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Alkaline denaturation and partial refolding of pepsin investigated with DAPI as an extrinsic probe

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Abstract

The binding parameters of DAPI to porcine stomach pepsin have been described in the previous article in this issue (A. Mazzini et al.). Here we exploit the differences in the spectroscopic (fluorescence and circular dichroism) properties of DAPI bound to either native or alkali denatured pepsin. We follow the kinetics of pepsin denaturation around neutrality (pH range 6.8–7.4), at several phosphate buffer ionic strengths (range 0.02–0.25). The dependence of the apparent dissociation rate constant on pH clearly shows that the rate limiting step follows the dissociation of about three acidic protein residues. The accelerating effect by ionic strength we observed can be accounted for by a simple treatment based on both transition state theory and Debye-Hueckel's limiting law. Furthermore, when a solution of pepsin, rapidly denatured at pH 7, is reacidified to a pH between 4.5 and 5.5, a substantial recovery of protein secondary structure, with no enzymatic activity, is observed, judging by the far UV circular dichroism of the protein. This process of partial refolding can easily be followed using DAPI as an extrinsic reporter group, able to monitor the kinetics of formation and decay of a highly fluorescent intermediate. This process becomes faster at a lower pH, at least in the limited range investigated (pH 4.5–5.5), in which the refolded protein does not aggregate, but, in contrast to unfolding, is almost independent in ionic strength. © 1997 Elsevier Science B.V.

Keywords: Pepsin, 4′,6-diamidino-2-phenylindole (DAPI); Denaturation; Unfolding; Refolding; Kinetics; Fluorescence; Circular dichroism

1. Introduction

The drug DAPI (4′,6-diamidino-2-phenylindole) has largely been used as a DNA specific probe in flow cytometry, chromosome staining and for DNA visualization in histochemistry and biochemistry [1].

DAPI has also been used as a fluorescent probe to monitor both structural and functional events occurring in a variety of proteins, as documented in a series of papers by us [2,3] and others [4–6]. Preliminary results on the interaction of this dye with pepsin as a function of pH and ionic strength have been published [7], whereas a deeper investigation on the use of DAPI as a sensitive extrinsic structural probe of this protein is described in the preceding paper [8].

Porcine pepsin is a gastric aspartic proteinase characterized by a single chain, with a secondary structure almost entirely consisting of pleated sheets

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole

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[9]. The X-ray diffraction structural analysis, at 1.8 Å resolution, shows that the substrate binding cleft is located between two homologous portions, the N-terminal lobe (residues 1–172) and the C-terminal lobe (residues 173–327) [10]. It has long been known that the protein spontaneously inactivates in neutral or alkaline solution, with kinetics strongly dependent on pH and ionic strength [11]. Recently, it has been proposed that this irreversible process, called 'alkaline denaturation', specifically involves the N-terminal lobe with ionization of some buried carboxyl groups, mainly Asp^{11} and Asp^{159}, with anomalous pK values near neutrality [12].

Here we describe how the process of alkaline denaturation of pepsin, already extensively studied in the past by monitoring changes of some protein intrinsic properties with calorimetry [13], viscosity [14], light scattering [14] and proton liberation [15], can also be investigated by following the spectroscopic properties of an extrinsic probe. In our case, we have exploited the fluorescence and circular dichroism changes of DAPI, in the presence of pepsin, accompanying the neutral denaturation, both kinetically and under equilibrium conditions. The strong dependence of this process on both pH and ionic strength, observed with DAPI, closely resembles that previously described [11].

It has previously been observed that, upon re-acidification, alkali unfolded pepsin shows a substantial recovery of its secondary structure, as judged by the protein far UV-CD spectrum, with no recovery of enzyme activity and tertiary structure [16]. We have re-investigated this process of partial refolding in more detail, by monitoring the spectroscopic properties of DAPI, here too, acting as an interesting reporter group.

2. Materials and methods

2.1. Porcine stomach pepsin

The protein has been purchased from Sigma and chromatographically purified, as previously described [8]. 4′,6-Diamidino-2-phenylindole (DAPI.2-HCl) from Fluka was used without further purification. All other reagents were of analytical purity grade. High resistivity water, as obtained from a Millipore Milli-Q purification system, was used throughout.

