Structural dissection of alkaline-denatured pepsin

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Abstract. Pepsin, a gastric aspartic proteinase, is a zymogen-derived protein that undergoes irreversible alkaline denaturation at pH 6–7. Detailed knowledge of the structure of the alkaline-denatured state is an important step in understanding the mechanism of the formation of the active enzyme. It has been established in a number of studies that the alkaline-denatured state of pepsin (the I_p state) is composed of a compact C-terminal lobe and a largely unstructured N-terminal lobe. In the present study, we have investigated the residual structure in the I_p state in more detail, using limited proteolysis to isolate and characterize a tightly folded core region from this partially denatured pepsin. The isolated core region corresponds to the 141 C-terminal residues of the pepsin molecule, which in the fully native state forms one of the two lobes of the structure. A comparative study using NMR and CD spectroscopy has revealed, however, that the N-terminal lobe contributes a substantial amount of additional residual structure to the I_p state of pepsin. CD spectra indicate in addition that significant non-native α -helical structure is present in the C-terminal lobe of the structure when the N-terminal lobe of pepsin is either unfolded or removed by proteolysis. This study demonstrates that the structure of pepsin in the I_p state is significantly more complex than that of a fully folded C-terminal lobe connected to an unstructured N-terminal lobe. The “misfolding” in this state could inhibit the proper refolding of the protein when returned to conditions that stabilize the native state.

Keywords: Pepsin, zymogen, denaturation, partially folded state, limited proteolysis, NMR, SAXS

Abbreviations

CD, circular dichroism; UV, ultraviolet; NMR, nuclear magnetic resonance; ppm, parts per million; DSS, 2,2-dimethyl-2-silapentane-5-sulfonic acid; 1D, one-dimensional; NOE, nuclear Overhauser effect; SAXS, small-angle X-ray scattering; ANS, 8-anilino-1-naphthalene-sulfonic acid; I_p, the alkaline-denatured state of pepsin at pH 8.0 and 25°C; C fragment, the pepsin fragment designated by α -chymotrypsin and chromatographically purified; N_p, the native state of pepsin at pH 5.6 and 25°C; D_{PU}, the urea-denatured state of pepsin at pH 8.0, 4 M urea and 25°C; D_{PH}, the denatured state of pepsin at pH 8.0 and 70°C; D_{PA}, the denatured state of pepsin at pH 12.0 and 25°C; R_G , radius of gyration.

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1. Introduction

Extensive structural studies have been carried out recently on denatured and other non-native states of proteins, largely motivated by a desire to probe the mechanisms of protein folding and aggregation or to understand the physiological roles of non-native structures [1–9]. An important group of non-native states of proteins includes those of several zymogen-derived enzymes which unfold irreversibly and become trapped in partially denatured states [10–14].

Aspartic protease (EC 3.4.23), found in animals, plants, fungi, yeast, some bacteria and viruses, constitute one of the four distinct superfamilies of proteolytic enzyme ([15,16] and Fig. 1). All non-viral aspartic protease are synthesized as inactive precursors (zymogens), in which the N-terminal propeptide is bound to the active site cleft, thus preventing undesirable degradation during intracellular transport and secretion [17,18]. In addition to their inhibitory function, prosegments play an important role in the correct folding, stability and intracellular sorting of many zymogens [19]. There is, however, relatively little detailed information about the structures of the denatured states of these and other zymogen-derived proteins. Defining the structural characteristics of such proteins should give insights not only into the functional properties of this very important family of enzymes involved in proteolysis, but also into the general factors defining protein structure, folding and activity [14,20].

The gastric aspartic proteinase, pepsin (porcine pepsin, molecular weight = 34623) is one of the aspartic protease that has been the subject of extensive study [21–23]. X-ray diffraction analysis shows that the substrate-binding cleft is located between two homologous portions of the structure, the N-terminal lobe (residues 1–172) and the C-terminal lobe (residues 173–327) (Fig. 2B and [24,25]). Pepsin undergoes a conformational transition from the native (at acidic pH) to the denatured (at alkaline pH) state in a narrow pH range (between 6 and 7). This alkaline denaturation process appears to be almost completely irreversible [10,26] although the unfolding of the zymogen, pepsinogen, is reversible under carefully controlled conditions [27]. Recently, refolding of an immobilized form of the denatured pepsin was achieved without the pro-sequence [28], but its refolding mechanism is still unsolved. Structural knowledge of the alkaline-denatured state of pepsin (designated “the I_P state” for convenience) is essential not only for understanding the folding behaviour of pepsin, but also for elucidating the mechanisms that govern the observed strong interaction of the I_P state of pepsin with species such as molecular chaperones [29] or amyloid fibrils [30].

