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

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Received 18 December 2002; accepted 27 January 2003
First published online 20 February 2003
Edited by Thomas L. Jones

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.

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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 cathepsin 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 pf. 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 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-GGGGNNH₂ 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 thermostatted Amino-Bowman Series 2 spectrophotometer from Spectronic Instruments, using a 1 cm cuvette. The cuvette was sealed for
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 pre-equilibrated 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:

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

where D is the denatured conformation above pH 7 that takes up n protons to become the I, intermediate conformation, that takes up m protons to become 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; $a = m(pH - pK_1)$; and $b = n(pH - pK_2)$.

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

$$F = \frac{F_D + F_0 10^{-b}}{1 + 10^{-b}}$$

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 alkaline region, there is a sudden increase in fluorescence intensity with an apparent $pK_1$ 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 $pK_2$ 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 $pK_2$ 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...
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 $pK_a$s. In addition, we have noticed that short incubation times up to 2 h are not enough to

<table>
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<tr>
<th>Technique</th>
<th>Pepsin</th>
<th>+Control peptide</th>
<th>+Pepstatin</th>
<th>Pepsin +Pepstatin</th>
<th>+Control peptide</th>
<th>+Pepstatin</th>
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<td>$pK_2$ (protons)</td>
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<td>Apparent size</td>
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<tr>
<td>Fluorescence intensity</td>
<td>6.79±0.03 (4.8±0.4)</td>
<td>6.76±0.03 (2.7±0.4)</td>
<td>6.76±0.04 (2.7±0.8)</td>
<td>7.33±0.03 (2.6±0.4)</td>
<td>4.40±0.21 (1.0±0.4)</td>
<td>4.18±0.10 (0.9±0.2)</td>
</tr>
<tr>
<td>Fluorescence maximum</td>
<td>3.52±0.32 b (1.0±0.1)</td>
<td>3.31±0.27 b (0.6±0.1)</td>
<td>3.17±0.10 (0.9±0.2)</td>
<td>3.79±0.08 (1.0±0.2)</td>
<td>6.78±0.04 (3.2±0.4)</td>
<td>6.69±0.04 (3.7±1.6)</td>
</tr>
<tr>
<td>Far-UV CD</td>
<td>4.40±0.21 (1.0±0.4)</td>
<td>3.17±0.10 (0.9±0.2)</td>
<td>4.18±0.10 (0.9±0.2)</td>
<td>3.79±0.08 (1.0±0.2)</td>
<td>6.78±0.04 (3.2±0.4)</td>
<td>6.69±0.04 (3.7±1.6)</td>
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<td>aErrors provided by the fitting programme. bMean of two determinations±standard deviation.</td>
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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: Neish-UV CD spectra 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). The pepsin and pepstatin concentrations used were 10 and 30 μM, respectively, and the buffer: 1 mM sodium borate, 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 inter-

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 $pK_a$s 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:

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

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, $1.3 \times 10^{-8}$ [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 $\lambda_{max}$ 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 specific by studying a
The control peptide can be superimposed to that of free pepsin. and, indeed, the pH-titration curve of pepsin mixed with the 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 ($pK_a$) 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 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 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.

References