2.2. Spectroscopic measurements

Fluorescence and circular dichroism measurements have been performed with a Perkin-Elmer LS-50 spectrofluorimeter and a Jasco J500-A spectropolarimeter, as previously described [8].

2.3. Kinetic measurements

Pepsin unfolding was studied at 20°C by a pH jump from 5.5 to 6.8 or higher, by monitoring either fluorescence or CD signals of DAPI, at different concentrations of phosphate buffer. The relative ion strength values were taken from a reference table [17].

Partial refolding of pepsin was induced by a pH jump from 7.2 to a value between 4.5 and 5.6 and monitored by following the changes occurring in both the protein far UV-CD and DAPI fluorescence spectra (no CD signal of the dye at neutral pH or upon re-acidification was detected). The protein used for refolding studies was first denatured at a neutral pH as quickly as possible, in order to prevent autolytic effects [8], then brought to a low pH, in any case above 4.3 to avoid precipitation of the protein.

Unfolding and refolding kinetic traces were best fitted using the iterative procedure based on the Marquard-Levemberg algorithm [18]. Depending on pH and phosphate buffer concentration, the kinetic traces were best fitted by either one or two exponentials.

3. Results

3.1. Alkaline denaturation of pepsin

This process has been studied both at equilibrium and kinetically, by exploiting the fluorescence and circular dichroism properties of DAPI as a reporter group, at several pH and ionic strength values.

3.1.1. Fluorescence and CD of DAPI with pepsin at equilibrium

The fluorescence intensity of DAPI, in the presence of pepsin, has been found to be strongly depen-

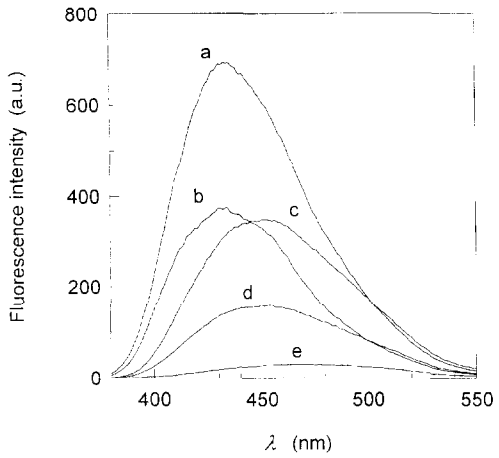


Fig. 1. Fluorescence spectra of DAPI in the presence of pepsin. Conditions used: 4.6 μM DAPI, 24 μM pepsin, $\lambda_{ex} = 350$ nm, 20°C. (a) pH 5.6, 20 mM phosphate; (b) pH 5.6, 100 mM phosphate; (c) pH 7.2, 20 mM phosphate; (d) pH 7.2, 100 mM phosphate and (e) DAPI alone at pH 5.6, 20 mM phosphate (spectrum invariant with respect to either pH or ionic strength).

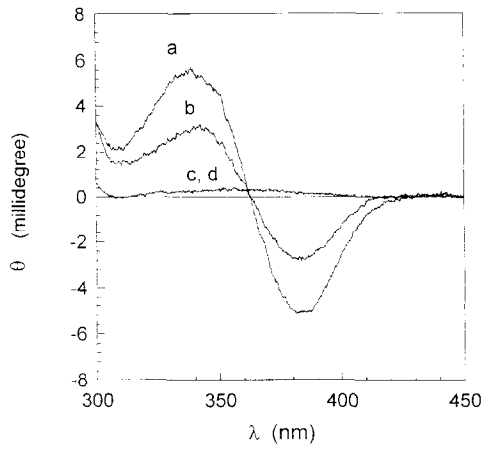


Fig. 2. CD spectra of DAPI in the presence of pepsin. Experimental conditions: 32 μM DAPI, 32 μM pepsin, cell pathlength 1 cm, 20°C. (a) pH 5.6, 20 mM phosphate; (b) pH 5.6, 100 mM phosphate; (c) pH 7.2, 20 mM phosphate and (d) pH 7.2, 100 mM phosphate. DAPI alone is not dichroic.

