The active site of pepsin is formed in the intermediate conformation dominant at mildly acidic pH

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Abstract Pepsin is an aspartic protease that acts in food digestion in the mammal stomach. An optimal pH of around 2 allows pepsin to operate in its natural acidic environment, while at neutral pH the protein is denatured. Although the pH dependence of pepsin activity has been widely investigated since the 40s, a renewed interest in this protein has been fuelled by its homology to the HIV and other aspartic proteases. Recently, an inactive pepsin conformation has been identified that accumulates at mildly acidic pH, whose structure and properties are largely unknown. In this paper, we analyse the conformation of pepsin at different pHs by a combination of spectroscopic techniques, and obtain a detailed characterisation of the intermediate. Our analysis indicates that it is the dominant conformation from pH 4 to 6.5. Interestingly, its near UV circular dichroism spectrum is identical to that of the native conformation that appears at lower pH values. In addition, we show that the intermediate binds the active site inhibitor pepstatin with a strength similar to that of the native conformation. Pepsin thus adopts, in the 6.5–4.0 pH interval, a native-like although catalytically inactive conformation. The possible role of this intermediate during pepsin transportation to the stomach lumen is discussed.

Key words: Pepsin; pH dependence; Intermediate

1. Introduction

Pepsin is a well-known enzyme whose activity has been extensively studied since Northrop crystallised it in 1929 [1]. It belongs, together with catepsin D, quinosine, renine and the HIV-protease amongst others, to the family of the aspartic proteases, that display a high degree of structural homology [2]. The recognition of the HIV-protease as a member of this family [3] has renewed the interest in this type of enzymes and in their inhibition [4]. There is a wide range of specific inhibitors that can bind to the active site and effectively remove the activity of pepsin. One of the best known ones is pepstatin [5], that, at acidic pH, tightly binds to the catalytic site of both pepsin and its precursor: pepsinogen [6,7]. The pepstatin–pepsinogen complex, however, cannot be formed above pH 3 because the active site is blocked by a propeptide sequence [6,8,9]. Below pH 5, pepsinogen is self-cleaved to produce active pepsin [10].

Pepsin is a monomeric, two domain, mainly β-protein, with a high percentage of acidic residues (43 out of 327) leading to a very low p*I*. The catalytic site is formed by two aspartate residues, Asp32 and Asp215, one of which has to be protonated, and the other deprotonated, for the protein to be active [11]. This occurs in the 1–5 pH interval [12,13]. Above pH 7, pepsin is in a denatured conformation that retains some secondary structure [14,15]. This denaturation is not fully reversible [16], the lack of reversibility being attributed to the N-terminal domain [17]. In the 5–7 pH interval the conformation of pepsin is poorly characterised. Although some conformational changes experienced by pepsin in the acidic region have been reported [16,18], the intermediate conformations associated to them have not been studied in detail. We present here a detailed spectroscopic characterisation of the structure of pepsin and of its competence towards pepstatin binding in the 1–10 pH interval. We identify an intermediate pepsin conformation dominant at mildly acidic pH that, although catalytically non-competent, is essentially native and binds pepstatin. Based on this, we hypothesise that this intermediate could play a role during pepsin transportation to the stomach lumen.

2. Materials and methods

2.1. Protein and peptides

Crystallised, highest grade pepsin was purchased from Sigma and used without further purification. Its concentration was calculated using an extinction coefficient of 51 mM^{-1} cm^{-1} and was of 5 μM in fluorescence experiments, 10 μM in near- and in far-UV circular dichroism (CD), and 30 μM in molecular exclusion experiments. Pepstatin was purchased from USB Corp. (Cleveland, OH). A 30 μM concentration was used for the CD and fluorescence experiments. The control peptide Ac-GGGGGG-NH_2 was ordered to *Sigma Genosys* and used to examine whether the effects exerted by pepstatin on the pH-related pepsin transitions where specific.

