Acid induced equilibrium unfolding of annexin V wild type shows two intermediate states

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Abstract Annexin V is an α-helical protein which shows anticoagulatory and antiinflammatory activity. It is supposed to be involved in membrane fusion and exocytosis. In this study acid-induced equilibrium unfolding of the human annexin V is investigated by fluorescence and circular dichroism spectroscopy. The spectroscopic data indicate that at least two intermediate states are involved in unfolding. One of the proposed intermediate states exhibits properties similar to those observed with annexin V wild type saturated with calcium, another may be regarded as 'molten globule'.

Key words: Protein folding; Intermediate; Annexin V

1. Introduction

The annexins form a family of cytosolic, water-soluble proteins that bind to phospholipid bi- or monolayers in a calcium-dependent manner [1]. The crystal structure of annexin V wild type (wt) and its calcium binding sites are well characterized [2,3].

Annexin V from human placenta contains 319 amino acids with a molar mass of 35 800 Da. The protein exhibits four homologous domains which consist of five α-helices each wound into a right-handed superhelix. The four domains are arranged in a cyclic, nearly planar manner. Calcium binding sites are located on the convex face of the protein. Annexin V wt has 12 tyrosine residues in domains I, II and IV and one tryptophan residue in domain II at position 187, that can be used for fluorescence studies.

Preliminary investigations have indicated that acid unfolding of annexin V may not proceed as a 'two-state' process [4].

Similar non-two-state behavior has been observed with apomyoglobin [5] and staphylococcal nuclease [6]. These proteins show an asynchronous disappearance of tertiary and secondary structure with decreasing pH.

The present fluorescence and circular dichroism (CD) studies provide evidence that acid-induced unfolding of annexin V wt is indeed associated with the occurrence of intermediate states between pH 4 and 1.2 before the protein collapses into an aggregated state below pH 1.0.

2. Materials and methods

2.1. Materials

Annexin V wt was prepared according to Burger et al. [7]. As standard buffer a solution of 20 mM citric acid, 100 mM KCl, 1 mM NaN_3 was used at all pH values. Citric acid, KCl, NaN_3, NaOH, and HCl were purchased from Merck (Darmstadt) and were of reagent grade purity.

2.2. Methods

2.2.1. pH measurement. pH values were measured using a 'Schott' pH electrode and a WTW pH meter model pH-DIGI 510. Electrodes were routinely calibrated before each measurement by appropriate calibration solutions.

The pH values of the buffer solutions have been adjusted in the following manner. A stock solution of 20 mM citric acid, 100 mM KCl, 1 mM NaN_3, pH 2.5 was titrated to the appropriate pH by adding μl samples of either 1 M HCl or 1 M NaOH solutions each containing 20 mM citric acid, 100 mM KCl, 1 mM NaN_3. When the final pH had been obtained 100 μl of a protein stock solution [c(protein) = 0.57 mg/ml] in 20 mM citric acid, 100 mM KCl, 1 mM NaN_3, pH 7.0 was added to 900 μl of the respective buffer solution to perform the fluorescence studies. For CD measurements a protein stock solution of 3.2 mg/ml was used. The exact pH values of the solutions were registered after the spectroscopic measurement.

2.2.2. Ultraviolet absorption. Protein concentration was determined by absorption measurements at 280 nm using a diode array spectrophotometer X-Dap (1024) from IKS. The extinction coefficient $\varepsilon = 0.6\ mg^{-1}\ cm^2$ at $\lambda = 280$ nm for annexin V wt was calculated according to Gill and von Hippel [8].

Temperature-induced aggregation measurements were carried out at $\lambda = 450$ nm using a cuvette holder equipped with a Peltier element for temperature control. The heating rate employed in the studies was 1 K/min.