dent upon pH and ionic strength: e.g. at a given phosphate buffer concentration, it is higher at pH 5.6 than 7.2 (compare spectra a and c, or b and d, in Fig. 1). Conversely, at a given pH, the intensity is higher at 20 than 100 mM phosphate (compare spectra a and b, or c and d, in Fig. 1). Furthermore, as a consequence of pepsin alkaline denaturation, the emission spectrum of DAPI at pH 7.2 appears to be slightly red-shifted with respect to that at pH 5.6 (emission maxima at about 450 for spectra c and d, and 435 nm for a and b). In contrast, the emission spectrum of DAPI alone is unaffected by either pH or ionic strength, with its emission maximum at about 465 nm (spectrum e).

Although DAPI is a symmetrical molecule, at an acidic pH and in the presence of pepsin it becomes dichroic, with a CD spectrum split in two component bands of similar intensity but opposite signs (Fig. 2). Furthermore, the CD intensity is strongly dependent on the ionic strength at acidic pH (compare spectra a and b), as also observed in fluorescence, whereas at a neutral pH it is almost undetectable either ionic strength (spectra c and d).

The overall pH dependence of both fluorescence and CD intensity of DAPI, in the presence of pepsin, at 100 mM phosphate, is shown in Fig. 3. The fluorescence intensity is strongly enhanced at any pH above 4, with respect to that of DAPI alone, with a maximum at pH 6, whereas between pH 6 and 7 it decreases markedly, still remaining considerably higher than that of DAPI alone. Moreover, the pH profile of the CD intensity at 335 nm of DAPI in the

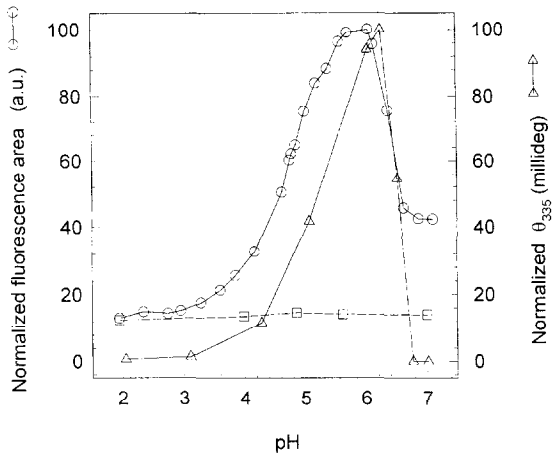


Fig. 3. Normalized fluorescence and CD of DAPI in the presence of pepsin as a function of pH. Conditions used: 100 mM phosphate buffer, 20°C. (– ∘ –) Fluorescence of 5.3 μM DAPI with 15 μM pepsin; (– △ –) CD of 77 μM DAPI with 33 μM pepsin; (– □ –) fluorescence of 5.3 μM DAPI alone. Due to the slowness of the denaturation process, measurements above pH 6 were done after several hours of preincubation of pepsin at each corresponding pH, in order to allow for complete equilibration.

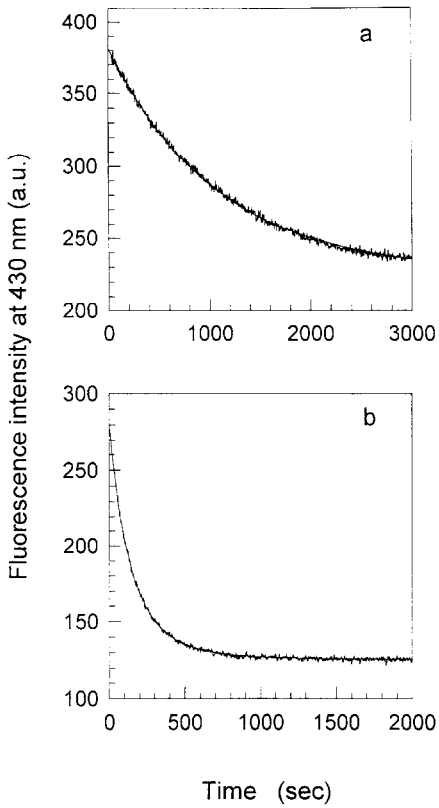


Fig. 4. Kinetics of the alkaline denaturation of pepsin at pH 7 and different phosphate concentrations, as revealed by DAPI fluorescence. Experimental conditions: 4.6 μM DAPI with 25 μM pepsin, λ_{ex} = 350 nm, 20°C. (a) 20 mM, (b) 100 mM phosphate. Both curves were best fitted by a single exponential with lifetime values of 1070 and 167 s, respectively.

presence of pepsin is very similar to that observed in fluorescence, except that it is shifted to the right by about 0.5 pH units, and becomes negligible above pH 6.8.