2.2. pH measurements

A 1 mM sodium borate, 1 mM sodium citrate, 1 mM sodium phosphate and 25 mM sodium chloride buffer was used, adjusted to different pH values as required. This buffer allows to work at a constant ionic strength in a wide pH interval. The pH was measured with a Crison micro pH-meter calibrated before each experiment. The pH of every solution was measured immediately before and after each spectroscopic measurement, and the two values were always the same within experimental error (−0.03 pH units).

2.3. Fluorescence and CD measurements

Fluorescence measurements were performed at 25.0 ± 0.1°C in a thermostated Aminco-Bowman Series 2 spectrofotometer from Spectronic Instruments, using a 1 cm cuvette. The cuvette was sealed for

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$$N \underset{m}{\overset{pK_1}{\longleftrightarrow}} I \underset{n}{\overset{pK_2}{\longleftrightarrow}} D$$

Scheme 1.

measurement in order to minimise changes in pH. Fluorescence pH titration curves were acquired at an emission wavelength of 355 nm (with excitation at 280 nm). CD measurements have been made in a JASCO 710 spectropolarimeter thermostatised at 25 ± 0.1°C. For the far- and near-UV CD measurements a 10 μM pepsin solution was used in a 1 mm or 1 cm cuvette, respectively. Far- and near-UV titration curves were recorded at 215 and 290 nm, respectively, as the greatest changes occurred at those wavelengths.

2.4. Molecular exclusion chromatography measurements

Molecular exclusion experiments were performed in an FPLC system with a Superose 12 HR 10/30 column from Pharmacia, calibrated with proteins of known molecular weight. In all experiments a 30 μM pepsin solution was injected in a preequilibriated column and then eluted with the same buffer.

2.5. Data fitting

Depending on the spectroscopic technique used, one or two pH-related transitions are observed whose slopes are related to the number of protons involved in the process. The fluorescence and far-UV CD curves showed two transitions that were fitted to a three-state model (Scheme 1) using Eq. 1:

$$F = \frac{F_D + F_I\,10^{-a} + F_N 10^{-(a+b)}}{1 + 10^{-a} + 10^{-(a+b)}} \quad (1)$$

where D is the denatured conformation above pH 7 that takes up n protons to became the I, intermediate conformation, that takes up m protons to became the N, native conformation at low pH; F_D, F_I and F_N are the spectroscopic signals of the denatured, intermediate and native state, respectively; $a = m(\mathrm{pH} - \mathrm{p}K_1)$; and $b = n(\mathrm{pH} - \mathrm{p}K_2)$.

The near-UV CD and molecular exclusion curves can be fitted to a two-state transition (Scheme 2) using Eq. 2:

$$F = \frac{F_D + F_I\,10^{-b}}{1 + 10^{-b}} \quad (2)$$

where everything has the same meaning as in Scheme 1 and Eq. 1.

3. Results and discussion

3.1. pH-linked fluorescence transitions in pepsin

The fluorescence intensity of pepsin at 355 nm has been monitored from pH 9 to 2, at 25.0 ± 0.1°C in a buffer of constant ionic strength. Two evident transitions are observed (Fig. 1a). As the pH approaches neutrality from the alcaline region, there is a sudden increase in fluorescence intensity with an apparent pK_a of 6.8 (Table 1), and then a plateau is reached that extends from pH 6.5 to 4.5. At lower pH values a second transition occurs, with a pK_a of 3.5, that lowers the intensity. The two transitions are perfectly mimicked by changes in the wavelength of maximal fluorescence emission (Fig. 1b) from where pK_as of 6.8 and 3.7 can be calculated. These double transitions reveal the accumulation of an intermediate pepsin conformation that is dominant in the 6.5 to 4.0 interval. In general, little structural insight can be obtained from protein fluorescence intensity curves because the fluorescence intensity of a polypeptide, as it becomes more tightly packed, can both increase or decrease. In addition, fluorescence intensity changes depend on the wavelength used to monitor the transitions. In contrast, the wavelength of maximal emission is usually more informative as it is related to the degree of solvent exposure of tryptophan residues. The data in Fig. 1b indicate that the pepsin tryptophan residues are, in the intermediate, more buried than in the unfolded state but less than in the active conformation at very low pH. The wavelength changes are however quite small (specially that from the intermediate to the active form) and could even be due to titration of acidic residues close to fluorescent ones. It should be pointed out that the denatured conformation at pH 9.0 is not fully unfolded according to its wavelength of maximal