2.2.3. Fluorescence. Fluorescence spectra were recorded at 20°C with a 'Spex' spectrofluorimeter model 'FluoroMax', using protein concentrations of approximately 0.05 mg/ml. Excitation wavelength were respectively 278 nm for the tyrosine residues and 295 nm for the tryptophan residue. The fluorescence was recorded between 300 and 365 nm. The slitwidth was set at 1 nm which corresponds to an excitation range of $\Delta\lambda$ +2.13 nm. All spectra were accumulated three times with an integration time of 0.5 s. Fluorescence values were corrected and standardized in the following manner. To eliminate the intrinsic pH dependence of fluorescence a solution containing a mixture of *N*-acetylated ethyl esters of tyrosine and tryptophan in the same concentration as the protein solutions were employed. The low solubility of the *N*-acetylated ethyl esters of the amino acids did not interfere with the control fluorescence studies, since the concentrations used in the measurements were below the concentrations that caused detectable aggregation. The procedures used were the following. Samples of *N*-acetyl-tryptophan ethyl ester ($c = 0.01$ mol/l) and *N*-acetyl-tyrosine ethyl ester ($c = 0.004$ mol/l) in buffer were sonified at 0°C to clear solution using molar extinction coefficient of $\varepsilon_{280nm} = 5600$ l cm mol^{-1} for *N*-acetyl-tryptophan ethyl ester and $\varepsilon_{274nm} = 1400$ l cm mol^{-1} for *N*-acetyl-tyrosine ethyl ester [9] for concentration determination. 16 μl of *N*-acetyl-tryptophan ethyl ester plus 500 μl of the *N*-acetyl-tyrosine ethyl ester solution were diluted with buffer to yield 10 ml of the solution employed in the fluorescence control measurements (*N*-acetyl-tryptophan ethyl ester: $c = 1.6 \times 10^{-5}$ M and *N*-acetyl-tyrosine ethyl ester: $c = 2.0 \times 10^{-4}$ M). The solution showed reproducible and constant fluorescence with time and exhibited neither light scattering nor precipitation. The fluorescence of this control solution was used at various pH values for correction of the fluorescence signal of the annexin solutions. This procedure rendered possible a separation of fluorescence changes originating from conformational changes from those due solely to pH changes. Subtraction of the fluorescence

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Abbreviations: wt, wild type; CD, circular dichroism

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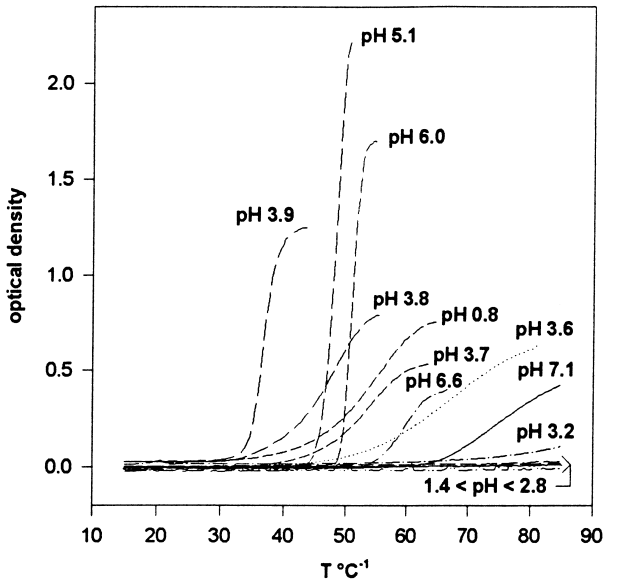

Fig. 1. Aggregation tendency of annexin V wt. It was measured as a function of temperature by UV scattering of transmitted light ($\lambda = 450$ nm) at the various pH values indicated near the curves. Measurements were carried out in 20 mM citric acid, 100 mM KCl, 1 mM NaN_3 with a heating rate of 1 K/min.