Privalov et al. [31] isolated the C-terminal lobe using limited proteolytic digestion of native pepsin and showed that the C-terminal lobe has higher stability than the N-terminal lobe in the native state. Lin et al. [26] demonstrated regeneration of the enzymatic activity of alkaline-inactivated pepsin by addition of the recombinant N-terminal lobe but not by addition of the C-terminal lobe. Both lines of evidence suggest the C-terminal lobe has higher stability than the N-terminal lobe and that the I_P state has a folded C-terminal lobe and a largely unstructured N-terminal lobe. A theoretical calculation by Lin et al. [26] and a recent mutational experiment by Tanaka and Yada [32] have resulted in similar conclusion. However, we demonstrated that a histidine residue located in the N-terminal lobe of the pepsin molecule is located near the folded region of the I_P molecule [33]. This observation suggests that there is a significant contribution by residues in the N-terminal lobe to the residual structure of the I_P state and that the structure of pepsin in the I_P state could be more complex than that of a natively folded C-terminal lobe connected to an unstructured N-terminal lobe. In order to obtain more detailed structural information on the I_P state of pepsin we have carried out experiments designed to isolate the folded core of the molecule, by purifying the material obtained from limited proteolysis of the alkaline denatured

Fig. 1. Aspartic proteases: proteins which is similar to pepsin was serched using The Dali server (<http://www2.ebi.ac.uk/dali/>) and found more than 100 structures in the database. All of them belonged to aspartic protease. The representative structures are shown in this figure. This figure was generated with MOLSCRIPT [40].

Fig. 2. (A) Far ultraviolet CD spectra of pepsin at 25°C. pH 5.6 (thin solid line); pH 8.0 (bold solid line); 4 M urea and pH 8.0 (broken line). (B) Fluorescence spectra of ANS (40 μ M) at pH 8.0. The concentration of pepsin is 0 (broken line) and 6 μ M (solid line).

Table 1
Conformational states of pepsin in aqueous solution^a

	Secondary structure	Tertiary structure	R_G (Å) ^b	$R_G/R_{G(N)}$ ^c	SAXS pattern
N _P	++	++	24.4 ± 0.5	1	native-state pattern
I _P	+	+	30.9 ± 1.4	1.27	compact-denatured pattern
D _{PA}	—	—	n.d. ^d	n.d. ^d	n.d. ^d
D _{PH}	±	—	n.d. ^d	n.d. ^d	n.d. ^d
D _{PU}	—	—	39.9 ± 2.6	1.64	highly-denatured pattern

^aThe residual structure in large (++) , significant (+) , marginal (±) amounts, or its absence (—).

^b R_G was determined from the scattering curve at infinite dilution.

^c $R_G/R_{G(Native)}$ is the ratio of the R_G value of each state relative to that of the native state.

^dn.d.: not determined because of technical difficulties.

protein. We have characterized the structure of this folded core spectroscopically and we compare it with that of the full-length I_P state of pepsin [34].

2. The alkaline denatured state of pepsin at pH 8 (I_P) has a highly folded region and flexible region

At pH 5.6, where pepsin is in its native state, the far-UV CD spectrum has a shape typical of a β -sheet-rich protein (Fig. 2A, thin solid line). At pH 8.0, where pepsin is denatured, the spectrum indicates a partial loss of secondary structure and the emergence of intensity characteristic of random coil regions of structure (Fig. 2A, bold solid line; [22,32,35]). The transition between the two spectra occurs in the pH range of 6–7, and is not reversible when the pH is decreased from 8.0 to below 6.0. Addition of 4 M urea at pH 8.0 leads to a further change in the spectral shape, typical of a highly unstructured conformation (Fig. 2A, broken line). For convenience, we denote the native state as N_P, the alkaline-denatured state as I_P, and the denatured state in the presence of 4 M urea as D_{PU}, respectively (Table 1). Existence of further structural transition of I_P by changing of pH (pH 11–12 at 25°C), temperature (45–60°C at pH 8.0) and addition of urea confirm the presence of extensive residual secondary structure. Residual tertiary structure is also clearly evident from the presence of well resolved and shifted NMR signals (Fig. 3C). The small-angle X-ray scattering (SAXS) analysis also demonstrates that the I_P state contains a globular component (Fig. 4A), and its overall dimensions are intermediate between those of N_P and those of D_{PU} (R_G in Table 1).