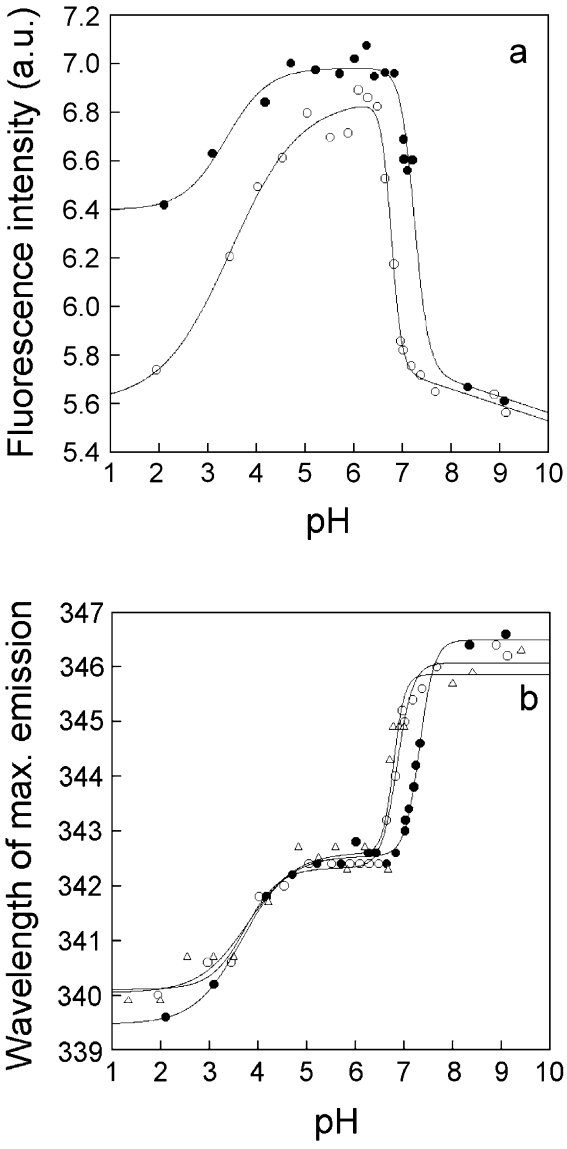

Fig. 1. a: Fluorescence intensity at 355 nm of pepsin (open circles) and of a pepsin–pepstatin complex (solid circles) as a function of pH, fitted to a three-state equation. b: Wavelength of maximal fluorescence emission of pepsin (open circles), of a pepsin–pepstatin complex (closed circles) and of a mixture of pepsin with a control peptide not expected to bind pepsin (open triangles) as a function of pH, fitted to a three-state equation. The spectra were measured from 300 to 400, with excitation at 280 nm. Pepsin, pepstatin and control peptide concentrations were 5, 30 and 30 μM, respectively, and the buffer: 1 mM sodium borate, 1 mM sodium citrate, 1 mM sodium phosphate and 25 mM sodium chloride.

$$I \underset{n}{\overset{pK_2}{\longleftrightarrow}} D$$

Scheme 2.