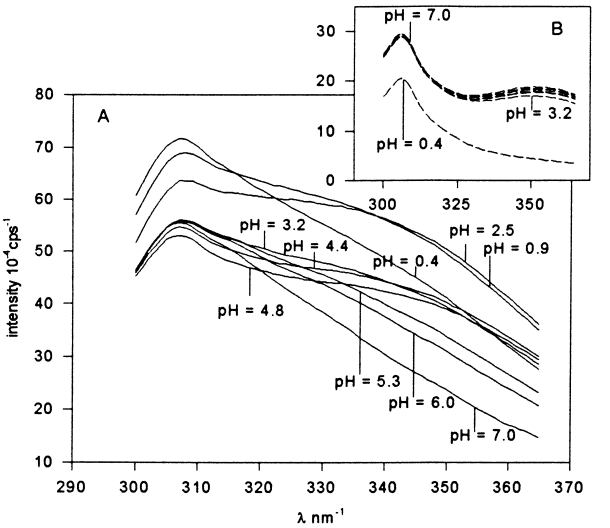

Fig. 2. A: Fluorescence emission spectra of annexin V wt. Spectra were taken exiting the samples (c[annexin V wt] = 0.057 mg/ml) at 278 nm and 20°C in 20 mM citric acid buffer, 100 mM KCl, 1 mM NaN_3. B: Fluorescence emission spectra of a corrective mixture of the free amino acids (*N*-acetyl-tyrosine ethyl ester and *N*-acetyl-tryptophan ethyl ester), so that the molarity of the aromatic side chains is identical to the protein sample.

intensity of the control solution from the protein sample results in a value which we call Δ(intensity). To obtain normalized graphs we plotted Δ_{rel}(intensity). This quantity is defined as Δ_{rel}(intensity) = {Δ(intensity)−Δ(intensity pH 7.0)}/{Δ(intensity pH 1.4)−Δ(intensity pH 7.0)}.

2.2.4. Circular dichroism. CD spectra were recorded on a Jobin-Yvon (Paris) spectropolarimeter model 'CD-6' equipped with a thermostatted cell holder. A Haake circulating water bath model C25 with a controller unit F6 was used for temperature control. The spectra were recorded at 20°C. The protein concentrations used ranged from 0.2 to 0.6 mg/ml. A quartz cell of 0.1 mm path length was employed.

The results were expressed as mean residue ellipticity, $[\Theta]_{MRE} = (100 \bullet MRW \bullet \theta_{obs}/c \bullet l)$, where θ_{obs} is the observed ellipticity in mdeg at the respective wavelength, MRW is the mean residue weight of annexin V wt (117.55 Da), l is the cuvette path length in cm and c is the protein concentration in mg/ml.

3. Results and discussion

3.1. Aggregation characteristics

A major problem when investigating the pH dependence of structural and energetic parameters of annexin V wt is its tendency to aggregate. This is illustrated in Fig. 1, which shows ultraviolet light scattering measurements as a function of temperature. Inspection of the curves shows that at pH 7.0 and between pH 3.2 and pH 1.4 aggregation starts only at around 60°C. Pronounced aggregation occurs at lower temperatures between pH 4.0 and pH 6.0 and below pH 1.0. The former range includes the isoelectric point at pH = 4.8 [10]. These pH ranges are shown hatched in all graphs to remind the reader of the possible problems aggregation might cause in the spectroscopic studies. It should be stressed, however, that at room temperature and low protein concentrations as used in the fluorescence experiments aggregation does not interfere with the measurements. Due to the higher protein concentration required for the CD studies, scattering of the results is much more pronounced in the same pH range.

3.2. Fluorescence spectroscopy

Environmental changes of the aromatic side chains tyrosine and tryptophan resulting from conformational changes of the tertiary structure can be measured very sensitively by fluorescence spectroscopy. A change in the degree of solvent exposure of these amino acids induces a change in fluorescence emission intensity of both aromatic amino acids and additionally a shift in the emission maximum of the spectrum of tryptophan [11].

