboxyl groups of the Asp and Glu side chains (pK_a=4.5) will be protonated, disrupting the salt bridges and causing repulsion between the uncompensated positive charges on the Arg and Lys groups. Both these factors can contribute to the unfolding of the protein, as observed here. It is interesting to note in this connection that the p*I* of bovine prothrombin was estimated to be 4.1 from measurements of variation in its electrophoretic mobility with pH [23]. The closeness of the p*I* value, the pH at which the protein is expected to be in the charge neutral state, and the pH of the maximally unfolded state observed in our work reinforces the idea that change of ionisation state of the surface residues might indeed play a role in the loss of compactness. Decreasing the pH further will add both protons and anions to the solution. The added anions would interact with the positively charged centres on the protein, thus effectively shielding the repulsive forces and causing the protein to fold up again [2]. It should be noted that the presence of the large number (12) of disulfide bonds must also act as a deterrent to the complete unfolding of the molecule, preventing further expansion of the partially unfolded form.

Partially unfolded states of proteins, like the one reported here, have been implicated for non-specific aggregation of proteins, especially those made up of multiple domains or subunits [24]. These states are characterized by exposed hydrophobic domains (as evidenced by enhanced binding of the hydrophobic probe ANS), which are conducive to self-association of the protein molecules to form oligomers ranging from dimers at low concentration to possible larger particles at higher concentration. Such a scenario is consistent with the light scattering data reported here (Fig. 9) and the observed precipitation at higher concentrations, as mentioned above.

The compact, refolded structure adopted by prothrombin below pH 3 resembles the so-called A-state, which has been

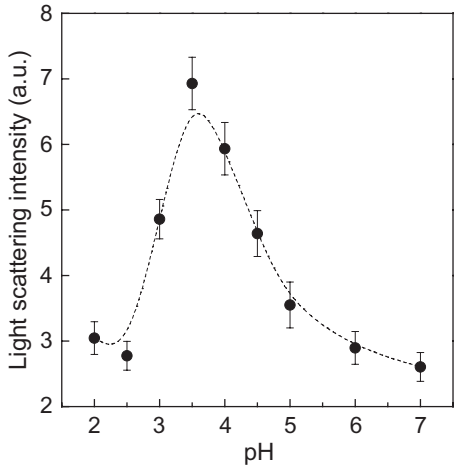

Fig. 9. Intensity of Rayleigh scattering of 450-nm light from prothrombin solution incubated at various pH. The dotted line is a smooth curve showing the trend of the data.

observed in a number of other proteins at around this pH [1–3]. It has a small hydrodynamic radius and partially retains native-like secondary structure, as shown by the light scattering data (Fig. 9) and the CD spectra (Fig. 7), respectively. The acid-induced unfolding behaviour of prothrombin can be represented by the following scheme:

$$N(\text{Native}) \rightarrow U_A(\text{Acid unfolded}) \rightarrow A(\text{Compact molten globule}).$$

According to the classification of proteins proposed by Fink et al. based on the pattern of their acid-denaturation behaviour, prothrombin is a type I protein, other notable examples of which are apomyoglobin, β-lactamase and cytochrome *c* [3]. The structural characteristics of the low pH refolded state adopted by these three proteins, which are also shared by prothrombin, resemble those of intermediate states on the denaturant-induced unfolding pathway and known as molten globules [19]. Further work is under way in our laboratory to compare and contrast the properties of the low pH compact state (A-state) of prothrombin with those of other intermediate states that may be formed during its unfolding by chemical denaturants (urea or GdmCl).

Acknowledgements

The authors thank Dr. Abhijit Chakrabarti and Sekhar Bhattacharya for their help with the isolation of prothrombin.