Table 1
pH-linked conformational transitions of pepsin[a]

Technique	Pepsin		+Pepstatin		+Control peptide	
	pK_1 (protons)	pK_2 (protons)	pK_1 (protons)	pK_2 (protons)	pK_1 (protons)	pK_2 (protons)
Apparent size	nd	6.79 ± 0.03 (4.8 ± 0.5)	nd	nd	nd	nd
Fluorescence intensity	3.42 ± 0.06[b] (0.9 ± 0.4)	6.72 ± 0.06[b] (3.2 ± 1.3)	3.22 ± 0.11[b] (0.7 ± 0.2)	7.26 ± 0.07[b] (1.5 ± 0.1)	3.19 ± 0.46 (0.6 ± 0.4)	6.69 ± 0.04 (3.7 ± 1.6)
Fluorescence maximum	3.52 ± 0.32[b] (1.0 ± 0.1)	6.78 ± 0.01[b] (2.9 ± 1.1)	3.31 ± 0.47[b] (0.8 ± 0.1)	7.25 ± 0.03[b] (2.4 ± 0.9)	3.79 ± 0.45 (0.9 ± 1.0)	6.78 ± 0.10 (2.4 ± 1.2)
Far-UV CD	4.40 ± 0.21 (1.0 ± 0.4)	6.76 ± 0.04 (2.7 ± 0.8)	4.18 ± 0.10 (0.9 ± 0.2)	7.38 ± 0.02 (2.8 ± 0.4)	nd	nd
Near-UV CD	–	7.33 ± 0.03 (2.6 ± 0.4)	–	7.36 ± 0.03 (2.6 ± 0.4)	nd	nd

[a]Errors provided by the fitting programme.
[b]Mean of two determinations ± standard deviation.

emission, far from the typical 355 nm of polypeptides with fully exposed tryptophan residues. One further transition occurs above pH 9.0 (not shown) that brings the emission maximum close to 355 nm. One interesting detail is that, although the transition from the native to the intermediate state is fully reversible, the higher pH transition, from intermediate to denatured, is only partially reversible (data not shown). This means that the analysis of the second transition yields only an approximation of the pK_as. In addition, we have noticed that short incubation times up to 2 h are not enough to

Fig. 2. CD spectra of pepsin and of a pepsin–pepstatin complex at several pH values. a: Far-UV CD spectra from 250 to 200 nm: pepsin at pH 9.46 (solid triangles), pH 5.52 (open triangles) and pH 1.81 (half-filled triangles); pepsin–pepstatin complex at pH 9.00 (solid circles), pH 5.51 (open circles) and pH 1.78 (half-filled circles). b: Near-UV CD spectra of pepsin from 310 to 260 nm: pH 8.70 (solid circles), pH 7.39 (open circles), pH 6.65 (solid triangles), pH 5.62 (open triangles), pH 4.26 (solid stars) and pH 2.21 (open stars). The pepsin and pepstatin concentrations used were 10 and 30 μM, respectively, and the buffer: 1 mM sodium borate, 1 mM sodium citrate, 1 mM sodium phosphate and 25 mM sodium chloride.

equilibrate the samples. For this reason we have used longer equilibration times (5–12 h) to gather the curves shown in Figs. 1 and 3. After 5 h of equilibration the curves are independent of the equilibration time, which suggests they are true equilibrium curves and therefore the reported pK_a values for the higher pH transition are essentially correct.

3.2. pH-linked far- and near-UV CD transitions in pepsin

Using a combination of techniques to monitor transitions associated to changes in solution conditions (pH, temperature, denaturant concentration, etc.) can help detect conformations that may pass undetected by using a single technique and often provides useful insight into the structure of the intermediates [19,20]. To further investigate the conformation of pepsin at different pH values, we have recorded far- and near-UV CD spectra and pH titration curves from pH 9 to 2. The far-UV CD spectra are shown in Fig. 2a. As already suggested by the fluorescence data, the high pH spectrum is consistent with some residual secondary structure being present in the denatured conformation. In addition, a titration curve has been recorded following the ellipticity at 215 nm, a wavelength that can, in principle, report on the content of beta structure. The curve, shown in Fig. 3a, is similar to the fluorescence curves in that it shows two transitions. The calculated pK_a for the higher pH transition (6.8) agrees very well with those found by fluorescence intensity and fluorescence wavelength. As for the lower pH transition, the agreement is only qualitative as it is a bit higher (4.4) than those found by fluorescence. The higher pH transition is associated with an increase (in absolute value) of the ellipticity at 215 nm and a decrease of the 205 minimum (Fig. 2a), which is consistent with an increase in secondary structure content. The lower pH transition, where the ellipticity decreases (in absolute value) may be interpreted as a debilitation of non-native secondary structure elements present in the intermediate conformation but it could also reflect a change in the contribution of aromatic residues to the far UV CD spectrum [21].