In this study the fluorescence emission of the tyrosines and

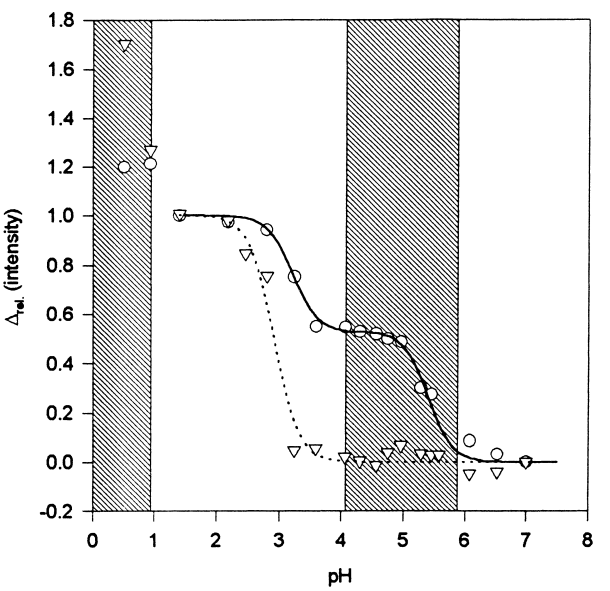

Fig. 3. pH dependence of the standardized fluorescence signal Δ_{rel}(intensity) of tryptophan (solid line) ($\lambda_{ex} = 295$ nm; $\lambda_{em} = 350$ nm) and tyrosine (dotted line) ($\lambda_{ex} = 278$ nm; $\lambda_{em} = 305$ nm) residues of annexin V wt. The standardizing procedure is described in Section 2.

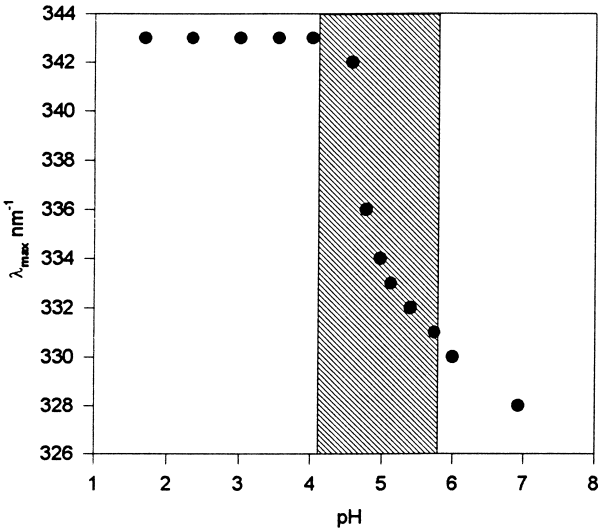

Fig. 4. Shift with pH of the emission maximum of the tryptophan residue 187 of annexin V wt. Spectra were taken at 20°C in 20 mM citric acid buffer, 100 mM KCl, 1 mM NaN_3 using a protein concentration of 0.03 mg/ml. The excitation wavelength was 295 nm.

of the single tryptophan (at position 187) was investigated to characterize changes in the tertiary structure.

Fig. 2A shows emission spectra of annexin V wt at various pH values. The excitation wavelength was 278 nm.

In the emission range of tryptophan between 330 nm $< \lambda <$ 360 nm a decrease in pH is associated with an increase in fluorescence intensity. It is remarkable that tyrosine emission remains unaltered during this process as indicated by the constancy of the emission maximum at 305 nm. When the pH is decreased below 3.5 a second increase of tryptophan fluorescence intensity is observed, and this time