References

[1] Y. Goto, N. Takahashi, A.L. Fink, Mechanism of acid-induced folding of proteins, Biochemistry 29 (1990) 3480–3488.
[2] Y. Goto, L.J. Calciano, A.L. Fink, Acid-induced folding of proteins, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 573–577.
[3] A.L. Fink, L.J. Calciano, Y. Goto, T. Korotsu, D.R. Palleros, Classification of acid denaturation of proteins: Intermediates and unfolded states, Biochemistry 33 (1994) 12504–12511.
[4] F.B. Sheinerman, B. Honig, On the role of electrostatic interactions in the design of protein–protein interfaces, J. Mol. Biol. 318 (2002) 161–177.
[5] C.N. Pace, R.W. Alston, K.L. Shaw, Charge–charge interactions influence the denatured state ensemble and contribute to protein stability, Protein Sci. 9 (2000) 1395–1398.
[6] K.G. Mann, Prothrombin, Methods Enzymol. 45 (1976) 123–156.
[7] C.M. Jackson, Y. Nemerson, Blood coagulation, Annu. Rev. Biochem. 49 (1980) 765–811.
[8] G.L. Nelsestuen, Role of γ-carboxyglutamic acid. An unusual protein transition required for the calcium-dependent binding of prothrombin to phospholipid, J. Biol. Chem. 251 (1976) 5648–5656.
[9] M. Huang, A.C. Rigby, X. Morelli, M.A. Grant, G. Huang, B. Furie, B. Seaton, B.C. Furie, Structural basis of membrane binding by Gla domains of Vitamin K-dependent proteins, Nat. Struct. Biol. 10 (2003) 751–756.
[10] O.P. Malhotra, H.-Y.C. Chang, K.A. Koehler, The pH-dependence of the equilibrium between the fast- and slow-folding forms of bovine prothrombin fragment: 1. A role for pH-induced self-association, Biochem. Biophys. Res. Commun. 206 (1995) 742–747.
[11] D. Debnath, PhD thesis, Jadavpur University (2003).
[12] J.S. Ingwall, H.A. Scheraga, Purification and properties of bovine prothrombin, Biochemistry 8 (1969) 1860–1869.
[13] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin Phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[14] C. Monoto, A. Yamagishi, T. Oshima, Urea-induced unfolding and conformational stability of 3-isopropylmalate dehydrogenase from the *Thermophile thermus thermophilus* and its mesophilic counterpart from *Escherichia coli*, Biochemistry 38 (1999) 1332–1337.
[15] M.R. Eftink, C.A. Ghiron, Fluorescence quenching of indole and model micelle systems, J. Phys. Chem. 80 (1976) 486–493.
[16] K. Mukhopadhyay, S. Basak, Structure induction in melanotropic peptides by trifluoroethanol: fluorescence and circular dichroism study, Biophys. Chem. 74 (1998) 175–186.
[17] P. Bevington, Data Reduction and Error Analysis in the Physical Sciences, McGraw Hill, New York, 1983.
[18] K. Bhattacharyya, S. Basak, Somatostatin in a water-restricted environment: fluorescence and circular dichroism study in AOT reverse micelles, Photochem. Photobiol. 62 (1995) 17–23.
[19] L. Stryer, The interaction of a naphthalene dye with apomyoglobin and apohemoglobin, J. Mol. Biol. 13 (1965) 482–495.
[20] O.B. Ptitsyn, in: T.E. Creighton (Ed.), The Molten Globule State, Protein Folding, W. H. Freeman, New York, 1992, p. 243.
[21] M.R. Eftink, C.A. Ghiron, Fluorescence quenching studies with proteins, Anal. Biochem. 114 (1981) 199–227.
[22] T.P. Seshadri, A. Tulinsky, E. Skrzypczak-Jankun, C.H. Park, Structure of bovine prothrombin fragment 1 refined at 2.25 Å resolution, J. Mol. Biol. 220 (1991) 481–489.
[23] G.H. Tishkoff, L.C. Williams, D.M. Brown, Preparation of highly purified prothrombin complex: crystallization, biological activity and molecular properties, J. Biol. Chem. 243 (1968) 4151–4167.
[24] J.-R. Garel, in: T.E. Creighton (Ed.), Folding of Large Proteins: Multidomain and Multisubunit Proteins, Protein Folding, W. H. Freeman, New York, 1992, pp. 405–454.