3.1.2. *Kinetics of pepsin alkaline denaturation monitored through DAPI*

The kinetics of pepsin alkaline denaturation has been studied at several pH and ionic strength values, by monitoring the fluorescence and CD signals of DAPI. As an example, two representative fluorescence kinetic curves, relative to pH 7 and different ionic strength, are shown in Fig. 4 (similar kinetics have been obtained with CD, data not shown). All fluorescence kinetic traces have been best fitted by a

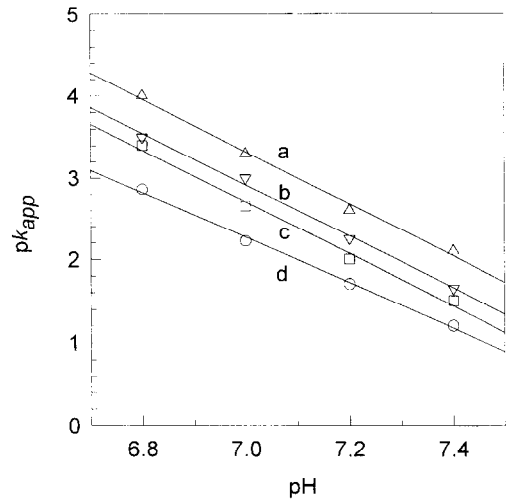


Fig. 5. Apparent rate constants of pepsin alkaline denaturation, as a function of pH, at different ionic strengths, as monitored through DAPI fluorescence kinetics. Conditions used: 4.6 μM DAPI with 25 μM pepsin, λ_{ex} = 350 nm, 20°C. Phosphate concentrations: (a) 10 mM, (b) 20 mM, (c) 50 mM and (d) 100 mM.

single exponential, and the relative apparent denaturation rate constants are shown in Fig. 5 as a function of pH, at several buffer ionic strengths, and in

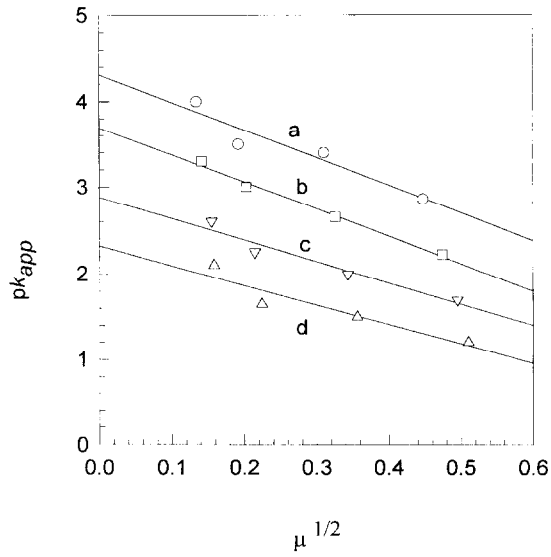


Fig. 6. Apparent rate constants of pepsin alkaline denaturation, as a function of ionic strength, at different pHs, as monitored through DAPI fluorescence kinetics. Conditions used: 4.6 μM DAPI with 25 μM pepsin, λ_{ex} 350 nm, 20°C. (a) pH 6.8, (b) 7.0, (c) 7.2 and (d) 7.4.

Table 1
Alkaline denaturation of pepsin, at different pH and ionic strength

Phosphate (mM)	n^a	pH	Slope[b]
10	3.2	6.8	−3.2
20	3.1	7.0	−3.1
50	3.2	7.2	−2.5
100	2.8	7.4	−2.3

[a] The number of protons (n), released by the acidic residues in the rate limiting step of denaturation, are derived from the negative slopes of the straight lines of pk_{app} vs. pH (Fig. 5), as theoretically given by Eq. (3).
[b] Values derived from the straight lines of pk_{app} vs. $\sqrt{\mu}$ (Fig. 6), as theoretically given by Eq. (12).