More revealing is the evolution of the near-UV CD spectrum as a function of pH. The spectra in Fig. 2b indicates that, as the pH is lowered from the alcaline region, a well-defined near-UV CD spectrum develops reflecting that some aromatic residues of pepsin become fixed in a stable asymmetric environment characteristic of well-folded conformations. Interestingly, there is no noticeable change from pH 6 to 2, which suggests that the delicate environment of the aromatic residues that give rise to the spectrum is the same in the intermediate and in the low pH active conformation. This is confirmed by the titration curve (Fig. 3b) that can be fitted to a single two-state transition with a pK_a of 7.3, not identical but close to that found for the first transition by the other techniques used. It is not clear why the near UV CD spec-

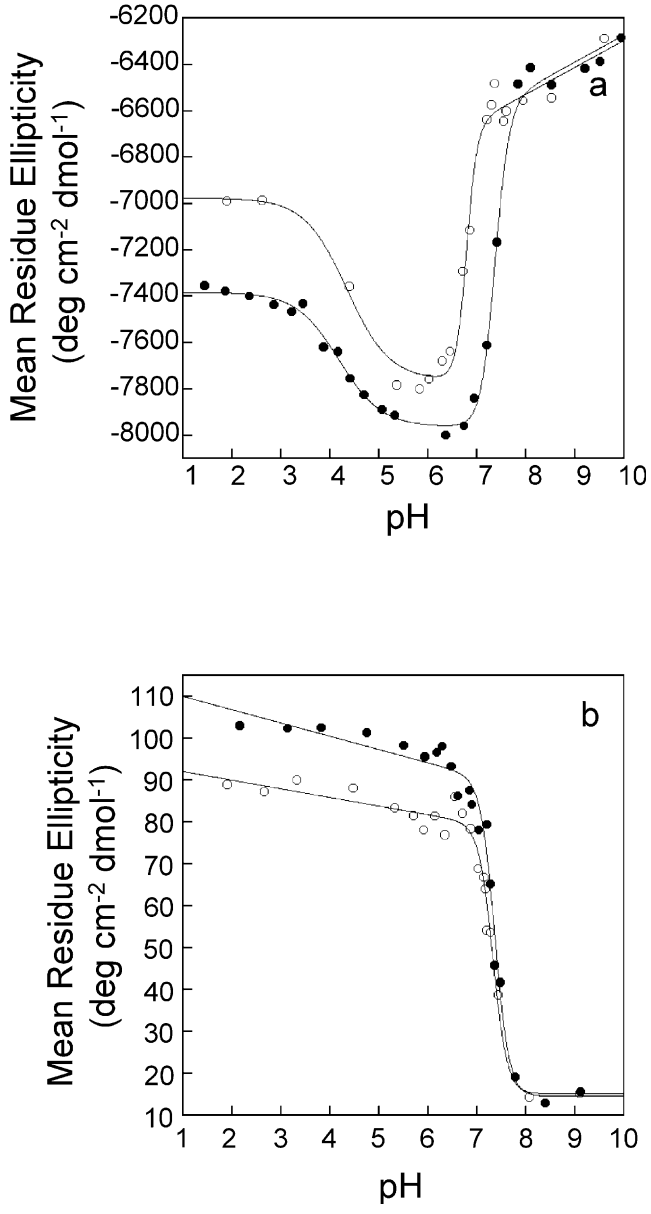

Fig. 3. Far-UV CD at 215 nm (a) and near-UV CD at 290 nm. (b) of pepsin (open circles) and of a pepsin–pepstatin complex (solid circles) as a function of pH, fitted to a three-state and to a two-state equation, respectively. The pepsin and pepstatin concentrations were 10 μM and 30 μM, respectively, and the buffer: 1 mM sodium borate, 1 mM sodium citrate, 1 mM sodium phosphate and 25 mM sodium chloride.