Fig. 3. 750 MHz ^1H NMR spectra of the native state of pepsin at pH 5.6 in H_2O (A), the C fragment at pH 8.0 (B), the I_P state at pH 8.0 (C) and the urea-denatured state in 4 M urea at pH 8.1 (D). The signals labeled (*), (x) and (a) are those of the reference molecule DSS, an impurity, and a peak used for pH titration and 1D NOE experiments shown in Fig. 4, respectively.

Fig. 4. Kratky plots (A) and $P(r)$ functions (B) of pepsin at pH 5.6 ($\circ$), pH 8.0 ($\bullet$) and pH 8.0 in the presence of 4 M urea ($\times$) at 25°C , 5 mg/ml pepsin.

A well established form of a compact partially folded denatured state is the molten globule state [35–38]. However, the rigid tertiary structure observed by NMR is not characteristic of molten globule states. Molten globule-like compact denatured states that are loosely packed show NMR signals significantly broader [3,4,35,39] than those observed for the I_P state. The partially folded region of I_P is also highly stable, since very slowly exchanging NH protons were found in this structure, again not charac-

teristic of molten globules. In addition, the fluorescence dye, ANS, does not bind to the I_P state (Fig. 2B) although ANS binding to the incompletely collapsed hydrophobic core is an indicator of molten globule character [36,38]. These results therefore suggest that the globular component of I_P originates from a native-like packing of the protein chain, not from a molten globule-like conformation. The fact that the some regions of NMR spectrum of I_P have similarities to that of D_{PU} is, however, an indication that part of the protein chain is in a highly unfolded structure (Fig. 3). Thus, the alkaline-denatured state of pepsin appears to have a tightly folded region and a flexible unstructured region. This conclusion is also consistent with the shape of the $P(r)$ function of I_P , which has a peak characteristic of a relatively compact structure and a very extended tail to much higher dimensions (Fig. 4B).

3. The folded structure of the I_P state corresponds primarily to the C-terminal lobe

We employed limited proteolysis using α -chymotrypsin to isolate the folded part of pepsin in the alkaline denatured (I_P) state. Our purpose is to study the structure of pepsin in the denatured state rather than the folded form, in contrast to the proteolytic experiment described by Privalov et al. [31] which digested diazoacetyl-inhibited pepsin in its natively folded state. Digestion of the I_P state of pepsin for 1–2 hours under our experimental conditions by α -chymotrypsin resulted in the transient accumulation of a fragment with a molecular mass of ~ 14 kDa. Mass spectroscopic and N-terminal sequencing analyses of the fragment revealed that its mass is 15895 ± 5 Da and its N-terminal amino acid sequence is TGSLNWVPVS; this information shows that the fragment corresponds to residues 176–327 of the protein representing the C-terminal lobe of the overall structure (and henceforth is designated the “C fragment”). This cleavage site is shown by an arrow on the pepsin amino acid sequence (Fig. 5A) and structure (Fig. 5B). It is also worth noting that there are 23 potential sites for chymotrypsin digestion within this C fragment, implying protection conferred by a stably folded structure.

This result strongly supports the conclusion that the I_P state has a structure consisting of both of fully folded and highly denatured regions. This proteolytic experiment reveals definitively that the tightly folded fragment corresponds to the C-terminal lobe of pepsin. This finding, together with the similarity of the thermal denaturation curve above 25°C for the I_P state and the C fragment monitored by far-UV CD spectroscopy (Fig. 6B), indicates that the folded cooperative core of the I_P state corresponds primarily to that of the C-terminal lobe of intact pepsin. These results confirm the previous suggestion by Lin et al. [26] and also agree with the conclusion that the C-terminal lobe of pepsin is more stable against heat or proteolytic digestion than the N-terminal lobe in the native-state [31].

4. Contributions of the N-terminal part to the residual structures of the I_P state and non-native structures in the I_P state

Although the folded structure of the I_P state corresponds primarily to the C-terminal lobe as shown above, some evidences, however, indicate that the structure in the I_P state does not correspond simply to the structure of the C-terminal lobe of the native protein in all its features. pH-dependent behavior of the upfield-shifted signals is directly coupled with the titration of His53 which is in the N-terminal lobe (Fig. 7A). Moreover, NOEs between the His53 C2 proton and the upfield-shifted signals are observed (Fig. 7B and C). These evidences indicate that the folded structure include some residues in the vicinity of His53. The differences in the ellipticity change at low temperatures ($<25^\circ\text{C}$) between the C fragment and the I_P state of pepsin also indicate a degree of non-random conformation in the N-terminal lobe

Fig. 5. (A) Amino acid sequence of pepsin. All potential cleavage sites digested by α -chymotrypsin are shown in bold. The cleavage site to produce the C fragment is shown by an arrow. (B) Main-chain trace of native pepsin (structure 4pep) showing the N-terminal lobe of the protein (residues 1–172) in light gray, the C-terminal lobe (residues 173–326) in dark gray, and His53 in black. The cleavage site to produce the C fragment is shown by an arrow. This figure was generated with MOLSCRIPT [40].