Fig. 6 as a function of the ionic strength, at several pH values. Results are summarized in Table 1.

3.2. Partial refolding of pepsin

As for alkaline denaturation, DAPI has been exploited to follow partial refolding, but only for what concerns its fluorescence, since its CD is irreversibly lost once the protein is denatured at a neutral pH.

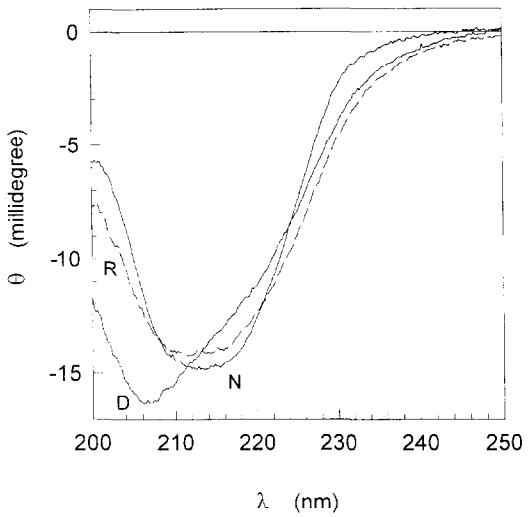


Fig. 7. Far UV-CD spectrum of native, alkali denatured and acid-induced partially refolded pepsin. Experimental conditions: 100 mM phosphate buffer, 54 μM pepsin: native (N) at pH 5.4, denatured (D) at pH 7.2, partially refolded (R) (from pH 7.2 to pH 4.7), after several hours of refolding. The methods used to obtain alkali denatured and partially refolded protein are described in Section 2.

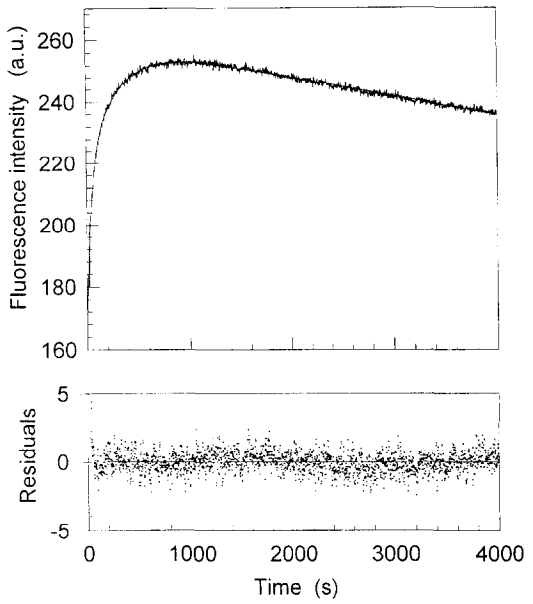


Fig. 8. Kinetics of acid-induced partial refolding of pepsin, as monitored through DAPI fluorescence. Conditions used: 100 mM phosphate buffer, 5 μM DAPI with 15 μM pepsin, 20°C, λ_{em} = 430 nm, λ_{ex} = 350 nm, 1 cm cell path length. The best fit to the kinetic trace, recorded after the pH jump from 7.2 to 4.6, is given by the following equation: $F = 51(1 - e^{-t/41}) + 44(1 - e^{-t/279}) - 0.0061t + 166$.

The partial refolding has also been monitored with the far UV-CD spectrum of pepsin itself.

3.2.1. CD of partially refolded pepsin

The far UV-CD spectrum of pepsin, rapidly denatured at pH 7 according to the procedure described under Section 2 is shown in Fig. 7 as spectrum D. Spectrum R and N refer to re-acidified (from pH 7 to 5) and native (at pH 5) pepsin, respectively. As judged by its spectral shape, neutral pepsin shows a consistent shift towards blue with respect to native pepsin, which can be ascribed to partial denaturation, whereas re-acidified pepsin clearly shows a large recovery of secondary structure, closely resembling native pepsin, apart from a slightly lower intensity and larger band width.