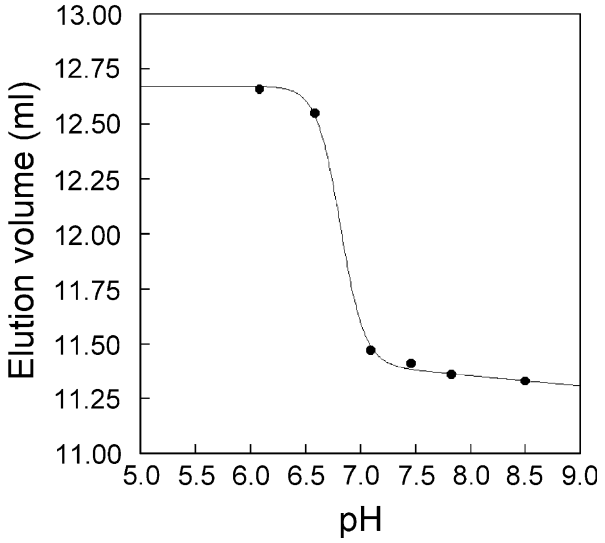

Fig. 4. Apparent size of pepsin as a function of pH, fitted to a two-state equation. Pepsin concentration was 30 μM in buffer 1 mM sodium borate, 1 mM sodium citrate, 1 mM sodium phosphate and 25 mM sodium chloride.

trum, usually very sensitive to conformational changes, does not significantly vary from pH 6 to 2, especially when both the far-UV CD and the fluorescence spectra clearly change in this region. One possible explanation is that the change in conformation in going from intermediate to native is confined to one of the two pepsin domains while the specific aromatic residues responsible for the near-UV CD spectrum (at 290 nm some of the tryptophan residues) are confined to the other. Since both the N- and C-terminal domains contain tryptophan residues (two and three respectively) it is not possible to assess which domain is being affected by the low pH conformational transition.

3.3. Pepsin apparent size as a function of pH

The apparent size of pepsin as a function of pH is shown in Fig. 4. At low pH the protein exhibits unrealistically high elution volumes (too low apparent molecular masses), indicative of an interaction with the column matrix (not shown). This precludes the characterisation of the lower pH transition in terms of apparent size. Above pH 6, the interaction with the column is less strong and pepsin is eluted at volumes consistent with its molecular mass. From pH 6.5 to higher values, the elution volume decreases as the pH increases, reflecting the expansion associated to the denaturation transition. The elution volume data can be fitted (Fig. 4) to a sigmoidal curve with an apparent pK_a of 6.8 (Table 1).

3.4. Conformational states of pepsin in different pH regions

The pK_a data obtained using fluorescence intensity, fluorescence wavelength of maximal emission, near-UV CD, far-UV CD and size exclusion chromatography (Table 1) offer a very clear picture. Above pH 7, pepsin is in an expanded conformation, devoid of well-defined tertiary interactions, with a reduced secondary structure content but still compact enough to manifest significant tryptophan burial and to contain some secondary structure. This conformation is enzymatically inactive and has been reported to experience a further unfolding event with an apparent pK_a of 11.5 [15]. At around pH 6.8 pepsin folds to an intermediate conformation, as shown by the five different techniques used to monitor the transition. The slight difference between the pK_a calculated by near-UV CD and by the other techniques suggests the transition may not be two-state. Compared to the denatured state, the intermediate displays a higher content of secondary structure, and a higher degree of tryptophan burial. Most revealingly, its near-UV CD spectrum is consistent with that of a well-folded conformation and, indeed, it is identical to that of the native active conformation at low pH. The size of the intermediate is also consistent with its postulated well-folded structure. All this together is a strong indication that the intermediate adopts a conformation very similar to the native one and that its lack of enzyme activity is only due to an inappropriate ionisation state of one of the active site residues. At lower pH values (around pH 3.5), the intermediate evolves to the native, active conformation. As in the case of the higher pH transition, a small but significant discrepancy between the pK_as calculated from the far-UV CD curves and from the other techniques suggests the intermediate to native transition may involve additional species in the transition region. Our analysis, together with the data in ref. [15] allows us to propose the following scheme that summarises the conformational states adopted by pepsin in different pH regions:

$$A \xleftrightarrow{pK_a\,3.5} I \xleftrightarrow{pK_a\,6.8} D_1 \xleftrightarrow{pK_a\,11.5} D_2$$

Scheme 3.