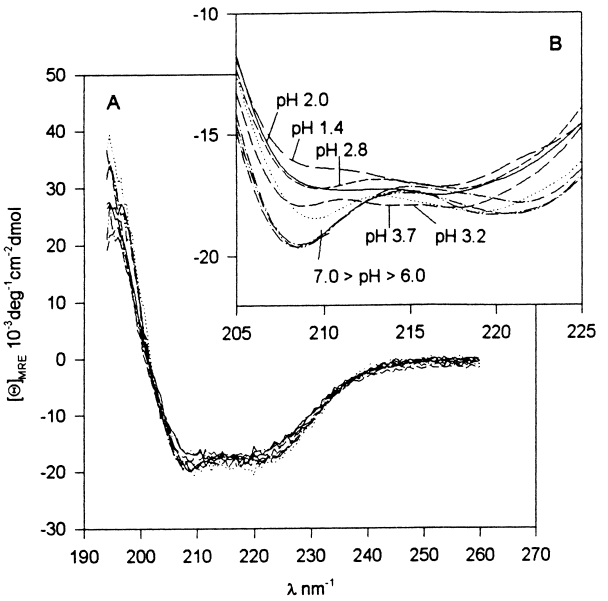

Fig. 5. A: Far-UV CD spectra of annexin V wt at various pH values. Spectra were taken at 20°C in 20 mM citric acid buffer, 100 mM KCl, 1 mM NaN_3. The concentration of protein was 0.3 mg/ml. The spectra are buffer corrected and unsmoothed. B: Enlarged view of the spectral range 205 nm to 225 nm; the spectra are smoothed.

it is accompanied by an increase in the tyrosine fluorescence at 305 nm.

The decrease in fluorescence intensity of tryptophan below pH 1.0 seen in Fig. 2A is only apparent and does not reflect any further conformational changes. This can be concluded from Fig. 2B which shows the control studies using the mixture of *N*-acetylated ethyl esters of tyrosine and tryptophan. The fluorescence of these fully hydrated amino acid derivatives is also strongly decreased at pH values < 1.0. If this correction is applied, fluorescence intensity is seen to increase according to aggregation as one would expect from interference by Raleigh scattering in the wavelength range studied.

Fig. 3 illustrates that three conformational states are occupied in the course of pH decrease. Between pH 7.0 and 6.0 the native state prevails. A second conformational state with increased tryptophan fluorescence emission is observed between pH 4.6 and 4.0. The increase of fluorescence intensity by about 20% of the absolute signal intensity (cps) is caused by a red shift of the emission maximum from $\lambda = 328$ nm to 343 nm. This conformational change is completed at pH 4.0 (Fig. 4). The present observations are analogous with those made by Meers and Mealy [12]. These authors reported a red shift of fluorescence emission and an increase of intensity by 20% when calcium was added to annexin V wt at pH 8.0. Therefore we conclude that the pH change to pH 4.0 induces the same overall solvent exposure of the tryptophan residue as calcium does in the binding process.

A third conformational state can be identified between pH 2.5 and 1.0. Here both amino acids reach their maximum intensity. This suggests that also the tyrosine residues undergo a change in solvation in this pH range.

The further increase of the emission signal can be assigned to light scattering effects because of aggregation.

3.3. Circular dichroism measurements

The far ultraviolet CD spectra shown in Fig. 5 indicate that the secondary structure of annexin V wt is nearly native even at a pH 1.4. Slight alterations are indicated by the findings, visible in Fig. 5B, that both the minima at $\lambda = 208$ nm