3.2.2. Kinetics of pepsin partial refolding monitored through DAPI fluorescence

Kinetic measurements of pepsin partial refolding have been performed as a function of final pH at a

Table 2
Apparent rate constants and amplitudes of pepsin partial refolding as a function of pH

pH	τ_1 (s)	τ_2 (s)	A_1 (a.u.)	A_2 (a.u.)	A_0 (a.u.)
4.5	41	279	51	44	166
4.8	73	575	21	31	124
5.3	155	844	18	9	115
5.6	95	2523	7	4	104

All lifetimes (in seconds) and amplitudes (in a.u.) are the best values from two-exponential fittings to the observed fluorescence kinetics, according to the following equation: $F = A_1(1-e^{-t/\tau_1}) + A_2(1-e^{-t/\tau_2}) + Bt + A_0$, where B represents the slope of the slow falling part of the kinetics (values not shown) and A_0 the initially recorded fluorescence value.
All measurements refer to 0.1 M phosphate buffer and 20°C.

given ionic strength (0.1 M phosphate buffer), using DAPI as a reporter group. A typical trace, relative to pH 4.6, is shown in Fig. 8 and all the experimental parameters are reported in Table 2.

The kinetics observed has been found to be strongly dependent on the final pH, but only marginally on ionic strength, in contrast to the unfolding process described above (data not shown). In all cases investigated, the highly fluorescent intermediate is formed with a biphasic kinetics and decays into the less fluorescent partially refolded form on a much longer timescale.

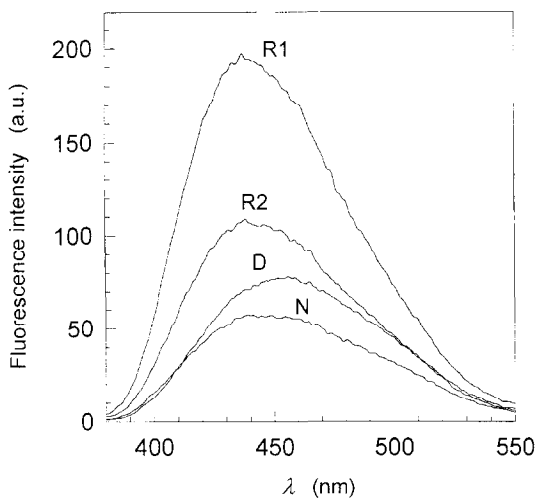


Fig. 9. Effect of acid-induced partial refolding of pepsin on the fluorescence spectrum of DAPI. Conditions used: 100 mM phosphate buffer, 5.3 μM DAPI with 15 μM pepsin 20°C. Spectra of DAPI were obtained with: (N) native pepsin at pH 4.5, (D) denatured pepsin at pH 7.1, (R1) and (R2) partially refolded pepsin, after 15 min and 16 h of refolding at pH 4.5, respectively.

3.2.3. Fluorescence of DAPI bound to partially refolded pepsin

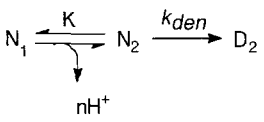
Partial refolding has also been monitored by looking at the fluorescence of DAPI, indicating the formation of a relatively stable intermediate state (Fig. 9). Spectrum R1, corresponding to a refolding time of about 15 min, is to be attributed to a highly fluorescent intermediate species, slowly decaying to a stable species, with a spectrum of almost halved intensity, denoted as R2. Both spectra R1 and R2 are considerably blue shifted in comparison to that of DAPI with alkaline denatured pepsin (D) and more similar to that with native pepsin (N). As already mentioned and in contrast with fluorescence, no CD signal of DAPI with partially refolded pepsin has been recovered.

4. Discussion

The fluorescence intensity of DAPI is enhanced by the presence of pepsin, in a way strongly dependent upon pH and ionic strength. Furthermore, native, but not alkali denatured, pepsin induces circular dichroism on the symmetrical DAPI molecule bound to it.