In Scheme 3, A denotes the active conformation, I the intermediate conformation (inactive but native-like), D_1 the denatured conformation that appears at neutral pH (and that still contains some secondary structure), and D_2 the more unfolded conformation at high pH.

3.5. Pepsin/pepstatin binding to the intermediate and native conformations

The native-like characteristics found for the intermediate conformation dominant from pH 6.5 to 4.5 prompted us to question whether it would contain a well-formed substrate binding site. To address this issue we have pH-titrated pepstatin/pepsin mixtures and followed their spectroscopic properties in parallel to those of pepsin. According to the dissociation constant of the complex at pH 2, 1.3×10^{-8} [22], the concentrations of enzyme and inhibitor used ensure that more than 99% of the pepsin molecules are bound to pepstatin at this pH value.

The binding of pepstatin to the native and to the intermediate pepsin conformations, but not to the denatured state, is reflected by an increase in the ellipticity at 215 nm (Fig. 2a) in the presence of the inhibitor. In contrast, the spectrum of the denatured conformation, at pH 9, does not change at this wavelength upon addition of pepstatin (the difference between the two spectra that can be observed at 205 nm is just the signal of pepstatin).

The best way, however, to assess whether pepstatin binds to the intermediate is to compare the spectroscopic pH profiles of pepsin and of the pepsin/pepstatin mixtures. The fluorescence intensity, fluorescence wavelength of maximal emission, near-UV CD and far-UV CD profiles shown in Figs. 1 and 3 display the same pattern: the signal of the denatured conformation is never altered by the presence of pepstatin, which strongly suggests pepstatin does not bind to the denatured conformation. In contrast, all the spectroscopic signals of the intermediate and native conformations are modified in the presence of the inhibitor (except the emission wavelength of the intermediate).

The binding of pepstatin to the intermediate is also indicated by the significant pK_a-shifts induced in the higher pH transition, as can be observed in the far-UV CD and in the fluorescence intensity and λ_{max} pH profiles (Figs. 1 and 3 and Table 1). Pepstatin increases these pK_a values by around 0.5 pH units and sets them quite close to the pK_a of the near-UV CD curve (Table 1), which suggests that in the presence of pepstatin the high pH transition becomes two-state. The observed shifts in pK_a values are certainly expected if pepstatin binds to the intermediate but not to the denatured state because the presence of pepstatin in the solution will shift the D–I equilibrium and favour the intermediate conformation over the denatured one in a wider pH interval. As for the low pH transition, the binding of pepstatin to the intermediate and native states only produces small pK_a changes, which suggests that the strength of the native and of the intermediate pepstatin complexes is similar.

Finally, we have checked that the pepstatin-induced spectroscopic and pK_a changes are not unspecific by studying a