Fig. 6. Far-UV CD spectra (A) of the C fragment (thick solid line), the Ip state (pH 8.0; thin solid line) and the native state (pH 6.5; thick broken line) of pepsin. The unit of ellipticity is and per residue for (B). Thermal denaturation curves (B) of the C fragment (filled circles) and the Ip state (open circles) of pepsin monitored by $[\theta]_{220 \text{ nm}}$. The ellipticity in (B) was normalized to the maximum ellipticity change in the temperature range shown in the figures.

Fig. 7. (A) pH dependent changes in the chemical shift of the C2 proton of His53 (○) and the upfield-shifted signal “a” of Fig. 2C (●) in D₂O solution. (B) 1D NMR spectrum of the alkaline denatured state I_p of pepsin in D₂O solution at pH* 8.9. (C) NOE difference spectrum resulting from saturation of the signal labeled “x” in Fig. 3B. The label “His” indicates the C2 proton signal of His53 of pepsin.

of the I_p state (Fig. 6B). Moreover, the NMR spectra show significant differences between the tertiary structure of the C fragment and the residual structure present in the I_p state (Fig. 3).

There is also some evidence that the residual structure in the I_p state and in the C fragment contains structure not present in the native state. Analysis of the far-UV CD spectra indicates that the I_p state and the C fragment have a larger α -helical and a smaller β -sheet content than that observed in the native state of pepsin (Fig. 6A and Table 2). One possibility is that some of the β -sheet structure localized at the interface between the N- and C-terminal lobes of folded pepsin [24,25] is disrupted by the removal or unfolding of the N-terminal lobe and replaced by α -helical structure.

Our study therefore indicates a relatively complex structure of the I_p state of pepsin. It contains the tightly-folded C-terminal lobe with a substantial amount of non-native secondary and tertiary structures, and additional contributions to the residual structure of the I_p state from the N-terminal lobe.

5. Implications for the folding mechanisms of pepsin

It is interesting to speculate that the observed non-native characteristics of the structure of pepsin in the I_p state could play a role in the folding of pepsin or its precursor pepsinogen. As formation of the interfacial β -sheet structure between the two structural lobes is likely to be a crucial step for the

Table 2
Secondary structure content of pepsin and the C fragment estimated from the CD spectra

	α -helix	β -sheet	Turn	Random	$R_{\alpha/\beta}$ ^a
Native pepsin (pH 5.6, 25°C)	11.3 ± 2.6	33.9 ± 2.7	23.2 ± 1.1	32.3 ± 2.6	0.33 ± 0.06
I _P state (pH 8.0, 25°C)	9.6 ± 1.9	21.9 ± 2.0	18.6 ± 2.1	50.6 ± 3.4	0.44 ± 0.05
C fragment (pH 8.0, 25°C)	19.0 ± 0.8	24.8 ± 1.2	20.5 ± 1.2	35.5 ± 3.0	0.77 ± 0.05
Pepsinogen (pH 8.0, 25°C)	16.5 ± 0.5	29.4 ± 2.7	21.5 ± 0.4	32.7 ± 2.1	0.57 ± 0.05

Each value is the average of six independent calculations, based on the CONTINLL, SELCOM3 and CDSSTR programs and two reference data sets supplied with the CDpro package [41]. Two independent runs for each of the three programs were performed using the two different database sets. Thus six independent predictions were averaged to obtain the values in this table. The error values are those obtained in this averaging process. The spectral data used in this analysis ranged in wavelength from 187 to 240 nm.

^a $R_{\alpha/\beta} = (\alpha\text{-helical content})/(\beta\text{-sheet content})$.

proper folding of the protein, it is possible that such structure can only be achieved efficiently when the N-terminal lobe folds in the presence of the pro-sequence. Alternatively, the non-native structural elements in the partially folded I_P state of pepsin at pH 8.0 could stabilize this structure relative to the native state, in other words “misfolding” in this state could inhibit the proper refolding of the protein when returned to conditions that stabilize the native state. Whether or not such speculation is correct, the present study suggests that further investigation of the partly folded states is likely to be of substantial importance in understanding the nature of their folding of zymogen-derived proteins and the manner in which their activity is controlled and regulated.

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