These preliminary observations suggested the use of DAPI as a possible structural probe to monitor conformational changes in the protein and prompted us to investigate these effects as a function of pH and ionic strength more extensively. Actually, as shown in Fig. 3, an abrupt decrease of both fluorescence and CD of DAPI, in the presence of pepsin, has been observed near neutral pH, which can be well correlated with the partial unfolding of the protein, known as alkaline denaturation [11]. Furthermore, when the pepsin is in its native conformation, i.e. at pH ≤ 6, DAPI binds to it in a chiral conformation, while, at neutral pH, where the N-terminal lobe of the protein is unfolded [12], is it still bound in a non-chiral conformation.

The alkaline denaturation of pepsin has also been studied kinetically, by following over a period of time the change in the spectroscopic properties of



Scheme 1.

DAPI, as a function of pH and ionic strength (Fig. 4).

In principle, at least two possible alternative mechanisms of denaturation can be considered for the denaturation process, one in which an irreversible denaturation step follows a reversible proton release step (Scheme 1) where N_1 stands for the native state before proton release, N_2 for a transient native state after proton release, with n more negative charges, D_2 for the final stable state of the alkali denatured protein, K is the dissociation constant and k_{den} the denaturation rate constant.

In the other mechanism the opposite is true, namely an irreversible proton release step follows a reversible protein conformational transition step (Scheme 2):where K is the equilibrium constant of the reversible denaturation step and k_d is the rate constant for the irreversible proton release step.

However, only Scheme 1 appears to be adequate to our case, because it is the only one including a pH dependence of the denaturation rate, as experimentally observed. In fact, we have:

$$v_{den} = k_{den}[N_2] = k_{app}[N_1] \quad (1)$$

where:

$$k_{app} = \frac{k_{den} K}{[H^+]^n} \quad (2)$$

hence:

$$pk_{app} = p(k_{den} K) - npH \quad (3)$$

On the contrary, the rate equation for Scheme 2:

$$v_{den} = k_d [D_1] = k_d K [N_1] = k_{app} [N_1] \quad (4)$$

gives $k_{app} = k_d K$, which is pH independent.

The experimentally found values of the reaction order with respect to $[H^+]$ are between -2.8 and -3.3 (Table 1), in accordance with literature data [11]. These kinetic results further support the view that DAPI can be considered an interesting structural probe, able to detect the transition from native to alkali denatured pepsin, following the dissociation of a few carboxyl residues.

Furthermore, the fact that the apparent denaturation rate constant is accelerated by the ionic strength implies that the reaction involves charged species of the same sign, very likely two or more negative protein residues, as suggested by the values of n obtained from Fig. 6 and reported in Table 1.

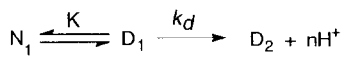
However, values of $n < -1$ may not necessarily imply the involvement of several dissociable acidic groups, but may be a consequence of a lower value of the dielectric constant, near the surface of the protein, with respect to that in the bulk, as put forward for the interaction between intestinal fatty acid binding protein and 8-anilino-naphtalene sulphonate (ANS) [19]

A more complete description of the dependence of the denaturation rate on the ionic strength is given in the Appendix A.

As already mentioned, partial refolding of pepsin has been studied using a pH jump of alkali denatured pepsin from 7 to a value between 5.6 and 4.5. Under such conditions, a consistent recovery of secondary structure can be deduced from the far UV-CD spectrum of the protein (Fig. 7).

Kinetics of partial refolding shows the formation of a highly fluorescent intermediate, which rearranges very slowly with a decrease of fluorescence (Fig. 8) and kinetic parameters strongly dependent on the final pH (lifetimes and amplitudes relative to the two-step formation of the highly fluorescent intermediate, are reported in Table 2).

The trend observed, namely lower lifetimes and higher amplitudes at lower pH, can be explained considering that the lower net negative charge on the protein can favour refolding. Moreover, since the fluorescence value of pepsin at pH 7 is always lower than that observed at the beginning of each kinetics, at any pH of refolding, one can infer the presence of a hidden step, attributable to a hydrophobic collapse of the alkali denatured N-terminal lobe of pepsin, as usually observed during protein refolding [20]. Parameter B (see Table 2) which defines the slope of



Scheme 2.

the slow decay step, leading to partially refolded protein, is very small and almost pH-independent.