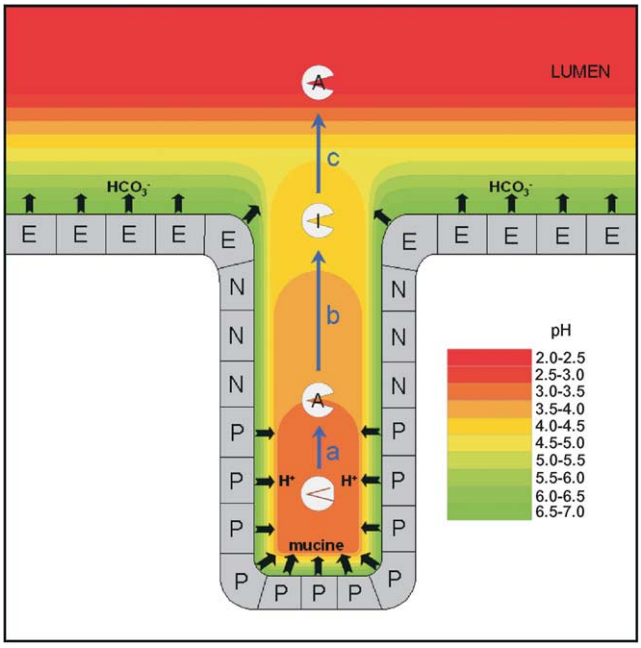

Fig. 5. Scheme of the pH gradients inside the crypts of the stomach and of the transit of pepsin from the parietal cells (P) to the stomach lumen. In addition, the neck and the epithelial cells are labelled as (N) and (E) respectively. The pepsinogen liberated into the crypt is converted at pH 3 (a) into active pepsin, A. On its way to the lumen, it reaches areas of higher pH were it evolves (b) into the inactive but well-folded intermediate, I, that can bind the mucus proteins without degrading them, and is transported bound to the mucus. Finally, it reaches areas of lower pH near the lumen and it becomes active again (c) and starts degrading the mucus layer.

control peptide not expected to bind at the pepstatin binding site. As shown in Fig. 1b, the control peptide fails to produce the accurately determined change in pK_a of the denatured/intermediate transition (pK_2) that is induced by pepstatin and, indeed, the pH-titration curve of pepsin mixed with the control peptide can be superimposed to that of free pepsin. The pK_a of the denatured/intermediate transition derived from the fluorescence intensity curve is also the same as that of free pepsin (Table 1).

3.6. *Participation of the intermediate in pepsin transportation to the stomach lumen*

The stomach of mammals uses a complex system to generate an acidic environment in the gastric lumen and to maintain a pH gradient from the epithelial surface to the lumen [23,24]. It is clear that pepsin at acidic pH can damage the stomach cells. To avoid this harmful contact, there are cells in the epithelial surface that, on the one hand, synthesise and liberate a protective mucus shield whose glycoproteins capture the protons produced by parietal cells, and, on the other, release HCO_3^- that increases the pH [25,26]. As a consequence, although the gastric lumen has a pH of around 2, the combined effect of proton release, neutralisation by the glycoproteins, secretion of HCO_3^- in the epithelium and back diffusion of protons from the lumen, establishes a 7–2 pH gradient across the mucus. When the mucus proteins reach the lumen, pepsin degrades them and protons are released [26]. Pepsin thus performs a double function: degradation of ingested proteins and degradation of the mucus, with a concomitant release of protons.

Pepsin is produced from pepsinogen by intramolecular or intermolecular cleavage. Between pH 5 and 4 the intermolecular cleavage dominates, while at lower pHs the activation tends to be intramolecular [27]. In its path to the lumen (Fig. 5), pepsinogen is secreted to gastric crypts at low pH (around 3) [28] where it can become enzymatically active and generate pepsin, that continues its trip to the lumen. As soon as pepsin reaches an area of pH above 4, it will adopt the enzymatically inactive but well-folded intermediate conformation studied in this work. Since this intermediate retains a native-like peptide binding site, we propose that it will get bound to the proteins of the mucus, not degrading them, and will thus be actively carried towards the lumen in this bound state. Moreover, since above pH 4 the intramolecular mechanism of activation is slow and since the intermolecular mechanism is not operative because the pepsin molecules are sequestered by their interaction with the mucus proteins, the activation of pepsin will be effectively halted near the crypts openings. Then, as bound pepsin is transported towards the lumen, encompassed with the movement of the mucus, it will reach again areas of lower pH where it will become readily active upon protonation because it is already in a native-like conformation. In addition if some molecules enter in a higher pH area near the epithelial cells, the protein will remain folded and bound to the mucus and will recover its activity as soon as it reenters a region of lower pH.

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