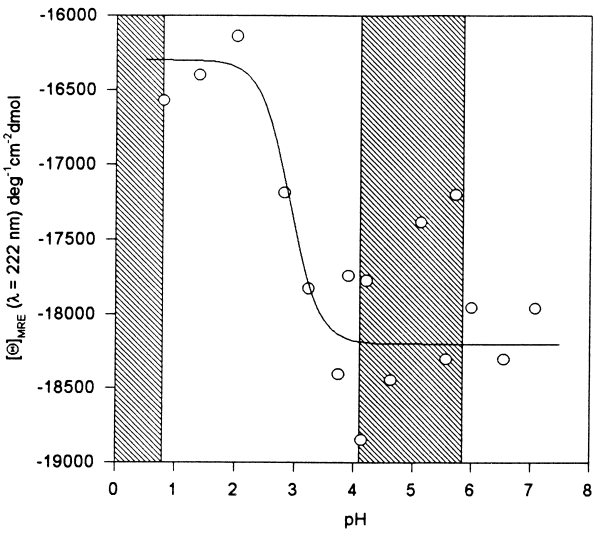

Fig. 6. Variation of the CD signal at $\lambda = 222$ nm with increasing pH. Spectra were taken at 20°C in 20 mM citric acid buffer, 100 mM KCl, 1 mM NaN_3. The concentration of protein was 0.57 mg/ml.

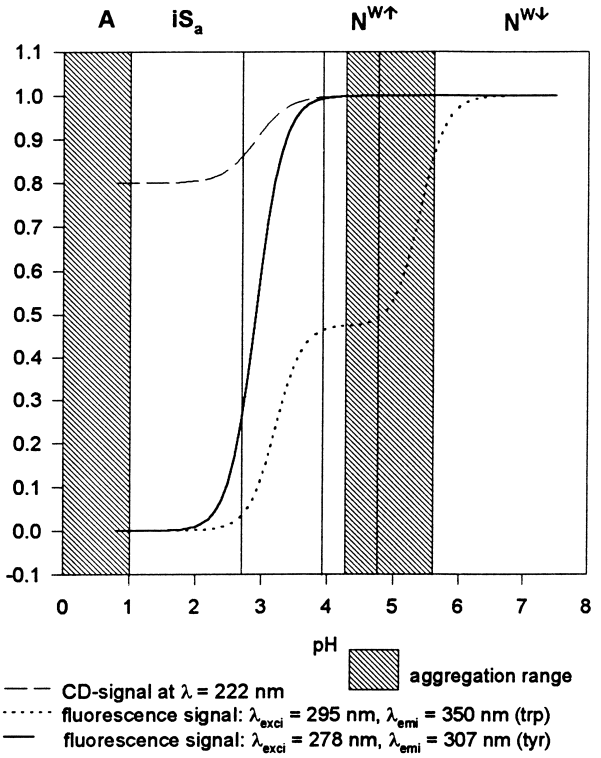

Fig. 7. pH dependence of the fluorescence and CD signals of annexin V wt. The signals are all given as normalized values. A value of 1.0 has been assigned to the native state at pH = 7.0 ($N^{W\downarrow}$), and a value of 0.0 to the state at pH 1.5 (iS_a).

and at $\lambda = 222$ nm suggestive of α-helical structure become flatter.

The pH dependence of the CD signal is shown in Fig. 6. It is obvious that with decreasing pH a transition can be observed between pH 3.5 and 2.0. The increase in the CD signal is small but significant.

These findings demonstrate that there is a large amount of secondary structure left even at pH < 2.0. The signal ($[\Theta]_{MRE} = -16\,700$ deg cm^2 dmol^{-1}) has nearly 80% of the native intensity which is $[\Theta]_{MRE} = -18\,300$ deg cm^2 dmol^{-1}. The native state CD remains practically unchanged until pH 4.0. Only at lower pH values does the transition become evident.

3.4. Conclusions

A summary of spectroscopic findings concomitant with the unfolding behavior of annexin V wt has been plotted in Fig. 8. The value of 1.0 has been assigned to the native state at pH 7.0, the value of zero to the signals at pH 1.5. The hypothetical unfolded state of annexin V wt, which has not been reached in our studies, has been observed to be characterized by a $[\Theta]_{MRE}$ value of −3000 deg cm^2 dmol^{-1} [13]. Using this value a $[\Theta]_{MRE}$ of −16 700 deg cm^2 dmol^{-1} corresponds to 80% of residual structure.