In Fig. 9 the fluorescence spectra of DAPI, relative to the intermediate (R1) and final partially refolded (R2) pepsin, are compared to those relative to native (N) and alkali denatured (D) pepsin. Since both spectra R1 and R2 are more similar to N than D, being more blue shifted, this suggests that DAPI binds to partially refolded pepsin in a hydrophobic environment more similar to that of native than denatured pepsin. On the other hand, the red-shift in spectrum D with respect to N suggests that DAPI binds in rather a different way to denatured than to native pepsin, perhaps more electrostatically on the highly negatively charged protein surface, as previously discussed [8].

Finally, the enhanced fluorescence intensity of DAPI to pepsin, during partial refolding, may be reminiscent of a similar effect observed when ANS binds to proteins, particularly to molten globular states [21]. Therefore, considering that DAPI is also hydrophobic, but of opposite charge with respect to ANS, we suggest trying to use this dye as a probe to monitor molten globules of acidic proteins, in parallel with or as an alternative to ANS, especially in those cases where the latter probe may prove ineffective.

Appendix A

Here we show how the observed dependence of pepsin alkaline denaturation rate on the ionic strength can be well accounted for by a simple treatment based of the transition state theory and Debye-Hueckel limiting law.

According to Scheme 1, shown above, after the reversible release of n protons, an irreversible denaturation step occurs. This second step can be assumed to involve the approach of two negative residues, say A and B, of the deprotonated but still native protein molecule to form a transition state species X^* before giving the irreversibly denatured form D_2:Scheme 3 where X^* stands for the transition state. Since the two negative groups belong to the same molecule, their distance varies, during the reaction, as their effective concentration. According to

$$N_2 \underset{}{\overset{K^*}{\rightleftharpoons}} X^* \xrightarrow{k^*} D_2$$

Scheme 3.

the transition state theory, the rate of denaturation ban then be written as:

$$v_{den} = k^*[X^*] \tag{5}$$

where $[X^*]$ is given by:

$$K^* = \frac{[X^*]\, y_{X^*}}{[N_2]\, y_{N_2}} \tag{6}$$

where K^* is the transition state equilibrium constant and y_{X^*} and y_{N_2} are activity coefficients. Now, called $[A/B]_{N_2}$ and $[A/B]_{X^*}$ the effective concentrations of the two negative groups on N_2 and X^*, Eq. (6) becomes:

$$K^* = \frac{[A/B]_{X^*}\, y_{X^*}}{[A/B]_{N_2}\, y_{N_2}} \tag{7}$$

hence:

$$[A/B]_{X^*} = \frac{K^*[A/B]_{N_2}\, y_{N_2}}{y_{X^*}} \tag{8}$$

Therefore:

$$v_d = k^*[X^*] = k^* k[A/B]_{X^*}$$

$$= k^* k K^*[A/B]_{N_2}\frac{y_{N_2}}{y_{X^*}} = k^*_{app}[A/B]_{N_2} \tag{9}$$

where k is a proportionality constant between $[X^*]$ and $[A/B]_{X^*}$ and:

$$k_{app} = k^* k K^* \frac{y_{N_2}}{y_{X^*}} \tag{10}$$

Now, by substituting $y_{N_2} = \sqrt{y_A\, y_B}$, we have:

$$pK_{app} = p(k^* k K^*) + \log y_{X^*} - \tfrac{1}{2}\log(y_A\, y_B) \tag{11}$$

According to Debye-Hueckel's limiting law: $\log y = -Bz^2\sqrt{\mu}$, Eq. (11) becomes:

$$pk_{app} = p(k^* k K^*) - B\left[z^2_{X^*} - \tfrac{1}{2}\left(z^2_A + z^2_B\right)\right]\sqrt{\mu} \tag{12}$$

where $B = 0.51$.

Since the experimental slope of pk_{app} versus $\sqrt{\mu}$ is between -3.2 and -2.3 (Table 1), a reasonable solution can be obtained by assuming $z_A = -1$ and $z_B = -2$, so that $z_X = -3$ and the slope becomes -3.3, in accordance with the experiment. The fact that the number of negative groups, responsible for the denaturation rate, decreases slightly with pH (from -3.2 at 6.8 to -2.3 at 7.4), while the denaturation rate increases considerably, can be attributed to an increase of the negative electrostatic potential on the protein, which depresses the dissociation of ionizable acidic residues.

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