It is evident from inspection of Fig. 7 that annexin V wt assumes at least two intermediate states during acid unfolding.

The conformational state below pH 1.0 is most difficult to characterize. Its major feature is the tendency to aggregate. Therefore we call it the aggregational state (A).

A second state can be differentiated between pH 1.0 and 2.0. In this pH range one does not observe fluorescence or CD changes and we call this conformational state an acid-induced intermediate state (iS_a). This state has no tertiary structure on the basis of the fluorescence signal but it retains a significant amount (∼80%) of native secondary structure as evident by the far ultraviolet CD.

A third state can be observed in the range of 4.5 > pH > 4.0. Here the secondary structure resembles that of the native state and also the tertiary structure. The only difference with the native state is a total solvent exposure of the tryptophan residue at position 187.

This result is highly interesting in view of a second crystallographic structure that has been reported for annexin V wt at high calcium concentrations [3]. This structure differs mainly in the position of tryptophan-187. With no or a low concentration of calcium residue 187 is buried in a hydrophobic core of the protein whereas at high calcium ion concentrations this residue changes its position to a solvent-exposed location. We suggest that the conformational change that leads to the effect on tryptophan fluorescence observed in weakly acidic medium reflects a similar structural reorganization of annexin V as that caused by calcium binding. Therefore this state is to be regarded as a native state with tryptophan-187 exposed ($N^{w\uparrow}$).

The native state at pH 7.0 differs from this state by the fact that tryptophan-187 is buried. We indicate this by introducing the symbol $N^{w\downarrow}$.

The findings reported above support the view that acid-induced unfolding of annexin V follows a multi-state pathway where secondary and tertiary unfolding as well as tryptophan-187 exposure can be differentiated. These results have been summarized in the reaction scheme shown in Fig. 8.

Remarkably, exposure of tryptophan-187 without further conformational changes could be identified by a comparison of tyrosine and tryptophan fluorescence data. This conformational change is identical to the calcium-induced structural change that has been found to be of importance for the binding process of annexin V to lipid membranes. Annexin V has been reported by Köhler et al. [14] to bind to large unilamellar vesicles in the absence of calcium at pH values of 5.0–4.0. The intriguing finding of the lack of calcium requirement for membrane binding of annexin V at these low pH values can be

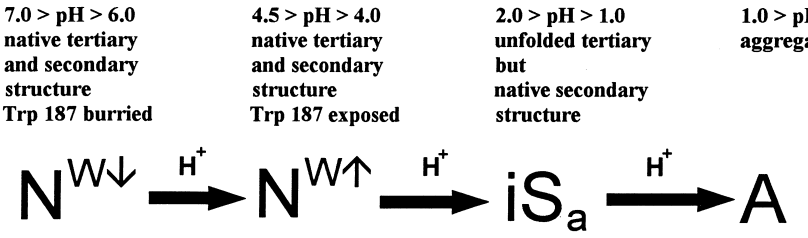

Fig. 8. Unfolding scheme of annexin V wt derived from fluorescence and CD data.

rationalized in view of our present result and the observations by Meers [15]. Meers has suggested that the exposed tryptophan-187 is instrumental in membrane interaction by getting immersed into the membrane. If the decrease in pH induces the same structural change as the binding of calcium ions, the protein-membrane interaction can be understood to take place in the absence of metal ions. A further interesting phenomenon, giving our studies biological significance, was reported by van der Goot et al. [16]. These authors observed that on the surface of membranes the pH value is lower by about 1.5 units than the pH in the solution. This opens up the interesting hypothesis that the conformational change required for binding is induced not only by calcium but also by the decreased local pH value.

The acid-induced state (iS_a) was characterized by maximal fluorescence and retention of almost 80% of its native state CD signal. It is pertinent to note that complete unfolding could not be achieved even at pH 0.4. Future kinetic studies are intended to reveal whether aggregation under these conditions is preceded by total unfolding.

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